For the correct identification of the taxonomic position of cultures, a complex of morphological criteria are proposed by authors: presence and morphology of teleomorph stage; colour, morphology, and growth rate of mycelial colony; type of anamorph; presence, dislocation, and morphology of clamp connections; special hyphal structures and other characteristics. Using scanning electron microscopy, new data were obtained on microstructures of vegetative mycelium in more than 100 species.

Keywords: scanning electron microscopy, clamp connections, conidial sporulations

INTRODUCTION
Mushroom cultures are widely applied in biotechnology (production of fruit bodies, cultural mycelium, pharmaceutical substances, enzymes, etc.) and in various aspects of fundamental mycological studies [1, 2, 3, 4, 5, 6a]. Like in most other fungi, the vegetative mycelium of mushroom cultures is a complex of branched hyphae, which differ only within narrow limits of width, length, number of nuclei, thickness of cell walls, and the character of branching. On the basis of statistical evaluation, some authors [7] conclude that the vegetative mycelium is similar in different groups of fungi, but its characteristics cannot be used as reliable taxonomic features. However, continuous accumulation of information on an increasing number of fungal species provides new material for study and allows the comparison of morphological characters and for the estimation of their potential use for taxonomic purposes and purity control in biotechnological processes [1, 8, 9, 6b].

Culture collections today store important components of medicinal mushrooms for carrying out fundamental studies and biotechnological application of medicinal as well as culinary mushrooms. A long-term study was carried out using strains from the Culture Collection of Mushrooms (IBK) of the M.G.Kholodny Institute of Botany, National Academy of Sciences of the Ukraine (Kiev), and the Culture Collection of Higher Basidiomycetes of the International Center for Cryptogamic Plants and Fungi, Institute of Evolution, University of Haifa (HAI), Israel. [10, 11].

Good representation of various species in these culture collections include the following genera: Agaricus, Pleurotus, Lentinus, Coriolus, Coprinus, Morchella, Ganoderma, Lycoperdon, Piptoporus, Oudemansiella, Flammulina, Hericium, etc. [10].

MATERIALS AND METHODS
Vegetative mycelial microstructures were studied using a scanning electron microscope (SEM). Mushroom cultures were grown on wort agar or malt-extract agar in Petri dishes. On inoculation of a Petri dish, five-to-seven sterilized square 4×4 mm cover glasses were aseptically placed 1–6
cm away from the inoculum. Petri dishes were incubated at 26°C. When the cultural mycelium grew over the cover glass surfaces, the cover glasses were removed from the surface of the agar media and transferred to microscope slides. The microscope slides were then placed into a sealed glass vessel to fix the mycelium with osmium tetroxide vapour (1% solution) for 96 h. On fixation, the slides were transferred to an empty Petri dish to dry for 72 h. After drying, samples were covered with gold in a vacuum spray gun JII–4X with rotation. The specimens were examined using a Scanning Electron Microscope JEOL JSM-6060 LA (Jeol, Japan) and studied at a magnification from × 100 to × 18.000 [8].

RESULTS AND DISCUSSION

Vegetative mycelium of mushroom species investigated in pure culture consisted of thin-walled, septated, and branched hyphae. The diameter of generative hyphae varied between 1.5 to 7.5 μm. In Agrocybe aegerita (V. Brig.) Singer, Auricularia auricula-judae (Bull.) Quél, A. polytricha (Mont.) Sacc., Coriolus zonatus (Nees) Quél., etc., cultures were thin (≤ 1μm wide), with no branching hyphae. In the younger part of Grifola frondosa (Dicks.) Gray mycelial colonies that branched, thin (≤ 1μm wide) hyphae (dichohyphidia) were formed. In the older parts of a mycelial colony, thin (≤ 1μm wide) non-branched hyphae and generative hyphae 3-7 μm wide were observed. Also, non-branched, aseptate, or secondary septa sceletal and sceletoid hyphae without clamp connections and with thick-walled cells occurred.

In mushroom cultures, a great diversity in hyphal morphology was described and some of the forms observed may have taxonomic importance (Fig. 1, 2). A few suggestions for classification of hyphae on the basis of their physiological role, type of branching, cell wall thickness, presence of aggregates on the surface or inside the cells, etc., were made. On the mycelia, different types of bristles, spines, swellings, bulbs, hyphal tangles, monilial hyphae, and gloeocystidia formed, some of which may be useful for the morphological characterization of cultures permitting the identification of fungal species. Stalpers [9] presented a description of 26 types of hyphal modifications, though many of them, in our opinion, are hardly distinguishable.

The presence of dolipore septa was an important criterion for the identification of cultures belonging to the higher Basidiomycetes. Dolipore septa were present between the cells. For the dikaryotic mycelia, the occurrence of clamp connections was typical. Clamp connections were
absent on primary monokaryotic mycelia that started from single spores or may have disappeared under favorable conditions of cultivation in liquid media [1].

Anastomoses formed between hyphae in all investigated species and strains. In some cases, numerous anastomoses were expected. In old parts of mycelial colonies, anastomoses between hyphae and clamps were typical. In our opinion, anastomoses are of no taxonomical significance.

Hyphal ornamentation from the genus *Lyophyllum*, observed under SEM, may serve as taxonomic characters. Warty ornamentations were detected in *Oudemansiella brunneomarginata* Lj. N. Vassiljeva and *O. mucida* (Schrad.) Höhn. on hyphae forming loops. Very typical lanunose structured hyphae were described in some species of *Morchella* (Ascomycetes) *M. angusticeps* Peck, *M. conica* Pers., *M. crassipes* (Vent.) Pers., *M. esculenta* (L.) Pers, *M. semilibera* DC.

In cultures of *Coprinus cinereus* (Schaeff.) Gray, *Crinipellis shevczenkoi* Buchalo, *Agaricus gennadii* (Chat. et Boud.) P.D. Orton, *Leucocoprinus bresadolianus*, etc. sclerotia of various shapes, sizes, and structural forms were present.

Strand-like mycelial cords were found in cultures of some species of *Agaricus*, *Macrolepiota*, *Omphalotus olearius*, and some Gasteromycetes species (*Phallus impudicus* L., *Tulostoma brumale* Bertero).

So called hyphae coils were detected in the *Oudemansiella brunneomarginata*, *O. mucida*, and *Tricholoma mongolicum* S. Imai mycelial cultures. The occurrence of coils in the mycelium is a new characteristic that has not yet been described in the literature. It is possible that similar structures will be found in other groups of fungi.

The presence of crystals on hyphae of mushroom cultures was reported in the literature [1, 8, 11, 12, 9]. Calcium oxalate crystals were formed on hyphae under cultivation in different nutritional media (agar and liquid media, grain, compost, etc.) and represented a relatively stable characteristic of the cultures. Oxalic acid represents one of the main metabolites of the Krebs cycle in living organisms [12]. Crystal formation was observed in all species of *Agaricus* investigated. Two types of cristals on a hyphae of *Agaricus brasiliensis* Wasser, M. Didukh, Amazonas & Stamets crystals were obsirved (Fig. 3 A,B). The density of crystals on the surface of hyphae may vary. Different stages of crystal formation could also be observed. As a rule, crystals cover the hyphae and were rarely found separated from the cells.

The morphology of the crystals was very different. We observed cubic, hexahedral, pyramidal, bipyramidal, prismatic, rod-shaped, and acicular crystals.

Polygonal crystals and crystals of other shapes were observed in *Armillariella mellea* (Vahl) P. Karst., *Hericium erinaceus* (Bull.) Pers., *Hypsizygus marmoreus* (Peck) H.E. Bigelow, *Kuehneromyces mutabilis* (Schaeff.) Singer & A.H. Sm., *Lentinus edodes*, *Omphalotus olearius*, *Pholiota adiposa* (Batsch) P. Kumm. etc.

On *Agaricus subfloccosus*, *Coprinus comatus* (O.F. Müll.) Gray and *Montagnea arenaria* (DC.) Zeller hyphae, thin filamentous hair-like crystals were observed.

In *Lentinus edodes*, crystals formed on the hyphae under cultivation on different nutritional media (agar and liquid media) and presented a relatively stable characteristic of cultures. The morphology of the crystals varied, and could be rhomboind and amorphous. Varioush shaped crystals (needle-like, rod-shaped, cubic-like, etc.) formed on hyphae in different, mainly aged, parts of *Omphalotus olearius* colonies.

Clamp connections are characteristic features of dikaryotic mycelia of many Basidiomycetes. The presence and dislocation of clamp connections on hyphae are essential taxonomic characteristics for some species. In addition to the presence of clamps in identifying Basidiomycetes cultures, form, size, and frequency of occurrence were also considered. Clamps can be divided into large or small, long or short, gentle or abrupt, and curved or medallion-types based on the ratio of clamp size to hyphal diameter, the angle of a clamp and hyphae, and the
presence or absence of a slit between a clamp and a septum [9]. Some species have clamps of an original form, namely, Auricularia auricula-judae, Lyophyllum decastes (Fr.) Singer, Lyophyllum ulmarium (Bull.) Kühner (Fig. 4), Oudemansiella mucida, Panus tigrinus (Bull.) Singer, Piptoporus betulinus (Bull.: Fr.) P. Karst. etc., and were characterized with clamp connections of various forms and sizes. In L. decastes and Piptoporus betulinus, besides single clamps, whorls of clamps, coupled clamps, and sprouted clamps were observed. Clamps on P. betulinus hyphae were rather variable in shape and size. Sprouted, coupled, and single clamps were also found on the mycelium of Pleurotus ostreatus (Jacq.) P. Kumm. In Panus tigrinus, the clamps were mostly single or coupled, and only seldom sprouted clamps were present; some clamps were asymmetrical.

In Coprinus comatus, a representative of Agaricales, clamps were mostly single, of medallion type and seldom without a slit. Their form was rather stable and uniform. In Marasmius oreades (Bolton) Fr., belonging to the same order, mostly single clamps of a relatively stable form were observed. The medallion type clamps occurred rather frequently.

Single clamps (except in pairs and whorls) were characteristic for the mycelium of Cyathus olla (Batsch) Pers. and C. striatus (Huds.) Willd. Some clamps forming anastomoses with adjacent hyphae occurred.

It is widely accepted, however, that clamps are not common in all species of Agaricales. They are constant in cultures of Pleurotus, Coprinus, Oudemansiella, Panus, Lentinus, and Pholiota. Clamp connections were observed in Agaricus campestris L., A. subperonatus (J.E. Lange) Singer, A. arvensis Schaeff., A. bernardii Quél., A. comtulus Berk. et Broome. The majority of authors noted that clamp connections occurred very rarely in vegetative mycelium of Agaricus. Clamps were found in Agaricus brasiliensis Wasser et al.,

Figure 3: Agaricus brasiliensis Wasser, M. Didukh, Amazonas & Stamets. SEM (× 4800)
Figure 4: Lyophyllum ulmarium (Bull.) Kühn. Clamp connections. SEM (× 10000)

A. campestris L., A. gennadii, A. nevoi Wasser etc. Clamps seldom occurred, they were mostly of a classical shape, often without a slit. However, in many species, even the SEM is unable to detect any specific morphological features of a clamp.

Species of macromycetes form different structures of asexual reproduction (anamorphs). Anamorphs, mainly conidial sporulation, may serve as taxonomic criteria for species or
sometimes at higher taxonomic levels. Even though the first study on asexual reproduction of mushrooms was provided by Brefeld [1], more detailed studies only began relatively recently. For most macrofungi species, anamorphs have not been revealed yet. Taking into account various taxons, the most attention was given to Agaricales, Boletales, Gasteromycetes and Pezizales were not studied in detail. In general, chlamydospores and arthrospores were the most common asexual reproduction structures of Basidiomycetes [13, 14, 15, 9, 16].

Anamorphs in cultures of macrofungi have been studied mostly using light microscopy. We presented a more detailed description of anamorphic structures using scanning electron microscopy [1, 17, 18, 19, 10, 8, 20, 21].

Arthroconidia have been found in the mycelial cultures of Oudemansiella brunneoincarnata. Conidial sporulations (arthroconidial structures) were also found for Agaricus arvensis (Jul. Schäff. & Steer) Pilát, A. fisuratus (F.H. Möller) F.H. Möller, A. maskae Pilát, A. silvaticus, Hypsizygus marmoreus, Lepista nuda, Lyophyllum ulmarium (Bull.) Kühner, and others (Fig. 5, 6, 7). Arthroconidia were formed by the increase in age of the protoplast.

Two coremia-forming species (Pleurotus abalonus Y.H. Han, K.M. Chen & S. Cheng and P. cystidiosus O.K. Mill.) were studied using the SEM. In our ultrastructural study, we found no difference between the imperfect states of P. abalones and P. cystidiosus, which were identical to Anthromycopsis broussonetiae. Coremia formation on colony surfaces began as a little tangle of sterile hyphae which turned into a clavarioid form. In the following stages, the growing coremia differentiated into the head and the stipe, and on the head chains of alantoid conidia 12-20×4-7 μm formed. No conclusive difference in the process of coremia formation; their size and shape as well as the shape and size of conidia between the two studied organisms were determined [21].

The blastic type of the anamorphic stage was more common in Agaricales. Blastoconidia in Fistulina hepatica Schaeff.: Fr. were formed on conidiophores either individually or in a chain (Fig. 8); chlamydospores were intercalary, lemon-shaped. Pholiota adiposa (Batsch) P. Kumm. anamorphs were similar to the conidia described from Ph. aurivella Singer and Ph. nameko (T. Ito) S. Ito et S. Imai cultures [15, 16]. Ph. adiposa conidia developed on short branches arising from hyphae. Our observation of conidia formation in P. adiposa confirms the opinion that they are arthroconidia.

In Coprinus spp the branched conidiophores bearing conidial structures were described (Fig. 9). We studied the fine structure of those “conidia” and showed that at the tips of conidiophore branches, no real conidial cells were formed. The tips of conidial branches ended with a tuft of fine, radially outgrowing hairs that gave at low magnification the impression of round verrucose conidia. However, the real nature of those structures was revealed at higher magnification. The possible role of the structures has yet to be elucidated.

Single globose conidia, which are termed by some authors as blastoconidia, pseudoconidia, or excretory conidia [22] on simple conidiophores, resembling a sterigmata of the basidium, were laterally formed on hyphae in cultures of Pleurotus spp. and Schizophyllum commune Fr.: Fr. They were globose, 3-5 μm in diameter.

Terminal and intercalary chlamydospores in dichotomous Hericium erinaceus cultures, and dichohyphidia and intercalary chlamydospores in the dichotomous vegetative stage of Grifola frondosa were of taxonomic significance [18, 8].

Conidial sporulations of the oidium type (budding cells) were characteristic of some species belonging to Morchellaceae (Ascomycota) (Fig. 10).
Figure 5: *Lepista nuda* (Bull.) Cooke. Conidial sporulation. SEM (× 1600)

Figure 6: *Laetiporus sulphureus* (Bull.) Murr. Conidial sporulation. SEM (× 1800)

Figure 7: *Morchella esculenta* (L.) Pers. Conidial sporulation. SEM (× 940).

Figure 8: *Fistulina hepatica* (Schaeff.) With. Conidial sporulation. SEM (× 4000)

Figure 9: *Coprinus cinereus* (Schaeff.) Gray. Sporangium-like anamorphic structure. SEM (×4000)

Figure 10: *Morchella conica* Pers. Budding cells on lacunose hyphae. SEM (× 2000)
Chlamydospores were also discovered in cultures of Agaricus arvensis, A. bisporus (J.E. Lange) Imbach, Auricularia auricula-judae, A. polytricha, Boletus edulis Bull., Calvatia excipuliformis (Scop.) Perdeck, Clitocybe gigantea (Sow.: Fr.) Quél., Coriolus zonatus, Hypsizygus marmoreus.

In the Ukrainian culture collection of edible and medicinal mushrooms, a diversity of species (about 200), genera (about 100), and strains (about 800) is represented, including over 100 species of medicinal mushrooms. Using scanning electron microscopy about 100 species were studied and the taxinomycal significance of different microstructures is discussed.

REFERENCES


