Phytopathol. Mediterr. (2006) 45, S110–S116

Characterization and identification of the basidiomycetous fungus associated with 'hoja de malvón' grapevine disease in Argentina

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Summary. *Inocutis jamaicensis* (Murrill) Gottlieb, J.E. Wright & Moncalvo was identified as the basidiomycetous species associated with 'hoja de malvón' grapevine disease in Argentina. Macro and micro-morphological characteristics of fruit bodies corresponded to those described for the white-rotting fungus associated with native plant species and *Eucalyptus globulus* Labill. planted in Uruguay. Monokariotic isolates were obtained from basidiospores produced by fruit bodies of *I. jamaicensis* collected from *Vitis vinifera* L. and *E. globulus*. Dikaryons and fruit bodies produced by pairing monokaryotic mycelium suggest that all these isolates belong to the same species. The analysis of RFLP of the dikaryon produced by pairing monokaryons derived from *V. vinifera* and *E. globulus* revealed fragments that corresponded to each monokaryon, confirming that isolates from *Vitis* mated with those from *Eucalyptus*. In order to compare grapevine and Uruguayan isolates, RFLPs from ITS region generated by restriction digestion with *Alu* I, *Hae* III, *Hha* I, *Msp* I and *Taq* I were performed. Differences found in some restriction pattern could reflect a certain degree of variability between dikariotic isolates, probably related with a particular lifestyle, host specificity or geographic origin.

Key words: *Inocutis*, *Vitis vinifera*, *Eucalyptus globulus*, wood diseases.

Introduction

'Hoja de malvón' is the name of a grapevine trunk disease that is widespread in Mendoza and San Juan, the main grape production areas of Argentina. Its increased spreading has caused economical losses and important damage to the vineyards (Gatica *et al*., 1999). Studies carried out to identify the causal agent(s) have shown that a basidiomycetous fungus provisionally classified as *Phellinus* sp., *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) Crous and 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 *Botryosphaeria* species are associated with 'hoja de malvón' (Gatica *et al*., 2000; Dupont *et al*., 2002).

The basidiomycetous fungus was found to be the most frequent fungus of the complex of organisms associated with 'hoja de malvón' symptoms on the leaves and wood (Césari and Gatica, 2001; Gatica *et al*., 2004). It was determined to belong to the *Hymenochaetaceae* family (Aphyllophorales), but was not assignable to any European basidiomyco-

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ta associated with esca. A discussion about its identification was reported by Gatica *et al*. (2004).

Using molecular methods, Auger *et al*. (2002) confirmed that the symptoms of curl chlorotic disease of grapevines in Chile, similar to those described for 'hoja de malvón' in Argentina, could not be linked with either *Fomitiporia punctata* (P. Karst.) Murrill or *Phellinus igniarius* (L.: Fr.) Quél. At the same time, Aguilera *et al*. (2002) used molecular methods to determine that the fungus associated with curl chlorotic disease was a species of *Inocutis* (*Inonotus rheades* group). Recently, Auger *et al*. (2003) associated *Fomitiporella vitis* with similar symptoms in Chile and Fischer *et al*. (2005) found that *Fomitiporia australiensis* M. Fisch., J. Edwards, J. Cunnington & I. Pascoe was the basidiomycete associated to grapevine in Australia.

The aim of this work was to characterize and identify the fungus associated with 'hoja de malvón' grapevine disease in Argentina.

Materials and methods

Fungal isolates

The basidiomycetous fungus was isolated from decayed woody tissues of grapevine cv. Chenin showing 'hoja de malvón' symptoms. Cultures were maintained on malt extract agar (MEA) at 4ºC. Its pathogenicity was recently tested (Gatica *et al*., 2004).

Four isolates, MVHC 11510, MVHC11511, MVHC11512 and MVHC 11518 from *Vitis vinifera* and two isolates MVHC 11297 and MVHC 11394 from *Eucalyptus globulus* (Fungal Collection of Laboratorio de Micología, Facultad de Ciencias/ Ingeniería, Universidad de la República, Montevideo, Uruguay) were used in this study.

Basidiocarps that had developed on trunks of some infected vines and also on MEA were described and identified according to Gilbertson and Ryvarden (1986). Single spore isolates from MVHC 11511, MVHC 11518 and MVHC 11297 were obtained from dikaryotic parental basidiocarps developed in cultures (Table 1).

Pairing of monokaryotic isolates

Monokaryotic mycelium derived from basidiospores obtained from fruit bodies developed on *V. vinifera* and *E. globulus* were paired on 2% MEA.

Dikaryotic mycelium resulting from compatible monokaryotic isolates was transferred to fresh medium.

DNA extraction

DNA was obtained from fresh aerial mycelia by extraction with cetyltrimethylammonium bromide (CTAB), followed by organic extraction and isopropanol precipitation of the DNA using a modified version of the method described by Lee and Taylor (1990). Ground material obtained by mechanical pulverization using a cordless drill was transferred to a sterile Eppendorf tube. Then 400 μl of extraction buffer (Tris HCl pH 8.0, EDTA Na_2, SDS) and 5 μl proteinase K (2% [wt/vol]) was added. The sample was mixed to resuspend the ground material in the buffer and incubated at 60ºC for 30 min. Then 112 μl NaCl 5M and 52 μl CTAB 10× (CTAB 10% [wt/vol]; Tris HCl pH 8.0, 100 mM; NaCl 1.4 M; EDTA 20 mM; β-mercaptoethanol 0.2%[vol/vol]) was added and the tubes were incubated at 65ºC for 10 min. Samples were extracted using 570 μl chloroform-isoamyl alcohol (25:24:1), vortexed, incubated at –20ºC (30 min) and centrifuged at 10,000×*g* for 10 min. The upper phase was transferred to a 1.5 ml Eppendorf vial, and DNA was precipitated using 3.0 M ammonium acetate and 600 μl ice-cold isopropanol and placed at –20°C for 1 h. DNA was pelleted by centrifugation at 10,000×*g* for 10 min and was washed with 800 μl 70% (vol/

Table 1. Monokaryotic isolates obtained from dikaryotic isolates of *Vitis vinifera*[a] and *Eucalyptus globulus*[b].

Dikaryotic isolates	Monokaryotic isolates					
MVHC 11511[a]	MVHC 11769	MVHC 11770	MVHC 11771	MVHC 11772	MVHC 11773	
MVHC 11518[a]	MVHC 11735	MVHC 11736	MVHC 11737	MVHC 11738	MVHC 11739	MVHC 11740
MVHC 11297[b]	MVHC 11797	MVHC 11798	MVHC 11819	MVHC 11827	MVHC 11830	

vol) ethanol. The pellet was dried in a Speed-vac for 10 min and resuspended in Tris–EDTA (10 mM Tris-HCl (pH 8), 1 mM EDTA). DNA extractions were visualised in 0.8% agarose gel. DNA was diluted 1:10 and stored at –20°C.

PCR amplification with universal primers ITS-1F and ITS-4

The ribosomal DNA ITS regions 1 and 2 and 5.8S were amplified by the polymerase chain reaction (PCR) using universal primer pair ITS-1F/ITS-4 (White *et al*., 1990; Gardes and Bruns, 1993). PCR was performed in 25 μl volume containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM Mg Cl_2 (pH 8.3); 50 μM of each deoxiribonucleotide triphosphate; 1 μM of each primer; and 1 unit of Taq polymerase (ATGen) on a Gene-tech SPCR1 MKII-Termoblock. The following parameters were used: 35 cycles of 70 s at 94ºC, 45 s at 52ºC and 90 s at 72ºC preceded by 5 min at 94ºC and ending with a 5 min elongation step at 72ºC.

Restriction digestion of PCR products

Aliquots of 10 μl of the amplified DNA were digested directly without further purification with restriction endonucleases to obtain ITS-RFLPs; each sample was digested with 2 units of *Alu* I, *Hae* III, *Hha* I, *Msp* I and *Taq* I (BioLabs, Beverly, MA, USA) in single-enzyme digest. The restriction fragments were separated by electrophoresis in 2% (wt/vol) agarose gel (USB), stained with ethidium bromide 10 $\mu g\ \mu l^{-1}$, and were visualized and photographed under UV light . The molecular size marker was the 100 bp DNA ladder (BioLabs).

Results

Fruit body characteristics

Basidiocarps annual, first nodulose and resupinate, then effuse-reflexed, with a dimidiate portion, triquetrous in section, imbricate, 1–2×3–6×0.5–1.0 cm. Upper surface yellow brown, finely tomentose and slightly zonate. Pore surface first creamy brown, then brown ochre, pores 3–5(–6) per mm, circular to angular. Tubes concolorous or lighter in colour than context, up to 0.5 cm deep. Context thin, up to 5 mm thick, golden brown to dark cinnamon brown. Tomentum present in young parts, golden brown (Fig. 1). Hyphal system monomitic, generative hyphae with simple septa, yellowish to rusty brown, thin to thick walled, 2–7 μm wide. Contextual hyphae with simple septa, yellow brown to dark reddish brown 4–10 μm wide, thick walled, with a wide lumen. Setal hyphae absent. Setae absent. Basidia subclavate, 4-sterigmate, hyaline, 12–18×5–6 mm. Basidiospores ellipsoid, thick-walled and with a straight side, rusty to umber or reddish brown, guttulate, 5.5–7.0 (–7.5)×4–5 μm, IKI and CB negative.

Hymenophores with microscopic characteristics similar to those of the collected specimens were sometimes produced on 2% MEA.

The macro- and micromorphological characteristics of fruit bodies collected from grapevine trunk corresponded to *Inocutis jamaicensis* (Murrill) Gottlieb, J.E. Wright and Moncalvo. In addition, cultural characters of the isolates analyzed also corresponded with isolates from *E. globulus*.

Pairing of monokaryotic isolates

Monokaryotic mycelia derived from basidiospores of fruit bodies from different origins (*Vitis* and *Eucalyptus*) were paired (Table 1). The resulting dikaryotic mycelium produced fruit bodies on fresh medium (Fig. 2). The growth rate of monokaryons (MVHC 11798: 2.3 cm and MVHC 11772: 3.0 cm) was lower than that of the dikaryon (4.8 cm) evaluated at 10 days.

Fig. 1. Basidiocarps of *Inocutis jamaicensis* on the trunk of a diseased vine.

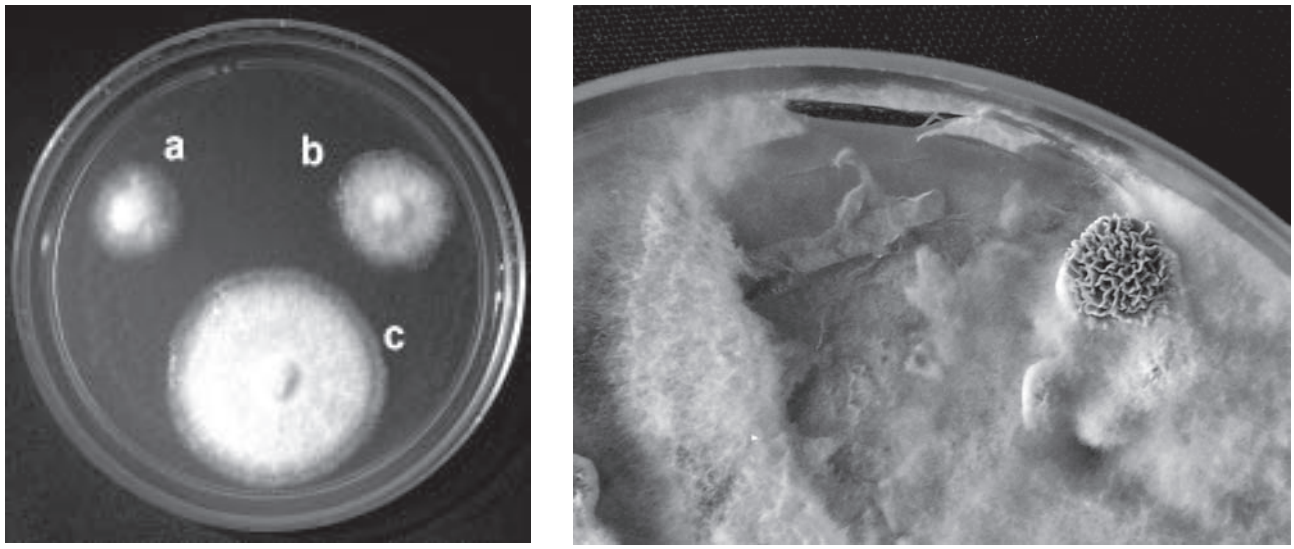

Fig. 2. Monokaryons and dikaryon (left). Monokaryon a, MVHC 11798 from *E. globulus* and monokaryon b, MVHC 11772 from *V. vinifera*; dikaryon c, obtained from pairing a and b. Fruit body formed from the dikaryon (right).

Molecular analysis

In order to compare the dikaryotic isolates, polymerase chain reaction (PCR) of internal transcribed spacer (ITS) regions was performed. The amplification of the ITS sequences from each isolate produced a fragment of approximately 850 bp.

ITS amplicons from all isolates were submitted to PCR-RFLP analyses, homo- and heterozygotes from the presence of restriction site polymorphisms were scored. In the heterozygotes the sum of fragments is longer than the original PCR products (Table 2).

The analysis of RFLP showed variability in the ITS region of the isolates (Table 2).

Taq I was the unique endonuclease that produced the same profile (*ca* 400 and 450 bp fragments) in all isolates, showing only one restriction site.

The same profile could be shown in all *V. vinifera* isolates and in one isolate from *E. globulus* using *Msp* I endonuclease. Using *Alu* I, a profile with three fragments of nearly 75, 250 and 600 bp was present in the isolates MVHC 11518 and MVHC 11510 from *V. vinifera*. A different profile with 175 bp and without the 250 bp fragments was

Table 2. ITS restriction fragments obtained with endonucleases.

Collection No.	Locality	Host	*Taq* I	*Hha* I	*Msp* I	*Alu* I	*Hae* III
MVHC 11297	Uruguay, Maldonado	*E. globulus*	400-450	100-150-250-350-350-400	50-100-700	75-175-600	200-650-850
MVHC 11394	Uruguay, Lavalleja	*E. globulus*	400-450	100-350-400	50-150-650	75-175-600	850
MVHC 11510	Argentina, Mendoza	*V. vinifera*	400-450	100-150-250-350	50-150-650	75-175*-250-600	850
MVHC 11511	Argentina, Mendoza	*V. vinifera*	400-450	100-150-250-350	50-150-650	75-175-600	100-750-850
MVHC 11512	Argentina, Mendoza	*V. vinifera*	400-450	100-150-250-350	50-150-650	75-175-600	100-750-850
MVHC 11518	Argentina, Mendoza	*V. vinifera*	400-450	100-150-250-350	50-150-650	75-175[a]-250-600	850

[a] 175 bp band was inferred from the progeny corresponding to the heterokaryon MVHC11518 (Fig. 5, lanes 2, 4, 5, 6 and 7).

present in the remaining isolates (Fig. 3), allowing homozygote isolates from both hosts to be distinguished from heterozygote isolates from *V. vinifera*. The absence of 175 bp band in Fig. 3 (lanes 5 and 8) is due to the DNA heteroduplex formation that causes a masked restriction site. Figure 5 clearly shows the presence of this band in the progeny of dikaryotic MVHC11518 (lanes 2, 4, 5, 6 and 7).

Hae III gave three profiles with three, two and one fragment in some isolates from both hosts (Fig. 4). The presence of an 850 bp fragment corresponded to the absence of restriction sites and it was present in all dikaryotic isolates (Fig. 4, lanes 2–7), and two 650 and 200 bp fragments were only present in one heterozygote isolate associated with *E. globulus* (Fig. 4, lane 2). The third profile of 100 and 750 bp was present in two heterozygote isolates from *V. vinifera* (Fig. 4, lanes 5 and 6). All isolates from *V. vinifera* showed the same profile using *Hha* I. Conversely, two different profiles were found for isolates from *E. globulus*. These differences represented homozygote and heterozygote genotypes in the parental isolates (Table 2).

The presence of heterozygosity was confirmed in allele segregation of single spore isolates. The progeny of MVHC 11518 showed two genotypes (Fig. 5, lanes 2 and 3) with *Alu* I endonuclease and only one genotype with *Hae* III (Fig. 5, lanes 14 to 19).

On the other hand, the progeny of MVHC 11511 showed only one genotype (Fig. 5, lanes 8 to 12) with *Alu* I and two genotypes with *Hae* III (Fig. 5, lanes 20 to 24).

The analysis of RFLP carried on the dikaryon produced by pairing monokaryons from *V. vinifera* and *E. globulus* hosts revealed the presence of fragments corresponding to each monokaryon, confirming that isolates from *Vitis* mated with those from *Eucalyptus* (Fig. 6).

Discussion

Species of *Inocutis* Fiasson & Niemelä and *Inonotus* P. Karst., such as *Inocutis jamaicensis* (Murrill) Gottlieb, J.E. Wright & Moncalvo, *Inonotus rickii* (Pat.) D.A. Reid and *I. patouillardii* (Rick)

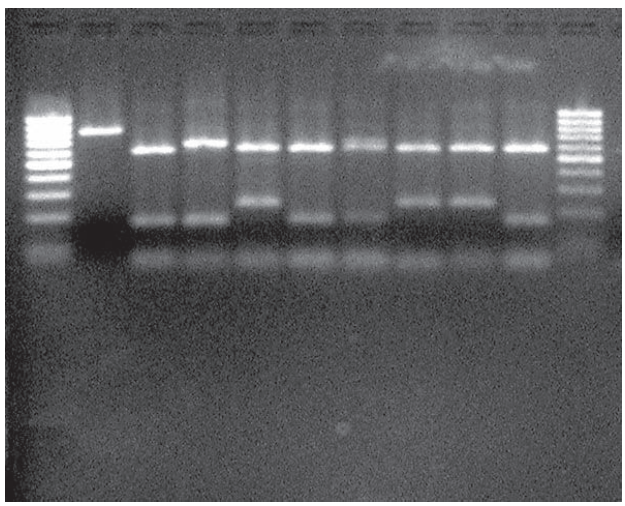

Fig. 3. ITS-RFLP patterns of *I. jamaicensis* using the restriction endonuclease *Alu* I. Lane 1 and 11, size marker (high-intensity: 500 and 1000 bp); lane 2, amplicon; lane 3, MVHC 11297; lane 4, MVHC 11394; lane 5, MVHC 11510; lane 6, MVHC 11511; lane 7, MVHC 11512; lane 8, MVHC 11518; lane 9, MVHC 11736; lane 10, MVHC 11769.

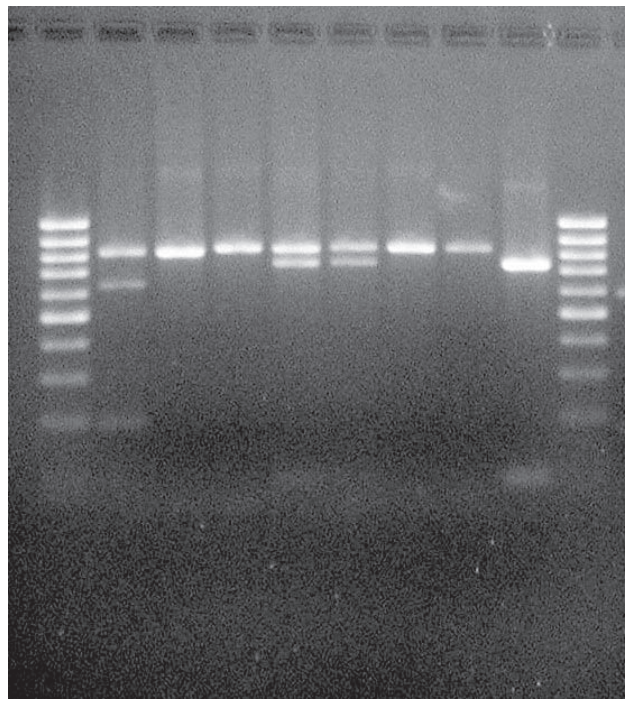

Fig. 4. ITS-RFLP patterns of *I. jamaicensis* using the restriction endonuclease *Hae* III. Lane 1 and 10, size marker (high-intensity: 500 and 1000 bp); lane 2, MVHC 11297; lane 3, MVHC 11394; lane 4, MVHC 11510; lane 5, MVHC 11511; lane 6, MVHC 11512; lane 7, MVHC 11518; lane 8, MVHC 11736; lane 9, MVHC 11769.

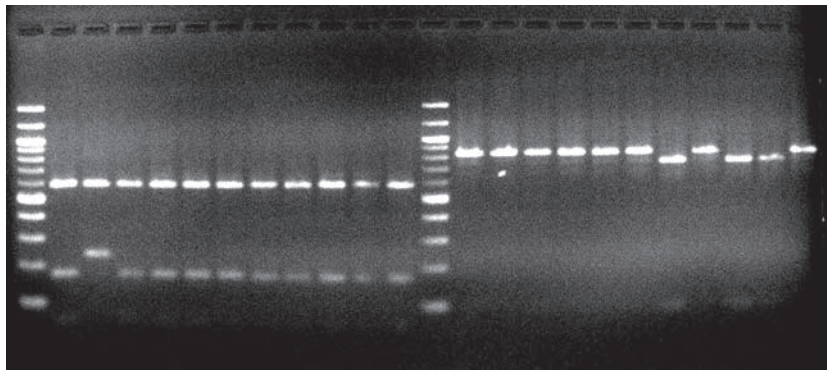

Fig. 5. ITS-RFLP patterns of single spore isolates of *I. jamaicensis* using restriction endonucleases *Alu* I (Lanes 2–12) and *Hae* III (Lanes 14–24). Lane 1 and 13, size marker 100 bp ladder (high-intensity: 500 and 1000 bp); lane 2, MVHC 11735; lane 3, MVHC 11736; lane 4, MVHC1 1737; lane 5, MVHC 11738; lane 6, MVHC 11739; lane 7, MVHC 11740; lane 8, MVHC11769; lane 9, MVHC 11770; lane 10, MVHC 11771; lane 11, MVHC 11772; lane 12, MVHC 11773; lane 14, MVHC 11735; lane 15, MVHC 11736; lane 16, MVHC 11737; lane 17, MVHC 11738; lane 18, MVHC 11739; lane 19, MVHC 11740; lane 20, MVHC 11769; lane 21, MVHC 11770; lane 22, MVHC 11771; lane 23, MVHC 11772; lane 24, MVHC 11773.

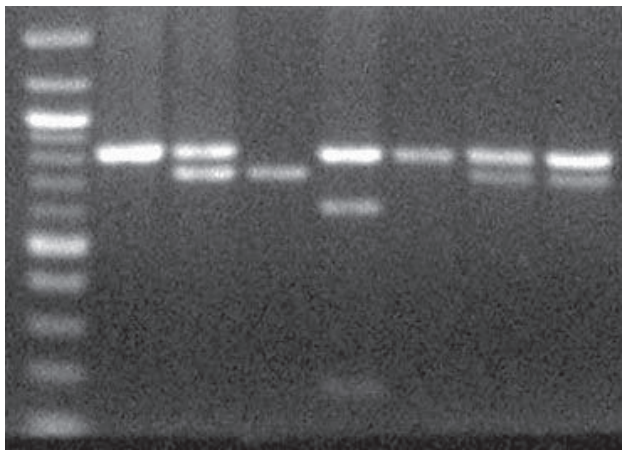

Fig. 6. ITS-RFLP patterns of single spore and dikaryotic isolates of *I. jamaicensis* using restriction endonuclease *Hae* III. Lane 1, size marker 100 bp ladder (high-intensity: 500 and 1000 bp); lane 2, amplicon; lane 3, MVHC 11511; lane 4, MVHC 11772; lane 5, MVHC 11297; lane 6, MVHC 11830; lane 7, heterokaryon from MVHC 11830+ MVHC 11772; lane 8, heterokaryon from MVHC 11771+ MVHC 11819.

Imazeki, have been cited as important wood rotting fungi in South America (Gottlieb *et al*., 2002; Martínez, 2005). Both genera are closely related and some authors preferred to maintain the above mentioned species in the heterogeneous genus *Inonotus* P. Karst. (Gilbertson and Ryvarden, 1987). However, using DNA sequence data Gottlieb *et al*. (2002) and Wagner and Fischer (2002) placed *Inonotus jamaicensis* in the genus *Inocutis*.

The specimens collected from grapevines associated with 'hoja de malvón' symptoms have similar micromorphological and cultural characteristics to those of *Inocutis jamaicensis* isolated from a heart rot in *E. globulus* in Uruguay (Bettucci, 2003; Bettucci *et al*., 2004; Martínez, 2005).

Moreover, the dikaryon and fruit body produced by pairing monokaryons derived from *V. vinifera* and *E. globulus* provides further evidence that these isolates belong to the same species.

Differences found in some restriction patterns could reflect a certain degree of variability between isolates, probably related with geographic origin. Morphological and molecular characteristics correspond to that of *Inocutis jamaicensis*, the basidiomycete associated with 'hoja de malvón' in grapevines of Mendoza, Argentina. *Inocutis jamaicensis* is broadly distributed in America from Arizona to Patagonia. In Argentina and Uruguay it is present on several plant species belonging to different families (Rajchenberg and Wright, 1998; Deschamps and Wright, 2000; Lupo *et al.,* 2005; Martínez, 2005). The presence of *I. jamaicensis* in grapevines could be due to the infection of basidiospores from fruit bodies developed on native plants or *Eucalyptus* plantations. Pruning wounds could be an important infection court. Recently *I. jamaicensis* was also found infecting grapevines in Uruguay (unpublished data).

Acknowledgements

The authors would like to thank the EUFORESSA-PDT for the financial support of the project *Inocutis jamaicensis* in standing *Eucalyptus globulus* and the Instituto de Sanidad y Calidad Vegetal de Mendoza (ISCAMEN), Argentina, for the project 'hoja de malvón' grapevine disease.

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Accepted for publication: December 12, 2005