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SHORT COMMUNICATION

Cobweb disease on oyster culinary-medicinal mushroom (*Pleurotus ostreatus*) caused by the mycoparasite *Cladobotryum mycophilum*

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Abstract

In autumn 2016, symptoms of cobweb disease were observed on cultivated *Pleurotus ostreatus* crops in Spain. Based on morphological and genetic analyses, the causal agent of cobweb was identified as *Cladobotryum mycophilum*. Two cropping trials, inoculated with *C. mycophilum*, were set up to evaluate the pathogenicity of this causal agent of cobweb. Two different inoculation methods were used: (i) an agar plug was taken from the growing edge of a *C. mycophilum* isolate and placed in the centre of each hole in the block of *P. ostreatus* substrate (IP), and (ii) spraying each hole with a conidial suspension (ISC). In both trials, there were significant differences in disease incidence between the controls and the inoculated samples, but there were no significant differences between the two inoculation treatments. Between 75 and 87.5% of the blocks of the IP treatments and 100% of the blocks of the ISC treatments showed cobweb symptoms. *Cladobotryum mycophilum* was consistently re-isolated from the inoculated blocks (100%). These findings suggest that *C. mycophilum* can equally cause cobweb disease in *A. bisporus*, *P. eryngii*, and *P. ostreatus* mushroom crops.

Keywords Oyster mushroom disease · Phylogenetic analysis · Pathogenicity · Yield loss

Pleurotus ostreatus (Jacq.) P. Kumm., commonly known as the oyster mushroom, is one of the most widely cultivated and consumed edible mushrooms in the world. This species possesses nutritional and medicinal benefits and some attractive culinary features such as high fibre and low-fat contents. Oyster mushroom also has antioxidant, antiviral, antimicrobial, antitumor, antimutagenic, antihypercholesterolemic, antihyperglycemic, and hepatoprotective activities (Wasser and Weis 1999; Iwalokun et al. 2007; Patel et al. 2012; Rodríguez Estrada and Pecchia 2017). *Pleurotus ostreatus* is a very versatile mushroom because it can use substrates with a C/N ratio ranging between 30 and 300:1 (Muez and Pardo 2001). It is often viewed as one of the easiest and most cost-effective mushrooms to cultivate at different commercial and experimental scales due to its capability to grow in a wide range of agricultural and forest wastes using different production methods (Bonatti et al. 2004; Chang and Miles 2004; Mandeel et al. 2005; Sánchez 2010; Rocha Vieira and Nogueira de Andrade 2016). In Spain, *P. ostreatus* is grown on pasteurized lignocellulosic substrates made of wheat and barley straw supplemented with delayed-release nutrients (protein-rich supplements) and packed in black plastic blocks (18–20 kg) with pre-punched holes (Muez and Pardo 2001; Picornell et al. 2017). Oyster mushroom production is estimated to be more than 16,800 tons per year in Spain.

In autumn 2016, symptoms of cobweb were observed affecting several clusters of mature fruitbodies on cultivated oyster mushroom farms in Castilla-La Mancha (Spain). Cobweb disease is found in all mushroom-growing countries worldwide and generally causes major crop losses, especially in white button mushroom [*Agaricus bisporus* (Lange) Imbach)] (Carrasco et al. 2017a; Verma 2017). Recently, *Cladobotryum mycophilum* (Oudemans) W. Gams & Hoozemans has been identified as the causal agent of cobweb in *A. bisporus* Spanish mushroom crops (Gea et al. 2012; Carrasco et al. 2016) and cultivated king oyster mushroom [*Pleurotus eryngii* (DC.: Fr.) Quél.] in Spain and Korea (Gea et al. 2011; Back et al. 2012; Kim et al. 2012). The aims of this

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study were to identify and characterize the pathogen responsible for cobweb disease in *P. ostreatus* Spanish crops and to test the pathogenicity of the cobweb causal agent.

Cobweb appeared at the first flush, growing first at the base of the fruitbodies, among the stems that form part of the cluster, subsequently spreading to the rest of the fruitbody by means of a fine grey-white mycelium, finally sporulating to produce masses of dry spores. The pinheads and fruitbodies concerned turned pale yellow and eventually rotted. The mycelium quickly covered oyster mushroom debris, pinheads, stalks, pileus and gills, resulting in decomposition of the entire fruitbody (Fig. 1). Unlike *A. bisporus*, no symptoms of cap spotting were seen on the fruitbodies of *P. ostreatus*, although some oyster mushrooms showed colonies of the mycopathogenic fungus growing over their surface.

Samples were collected in autumn 2016 from an oyster mushroom farm situated in Castilla-La Mancha (Spain). Symptomatic portions of fruitbodies of *P. ostreatus* were placed on potato dextrose agar (PDA; Oxoid, England) medium at 22 °C in darkness. Two isolates were used in this study. Fungal structures were mounted on glass slides with lactic acid for microscopic examination. Measurements of all taxonomically relevant characters (conidium size, number of septa per conidium) were performed using Nikon software (NIS-Elements Advanced Research, Nikon, Japan). One hundred conidia were measured from each isolate. The presence or absence of phialide extensions or rachides was recorded. Chlamydospore and/or microsclerotium production, the colony reverse colour and the odour detectable upon lifting the lid of the Petri dish was also annotated. The isolates were then identified according to the descriptions from Gams and Hoozemans (1970), Rogerson and Samuels (1994), Carrasco et al. (2016, 2017a) and Gea et al. (2017).

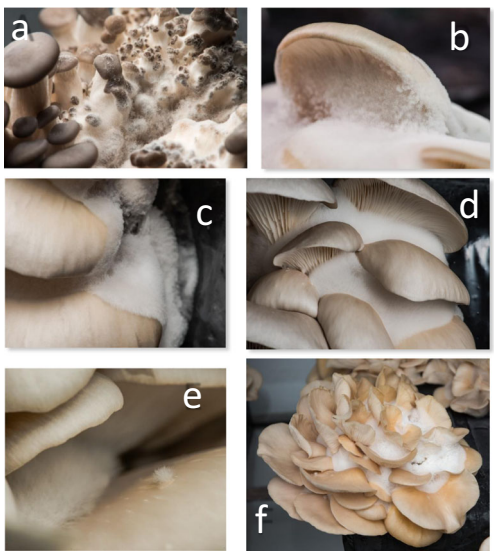

Fig. 1 Symptoms of cobweb in *Pleurotus ostreatus* mushroom crops: **a** Cobweb mycelium (*Cladobotryum mycophilum*) growing over *P. ostreatus* primordia; **b**-**c**-**d** Cobweb mycelium growing over *P. ostreatus* fruit bodies; **e** *Cladobotryum mycophilum* colonies growing over the surface of the oyster mushrooms; **f** *Pleurotus ostreatus* fruit bodies attacked by cobweb mycelium (*Cladobotryum mycophilum*) turned pale yellow and eventually rotted

Aerial and cottony mycelium spreads rapidly on PDA from the inoculation plug. The whitish to buff mycelium sporulates profusely in a few days, mainly at the edge of the colony. The colonies acquire yellow hues and then turn pink, before evolving to a strong blood red when old because the pigment aurofusarin is copiously secreted by the hyphae submerged in the medium while the aerial mycelium remains white (Põldmaa 2011). These isolates produce chlamydospores and some microsclerotia (Lane et al. 1991; McKay et al. 1999), and the cultures lack the distinctive camphor odour, normally associated with *C. mycophilum* (Gams and Hoozemans 1970; Carrasco et al. 2016, 2017a; Gea et al. 2017).

Conidiogenous cells were with no evident rachis. Conidia were hyaline, cylindrical to ellipsoidal, sometimes ovoid, with a prominent and central hilum, (12.2)15.0–29.8(34.8) μm long, (4.0)5.8–14.8(16.7) μm wide, 0- to 2-septate, with a predominance of 2-celled (80%) conidia. The isolates were identified as *Cladobotryum mycophilum* based on the above characteristics.

Genomic DNA from the two fungal cultures (PO1 and PO2) was isolated using Extract-N-Amp (Sigma) and the Internal Transcribed Spacer (ITS) region amplified using the primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). PCR products were purified using ExoSAP-IT (GE Healthcare) and sequenced bidirectionally using a BigDyeVR v.3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI3730 Genetic Analyzer. DNA sequences were edited in Sequencher v.4.2 (Gene Codes Inc.) and aligned together with selected sequences from *C. mycophilum*, *C. varium* and *C. dendroides* downloaded from GenBank (Gea et al. 2017, 2018), using the algorithm Q-INS-i implemented in MAFFT v7.164b (Katoh et al. 2002). Phylogenetic analyses were carried out under the maximum likelihood criterion using RAxML (Stamatakis 2014) implemented in raxmlGUI v1.3.1 (Silvestro and Michalak 2012). The GTRGAMMA model of evolution was used and branch support was assessed using nonparametric bootstrap with 10,000 replicates. The resultant tree was rooted with *Sepedonium* sp. (HQ604857).

Identical ITS sequences were obtained from PO1 and PO2 fungal isolates (GenBank accession Nos. MH042302 and MH042303). BLAST searches showed highest similarity with ITS sequences of *C. mycophilum* (teleomorph *Hypomyces odoratus*). Phylogenetic analyses showed that the isolates from *P. ostreatus* clustered with isolates belonging to the *C. mycophilum* Group I as defined by Gea et al. (2017), confirming their identification (Fig. 2).

Two cropping trials (A and B) were set up in an experimental mushroom growing room following the standard

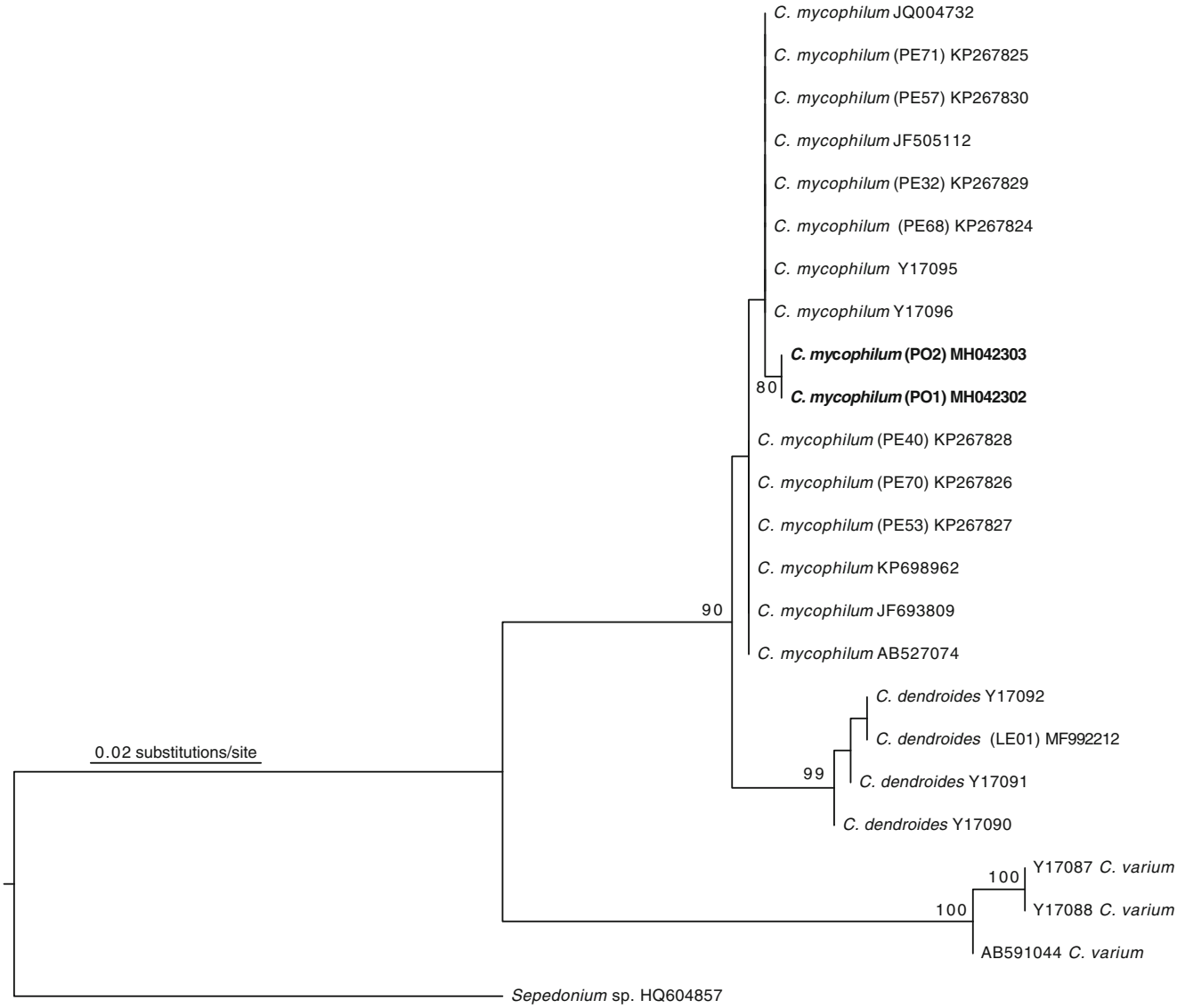

Fig. 2 Maximum likelihood tree using full nuclear ribosomal internal transcribed spacer (ITS) sequences from *Cladobotryum* spp. Only nonparametric bootstrap values over 70 are shown above or below branches. Sequences obtained in this study from isolates PO1 and PO2 growing on *Pleurotus ostreatus* are highlighted in bold. *Sepedonium* sp. was used to root the tree

practices used in Spanish oyster mushroom farms. Each pathogenicity trial was performed using 28 blocks (18.4 kg, 5 prepunched holes, 2.5 cm in diameter) containing pasteurized, spawned (strain PLC-35, Champinter Soc. Coop., Villamalea, Albacete, Spain), supplemented (50 g Super Champ block^{-1}) and incubated *P. ostreatus* substrate. Two different inoculation methods were carried out using *C. mycophilum* isolate PO2: IP and ISC. In the IP method all the holes of the eight blocks were inoculated fourteen days after starting the crop cycle (when the mycelium of *P. ostreatus* had colonized the whole substrate) with a 1 cm agar plug taken from the growing edge, inverted and placed in the centre of each hole. In the ISC method, all the holes of the eight blocks were inoculated nineteen days after starting the crop cycle, when primordia had formed, with a conidial suspension (1×10^6 conidia ml^{-1}, 10 ml per block). The holes of the six remaining blocks were sprayed with sterile distilled water as a control, and another six blocks were inoculated with 1 cm agar plug without *C. mycophilum* mycelium, inverted and placed in the centre of each hole.

The environmental conditions maintained throughout cropping were: temperature 15–18 °C, 80–90% relative humidity, 600–900 ppm CO_2 and cool-white fluorescent light. *Pleurotus ostreatus* fruitbodies were harvested daily for each treatment during the yield period. The numbers of clusters per block and the total weight of the fruiting bodies were recorded for each treatment. Harvested mushrooms were classified as either healthy or infected by *C. mycophilum*. The effect of cobweb disease on oyster mushroom productivity was evaluated by the disease incidence, calculated as the ratio of the

fresh weight of total yield of diseased fruiting bodies to the total weight of harvested mushrooms (healthy and diseased). Blocks were also inspected daily for cobweb symptoms, and Koch's postulates were verified by re-isolating the pathogen on PDA from the artificially inoculated fruitbodies.

An analysis of variance (ANOVA) was used to test for the effect of treatment based on the different yield parameters. Data were analyzed separately in each trial. A Fisher's LSD (least significant difference) means separation test was used to compare means ($P < 0.05$). Percentages were arcsine square-root transformed before analysis. Data are reported as back-transformed means. Statistical analyses were carried out using Statgraphics Plus 5.1 (Statistical Graphics Corp., Princeton, NJ).

In both trials, the first symptoms of cobweb observed in IP treatments were noticed seven days after inoculation, while in the ISC treatments the first symptoms of cobweb were noticed six days after inoculation. In both cases the symptoms were characterized by the mycelium of *C. mycophilum* growing at the base of the fruitbodies. Another symptom initially observed in the ISC treatments was the presence of small colonies of *C. mycophilum* growing on the surface of the fruitbodies. The control blocks remained symptomless.

In trial A, 87.5% of the blocks of the IP treatment and 100% of the ISC treatment showed cobweb symptoms and there were significant differences between the control (C) and the inoculation treatments (IP, ISC) in the percentage of clusters of oyster mushroom affected by cobweb, in the disease incidence, and in the yield of diseased oyster mushrooms (Table 1). There were no significant differences between the two inoculation treatments (IP, ISC) in any case.

In trial B, 75% of the blocks of the IP treatment and 100% of the ISC treatment blocks showed cobweb symptoms and there were significant differences between the control (C) and the inoculation treatments (IP, ISC) in the percentage of clusters of oyster mushroom affected by cobweb, in the disease incidence and in the yield of diseased oyster mushrooms (Table 1). There were no significant differences between the two inoculation treatments (IP, ISC) in any case.

The symptoms of cobweb observed on cultivated oyster mushroom are very similar to those described for king oyster fruitbodies (Gea et al. 2017). In both cases, *C. mycophilum* has been identified as the causal agent of cobweb disease in Spanish *P. ostreatus* and *P. eryngii* mushroom crops. Both the morphological characteristics and the sequenced ITS regions of the *C. mycophilum* isolates collected from *P. ostreatus* crops in Spain are similar to those described for isolates of *C. mycophilum* collected from Spanish *A. bisporus* and *P. eryngii* mushroom crops (Carrasco et al. 2016; Gea et al. 2017). Phylogenetic analyses confirmed the identification and showed that the isolates from *P. ostreatus* clustered with isolates belonging to the *C. mycophilum* Group I, as defined by Gea et al. (2017) and isolates from *A. bisporus*. These findings seem to suggest that *C. mycophilum* can indifferently cause cobweb disease in *A. bisporus*, *P. eryngii*, and *P. ostreatus* mushroom crops. Recently, symptoms of cobweb have also been observed on cultivated shiitake [*Lentinula edodes* (Berk.) Pegler] mushroom crops in Castilla-La Mancha (Spain), although the causal agent was identified as *Cladobotryum dendroides* (Bull.: Fr.) W. Gams & Hoozemans (Gea et al. 2018). Therefore, an accurate identification of the cobweb causal agent is necessary

Table 1 Effect of two inoculation methods of *Cladobotryum mycophilum* isolate PO2 on the total oyster mushroom yield in two pathogenicity trials (A and B). Values followed by different letters in the columns (comparing each trial) are significantly different according to Fisher's LSD (least significant difference) at $P = 0.05$

Trial	Treatment[a]	Clusters of oyster mushroom affected by cobweb (%)	Disease incidence (%)	Yield of diseased oyster mushrooms (kg/block)
Trial A	CP	0.0 a	0.0 a	0.0 a
	CSC	0.0 a	0.0 a	0.0 a
	IP	70.8 ± 35.2 b	64.3 ± 35.0 b	2.6 ± 1.6 b
	ISC	82.1 ± 24.4 b	85.9 ± 31.6 b	3.4 ± 1.3 b
		$P = 0.0000$	$P = 0.0000$	$P = 0.0000$
		$F_{3,25} = 22.89$	$F_{3,25} = 23.08$	$F_{3,25} = 45.44$
Trial B	CP	0.0 a	0.0 a	0.0 a
	CSC	0.0 a	0.0 a	0.0 a
	IP	84.4 ± 35.2 b	82.9 ± 35.9 b	3.1 ± 1.4 b
	ISC	69.1 ± 34.2 b	80.6 ± 32.2 b	2.9 ± 1.2 b
		$P = 0.0000$	$P = 0.0000$	$P = 0.0000$
		$F_{3,26} = 21.17$	$F_{3,26} = 23.81$	$F_{3,26} = 21.27$

[a] CP: Control consisting on oyster mushroom blocks inoculated with a 1 cm diam potato-dextrose agar plug without *C. mycophilum* mycelium and placed in the centre of the pre-punched holes in the blocks; CSC: Control consisting on oyster mushroom blocks sprayed with sterile distilled water; IP: Oyster mushroom blocks inoculated with a 1 cm diam potato-dextrose agar plug taken from the growing edge of the *C. mycophilum* isolate and placed in the centre of the pre-punched holes in the blocks; ISC: Oyster mushroom blocks inoculated with a conidial suspension of 10^6 conidia ml^{-1}

for effective management of the disease, especially in view of the propensity of *C. mycophilum* to develop fungicide resistance (McKay et al. 1998; Grogan 2006; Carrasco et al. 2017b).

The results obtained in the two pathogenicity cropping trials indicate that cobweb successfully established itself with the two different inoculation methods used in the inoculated blocks but was not detected in the control blocks. The findings confirm that the fragments of mycelium and conidia of *C. mycophilum* can infect the substrate completely colonized by the vegetative mycelium of *P. ostreatus*, and that air-borne conidia can infect *P. ostreatus* primordia and fruitbodies. These observations agree with those of Adie et al. (2006) in *A. bisporus* mushroom crops, who stressed the possibility of a rapid and widespread dispersal of conidia within the facility.

The present study widens the list of culinary-medicinal mushrooms that may be affected by cobweb disease and underlines the fact that *C. mycophilum* is now probably the most common causal agent of cobweb disease in cultivated mushrooms crops worldwide.

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