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PHENOTYPIC CHARACTERISTICS, PATHOGENICITY, AND MOLECULAR IDENTIFICATION OF *HYPOMYCES PERNICIOSUS* CAUSING WET BUBBLE DISEASE OF EDIBLE MUSHROOMS

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ABSTRACT

The research aimed to determine the phenotypic characteristic, pathogenicity and molecular characterization of *Hypomyces perniciosus* isolates responsible for wet bubble disease of the white button mushroom *Agaricus bisporus*. Phenotypic characteristics such as colony appearance, mycelium texture, and pigmentation were studied on four different agar media, i.e., potato dextrose agar (PDA), malt extract agar (MEA), Czapek-Dox yeast agar (CYA), and Sabouraud dextrose agar (SDA), after eight days of incubation. Additionally, the growth rate of the tested isolates was studied depending on the pH of the medium. Fungal isolates showed the highest mycelial growth on MEA and SDA at pH 6.0. However, on CYA at pH 7.0, the mycelium exhibited the worst growth. Isolate identification and genetic relationship analysis were carried out using internal transcribed spacer region sequencing and the random amplified polymorphic DNA method. The research confirmed that all Polish isolates belong to the species *H. perniciosus*, and the genetic diversity is relatively low. Phylogenetic analyses revealed three subgroups of *H. perniciosus* isolates. The first group included three genetically distinct isolates with a similarity coefficient in the range of 0.76–0.85 to isolates CBS 815.73 and CBS 322.52. The second group was divided into two subgroups and included 16 isolates with a genetic similarity range of 0.91 to 1.0 to CBS 815.73 and CBS 322.52 isolates. Furthermore, the eight genetically similar isolates exhibited the greatest pathogenicity towards *A. bisporus*.

Key words: RAPD analysis, *Agaricus bisporus*, wet bubble disease, fungal pathogen

INTRODUCTION

The white button mushroom (*Agaricus bisporus*) is the world's most common cultivated mushroom species. It is susceptible to numerous fungal and bacterial diseases, including wet bubble disease caused by the mycopathogen *Hypomyces perniciosus* [syn. *Mycogone perniciosa* (Magnus) Delacroix], is considered one of the most significant mushroom diseases. This pathogen can cause significant losses in mushroom production, ranging from 10% to 60% (Ślusarski et al. 2012; Kouser et al. 2013). In severe cases, it can even lead to a complete loss of yield (Maszkiewicz & Dyki 1988; Sharma & Kumar 2000; Fu et al. 2016).

The main symptoms of wet bubble disease are undifferentiated forms of mushroom tissue (called sclerodermoid mushrooms), cap spots, and the development of amber liquid droplets on the distorted mushrooms. The primary infection of primordia resulted in the development of large shapes and lumps of fungal tissue with no signs of mushroom differentiation. The white and fluffy mycelium of the pathogen can be observed on infected mushrooms, which turns creamy brown after a few days. Infected bubbles emit an unpleasant odor typical of this disease (Fletcher & Gaze 2008). Zhang et al. (2017a, b) showed that *H. perniciosus* has no pathogenic activity in the mycelial stage of *A. bisporus*. However, it can infect *A. bisporus* basidiomes at any development phase of the fruitbody.

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Moreover, bacteria probably participate in the degradation of diseased basidiomes after fungal infection. Pathogen can be spread by contaminated compost and casing soil. Mushrooms can also be infected via dust, flies, or picking personnel (Umar et al. 2000; Sharma & Kumar 2000; Kouser et al. 2015).

Effective control strategies based on early pathogen detection and strict farm hygiene, combined with chemical fungicides, can reduce the incidence of mushroom diseases. Currently, only one fungicide (metrafenone) is registered for controlling fungal diseases in Poland. During an intense disease outbreak, the effectiveness of the fungicide may be low, according to Navarro et al. (2023). Even under controlled conditions, wet bubble disease is frequently observed (Szumigaj-Tarnowska et al. 2015). Therefore, it is crucial to quickly identify the causative agent to control this infection in white button mushroom crops effectively. Furthermore, it is being determined whether the differences in colony morphology and pathogenicity of the isolates are linked to their phenotype or geographical origin, as they were obtained from various mushroom farms.

Due to the limited knowledge of the phylogenetic diversity of Polish isolates of *H. perniciosus*, this study aims to examine the morphological characteristics, pathogenicity, and genetic variability of seventeen isolates collected from Polish mushroom farms. Our research may help in the effective control of wet bubble disease in the cultivation of a common edible mushroom.

MATERIALS AND METHODS

Isolates

The research material consisted of 17 isolates of *H. perniciosus* obtained from diseased fruitbodies of *A. bisporus* showing typical symptoms of wet bubble disease. The infected mushrooms were obtained from mushroom farms in different regions of Poland (Table 1). The two reference isolates *H. perniciosus* (CBS 322.52 and CBS 815.73) as well as *Mycogone rosea* CBS 527.80 from Centralbureau voor Schimmelcultures (Utrecht, the Netherlands) were also examined.

Table 1. Polish *Hypomyces perniciosus* isolates used in this study

Isolate code	Locality of mushroom farms in Poland (city, region)	Isolation date
M7	Żeliszów (dolnośląskie)	03.2008
M6	Żeliszów (dolnośląskie)	03.2008
5A	Iwiny (dolnośląskie)	03.2011
M9	Mogielnica (mazowieckie)	03.2008
M8B	Wodziczna (mazowieckie)	03.2008
M19	Skierniewice (łódzkie)	11.2008
M18	Skierniewice (łódzkie)	10.2008
M20	Skierniewice (łódzkie)	12.2008
B1	Skierniewice (łódzkie)	09.2012
M4.06	Maków-Kolonia (łódzkie)	06.2013
M26.4	Maków-Kolonia (łódzkie)	04.2013
M5.03	Maków-Kolonia (łódzkie)	03.2013
M10	Głogów Małopolski (podkarpackie)	03.2008
M11A	Nowe Iganie (siedleckie)	03.2008
M11B	Nowe Iganie (siedleckie)	04.2008
M12B	Żelków-Kolonia (siedleckie)	04.2008
M18.4x	Nowosielec (siedleckie)	06.2010

For isolation of the pathogen, 5 mm discs of infected sporophore tissue, with the help of a sterilized scalpel, were taken and placed on potato dextrose medium (PDA) supplemented with 50 $mg \cdot l^{-1}$ of streptomycin and 5 $mg \cdot l^{-1}$ rifampicin to prevent bacterial contamination. Mycelium was grown at 23–24°C for seven days. For further studies, the isolates were maintained for short term on a PDA medium at 4°C and on a PDA medium placed in 15% v/w glycerol at −75°C for extended storage. Before analysis, the isolates were activated on a PDA medium at a temperature of 23−24°C. Once a year, fungal isolates were inoculated on the white button mushroom to enhance their pathogenicity.

Mycelial growth on various media and pH values

Culture characteristics such as colony appearances, mycelia textures, and pigmentations were examined on different agar media after 8 days of incubation at 24°C. Agar plugs (5 mm diameter) were placed in the centers of 9 mm diameter Petri dishes containing different media from the edges of 7-day-old fungal colonies of these isolates. Four replicates (Petri dishes) of each isolate were incubated, and the colony diameter (mm) was measured at 24-hour intervals for 8 days to estimate fungal growth. Trials were repeated twice. This morphological characterization of the isolates was carried out on the following media: potato dextrose agar (PDA), malt-extract agar (MEA),

Czapek-Dox yeast agar (CYA), and Sabouraud dextrose agar (SDA). Species characteristics such as conidia shapes, conidiophores, branching patterns, and chlamydospores were examined under the light microscope (Nikon Eclipse 50i) with a camera connected to a computer. Fungi were identified to genus level according to Barnett and Hunter (1999) and Marcinkowska (2012). To determine the optimal pH for the mycelial growth of isolates, media were prepared at four pH values (pH 4.5, 5.0, 6.0, and 7.0).

Studies on the influence of different medium and pH values on the rate growth of *H. perniciosus* isolates were performed twice in three replications. The growth rate (mm per day) of isolates on different media (pH 5.0) was assessed using two-way analysis of variance. The first factor – A – was the isolates, and the second factor – B – was four different media. The average mycelial growth of isolates on different media at different pH was assessed using the same statistical analysis. Factor A was the medium and factor B was the pH of the medium.

The means were compared using Tukey test at a significance level of $p = 0.05$. The results are expressed as mean values and standard deviation.

Molecular identification

Molecular analyses were carried out to confirm the identification of the pathogen. DNA was isolated from mycelial cultures grown on malt extract agar (MEA) using the extraction protocol of Aljanabi and Martinez (1997). The concentration and purity of the extracted DNA were measured spectrophotometrically (Biophotometer, Eppendorf) and on 0.8% agarose gel after ethidium bromide staining.

For amplification of the internal transcribed spacer region of ribosomal DNA (ITS), ITS1 and ITS4 primers were used. The reaction mixture contained in 20 µl of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM each of dNTP (Thermo Scientific), 0.5 µM each of ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers, 1 U Taq DNA polymerase (Thermo Scientific) and 40 ng genomic DNA. The reaction was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystem) with PCR condition consisting of 94°C for 1 min, followed by 35 cycles of 94°C – 60 s, 55.5°C – 2 min, 72°C – 2 min and final extension of 7 min at 72°C. A 600-bp PCR product was amplified in all isolates (data not shown). Sequencing of PCR products were performed at Genomed (Warsaw, Poland). Nucleotide sequences derived from amplicons sequencing were compared using the BLAST algorithm available on NCBI (the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov). Random amplified polymorphic DNA (RAPD) analysis was performed using sixty arbitrary 10-mer primers from Operon Technologies (Alameda, USA): OPA, OPB, and OPC. Amplification was done in a total reaction mixture of 20 µl containing 1x PCR buffer, 0.001% gelatin (Sigma Aldrich), 0.3 µM primer, 0.1 mM each of dNTP (Thermo Scientific), 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Thermo Scientific), 20 ng genomic DNA. The thermal parameters of the cycles were: 94°C for 1 min, followed by 45 cycles of 15 sec at 92°C, 25 sec at 36°C, 74 sec at 72°C and final elongation 5 min at 72°C. After ethidium bromide staining PCR products were separated on 1.4% agarose gel electrophoresis. Polymorphic amplicons were used to construct a binary matrix (0 – lack of polymorphic product, 1 – presence). Phylogenetic analysis was performed using NTSYS-pc 2.10m software (Exeter Software). The genetic distance was calculated according to the model described by Nei and Li (1979).

Partial nucleotide sequences were obtained. The sequences were then subjected to BLAST analysis, and the identity of the cultures was determined based on the nearest neighbor with the highest percent identity.

The sequences of the isolates were compared with the sequences of *H. perniciosus* CBS 648.82 and PPRI5784 deposited in the Gene Bank (NCBI) (Meyer & Korsten 2008).

Pathogenicity tests

The pathogenicity tests were carried out in 22 cm diameter pots (surface area 0.04 m^2) filled with 1.7 kg of phase III compost, inoculated with the A15 fungal strain characterized as moderately susceptible to wet bubble disease (Szumigaj-Tarnowska et al. 2013). The surface of the compost was then covered with a 4 cm casing layer. The conditions in the mushroom house chamber were fully controlled as in routine production, i.e., the temperature was 24°C, carbon dioxide concentration was 3000 $mg \cdot l^{-1}$, and relative humidity was 95%. After eight days, when the mycelium reached the surface of the casing, the temperature was lowered to 18°C, and the carbon dioxide concentration was reduced to 800 $mg \cdot l^{-1}$.

The casing surface was then sprayed with 5 ml of aleuriosporic suspension containing 1×10^3 spores per ml (Van Zaayen & Van Adrichem 1982), corresponding to 1.3×10^5 spores per 1 m^2 of casing. Controls were not inoculated. The pathogenicity of the isolates was assessed based on yield reduction in the first flush. The assessment of yield reduction by different isolates was subjected to one-way analysis of variance, and the means were compared using the Tukey test at the significance level of $p = 0.05$.

RESULTS

Growth characteristic

Growth characteristics of *H. perniciosus* isolates, such as growth rate, pigmentation, colony pattern, and texture, varied according to the medium (Fig. 1). On MEA and SDA, colonies of isolates were very regular, with floccular and dense aerial mycelia with a velvety texture. However, on PDA and CYA colonies were rarer and colorless or light brown, with a sparse and thin texture. On MEA and SDA, most isolates produced abundant, flocculent aerial mycelia, which turned light or dark brown after five days of incubation with the production of chlamydospores. Figure 1 shows the morphological characteristics of selected isolates, which ranged from white to dark brown mycelium depending on the growth medium for ten days at 25°C. The colony color of all isolates was initially white but later changed to brown or light brown. Some isolates did not change colony color and remained cream or white, e.g., CBS322.52 (Fig. 1).

Molecular identification

Analysis of the nucleotide sequences of the isolates showed a genetic similarity of 98–99% with *H. perniciosus* CBS 648.82 and PPRI5784 deposited in the gene bank (NCBI). The similarity of isolates from Polish mushroom farms to reference isolates CBS 322.52 and CBS 815.73 was 96.3–99.1% and 96.1–97.9%, respectively. RAPD analysis was performed using sixty arbitrary 10-mer primers from the Operon Technologies (Alameda, USA) kits: OPA, OPB, and OPC.

The highest genetic similarity to isolates CBS 815.73 and CBS 322.52 (genetic similarity coefficient ranging from 0.91 to 1.0) was observed for fourteen Polish isolates, while the lowest genetic similarity (coefficient ranging from 0.76 to 0.85) was found for three isolates (Table 2).

60 RAPD primers were used to assess the genetic diversity of *H. perniciosus* isolates. Of the primers tested, 13 were selected for analysis, which gave reproducible and polymorphic amplification products specific to the isolates. A total of 34 polymorphic amplification products were identified.

Phylogenetic analysis identified three subclades (Fig. 2). The first group included isolate *M. rosea*, which was the most different from the others. The second group included three isolates: M19, M20, and M4.06. These three isolates differed most from other *H. perniciosus* isolates from Polish mushroom farms. The third group was the largest and included 16 isolates. This group was divided into two subgroups. The first included seven isolates, i.e., CBS 322.52, M26.4, M10, M18 (which were genetically identical) and M18.4x, M11B, M5.03. The following isolates belonged to the second group: CBS 815.73, B1, M6 (genetically identical – genetic similarity coefficient was 1.0), M7, M11A, M12B, 5A, M9, and M8B. The isolates M8B, M9, 5A, M12B, M11A, and M7 did not distinguish between themselves (Fig. 2).

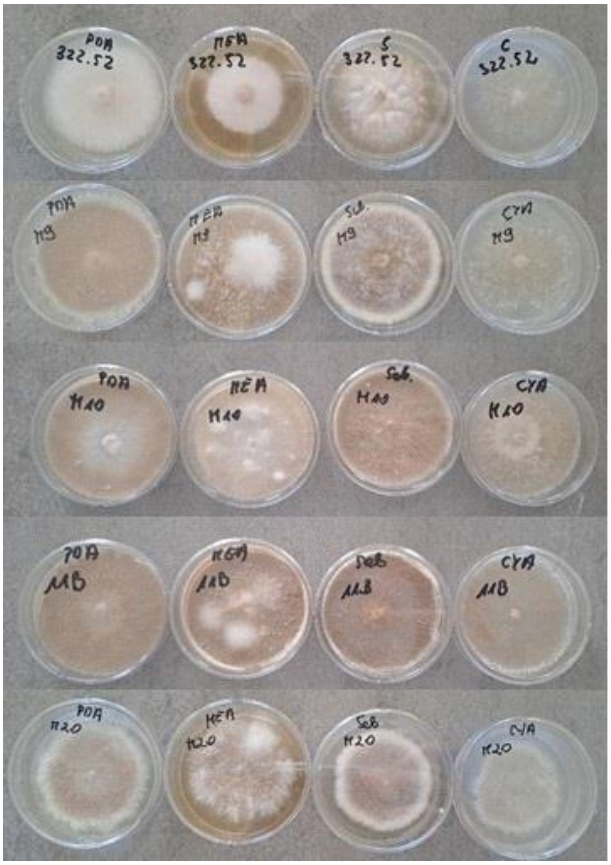

Figure 1. Mycelial growth of selected *H. perniciosus* isolates on various media

Table 2. Genetic similarity coefficient of *H. perniciosus* isolates based on RAPD analysis

Isolate no.	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	-																		
2	0.94	-																	
3	0.94	1.0	-																
4	0.97	0.97	0.97	-															
5	0.97	0.97	0.97	0.94	-														
6	0.94	1.0	1.0	0.97	0.97	-													
7	0.97	0.97	0.97	1.0	0.94	0.97	-												
8	0.76	0.82	0.82	0.79	0.79	0.82	0.79	-											
9	0.97	0.97	0.97	0.94	1.0	0.97	0.94	0.79	-										
10	1.0	0.94	0.94	0.97	0.97	0.94	0.97	0.76	0.97	-									
11	0.97	0.91	0.91	0.94	0.94	0.91	0.94	0.74	0.94	0.97	-								
12	0.82	0.82	0.82	0.79	0.85	0.82	0.79	0.88	0.85	0.82	0.79	-							
13	0.97	0.97	0.97	0.94	1.0	0.97	0.94	0.79	1.0	0.97	0.94	0.82	-						
14	0.97	0.97	0.97	0.94	1.0	0.97	0.94	0.79	1.0	0.97	0.94	0.85	1.0	-					
15	0.79	0.85	0.85	0.82	0.82	0.85	0.82	0.91	0.82	0.79	0.76	0.94	0.82	0.82	-				
16	1.0	0.94	0.94	0.97	0.97	0.94	0.97	0.76	0.97	1.0	0.97	0.82	0.97	0.97	0.79	-			
17	0.97	0.97	0.97	0.94	1.0	0.97	0.94	0.79	1.0	0.97	0.94	0.85	1.0	1.0	0.82	0.97	-		
18	0.97	0.97	0.97	0.94	1.0	0.97	0.94	0.79	1.0	0.97	0.94	0.85	1.0	1.0	0.82	0.97	1.0	-	
19	1.0	0.94	0.94	0.97	0.97	0.94	0.97	0.76	0.97	1.0	0.97	0.82	0.97	0.97	0.79	1.0	0.97	0.97	-

*1 – CBS322.52, 2 – CBS815.73, 3 – B1, 4 – M18.4x, 5 – M7, 6 – M6, 7 – M11B, 8 – M4.06, 9 – M8B, 10 – M26.04, 11 – M5.03, 12 – M20, 13 – M9, 14 – M12B, 15 – M19, 16 – M 10, 17 – M5A, 18 – M11A, 19 – M18

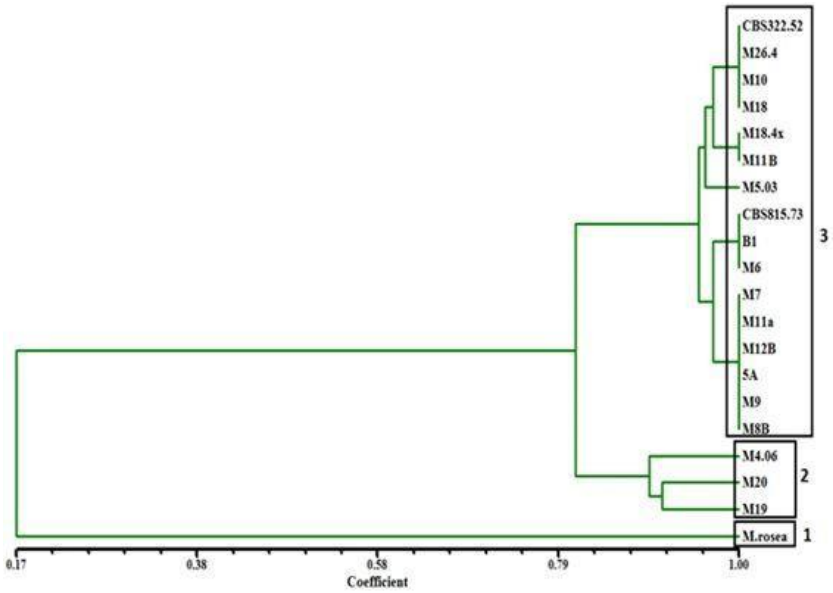

Figure 2. UPGMA dendrogram showing genetic relationship of *Hypomyces* isolates on the basis RAPD analysis

Mycelial growth on different media and pH values

Table 3 shows the results of the mycelial growth rate of isolates on different media. The order of isolates in Table 3 corresponds to the order obtained by molecular analysis in Figure 2. Analysis of variance showed that mycelium growth of *H. perniciosus* differed significantly between media. The highest mycelial growth rate was obtained on MEA and SDA (Table 3). The average growth rate of isolates on these media was 7.4 and 7.0 mm daily, respectively. The mycelial growth of individual isolates varied from 5.4 to 10.2 mm per day on SDA from 5.2 to 8.5 mm on MEA. Most isolates showed slower mycelial growth on CYA than on the other media tested. The average mycelial growth rate of the isolates was the lowest on CYA at 6.3 mm per day. Isolates M18 and M20 exhibited the highest radial growth rates, exceeding 8.0 mm per day on each medium, whereas M6 and M4.06 showed the lowest growth rates, below 6.0 mm per day (Table 3).

Table 4 shows the mycelial growth diameter results for various media and pH levels. Statistical analysis showed that the pH value and the medium had a significant impact on the mycelium growth of the isolates. The results indicated that SDA and MEA were most suitable for mycelial growth when cultured at pH 5.0 and 6.0. However, these two factors had no significant interaction (Table 4).

Pathogenicity of isolates

Depending on *H. perniciosus* isolates, the range of yield reduction in the first flush varied from 20% to 100%. The results are shown in Figure 3. The order of isolates in Figure 3 corresponds to the order obtained by molecular analysis.

Inoculation with B1, M11A, M12, M5A, M9, and M8B resulted in the highest yield reduction of 100%, while the lowest yield reduction of 20–30% was observed with CBS322.52 and M19 isolates.

Phylogenetic analysis identified three subclades of *H. perniciosus* isolates. The average yield reduction for these groups was calculated to demonstrate the relationship between pathogenicity and phylogenetic characteristics. The results showed that the following isolates – CBS 815.73, B1, M6, M7, M11A, M12B, 5A, M9, and M8B – belonged to one group and were most genetically similar. This group also exhibited the highest pathogenicity against *A. bisporus*. The average yield reduction in these trials was 90.5%, significantly higher than the 50–60% reduction observed in the remaining isolate groups.

Table 3. Mycelial growth rate (mm per day) of *Hypomyces perniciosus* isolates on different media (n = 6)

Isolate	Medium			
	PDA	**MEA**	**SDA**	**CYA**
CBS322.52	6.4 ± 0.29	7.0 ± 0.16	8.3 ± 0.17	6.8 ± 0.17
M26.4	6.7 ± 0.29	7.1 ± 0.09	9.4 ± 0.26	6.7 ± 0.17
M10	6.7 ± 0.21	5.4 ± 0.29	5.7 ± 0.34	5.6 ± 0.33
M18	8.7 ± 0.12	8.5 ± 0.29	10.2 ± 0.22	8.4 ± 0.29
18.4x	7.1 ± 0.29	7.1 ± 0.17	6.9 ± 029	6.0 ± 0.16
M11B	7.0 ± 0.33	6.6 ± 0.25	7.3 ± 0.24	5.1 ± 0.11
M5.03	5.0 ± 0.16	7.0 ± 0.12	7.3 ± 0.24	6.9 ± 0.09
CBS815.73	6.6 ± 0.22	7.1 ± 0.33	7.6 ± 0.33	6.8 ± 0.21
B1	7.3 ± 0.26	8.0 ± 0.22	6.8 ± 0.21	6.5 ± 0.12
M6	5.6 ± 0.12	5.4 ± 0.14	5.4 ± 0.09	4.9 ± 0.12
M7	6.1 ± 0.08	6.0 ± 0.16	6.5 ± 0.41	6.8 ± 0.20
M11A	7.3 ± 0.37	8.2 ± 0.21	7.0 ± 0.17	6.1 ± 0.22
M12B	7.6 ± 0.12	8.1 ± 0.19	8.8 ± 0.24	6.3 ± 0.25
M5A	6.2 ± 0.16	6.2 ± 0.08	6.4 ± 0.19	5.5 ± 0.24
M9	5.3 ± 0.25	7.0 ± 0.12	7.1 ± 0.29	6.1 ± 0.12
M8B	7.0 ± 0.22	6.5 ± 0.05	7.0 ± 0.22	5.5 ± 0.17
M4.06	5.0 ± 0.25	5.2 ± 0.14	5.5 ± 0.12	4.1 ± 0.10
M20	8.1 ± 0.17	8.0 ± 0.10	8.5 ± 0.05	7.6 ± 0.08
M19	6.7 ± 0.21	7.0 ± 0.16	7.0 ± 0.21	6.8 ± 0.19
Mean	**6.7 ± 0.21 AB**	**7.0 ± 0.17 A**	**7.4 ± 0.22 A**	**6.3 ± 0.18 B**

Means in rows followed by the same letter are not significantly different using Tukey test at p = 0.05

Table 4. Average colony diameters (mm) of *Hypomyces perniciosus* isolates after 8 days of incubation depending on the medium composition and pH value, n = 57

Medium	**pH value**				
	4,5	**5,0**	**6,0**	**7,0**	**Mean**
PDA	71.3 ± 3.2	73.7 ± 4.7	71.5 ± 4.8	66.0 ± 5.4	70.6 ± 4.5 B
MEA	74.3 ± 3.1	77.8 ± 2.5	76.5 ± 2.6	72.3 ± 2.8	75.2 ± 2.8 A
SDA	75.6 ± 2.2	80.2 ± 2.2	77.4 ± 2.8	71.4 ± 3.0	72.2 ± 2.6 A
CYA	61.2 ± 4.0	66.7 ± 4.8	65.3 ± 3.7	60.2 ± 3.5	63.4 ± 4.0 C
Mean	70.6 ± 3.1 B	74.5 ± 3.6 A	72.7 ± 3.5 A	67.5 ± 3.7 C	-

Means in columns followed by the same letter are not significantly different using Tukey test at p = 0.05;
Means in rows followed by the same letter are not significantly different using Tukey test at p = 0.05

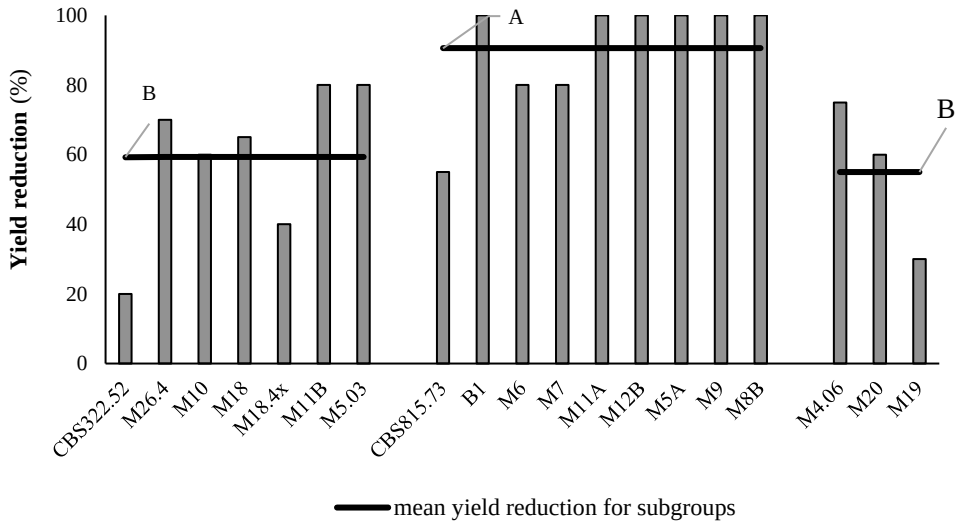

Figure 3. Pathogenicity of tested *Hypomyces perniciosus* isolates

DISCUSSION

Wet bubble disease caused by *H. perniciosus* is considered one of the severe diseases of white button mushrooms (Fletcher et al. 1995; Sharma et al. 2011; Shi et al. 2020). This study examined the morphological characters, pathogenicity, and genetic variability of *H. perniciosus* obtained from Polish mushroom farms was examined. Seventeen isolates were identified based on colony morphological characteristics and confirmed by molecular characterization using the ITS region.

The differences in the morphology of the colonies and the growth rate according to the medium were observed between isolates. The fungal colonies first produced floccular or velvety white mycelium, which later turned brown or remained colorless, depending on the growth medium tested. The same morphological characteristics of *H. perniciosus* were shown by Fletcher et al. (1995), Glamočlija et al. (2008), and Kouser et al. (2015). Colorless colonies of *H. perniciosus* may indicate colorless conidia and a lower sporulation rate (Li et al. 2019b). Albouy and Lapierre (1972) found that some highly pigmented isolates that grew more slowly on agar produced more aleuriospores and were more virulent. Other isolates were weakly pathogenic, producing more vegetative growth than before, with slight pigmentation. Fletcher et al. (1995) also reported that nonpigmented white isolates of *H. perniciosus* were less pathogenic than the pigmented isolates. The moderate to high virulence of the yellow to brown *H. perniciosus* isolates may be due to high conidia production, and the production of secondary metabolites (such as mycotoxins) plays a vital role in pathogenesis. We showed that isolate CBS322.52, characterized by a slight pigmented colony, was weakly pathogenic against *A. bisporus*, whereas M9, characterized by a brown pigmented colony, had a high level of virulence. On the other hand, isolate M20 with brown pigmented mycelium was weakly pathogenic. This result shows that the pigmentation of the *H. perniciosus* colony does not indicate the pathogenicity of the isolate. Atkey et al. (1976) also found that all isolates examined were highly pathogenic, regardless of growth rate.

All media tested supported the growth of *H. perniciosus* isolates to varying degrees. The best radial growth of isolates was obtained on MEA, followed by PDA, Sabouraud, and CYA. MEA medium likely provides a good carbon and nitrogen source for most fungal growth (Su et al. 2012). However, isolates on PDA also reached the edges of the Petri dishes very quickly. Different colony characteristics and good mycelial growth of *H. perniciosus* on potato dextrose agar was also confirmed in the literature (Fletcher et al. 1995; Kouser et al. 2015). In the present study, SDA and MEA media were identified as the best media for the mycelial growth of isolates, while CYA medium was the worst. The mycelial growth rate on SDA medium

ranged from 5.5 to 10.2 mm per day and on MEA from 5.0 to 8.2 mm. Li et al. (2019b) reported that the average growth rate of *H. perniciosus* colony on PDA was 11.7 mm per day, which was inconsistent with the present result. In the present study, the mycelial growth rate of *H. perniciosus* isolates on a PDA medium ranged from 5.0 to 8.7 mm per day. Sharma and Kumar (2000) also studied the influence of different culture media on mycelial growth, colony characteristics, and sporulation rate of *H. perniciosus* isolates. Contrary to our results, they showed that the best radial growth of isolates was obtained on CYA medium, followed by PDA, MEA, and SDA. On the other hand, according to Sharma and Kumar (2000), the best spore production by *H. perniciosus* isolates was obtained on MEA, followed by SDA, PDA, and CYA. This finding correlates with the data obtained for colorless colonies on CYA medium, indicating poor or no sporulation of the tested fungi. This phenomenon may be caused by chloride ions in the CYA medium (Sharma & Pandey 2010). Differences in the mycelium growth of *H. perniciosus* isolates depending on the pH of the medium were also recorded. The best mycelial growth was observed at pH 6.0, which consistent with the results of Siwulski et al. (2011). This study investigated the pathogenicity of different isolates of *H. perniciosus* and their effect on the yield of mushrooms. The most significant yield reduction (100%) was observed in trials infected with B1, M11A, M12, M5A, M9, and M8B, which were genetically similar according to phylogenetic analysis. The lowest yield reduction (20–30%) was observed in trials infected with CBS322.52 and M19 isolates.

Our finding of the high severity of wet bubble disease is in agreement with the results recorded by Sharma and Kumar (2000), Bhatt and Singh (2002), and Kouser and Shah (2013). They also observed yield losses of 65.6%, 80.1%, and 100% after inoculating the casing with *H. perniciosus* isolates. On the other hand, Gea et al. (2010) reported that the disease incidence was 30.6% in the first flush. What is important is that the concentration of spores in our study was lower than that reported by Gea et al. (2010). It shows that tested isolates of *H. perniciosus* in our experiments were highly pathogenic to *A. bisporus*.

Studies on the genetic diversity of *H. perniciosus* fungi were reported in the literature (Wen et al. 2010; Wang et al. 2016; Li et al. 2019b). In addition, Li et al. (2019a) investigated the whole genome sequence of *H. perniciosus* using the PacBio Sequel platform. They estimated the evolutionary time of the genome and performed a phylogenomic study of the order Hypocreales. The *H. perniciosus* genome size was found to be within the range observed for the Hypocreaceae family and consists of 25.27% repeat sequences, the largest in the Hypocreaceae family and other fungal plant pathogens.

The study found low genetic diversity between isolates collected from Polish mushroom farms, regardless of their origin. The RAPD method was used for analysis, and phylogenetic analysis identified three subclades. Similar results were obtained by Wang et al. (2016), who examined the genetic variation among 49 strains of *H. perniciosus* collected from different areas of Fujian Province in China. Analysis of DNA sequence polymorphism revealed two major distinct groups. Wen et al. (2010) conducted ISSR analysis and found that *H. perniciosus* strains from Chinese mushroom crops could be divided into three cladograms corresponding to the morphological characteristics of the colonies, which could be divided into three categories. Zhang et al. (2017c) also achieved a high similarity between the *H. perniciosus* isolate obtained from diseased mushrooms and the reference isolate. It was determined by amplifying the rDNA-ITS gene region of the tested isolate and sequencing it using the ITS1/ITS4 primers.

We calculated the average yield reduction for three subgroups to demonstrate the relationship between phylogenetic characteristics and pathogenicity isolates. Our findings indicate that the eight genetically similar isolates (CBS 815.73, B1, M6, M7, M11A, M12B, M5A, M9, and M8B) exhibited the highest pathogenicity against *A. bisporus*, resulting in an average yield reduction of 90.5% in infected trials. In contrast, the remaining groups of isolates resulted in a yield reduction of 50–60%. Zhang et al. (2021) also reported a similar finding of low genetic diversity among *H. perniciosus* isolates. Zhang et al. (2021) also reported a similar finding of low genetic diversity among *H. perniciosus* isolates. However, they identified two groups of isolates with differing pathogenicity.

The study showed that the pathogen isolated from commercial mushroom crops showing symptoms of wet bubble disease belongs to *H. perniciosus*. Constant monitoring of mushroom farms throughout Poland is necessary to develop a representative collection of fungus isolates that can be used for research on pathogenicity markers and help to manage the protection of white mushrooms against wet bubble disease.

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