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RESEARCH PAPER

Contribution for a better understanding of grapevine fungal trunk diseases in the Portuguese Dão wine region

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Summary. Esca and Petri disease, two of the most important fungal trunk diseases of grapevine, are responsible for significant losses by causing premature decline and dieback in vineyards worldwide. The Portuguese Dão wine region is no exception. Local winegrowers' knowledge on Grapevine Trunk Diseases (GTD) in general and of esca and Petri disease in particular is incomplete. The real scope of those problems has been based largely on individual perceptions rather than on a methodical evaluation of the situation. In order to get a full picture of the diseases impact, a leaflet with color pictures was produced and issued to winegrowers, accompanied by a simple questionnaire. The results of this survey represent a first indication of the extent of grapevine trunk diseases in the Dão wine region, specifically its economic impact and relevance to the local wine industry. In conjunction with the survey, several samples of wood collected from esca and Petri disease symptomatic vines, identified during the survey throughout the entire region, were processed and a collection of isolates of *Phaeomoniella chlamydospora* obtained. To determine the intra-specific variability among these isolates, morphological, cultural and molecular characteristics were evaluated. A protocol to study the pathogenicity with *P. chlamydospora* was conducted, consisting on the inoculation of cv. Touriga Nacional's spurs with a previously studied *P. chlamydospora* isolate.

Key words: *Vitis vinifera*, *Phaeomoniella chlamydospora*, Touriga Nacional, variability, pathogenicity.

Introduction

The Dão wine region has a total area of 376,000 ha, from which less than 20,000 ha are dedicated to grapevine (Figure 1). Wine production there might go back to Roman times (Loureiro and Cardoso, 1993). However the oldest signs of wine production date from the VI century CE, including wine presses carved on solid granite (Falcão, 2012), found throughout the entire region and some are still in use. The Dão region is distinguished by its complex orographic features, soils that are typically low-pH sandy granite with low levels of organic matter, and traditional cultivars, most of them of Portuguese origin. Grown throughout Portugal, cv. Touriga Nacional may originate from this region and due to a clonal selection program (Faustino 2011), is starting to internationalize specifically in Australia (Robinson, 1996; Ambrosi *et al*., 1997). This unique region remains a reservoir for Portuguese grape cultivar diversity thanks to specific grapevine preservation projects. In addition, the region hosts an important R&D facility – Centro de Estudos Vitivinícolas do Dão – one of the most important producers of Portuguese grapevine cultivars.

Research to improve knowledge about Grapevine Trunk Diseases (GTD) is taking place around the world. In France, there has been intense work on the evaluation of GTD with the establishment in 2003 of the "Observatoire Nationale des Maladies du Bois de la Vigne", that produces annual reports on GTD surveillance (MAAPAR, 2004). In Portugal lo-

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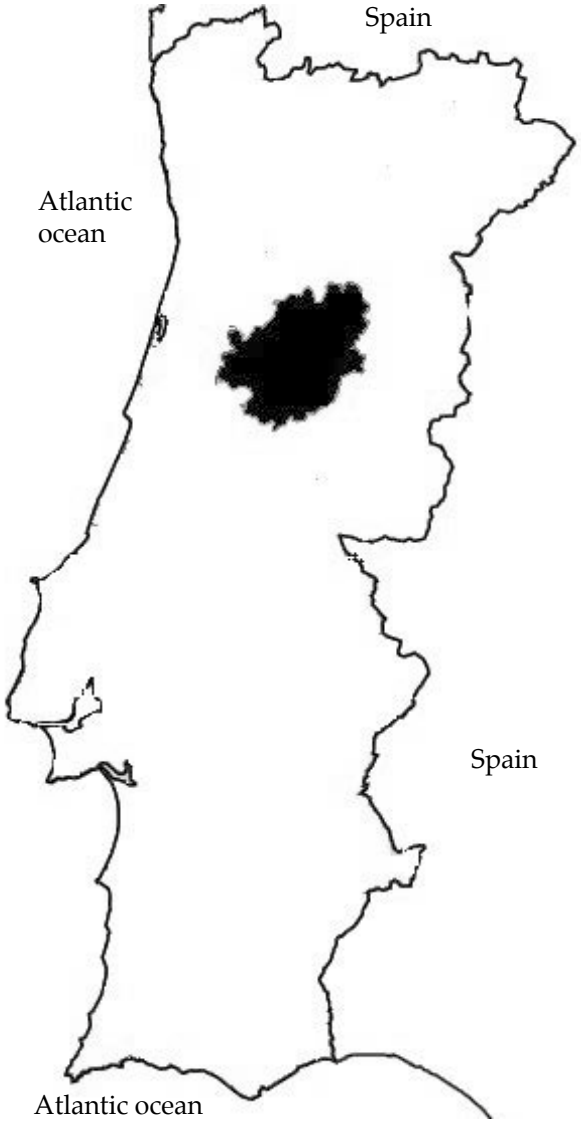


Figure 1. Map of Portugal with the Dão wine region in black.

cal growers are often incapable of understanding the causes for economic losses due to grapevine decline and mortality in their vineyards, which are often due to GTD. The observed symptoms are often confused with other diseases (e.g. viruses), occasional plagues (e.g. *Empoasca* leafhoppers), nutritional and water deficits, thereby misleading them on management solutions. The most common GTD in the Dão, leading to substantial economic losses are esca, Phomopsis cane and leafspot, black dead arm (BDA) and young grapevine declines. Typical foliar symptoms of esca - tiger stripped leaves (Mugnai *et al*., 1999) - are obvious, but foliar symptoms of BDA, disease associated with several species of Botryosphaeriaceae fungi, might resemble those of esca appearing earlier in the season (Larignon *et al*., 2009). BDA is also associated with wood necrosis, being able to infect both young and mature tissues as well as green shoots causing cankers, vascular discoloration, and/or otherwise dark streaking of the wood (Úrbez-Torres and Gubler, 2009). BDA symptoms in wood may be misattributed to *Eutypa* spp., and on herbaceous organs may be confused with Phomopsis cane and leafspot. This panoply of very similar symptoms, sometimes over the same organs, baffles the winegrower.

In previous related studies done at the Dão wine region, *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams was often isolated both from black and red-brown wood discolouration patches found inside esca affected grapevines and from field spore traps (Sofia *et al*., 2006). In the present work, the first objective was to increase the knowledge of winegrowers on GTD, while evaluating its frequency in the Dão wine region, centered on a survey among local winegrowers. The results of the survey will, in a near future, be integrated with field data to provide a more accurate picture of the Dão's GTD situation. In order to determine the intra-specific variability among *P. chlamydospora* isolates from Dão wine region, morphological, cultural and molecular characteristics have been evaluated. Finally, an experiment, using a studied isolate of *P. chlamydospora* was conducted on cv. Touriga Nacional, one of the most important of the Dão wine region, to validate a pathogenicity test procedure for grapevine infection with this fungus, which can become an useful tool to help detecting pathogenic variability within the collected isolates of *P. chlamydospora*, or to help on detection of different cultivars' susceptibility to *P. chlamydospora*.

Materials and methods

Leaflet and survey

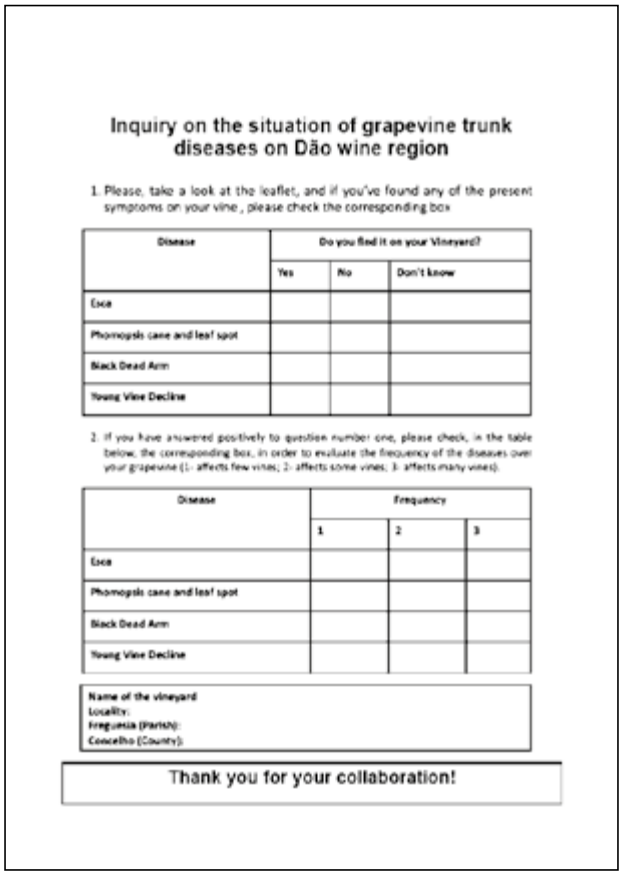
A four page color leaflet was produced with the key symptoms associated with the main GTD commonly found in the Dão wine region – esca, Phomopsis cane and leafspot, BDA and young grapevine declines - to promote the growers knowledge on GTD (Figure 2). Simultaneously, local growers were invit-

Figure 2. Leaflet produced for divulgation of the main symptoms of grapevine trunk diseases affecting the Dão wine region (English version).

ed to fulfill an easy three step questionnaire (Figure 3) where the first step was a question acknowledging the existence of any of the four GTD on their vineyards; the second step was also a question meant to evaluate the frequency of the disease(s) based on three numerical boundary categories (level 1: few vines affected; level 2: some vines affected; level 3: many vines affected) and the third step concerned the location of the vineyard within the region.

Fungal isolates

Eighteen of the vineyards identified during the survey were studied, and the frequency of GTD evaluated, in order to determine the accuracy of the answers given and also to collect some wood samples from esca and Petri disease symptomatic grapevines. From these samples, cross sections were cut from the trunk, and typical dark brown to black discolored fragments, usually associated to *P. chlamydospora* were extracted. They were surface disinfected by immersion in an 8% solution of NaOCl for 1 min., rinsed with sterile distilled water (SDW), dried with filter paper and placed in Petri dishes containing potato dextrose agar (PDA; Difco, Beckton, Dickinson and Co, Sparks, MD, USA) amended with 250 mg L^{-1} of chloramphenicol (BioChemica, AppliChem, Germany). Inoculated plates were incubated in the dark for eight days, at 25 ± 1°C. After this period, suspected colonies of *P. chlamydospora* were transferred to PDA in order to get pure cultures.



Inquiry on the situation of grapevine trunk diseases on Dão wine region

1. Please, take a look at the leaflet, and if you've found any of the present symptoms on your vine, please check the corresponding box

Disease	Do you find it on your Vineyard?		
	Yes	No	Don't know
Esca			
Phomopsis cane and leaf spot			
Black Dead Arm			
Young Vine Decline			

2. If you have answered positively to question number one, please check, in the table below, the corresponding box, in order to evaluate the frequency of the diseases over your grapevine (1- affects few vines; 2- affects some vines; 3- affects many vines).

Disease	Frequency		
	1	2	3
Esca			
Phomopsis cane and leaf spot			
Black Dead Arm			
Young Vine Decline			

Name of the vineyard
Locality:
Freguesia (Parish):
Concelho (County):

Thank you for your collaboration!

Figure 3. Questionnaire used to evaluate the situation of grapevine trunk diseases in the Dão wine region (English version).

Phenotypic characterization

A collection of twenty *P. chlamydospora* isolates obtained from different locations and different scion/rootstock combinations was used: 17 isolates were collected within the Dão wine region plus three studied and classified isolates obtained from Vidigueira, Alentejo (Ph19), from Arruda dos Vinhos, Extremadura (Ph24) and from a Dão's wine region (Ph30) (Table 1).

All isolates were grown in triplicate on PDA, at 25 ± 1°C, in the darkness for 15 days and phenotypic features (texture, colour, growing margin zonation and hyphal morphology) were described according to Crous and Gams (2000) and González and Tello (2011).

Daily growth and colony mean diameters were obtained after 25 days by measuring two perpendicular diameters for each colony and calculating mean diameters. For each isolate, six repetitions were taken. The number of conidia produced was evaluated according to the method described by Whiting *et al*. (2001).

Molecular characterization

For each isolate, DNA was extracted from mycelium grown on potato dextrose broth (PDB; Difco) using the protocol of Cenis (1992) adapted by Nascimento *et al*. (2001). To study the genetic diversity among *P. chlamydospora* isolates the inter-simple sequence repeat (ISSR) analysis was used. The ISSR primers $(AG)_8YT$ (Fang and Rose, 1997), $(CAG)_5$ (Rodriguez and Yoder, 1991), $HVH(TG)_7$ (Gilbert *et al*., 1999) and MR (5'-GAGGGTGGCG-

Table 1. *Phaeomoniella chlamydospora* isolates studied.

Isolate	Year of isolation	Geographical origin		Host scion/rootstock combination
		Location	Wine region	
Ph19[a]	2008	Vidigueira	Alentejo	Petit Verdot/400VO
Ph24[a]	2011	Arruda dos Vinhos	Estremadura	Touriga Nacional/-
Ph26	2011	Lousã	Dão	Cerceal/-
Ph27	2011	Nelas	Dão	Jaen/-
Ph28	2011	Mangualde	Dão	Jaen/-
Ph29	2012	Mangualde	Dão	Touriga Nacional/-
Ph30[a]	2012	Nelas	Dão	Jaen/S04
Ph31	2012	Nelas	Dão	Tinta Roriz/S04
Ph32	2012	Nelas	Dão	Alfrocheiro/-
Ph33	2012	Seia	Dão	Jaen/-
Ph34	2012	Tondela	Dão	Aragonês/-
Ph35	2012	Mangualde	Dão	Touriga Nacional/-
Ph36	2012	Mangualde	Dão	Encruzado/-
Ph37	2012	Gouveia	Dão	Gouveio/-
Ph38	2012	Nelas	Dão	Touriga Nacional/-
Ph39	2012	Gouveia	Dão	Jaen/-
Ph40	2012	São Martinho da Cortiça	Dão	Baga/-
Ph41	2012	Viseu	Dão	Encruzado/-
Ph42	2012	Mangualde	Dão	Jaen/-
Ph43	2012	Viseu	Dão	Jaen/-

[a] Isolates formerly identified and characterized.

GTTCT-3′) (Bridge *et al*., 1997) were used. Each PCR reaction contained 1× PCR buffer, 3 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 0.8 U of DreamTaq DNA Polymerase (MBI Fermentas, Vilnius, Lithuania), and 3 μL of diluted template DNA in a final volume of 20 μL. Amplifications were performed in a "Biometra T-Gradient", with an initial step of 4 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C $(CAG)_5$ and MR or 52°C $(AG)_8YT$ and $HVH(TG)_7$ for 45 s, and an elongation at 72°C for 2 min. A final extension was performed at 72°C for 10 min (Talhinhas *et al*., 2003). Reactions without DNA were used as negative controls, and each reaction was repeated at least once. Amplification products were separated by electrophoresis in 2% agarose gels in 0.5 × TBE buffer at 40 V for 19 h. A GeneRuler™ 100 bp Plus DNA Ladder (MBI Fermentas) was used as a molecular weight marker. Gels were stained with ethidium bromide and visualized under UV light, followed by digital image capturing using an UVIdoc system (UVItec Limited, Cambridge, UK). The banding patterns were analyzed with GelCompar II Version 5.10 software package (Applied Maths, Saint-Martens-Latem, Belgium). DNA bands detected by the software were verified by visual examination to correct unsatisfactory detection, and the presence (1) or absence (0) of bands was recorded in a binary matrix.

Genetic similarities were calculated using the Dice coefficient and dendrograms obtained by clustering according to the unweighted pair-group method using arithmetic averages (UPGMA). The robustness of the branches was assessed by bootstrap analysis with 2,000 replicates.

Pathogenicity experiment

A pathogenicity test was carried out in a vineyard of cv. Touriga Nacional, five years old, trained on bilateral cordon with six spurs. During winter, two spurs in each vine were left with three buds and were inoculated immediately after pruning by depositing a droplet of 40 μL of a 10^5 spores mL^{-1} conidial suspension of strain Ph19. Conidial suspension was obtained by plunging a 3 mm diameter micelial disk of the isolate in a 250 mL Erlenmeyer flask containing PDB and placed at 20°C, under darkness, in a reciprocal shaker (90 strokes min^{-1}), for 15 days. The inoculation site was sealed during one week with Parafilm (Parafilm® "M", Pechiney Plastic Packaging, Menasha, USA). Thirty repetitions were made. Control canes were similarly treated but SDW was used instead of inoculum.

Eight months after the inoculation, brown internal discolorations were visible along a longitudinal cut of last year inoculated spurs. Parameters evaluated were thickness (cm) and length from cut to the base (cm) of inoculated canes and length of necrosis (cm). Four pieces of wood were excised from the border of the necrosis and reisolation procedures were performed as described before. The percentage of reisolation was calculated as the proportion of wood pieces from which *P. chlamydospora* colonies were recovered, versus the total number of pieces of wood plated for each plant. Data obtained were compared using an unpaired T-test, type 2. Calculations were performed with the Statistica 6.1 software package (Statsoft Inc., Tulsa, OK, USA).

Results

Results of the GTD survey

During the 2011/2012 survey a total of 62 questionnaires were considered completely fulfilled and validated. It was clear from results that esca was the most well-known GTD of the four explained on the leaflet, with positive identification of its presence in more than 88% of the vineyards (Table 2). Merely 12% of the inquired winegrowers answered that they had never noticed the disease on their vineyards. Concerning the frequency of esca, level 1 of disease frequency was recorded in 80% of the vineyards, level 2 in 16% and level 3 only in 5% of the vineyards (Table 3).

The second most recognizable disease among the inquired was Phomopsis cane and leafspot with 82% of the fulfilled forms confirming its presence. Only 16% of the inquired winegrowers answered that they had never noticed the disease on their vineyards and 2% did not know the disease (Table 2). Regarding frequency of Phomopsis cane and leafspot, 46% of the inquired winegrowers considered it present although affecting a scarce number of vines (level 1),

Table 2. Survey on the situation of grapevine trunk diseases in Dão wine region.

Disease	Do you find it in your vineyard? (%)[a]		
	Yes	No	Don't know
Esca	88	12	0
Phomopsis cane and leafspot	82	16	2
Black dead arm	58	34	8
Young vine decline	30	60	10

[a] Results of a total of 62 questionnaires considered completely fulfilled and validated.

Table 3. Frequency of grapevine trunk diseases in Dão wine region: level 1 - affects few vines; level 2 - affects some vines; level 3 - affects many vines.

Disease	Frequency (%)[a]		
	Level 1	Level 2	Level 3
Esca	80	16	5
Phomopsis cane and leafspot	46	41	12
Black dead arm	72	24	3
Young vine decline	87	13	0

[a] For each of the diseases, frequency was determined considering only the number of questionnaires that acknowledged the existence of the disease. Figures rounded to the next integer.

41% considered it was affecting an important number of plants (level 2) and 12% considered it a serious problem (level 3) (Table 3).

The third identifiable disease for the inquired was BDA with 58% of the fulfilled forms confirming it presence; 34% of the inquired winegrowers answered that they had never noticed the disease on their vineyards and 8% did not know the disease (Table 2). Concerning the frequency of BDA, 72% of the surveyed winegrowers considered it present on their vineyards, but affecting a small number of plants (level 1), for 24% it was affecting some of plants (level 2) and only 3% considered it a severe problem for their vineyards (level 3) (Table 3).

Finally, for young vine decline, 30% of the winegrowers recognized its presence on their vineyards, while 60% never acknowledged the disease on their vineyards and 10% where not familiar with the disease (Table 2). In relation to frequency of young vine decline, 87% of the inquired considered that it was affecting a negligible number of plants (level 1) and 13% considered it present in some plants (level 2) (Table 3).

Phenotypic characterization of the obtained isolates

After 25 days of growth, *P. chlamydospora* isolates produced the characteristic colonies with a felty texture and an absent zonation. However, it was noticeable that the morphology of the colonies was found to be variable among the 20 isolates under study leading to the establishment of four morphological groups (Table 4). Group I shared colony characters such as an olive-grey color, an even growing margin and the existence of predominant filamentous somatic hyphae in PDA. Colonies of group II exhibited olive-grey to white color towards the edge, an even growing margin, producing filamentous, aerial somatic mycelium. Isolates from group III had olive-grey to white color towards the edge, an uneven growing margin and they produced filamentous, aerial somatic mycelium. Finally, group IV had an olive-grey color with the pigment concentrically distributed; an even growing margin and it produced filamentous, aerial somatic mycelium. Mycelial growth rates did not differ significantly among *P. chlamydospora* isolates, and not even within the four mentioned groups.

Phaeomoniella chlamydospora isolates produced the characteristic conidia and chlamydospora-like structures of such species. Sporulation rates of the different isolates (Table 5) showed a broad range of variation (from 2.0 to 14.6 × 10^6 conidia mL^{-1}). Daily growth rate at 25°C ranged from 0.70 to 1.40 mm and the growth diameter at 25°C, after 25 days varied from 17.30 to 34.30 mm.

Molecular characterization

The four ISSR primers tested were able to generate amplification products for all isolates of *P. chlamydospora*. A consensus dendrogram was generated from analysis of all markers (Figure 4). The isolates studied were clustered with *P. chlamydospora* isolates Ph19 and Ph24 with about 82% similarity. This confirms that all the isolates collected in Dão wine region belong to the same species. *Phaeomoniella chlamydospora* isolates were clustered into two groups supported by low bootstrap values, 48% and 54% respectively. The similarity level between groups, around 87%, indicates a low intra-specific genetic diversity. No relationship was found between ISSR band patterns and origin or scion/rootstock combination of isolates and the different groups formed.

Pathogenicity experiment

No significant differences were found among thickness and length (from cut to the base) of the control and inoculated spurs (Table 6). Although *P. chlamydospora* was not recovered from the control spurs, some necroses were noticeable on the tissues of those plants (Table 6). Significant statistic differences were found in the extension of necrosis among control and inoculated canes. It ranged from 2.47 cm in controls to 8.95 cm in inoculated canes. The reisolation percentage reached 72.4% of the canes inoculated.

Discussion

After one year of public education with the GTD leaflet, it was our perception, based on observation of cultural practices like flagging, removing and destruction of symptomatic vines, or in the number of questions on the subject, that winegrowers within the Dão wine region have improved their knowledge on GTD symptoms and general management of the diseases.

Previous works, based on a survey on grapevine trunk diseases (Tomaz *et al.*, 1989), considered esca

Table 4. Distribution of the twenty *Phaeomoniella chlamydospora* isolates among the four morphological groups according to several phenotypic characteristics for each group (after 15 days at 25ºC in PDA).

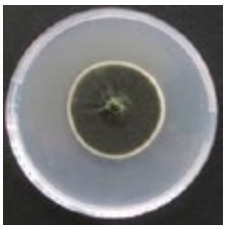
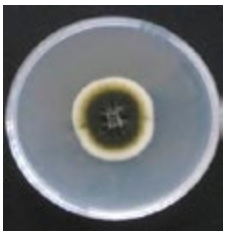
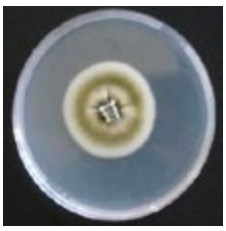
Group	Isolates	Phenotype in PDA culture	Texture	Colour	Growing margin	Zonation	Hyphal morphology
I	Ph19, Ph24, Ph26, Ph28, Ph33, Ph34, Ph37, Ph40, Ph41		Felty	Olive-grey	Even	Absent	Filamentous somatic hyphae predominant
II	Ph27, Ph32, Ph35, Ph36, Ph39		Felty	Olive-grey to white towards the edge	Even	Absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
III	Ph38, Ph42, Ph43		Felty	Olive-grey to white towards the edge	Uneven	Absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
IV	Ph29, Ph30, Ph31		Felty	Olive-grey; pigment distributed concentrically	Even	Absent	Filamentous somatic hyphae predominant

Table 5. Mean, maximum and minimum values of the phenotypic variables studied for all the *Phaeomoniella chlamydospora* isolates.

Phenotypic variable	Mean[a]	Maximum	Minimum
Sporulation ($\times 10^6$ conidia mL^{-1})	5.9	14.6	2.0
Daily growth rate (mm) at 25°C	1.2	1.4	0.7
Growth (mm) at 25°C, after 25 d	30.0	34.3	17.3

[a] Mean of two independent sets of six replicates for each isolate.

as the main GTD in Dão, having also pointed out for that region the importance of Phomopsis cane and leafspot caused by *Phomopsis viticola* (Sacc.) Sacc. Also, a new emerging disease, designated as european excoriose, caused by *Macrophoma flaccida* (Viala & Ravaz) Cavara (*Fusicoccum aesculli* Corda) was also identified in the area (Tomaz and Rego, 1990).

The survey has provided an overview of the phytosanitary status of grapevines within the Dão wine region especially concerning GTD. The occur-

Table 6. Results of pathogenicity experiment carried out in a vineyard of cv. Touriga Nacional.

Treatments	Thickness (cm)	Length from cut to the base (cm)	Length of necrosis (cm)	Reisolation (%)
Control	3.06a[a]	23.60a	2.47a	0.00a
Inoculated	3.10a	23.70a	8.95b	72.41b

[a] Mean values followed by the same letter are not statistically different at the level 5% .

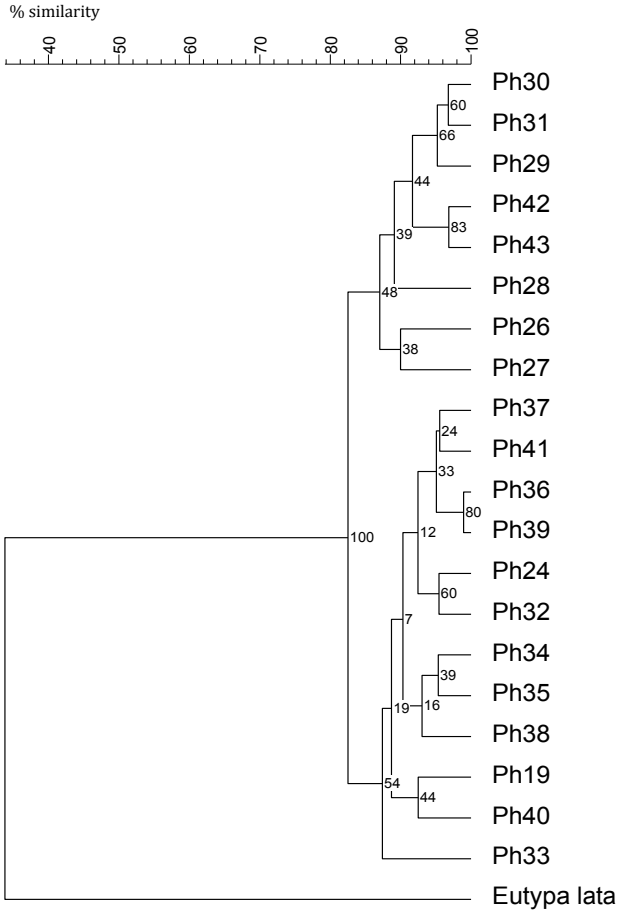


Figure 4. UPGMA cluster analysis based on Dice coefficient of ISSR fingerprints from a collection of isolates of *Phaeomoniella chlamydospora* with the primers $(AG)_8YT$, $(CAG)_5$, $HVH(TG)_7$ and MR. The numbers at the nodes represent bootstrap support values (2,000 replicates). An *Eutypa lata* isolate was used as outgroup.

rence of these fungal diseases in Dão's vineyards is confirmed, although its frequency and incidence in the vineyards is not high enough to become a matter of urgent concern. Esca and Phomopsis cane and leafspot are better known due to published research (Tomaz *et al*., 1989; Tomaz and Rego, 1990). However, BDA caused by Botryosphaeriaceous fungi is not as well known. The abandon of viticulture and the relatively few new plantations registered in this wine region recently (Falcão, 2012; IVV, 2012), has reduced the potential appearance of young plants showing young vine decline symptoms.

Worldwide, *P. chlamydospora* has been regarded as the most important fungus associated with esca and Petri disease (Ridgway *et al*., 2005; Tello *et al*., 2010) together with *Phaeoacremonium* spp. In Portugal, several studies have been focused on *P. chlamydospora* isolates (Chicau *et al*., 2000; Rego *et al*., 2000; Cruz *et al*., 2005; Santos *et al*., 2006). In the Dão wine region, this fungus is usually isolated from esca symptomatic grapevines (Sofia *et al*., 2006). Nevertheless, there is a lack of information about phenotypical and molecular variability of such species.

In our study, analysis of phenotypic characteristics showed that variation among morphological features in culture, such as texture or zonation, concerning *P. chlamydospora* isolates was low. This pattern was consistent with previous studies (Dupont *et al*., 1998; Whiting *et al*., 2005; Tello *et al*., 2010) in which homogeneity was recorded. However, features like colony colour, growing margin or hyphal morphology were found to be variable, allowing the recognition of four groups of *P. chlamydospora* isolates. Within the four recognized phenotypic groups, the variation of phenotypic characteristics was found to be independent of *P. chlamydospora* isolates geographical origin or scion/rootstock combination. The sporulation and the daily growth rate at 25°C of *P. chlamydospora* isolates were similar to the obtained by Tello *et al*. (2010).

In the ISSR primers analysis, *P. chlamydospora* isolates clustered into two groups although no bootstrap support was found for such grouping. Similar results were previously obtained by Tegli *et al*. (2000) and Mostert *et al*. (2006).

The lack of diversity established among the studied isolates might be justified by the short period of time in which the isolates where obtained and by the genetic structure of the population based in asexual reproduction in natural ecosystems (Tegli *et al*., 2000; Pottinger *et al*., 2002; Mostert *et al*., 2006). Moreover, ISSR tools did not detect a significant genetic variability which confirms that sexual reproduction does not occur. Further research based on an enlarged collection of isolates and in other molecular tools is needed to confirm the low genetic diversity within *P. chlamydospora* population in Dão region.

Concerning the results obtained in the pathogenic experiment, the use of the necrosis length in inoculated plants, as a measure of disease severity (Adalat *et al*., 2000; Halleen *et al*., 2007; Laveau *et al*., 2009; Gramaje *et al*., 2010), proved to be an accurate method to evaluate pathogen virulence. The high values obtained on the length of the necrosis formed, agrees with the conclusions of Laveau *et al*. (2009) in which *P. chlamydospora* is considered one of the most aggressive pathogens associated with esca. Taking in account that in Dão region, canes are usually pruned to one to two bud spurs on cordons, the extension of the obtained necrosis and the high frequency of reisolation of *P. chlamydospora* strengthens the idea that recently pruned canes may be a potential entrance for *P. chlamydospora* to the main structure of grapevine, in a short period of time. No esca or Petri disease typical foliar symptoms were observed in inoculated vines. This observation is in accordance with results previously reported by Halleen *et al*. (2007) and Gramaje *et al*. (2010). The inoculation method tested in this pathogenicity test proved to be a successful, simple and practical method to infect plants in field experiments. The known feasibility and simplicity of this method means it will be used in further studies of cultivars' susceptibility to all *P. chlamydospora* isolates obtained, in order to add other criteria for separation of *P. chlamydospora*'s strains. Thus, performing a pathogenicity test with the entire collection could lead to a characterization of hypovirulent or less pathogenic isolates and to a correlation of these data with phenotypic features, geographical origin or ISSR clustering.

Literature cited

Adalat K., C. Whiting, S. Rooney and W.D. Gubler, 2000. Pathogenicity of three species of *Phaeoacremonium* spp. on grapevine in California. *Phytopathologia Mediterranea* 39, 92–99.

Ambrosi H., E. Dettweiler-Münch, E.H. Rühl, J. Schmid and F. Schumann, 1997. *Guide des Cépages. 300 Cépages et leurs Vins*. Ed. Les Editions Eugen Ulmer, Paris, France, 320 pp.

Bridge P.D., D.A. Pearce, A. Rivera and M.A. Rutherford, 1997. VNTR derived oligonucleotides as PCR primers for population studies in filamentous fungi. *Letters in Applied Microbiology* 24, 426–430.

Cenis J.L., 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* 20, 23–80.

Chicau G., M. Aboim-Inglez, S. Cabral and J.P.S. Cabral, 2000. *Phaeoacremonium chlamydosporum* and *Phaeoacremonium angustius* associated with esca and grapevine decline in Vinho Verde grapevines in north-west Portugal. *Phytopathologia Mediterranea* 39, 80–86.

Crous P.W. and W. Gams 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri grapevine decline and esca. *Phytopathologia Mediterranea* 39, 112–118.

Cruz N., C. Rego, J.M. Afonso and H. Oliveira, 2005. Doenças do lenho da videira: Resultados de uma prospecção realizada na sub-região de Monção na casta "Alvarinho". In: *A Produção Integrada e a Qualidade e Segurança Alimentar – Actas do VII Encontro Nacional de Protecção Integrada-*, Ed. IPC, Coimbra, Portugal, vol I, 200–208.

Dupont J., W. Laloui and F. Roquebert, 1998. Partial ribosomal DNA sequences show an important divergence between *Phaeoacremonium* species isolated from *Vitis vinifera*. *Mycological Research* 102, 631–637.

Falcão R., 2012. *Cadernos do Vinho, Dão*. Público Comunicação Social S.A. ed., Maia, Portugal, 147 pp.

Fang D.Q. and M.L. Rose, 1997. Identification of closely related citrus cultivars with inter-simple sequence repeat markers. *Theoretical and Applied Genetics* 95, 408–417.

Faustino R. (ed.), 2011. *Instituto da Vinha e do Vinho, Catálogo das Castas para Vinho Cultivadas em Portugal***.** 1st edition, vol. I., Chaves Ferreira Publicações S.A., Lisboa, Portugal, 200 pp.

Gilbert J.E., R.V. Lewis, M.J. Wilkinson and P.D.S. Caligari, 1999. Developing an appropriate strategy to assess genetic variability in plant germplasm collections. *Theoretical and Applied Genetics* 98, 1125–1131.

González V. and M.L. Tello, 2011. Genetic variations in Spanish isolates of *Phaeomoniella chlamydospora*, the causal etiological agent of Petri disease of grapevine. *Phytopathologia Mediterranea* 50, S191–S203.

Gramaje D., J. García-Jiménez and J. Armengol, 2010. Field evaluation of grapevine rootstocks inoculated with fungi associated with Petri disease and esca. *American Journal of Enology and Viticulture* 61, 512–520.

Halleen F., L. Mostert and P.W. Crous, 2007. Pathogenicity testing of lesser-known vascular fungi of grapevines. *Australasian Plant Pathology* 36, 277–285.

IVV, Instituto da Vinha e do Vinho I.P., 2012. *Regime de Apoio à Reestruturação e Reconversão das Vinhas*. Available at

http://www.ivv.min-agricultura.pt/np4/246 (consulted 15th October 2012).

Larignon P., F. Fontaine, S. Farine, C. Clément and C. Bertsch, 2009. Esca et black dead arm: deux acteurs majeurs des maladies du bois chez la vigne. *Comptes Rendus Biologies* 332, 765–783.

Laveau C., A. Letouze, G. Louvet, S. Bastien and L. Guérin-Dubrana, 2009. Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* 48, 32–46.

Loureiro V. and A.H. Cardoso, 1993. *Colecção Enciclopédia dos Vinhos de Portugal - Os vinhos do Dão*. Chaves Ferreira Publicações S.A., Lisbon, Portugal, 160 pp.

MAAPAR, Ministère de l'agriculture, de l'Alimentation, de la pêche et des Affaires Rurales, 2004. *Observatoire National des Maladies du Bois de la Vigne – Année 2004*. Note de Service DGAL/SDQPV/N2004-8126 April, 24th 2004, Paris, France, 4 pp. Available at http://agriculture.gouv.fr/IMG/pdf/dgaln20048126z-2.pdf (consulted 11th October 2012)

Mostert L., E.C.A. Abeln, F. Halleen and P.W. Crous, 2006. Genetic diversity among isolates of *Phaeomoniella chlamydospora* on grapevines. *Australasian Plant Pathology* 35, 453–460.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.

Nascimento T., C. Rego and H. Oliveira, 2001. Detection of *Cylindrocarpon* black-foot pathogens in grapevine by nested PCR. *Phytopathologia Mediterranea* 40, S357–S361.

Pottinger B., A. Steward, M. Carpenter and H.J. Ridgway, 2002. Low genetic variation detected in New Zealand populations of *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 41, 199–211.

Rego C., H. Oliveira, A. Carvalho and A. Phillips, 2000. Involvement of *Phaeoacremonium* spp. and *Cylindrocarpon destructans* with grapevine decline in Portugal. *Phytopathologia Mediterranea* 39, 76–79.

Ridgway H.J., J.M. Steyaert, B. Pottinger, M. Carpenter, D. Nicol and A. Steward, 2005. Development of an isolate specific marker for tracking *Phaeomoniella chlamydospora* infections in grapevines. *Mycologia* 97, 1093–1101

Robinson J. 1996. *Jancis Robinson's Guide to Wine Grapes*. 1st edition, Oxford University Press, USA, 240 pp

Rodriguez R. and O. Yoder, 1991. A family of conserved repetitive DNA elements from the fungal plant pathogen *Glomerella cingulata* (*Colletotrichum lindemuthianum*). *Experimental Mycology* 15, 232–242.

Santos C., F. Fragoeiro, H. Valentim and A. Phillips, 2006. Phenotypic characterisation of *Phaeoacremoium* and *Phaeomoniella* strains isolated from grapevines: enzyme production and virulence of extra-cellular filtrate on grapevine calluses. *Scientia Horticulturae* 107, 123–130.

Sofia J., M.T. Gonçalves and H. Oliveira, 2006. Spatial distribution of esca symptomatic plants in Dão vineyards (Centre Portugal) and isolation of associated fungi. *Phytopathologia Mediterranea* 45, S87–S92.

Talhinhas P., J. Neves-Martins and J. Leitão, 2003. AFLP, ISSR and RAPD markers reveal high levels of genetic diversity among *Lupinus* spp. *Plant Breeding* 122, 507–510.

Tegli S., E. Bertelli, E. Santilli and G. Surico, 2000. Genetic variation within *Phaeoacremonium aleophilum* and *P. chlamydosporum* in Italy. *Phytopathologia Mediterranea* 39, 125–133.

Tello M.L., D. Gramaje, A. Gómez, P. Abad-Campos and J. Armengol, 2010. Analysis of phenotypic and molecular diversity of *Phaeomoniella chlamydospora* isolates in Spain. *Journal of Plant Pathology* 92, 195–203.

Tomaz I.L. and M.C. Rego, 1990. Fungos do complexo responsável pelo declínio das videiras em Portugal. *Vida Rural* 1493, 12–20.

Tomaz I.L., M.C. Rego and P.A Fernandes, 1989. A esca, principal doença do lenho na região dos vinhos do Dão. *Vida Rural* 1474, 10–16.

Úrbez-Torres J.R. and W.D. Gubler, 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cankers in California. *Plant Disease* 93, 584–592.

Whiting E.C., A. Khan and W.D. Gubler, 2001. Effect of temperature and water potential on survival and mycelial growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. *Plant Disease* 85, 195–201.

Whiting E.C., M.G. Cunha and W.D Gubler, 2005. *Phaeomoniella chlamydospora* and *Phaeoacremonium* species distinguished through cultural characters and ribosomal DNA sequence analysis. *Mycotaxon* 92, 351–360.

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