CLADOBOTRYUM MYCOPHILUM, CAUSAL AGENT OF COBWEB DISEASE ON COMMERCIAL *AGARICUS BISPORUS* AND *PLEUROTUS ERYNGII* CROPS IN SPAIN

GEA FJ[1], CARRASCO J[1]*, SANTOS M[2], DIÁNEZ F[2] AND NAVARRO MJ[1]

[1]Centro de Investigación, Experimentación y Servicios del Champiñón (CIES), 16220 Quintanar del Rey, Cuenca Spain
[2]Departamento de Agronomía, EscuelaPolitécnica Superior, Universidad de Almería, 04120 Almería, Spain
jaime.carrasco@uam.es /fjgea.cies@dipucuenca.es

ABSTRACT

Between 2008 and 2011, outbreaks of cobweb were observed in commercial white button and king oyster mushroom crops in Castilla-La Mancha (Spain) based on morphological and genetic analysis the casual agent was identified as *Cladobotryum mycophilum*. Two *Agaricus bisporus* mushroom cropping trials inoculated with *C. mycophilum* were performed. The total area of the crop affected by cobweb was 30% in the inoculated blocks of trial A and 45% in trial B. The non-inoculated blocks remained healthy. Compared with the uninoculated controls, a decrease in the yield of 10.7% was observed in trial A and 9.1% in trial B. Pathogenicity trial was also performed using blocks containing sterilized, spawned and incubated *P. eryngii* substrate. The first cobweb symptoms developed 23 days after inoculation and *C. mycophilum* was consistently re-isolated from nine (37.5%) of the inoculated blocks. Non-inoculated blocks remained healthy. In a second test, conidial suspensions of three isolates of *C. mycophilum* were inoculated onto *P. eryngii* fruit bodies. *C. mycophilum* grew between 80 and 85% of the inoculated fruit bodies, while the control fruit bodies remained symptomless.

Keywords: mushroom disease, pathogenicity, white button mushroom, king oyster mushroom

INTRODUCTION

Several fungal diseases can affect *A. bisporus* and *P. eryngii* commercial crops [1, 2]. Among them, cobweb is a common disease of mushroom in every country that causes yield reduction and subsequently economic losses [3]. Different taxa from the genus *Cladobotryum* Nees can generate the pathology in *A. bisporus* and *P. eryngii*. *Cladobotryum dendroides* (Bull.: Fr.) W. Gams & Hoozem. (conidial state of *Hypomycesrosellus*) has hitherto been considered to be the most common cause of cobweb in *A. bisporus* crops. However, the most common causal agent is now *Cladobotryum mycophilum* (Oudem.) W. Gams & Hoozem.(conidial state of *Hypomycesodoratus*). This cobweb pathogen varies somewhat from published descriptions of *C. mycophilum* in conidial septation and the cultures lack the characteristic camphor-odour [1, 4, 5]. Other species like *C. multiseptatum*, *C. varium* and *C. verticillatum* from the same genus have been reported causing the disease in white button mushroom crops [6-8]. *C. dendroides* has been reported as pathogen to *Pleurotus eryngii* [9] and recently, *C. mycophilum* has also been identified as the causal agent of cobweb in cultivated king oyster mushroom in Spain and Korea [10-13].

Between 2008 and 2011 a strong presence of the cobweb disease were observed in *A. bisporus* mushroom crops in Castilla-La Mancha (Spain). A fluffy white mycelium grows over the casing or carpophores that quickly decay and become rotten. This feathery mycelium becomes denser due to a profuse sporulation occasionally acquiring yellow and pink hues when aged. Brown or grey spotting can also affect the mushroom caps. The first kind of spots, with a fuzzy outline, appear over the infected caps when a harmful airborne spore lands over a button mushroom and germinates into its surface, while the grey spots are due to a infection by the pathogenic mycelium (Fig. 1 a-d).

In the same way, symptoms of cobweb were also observed in cultivated *P. eryngii* during 2010. A soft-light cobweb-like mycelium appeared at the end of the crop cycle affecting substrate cultivated. Small white patches on the casing soil at first, subsequently spreading to the nearest king oyster mushroom by means of a fine gray-white mycelium, and eventually sporulating to produce masses of dry spores. The mycelium can quickly cover pinheads, stalks, pileus, and gills, eventually resulting in decomposition of the entire fruit body (Fig. 1 e, f).

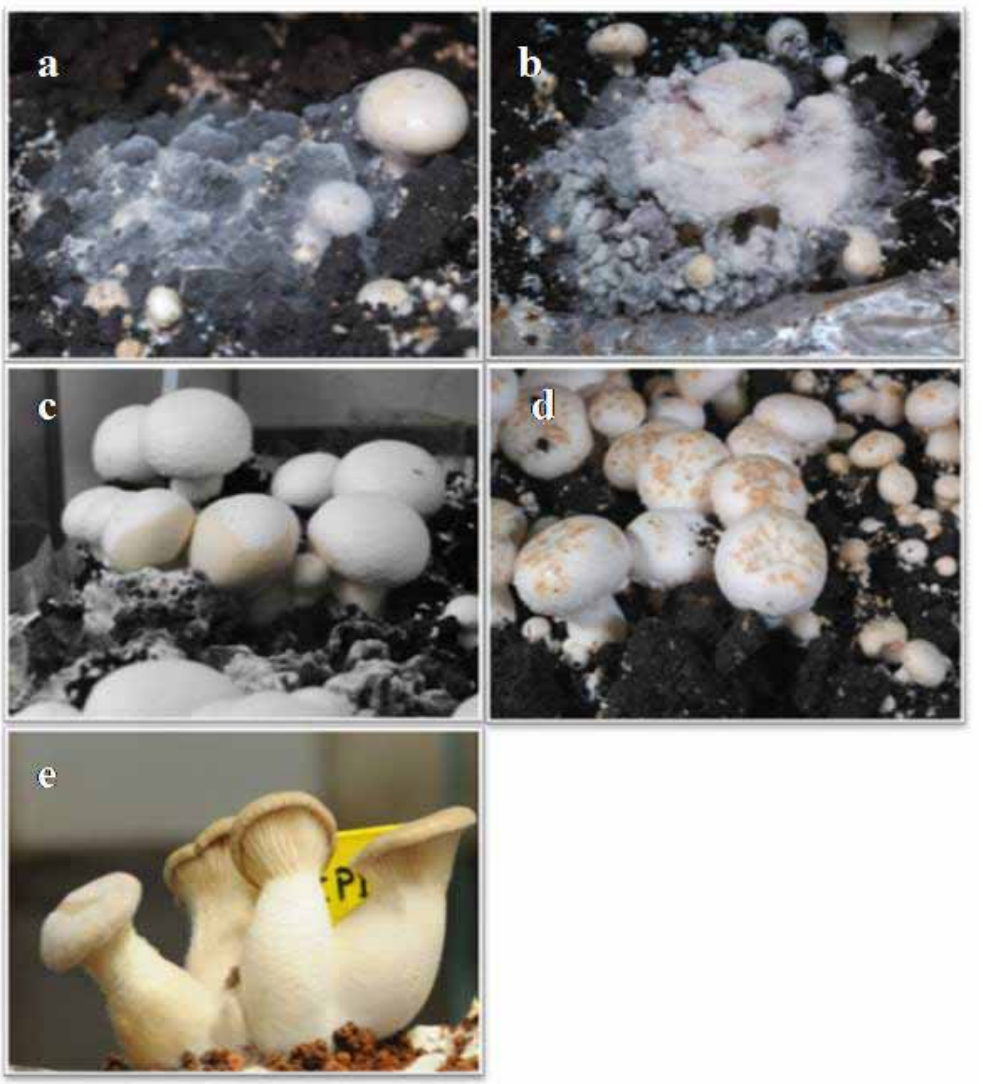

Figure 1. Cobweb symptoms on diseased crops of *A. bisporus* and *P. eryngii*. **a)** Fluffy mycelium over the casing. **b)** Dense mass of sporulation over the casing engulfing diseased fruit bodies. **c)** Regular grey spots due to a infection *via* mycelium. **d)** Irregular brown spots over the caps. **e)** Fluffy mycelium growing over *P. eryngii*

The objectives of the current work were to identify and characterize the pathogen responsible for cobweb disease in *A. bisporus* and *P. eryngii* Spanish crops. The pathogenicity of the cobweb causal agent has been also studied.

MATERIALS AND METHODS

Several isolates of *Cladobotryum* were recovered from *A. bisporus* and *P. eryngii* crops showing disease symptoms (Table 1). Samples were collected between 2009 and 2010 from mushroom farms located in Castilla-La Mancha (Spain), plated onto potato dextrose agar (PDA; Oxoid, Basingstoke, England) culture medium and maintained in growth chamber at 22 ºC in darkness.

Morphological characterization

Fungal structures were mounted on glass slides with lactic acid for microscope examination. Measurements of all taxonomically relevant characters (conidia and conidiogenous cells size, number of septa per conidium) were performed using Nikon software NIS-Elements Advanced Research (Nikon, Japan). Two hundred conidia and one hundred conidiogenous cells were measured from each isolate. The presence or absence of a conspicuous basal hilum on the conidia and phialide extensions or rachises was observed. Chlamydospore and/or microsclerotium production, the colony reverse colour side

Table 1. Strains collected from mushroom crops

Strain	Host	Isolation date	Casing	Flush
CL80	*A. bisporus*	28-1-2010	Mineral	3rd
CL55	*A. bisporus*	28-12-2009	Mineral	2nd
CL30	*A. bisporus*	4-2-2009	Mineral	3rd
PE26	*P. eryngii*	4-10-2010	Mineral	1st
PE40	*P. eryngii*	29-4-2010	Peat	1st
PE72	*P. eryngii*	29-4-2010	Mineral	1st

and the odour detectable upon lifting the lid of the Petri dish was also noted. The isolates were then identified according to the descriptions from Gams and Hoozemans [14].

Genetic characterization

DNA from two *Cladobotryum* isolates, CL80 and PE72, collected from *A. bisporus* and *P. eryngii* infected crops respectively, was extracted using the E.Z.N.A. fungal DNA commercial kit (Omega Bio-Tek, Doraville, USA) without mercaptoethanol. A 0.5 cm^{-2} fragment of mycelium growing on PDA medium was taken from the edge of the colony for identification of each isolate. Polymerase chain reaction (PCR) was performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with primers ITS1F and ITS4 [15, 16] following cycling conditions proposed by Martín and Winka [17]. Amplicons were visualized in 2% agarose gels stained with SYBR® safe (Invitrogen, Eugene, OR, USA) under visible light. PCR bands were excised from the agarose gels, cleaned by the QIA quick DNA gel extraction (Qiagen, CA, USA) and sequenced in an ABI Prism 3130 Analyzer (Applied Biosystems). The DNA sequences were edited in Sequencher v.4.2 (Gene Codes Inc., Ann Arbor, MI, USA) and identification was achieved by conducting BLASTn searchs on GenBank (NCBI).

Pathogenicity trials in *Agaricus bisporus* mushroom growing rooms

Two cropping trials (A and B) were placed in experimental mushroom growing rooms, according to the standard practices used in mushroom farms in Spain. *A.bisporus* was cultivated in experimental trays (0.15 m^2 in area) filled with 10 kg of commercial mushroom phase III compost spawned at 1% (Gurelan 45 strain, Gurelan S. Coop., Huarte, Pamplona, Spain). On day 0 of the cropping cycle, trays of spawn-run compost were cased with a 30 mm of a peat based casing layer (5.5 l per block) (EuroveenB.V., BVB Substrates, Grubbenvorst, Limburg, Holland). Proper culture practices of peat cased crops were set up during the cropping cycle. A randomized block design of 24 blocks per trial was used and the assay was carried out in duplicate (A and B). Nine days after casing, a spore suspension of *C. mycophilum* ($7.5x10^3$spores ml^{-1}) was sprayed onto the surface of the casing layer (20 ml per block) of 12 blocks at 10^6 conidia m^{-2}. 12 blocks were sprayed with sterile distilled water as a control treatment. Blocks were maintained at 17.5 °C and 90% relative humidity. The effect of cobweb on mushroom productivity during three flushes was evaluated by the biological efficiency of the cultures inoculated with the pathogen [calculated as the ratio of the fresh weight of total yield of harvested mushrooms (healthy and diseased) to the weight of dry substrate at spawning and expressing the fraction as kg 100 kg^{-1} compost], as well as by the number of infected blocks, earliness of infection and final crop area affected.

Pathogenicity trials in *Pleurotus eryngii* mushroom growing rooms

A pathogenicity test was performed using 24 blocks containing sterilized, spawned (M2600, Mycelia bvba, Nevele, Belgium) and incubated *P. eryngii* substrate (3.6 kg, 352 cm^2 in area). Twelve blocks were cased with a 20 mm layer of a casing soil (0.7 l/block) made with mineral soil + sphagnum peat 4:1 (v/v) and the other twelve blocks were cased with a 20 mm layer of Topterra® (0.7 l/block). Five days after casing, six blocks from each casing type were inoculated with a conidial suspension (7×10^3 conidia ml^{-1}) of a *C. mycophilum* strain. Each block was sprayed (5 ml per block) onto the

surface of the casing layer at a rate of 10^6 conidia/m^2. Twenty-two blocks were sprayed with sterile distilled water as a control. A temperature of 16-17 ºC, 90% relative humidity, 900 ppm de CO_2 and cool-white fluorescent light were maintained throughout cropping. *P. eryngii* fruit bodies were harvested daily for each treatment over a period of eight days. Blocks were also prospected daily for cobweb symptoms.

Pathogenicity test on *A. bisporus* and *P. eryngii* fruit bodies

Three selected isolates (PE26, PE40 and PE72) were used for the pathogenicity test. Conidial suspensions (ca. 10^5 conidia/ml) of each isolate were inoculated onto 20 *A. bisporus* and 20 *P. eryngii* fruit bodies. *A. bisporus* carpophores were externally inoculated over the mushroom cap with 50 µl of conidial suspension, a scalpel incision of 0.5 cm in a cross shape was previously made in the inoculation point. Ten fruit bodies of *P. eryngii* were inoculated externally, while the other ten fruit bodies were cut in half and inoculated internally with 50 µl of conidial suspension per fruit body. Sterilized distilled water was used as a control. All the fruit bodies were then incubated in a moist chamber at 22 ºC in darkness. Assays were conducted twice and the results were recorded after seven days. Sterilized distilled water was used as a control. Koch´s postulates were verified by re-isolating the pathogenon PDA from the mushrooms artificially inoculated.

RESULTS AND DISCUSSION

Morphological characterization

On PDA, whitish to buff mycelium develops fast after inoculation. The mycelium sporulates profusely in a few days, mainly in the edge of the colony. Usually in 3-4 days, the colonies turn into pink hues that evolves toward strong red-blood when the isolate gets old (Fig. 2). This change in shade is more remarkable in the lower side of the plate because this pigment is copiously secreted by the hyphae submerged in the media while the aerial mycelium remains white [19]. These isolates produce microsclerotia and chlamydospores (Fig. 3b)[5] and the cultures lack characteristic camphor odour, normally associated with *C. mycophilum* [4].

Conidia hyaline, ellipsoidal, globose to subglobose, sometimes slightly curved, with a central hilum. Conidia measurements (Table 2) are 17.6-23.1 x 9.1-9.3 µm in isolates obtained from *A. bisporus* crops, and 20.9-22.2 x 8.8-10.1 µm in isolates from *P. eryngii* crops. Conidia septated (0-3 septa), majority of the spores were 2 celled. Conidiogenous cells hyalines, subulatedto almost cylindrical (Fig. 3a). Conidiogenous cells measurements (Table 2) in isolates obtained from *A. bisporus* crops are 25.4-34.8 µm long, 5.3 µm wide near base, attenuating gradually to 2.6-3.0 µm at the tip; and 29.0-30.5 µm long, 5.6-6.0 µm wide near base, and 2.7-3.1 µm at the tip, in isolates from *P. eryngii* crops.

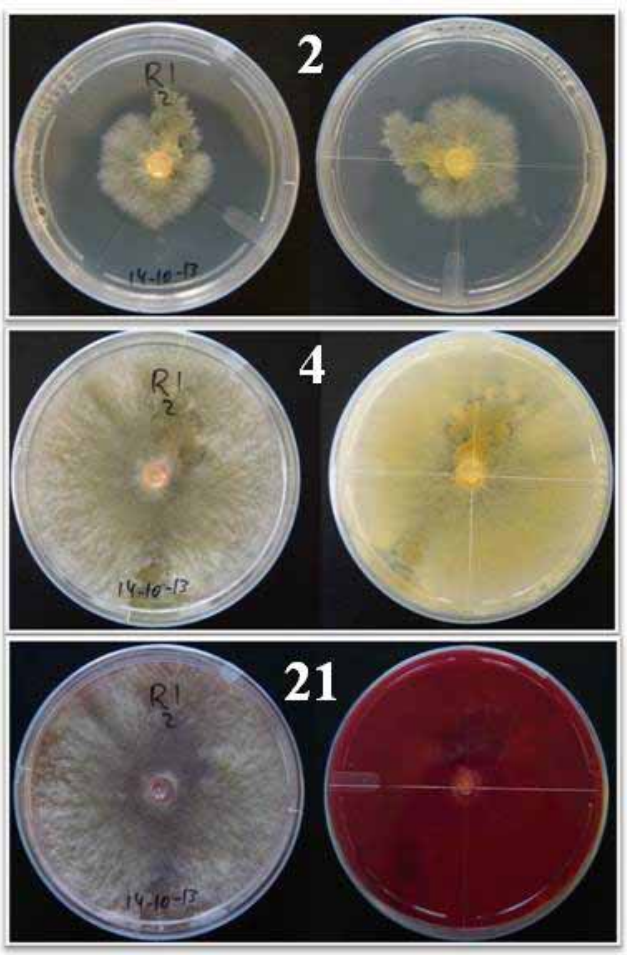

Figure 2. *C. mycophilum* on PDA, temporary evolution. Front and back side of the plates. *2, 4, 21: Age in days from inoculation

Table 2. Fungal structures measurement (μm) of studied strains

Strain	Conidia measurements (n=200)			Phyalide measurements (n=100)		
	Length	Width	Septa	Length	Base width	Apex width
CL80	23.1±0.3	9.1±0.1	1 (51%)	33.1±0.7	5.3±0.1	2.6±0.1
CL55	19.6±0.3	9.1±0.1	1 (62%)	25.4±0.7	5.3±0.1	3.0±0.1
CL30	21.7±0.3	9.3±0.1	1 (72%)	34.8±0.7	5.4±0.1	2.7±0.1
PE26	22.1±0.3	10.1±0.1	1 (68%)	30.5±0.7	6.0±0.1	3.1±0.1
PE40	21.6±0.3	9.3±0.1	1 (60%)	30.0±0.7	5.8±0.1	2.8±0.1
PE72	20.9±0.3	8.8±0.1	1 (81%)	29.0±0.7	5.6±0.1	2.7±0.1

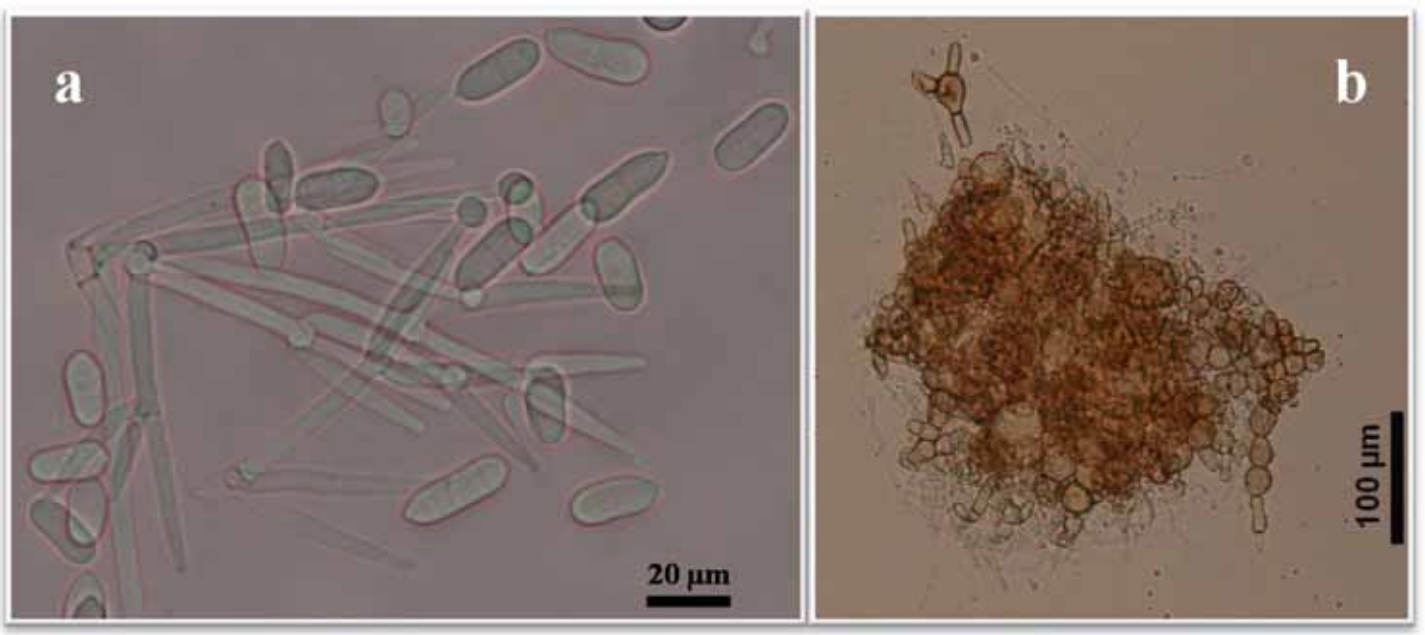

Figure 3. Morphological characteristics of *C. mycophilum* isolated from infected fruit bodies. **a)** Conidia and phyalides. **b)** Microsclerotium

Genetic characterization

The amplicon of the PE72 isolate (GenBank Accession Nº JF505112), showed 100% similarity of the obtained ITS sequence with two sequences of *Cladobotryum mycophilum* (teleomorph *H. odoratus*) (GenBank Accession Nos. Y17096 and Y17095) [5]. The amplicon of the CL80 isolate (GenBank Accession Nº JQ004732) also revealed highest similarity (99 and 100%) of the ITS sequence to four ITS sequences of *C. mycophilum* (teleomorph *H. odoratus*) (GenBank Accession Nos. AB527074, JF505112, Y17095, and Y17096) [10, 18].

Pathogenicity trials in *Agaricus bisporus* mushroom growing rooms

In the *A. bisporus* crop assays, the first cobweb symptoms developed 25 days after inoculation, between the second and third breaks in trial A; and after 11 days, between the first and second breaks in trial B (Fig. 3). *C. mycophilum* was consistently reisolated from eight inoculated blocks (67%) in trial A, and 11 inoculated blocks (92%) in trial B. As the growing cycle progressed the area colonized by the pathogen increased; by the end of the cycle, the total area of the crop affected by cobweb was 30% in inoculated blocks in trial A and 45% in trial B. As a result it is remarkable that the sooner the infection appears the heavier is the dispersion through the crop. The noninoculated blocks remained healthy. Compared with the noninoculated control blocks, a 10.7% decrease in yield of mushrooms was observed in trial A and 9.1% in trial

B. Production was lower in the second trial than in the first; that could be as well conditioned in some degree for the widespread of the outbreak.

Pathogenicity trials in *Pleurotus eryngii* mushroom growing rooms

The first cobweb symptoms developed 23 days after inoculation of *C. mycophilum* in the *P. eryngii* crop trial (Fig. 3). The pathogen was consistently reisolated from nine (37.5%) of the inoculated blocks. Noninoculated blocks remained healthy.

Pathogenicity test on *A. bisporus* and *P. eryngii* fruit bodies

The isolates tested were able to infect *P. eryngii* and *A. bisporus* carpophores. The strains revealed higher pathogenicity against *A. bisporus*, infecting 100 % of inoculated mushrooms. A radial mycelium grew from the point of inoculation and quickly evolved to a profuse sporulation mass within few days. The characteristics brown spots appeared over some of the infected mushrooms, which may suggest conidia dispersion through the moist chamber. In addition, they also resulted pathogenic against *P. eryngii* sporophores, 85% of the internally inoculated king oyster mushrooms and the 40% of those externally treated showed disease symptoms. So it is noticeable that external tissue of *P. eryngii* is less sensitive to the pathogen, and thereby it should be advisable to remove stipes wastes from the crop after harvest. A white fluffy mycelium grew over the *P. eryngii* infected fruit bodies from the inoculation point that started to sporulate within one or two days, although this sporulation was lighter than for the *A. bisporus* infected carpohores. The control mushroom remained healthy. These results satisfied Koch´s postulates, because the pathogen was re-isolated from the diseased tissues.

In light of the report findings, the same pathogenic specie is able to produce the cobweb disease over different kind of edible mushroom cultivated in Spain.

CONCLUSION

On the basis of the results, the causal agent of cobweb disease in *A. bisporus* and *P. eryngii* Spanish crops is *Cladobotyum mycophilum* (Oudem.) W. Gams & Hoozem. Pathogen isolated from diseased *P. eryngii* fruit bodies was able to infect *A. bisporus* and *P. eryngii* carpophores. Cobweb disease provokes yield decrease and reduces the crop area by colonizing the casing surface. Punctual outbreaks are adequately removed by covering the patches with a damp paper to avoid conidia dissemination and applying salt generously over it [3].

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REFERENCES

[1] Fletcher JT and Gaze RH. (2008). Fungal diseases. In: Mushroom Pest and Diseases Control, pp 63-92, ISBN: 978-84076-083-5.

[2] Gea FJ. (2001). Plagas y enfermedadesdelgénero *Pleurotus* spp. In: La biología y el cultivo de *Pleurotus* spp., pp 205-224, ISBN 968-18-6357-7.

[3] Adie B *et al*. (2006). Temporal and spatial dispersal of *Cladobotryum* conidia in the controlled environment of a mushroom growing room. *Appl Environ Microbiol*. 72 (11): 7212-7217.

[4] Grogan HM. (2005). Cobweb confusion-Cobweb isolates explained. *Mushroom J*. 660: 21-33.

[5] McKay GJ *et al.* (1999). Genetic and morphological characterization of *Cladobotryum* species causing cobweb disease of mushrooms. *Appl. Environ. Microbiol*. 65: 606-610.

[6] Sinden JW. (1971). Ecological control of pathogens and weed moulds in mushroom culture. *Annu. Rev. Phytopathol*. 9: 411-432.

[7] De Hoog GS. (1978). Notes on some fungicolus hyphomycetes and their relatives. *Persoonia.*10: 33-81.

[8] Sharma SR *et al.* (2007). Fungal diseases and competitor moulds. In: Diseases and competitor moulds of mushrooms and their management. Technical Bulletin.National Research Centre for Mushroom. Indian Council of Agricultural Research.Chambaghat, Solan. pp. 2-43.

[9] Gioia T *et al.* (2007). Morpho-productive compariso among some wild and commercial strains of *Pleurotus eryngii* (D.C.:Fr.) Quél, in protected cultivation. *Micologia Italiana*. 36 (1): 35-46.

[10] Gea FJ and Navarro MJ. (2011). First report of *Cladobotryum mycophilum* causing cobweb on cultivated king oyster mushroom in Spain. *Plant Dis.* 95(8): 1030.

[11] Back CG *et al.* (2012). Characterization of species of *Cladobotryum* which cause cobweb disease in edible mushrooms grown in Korea. *Mycobiology*. 40(3): 189-194.

[12] Kim MK *et al*. (2012). First report of cobweb disease caused by *Cladobotryum mycophilum* on the edible mushroom *Pleurotuseryngii* in Korea. *Plant Dis*. 96(9): 1374.

[13] Kim MK *et al.* (2014). Fungicide sensitivity and characterization of cobweb disease on a *Pleurotuseryngii* mushroom crop caused by *Cladobotryum mycophilum*. *Plant Pathol. J.* 30 (1): 82-89.

[14] Gams W and Hoozemans ACM. (1970). *Cladobotryum*-konidienformen von *Hypomyces*-arten. *Persoonia*. 6(1): 95-110.

[15] Gardes M and Bruns TD. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol. Ecol*. 2: 113-118.

[16] White TJ *et al.* (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a Guide to Methods and Applications*, pp 315-322, ISBN: 978-0123721815.

[17] Martín MP and Winka K. (2000). Alternative methods of extracting and amplifying DNA from lichens. *Lichenologist*. 32: 189-196.

[18] Back CG *et al.*(2010). Cobweb disease on *Agaricusbisporus* caused by *Cladobotryum mycophilum* in Korea. *J. Gen. Plant Pathol.* 76: 232-235.

[19] Põldmaa K. (2011). Tropical species of *Cladobotryum* and *Hypomyces* producing red pigments. *Stud Mycol*. 68: 1–34.