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Morphological and Pathogenic Characteristics of the Fungus *Cladobotryum dendroides*, the Causal Agent of Cobweb Disease of the Cultivated Mushroom *Agaricus bisporus* in Serbia

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SUMMARY

Twenty isolates were isolated from diseased fruiting bodies of *Agaricus bisporus* collected from Serbian mushroom farms during 2003-2007. The isolates formed white, cottony, aerial colonies on agar media. With age, conidia and colonies turned yellow and redish. Pathogenicity of these isolates was confirmed by inoculation of harvested basidiomes of *A. bisporus* and by casing inoculation. Symptoms similar to natural infection were recorded. Based on pathogenicity tests and morphological characteristics, the isolates were identified as *Cladobotryum dendroides* (Bulliard : Fries) W. Gams & Hoozemans.

Keywords: *Cladobotryum dendroides*; *Agaricus bisporus*; Morphological characteristics

INTRODUCTION

A decade ago, *Verticillium fungicola* and *Mycogone preniciosa*, the causing agents of bubble diseases, were the most important pathogens of cultivated mushroom (*Agaricus bisporus* (L)) in Serbia (Potočnik, 2006). Today, cobweb disease is one of the most serious diseases in Serbian mushroom farms affecting both quality and yield (Potočnik et al., 2007). Cobweb disease of *A. bisporus* is caused by three *Cladobotryum* species: *C. dendroides* (Bulliard : Fries) W. Gams & Hoozemans (teleomorph *Hypomyces rosellus* (Albertini & Schweinitz : Fries) Tulasne and C. Tulasne), *C. mycophilum* (Oudemans) W. Gams & Hoozemans (teleomorph *Hypomyces odoratus* G. R. W. Arnold) and *C. varium* Nees : Fries (teleomorph *Hypomyces aurantius* (Persoon) Tulasne) (Eicker and Van Greuning, 1991). All three species cause more or less similar symptoms: cottony fluffy, white/greyish colonies on mushroom casing, rapid colonization of casing surface and covering of host basidiomata by mycelia, and its decay. With time, mycelium becomes yellowish or redish/pink in colour (McKay et al., 1998). The red colouration is due to the pigment aurofusarin (Rogerson and Samuels, 1993). *C. varium* does not exhibit the typical pinkish red coloration of the mycelium as *C. dendroides* and *C. mycophilum*. *C. dendroides* produces phialide extensions/rachises and conidia with conspicuous basal hilum. *C. mycophilum* does not share these characteristics and its colonies have a typical camphor odor. *Cladobotryum* species are soil-inhabiting cosmopolitan fungi found in all mushroom-growing countries worldwide (Van Zaayen and Van Andrichem, 1982; McKay et al., 1998, 1999). They also occur on other mushroom species growing in uninhabited regions (Rogers and Samuels, 1989, 1993, 1994).

Cladobotryum spp. produce verticillately or irregularly branched conidiophores. Conidiophore carriers are branched into three to four phyalides. The conidiophores are hyaline, initially single-celled and later have 1 to 3 septae. The spores are approximately 21-30 x 9-10.5 µm and have a symmetrically placed basal scar where they had previously joined the phialide. Mycelia produce dark microsclerotia (Hughes, 1978). Ordinarily, *Cladobotryum* spp. spores can survive for a maximum of seven days in sterile water. The survival rate of microsclerotia is considerably higher. Saved in sterile water, 100% of microsclerotia still have germinating power after four months. It is noted that even when the casing layer is strongly infested by spores, symptoms usually appear during the last flushes. When the casing layer is infested by mycelium of the pathogen, disease symptoms are observed just before the first flush, when pins begin to develop. Very often, the disease begins developing on mushroom stipes that have been left after harvesting, or on dead *A. bisporus* fruit bodies. Cobweb spores are easily dislodged by air and are carried to considerable distances by air (Adie and Grogan, 2000). Flies, people and equipment are also vectors of the pathogen. The pathogen thrives under warm moist conditions and grows rapidly under ideal mushroom growing conditions. Higher casing moistures and lower evaporation rates provide conditions more conducive to disease development.

The aims of this study were to isolate and identify the causal agent of cobweb disease of the cultivated mushroom *A. bisporus* in Serbia, and examine pathogen variations as evidenced by the morphology of its colonies under different growth conditions and their pathogenic characteristics.

MATERIAL AND METHODS

Isolates and growth conditions

The isolates of *Cladobotryum* spp. collected from diseased *A. bisporus* fruiting bodies in Serbia during 2003-2007 are shown in Table 1.

Table 1. List of isolates of *Cladobotryum* spp. and their origin
Tabela 1. Lista izolata *Cladobotryum* spp. i njihovo poreklo

Oznaka izolata Isolate code	Poreklo izolata Origin of isolate	Godina skupljanja Year of collection
SP_1C_4	Smederevska Palanka	2003
P_3C_1	Požarevac	2003
Ba_1C_1	Beograd – Banjica	2004
B_1C_1	Beograd – Savski Venac	2004
Ku_1C_1	Kurjače	2004
NSl_1C_1	Novi Slankamen	2004
OB_1C_2	Ovčar Banja	2004
OB_1C_3	Ovčar Banja	2004
P_7C_1	Požarevac	2004
Res_1C_1	Resnik	2004
VG_3C_2	Vračev Gaj	2004
$Beč_1C_1$	Bečej	2006
Jak_1C_1	Jakovo	2006
Kal_1C_1	Kaluđerica	2006
NSl_2C_1	Novi Slankamen	2006
$Beč_2C_1$	Bečej	2007
Veg_2C1	Veliko Gradište	2007
VG_2C2	Vračev Gaj	2007
NSl_3C_1	Novi Slankamen	2007
NSl_4C_1	Novi Slankamen	2007

Isolation was done by taking small pieces (2 x 2 x 5 mm) of fruiting bodies with disease symptoms, immersing them in a 1% sodium hypochlorite solution for 1 min, and placing onto Potato Dextrose Agar (PDA). The isolates were kept on PDA, at 5°C, in the culture collection of the Institute of Pesticides and Environmental Protection, Belgrade. Colony morphology was studied after three days of cultivation on Malt Extract Agar (MEA) at 25°C. Conidium size, the number of septa per conidium, the presence or absence of phialide extension/rachis, and the conspicuous basal hilum on the conidia were studied. Chlamidospore and microsclerotium production was also noted. The influence of temperature on growth was studied by growing isolates on MEA at 10°C, 13°C, 18°C, 20°C, 25°C, 28°C and 30°C after three days. Optimal pH for pathogen growth was studied on Potato Dextrose Agar (PDA) by adjusting the pH on a scale of 5-9 at 18°C. The influence of different agar media: PDA, MEA, Czapek agar (CzA), modified Mushroom Dextrose Agar (mMDA) and Water Agar (WA) was examined at 18°C. Each plate was inoculated with an inverted mycelium agar disc (10 mm), taken from the edge of four-day-old cultures of *Cladobotryum* spp. isolates placed centrally onto the agar media. Colony diameter was measured after three days of cultivation. Three replicates per each treatment and isolate were submitted to statistical analyses. Data were analysed separately for each trial using ANOVA and the means were separated by Duncan's multiple range test (EPPO, 1997a, 1997b).

Pathogenicity test I

Pathogenicity assay was performed on harvested basidiomes of *A. bisporus* by a modified method of Collopy at al. (2001). Approximately 1 ml of spore suspensions containing 3 x 10^6 conidia mL^{-1} were prepared out of four-day-old cultures of all tested *Cladobotryum* spp. isolates. Pilei were converted and inoculated at a site of previously removed stipes. Pilei treated with 1 ml of sterile H_2O were used as a negative control. Inoculated pilei were incubated at room temperature (22 ± 2°C) for four days and the development of symptoms was observed.

Pathogenicity test II

Spawn-run compost (*A. bisporus* Italspown F 56), produced by Uča & Co., Vranovo, Serbia, was used for the pathogenicity test. Compost bags were cased with a 40-50 mm layer of black peat/lime casing („Makadam" Co., Belgrade), which was artificially inoculated with the studied *Cladobotryum* spp. isolates. Casing inoculation was done by spore suspension spraying (approximately 10^6 conidia/ml) three days after casing. The bags were incubated at 25°C during spawn-running of casing (for seven days) and then temperature was decreased to 18°C (Grogan et al., 2000). Pathogen reisolation from the infected fruiting bodies of *A. bisporus* was performed on PDA in order to confirm pathogenicity.

RESULTS AND DISCUSSION

Diseased fruiting bodies of *A. bisporus* with symptoms resembling cobweb disease were observed on 13 Serbian mushroom farms. Early symptoms were round, fleshy, yellowish brown lesions on *A. bisporus* caps (Figure 1). Late symptoms progressed when the parasitic fungus formed white cobweb-like circular colonies on dead or damaged pinheads, spread on the surface of the casing and covered entirely *A. bisporus* fruiting bodies (Figure 2). With age, the fluffy mycelia became thicker and granular, taking on pinkish hue. *A. bisporus*

Figure 1. Early symptoms of cobweb disease on naturally-infected *Agaricus bisporus* caps by fungus *Cladobotryum dendroides*
Slika 1. Rani simptomi paučinaste plesni na šeširima *Agaricus bisporus* nakon prirodne zaraze gljivom *Cladobotryum dendroides*

Figure 2. Progressed symptoms of cobweb disease on naturally-infected *Agaricus bisporus* fruiting bodies by fungus *Cladobotryum dendroides*
Slika 2. Kasni simptomi paučinaste plesni na plodonosnim telima *Agaricus bisporus* nakon prirodne zaraze gljivom *Cladobotryum dendroides*

caps turned dark brown and eventually shrunk due to soft rot. The described symptoms fit those caused by *Cladobotryum* spp.

Pathogenicity assay on mushroom pilei showed that each of the twenty isolates had high virulence level for *A. bisporus*. The symptoms were not produced on pilei treated with sterile H_2O that was used as a negative control. All isolates induced severe disease symptoms on *A. bisporus* pilei. The growth of pathogen mycelia was recorded two days after inoculation. White cobweb mycelium extended beyond the inoculation site. Three days after inoculation, the sporocarps were completely covered with white cottony mycelium and profuse sporulation was noted, resembling the symptoms of natural infection. The pilei were completely rotten, soft and decayed on the fourth day of incubation (Figure 3). There were no significant differences in the levels of symptom development among the different isolates.

The first symptoms were noticed twelve days after artificial inoculation of casing layer with the investigated *Cladobotryum* spp. isolates. The white fluffy mycelium first appeared on the casing layer and covered the fruiting bodies of *A. bisporus*. Colony was initiated as small, circular patches of infection on casing soil. The diameter of infection was usually no larger than 3 to 4 centimetres. Infection spreaded from dead pinheads and stalks. The mycelium quickly overwhelmed *A. bisporus* fruiting bodies. The infected mushrooms were brown-coloured and decayed.

Cobweb mycelium was initially white or grayish (Figure 4). Later, the mycelium and infected mushrooms assumed reddish colour. Colonies growing on the casing were circular and overwhelmed mushrooms, causing rapid decay. As the cobweb mycelia became thicker, taking on pinikish hue, *A. bisporus* fruiting bodies turned dark brown from soft rot. There were no statistical differences among the studied iso-

Figure 3. Left – *Agaricus bisporus* sporocarp three days after artificial infection by fungus *Cladobotryum dendroides*, isolate B_1C_1; right – negative control
Slika 3. Levo – Izgled sporokarpa *Agaricus bisporus* tri dana nakon veštačke zaraze gljivom *Cladobotryum dendroides*, izolat B_1C_1; desno – negativna kontrola

Table 2. Conidial size of investigated *Cladobotryum* dendroides isolates
Tabela 2. Veličina konidija ispitivanih izolata *Cladobotryum* dendroides

Isolate Izolat	Conidial length µm Dužina konidije µm	Conidial width µm Širina konidije µm
SP_1C_4	18.99 a (12.3-26.06)	10.33 a (7.38-11.07)
P_3C_1	19.99 a (12.3-26.06)	9.53 a (7.38-11.07)
Ba_1C_1	22.24 a (12.3-26.06)	9.05 a (7.38-11.07)
B_1C_1	17.83 a (12.3-26.06)	11.07 a (6.15-11.07)
Ku_1C_1	19.37 a (12.3-26.06)	9.34 a (7.38-11.07)
NSl1C1	20.29 a (12.3-26.06)	10.46 a (7.38-11.07)
OB_1C_1	22.45 a (12.3-26.06)	8.61 a (7.38-11.07)
OB_1C_2	20.27 a (12.3-26.06)	9.25 a (7.38-11.07)
OB_1C_3	21.22 a (12.3-26.06)	9.25 a (7.38-11.07)
P_7C_1	20.60 a (12.3-26.06)	8.92 a (7.38-11.07)
Res_1C_1	19.97 a (12.3-26.06)	9.15 a (7.38-11.07)
VG_3C_2	17.53 a (12.3-26.06)	9.84 a (7.38-11.07)
$Beč_1C_1$	20.91 a (12.3-26.06)	8.91 a (6.15-11.07)
Jak_1C_1	19.68 a (12.3-26.06)	8.61 a (7.38-11.07)
Kal_1C_1	18.76 a (12.3-26.06)	9.53 a (7.38-11.07)
NSl_2C_1	19.68 a (12.3-26.06)	9.84 a (6.15-11.07)
$Beč_2C_1$	21.22 a (12.3-26.06)	8.92 a (7.38-11.07)
Veg_2C_1	19.98 a (12.3-26.06)	9.84 a (7.38-11.07)
VG_2C_2	19.37 a (12.3-26.06)	8.61 a (7.38-11.07)
NSl_3C_1	20.30 a (12.3-26.06)	9.25 a (7.38-11.07)

*mean values in columns followed by the same letter do not differ significantly, p=0.05
* ista slova u koloni označavaju da nema statistički značajne razlike, p=0,05

Figure 4. Fruiting bodies of *Agaricus bisporus* artificially-infected by fungus *Cladobotryum dendroides*, isolate SP_1C_4
Slika 4. Inokulisana plodonosna tela *Agaricus bisporus* izolatom SP_1C_4 gljive *Cladobotryum dendroides*

lates. Pathogenicity of all investigated isolates was confirmed, on which occasion symptoms had attributes of cobweb disease. The results were in accordance with those recorded by Bhatt and Singh (1992) and Beyer and Kremser (2001).

The isolates formed white, cottony, aerial mycelium on MEA at 25°C. The mycelia produced spores four days after inoculation and changed the colour from white to yellow (Figure 5). After nine days the colour of colonies turned pink, and after 12 days red. The maximum mycelial growth of the Serbian *Cladobotryum* spp. isolates was noted on MEA at 25°C when radial growth rate was in the range between 14 and 20 mm day^{-1}. No growth of the pathogen was recorded at 10°C. Beyer and Kremser (2001) reported radial growth rate ranging from 15 to 20 mm day^{-1} at optimal temperature of 25°C on MEA. Among the media evaluated, the best growth of the investigated Serbian *Cladobotryum* spp. isolates was recorded on PDA (44.20 mm), followed by CzA (43.18 mm), mMDA (38.48 mm), MEA (33.60 mm) and WA (30.38 mm) three days after inoculation at 18°C. Consistent with observations of Dhar and Seth (1992) and Bhatt and Singh (1992), the optimal pH for pathogen growth was 7.0. The hyphae were hyaline, septate and prostrate, with 3-4 pointed and oppositely placed branches. The conidiophores were erect, hyaline, simple, arising from aerial mycelium. They were branching verticillately, terminating in groups of phialides that tapered toward the apex. Conidia were hyaline, oblong, and had one to three septa, with centrally or laterally placed conspicuous basal hilum (Figure 6). Secondary extension (rachis) was evident on the phialides. Their dimensions were 6.15 – 9.38 - 11.07 µm x 12.30 – 19.96 - 27.06 µm. Similar observations were reported by Bhatt and Singh (1992), Rogerson and Samuels (1993) and McKay et al. (1998). Chlamidospores and microsclerotia were present. Chlamydospores and microsclerotia are regularly produced both by *C. dendroides* and *C. mycophilum,* but conidia with conspicuous basal hilum and a secondary extension on the phialides are typical of *C. dendroides* (McKay et al., 1998). According to the investigated morphological and pathogenic characteristics, the microfungal isolates from screened Serbian *A. bisporus* farms were identified as *C. dendroides* (Bulliard : Fries) W. Gams & Hoozemans.

Figure 5. Colony of fungus *Cladobotryum dendroides*, isolate SP_1C_4
Slika 5. Izgled kolonije gljive *Cladobotryum dendroides*, izolat SP_1C_4

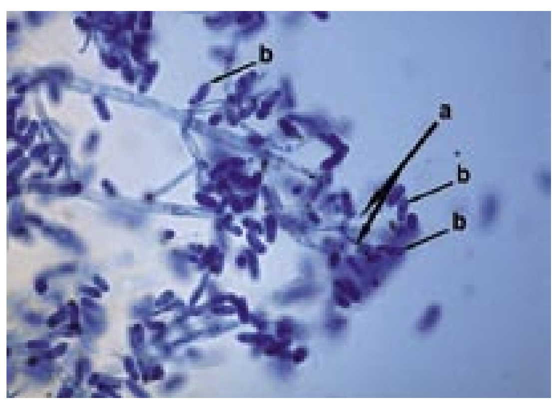

Figure 6. Conidiophores of fungus *Cladobotryum dendroides*, isolate VG_2C_2, terminating in groups of phialides (a); conidia had one to three septa (b)
Slika 6. IIzgled konidiofore i konidija gljive *Cladobotryum dendroides*, izolat VG_2C_2: fijalide u grupama na završecima konidiofora (a); konidije sa jednom do tri septe (b)

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Morfološke i patogene karakteristike gljive *Cladobotryum dendroides*, prouzrokovača paučinaste plesni šampinjona *Agaricus bisporus* u Srbiji

REZIME

Dvadeset mikrofungalnih izolata je dobijeno iz obolelih plodonosnih tela *Agaricus bisporus* prikupljenih iz gajilišta u Srbiji u periodu od 2003. do 2007. godine. Izolati su obrazovali bele, vazdušne kolonije na krompir-dekstroznoj podlozi. Nakon nekoliko dana kolonije su poprimile žutu i ružičastu boju. Veštačkim inokulacijama ubranih plodonosnih tela *A. bisporus* i pokrivke za gajenje šampinjona, pojavili su se simptomi paučinaste plesni. Izolati su identifikovani na osnovu morfoloških i patogenih osobina kao *Cladobotryum dendroides* (Bulliard : Fries) W. Gams & Hoozemans.

Ključne reči: *Cladobotryum dendroides*; *Agaricus bisporus*; morfološke karakteristike