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ORIGINAL ARTICLE

Influence of pH values of a nutrient medium on growth and morphological properties of strains *Cladobotryum sp*

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Isolates of the plant pathogenic fungus *Cladobotryum sp*. were isolated from white button mushroom carpophores affected by cobweb mould, which were collected in different commercial mushroom farms of Ukraine. Influence of nutrient medium pH values on accumulation of mycelium biomass of 11 strains of *Cladobotryum sp*. was determined. It was determined that the optimal pH value for the growth of all strains is 6.9. It was found that depending on the acidity of the nutrient medium, the majority of strains synthesize pigments that stain mycelium of the fungus. Using the methods of confocal microscopy, we have studied the colour and conducted spectral analysis of autofluorescence of mycelial hyphae of different strains of *Cladobotryum sp*.

Keywords: *Cladobotryum sp*.; pH medium; mycelium; biomass; autofluorescence; hypha

Introduction

Commercial cultivation of edible mushrooms is an important area of biotechnology, especially at the present stage, in the face of the global food shortage in the world. In Ukraine, like in most European countries, *Agaricus bisporus* (J.E. Lange) Imbach (white button mushroom) ranks first in the volume of the commercial production. A significant problem during the cycle of commercial cultivation of button mushrooms lies in affection of fungal fruits with cobweb mould, which is caused by mycophilous fungus *Cladobotryum sp*., resulting in significant loss of yield and reduction in profitability of production (Carrasco et al., 2017; Bhatt & Sing, 1992; Chakwiya et al., 2015). Elaboration of effective and environmentally safe methods for fighting cobweb mould of *A. Bisporus* requires clarification of biologic characteristics of *Cladobotryum sp*. strains and investigation of the role of abiotic factors, including the pH value, on the development of the pathogen of cobweb mould disease.

Literature data on the influence of the pH value on growth of mycophilous fungi *Cladobotryum sp*. in culture are quite limited. There are no data about the influence of the pH value on growth of strains of *Cladobotryum sp*. affecting button mushrooms in commercial mushroom farms of Ukraine. The lack of knowledge on this problem determines the relevance of such study.

The purpose of this study was to determine the influence of pH values of a nutrient medium on accumulation of biomass and morphological features of mycelium of *Cladobotryum sp*. Strains.

Materials and methods

The object of the study is represented by pure cultures of 11 different strains of *Cladobotryum sp*. isolated from white button mushroom carpophores affected by cobweb mould, which were collected in different commercial mushroom farms of Ukraine according to the methods (Naumov, 1937; Bilay, 1982).

To determine taxonomical characteristics of the genus, we used identification guides (Gaze, 1995; Gams & Hoozemans, 1970; McKay et al. 1999; Mutumeenakshi & Mills, 1995).

The study of the influence of pH values on synthesis of biomass of isolated strains of *Cladobotryum sp*. was conducted in sterile plastic containers with a volume of 120 mL with 40 mL of glucose-peptone-yeast nutrient medium (GPY). Composition of GPY, g/dm3: glucose-25 (Khimlaborreaktyv, Kyiv), peptone-3 (Sigma), yeast extract-3 (Sigma), K2HPO4-1 (Khimlaborreaktyv, Kyiv), KH2PO4-1 (Khimlaborreaktyv, Kyiv), $MgSO_4 \cdot 7H_2O$-0.25 (Khimlaborreaktyv, Kyiv), distilled water-1 dm^3. Acidity of the medium was adjusted to the necessary level (6; 6.5; 7; 7.5) using a 10% solution of KOH and 1 N HCl. After autoclaving, pH values of the nutrient media changed to the following values: 6; 6.6; 6.9; 7.3, respectively. In order to obtain an inoculum, strains of *Cladobotryum sp*. were cultivated in GPYA (20 g of agar agar was added to GPY) inside Petri dishes for 20 days; spores were sampled using a loop and transferred to 1 ml of sterile water. The aqueous suspension of spores was transferred to containers with a sterile nutrient medium. 9-days cultivation in the dark was carried out at a temperature of 25 °C, which is

optimal for the growth of strains of *Cladobotryum sp*. as it had been determined in the previous study (Medvedev & Bisko, 2018).
Staining of mycelium of strains and the nutrient medium was evaluated on visual inspection. The obtained mycelial biomass was separated from the culture fluid through filtration followed by double washing off with distilled water. The weight of the biomass was determined by the standard method (Bilay, 1982). The final pH value was measured in the culture fluid.
Morphology of all strains of *Cladobotryum sp*. which were grown in GPYA in the dark at an optimal pH and temperature (25 °C) during 9 days was studied using the LSM510-META microscope (Carl Zeiss, Germany). To create a three-dimensional relief image of samples of hyphae and conidia, DIC (differential interference contrast) with Axio Obzerver Z1 (Carl Zeiss, Germany, Scale bars=20 μm) was used. For integral estimation of 11 strains of *Cladobotryum sp*. mycelium, the computer software AimImageExaminer was employed (Carl Zeiss MicroImaging).
Autofluorescence of hyphae and conidia was studied using the laser confocal microscope LSM-510 Meta (Carl Zeiss, Germany) with excitation by a diode laser with a wavelength of 405 nm. Statistical processing was performed using the standard Excel package (Microsoft, USA).

Results and discussion

The pH value of the nutrient medium is an important factor of growth of fungi.
The standard optimal acidity value for industrial compost of the second phase for cultivating *Agaricus bisporus* is 7.1-7.4. Progressively as it grow, and after compost is completely overgrown with mycelium of *A. Bisporus*, pH is reduced to 6.0-6.4, and the pH of the soil covering the compost initially is 7.2-7.5, and then decreases to 6.5-6.7 (after its complete overgrowth with button mushroom mycelium). Since fungal fruits of button mushrooms are affected by cobweb mould only on the surface of the covering soil, the nutrient medium pH range of 6-7.3 was chosen to study growth and cultural-morphological characteristics of mycelium of 11 strains of *Cladobotryum sp*.
The obtained results show that the optimal acidity value of the GPY nutrient medium for the growth of 11 strains of *Cladobotryum sp*. is pH 6.9 (Table 1). Findings of Indian scientists suggest that the optimal pH value of the nutrient medium for growth of mycelium of C. dendroides is 7.0 (Dhar & Seth, 1992; Bhatt & Sing, 1992). Alekseeva K.L. (2013) has found that under in vitro conditions, the most favourable for growth and sporulation of C. dendroides is the pH value within the range of 6.5 to 7.2, whereas another study states that the optimal medium acidity for growth and sporulation of this species ranges from 5 to 6 (Fletcher et al., 2007; Jandaik et al., 2004).
It was found that the increase of the initial pH of the nutrient medium up to 7.3 causes average reduction in the mycelial weight of all of the studied strains by 15-19% (Table 1). When pH reduced from 6.9 to 6.0, the reduction in biomass of all strains of *Cladobotryum sp*. in experiments on media was more significant compared to the reduction in the setting of pH increase from 6.9 to 7.3 (Table 1).
It was found that during the process of growth of all strains of *Cladobotryum sp*., nutrient medium pH is reduced compared to initial pH values (Table 2). However, the extent of the pH reduction varies depending on the initial pH value of the medium and biological features of the studied strains (Table 2). Thus, when the initial pH was 6.0, cultivation of all strains of *Cladobotryum sp*. caused reduction of the medium pH by 0.2 – 0.5 units. According to the reaction of strains to increase of the initial medium pH to 6.6, the studied isolates can be divided into several groups: strains 1 and 11 reduced pH by 1-1.1 units, strains 5 and 7 reduced pH by 0.5-0.7 units, other strains reduced it by 0.2 -0.4 units. As the result of cultivation of strains with the medium pH of 6.9 acidity of the medium significantly reduced by 1.1-1.3 units, only during growth of isolates 9 and 10 (Table 2). After cultivation of other strains, the pH value of the medium decreased by 0.1-0.5 units. Further increase of the medium pH up to 7.3 on the basis of the intensity of pH reduction resulted in the possibility of isolating a large group of isolates-strains 1, 4, 5, 6, 7, 8, 9, 10, 11 which decreased the pH value by 0.2-0.5, and two strains-2 and 3 which in the course of their growth decreased the pH value by 0.8 units (Table 2).

Table 1. Influence of the pH value of the GPY nutrient medium on accumulation of the biomass (g/L) by strains of *Cladobotryum sp*. (9 days of cultivation).

*pH_i	6	6.6	6.9	7.3
strain				
1	14.2 ± 0.6	15.1 ± 0.2	18.6 ± 0.2	15.7 ± 0.3
2	13.4 ± 0.1	15.0 ± 0.2	18.0 ± 0.3	15.4 ± 0.4
3	14.0 ± 0.1	15.5 ± 0.1	18.0 ± 0.4	16.4 ± 0.3
4	13.6 ± 0.2	15.4 ± 0.3	18.0 ± 0.3	15.6 ± 0.2
5	12.6 ± 0.1	14.8 ± 0.1	19.5 ± 0.3	15.8 ± 0.4
6	13.1 ± 0.3	15.6 ± 0.2	18.0 ± 0.4	15.7 ± 0.1
7	12.8 ± 0.1	15.3 ± 0.1	18.3 ± 0.5	16.1 ± 0.2
8	13.3 ± 0.2	15.4 ± 0.2	18.0 ± 0.2	15.9 ± 0.4
9	13.5 ± 0.2	15.5 ± 0.2	18.9 ± 0.3	16.2 ± 0.3
10	13.3 ± 0.3	14.9 ± 0.3	19.9 ± 0.4	16.4 ± 0.4
11	14.0 ± 0.3	14.7 ± 0.3	19.7 ± 0.5	15.9 ± 0.3

***pHi** - initial pHi value.

Table 2. Changes in pH values of the GPY nutrient medium and mycelium colour of strains of *Cladobotryum sp*. after 9-day cultivation.

Strain	pH_i 6.0	colour	pH_i 6.6	colour	pH_i 6.9	colour	pH_i 7.3	colour
	pH_f		pH_f		pH_f		pH_f	
1	5.5	r	5.6	r	6.8	w	6.8	r
2	5.7	r	6.5	r	6.4	w	6.5	r
3	5.8	r	6.4	w	6.6	w	6.5	y
4	5.7	w	6.5	w	6.5	w	6.9	w
5	5.6	w	5.8	o	6.6	r	7.1	r
6	5.7	r	6.2	o	6.4	r	7	r
7	5.6	r	5.9	o	6.7	r	6.8	w
8	5.6	r	6.3	r	6.8	r	7.1	r
9	5.7	w	6.5	o	5.6	w	7	y
10	5.7	r	6.6	r	5.8	w	6.8	r
11	5.7	r	5.5	o	6	w	7.1	r

Arbitrary notations: w – white; r – red; y – yellow; o – orange, pHi – initial pH value, pHf – final pH value

Simultaneously with changes in the medium pH in the course of cultivation, the majority of strains of *Cladobotryum sp*. were characterized by changes in mycelium colour. The white mycelium at the beginning of growing gradually turned into yellow, orange, or deep red by the end of cultivation (Table 2). It should be noted that only one strain No.4 did not change the mycelium colour despite the different pH value of the medium (Table 2). At the end of cultivation on the medium with pH 6 most strains, 8 of 11, changed mycelium colour to red (Table 2). Findings in Table 2 show that only upon cultivation on the medium with pH 6.6, mycelium colour of 5 out of 11 strains becomes orange. Cultivation of strains of *Cladobotryum sp*. in the nutrient medium with pH 6.9 resulted in preservation of white colour by most strains, 7 out of 11, by the end of cultivation (Table 2). Due to increase in medium pH up to 7.3, mycelium of two isolates (No.3 and No.9) became yellow, two isolates (No. 4 and No.7) remained white, and the majority of strains became red (Table 2). Thus, it can be argued that mycelium pigmentation of strains of *Cladobotryum sp*. largely depends on biological features of the isolate and the pH value of the culture medium.

Literature data confirm our results regarding the variability of pigment synthesis by isolates of *Cladobotryum sp*. (Potočnik et al., 2008).

The experiments conducted to study cultural-morphological characteristics of strains that have been cultivated for 9 days in the agarized GPYA nutrient medium at pH 6.9, which is optimal for biomass accumulation, have confirmed results obtained earlier in the liquid GPY (Table 2) regarding changes in the colour of mycelium of the studied isolates of *Cladobotryum sp*.

It is known that any physical, chemical or biological factor that negatively affects cellular physiology, growth, or survival causes a chain of reactions at different hierarchical levels, from the populational to molecular, which leads to induction of adaptive mechanisms within the genetic potential. Thus, in response to environmental stress (temperature, composition of the nutrient medium, fluorescent light) during mycelial growth, quinone-type pigment, aurofusarin, is synthesized by fungi of the Cladobotrium genus - C.varium, C.micophillum, C.semicirculore, C.heterosporum (Back et al., 2012; Poldmaa, 2011; Potočnik et al., 2008), which causes changes in the colour of mycelium, colour of the medium and morphological properties of cultures.

In order to study pigmentation of 11 isolates of the *Cladobotryum sp*. fungus, we have conducted a series of microscopic examinations of aerial mycelium and mycelium immersed in the nutrient medium.

It was found that white colonies of strain No.4 consist exclusively of colourless conidia and hyphae (Figure 1B). The increase of the duration of growth up to 20 days or a short-term effect of lowered temperatures (from 25 to 8 °C) for 4 hours followed by cultivation did not change the colour of mycelium, hyphae, and conidia. Thus, negative factors such as changes in pH (Table 2), temperature and reduction of nutrients during 20 days growth, and accumulation of metabolic products did not induce the synthesis of pigments in strain No. 4. It can be assumed that the absence of mycelium staining of this strain is genetically determined.

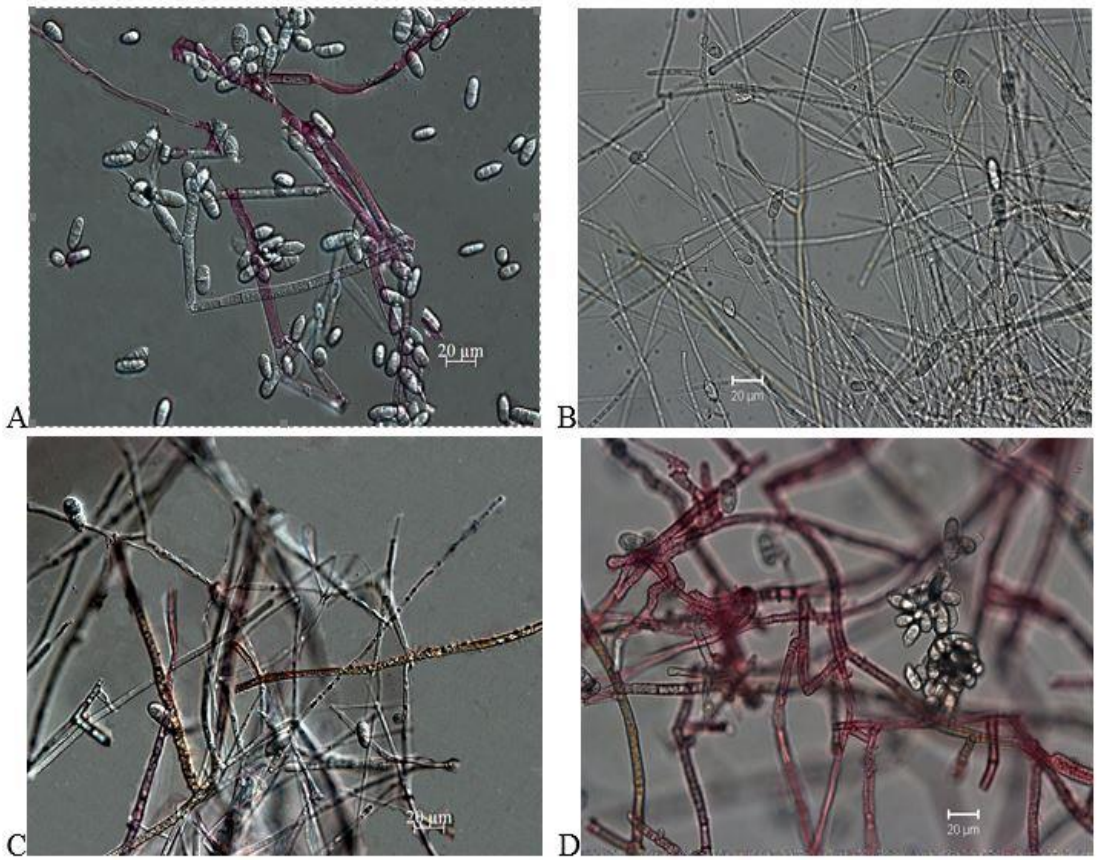

Figure 1. Microphotographs of hyphae and conidia of strains of *Cladobotryum sp*. (9 days of cultivation). Scale bars: 20 µm, A-strain No.7 (DIC); B-strain No.4 (DIC); C-strain No.11 (DIC); D-strain No.5 (light microscopy).

In samples of the remaining 10 strains (regardless of the colour of colonies), yellow, orange, or red hyphae are detected along with colourless hyphae. The ratio between colourless and coloured hyphae varied across different cultures-white mycelium of strains No. 1, 2, 3, 9, 10, 11 on the periphery of colonies consisted only of colourless hyphae and conidia, while occasional coloured hyphae were observed in samples collected from the centre of colonies (Figures 1A, 1C, 1D and Figure 2A). In red-coloured colonies of strains No. 5, 6, 7, 8 red hyphae are predominant (Figure 1D). A part of pigmented hyphae becomes thicker. This is partially due to encrustation of the cell membrane and the cell itself with pigments (Figures 1C and 2), and is also associated with biological properties of the strains.

According to scientific publications, hyphae of C. rubrobrunnescens and C. tenue immersed in the nutrient medium synthesize the red pigment aurofusarin (Back et al., 2012; Poldmaa, 2011). Findings of our study give grounds to suggest different staining of hyphae immersed in the culture medium, as well as of aerial mycelium of isolates of *Cladobotryum sp*. The difference in the evaluated colour of the strain colonies and hyphae (white, yellow, orange, red) depending on the medium pH is subjective in its nature, since it is evaluated visually. However, by studying autofluorescence of samples, it was determined that yellow hyphae do not have autofluorescence; red hyphae are identified in the range of 433 - 476 nm and 583 - 668 nm, colourless hyphae are identified in the range of 433 - 476 nm, with the excitation wavelength of 405 nm (Figure 2) A more detailed interpretation of the results will require further in-depth study of endogenous fluorophores of *Cladobotryum sp*., which may be promising and provide important information, since many of them play an important role in cell metabolism.

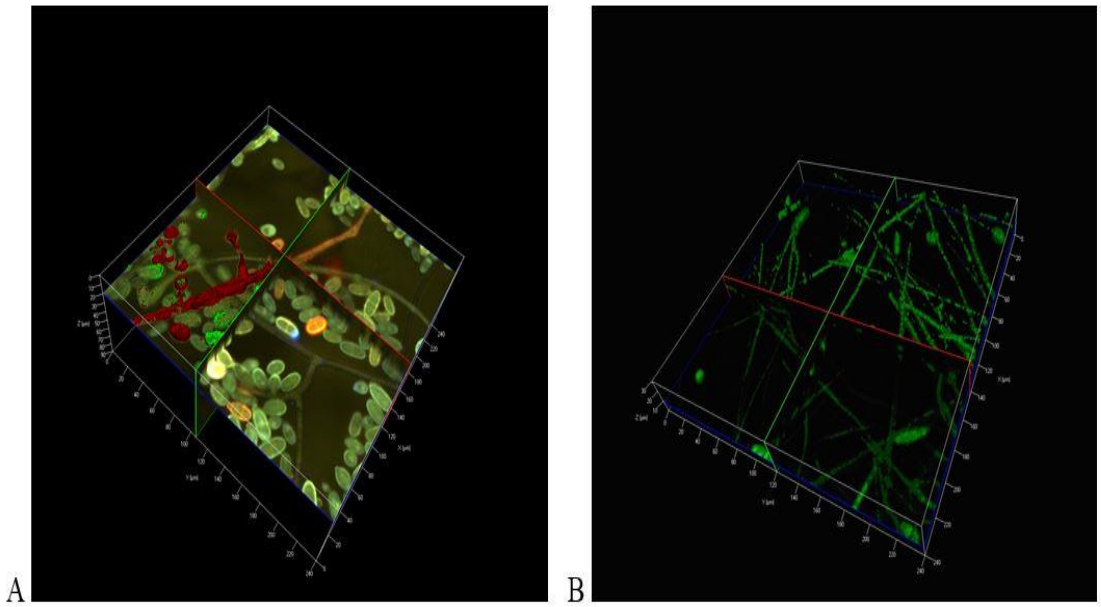

Figure 2. 3D image of hyphae and conidia of aerial mycelium obtained using the laser scanning microscope LSM510¬META: A-red, colourless, orange hyphae of *Cladobotryum sp*., strain 8; B-white hyphae of *Cladobotryum sp*., strain 4. 20XY scans with an increment size on the Z scale of 4.2 μm. The excitation wavelength is 405 nm. The power of the laser is 10 mW. Emission filters: blue 433-476 nm; green 433-476 nm; orange 561-583 nm; red 583-668 nm. The thickness of the sample is 80.6 μm. Scale bars: 20 μm.

Conclusion

The obtained results suggest that for biomass accumulation of 11 studied strains of *Cladobotryum sp*., the optimal pH value of the nutrient medium (GPY) is 6.9. It was found that when acidity parameters of the nutrient medium are close to unfavourable, mycelium becomes orange, hot-yellow, and then almost completely red. It was found that in the course of growth of all strains of *Cladobotryum sp*., the pH value of the nutrient medium decreases in comparison to initial values. Pigmentation of hyphae of mycelium of *Cladobotryum sp*. strains have been studied using a laser confocal microscope.

References

Alekseeva, K. L. (2013). Species of the genus Cladethrium causing diseases of cultivated edible mushrooms. Problems of mycology and phytopathology in the KSHI century. Materials of the international scientific conference, dedicated to the 150th anniversary of the Corresponding Member of the INSSR, Professor Artur Arturovich Yachevsky. National Academy of Mycology, BGS, Design Studio "Dozor". SPb. Copy-R Group, 91-93. (in Russian).

Bhatt, N., & Singh, R. P. (1992). Cobweb disease of Agariucus bisporus: incidence, losses and effective management. Indian Journal of Mycology and Plant Pathology, 22(2), 178-181.

Bilay, V. I. (1982). Methods of experimental mycology. Reference book of science. Dumka, Kiev (in Russian).

Back, C. G., Lee, C. Y., Seo, G. S., & Jung, H. Y. (2012). Characterization of species of Cladobotryum which cause cobweb disease in edible mushrooms grown in Korea. Mycobiology, 40(3), 189-194.

Carrasco, J., Navarro, M. J., & Alegría, F. J. G. (2017). Cobweb, a serious pathology in mushroom crops: A review. Spanish journal of agricultural Research, 15(2), 19.

Chakwiya, A., Van der Linde, E. J., & Korsten, L. (2015). In vitro sensitivity testing of Cladobotryum mycophilum to carbendazim and prochloraz manganese. South African Journal of Science, 111(11-12), 1-7.

Dhar, G. M., & Seth, P. K. (1992). Factors influencing cobweb disease of *Agaricus bisporus* caused by Cladobotryum dendroides. Indian Journal of Mycology and Plant Pathology, 22(2), 178-181.

Fletcher, J. T., & Gaze, R. H. (2007). Mushroom pest and disease control: a colour handbook. CRC Press.

Gams, W., & Hoozemans, A. C. M. (1970). Cladobotryum-konidienformen von Hypomyces-arten. Persoonia-Molecular Phylogeny and Evolution of Fungi, 6(1), 95-110.

Gaze, R. (1995). Dactylium or cobweb. Mushroom Journal, (546), 23-27.

Jandaik, S., Guleria, D. S., Parmar, Y. S. (2004). Influence of cultural parameters on the growth and sporulation of Cladobotryum dendroides: the pathogen of cobweb on mushrooms. Science and cultivation of edible and medicinal fungi XVI, Penn. State, 503-505.

Medviedev, D., Bisko, N. (2018). The Influence of Temperature on Growth and Vitality of Mycelia of Fungicolous Fungus *Cladobotryum dendroides* Strains. Lesia Ukrainka Eastern European National University Scientific Bulletin Series: Biological Sciences, 4(377), 44-47. (in Ukraine).

McKay, G. J., Egan, D., Morris, E., Scott, C. and Brown, A. E. (1999). Genetic and morphological characterization of *Cladobotryum sp*ecies causing cobweb disease of mushrooms. Applied and Environmental Microbiology, 65, 606-610.

Mutumeenakshi, S., Mills, P. R. (1995). Detection and differentiation of fungal pathogens of bisporus . Science and Cultivation of Edible Fungi, Elliott (ed.) Balkema, Rotterdam.
Naumov, N. A. (1937). Methods of mycological and phytopathological studies. Moscow-Le Selkhozgiz. (in Russian).
Põldmaa, K. (2011). Tropical species of Cladobotryum and Hypomyces producing red pigments. Studies in Mycology, 68, 1-34.
Potočnik, I., Rekanović, E., Milijašević, S., Todorović, B., & Stepanović, M. (2008). Morphological and pathogenic characteristics of the fungus *Cladobotryum dendroides*, the causal agent of cobweb disease of the cultivated mushroom *Agaricus bisporus* in Serbia. Pesticidi i fitomedicina, 23(3), 175-181.
Rudakov, O. L. (1981). Mycophilic fungi, their biology and practical significance. The Science. Moscow (in Russian).

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