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Armillaria species infesting vineyards in northwestern Spain

Olga Aguín-Casal[1], María J. Sáinz-Osés[2] and J. Pedro Mansilla-Vázquez[1,2*]
[1]*Estación Fitopatológica 'Do Areeiro', Subida a la Robleda s/n, E-36153 Pontevedra, Spain;*
[2]*Departamento de Producción Vegetal, Universidad de Santiago de Compostela, E-27002 Lugo, Spain;*
[*]*Author for correspondence (Phone: +34 986 841491; Fax: +34 986 864291; E-mail: pmansilla@efa-dip.org)*

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Abstract

Twelve vineyards in northwestern Spain were studied to assess the incidence of white root rot during 1995 and 1997. In both years, diseased plant material was collected and the *Armillaria* species responsible was identified on the basis of compatibility testing. Subsequently, restriction fragment length polymorphism (RFLP) analysis of the intergenic spacer region of the nuclear ribosomal RNA gene cluster was used to identify *Armillaria* species in another 45 symptomatic samples submitted for diagnosis from this area. During 1995, 9 of the 12 vineyards showed white root rot, although in eight cases less than 2% of plants were affected; in the remaining vineyard 17% of plants were diseased. During 1997, 10 of the 12 vineyards were affected and three showed a marked increase in the number of plants with white root rot (to 43%, in the vineyard in which 17% were affected in 1995). The compatibility method detected *Armillaria mellea* in samples from 10 of the 12 vineyards, and *Armillaria gallica* in samples from two vineyards. RFLP–PCR analysis detected four restriction patterns corresponding to *A. mellea*, *A. gallica* and *Armillaria cepistipes*: patterns mel 1 (*A. mellea*, 71% of samples), mel 2 (*A. mellea*, 18%), gal 1 (*A. gallica*, 9%), and cep 1 (*A. cepistipes*, 2%, i.e. a single sample). This is the first report of *A. gallica* and *A. cepistipes* infecting *Vitis* spp. The presence of these two *Armillaria* species may be related to the fact that the vineyards from which they were isolated were located on cleared forestry sites.

The genus *Armillaria* includes more than 40 species distributed throughout the world, some of which may cause a disease known as white root rot (Watling et al., 1991). In Europe, seven species have been described: *Armillaria borealis*, *Armillaria cepistipes*, *Armillaria ostoyae*, *Armillaria mellea*, *Armillaria gallica*, *Armillaria tabescens* and *Armillaria ectypa*. *A. mellea* and *A. ostoyae* are the most pathogenic and polyphagous, while the remaining species are considered saprophytes or weak parasites. *A. mellea* affects a wide range of hosts, including ornamental species, forest and fruit trees, and grape vines. *A. ostoyae* principally affects conifers.

There have been very few studies of the incidence of white root rot in grape (*Vitis* spp.). In grape, this disease is usually attributed to *A. mellea*, although it may also be caused by *Rosellinia necatrix* or *Roesleria hypogea* (Guillaumin, 1986), so that correct diagnosis and control requires appropriate tests to identify the pathogen present. In Europe, in 1880, Millardet reported for the first time white root rot in *Vitis* species samples from France, Hungary, Germany, Scotland, Bulgaria, Greece, Spain, Italy, England, Switzerland and Belgium, and attributed the disease to *A. mellea* (Raabe, 1962). Until the 1970s, most plant pathologists considered that *A. mellea* was the only species in the genus (Harrington and Wingfield, 1995). This was because species differentiation was based solely on the observation of morphological characters of the mycelium, rhizomorphs and fruiting bodies, which we now know to be ineffective for discriminating among the species of this genus. Furthermore, fruiting occurs

for only a short period in the year, and in some cases fruiting bodies are not present on infected material (Guillaumin and Berthelay, 1981).

The compatibility or pairing-test method proposed by Korhonen (1978) constituted a major advance in the study of *Armillaria*. Guillaumin (1986) was the first to apply this method to the identification of *Armillaria* species in grapevine samples with symptoms of white root rot from France, Italy and Portugal, in all cases finding *A. mellea*. In Switzerland, Prospero et al. (1998) likewise identified *A. mellea* in infected vines of the Riparia × Rupestris 3309 rootstock; these authors used the compatibility method and confirmed their diagnosis by the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of the IGS region of the nuclear ribosomal RNA gene cluster. They also performed a study of the incidence of this pathogen in 10 vineyards, finding that between 2% and 20% of plants were affected. This work was the first to apply molecular techniques to identify *Armillaria* spp. in grapevine. Specific primers for a number of *Armillaria* species had been previously proposed by Anderson and Stasovsky (1992), and Harrington and Wingfield (1995) first designed an identification procedure for *Armillaria* species based on polymorphisms in the amplified IGS region of the rDNA. As far as we know, there have been no other studies of the incidence or identification of species causing white root rot in European vineyards.

In Spain, viticulture is a pillar of the rural economy. As in many countries, white root rot has generally been attributed to *A. mellea*, but there have been no detailed studies to confirm that this is the case or to evaluate the incidence of *Armillaria*, in any of the major wine-producing areas. In Galicia (northwestern Spain), a region with five denominations of origin (D.O.), the last 10 years have seen a significant increase in diagnosis requests for samples affected by white root rot, and this disease – for which no effective control method is currently available – is causing very significant losses in a wide range of vineyards. Vineyards are frequently established on cleared forestry sites, which previously held mainly conifers (*Pinus*) or occasionally broad-leaved trees of *Eucalyptus* or *Quercus*. These tree genera are hosts not only for *A. mellea* but also for *A. ostoyae*, *A. gallica*, *A. cepistipes* and *A. tabescens* (Guillaumin et al., 1993). The aim of this study was to investigate the incidence of white root rot in vineyards of the D.O. Rías Baixas (a D.O. producing prestigious high-quality white wines), and to identify the *Armillaria* species present in infected plant material on the basis of compatibility testing of cultured isolates, and RFLP–PCR techniques.

Samples were obtained from 12 vineyards in the D.O. Rías Baixas (Galicia, NW Spain). A first inspection of grapevines affected by white root rot was performed in October 1995, and a second in October 1997 to assess the change of the disease over time. In addition, each vineyard was explored for fruiting bodies of *Armillaria*. The total area explored was 85.2 ha, containing a total of 138,380 plants, almost all of them on 196-17 Castel or 110 Richter rootstocks, between 2 and 12 years old (Table 1). In each vineyard, we inspected all dead grapevines and all grapevines showing evidence of nutritional deficiency or decay of aerial parts, such as chlorotic leaves, poor shooting and wilting. A small trench was dug around the vine to allow detailed examination of the collar and superficial roots, carefully detaching the bark with the aim of detecting mycelia. Root samples were collected from all weak or dead plants in which mycelia were detected, where possible from the wettest part of the trench. The samples were placed in plastic bags that were maintained in a cold chamber at 4 °C until isolation or analysis. For each sample, small fragments of root with mycelium were cut and then first disinfected by washing in: (a) distilled water for 15–30 min, changing the water several times when necessary; (b) a 1 : 1 mixture of distilled water and absolute ethanol for 5–10 min and (c) distilled water again for 15–30 min. The samples were allowed to dry, then seeded onto Petri dishes in malt-extract agar (AM) or benomyl–dichloran–streptomycin media (Worrall, 1991). Seeding was done with 4–5 root fragments per Petri dish, and three dishes per culture medium and sample. The entire seeding process was done under sterile conditions. The dishes were then sealed with Parafilm® and maintained at 24 ± 1 °C in the dark.

Species identification of field isolates was carried out by the compatibility method (Korhonen, 1978). The fungal material consisted of 16 diploid isolates obtained by the procedure described above. Each diploid isolate was compared with haploid reference strains of the six European species: *A. borealis*, *A. cepistipes*, *A. ostoyae*, *A. gallica*, *A. mellea* and *A. tabescens*. The reference strains were supplied by Dr. Kari Korhonen (Finnish Forest Research Institute, Finland) and Dr. Jean-Jacques Guillaumin (INRA, Clermont-Ferrand, France). Two haploid reference strains were used for each of the six *Armillaria* species. For the compatibility tests, two fragments of the diploid isolate and the haploid reference strain were cultivated

Table 1. Characteristics and infection levels of the twelve vineyards included in the study

Vineyard	Area (ha)	Rootstock	Age (years)	No. of plants	% affected	
					1995	1997
San Antón	1.5	196-17 Castel	10	1250	0	8
Xielles	1.5	110 Richter	6	2000	0	0
Iglesario Tremoedo	0.5	196-17 Castel	15	580	17.2	43.1
Cornazo	0.7	110 Richter, 196-17 Castel	4–8	650	1.5	15.3
Baión	2	110 Richter, 196-17 Castel	10	1200	0	0
Torre Quintáns	4	110 Richter	8	3000	2	3.3
Agro de Bazán	11	196-17 Castel	10	9500	0.1	0.05
Pazo Barrantes	7	196-17 Castel	8	20 000	0.1	0.07
Pazo Señoráns	3	196-17 Castel, 110 Richter	4–8	3700	0.5	1.3
Meder	2	196-17 Castel, 110 Richter	8	1500	0.5	1.6
Fillaboa	10	196-17 Castel, 41-B, 110 Richter	8	25 000	0.06	0.3
Lagar de Fornelos	42	110 Richter	2–12	70 000	0.01	0.03
Total	85.2			1 38 380		

on AM medium in 9-cm-diameter Petri dishes: i.e., each test was duplicated on a single Petri dish. The whole process was performed twice for each diploid isolate. A total of 768 tests were thus performed (16 diploid isolates × 12 haploid reference strains × 2 tests per Petri dish × 2 replications). The Petri dishes were maintained in a culture oven at 24 ± 1 °C in the dark. After 3–4 weeks, mycelial morphology was examined to decide whether the two strains were compatible, in accordance with the criteria of Korhonen (1978) and Guillaumin and Berthelay (1981).

The RFLP–PCR technique was used to analyse a total of 45 grapevine samples affected by white root rot, submitted for diagnosis by wine growers and agricultural extension technicians from D.O. Rías Baixas vineyards in 1998, 1999 and 2000. As controls, samples of haploid reference strains of *A. mellea*, *A. ostoyae*, *A. gallica*, *A. cepistipes*, *A. borealis* and *A. tabescens* were included. The material analysed in most cases was mycelium collected from the plant, although in a few cases rhizomorphs or mycelium obtained after pure culture isolation were used. PCR amplification and RFLP analysis of the IGS region of the rDNA for species identification was done by the method of Mansilla et al. (2000), which is based on the methods described by Harrington and Wingfield (1995) and Pérez et al. (1999), but modified to allow direct identification of field material without any need for pure culture isolation.

For PCR, rDNA was extracted with the EZNA Fungal DNA Miniprep kit (Omega Biotek), but without addition of either RNase or mercaptoethanol (Martín and Torres, 1998). The primers used were two oligonucleotides recommended for *Armillaria* by Anderson and Stasovsky (1992), and synthesized by Perkin-Elmer (Madrid): LR12R and O-1. The sequence of LR12R is 5′-TGAACGCCTCTAAGTCAGAA-3′ (Veldman et al., 1981), and that of O-1 is 5′-AGTCCTATGGCCGTGGAT-3′ (Duchesne and Anderson, 1990). One microlitre of the DNA solution was introduced into a microcentrifuge tube containing a Ready-To-Go PCR bead (Amersham-Pharmacia) and 0.5 µl of each primer (10 pmol/µl), and made up with sterile water to a final volume of 25 µl. PCR was performed in a Mastercycler Personal thermocycler (Eppendorf) under the conditions proposed by Harrington and Wingfield (1995) and modified by Pérez et al. (1999): initial denaturation for 95 s at 95 °C; then 35 cycles of annealing for 60 s at 60 °C, elongation for 120 s at 72 °C, and denaturation for 60 s at 95 °C; then final elongation for 10 min at 72 °C. All samples were analysed in duplicate. A negative control was included in all assays, to confirm the absence of contamination. The PCR products were separated and visualized by agarose gel electrophoresis. In each assay, a negative control and a 100-bp marker (marker XIV, Roche Diagnostics) were also run. The gels were stained with ethidium bromide

(0.5 μg/ml), and examined under a UV transilluminator. Having confirmed amplification, an RFLP analysis was performed. The amplified DNA was digested with the restriction endonucleases *Alu* I, *Nde* I and *Bsm* I (Roche Diagnostics). The fragments digested by the three enzymes were analysed by electrophoresis at 100 V in 1× TBE buffer on a 3% agarose gel, with ethidium bromide staining. In each assay a negative control and a 100-bp marker (marker XIV, Roche Diagnostics) were also run. Gel images were analysed with the 1D-Manager densitometry program (TDI, Madrid). For identification, the restriction pattern of each sample was compared with those described by Harrington and Wingfield (1995) and Pérez et al. (1999) for the different European and North American species of *Armillaria*.

Inspection of 12 vineyards in northwestern Spain revealed the widespread presence of white root rot. In 1995, the number of grapevines with aerial symptoms of disease, considering all 12 vineyards (Table 1), was 251 (1.8% of the total). In most vineyards, white root rot was observed in restricted areas affecting a small number of plants (less than 1% of the total). However, in two vineyards (Cornazo and Torre Quintáns) between 1.5% and 2% of the plants were affected, and in a third (Iglesario Tremoedo) 17.2% were affected. There were also three plantations (San Antón, Xielles and Baión) in which none of the plants showed symptoms. In 1997, although two vineyards (Xielles and Baión) remained unaffected, a moderate increase in the incidence of affected plants was observed in most vineyards, and a marked increase occurred in three vineyards: specifically, incidence increased from 17% in 1995 to 43% in 1997 in Iglesario Tremoedo; from 1.5% to 15.3% in Cornazo and from 0% to 8% in San Antón. The *Armillaria* isolates that infected the vines were obtained from infected plant material, whether rhizomorphs or mycelium located in the plant's roots or collar, since fruiting bodies were not found in any of the vineyards in either 1995 or 1997, despite the fact that inspections were carried out in October. It is frequently difficult to obtain fruiting bodies, since these are often present only in some years and only for a few days in autumn (Fox, 2000). In addition, grapevine plants severely affected by white root rot are often uprooted after diagnosis of the disease, preventing fruiting body production even when climatic conditions are suitable. This means that, until recently, definitive identification of the causal agent of the disease required pure culture isolation.

The compatibility tests indicated that fourteen isolates were compatible with the haploid reference strains of *A. mellea*, and two with *A. gallica*. However, identification was not always straightforward. Five isolates identified as *A. mellea* and the two isolates identified as *A. gallica* showed borderline compatibility with other *Armillaria* species. This probably reflects the fact that these were diploid–haploid confrontations, in which compatibility is perhaps less easily observed than in haploid–haploid confrontations (Korhonen, 1978; Guillaumin and Berthelay, 1981; Pérez et al., 2000).

When applying molecular techniques, the primers LR12R and O-1 amplified a product of ∼900 bp in all samples. After digestion of the amplified DNA with the enzyme *Alu* I, two or three smaller DNA fragments were obtained in each sample, with absolute repeatability. Four restriction patterns were detected, corresponding to those described by Harrington and Wingfield (1995) and Pérez et al. (1999) for *A. mellea* pattern mel 1, *A. mellea* pattern mel 2, *A. gallica* pattern gal 1 and *A. cepistipes* pattern cep 1. Therefore the restriction patterns obtained indicate the presence in our samples of three of the seven species of *Armillaria* described in Europe. Until now, only the presence of *A. mellea* and *A. ostoyae* had been recorded in Spain (Kile et al., 1991). In the majority of infected samples (89%) we identified *A. mellea*, one of the most polyphagous and pathogenic species of the genus. This result is in line with Guillaumin (1986) and Prospero et al. (1998), both of whom isolated only this species in grapevine, and who indicated it to be responsible for major economic losses in the major European wine-growing areas. Pérez et al. (1999) described two restriction patterns for *A. mellea*, mel 1 and mel 2, in a study of numerous woody genera (including *Acer*, *Betula*, *Ligustrum* and *Prunus*), indicating that within Europe this pathogen shows intraspecific variation. Prospero et al. (1998) found only the mel 1 pattern in Swiss vineyards. In the present study, most *A. mellea* isolates (32 samples, 71%) showed the mel 2 pattern (320 and 155 bp), but eight isolates showed the mel 1 pattern (320, 180 and 155 bp). As far as we know, this is the first detection of the mel 2 pattern in grapevine, which perhaps simply reflects the recentness of the introduction of molecular techniques in the identification of *Armillaria* species.

Restriction fragment length polymorphism analysis also revealed that two of the samples were infected with *A. gallica*, which showed a 400, 240 and 190 bp restriction pattern. This corresponds to the gal 1 pattern described by Pérez et al. (1999) in *Euonymus*,

Quercus, *Picea* and *Pinus*. These authors likewise observed intraspecific variation in European isolates of *A. gallica*, describing three restriction patterns, of which the most frequent seems to be gal 1. We also detected the species *A. cepistipes*, although only in one sample. The restriction pattern (400–200–190 bp) corresponds to that defined as cep 1 by Pérez et al. (1999), for isolates obtained from symptomatic *Picea* and *Pinus*. This species shows many morphological and behavioural similarities to *A. gallica*, which makes differentiation by traditional methods difficult, but which RFLP–PCR techniques resolve easily. The presence of *A. gallica* and *A. cepistipes* on grapevine has not been previously reported, and it would be interesting to investigate their impact in vineyards. *A. gallica* is considered a saprophyte or weak parasite, and is frequent in broad-leaved tree species (Smith et al., 1992). *A. cepistipes* is considered a weak parasite, and is found not only in broad-leaved species (Intini, 1997) but also in conifers (Korhonen, 1978). The presence of these two *Armillaria* species in vineyards must have been related to the fact that the vineyards from which they were isolated were located on cleared forestry sites. These results point out the need of a correct diagnosis of the *Armillaria* species causing white root rot in vineyards to undertake appropriate management and control methods.

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