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 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 (White-man *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.
*Phaeomoniella chlamydospora* and other tracheomycotic fungi (mainly *Phaeacremonium* spp.) are slow-growing fungi in culture media, and their detection and identification using molecular techniques have been intensively developed (Teiglie *et al.*, 2000; Pollastro *et al.*, 2001; Ridgway *et al.*, 2002; Retief *et al.*, 2006; Martin *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-LPVA”, 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 × 10⁸ 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 × 10⁸ 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 × 10⁸ 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.

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

(Continued)
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 × 10⁶ 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 cm² 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 ELA/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 H₂CO₃, 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 1. (Continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.ᵃ</th>
<th>Year</th>
<th>Geographical origin</th>
<th>Scion/rootstock</th>
<th>A₄₁₀ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diplodia seriata</td>
<td>CBS 112555</td>
<td>1997</td>
<td>Portugal</td>
<td>Unknown; Vitis vinifera</td>
<td>0.277 ghi</td>
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<tr>
<td>Botrytis cinerea</td>
<td></td>
<td>2010</td>
<td>Portugal</td>
<td>Unknown; Vitis vinifera</td>
<td>0.262 hi</td>
</tr>
<tr>
<td>Fusidium aesculi</td>
<td>CBS 110302</td>
<td>1996</td>
<td>Portugal</td>
<td>Unknown; Vitis vinifera</td>
<td>0.223 i</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.216 i</td>
</tr>
</tbody>
</table>

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.
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 ethanol-amine plus 1 mM MgCl₂ was used as a chromogen. The reaction was detected after incubation for 2 h at

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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).

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| 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⁺ | 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.
37°C and the absorbance (A410) 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 (A410 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 A410 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 (A410). 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<0.05) non-inoculated (average A410 0.205) from inoculated plants (average A410 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-
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.

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