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Genetic variations in Spanish isolates of *Phaeomoniella chlamydospora*, the causal etiological agent of Petri disease of grapevine

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Summary. Petri disease is currently considered as one of the most important mycoses of grapevine wood in terms of its incidence and extent, causing young grapevine decay in numerous vine-producing areas worldwide. One of its causal agents is the ascomycete *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingfield & Mugnai) Crous & W. Gams. Recently, several studies focusing on the genetic and variability of this species have reported that its genetic variability is low. However, studies on the intraspecific characterisation of *P. chlamydospora* by other than molecular means are scarce. In this study, 57 Spanish isolates of *P. chlamydospora* were characterised by integrating data from morphological, pathogenic, cytological and ecological techniques. It was found that there was a relationship between the high polymorphism in these isolates (melanised, intermediate and albino cultures), and a number of groups, distinguished by their nuclear number, pathogenicity or survival in soil. The non-melanised phenotypes were associated with less virulent strains, generally possessing mono- or binucleate hyphae with low survival in soils, whilst melanised, multinucleate isolates were more virulent and survived up to 12 months in grapevine soils under laboratory conditions. Using these other criteria to distinguish forms of this pathogen, should make it easier to detect hypovirulent or non-pathogenic isolates and to advance our understanding of the biology of the fungus, especially its modes of dispersal or its ecological range in the field. The adoption of these criteria should also make it easier to characterise intraspecific variations in those cases where the genetic methods do not reflect them, as with *P. chlamydospora*.

Key words: pathogen, phenotypes, nuclear staining, virulence.

Introduction

Grapevine wood diseases from fungi cause heavy economic losses worldwide. In the last ten to fifteen years the mortality of nursery vines has been increased significantly. In addition, the banning of fungicides such as sodium arsenite, traditionally used against esca, has further increased the spread, incidence and damage caused by these fungal diseases.

Recently, symptoms of Petri disease in the wood of young grapevines have been seen in numerous countries including Spain (Bertelli *et al.*, 1998; Armengol *et al.*, 2001; Gatica *et al.*, 2001; Aroca *et al.*, 2006; Giménez-Jaime *et al.*, 2006). These symptoms are caused by *Phaeomoniella chlamydospora* and several species of the genus *Phaeoacremonium*. Both *Phaeoacremonium* and *Phaeomoniella* are anamorphic ascomycetes, producing asexual fruiting bodies directly from the vegetative mycelium, normally without differentiating the conidiomata (pycnidia or acervuli). Some investigators, however, have reported the existence of a *Phoma*-like synanamorph in *P. chlamydospora* (Crous and Gams, 2000), or have associated the genus *Phaeoacremonium* with its teleomorph *Togninia*, an ascomycete genus in the order Diaporthales (Mostert *et al.*, 2003).

Petri disease causes heavy losses in new plantations. Symptoms range from a progressive decline, stunted growth, wilt, and leaf symptoms such as chlorosis or marginal necrosis, to the sudden col-

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lapse of the entire vine. Internally, there is a characteristic darkening of the xylem vessels which show longitudinal grooves, and even tyloses or gum formation (Del Rio *et al.*, 2001). Young vines infected with *P. chlamydospora* set more slowly or do not develop properly. Vineyards are worst affected when rootstocks are infected before grafting; this causes serious losses.

Numerous studies on the intraspecific variability of *P. chlamydospora* have been published; these studies mostly exploited molecular methods (Borie *et al.*, 2002; Pottinger *et al.*, 2002; Alves *et al.*, 2004; Comont *et al.*, 2010; Smetham *et al.*, 2010; Tello *et al.*, 2010). At the same time, several reliable microbiological (Rooney *et al.*, 2001) and PCR-based methods have been developed to track and detect this pathogen on several growth stages and in ecological niches relating to culture, such as nurseries (Ridgway *et al.*, 2002; Whiteman *et al.*, 2003), plant tissues (Overton *et al.*, 2004; Aroca and Raposo, 2007) and soil (Whiteman *et al.*, 2002; Ridgway *et al.*, 2005). However, few studies have applied non-molecular methods to study the intraspecific variation and the population structure of *P. chlamydospora*, nor have these characteristics been related to disease susceptibility (Feliciano *et al.*, 2004; Eskalen *et al.*, 2007; Díaz *et al.*, 2009; Anonymous, 2010), comparative anatomy of plant tissue degradation (Valtaud *et al.*, 2009), cultural requirements (Whiteman *et al.*, 2001; Tello *et al.*, 2009, 2010), *in vitro* susceptibility to fungicides (Jaspers, 2001), or extracellular enzyme production (Marchi *et al.*, 2001; Santos *et al.*, 2006). These studies have generally been limited to few isolates, and so far, no studies have integrated phenotypic, cytological and ecological characteristics from a large collection of fungal strains.

The aim of this study was to characterise intraspecific variability in a collection of Spanish isolates of *P. chlamydospora* using non-molecular criteria. The integration of the data allowed variants or functional groups to be detected based on the ecological ranges and/or physiological profiles of the entire collection of isolates.

Materials and methods

Fungal isolates

A collection of 57 monosporic isolates of *P. chlamydospora* isolated from diseased young grapevines and coming from different scion/rootstock combinations were used (Table 1). Strains were isolated from several vine-producing parts of Spain, particularly in central and Mediterranean areas.

Phenotypic characterisation

All isolates were grown in triplicate on PDA (Difco, Sparks, MD, USA) at 25°C for 12 days, to evaluate and describe the macroscopic features of the colonies. Mycelial growth and production of conidia were also recorded. Mycelial growth was evaluated by culturing isolates on 90 mm diam. dishes in darkness at 5, 10, 15, 20, 25, 30 and 35ºC, with four replicates per strain and temperature combination. Radial colony growth was recorded following Tello *et al.* (2010). Production of conidia was measured following Whiting *et al.* (2001). Each strain was grown in quadruplicate on 90 mm diam. PDA dishes and incubated at 25ºC for 15 days in the dark. Two agar plugs 4 mm in diam. excised from the margins of each colony and containing mycelia and spores were placed in 1.5 mL Eppendorf tubes with 1 mL of sterile deionised water (SDW). The tubes were vortexed for 5–10 seconds and then the concentration of conidia per mL was measured with a haemocytometer.

Nuclear condition

The *P. chlamydospora* isolates were processed to determine the nuclear number per somatic hyphal cell, according to Julián *et al.* (1997). Three mm^2 agar plugs from the margins of each fungal culture were placed in flasks containing Complete Medium (CM) (malt extract 0.5 %; yeast extract 0.5 %; glucose 0.5 %) and incubated for three days at 25ºC in the dark. Each resulting mycelial colony was transferred to a microscope slide with a drop of 50 $\mu g\ mL^{-1}$ Hoechst 33342 fluorophore (Molecular Probes Europe, Leiden, Netherlands). After 15 min of incubation at 37ºC in the dark, the staining solution was removed and a drop of 50% glycerol was added as mounting medium. A coverslip was placed on the samples and the number of nuclei was determined by fluorescence microscopy using an Olympus BHS microscope supplemented with a BH2-RFC reflected light fluorescence attachment, with dichroic mirrors BH2-DMV for UV excitation and BH2-DMB for blue excitation. Fungal nuclei were counted in both hyphal tips and subapical cells (50 counts each) randomly selected for each

Table 1.- Spanish *P. chlamydospora* isolates employed in the present study.

P. chlamydospora isolate [a]	Year of isolation	Geographical origin		Host scion/ rootstock
		Town	Province	
Pch 4 (s)	2001	Valdilecha	Madrid	Malvar/41 B M
Pch 9 (r)	2001	Autol	La Rioja	Tempranillo/110 R
Pch 20 (s)	2001	Argamasilla de Alba	Ciudad Real	Cabernet Sauvignon
Pch 23 (r)	2002	Gumiel de Mercado	Burgos	n.d.
Pch 24 (r)	2002	Villafranca Caballeros	Toledo	Tempranillo/110 R
Pch 26 (r)	2002	Navarrete	La Rioja	Tempranillo
Pch 40 (r)	2002	Lardero	La Rioja	Tempranillo/110 R
Pch 61 (n.d.)	2002	Villarrobledo	Albacete	n.d.
Pch 67 (r)	2002	Iniesta	Cuenca	Tempranillo/1103 P
Pch 68 (r)	2002	Villanueva Alcardete	Toledo	Tempranillo/110 R
Pch 71 (r)	2002	El Toboso	Toledo	110 R
Pch 74 (r)	2002	Sisante	Cuenca	Tempranillo/110 R
Pch 83 (r)	2002	Casas de Haro	Cuenca	Tempranillo/110 R
Pch 85 (n.d.)	2002	El Romeral	Toledo	Tempranillo/110 R
Pch 95 (r)	2002	O Rosal	Pontevedra	196-17 C1
Pch 106 (r)	2002	O Rosal	Pontevedra	196-17 C1
Pch 108 (r)	2002	O Rosal	Pontevedra	1103 P
Pch 139 (r)	2002	O Rosal	Pontevedra	196-17 C1
Pch 180 (r)	2003	Quero	Toledo	Tempranillo/110 R
Pch 184 (r)	2004	Sinarcas	Valencia	Tempranillo/110 R
Pch 185 (s)	2003	Los Ruíces	Valencia	Bobal
Pch 186 (r)	2004	Campo de Criptana	Ciudad Real	Tempranillo/140 Ru
Pch 187 (r)	2004	Onteniente	Alicante	Monastrell/110 R
Pch 188 (r)	2004	Laujar de Andarax	Almería	Tempranillo/110 R
Pch 189 (r)	2003	Sinarcas	Valencia	Bobal/110 R
Pch 190 (r)	2004	Haro	La Rioja	Tempranillo/110 R
Pch 191 (n.d.)	2004	Villagarcía del Llano	Cuenca	Bobal/161-49 C
Pch 192 (r)	2004	Fuendejalón	Zaragoza	Cabernet
Pch 193 (r)	2004	Las Monjas	Valencia	Tempranillo
Pch 196 (r)	2004	Ronda	Málaga	Syrah/110 R
Pch 197 (r)	2004	San Clemente	Cuenca	Tempranillo/110 R
Pch 199 (r)	2004	Andarax	Almería	Tempranillo/110 R
Pch 200 (r)	2003	Alcazar de San Juan	Ciudad Real	Syrah/SO4
Pch 201 (r)	2003	Montijo	Badajoz	Tempranillo
Pch 204 (r)	2004	Andarax	Almería	Tempranillo/110 R
Pch 205 (r)	2004	Mollina	Málaga	Pedro Ximenes/110 R
Pch 207 (r)	2004	Alicante	Alicante	Red Globe/140 Ru
Pch 208 (r)	2004	Turís	Valencia	Monastrell/161-49 C
Pch 211 (n.d.)	2004	Dos Barrios	Toledo	Tempranillo/41 B M
Pch 213 (r)	2004	Herencia	Ciudad Real	Tempranillo/1103 P
Pch 214 (r)	2004	Tarazona de la Mancha	Albacete	Tempranillo/110 R
Pch 217 (r)	2004	Pinoso	Alicante	Monastrell/110 R
Pch 218 (r)	2004	Los Ruíces	Valencia	Garnacha Tintorera
Pch 220 (r)	2004	Ronda	Málaga	Syrah/110 R
Pch 221 (r)	2004	Pinoso	Alicante	Airén/161-49 C
Pch 223 (r)	2004	Villarrubia de los Ojos	Ciudad Real	Tempranillo/110 R
Pch 224 (r)	2004	Ciudad Real	Ciudad Real	Tempranillo/110 R
Pch 226 (r)	2004	Cretas	Teruel	Garnacha/SO4
Pch 227 (r)	2004	Cózar	Ciudad Real	Tempranillo/41 B M
Pch 228 (n.d.)	2005	Hontayana	Cuenca	Tempranillo/1103 P
Pch 230 (n.d.)	2005	Las Monjas	Valencia	Tempranillo
Pch 243 (r)	2005	n.d.	Asturias	n.d.
Pch 221.0 (s)	2001	Valdilecha	Madrid	Tempranillo/110 R
Pch 9.43 (s)	2005	San Martín de Valdeiglesias	Madrid	Garnacha/110 R
Pch 5.04 (s)	2005	San Martín de Valdeiglesias	Madrid	Garnacha/110 R
Pch 9.27 (s)	2005	San Martín de Valdeiglesias	Madrid	Garnacha/110 R
Pch 259 (s)	2005	n.d.	Valladolid	n.d.

[a] The code in brackets indicates from which plant part the isolates were obtained: (r) rootstock; (s) scion; (n.d.) not determined.
[b] n.d., not determined

slide, and all isolate counts were repeated twice.

Pathogenicity

A bioassay was carried out to determine the pathogenic potential of five selected strains each representing one of the five main morphotypes recognised. Semi-lignified twigs of 6-year-old 'Tempranillo' grapevines growing in 15 L pots were mechanically inoculated. Agar plugs (5 mm diam.) from the margins of 15-day-old cultures of the five *P. chlamydospora* strains were inserted in a previous lesion made with a sterile punch at sufficient depth (0.4 cm) to reach the medullar zone (one plug per shoot, three shoots per vine, 10 vines per strain), covered with wet cotton and sealed. Vines were maintained in a greenhouse for 2 months at 20±2ºC. Then, the inoculated shoots were removed and 10-cm fragments were longitudinally sectioned to examine the length of the necroses produced. The length of the external necrosis around infection points, the length of the pith necrosis, the length of the inner vascular necrosis towards the apex (upper vascular necrosis) and the length of the inner vascular necrosis towards the base of the shoot (lower vascular necrosis) were recorded for each vine fragment. Ten vines inoculated with sterile agar plugs were used as controls.

Survival in soil

The survival of the five selected *P. chlamydospora* strains representing the main phenotypic groups was tested in the laboratory on three vineyard soils. Before inoculation, the soil samples were air-dried, sieved to remove coarse fragments (>2 mm diam.), autoclaved three times on successive days (120ºC, 20 min, 1 atm), and then 20 g of each soil was dispensed to 90 mm diam. Petri dishes, 18 dishes per soil sample. Five isolates (Pch 9, Pch 9.27, Pch 221.0, Pch 223 and Pch 9.43) were selected based on their possessing of certain stable features in culture, mainly features related to melanised and non-melanised patterns. Conidia were collected from 4-week-old colonies grown on PDA and suspended in SDW. Inoculum concentration was adjusted with a haemocytometer to 10^2 and 10^6 conidia per mL. Equal amounts of the adjusted suspensions from the five isolates were mixed and 1 mL of the final suspension was added to each soil dish (50 µL per gram of dry soil). Three replicates for each soil were incubated in the dark at one of three temperatures, 10, 25 and 30ºC, at three soil moisture contents, 10, 25 and 100% of field capacity respectively. SDW was regularly added as needed to maintain the desired soil moisture, and differences in the survival rates, dominance and relative abundance of the isolates were recorded. To determine recovery and survival rates, 0.2 g of soil per dish was removed at monthly intervals over 12 months. Each subsample was suspended in 400 µL of SDW, vortexed for 60s, diluted to 1/100 and then spread (20 µL per dish) on Petri dishes containing the semi-selective medium F1S0.5, a modification of the F10S medium described by Tello *et al*. (2009). In the modified medium the concentration of folpet (Folpan, Aragro, Madrid, Spain) was reduced to 1 mg L^{-1} and that of streptomycin sulphate (Duchefa Biochemie, Haarlem, Netherlands) to 0.5 g L^{-1}. The dishes were incubated at 25ºC in the dark, and the number of colonies of the five strains inoculated was screened and counted after 21 days of inoculation. The persistence and abundance of the five *P. chlamydospora* strains inoculated were estimated by the number of CFUs recovered per month and per treatment.

Data analysis

The variables colony-forming-units (CFUs, number of colonies grown per dish) and isolation frequency (number of dishes with at least one *P. chlamydospora* colony) were recorded monthly over 12 months. Data of vascular necroses obtained in the pathogenicity assays were subjected to analysis of variance (ANOVA) using SPSS 15.0 software (IBM Corporation, NJ, USA), with one factor, isolate type, and the four necroses, external, pith, upper vascular and lower vascular as dependent variables. The mean necrosis lengths were compared using the least significant difference (LSD) test at P=0.05.

Results

Phenotypic characterisation

Among the 57 Spanish *P. chlamydospora* isolates, five phenotypic variants or morphotypes were recognised by their macro- and micromorphological characteristics features in culture (Figure 1). These characteristics remained stable and reproducible during the three years of study and

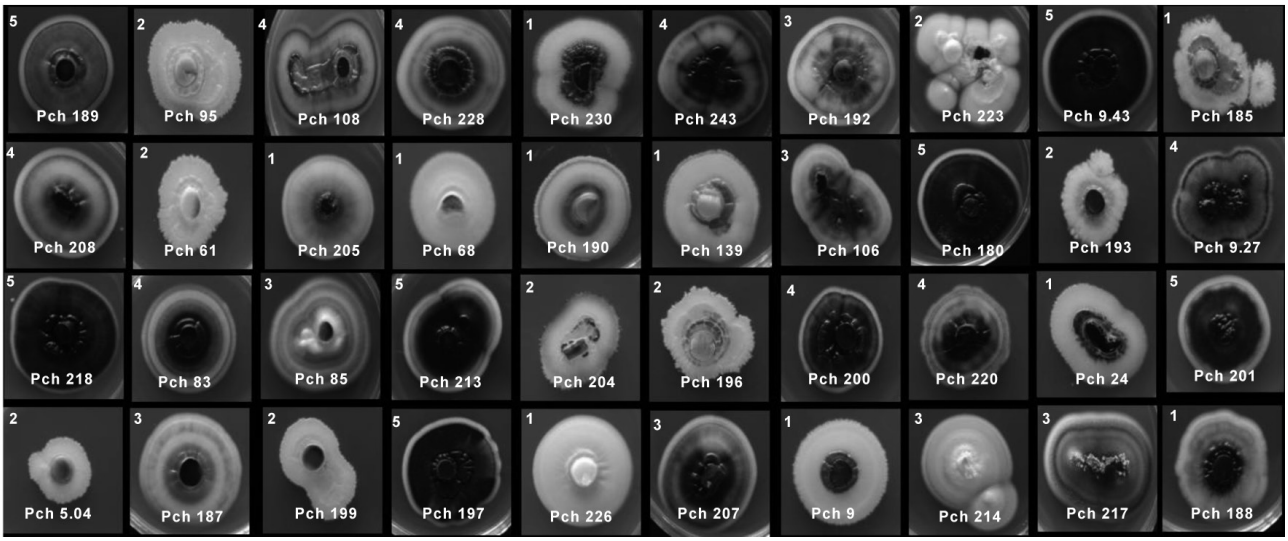


Figure 1. Macromorphological aspect in PDA of some of the *P. chlamydospora* isolates studied, showing the existence of different phenotypes. 1, albino type 1 (Pch9-like); 2, albino type 2 (Pch 223-like); 3, intermediate type (Pch 221.0-like); 4, melanised type 1 (Pch 9.27-like) and 5, melanised type 2 (Pch 9.43-like).

they comprised two albino, one intermediate and two melanised phenotypes (Figure 2). The relevant and diagnostic features of these morphotypes are also listed in Table 2. The albino variants (Pch 9 and Pch 223) shared morphological characters such as a great number of chlamydospore-like structures, they lacked a *Phoma*-like synanamorph and they had a predominance of yeast-like, rounded somatic hyphae in PDA. The intermediate forms (Pch 221.0) produced filamentous, aerial somatic hyphae, exhibited an intermediate melanisation pattern, with pigment distributed mostly in radial sectors along the entire colony, and lacked a *Phoma*-like synanamorph in PDA. The melanised forms (Pch 9.27 and Pch 9.43) had green to black-green pigments distributed evenly throughout the colony, with few presence of chlamydospores. They produced *Phoma*-like structures, and did not have any yeast-like cells in culture (Figure 2). All these diagnostic features were stable at all the temperatures assayed, regardless of the number of culture transfers performed, and even with cultures from long-term storage in stock collections. Mycelial growth did not differ significantly between *P. chlamydospora* strains, and not even between the five representative isolates.

Nuclear condition

There was a relation between the morphotypes defined and the number of nuclei in their somatic hyphae (Figure 3). The less-melanised or albino forms of *P. chlamydospora* tended to have mono- or binucleate somatic hyphae. Most of the intermediate forms constantly had two nuclei per somatic hyphal cell. Melanised strains of the fungus mostly possessed multinucleate somatic cells, ranging from 3 to 6 nuclei per cell, although some of the multinucleate hyphae had 2-nucleate cells.

Pathogenicity

The melanised strains tended to be more virulent than the albino strains. The melanised isolate Pch 9.43 caused the largest necroses in the pith and in the vascular and external tissues. The albino strains produced less severe and shorter lesions (Figure 4). Albino isolate Pch 223 produced the lowest rates for all types of necroses and its behaviour in terms of mean values was differed significantly from the other morphotypes in mean lesion values, except for lesions in the the pith. Isolate Pch 9 constitutes an exception (Figure 4) because, although being not melanised, it developed vascular necroses similar to those of the melanised

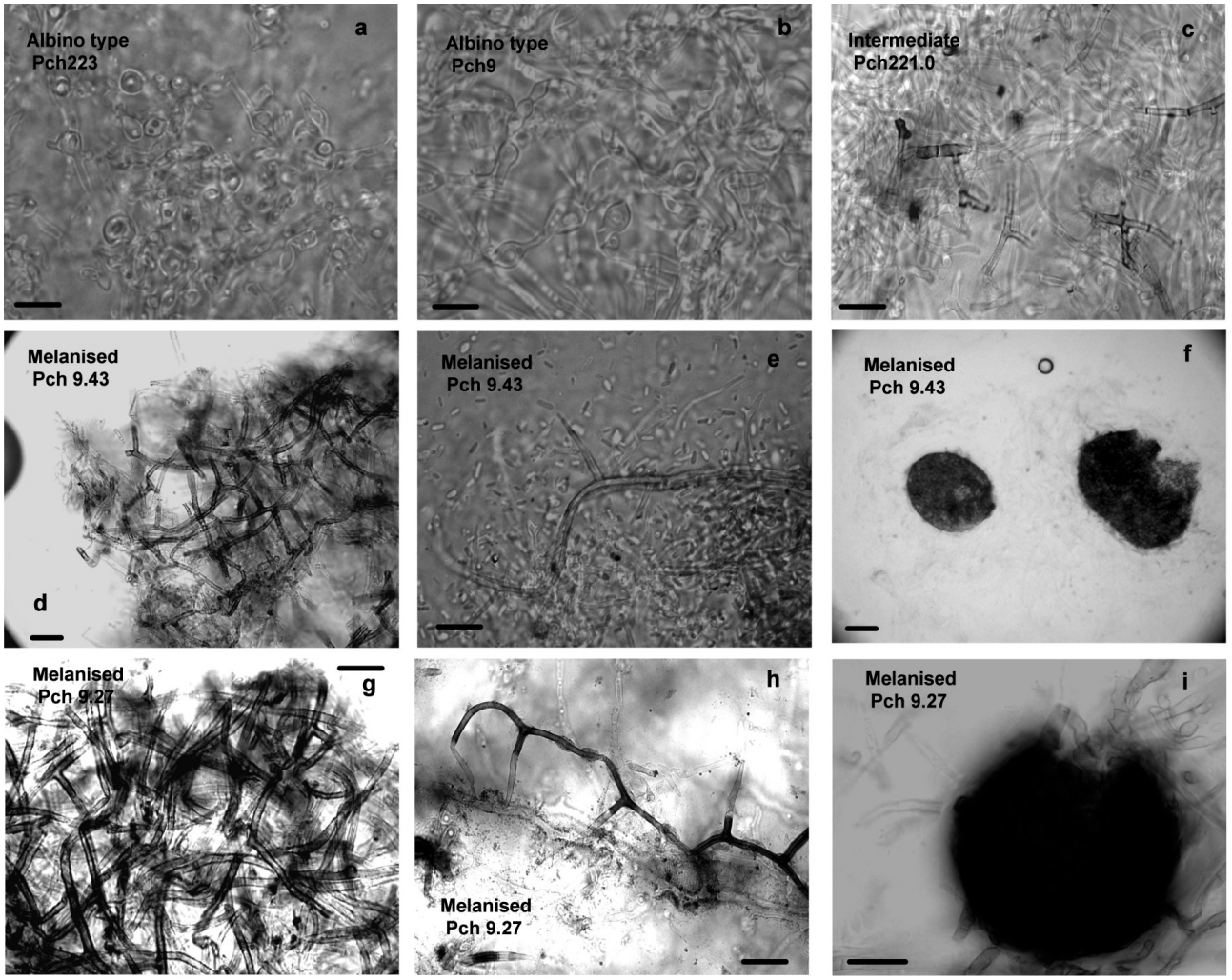


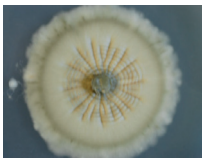
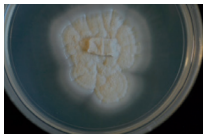
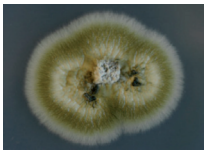
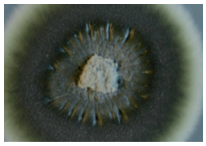
Figure 2. Micromorphological features of the several *P. chlamydospora* morphotypes recognized in the present study. a, Albino type (Pch 223) yeast-like somatic hyphae; b, albino type (Pch 9) yeast-like somatic hyphae; c, intermediate type (Pch 221.0) filamentous hyphae; d, e, f, melanised type (Pch 9.43) filamentous somatic hyphae, conidia production and *Phoma*-like synanamorph respectively; g, h, i, melanised type (Pch 9.27) filamentous somatic hyphae, conidiomata and *Phoma*-like synanamorph respectively. Bars = 10 μm.

isolates (Pch 9.27 and Pch 9.43). The intermediate morphotype Pch221.0 also behaved erratically in that it caused lesions smaller than those produced by the melanised isolates (Figure 4).

Survival in the soil

Phaeomoniella chlamydospora isolates survived in mechanically infected vineyard soils for up to 36 months, and the type of isolate represented a relevant variable (Figure 5). Recovery rates, expressed as the percentage of each strain re-isolated (Figure 6), differed with the phenotype. Some of the strains were dominant and were constantly recovered throughout the experiment, while others were scarcely detected after a few months or even disappeared altogether. The melanised strains (Pch 9.43 and Pch 9.27) were recovered in higher numbers than the albino strains (Pch 223 and Pch 9) or the intermediate (Pch 221.0) strains. More CFUs were recovered monthly from the melanised types (Pch 9.43), than from Pch 223 or Pch 9 (Figure 5).

Table 2. Strain name and diagnostic macro- and morphological features of representative strains of the five morphotypes recognised among a collection of 57 Spanish isolates of *Phaeomoniella chlamydospora*.

Isolate	Phenotype in PDA culture (after 12 days at 25ºC in PDA)	Diagnostic morphological features				
		Pigment	Chlamydo-spores	*Phoma*-like synanamorph	Hyphal morphology	Conidial production
(Albino) Pch 9		Weak; pigment restricted to the margin of the colonies	Numerous	Absent	Filamentous and yeast-like somatic hyphae in similar proportions	Low rates of conidial production
(Albino) Pch 223		Absent	Numerous	Absent	Yeast-like somatic hyphae predominant, aerial mycelium scanty	Low rates of conidial production
(Intermediate) Pch 221.0		Intermediate; pigment distributed in radial sectors	Scanty	Absent	Filamentous somatic hyphae predominant, aerial mycelium abundant	High rates of conidial production
(Melanised) Pch 9.27		Melanised; pigment distributed concentrically	Scanty	Present	Filamentous somatic hyphae predominant, aerial mycelium scanty	High rates of conidial production
(Melanised) Pch 9.43		Strong; pigment distributed evenly, lighter towards the outers	Scanty	Present	Filamentous somatic hyphae predominant	High rates of conidial production

The frequency of CFU isolation fluctuated approximately every 3–4 months, regardless of initial inoculum concentration. Cycles of sporulation were followed by cycles of mycelial development, and this alternation was also found when the five strains were examined individually. The alternation in numbers seemed to indicate that the strain was reproducing in autumn after having declined in summer.

The survival assays suggest that melanised, multinucleate isolates of *P. chlamydospora* survive better in the soil than albino or intermediate forms, those last had lower recovery rates, or even disappeared after a few months of sampling.

Discussion

This study deals with the feasibility of integrating some data other than molecular ones to recognise and characterise intraspecific variations in *P. chlamydospora*. A relationship was found between the high phenotypic polymorphism observed mostly under artificial conditions, in a collection of *P. chlamydospora* isolates (including melanised, intermediate and albino forms) and characteristics such as nuclear condition, virulence, or survival in the soil.

Studies on the population structure of this pathogen have so far been restricted to molecular methods and, to a lesser extent, on non-mo-

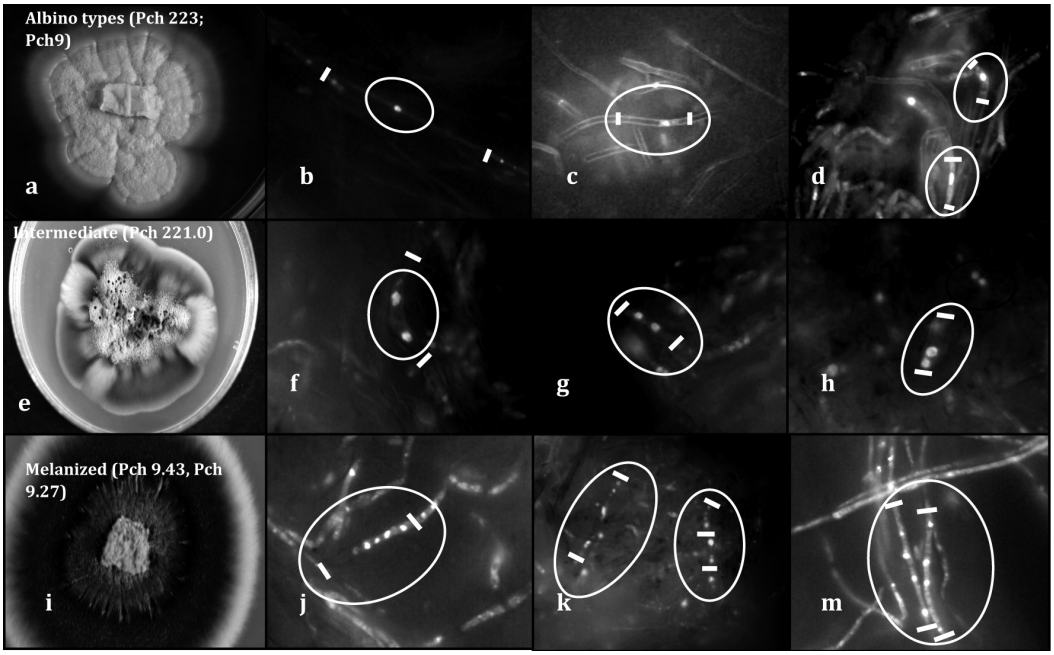


Figure 3. Nuclear condition of the strains representing the five morphotypes recognized among a collection of 57 Spanish isolates of *P. chlamydospora*; a-d, albino types; e–h, intermediate forms; i-m, melanised types. Septa of hyphal cell walls have been highlighted graphically.

lecular features. Studies on the genetic structure of *P. chlamydospora* populations have found different degrees of genetic variation depending on the molecular approach employed. When random amplified polymorphic DNA (RAPD) markers were used (Tegli *et al.*, 2000; Borie *et al.*, 2002, Tello *et al.*, 2010), genetic diversity was low. With AFLP results on the other hand, results differed depending on the wine-producing areas considered. Thus, Pottinger *et al.* (2002) and Mostert *et al.* (2006) reported low genetic variation in their studies on fungal populations isolated in more recently cultivated areas, such as New Zealand and South Africa, whereas studies by Comont *et al.* (2010) and Smetham *et al.* (2010) on fungal populations in France, which has a long tradition of vine cultivation, reported a certain amount of genotypic diversity. Smetham *et al.* (2010) compared the genetic structure of a population from France with a population from southeastern Australia, and concluded that the French population was genotypically more diverse than the more homogeneous Australian population. In Spain, Tello *et al.* (2010) also studied the phenotypic and molecular diversity of a collection of *P. chlamydospora* isolates. As with the French population in Comont *et al.* (2010), Tello *et al.* (2010) differentiated several groups by RAPD analysis, and found some genetic diversity in these Spanish populations. In summary, molecular studies on the population structure of the pathogen indicate that genetic diversity, was low in general terms, but was higher in areas with a longer tradition of vine cultivation (Europe) than in areas where vine cultivation started later (Australia, New Zealand), and where the rather recent introduction of the pathogen causing a kind of genetic bottleneck phenomenon, may have reduced the genetic variation in pathogen populations (Smetham *et al.*, 2010). As no teleomorph has ever been described for *P. chlamydospora*, further studies will have to focus on possible alternative forms of recombination and parasexual genetic exchange in order to explain the genetic diversity observed in this species, and the differences in pathogenic behaviour detected in the study.

The findings reported by Tello *et al.* (2010) that a collection of Spanish *P. chlamydospora* isolates exhibited little genetic variation are the basis of the present study, which attempted to integrate

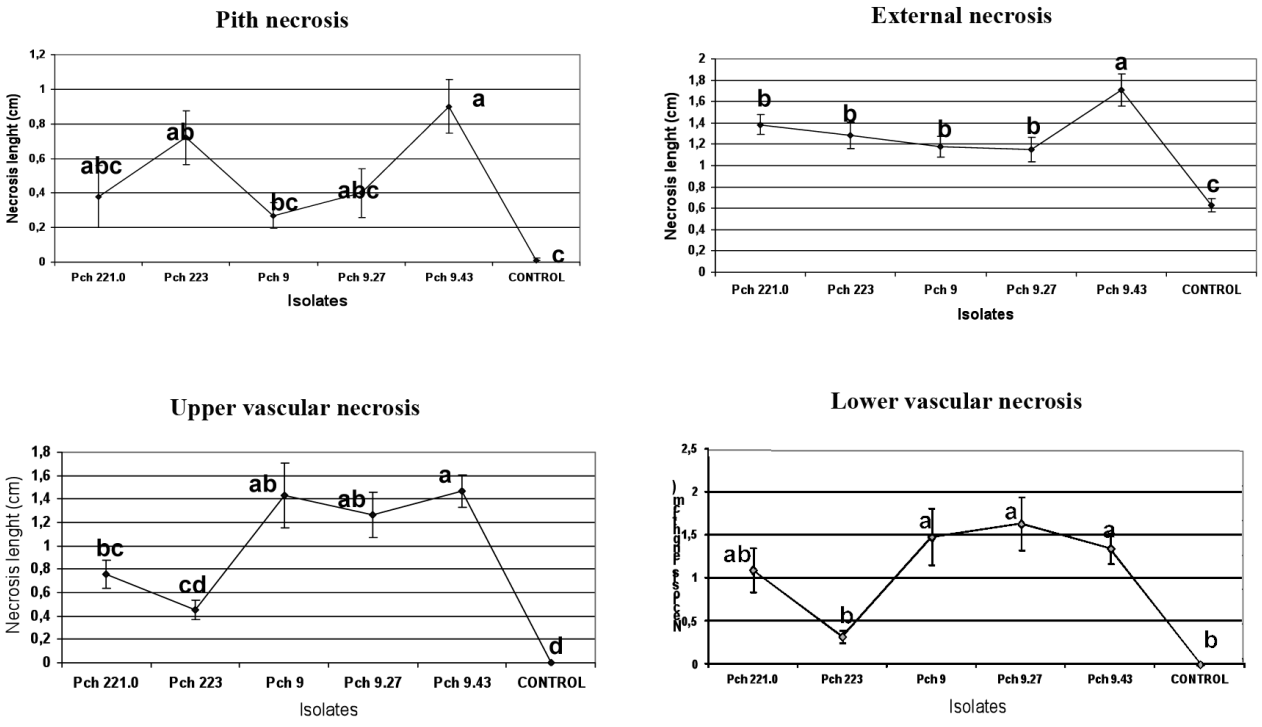


Figure 4. Necrosis length produced by the *P. chlamydospora* isolates selected as representatives of the main morphotypes recognized after artificial inoculation of grapevine shoots. Length values are expressed as means of 30 data. Bars represent standard deviations. Different letters refer to LSD test significance ($P<0.05$).

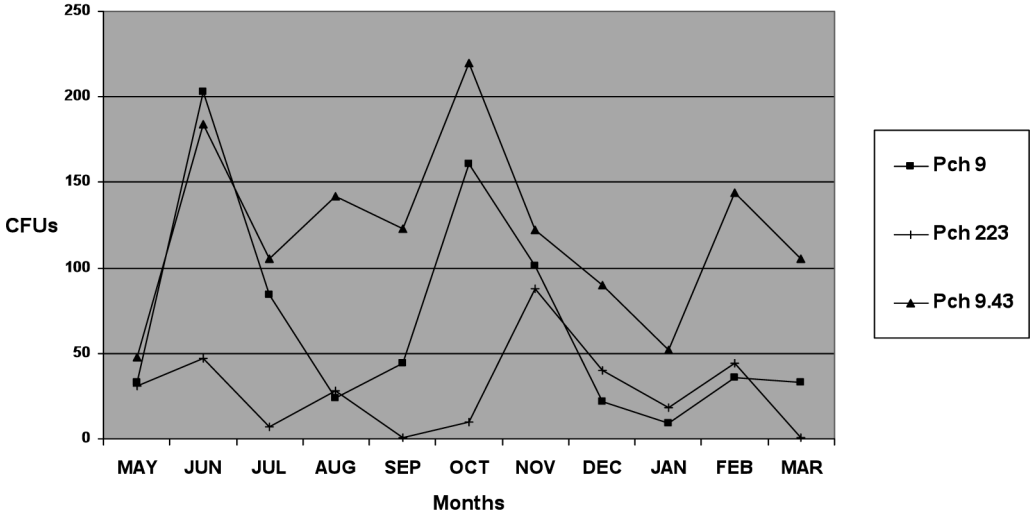


Figure 5. Evolution of recovery rates of three representative melanised (Pch 9.43) and albino (Pch 9 and Pch 223) isolates of *P. chlamydospora* artificially inoculated on grapevine soils. Recovery rates are expressed as total number of colony forming units (CFU) per month.

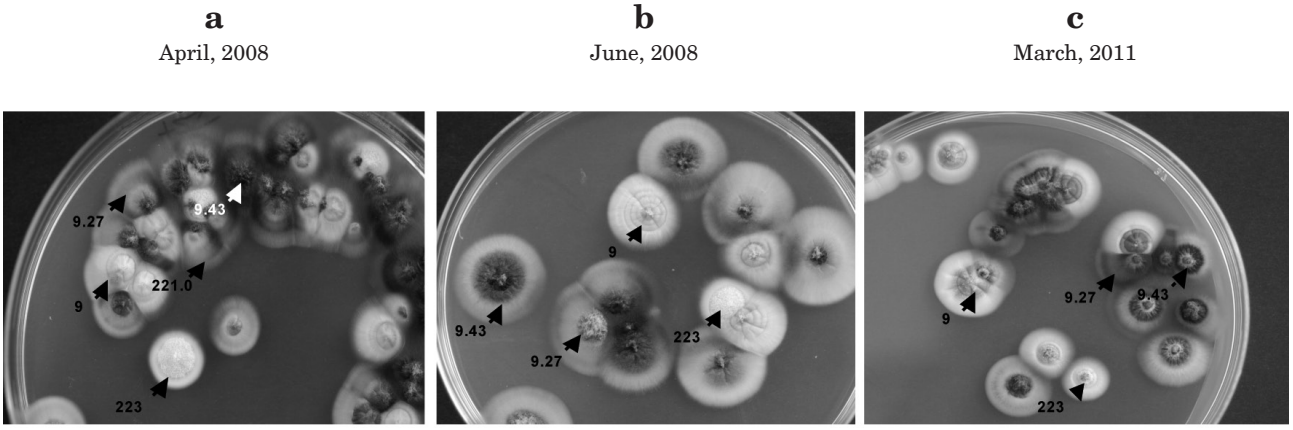


Figure 6. Recovery of *P. chlamydospora* in PDA dishes at several intervals after artificial inoculation on vineyard soils. Different types of colonies correspond with some of the five morphotypes inoculated (Pch 9, 223, 221.0, 9.27 and 9.43). a, 1 month after inoculation; b, 3 months after inoculation, and c, 36 months after inoculation.

other non-molecular criteria in order to better characterise these strains.

The low intraspecific variations related with culture parameters such as optimal growing temperature are consistent with previous studies (Dupont *et al.*, 1998; Whiteman *et al.*, 2001; Whiting *et al.*, 2005; Tello *et al.*, 2010), in which no significant differences were recorded. However, the 57 *P. chlamydospora* isolates were grouped in five morphotypes according to the macromorphological appearance they assumed in axenic culture. These phenotypic variants were constantly seen during at least three years of the experiment, and were established on the basis of several constant features such as melanisation pattern, the morphology of somatic hyphae, the nuclear number, the production of synanamorph in culture, and the presence or absence of chlamydospore-like structures. These morphotypes could not be distinguished with the molecular methods used for the same material by Tello *et al.* (2010). There are numerous other examples of well-known pathogenic fungal taxa having phenotypic variants, which usually have a different pathogenic behaviour like *Helgardia* (*Pseudocercosporella*) (Julian *et al.*, 1994), *Puccinia* (Mantovani *et al.*, 2010), *Rhynchosporium* (McDermott *et al.*, 1989), *Fusarium* (Jiménez-Gasco *et al.*, 2004), *Colletotrichum* (Sreenivasaprasad and Talhinhas, 2005) and *Botrytis* (Martínez *et al.*, 2009). This was also the case of the *Phaeomoniella* isolates in our study. In contrast, Santos *et al.* (2006) found some *P. chlamydospora* isolates with a different pattern of extracellular enzyme production, but the virulence of these isolates was unchanged.

The number of nuclei was not homogeneous across the collection. Mono-, bi- and multinucleate forms were found and they were related to other phenotypic features. A multinucleate form was mostly associated with the strongly pigmented isolates, while the albino and intermediate forms had one or two nuclei in their somatic hyphae. In fungal pathogens like the *Rhizoctonia* complex, a loss of melanin synthesis was also correlated with hypovirulence (González *et al.*, 2006). Several authors (Sneh *et al.*, 1985; Villajuan-Abgona *et al.* 1993) have pointed out that hyphae from hypovirulent strains of *Ceratobasidium* (a genus close to *Rhizoctonia*) are usually hyaline, whereas multinucleate hyphae from pathogenic isolates of *Rhizoctonia s. str.* usually have brown or grey pigments. Melanin is essential for pathogenicity in several phytopathogenic fungi such as *Pyricularia* or *Colletotrichum* (Bell and Wheeler, 1986; Suzuki *et al.*, 2001); it is involved in appressorium formation and in the maintenance of physical rigidity (Mendgen *et al.*, 1996). In other well-known pathosystems such as *Rhizoctonia solani* AG1/*Oryza sativa*, differences in mycelial melanisation were related to differences in pathogenic behaviour (Kim *et al.*, 2001). Our

study also showed a relationship between the type and pattern of pigmentation and the virulence of the isolates. Thus hypovirulent albino variants not easily detected by molecular techniques could well exist in the populations of *P. chlamydospora*.

Taking into account the low genetic diversity that several molecular methods detected in populations of *P. chlamydospora* and the fact that no sexual mechanism providing genetic variability has been identified, the study makes clear that other, non-molecular criteria are sometimes needed to describe the population structure of a large set of isolates in which it is desired to differentiate hypovirulent non-melanised strains from virulent, melanised strains. Further research on the role of natural soils in the spread and life cycle of this pathogen and on the molecular determinants of hypovirulence in *P. chlamydospora* is needed, and this should include the possible existence of transmissible extrachromosomal elements or the reversibility of such elements.

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