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Micromorphological characteristics of the species of *Pholiota* (*Strophariaceae*, *Basidiomycota*) in pure culture

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Abstract. The article presents results of the research on micromorphology of vegetative mycelia of eight species of the genus *Pholiota* from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine. Using scanning electron microscopy and light microscopy, the micromorphological structures specific to these species were studied in order to enable their identification in pure culture. New data on micromorphology of *Pholiota* species are given. A set of micromorphological structures was observed for this genus, namely clamp connections, chlamydospores, arthrospores, anastomoses, crystals on hyphae, hyphal rings, rhizomorphs, pellicle spots, hyphae ornamentation, and secretory hyphae. For the first time a detailed study of microstructures of *P. alnicola*, *P. limonella*, *P. nameko*, *P. populnea*, and *P. subochracea* was conducted. Secretory hyphae and vacuolized mycelia in pure culture were noticed only for *P. populnea*. For *P. subochracea,* various hyphae ornamentation on vegetative mycelium was demonstrated. New information about the presence of hyphal rings for three *Pholiota* species, *P. alnicola*, *P. limonella*, and *P. subochracea*, is presented.

Keywords: light microscopy, macrofungi, micromorphological structures, *Pholiota*, pure culture, scanning electron microscopy, vegetative mycelium

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Резюме. Представлені результати досліджень мікроморфології вегетативного міцелію восьми видів роду *Pholiota* з Колекції культур шапинкових грибів Інституту ботаніки ім. М.Г. Холодного НАН України (ІВК). За допомогою світлової мікроскопії та сканувальної електронної мікроскопії були вивчені мікроморфологічні структури, специфічні для цих видів та необхідні для їхньої ідентифікації в чистій культурі. Наведено нові дані з мікроморфології видів роду *Pholiota*. Відмічено сукупність мікроморфологічних структур, а саме: пряжки, хламідоспори, артроспори, анастомози, кристали на гіфах, гіфальні кільця, ризоморфи, міцеліальні плівки, секреторні гіфи на вегетативному міцелії, орнаментація гіф. В нашому дослідженні вперше проведено детальне вивчення мікроструктур *P. alnicola*, *P. limonella*, *P. nameko*, *P. populnea* та *P. subochracea* для уточнення морфологічних характеристик та надійної ідентифікації цих видів у чистій культурі. Лише для *P. populnea* було відмічено існування секреторних гіф і вакуолізованого міцелію. Для *P. subochracea* отримана інформація щодо орнаментації гіф на вегетативному міцелії. Гіфальні кільця вперше виявлені у трьох видів – *P. alnicola, P. limonella* і *P. subochracea*.

Ключові слова: вегетативний міцелій, макроміцети, мікроморфологічні структури, світлова мікроскопія, сканувальна електронна мікроскопія, чиста культура, *Pholiota*

Introduction

About 470 species of the genus *Pholiota* (Fr.) P.Kumm. are known worldwide (http://www.mycobank.org), 25 of them have been reported for Ukraine (Zerova, 1979; Dudka et al., 2009). Mushrooms received attention due to combination of nutritious properties with health-stimulating and medicinal effects (Kim et al., 2006). Anticarcinogenic, antioxidant, antimicrobial and immunomodulating properties of the components isolated from mycelia and fruit bodies of this genus have been established (Cho et al., 2003; Kim et al., 2006; Zhang et al., 2009). Using mushrooms as a source of preventive and therapeutic agents and their cultivation in the industrial scale have been realized after many years of fundamental research of biology of macrofungi, peculiarities of their growth and development, and nature and mechanisms of metabolic and enzymatic activity (Wasser, 2010). Fruit bodies of *P. adiposa* and *P. nameko* are cultivated in the countries of Southeast Asia in the industrial scale (Pegler, 2003; Gizaw, 2015).

Taxonomic features of macrofungi can be used for their identification in pure culture, therefore research

Table 1. **List of the studied *Pholiota* species and strains**

Species	IBK strain	Origin of culture, year
Pholiota adiposa (Batsch) P.Kumm.	2169	Ukraine, Kyiv, on *Populus*, 2011
Pholiota alnicola (Fr.) Singer	2406	Ukraine, Ivano-Frankivsk Region, Halych, Halych National Nature Park, 2015
Pholiota aurivella (Batsch) P.Kumm.	2605	Ukraine, Kyiv Region, Vasylkiv, 2018
Pholiota limonella (Peck) Sacc.	2335	Ukraine, Kamianets-Podilskyi, 2013
Pholiota nameko (T.Ito) S.Ito & S.Imai	2154	Obtained from TSAU (AM2), Melitopol, Ukraine, 2011
Pholiota populnea (Pers.) Kuyper & Tjall.-Beuk.	2602	Ukraine, Kyiv, on *Populus*, 2018
Pholiota squarrosa (Oeder) P.Kumm.	2010	Obtained from MSU (3935), Moscow, Russia, 2009
Pholiota subochracea (A.H.Sm.) A.H.Sm. & Hesler	2535	Ukraine, Kyiv Region, Kyyliv, on soil, 2017

MSU – Moscow State University, Moscow, Russia; TSAU – Tauria State Agrotechnological University, Melitopol, Ukraine

on their micromorphological structures is important. Micromorphological characteristics of Basidiomycetes include a set of microscopic features such as presence of clamp connections or pseudo-clamp connections, width and types of hyphae according to the traditional classification by Stalpers (1978), presence of anastomoses and various structures formed during differentiation of the hyphae in culture (hyphal rings, rhizomorphs, inlaid hyphae, crystals on hyphae, etc.), presence of asexual reproduction structures (Mykhaylova, 2014).

This study was focused on micromorphological structures of eight species of the genus *Pholiota* preserved in the IBK Mushroom Culture Collection.

The study of microstructures of the *Pholiota* species was traditionally conducted based on fruiting bodies collected in natural habitats (Sawyer, 1917; Hesler, 1968; Farr et al., 1977; Farr, 1985; Smith, Adamcik et al., 2006; Kirk et al., 2008; Chang, Hayes, 2013). In contrast, in pure culture these species were poorly studied. Microstructures only of few species from the genus *Pholiota* were investigated, *P. adiposa*, *P. aurivella*, *P. lenta*, *P. nameko*, and *P. squarrosa* (Yoshinori et al., 1999; Buchalo, Didukh, 2005; Buchalo et al., 2009, 2011; Dyakov et al., 2011). Our research was aimed at studying microstructures of some of these and other species of the genus.

Materials and methods

Eight strains of *Pholiota* species from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of the Ukraine were investigated (Bisko et al., 2016). Some of these strains were obtained in 2017–2018 (Table 1).

Mushroom cultures were grown on glucose peptone yeast agar media; g/l: glucose – 25; peptone – 3; yeast extract – 3; $MgSO_4$ – 0.25; KH_2PO_4 – 1; K_2HPO_4 – 1; agar-agar – 22; pH – 6.0) in Petri dishes at temperature 26 ± 1 °C. Mycelia were taken from the cultures in the phase of active growth (3–10 days) and from the long-term cultivation of *Pholiota* species (2 months). Vegetative mycelium microstructures were studied using Zeiss light microscope (LM) and scanning electron microscopy (SEM). Samples were prepared for light microscopy using distilled water, 10% KOH or preparative mixture (glycerin: ethanol: water = 1: 1: 1) (Bilay, 1982). Samples were prepared for SEM using the modified method of Quattelbaum and Carner (Quattelbaum, Carner, 1980). Four sterilized square 4×4 mm pieces of the cover glass were placed 1–3 cm away from inoculum into Petri dishes. The cover glasses were removed from the agar media, when mycelia overgrew the surface, and then transferred to a microscopic slide. The slide was then placed into a sealed glass vessel fixed with osmium tetroxide vapor (1% solution) for 8 hours. On fixation, the slides were transferred to an empty Petri dish to dry out for 72 hours. After drying out, samples were covered with gold in the vacuum spray gun JII-4X with rotation (Buchalo, Didukh, 2005). The specimens were examined using the Jeon JSM-6060 LA Scanning Electron Microscope (Japan) and studied at a magnification ×1000 – 10000.

Results and discussion

An important taxonomic feature for identification of macrofungi in pure culture is the presence of a unique structure that occur on the mycelium of many species of *Basidiomycota* clamp connections (Stalpers, 1978; Buchalo, Didukh, 2005). Though the clamp connections are exclusive to this phylum, not all species possess these structures. Regular clamp connections were observed for vegetative mycelia in all studied

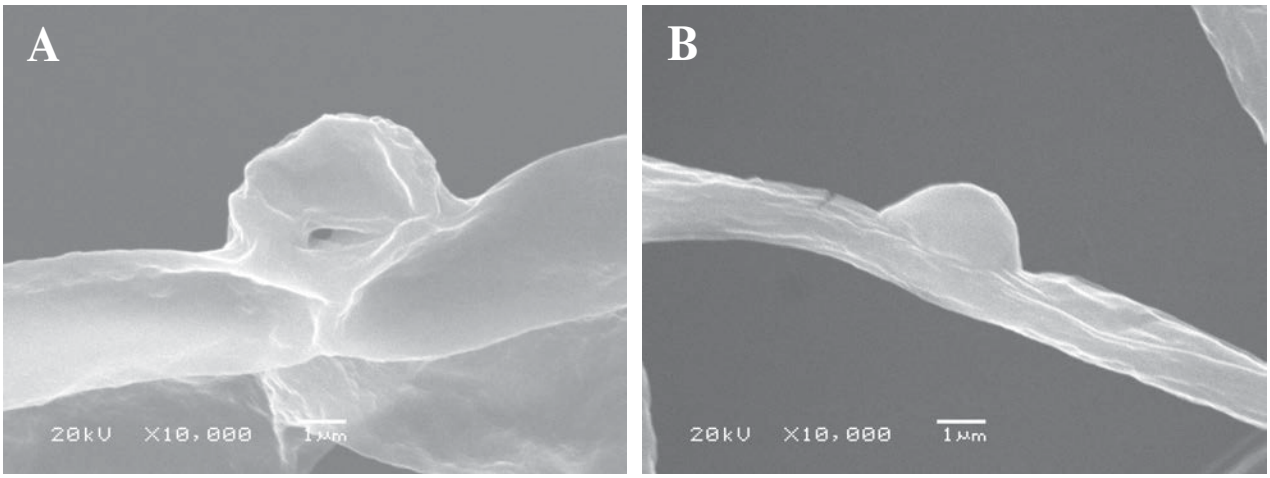

Fig. 1. Clamp connections of *Pholiota populnea* 2602 (A) and *Pholiota adiposa* 2169 (B), SEM (×10000)

species (Fig. 1), which agrees with the literature data (Buchalo, Didukh, 2005; Buchalo et al., 2009, 2011; Dyakov et al., 2011).

Vegetative mycelium of *Pholiota* species in pure culture consist of the thin-walled, moderately branched, regularly septated, uncolored hyphae of 1–3 μm in diameter, with anastomoses formed between hyphae (Table 2, Fig. 2, A) and pellicle spots on vegetative mycelium (Table 2, Fig. 2, B). Hyphae fusion is carried out through anastomoses and pellicle spots on vegetative mycelium, which is a well-known phenomenon in the phylum *Basidiomycota* (Buchalo et al., 2009).

The formation of secretory hyphae and vacuolised mycelium in old cultures were noticed only in *P. populnea* 2602 (Fig. 3). Similar secretory hyphae were found for *Fistulina hepatica* (Buchalo et al., 2009), *Lepista nuda* (Badalyan, Gharibyan, 2017) and vacuolised mycelia for *Coprinopsis strossmayeri*, *Fomitopsis pinicola* (Badalyan, Gharibyan, 2017) and *Ganoderma adspersum* (Badalyan et al., 2019). Hyphae ornamentation that was observed in *P. subochracea* cultures and hyphal rings in old cultures of *P. alnicola, P. limonella* and *P. subochracea* can be used as diagnostic taxonomic characters (Fig. 4).

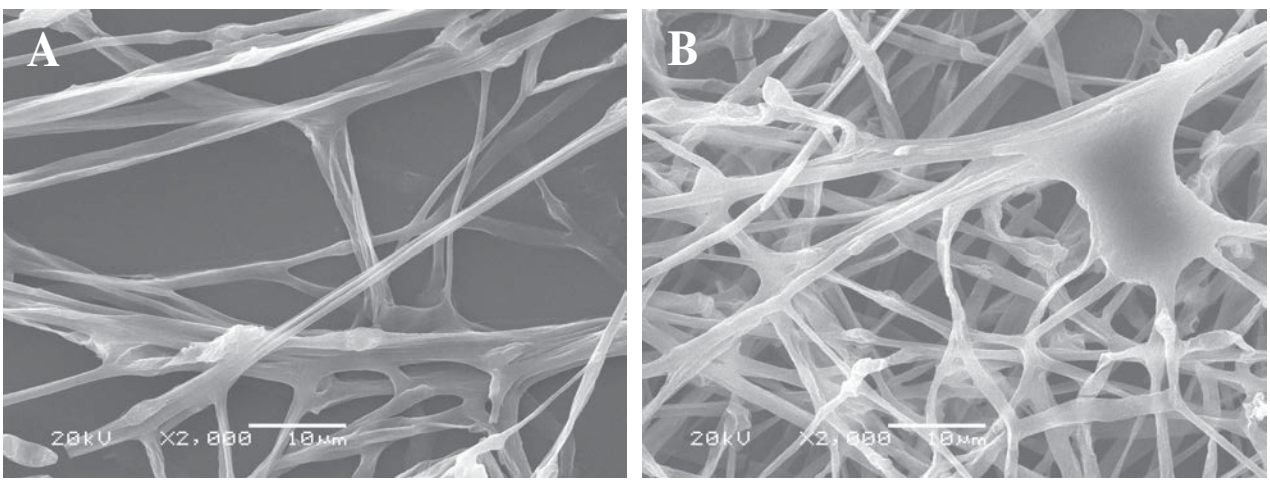

Fig. 2. Anastomoses of *Pholiota nameko* 2154 (A) and pellicle spot on vegetative mycelia of *Pholiota squarrosa* 2010 (B), SEM (×2000)

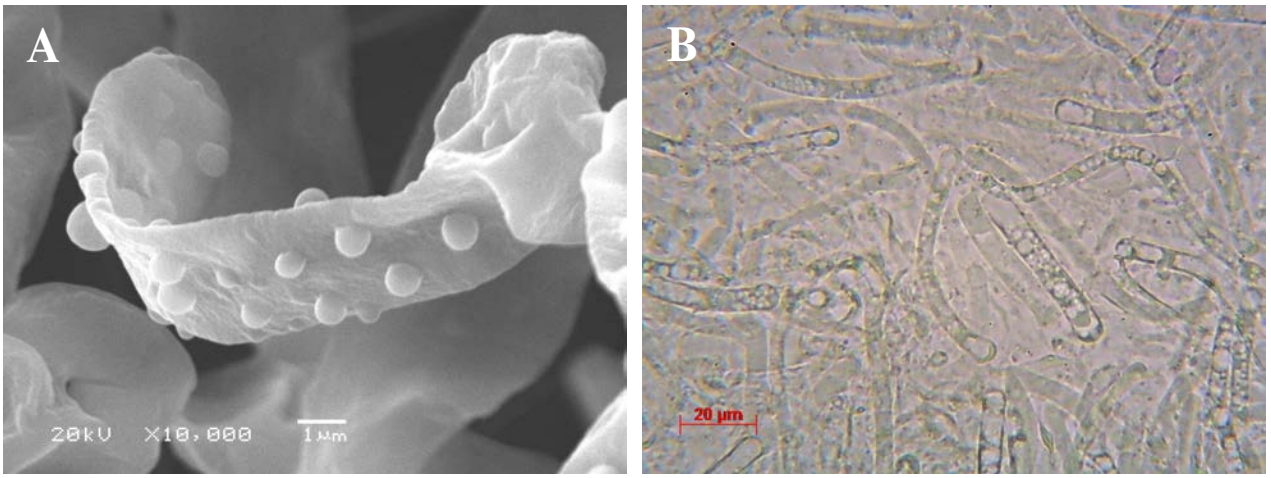

Fig. 3. Exudate on hyphae (A), SEM (×10000) and vacuolised mycelium (B), LM (×40) of *Pholiota populnea* 2602

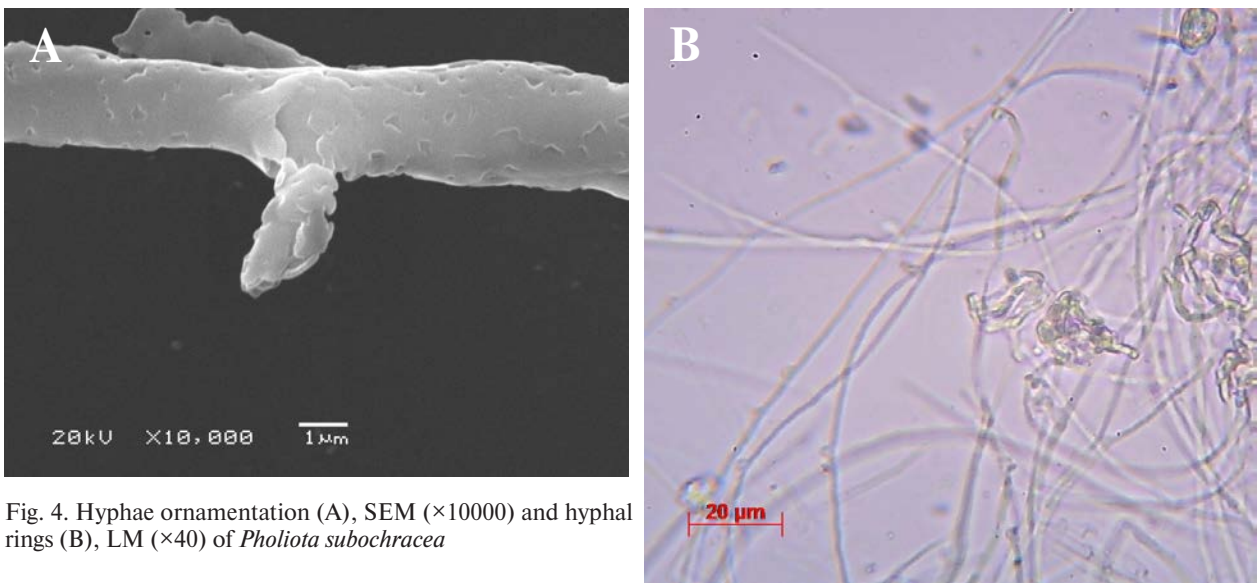

Fig. 4. Hyphae ornamentation (A), SEM (×10000) and hyphal rings (B), LM (×40) of *Pholiota subochracea*

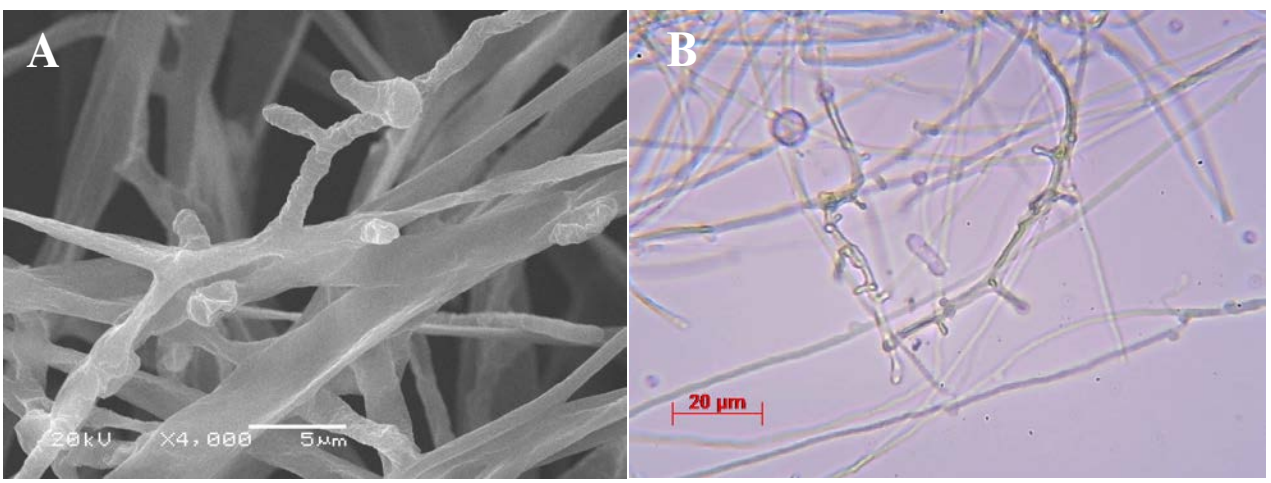

Fig. 5. Conidial sporulation of *Pholiota aurivella* 2605 (A), SEM (×4000), and *Pholiota subochracea* 2535 (B), LM (×40)

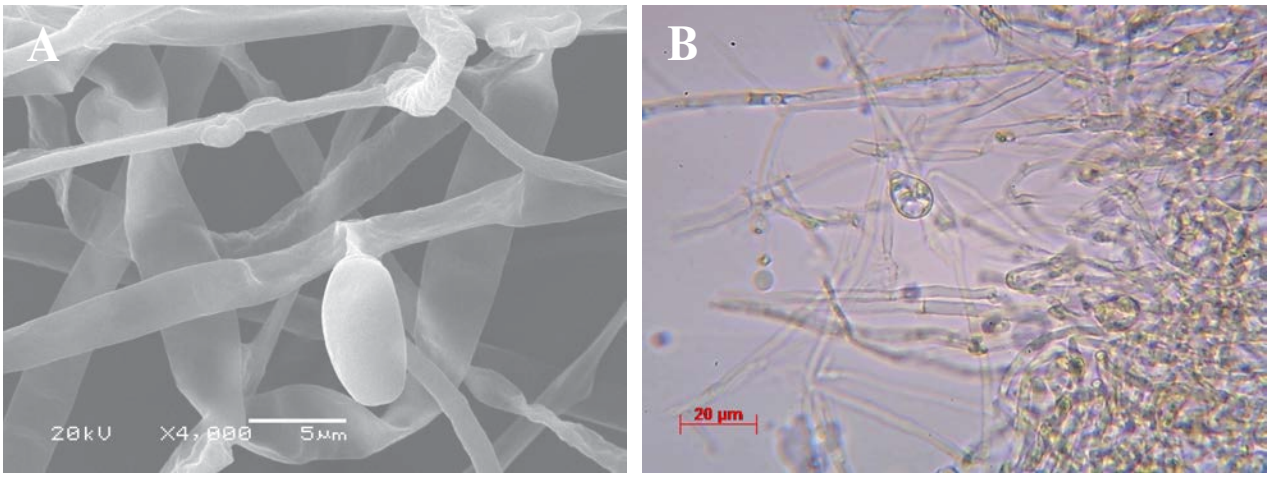

Fig. 6. Chlamydospores of *Pholiota limonella* 2335 (A), SEM (×4000), and *Pholiota alnicola* 2406 (B), LM (×40)

Species of macrofungi form different structures of asexual reproduction (anamorph structures). Chlamydospores and arthrospores are the most common asexual reproduction structures of *Basidiomycota* (Buchalo, Diduch, 2005; Buchalo et al., 2011). Anamorphs can be used as a taxonomic criterion at the species level, or sometimes for taxa of higher taxonomic ranks, but most macrofungi do not have these structures. We observed conidial sporulation in all studied cultures (Fig. 5), except *P. adiposa*.

Single intercalary chlamydospores were observed for six cultures – *P. adiposa*, *P. alnicola*, *P. aurivella*, *P. limonella*, *P. nameko*, and *P. subochracea* (Table 2, Fig. 6).

According to literature data (Buchalo, Didukh, 2005; Buchalo et al., 2009; Dyakov et al., 2011), vegetative mycelia of the strains of *P. adiposa*, *P. aurivella*, and *P. squarrosa* are able to form a structure of asexual reproduction, mitotic spores (arthrospores). We observed arthrospores in our experiment only for *P. aurivella, P. adiposa,* and *P. limonella* (Fig. 7).

The ability to form crystals is widely known for many species of *Basidiomycota* which sometimes also depends on the nutrient media and age of the mushroom cultures (Buchalo et al., 2009; Dyakov et al., 2011). Crystal formation was observed in almost all investigated species of *Pholiota* (Table 2, Fig. 8). The morphology of the crystals is very different. We observed prismatic, cubic, hexahedral, sometimes of the undefined shape crystals. Maximum length of the crystals was 12.2 µm, minimum – 0.5 µm, width – 0.3–6.1 µm.

Thus, presence of clamp connections, ability to form anastomoses and pellicle spots on vegetative mycelia,

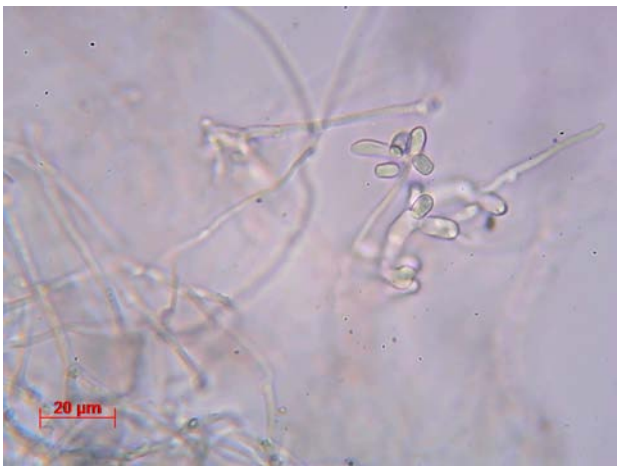

Fig. 7. Arthrospores of *Pholiota limonella* 2335, LM (×40)

formation of conidial sporulation, arthrospores and chlamydospores, ability to secrete crystals different in shape and size were observed for various investigated species. Our results confirm some earlier literature data (Buchalo, Didukh, 2005; Buchalo et al., 2009, 2011; Dyakov et al., 2011). Micromorphological characteristics of the studied *Pholiota* species are shown in Table 2.

Conclusions

The micromorphological features of eight species of *Pholiota* from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine were established.

For the first time we conducted a detailed study of microstructures of vegetative mycelia of such species

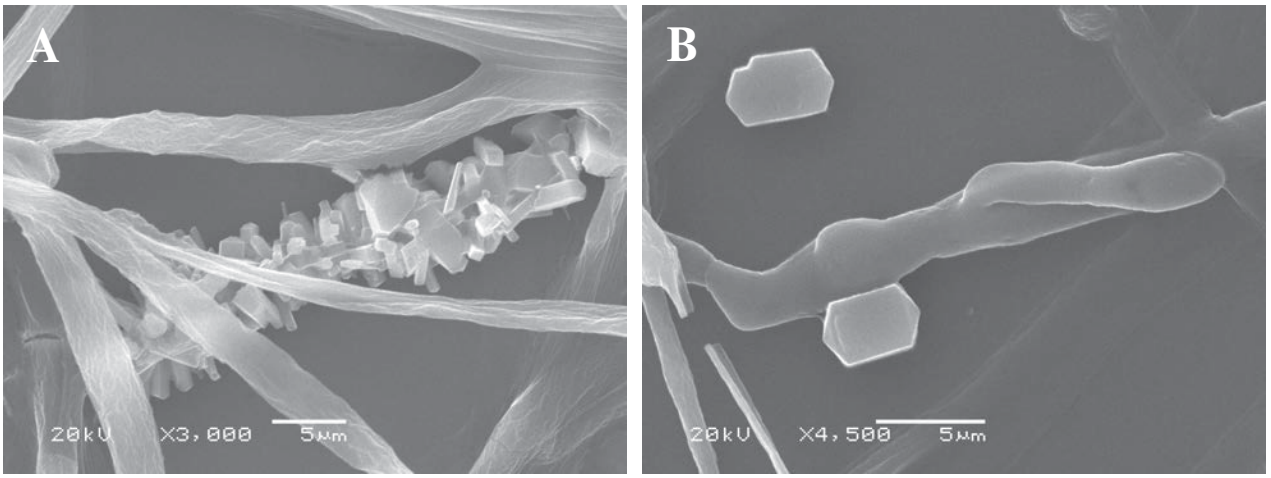

Fig. 8. Crystals of *Pholiota adiposa* 2169 (A), SEM (×3000) and *Pholiota alnicola* 2406 (B), SEM (×4500)

as *P. alnicola*, *P. limonella*, *P. nameko*, *P. populnea*, and *P. subochracea* for morphological characteristics and identification of these taxa in pure culture. The presence of all typical for the genus features has been noted in various studied species.

New data about micromorphological features of *Pholiota* species in pure culture were obtained. Only for *P. populnea* the existence of secretory hyphae and vacuolized mycelium and for *P. subochracea* – the hyphae ornamentation on vegetative mycelium were noticed. New information about the presence of hyphal rings for three *Pholiota* species, *P. alnicola, P limonella* and *P. subochracea*, is provided.

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Table 2. **Micromorphological features of vegetative mycelium of *Pholiota* species**

Species, strain	Anastomoses	Arthrospores	Chlamydospores	Clamp connections	Conidial sporulation	Crystals	Exudate on hyphae	Hyphal rings	Pellicle spots on vegetative mycelium
Pholiota adiposa 2169	+	+	+	+	–	+	–	–	–
Pholiota alnicola 2406	–	–	+*	+*	+*	+*	–	+*	–
Pholiota aurivella 2605	+	+	+	+	+	–	–	–	+
Pholiota limonella 2335	+*	–	+*	+*	+*	+*	–	+*	–
Pholiota nameko 2154	+*	–	+*	+*	+*	+*	–	–	+*
Pholiota populnea 2602	+*	–	–	+*	+*	+*	+*	–	–
Pholiota squarrosa 2010	+*	–	–	+	+	–	–	–	+
Pholiota subochracea 2535	+*	–	+*	+*	+*	+*	–	+*	+*

"+" structures found; "–" structures not found; * new data for this species

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