Phytopathologia Mediterranea (2014) 53, 1, 194–201
DOI: 10.14601/Phytopathol_Mediterr-11860

SHORT NOTES

Development of a monoclonal antibody TAS-ELISA assay for detection of *Phaeomoniella chlamydospora*

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Summary. *Phaeomoniella chlamydospora* is an important tracheomycotic fungus involved in different syndromes of the esca disease complex affecting young and mature grapevine plants. Although grapevine planting material is frequently infected by this pathogen, no regular screening to detect the fungus is carried out, mainly due to the lack of fast, sensitive and affordable methods for large-scale application. A monoclonal antibody-based triple antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA) was developed to specifically detect *Pa. chlamydospora* from grapevine wood tissues. This assay could be especially useful for large-scale application in nurseries, to ensure *Pa. chlamydospora*-free grapevine stocks, and thus contributing to the production of healthy propagation material.

Key words: grapevine wood streaking, Petri disease, leaf stripe disease, esca proper, *Vitis vinifera*.

Introduction

Phaeomoniella (*Pa.*) *chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams (Crous and Gams, 2000) is a tracheomycotic pathogen associated with different syndromes in the grapevine esca disease complex, including brown wood streaking (mainly affecting rooted cuttings), Petri disease and leaf stripe disease. In mature grapevines, the pathogen could be also associated with esca proper, a syndrome characterised by a combination of leaf stripe symptoms and white rot of vinewood. White rot is, however, attributed to *Fomitiporia mediterranea* and other basidiomycete species (Surico, 2009).

The presence of *Pa. chlamydospora* and other grapevine wood pathogens in planting material has been recognised as a major cause of failure of newly established vineyards (Gramaje and Armengol, 2011). These young plantations show poor growth and decline, and take increased time to provide satisfactory fruit yield or quality. As a result, sizeable areas of these affected vineyards have frequently to be uprooted, which represents severe losses for grape growers (Oliveira *et al*., 2004).

Infections incited by *Pa. chlamydospora* often begin in young shoots on mother vines and progress as the plants mature (Surico *et al*., 2006). Canes taken from these mother vines (scion or rootstock) are frequently asymptomatic, but they can carry the pathogen either as endophytes (Bertelli *et al*., 1998; Whiting *et al*., 2001) or as epiphytes, if airborne spores have landed on the wood surfaces. In addition, several reports have indicated that even healthy plant material may become contaminated during the practices carried out during grapevine propagation processes (Whiteman *et al*., 2003; Retief *et al*., 2006; Aroca *et al*., 2010; Gramaje and Armengol, 2011).

Although grapevine planting material is frequently infected by *Pa. chlamydospora*, no regular screening against this fungus is done, mainly due to the lack of fast, accurate and inexpensive detection methods for large-scale application.

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ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

Phaeomoniella chlamydospora and other tracheomycotic fungi (mainly *Phaeoacremonium* spp.) are slow-growing fungi in culture media, and their detection and identification using molecular techniques have been intensively developed (Tegli *et al.*, 2000; Pollastro *et al.*, 2001; Ridgway *et al.*, 2002; Retief *et al.*, 2006; Martín *et al.*, 2012). Regardless of the the sensitivity, accuracy and rapidity provided by these techniques, as compared to isolating techniques, they are still expensive, time-consuming and restricted to research laboratories.

Immunology based methods are extensively used to detect grapevine viruses; however they are of limited use for detecting fungal pathogens. Although less sensitive than molecular methods, they are better suited for the large-scale use, because they can be operated by relatively unskilled personnel. Recently, polyclonal antibodies were produced against exopolysaccharides (Andolfi *et al.*, 2009) and polypeptides (Fleurat-Lessard *et al.*, 2010) excreted by *Pa. chlamydospora*. Our approach, however, aimed to obtain monoclonal antibodies (mAb) against the pathogen, because they are more specific, they allow more reproducible results between experiments, and once hybridomas are produced, a constant and renewable source of mAb is available.

In the present study, we developed a monoclonal antibody-based triple antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA) able to detect specifically *Pa. chlamydospora* from grapevine wood, with potential to be used in large-scale pathogen-free certification programmes.

Materials and methods

One isolate of *Phaeomoniella chlamydospora* (Ph19) was employed for rabbit and mouse immunizations. Additional *Pa. chlamydospora* isolates were used for reactivity studies, whereas fungi commonly associated with grapevine wood were selected for cross reactivity tests (Table 1). Unless otherwise stated, all isolates used belong to the Collection of the "Laboratório de Patologia Vegetal Veríssimo de Almeida-LPVVA", Instituto Superior de Agronomia, Lisbon, Portugal, and were maintained on potato dextrose agar slants (PDA, Difco) at 5°C. All fungi were routinely incubated in the dark at 25°C.

To prepare the immunogen, isolate Ph19 was grown in potato dextrose broth (PDB, Difco) during 3 weeks. The mycelium was harvested by filtration using glass wool and cheesecloth, and washed three times with 200 mL of phosphate buffered saline (PBS), pH 7.4. Freeze-dried mycelium was stored at -80°C until further use. To produce conidia, isolate Ph19 was grown in PDA, during 14 d, and spores were harvested into 3 mL of PBS. The spore suspension was filtered through glass wool and centrifuged at 14,000 *g* for 2 min. The supernatant was discarded and the pellet washed twice by resuspending in PBS and centrifuging as above. The spore concentration was adjusted to 1×10^8 conidia mL^{-1} and dispensed in 1 mL aliquots in PBS with 20% glycerol and stored at -80°C.

Plant material consisted of grapevine grafted plants, Aragonez/1103P, which were artificially inoculated with *Pa. chlamydospora* (isolate Ph19) and maintained as described by Martin *et al.* (2009). The point of inoculation was located between the second and the third node of each cane. The controls were non-inoculated grapevine plants grown in the same conditions. After 6 months of incubation, canes from inoculated and control grapevine plants were collected, and wood fragments (25–50 mg) were taken from each cane above the inoculation point. All wood samples were stored at -20°C until further use.

Grapevine plants (Tinta Roriz/1103P, Alicante Bouschet/SO4) collected from one young vineyard (3-y-old) showing severe decline symptoms, and mature grapevine plants (Cabernet Sauvignon/110R) collected from one vineyard where esca proper symptoms have been recorded, were also included in the assays. Each plant was cut transversely in two different regions and examined for internal symptoms (Table 2). Wood fragments were collected from each section and stored as described above.

To obtain polyclonal antibodies, rabbits were immunized four times, at 1 month intervals, with a mixture of approximately 1×10^6 conidia mL^{-1} and mycelium (1 mg mL^{-1}) in incomplete Freund's adjuvant and monophosphoryl lipid A plus trehalose dicorynomycolate adjuvant (MPL®+TDM adjuvant, Sigma). The fourth immunization was performed without adjuvants and 3 d before the removal of blood. Before injection, the conidia stock (1×10^8 conidia mL^{-1}) was re-suspended and diluted 1:100 by volume in PBS and the dried mycelium was homogenized three times in PBS buffer, centrifuged at 10,000 *g* for 10 min, and the supernatant was discarded.

The serum was collected from 4 month immunized rabbits, diluted in glycerol to 50% and main-

Table 1. Absorbance values (A_{410}) for fungal species and isolates, in a test for reactivity and cross-reactivity of a monoclonal antibody (mAb116) produced against *Phaeomoniella chlamydospora*.

Species	Strain No.[a]	Year	Geographical origin	Scion/rootstock	A_{410}[b]	
Phaeomoniella chlamydospora	Ph20	2008	Portugal	Chardonnay/76PB	1.760	a
Phaeomoniella chlamydospora	Ph14	2007	Portugal	Viognier/1103P	1.665	ab
Phaeomoniella chlamydospora	Ph18	2008	Portugal	Petit Verdot/400MM	1.658	ab
Phaeomoniella chlamydospora	Ph13	2005	Portugal	Syrah/-	1.516	b
Phaeomoniella chlamydospora	Ph17	2008	Portugal	Cabernet Sauvignon/337MM	1.000	c
Phaeomoniella chlamydospora	Ph16	2008	Portugal	Cabernet Sauvignon/160VO	0.996	c
Phaeomoniella chlamydospora	Ph9	2000	Portugal	Castelão/99R	0.902	cd
Phaeomoniella chlamydospora	Ph19	2008	Portugal	Petit Verdot/400VO	1.102	cd
Phaeomoniella chlamydospora	Ph15	2007	Portugal	Arinto/1103P	0.770	de
Phaeomoniella chlamydospora	Ph21	2011	Portugal	Arinto/1103P	0.711	e
Truncatella sp.		2011	Portugal	Unknown; *Vitis vinifera*	0.454	f
Ilyonectria macrodidyma	Cy175	2004	Portugal	Touriga Nacional/1103P	0.452	f
Trichoderma sp.		2012	Portugal	Alicante Bouschet/-	0.449	f
Phaeoacremonium aleophilum	CBS 110713	2001	South Africa	Unknown; *Vitis vinifera*	0.434	fg
Fomitiporia mediterranea		2010	Portugal	Castelão/99R	0.425	fgh
Neofusicoccum parvum	CBS 110301	1998	Portugal	Unknown; *Vitis vinifera*	0.416	fgh
"Cylindrocarpon" pauciseptatum	Cy217	2007	Portugal	Gouveio/-	0.395	fgh
Colletotrichum sp.		2010	Portugal	Alicante Bouschet/1103P	0.395	fgh
Lasiodiplodia theobromae	CBS 124060		Italy	Insolia/140R	0.390	fgh
Neofusicoccum luteum	CBS 110299	1996	Portugal	Unknown; *Vitis vinifera*	0.371	fghi
Pestalotiopsis sp.		2011	Portugal	Unknown; *Vitis vinifera*	0.369	fghi
Diplodia mutila	CBS 112553	1997	Portugal	Unknown; *Vitis vinifera*	0.360	fghi
Eutypa lata	BX 1-10	1990	France	Cabernet Sauvignon/ -	0.359	fghi
Fusarium sp.		2010	Portugal	Alicante Bouschet/140Ru	0.352	fghi
Epicoccum sp.		2010	Portugal	Unknown; *Vitis vinifera*	0.344	fghi
Ilyonectria liriodendri	CBS 117526; Cy68	1999	Portugal	-/99R	0.341	fghi
Penicillium sp.		2012	Portugal	Touriga Franca/-	0.333	fghi
Ilyonectria robusta	CBS 129084; Cy192	2005	Portugal	Alicante/196-17	0.321	fghi
Ilyonectria macrodidyma	CBS 112615	2000	South Africa	Sultana/143-B Mgt	0.320	fghi
Dothiorella viticola	CBS 117006	2003	Catalonia, Spain	Garnatxa negra/-	0.293	fghi
Phomopsis viticola		2010	Portugal	Touriga Nacional/110R	0.293	fghi

(Continued)

Table 1. (Continued)

Species	Strain No.[a]	Year	Geographical origin	Scion/rootstock	A_{410}[b]
Diplodia seriata	CBS 112555	1997	Portugal	Unknown; *Vitis vinifera*	0.277 ghi
Botrytis cinerea		2010	Portugal	Unknown; *Vitis vinifera*	0.262 hi
Fusicoccum aesculi	CBS 110302	1996	Portugal	Unknown; *Vitis vinifera*	0.223 i
Buffer					0.216 i

[a] All strains are from Laboratório de Patologia Vegetal "Veríssimo de Almeida", Instituto Superior de Agronomia, except those referred as CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and BX, Bordeaux, France (P. Lecomte, INRA).
[b] Values (means of four replicates) followed by different letters are significantly different (P=0.05) according to the Tukey´s test.

tained at -20°C until use. The serum was tested by indirect ELISA (Crowther, 2009) to multiple isolates of *Pa. chlamydospora* and for other fungi frequently isolated from grapevine wood (Cardoso *et al*., 2011).

Five female BALB/c mice were immunized five to ten times at intervals of 2-4 weeks with the spore suspensions prepared as described above. From the fifth immunization, two out five mice were also immunized with fungal mycelium (100 μg 200 μL^{-1}). The serum was removed after 2 to 5 months, centrifuged at 10,000 *g* for 10 min, two-fold diluted in glycerol (87% v:v) and frozen at -20°C until used. Then, the serum was tested by indirect ELISA, as described above. BALB/c mice with the highest serum titres were selected for monoclonal antibody production (Mechetner, 2007). Three cell fusions were carried out between splenocytes and Sp2/0-AG14 cell partner, using polyethylene glycol (PEG). Hybridomas were cultured in HAT medium, and stable hybridomas were screened against the different isolates of *Pa. chlamydospora* by indirect ELISA. The hybridomas were initially screened by indirect ELISA for a total mix of *Pa. chlamydospora* extract (mycelium 100 μg mL^{-1}, spores 1×10^6 spores mL^{-1}) in PBS buffer and expanded in 24-well plates. A second screen was then performed and only those clones reacting to *Pa. chlamydospora* extract were expanded to 25 cm^2 flasks and frozen with dimethyl sulfoxide (DMSO, 10% v:v) in liquid nitrogen. These positive clones were then intensively screened by indirect ELISA against other fungi in order to remove the hybridoma producing mAbs that cross reacted with other fungi.

The Ig subclass of mAbs was determined with a commercial kit (Mouse Immunoglobulin Isotyping ELISA Kit, #550487, BD Pharmingen), according to the manufacturer's instructions. Hybridoma cell lines were cloned by limiting dilution, grown in a non-selective medium, preserved by slowly freezing in fetal bovine serum/DMSO (90:10, v:v), and maintained in liquid nitrogen. Monoclonal antibodies were purified from culture medium by PEG ammonium sulphate precipitation according to Brooks *et al.* (1992). After dialysis against sodium chloride-Tris-EDTA (STE) buffer, the antibodies were stored at 4°C in STE buffer plus 10 mM phenylmethylsulfonyl fluoride (PMSF). For the determination of the serum titre and mAb titre, the indirect ELISA method was used (Crowther, 2009).

Proteinase K and periodate oxidation of immobilized antigen was used to characterize the antigen, according to Thornton *et al*. (2002).

A modified protocol based on TAS-ELISA method (Custódio *et al*., 2006; Crowther, 2009) was used for specific detection of *Pa. chlamydospora* antibodies. Volumes of samples and reagents used in ELISA were 50 µL, and between each step the microtitre plates (Corning® 96 Well EIA/RIA, Medium Binding) were washed three times with washing buffer, PBST (PBS plus 0.05% Tween 20, pH 7.5). Wells were coated with selected mAbs at 5 µg mL^{-1} in antibody capture buffer (50 mM H_2CO_3, pH 8.4), and incubated for 1 h at 37°C. After washing, wells were coated with fungal extracts (100 µg mL^{-1}) or with wood fragments extracts (20 mg mL^{-1}) diluted in sample extraction buffer (PBS plus 0.1% Tween 20 and 1% BSA) for 1 h at 37°C.

The rabbit detection antibody was then diluted (1/500) in antibody dilution buffer (PBST plus 0.1% gelatine) and added to the wells and incubated for 1 h at 37°C. Finally, the anti-rabbit antibody alkaline phosphatase conjugate (Sigma A-3687) diluted

Table 2. Detection of Phaeomoniella chlamydospora in different samples of naturally infected grapevine plants, by nested PCR using species-specific primers, and TAS-ELISA using a monoclonal antibody (mAb116).

Sample No.	Symptoms on grapevine wood cross section/ sampling region	Scion/rootstock[a]	Nested PCR	TAS-ELISA A_{410}[b]	
9A	Asymptomatic; 2-y-old cane	Tinta Roriz/1103P	+	1.054	a
8A	Asymptomatic; 2-y-old cane	Tinta Roriz/1103P	+	1.013	a
5A	Asymptomatic; 2-y-old cane	Alicante Bouschet/SO4	+	0.975	ab
11A	Asymptomatic; 2-y-old cane	Tinta Roriz/1103P	+	0.951	ab
1A	Black spots; 2-3-y-old wood collected from the arm end	Cabernet Sauvignon/110R	+	0.909	ab
6A	Asymptomatic; 2-y-old cane	Alicante Bouschet/SO4	nd[c]	0.898	ab
1B	Black spots and necroses; mature wood collected from the trunk	Cabernet Sauvignon/110R	+	0.896	ab
3A	Asymptomatic; 2-y-old cane	Alicante Bouschet/SO4	+	0.870	abc
10A	Asymptomatic; 2-y-old cane	Tinta Roriz/1103P	+	0.868	abc
7B	Black spots and necroses; bottom of rootstock	Alicante Bouschet/SO4	+	0.838	abcd
4A	Asymptomatic; 2-y-old cane	Alicante Bouschet/SO4	+	0.836	abcd
8B	Black spots and necroses; bottom of rootstock	Tinta Roriz/1103P	+	0.787	bcde
10B	Black spots and necroses; bottom of rootstock	Tinta Roriz/1103P	+	0.656	cdef
Positive control	Inoculated plant, black spots; 1-y-old cane	Aragonez/1103P	+	0.655	cdef
11B	Black spots and necroses; bottom of rootstock	Tinta Roriz/1103P	+	0.628	def
3B	Black spots; bottom of rootstock	Alicante Bouschet/SO4	+	0.625	def
4B	Black spots; bottom of rootstock	Alicante Bouschet/SO4	+	0.609	ef
9B	Black spots and necroses; bottom of rootstock	Tinta Roriz/1103P	+	0.593	efg
5B	Black spots; bottom of rootstock	Alicante Bouschet/SO4	+	0.586	efg
7A	Black spots; 2-y-old cane	Tinta Roriz/1103P	+	0.497	fgh
2A	Black spots; 2-3-y-old wood collected from the arm end	Cabernet Sauvignon/110R	+	0.456	fgh
6B	Black spots and necroses; bottom of rootstock	Alicante Bouschet/SO4	+	0.374	ghi
2B	Black spots and necroses; mature wood collected from the trunk	Cabernet Sauvignon/110R	-	0.313	hij
Negative control	Non-inoculated, asymptomatic 1-y-old cane	Aragonez/1103P	-	0.185	ij
Buffer				0.120	j

[a] Young vines: Tinta Roriz/1103P and Alicante Bouschet/SO4; Mature vines: Cabernet Sauvignon/110R.
[b] Absorbance values (means of four replicates) followed by different letters are significantly different (P=0.05) according to the Tukey´s test.
[c] Not determined.

1/10000 in antibody dilution buffer was added. The plates were incubated for 1 h at 37°C and washed five times in washing buffer. The substrate 4-nitrophenyl phosphate at 1 mg mL^{-1} in 10 mM ethanolamine plus 1 mM $MgCl_2$ was used as a chromogen. The reaction was detected after incubation for 2 h at

37°C and the absorbance (A_{410}) was determined on BioRad plate reader Model 550, at 410 nm.

For naturally infected grapevine plants, the results of TAS-ELISA were verified by nested PCR. DNA extraction from plant material was carried out according to Nascimento *et al*. (2001). A primary PCR was carried out using the universal primers ITS4/ITSF1 (White *et al*., 1990; Gardes and Bruns, 1993). This was performed in a volume of 20 µL containing 1× PCR buffer, 1 µM of each primer, 100 μM of each dNTP, 1.0 U *Taq* polymerase (DreamTaq DNA Polymerase, Thermo Scientific) and 2 µL of DNA as template. Conditions consisted of a first denaturation phase at 95°C for 3 min, 30 cycles at 92°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR product (1 µL) was used as DNA template for the secondary PCR using the primer pair Pch1/Pch2 (Tegli *et al*., 2000). Concentration of reagents in a final volume of 20 µL was: 1× PCR buffer, 0.5 µM of each primer, 50 µM of each dNTP and 2.0 U *Taq* polymerase. Thermal conditions were 3 min at 95°C followed by 15 cycles of 20 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 2% (w:v) agarose gel and visualized under UV light following staining in ethidium bromide. The presence or absence of *Pa. chlamydospora* infection, evaluated by nested PCR, was recorded with +/- scoring system.

For each assay, the ELISA results (A_{410} values) were statistically analysed by one-way ANOVA and means compared using the Tukey´s test at a 5% significance level (STATISTICA 8.0). When a representative number of negative samples were available, the cut-off was calculated as the mean plus 3 standard deviations from the A_{410} values of these samples (Erlwein *et al*., 2011).

Results and discussion

A TAS-ELISA assay based on monoclonal antibodies was developed to specifically detect *Pa. chlamydospora* from grapevine wood tissues. In a first step, a polyclonal rabbit serum was produced, and the final titre for rabbit serum was 1/500 by indirect ELISA. The specificity of this serum to *Pa. chlamydospora* spores and mycelium was also assessed by indirect ELISA in which a group of other fungi frequently isolated from grapevine wood were also coated to the plates (Cardoso *et al*., 2011).

That serum was selected to be used in combination with the monoclonal antibody produced during this research to construct the final TAS-ELISA assay for *Pa. chlamydospora* detection.

The final titre of BALB/c mice serum which was used for mAb production was 1/1000. A total of four hybridoma clones were obtained, and isotyping revealed that all cell lines produced IgM antibodies. From these clones, mAb58 and mAb116 were capable of reacting specifically with *Pa. chlamydospora* isolates, with minimal or no cross-reaction with other fungi currently isolated from grapevine wood. Nonetheless, the mAb116 clone was selected for the final TAS-ELISA assays, based on its higher reactivity compared with mAb58 (data not shown).

The antigen characterization, made by proteinase K and periodate oxidation of immobilized antigen (Thornton *et al*., 2002), revealed that mAb116 was not affected by proteinase, which suggests that proteins are not part of the epitopes for this mAb. However, periodate oxidation of the immobilised antigen was immediate, as evaluated by indirect ELISA, suggesting that mAb116 recognizes a carbohydrate epitope (data not shown). However, the exact nature of the antigen recognized by mAb116 is still under study.

Results of reactivity and cross-reactivity for the different fungal species/isolates tested against mAb116 are shown in the Table 1. Absorbance values for *Pa. chlamydospora* isolates were an average of 1.187; while for the set of the other fungi used as negative controls the average absorbance was 0.359. For this experiment, the calculated cut-off value was 0.463 (A_{410}). From negative controls, *Truncatella* sp., one isolate of *I. macrodidyma* (Cy175) and *Trichoderma* sp. were the fungi which attained the greatest absorbance values. However, these were below the cut-off value and significantly less than the values recorded for *Pa. chlamydospora* isolates.

A similar procedure was used to evaluate the presence of *Pa. chlamydospora* in wood extracts obtained from grapevine plants, which had been inoculated 6 months before with the *Pa. chlamydospora* isolate Ph19. Results of absorbance values showed that mAb116 significantly differentiated ($P \leq 0.05$) non-inoculated (average A_{410}, 0.205) from inoculated plants (average A_{410}, 0.846). Absorbance values of positive samples ranged from 0.602 to 1.335, all above the cut-off value (0.512) (Figure 1).

In order to determine if the mAb116 was also able to detect *Pa. chlamydospora* under natural condi-

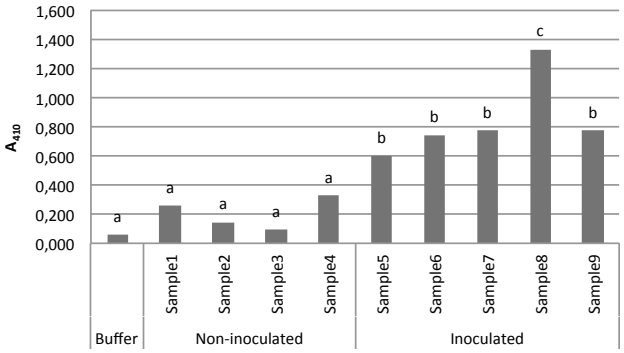

Figure 1. Mean absorbance values for TAS-ELISA detection of *Phaeomoniella chlamydospora* colonizing grapevine wood, using the monoclonal antibody mAb116. . Samples 1-4, healthy grapevine wood material; samples 5–9, inoculated grapevine wood material. Bars followed by different letters are significantly different (P=0.05) according to the Tukey´s test.

tions, we tested different grapevine samples of Tinta Roriz/1103P and Alicante Bouschet/SO4, collected from a young vineyard showing severe decline symptoms (Petri disease), and also different samples from a mature vineyard (Cabernet Sauvignon/110R) affected by esca proper disease. In parallel, these samples were tested by nested PCR, using in the second round amplification a primer pair that is *Pa. chlamydospora* specific (Tegli *et al*., 2000). Overall, results obtained either by nested PCR or TAS-ELISA were all in agreement, with only one exception regarding sample 6B. This sample tested positive by nested PCR, but the absorbance value was not significantly different from the negative control (Table 2). It is probable that *Pa. chlamydospora* may have been present in the sample at some level, but the PCR technique was more sensitive than TAS-ELISA.

Noticeable were the results obtained from young grapevine plants (Tinta Roriz/1103P and Alicante Bouschet/SO4) showing severe decline symptoms, where a trend was observed for higher absorbance values for grapevine canes than for rootstocks, even when asymptomatic wood was assayed. This finding is relevant, suggesting that infectious structures of *Pa. chlamydospora* (spores/mycelium) might be detected by this method before wood disease symptoms are visible. The presence of *Pa. chlamydospora* hyphae in grapevine xylem vessels is well documented, leading to xylem occlusion by tyloses and gums, and to the subsequent discolouration of vessels (Mutawilla *et al*., 2011). Also, there is evidence that the severity of wood discolouration and the presence of *Pa. chlamydospora* are positively correlated (Abbatecola *et al*., 2006). However, the results of Landi *et al*. (2012) using synthetic green fluorescent protein-transformed *Pa. chlamydospora* revealed the presence of the pathogen in grapevine tissues before the expression of any internal wood symptoms (dark-streaking) in inoculated grapevine cuttings. In the present study, it is possible that a slight discolouration of internal tissues corresponding to the onset of symptoms has been evaluated as asymptomatic, since the plant sections were only observed with the naked eye. However, it is also possible that mAb116 recognized *Pa. chlamydospora* structures, before symptoms were visible, thus indicating the usefulness of this antibody to locate the pathogen inside grapevine plants.

The new monoclonal antibody TAS-ELISA assay reported here could be especially useful for large-scale application in grapevine nurseries, to ensure that nursery stocks are free of *Pa. chlamydospora*. However, before mAb116 is generally used for detection of the pathogen, the antibody should be tested against a large number of samples, and results confirmed by PCR and/or isolation. Additionally, we aim to simplify the method towards a DAS-ELISA assay for practical application.

Acknowledgements

This research was supported by funds from Fundação para a Ciência e a Tecnologia, Portugal (projects PEst-OE/AGR/UI0245/2011 and PTDC/AGR-AAM/099324/2008).

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Accepted for publication: October 1, 2013