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Phellinus species inducing hoja de malvón symptoms on leaves and wood decay in mature field-grown grapevines

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Summary. Hoja de malvón is a common vine wood disease widely spread in grape production areas of Argentina which causes wood necrosis, decline, and the death of plants. Leaves are smaller than normal and chlorotic, with margins curled downwards. A basidiomycete, provisionally classified as *Phellinus* sp., is the fungus most frequently isolated from infected plants. The aim of this study was to determine the pathogenicity of this fungus in field-grown grapevines and to clarify its taxonomic positioning. The trunks and branches of five 13-year-old grapevines cv. Riesling were infected on October 1994. Six years later some of the infected grapevines showed foliar symptoms of hoja de malvón. The inoculated fungus was reisolated with relatively high frequency, together with other fungi from different necrotic areas near the inoculation sites. The fungus under study was ascertained to belong to the *Hymenochaetaceae* family (Aphyllophorales, Basidiomycota). Further inoculations of a much greater number of plants and with various associated fungi like species of the genera *Phaeoacremonium, Phaeomoniella* and *Botryosphaeria*, will be conducted.

Key words: *Vitis vinifera*, wood diseases, pathogenicity.

Introduction

Hoja de malvón is a widely spread vine wood disease in grape production areas of Argentina. The disease causes necrosis in the wood, decline and death of the plants. Leaves are smaller than normal and chlorotic, with margins curling downwards resembling a geranium leaf. Basidiocarps fleshy, pileate to effused reflexed, upper surface tawny when they are young and brownish when they are old, can be found on trunks of some infected vines.

Previous studies have shown that a number of fungi are associated with the disease: a *Phellinus* species, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) Crous & W. Gams, *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingfield & L. Mugnai, *Phaeoacremonium parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingfield and *Botryodiplodia* species are associated with hoja de malvón (Gatica *et al.*, 2000).

The *Phellinus* species was the most frequent fungus in the complex of organisms isolated (Césari and Gatica, 2001).

Phellinus punctatus (P. Karst.) Pil. (syn. *P. igniarius* var. resupinatus Bres.) and renamed *Fomitiporia puntacta* (P. Karst) Murrill, is considered to be a primary pathogen of grapevine causing in the wood a white decay. Together with other fungi associated with various types of wood discolouration, it is also reported to be one of the agents of

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esca in European and non-European vine growing countries (Chiarappa, 1959, 1997; Larignon and Dubos, 1997; Mugnai *et al*., 1999; Cortesi *et al*., 2000; Sparapano *et al*., 2000, 2001).

The aim of this study was to determine in field-grown grapevines the pathogenicity of the *Phellinus* species associated with hoja de malvón in vineyards of Argentina and to improve its taxonomic identification.

Materials and methods

Fungal strain

Strain Fl 1514 (Mycological collection, Departamento de Botánica, Facultad de Ingeniería, Universidad de la República, Montevideo, Uruguay) of the basidiomycetous fungus isolated from hoja de malvón diseased vines was used for pathogenicity tests. The strain had been isolated from decayed woody tissues of a cv. Chenin grapevine showing hoja de malvón foliar symptoms. It was maintained on 2% potato dextrose agar (PDA) at 4ºC.

The basidiomycetous fungus was sent to taxonomists for identification.

Other fungi, including *Phaeoacremonium* species, *Pa. chlamydospora* and *Botryosphaeria* species, were identified based on morphological and cultural characteristics (Punithalingam and Waller, 1973; Punithalingam, 1976; Crous *et al*., 1996; Dupont *et al*., 2000; Phillips, 2002).

Plant material

Ten 13-year-old grapevines cv. Riesling showing neither foliar symptoms of hoja de malvón nor any sign of external wood deterioration, were selected in 1994, at the Mendoza Experimental Station, INTA, Mendoza, Argentina, to be used for pathogenicity tests.

Pathogenicity tests

In October 1994 a 2-month-old fungal culture of strain Fl 1514, was used to inoculate selected vines. Five vines were inoculated with plugs of mycelium inserted into 5 mm drilled holes. In vines I_1 and I_3 the mycelium was inoculated into the pith of the main trunk near the primary branches, and in vines I_2, I_4 and I_5, it was inoculated at the base of one of the branches. After inoculation the drilled holes were filled with a humid cotton plug and sealed with Parafilm. The remaining five vines were used as controls: plugs of uncolonized PDA were inserted into the holes, which were sealed as those of the inoculated vines. In control vines C_1, C_2 and C_5 these plugs were drilled into the pith of the main trunk, and in control vines C_3 and C_4 at the base of one of the branches.

Vines were inspected for the development of foliar symptoms in the following years. In 2000, after an incubation period of six years, some hoja de malvón foliar symptoms were observed for the first time. Their severity was visually assessed, based on percentage of foliage affected according to the following scale: grade 0, no symptoms; 1, less than 10% of symptomatic leaves; 2, 10–25%; 3, 25–50%; 4, 50–75% and 5, 75–100%. One year later, reisolations of the inoculated fungus, and of concomitant fungi from different necrotic areas near the inoculation site of inoculated and control vines were performed from cross sections of the trunks.

Results

Pathogenicity tests

Hoja de malvón foliar symptoms (Fig. 1) in the vines inoculated in 1994 appeared for the first time in March 2000 (I_1; severity grade 4). In March 2001, another three vines (I_2, I_4 and I_5) showed the same symptoms (grade 3, 1, 1 respectively) and in December 2001, the fifth vine (I_3) also did so (grade 2) (Table 1).

In the control plants, only one vine (C_2) developed symptoms after March 2001 (Table 1). A transverse section across the inoculated trunk of this vine at the inoculation point revealed no internal decay in this area. Nevertheless the vine showed some spongy decay tissue at the site of a wound on the trunk, 10 cm below the inoculation point. This suggested that there was a natural infection here that had been asymptomatic at the time of the trial.

All the inoculated vines on the other hand showed internal symptoms around the inoculation site, similar to those observed in vines naturally affected by hoja de malvón (Fig. 2). No other decayed area was found anywhere along the trunk of inoculated vines except a little necrosis limited to pruning wounds. At the inoculation site the following types of necrosis all characteristic of the disease (Gatica *et al*., 2000) were detected: necrosis a

(soft white decay), necrosis b (black line surrounding the decay), necrosis c (brownish area of hard consistency surrounding a decayed area) and necrosis d (sectorial brown area of hard consistency).

Reisolations were done from only four of the inoculated vines (Table 2). The branch of the inoculated fifth vine (I_5), was inadvertently pruned so it could not be analysed. In the total number of

Fig. 1. Hoja de malvòn foliar symptoms in a vine inoculated (vine No. 3) with *Phellinus* sp.

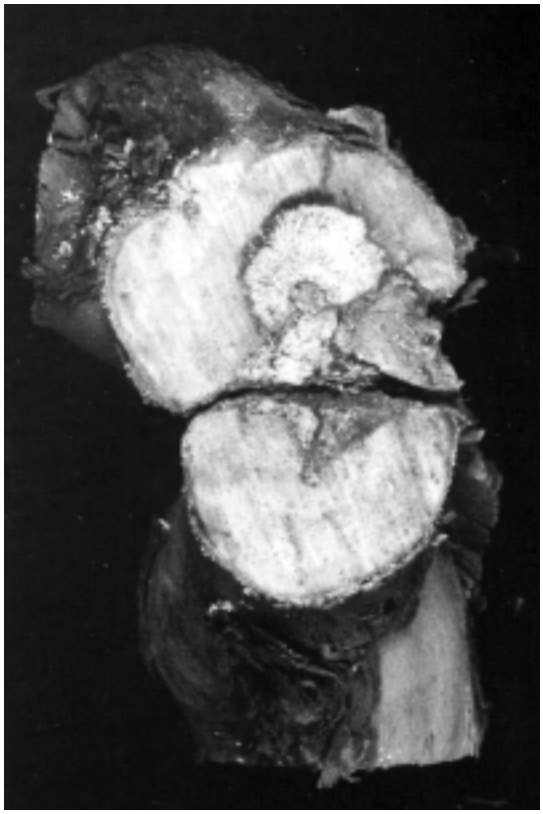

Fig. 2. Formation of wood decay at the inoculation point in a vine inoculated with *Phellinus* sp. (vine No. 3).

Table 1. Pathogenicity test with the basidiomycete species isolated from hoja de malvón affected vines (strain Fl 1514). Severity of symptoms on inoculated (I) and control (C) vines.

Vine	Disease severity grade [a]							
	March 2000		March 2001		December 2001		February 2002	
	I	C	I	C	I	C	I	C
1	4	0	4[b]	0	0[c]	0	0[c]	0
2	0	0	3[b]	3	0[c]	4	0[c]	3
3	0	0	0	0	2	0	4	0
4	0	0	1	0	2	0	2	0
5	0	0	1	0	0	0	2	0

[a] Grade 0, without symptoms; 2, 10–25%; 3, 25–50%; 4, 50–75% and 5, >75%.
[b] Vine removed for reisolations.
[c] Vine renewal without symptoms.

infected (1541) and non-infected (113) samples of woody tissues obtained from the inoculated vines, *Phellinus* sp. was always the most frequently isolated species, occurring in 38.2% of colonies from vine I_1 and in 34.3, 31.5 and 39.8% of the vines I_2, I_3 and I_4 respectively.

Among the concomitant lignicolous fungi isolated, *Pa. chlamydospora* was found in one inoculated vine (I_3, 1.3%), *Pm. parasiticum* in vine I_1 (0.2%), and other unidentified *Phaeoacremonium* species on two of the four vines (I_2, 0.4% and I_3, 8%) (Table 2).

With regard to the type of necrosis observed in inoculated vines, *Phellinus* sp. was most frequent in necrosis b and c (Table 3).

Reisolations from the four control vines including the symptomatic control vine, were also done. Cutting across the inoculation point showed non-discoloured wood in C_2 and C_5 and necrosis type b and d in C_3 and b in C_4. Necrosis types b, c, d and p

Table 2. Mycroflora isolated from discoloured wood in 4 vines (I_1, I_2, I_3, I_4) inoculated with *Phellinus* sp. (strain Fl 1514) and frequency of isolation (number of colonies of each species).

Fungus	I_1		I_2		I_3		I_4	
	No.[a]	%[b]	No.	%	No.	%	No.	%
Acremonium spp.	2	0.5	28	3.8	2	0.8	0	0.0
Alternaria spp.	33	8.0	24	3.3	2	0.8	18	11.2
Aspergillus spp.	75	18.2	0	0.0	1	0.4	0	0.0
Bacteria	85	20.7	124	17.0	8	3.4	4	2.4
Cladosporium sp.	1	0.2	1	0.1	4	1.7	6	3.7
Other fungi[c]	5	1.2	27	3.7	16	6.7	33	20.5
Penicillium spp.	6	1.5	132	18.1	54	22.7	22	13.7
Phaeomoniella chlamydospora	0	0.0	0	0.0	3	1.3	0	0.0
Phaeoacremonium parasiticum	1	0.2	0	0.0	0	0.0	0	0.0
Phaeoacremonium spp.	0	0.0	3	0.4	19	8.0	0	0.0
Phellinus sp.	157	38.2	251	34.3	75	31.5	64	39.8
Trichoderma sp.	0	0.0	0	0.0	3	1.3	0	0.0
Unknown fungi	46	11.2	141	19.3	51	21.4	14	8.7
Total No. of colonies	411		731		238		161	

[a] No. of colonies obtained for each species or group of micro-organisms.
[b] No. of colonies obtained for each species or group of micro-organisms as a percentage of the total number of colonies obtained.
[c] Other fungi species identified and occasionally isolated (*Fusarium*, *Monochaetia*, *Paecilomyces*, *Gliocladium*, *Epicoccum* and yeasts).

Table 3. Distribution of *Phellinus* sp. in the different types of necrotic areas in inoculated vines.

Inoculated vine	Frequency (%)[a]			
	Necrosis a[b]	Necrosis b[c]	Necrosis c[d]	Necrosis d[e]
I_1	25.4	40.2	73.7	0.0
I_2	42.2	23.1	0.0	8.7
I_3	19.4	54.5	28.1	0.0
I_4	15.5	76.4	0.0	0.0

[a] Percent values were calculated over the total number of colonies obtained in each type of necrosis.
[b] Soft white decay.
[c] Black line bordering a decayed area.
[d] Brownish area surrounding a decayed area of hard consistency.
[e] Sectorial brown area of hard consistency.

Table 4. Mycroflora isolated from discoloured wood in 4 control vines (C_2, C_3, C_4, C_5) and frequency of isolation as a percentage of the total No. of colonies of each species.

Fungus	Artificial inoculation drilled site					Natural wound sites										
	C_2[a]	C_3		C_4	C_5	C_2			C_3				C_4		C_5	
		b[c]	d[e]	b[c]		a[b]	b[c]	c[d]	b[c]	c[d]	d[e]	p[f]	c[d]	d[e]	b[c]	d[e]
Acremonium spp.	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	0.0
Alternaria spp.	3	7.2	49.4	2.9	6.5	1.2	1.5	2.4	30.4	0.0	3.2	3.3	7.7	72.8	24.7	9.5
Aspergillus spp.	0.0	16	0.0	2.9	0.0	0.0	0.0	0.0	3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteria	0.0	1.4	0.0	13.2	0.0	4.9	0.0	0.0	0.0	0.0	0.0	0.0	8.5	0.0	1.4	0.0
Botryodiplodia theobromae	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cladosporium sp.	0.0	0.0	0.0	0.0	0.0	1.2	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other fungi	0.0	18.8	20.5	5.9	0.0	2.4	4.4	4.2	27.7	0.0	0.0	0.0	11.5	12.1	8.1	0.0
Penicillium spp.	0.0	7.2	20.5	0.0	0.0	68.3	42	47.9	8.7	0.0	23.8	0.0	0.0	3	12.3	6.3
Phaeoacremonium aleophilum	0.0	3	0.0	1.5	3.2	0.0	1.5	0.0	4	2.8	0.0	0.0	0.0	0.0	8.2	1.6
Phaeomoniella chlamydospora	87	7.2	0.0	28	0.0	0.0	0.0	0.0	0.0	83.3	0.0	86.7	47	0.0	0.0	0.0
Phaeoacremonium parasiticum	0.0	0.0	2.4	39.7	0.0	0.0	0.0	0.0	1.5	0.0	66.7	0.0	0.7	0.0	0.0	0.0
***Phellinus* sp.**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**20.7**	**36.2**	**44.3**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
Sterile chips	10	8.7	0.0	2.9	90.3	0.0	5.8	0.6	8.7	8.3	1.6	10	20.8	0.0	1.4	0.0
Trichoderma sp.	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.6	0.0	0.0	0.0	0.0	0.0	3	0.0	0.0
Unknown fungi	0.0	27.5	6	1.5	0.0	0.0	7.2	0.0	14.5	5.6	3.1	0.0	3.8	6.1	37	82.6
Yeasts	0.0	3	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	1.6	0.0	0.0	0.0	1.4	0.0

[b], [c], [d], [e] See Table 3.
[f] Black spots.

(black spots) from decayed or discoloured tissues at the sites of other wounds, were also observed (Table 4). Necrosis a was detected only in C_2, the symptomatic vine, which was also the only one from which *Phellinus* sp. was recovered.

Phaeomoniella chlamydospora, *Pm. aleophilum* and *Pm. parasiticum* were isolated from the inoculation site or other wound sites of control vines (Table 4).

Discussion

The basidiomycetous fungus isolated with high frequency from diseased vines (Gatica *et al.*, 2001) and here used for artificial inoculations in asymptomatic vines can be considered a major causal agent of hoja de malvón in Argentina. This is the first report of the artificial induction of hoja de malvón symptoms on the leaves and wood in mature field-grown grapevines.

The fungus produced white rot, dark tissues and the characteristic hoja de malvón symptoms on the leaves. In re-isolations, the frequencies of *Phellinus* in necrotic areas b and c were consistent with data from previous studies (César and Gatica, 2001).

Nevertheless, other lignicolous plant pathogenic fungi, like *Pa. chlamydospora* and species of *Phaeoacremonium* were also encountered, both in inoculated and control vines.

Basidiomycetes are known to cause wood rot in different plant species (Shigo, 1963; Wright and Deschamps, 1972; Martínez *et al.*, 2002). In grapevine, the basidiomycetous fungus most often associated with esca is *P. igniarius* (*F. punctata*) (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Cortesi *et al.*, 2000; Fischer, 2000, 2002). A species classified as *P. punctatus* (*F. punctata*) caused internal spongy white decay (Chiarappa, 1997) and produced wood and foliar esca symptoms such as degradation of the wood, chlorosis and reddening of the leaf margins, and necrosis of large parts of the leaf lamina (Sparapano *et al.*, 2001).

Unfortunately the identification of the fungus studied here is still incomplete. This basidiomycetous fungus was first studied by Rajchenberg (*In*: Gatica *et al.*, 1998) (Table 5). Following these data

Table 5. Description of the culture of the basidiomycete species isolated from hoja de malvón diseased vines in 1998.

Macroscopic characters	Growth moderate, covering the Petri dish in the 3rd week. Margin regular; mat homogeneously felty, except the dish center and backwards, where the mat becomes subfelty to slightly felty; colour of the mat yellowish, beige to light chestnut (yellow 10YR 8/6-8/8-7/6); mat growing walls around the inoculum, densely felty, chestnut (reddish yellow 7.5YR 6/6-6/8). Odor none. Reverse bleaching.
Phenoloxidase reactions	Gallic acid: ++, 0 mm in diameter. Tannic acid: +++, 10 mm in diameter. Tyrosinases: -, 15 mm in diameter.
Microscopic characters	Margin with simple septate generative hyphae, 1–4 µm in diam., with thin to slightly thick walls, hyaline, branched or not. Aerial hyphae formed with similar generative hyphae and fiber hyphae; the latter 1.5–3.0 µm in diam., poorly branched, hyaline when formed but becoming thick-walled, the walls melleous to chestnut. Generative hyphae growing on the agar surface, 5–6 µm in diameter. Hyphae growing into the agar only when they are of the generative type, densely branched, 1–4 µm in diam., and with lateral, digitiform branches.
Species code following Nobles 1965	2.6.8.32.37.40.43.54
Remarks	In growth, colour, texture and microscopic features the species clearly belongs to the *Hymenochaetaceae* but it does not present distinct features that allow identification to species level.

the fungus was classified as belonging to the Hymenochaetaceae family (Aphyllophorales, Basidiomycota).

J.A. Stalpers (CBS, Utrecht, The Netherlands) identified the culture as *Phellinus* sp. in 1998 (*In*: Gatica *et al*., 2000). M. Fischer (University of Regensburg, Germany), reported that Argentinean isolates of this species were not assignable to any of the European *F. punctata* sequences (Fischer, personal communication). He compared those isolates with some other Hymenochaetales taxa and it came out as somewhat related to species of *Inonotus s.l*. (*Inocutis* Fiasson & Niemelä). However, no strict correlation with any European taxa of this group was found. Fischer did not find any differences between cultures from woody tissue and those from spores of fruiting bodies (Fischer, personal communication).

Also, the characteristics of fruiting bodies in some infected vines did not clarify the matter, since the fungus was considered an *Inonotus* sp. by Rajchenberg (*In:* Gatica *et al*., 1998) and *Phellinus fastuosus* (Lév.) Ryvarden by Samsom, in 1999 (personal communication). A study is now under way in Uruguay to compare it with other American taxa associated with *Eucalyptus* stem rot (Bettucci, 2002; Martínez *et al*., 2002).

In Australia, Pascoe and Edwards (2000), studying white decay in grapevine not showing foliar symptoms, have also found, as in Argentina, only basidiomycetes that are not related to any of the fungi occurring in Europe.

These preliminary data will be a basis for further studies on the taxonomic position of the fungus and its pathogenicity.

Further inoculation tests with this fungus in a greater number of plants and with other associated fungi such as *Phaeoacremonium, Phaeomoniella* and *Botryosphaeria,* that are involved in another grapevine diseases (Lehoczky, 1974; Mugnai *et al*., 1996; Larignon and Dubos, 1997; Bertelli *et al*., 1998; Larignon *et al*., 2001; Phillips, 2002), are being conducted in order to confirm the results obtained in this study and to elucidate the role of those fungi disease expression.

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