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Macro- and micromorphological characteristics of Ganoderma lucidum Karsten strains isolated in Hungary

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Abstract – Identification of *Ganoderma lucidum*, a cosmopolitan species of Ganodermataceae is not clear because of its similarity to other species of the *G. lucidum* species complex. Macroscopical and microscopical characteristics suitable for determination of the species and for making distinctions between different strains were investigated on 14 isolates originating in Hungary. It have been proved that Hungarian isolates are highly homogeneous but differ considerably from the strains known from literature. With 3 tables and 7 figures.

INTRODUCTION

Ganoderma lucidum (WILLIAM CURTIS: FRIES) KARSTEN (Ganodermataceae) is a common wood rotting tinder widespread on the northern hemisphere with considerable pharmaceutical interest. It has been used in traditional Far Eastern medicine for centuries. Recently more than a hundred of its lanostanoid compounds and a series of polysaccharides effective as antitumoral, antihepatotoxic, antiinflammatory and cardiotonic agents have been detected (JONG & BIRMINGHAM 1992). Great differences in morphological characteristics (ADASKAVEG & GILBERTSON 1988) and composition of pharmaceutically active substances (NISHITOBA *et al.* 1986) have been proved according to geographical distribution and habitat of the different isolates. The taxonomic status of *G. lucidum* and its distinction from other *Ganoderma* species is not clear, either.

The genus *Ganoderma* was established by KARSTEN in 1881 by the revision of *Boletus lucidus* with *Ganoderma lucidum* as the only species. The main characteristic feature of the Ganodermataceae family is the complex spore wall, which consists of an outermost primary layer, darkly stained interwall pillars surrounded by electron transparent regions and an innermost secondary pigmented layer (MIMS & SEABURY 1989). PATOULLIARD (1889) divided the genus into 48 species. MURRILL (1902) considered geographical distribution, host specificity and macromorphology of the fruit body (e. g. colour, shape) to be mostly suitable for differentiation within the genus. In the last decades STEYAERT (1972, 1980), CORNER (1983) and ADASKAVEG & GILBERTSON (1986, 1988) revised this genus taxonomically. The latter divide the *G. lucidum* species-complex into two parts: the *G. lucidum* group living on hardwood and the *G. tsugae* group living on

softwood. These groups can be distinguished by the size and cell wall structure of spores, the structure of pilocystidia, some cultural properties and interfertility tests. A Far Eastern team also tried to make distinctions by characterizing the mycelial cultures (WANG & HUA 1991). The macro- and micromorphology and enzymatic properties of 50 strains within 14 *Ganoderma* species had been investigated, partly with similar, partly with different results as ADASKAVEG & GILBERTSON (1986). Former investigations suggested that macromorphological characteristics of the fruitbody are not suitable for identification of the species as these show great variability with the environment and sometimes no fruitbodies, only micelium is available. Consequently taxonomic research continued into two directions: on one hand microscopical markers had been searched (ADASKAVEG & GILBERTSON 1988), on the other hand an attempt had been made to establish a commonly used uniform code-system for describing mycelial cultures (ADASKAVEG & GILBERTSON 1986, WANG & HUA 1991).

The main characteristics recently used for identification within the genus *Ganoderma* are as follows:

1. Host relationships. Some species grow on hardwoods while others on softwoods only.

2. Morphology of the fruitbody. Basidiocarp is stipitate or sessile. The position of the stipes can be central or excentric. Nevertheless, ADASKAVEG & GILBERTSON (1986) found that isolates from sessile fruitbodies can also produce stipitate fruitbodies.

3. Morphology of the pilocystidia from the outer layer of the pileus. HADDOW (1931), FURTADO (1965), RYVARDEN (1976) and ADASKAVEG & GILBERTSON (1988) found only slight, but important differences in the shape of pilocystidia.

4. Shape and size of basidiospore. Basidiospores are mainly ovated with truncated apex, except for *Ganoderma zonatum*. The size of the spores is characteristic but ranges overlap, therefore spore biometrics are not suitable for distinction of the species (ADASKAVEG & GILBERTSON 1988).

5. Structure of spore wall. The spore wall is composed of three layers. The middle interwall pillars vary in size and number within the genus, so the roughness of the outer spore-surface is also different (ADASKAVEG & GILBERTSON 1988, MIMS & SEABURY 1989).

6. Cultural characteristics. The optimal growth temperature, the speed of growth and some macroscopical and microscopical characteristics of the mycelial cultures are also suitable for identification of the species (NOBLES 1948, 1958, BAZZALO & WRIGHT 1982, ADASKAVEG & GILBERTSON 1986).

The species *Ganoderma lucidum* itself is believed to grow on hardwoods only. The basidiocarp is stipitate, with a pileus more or less imbricate. The surface of the pileus is covered with a dark-red laccate layer, in the case of young fruitbodies with a non-laccate yellow to white margin. The shape of basidiospores is ovate with truncated apex. The basidiospores have numerous, narrow inter-wall pillars and "smooth" wall. The pilocystidia are medium long, clavate, amyloid, thick-walled, with abruptly tapering shafts (ocassionally branched) intermixed with branching non-swollen hyphae in the mature pilear surface tissue. Different authors describe different spore sizes: according to PEGLER &

YOUNG (1973) these are 9–13 × 6–8 µm, as to ADASKAVEG & GILBERTSON (1988) 10.0–11.8 × 6.8–7.9 µm, and as to WANG & HUA (1991) 7.0–12.0 × 6.0–8.0 µm.

The hyphal system is trimitic.

Isolates of *Ganoderma lucidum* regularly form chlamydospores in mycelial culture and have an average growth rate of 7.8 mm/day at its optimum temperature range of 30–34 °C (ADASKAVEG & GILBERTSON 1986).

The aim of our work was to investigate the morphological and cultural characteristics of *Ganoderma lucidum* strains isolated from different habitats of Hungary and to compare them to isolates originating in other countries. The taxonomic value of characteristics regularly used to make distinctions between the species and between strains of the species itself were examined.

MATERIALS AND METHODS

Isolates

Basidiocarps of *Ganoderma lucidum* were collected from various regions of Hungary. Collection informations (host relationships, collection localities) are presented in Table 1. Mycelial cultures were obtained by cutting out small pieces from the inner layers of basidiocarps under steril conditions and were maintained at 26 °C on malt extract agar (MEA) or on potato dextrose agar (PDA). The strains were deposited in the Plant Collection of Hungarian Natural History Museum and in the Department of Plant Anatomy of Eötvös Loránd University.

Table 1. Sources of *Ganoderma lucidum* strains

Isolate	Locality
C17	Budakeszi
C64	Budakeszi
C114	Mátra Mts
C115	Pilis Mts
C116	Pilis Mts
C118	Kópháza–Nagycenk
C119	Kópháza–Nagycenk
C120	Budakeszi
C122	Buda Mts: Normafa
C123	Mátra Mts
C124	Mátra Mts
C125	Mátra Mts
C126	Mátra Mts
C127	Buda Mts: Farkas-völgy
C129	Buda Mts

Light microscopy

Preparates for light microscopy were made from the fresh mycelium grown on the agar slides or spores taken from the fruitbody. One sample from both the margin (young part) and the middle (old part) of two- and six-week-old cultures grown on MEA and PDA was mounted in lactophenol (lactic acid:glycerol:phenol:distilled water, 2:4:2:2), covered with cover slips and sealed. In some cases staining with 1% anilin-blue was applied. The preparates were examined by Nomarski interference contrast microscope (Olympus). Photos were taken using an immersion objective of 100× magnitude.

Scanning electron microscopy (SEM)

Mycelium, basidiospores and pilocystidia were examined by Hitachi–2360–N scanning electron microscope.

For SEM investigations mycelium on agar cubes were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, washed in 0.07 M phosphate buffer (pH 7.2), dehydrated stepwise in ethanol (25%, 50%, 75%, 90%, 96%, abs.) and amylacetate. The samples were critical point dried and coated with carbon and gold layers. Spores were air dried from xylene before coating. Spore

size was measured by the average of about ten basidiospores. Pilocystidia were prepared by diluting the laccate layer by placing a small piece of the surface tissue from the pileus into acetone for 24, 48 and 72 hours and coated.

Optimal growth temperature

Optimal growth temperature of the strains was determined on malt agar (MEA) and potato dextrose agar (PDA) growing for 7 days at 14, 18, 22, 26, 30, 34 and 38 °C. The experiment was repeated twice in three replications.

RESULTS

Morphology of the fruitbodies

The fruitbodies of our *Ganoderma lucidum* isolates were stipitate. Each pileus was more or less imbricate. The colour of the pileus surface was dark red and laccate. The young fungus had a non-laccate yellow to white margin. At the beginning the context colour was pale buff, later dark brown. The stipes was excentric, woody, with different length. The thickness of the context ranged from 5 to 30 mm.

Temperature studies

Temperature studies showed different optimal temperatures on MEA and PDA (Fig. 1, Table 2). In most cases growth of our strains on 14 °C was similar to that on 30 °C, mainly about the third or half of the optimal growth. Considerable growth on 14 °C had been proved also by ADASKAVEG & GILBERTSON (1986), but WANG & HUA (1991) had demonstrated slight growth in only one case under such conditions. Although according to the data by ADASKAVEG & GILBERTSON (1986) and WANG & HUA (1991) growth on 34 and 38 °C is vigorous, none of our strains grew at all at these temperatures. As to the investigations of ADASKAVEG & GILBERTSON (1986) *G. lucidum* can grow in an interval of greater than 30 °C, according to WANG & HUA (1991) in an interval of 25 °C, and our

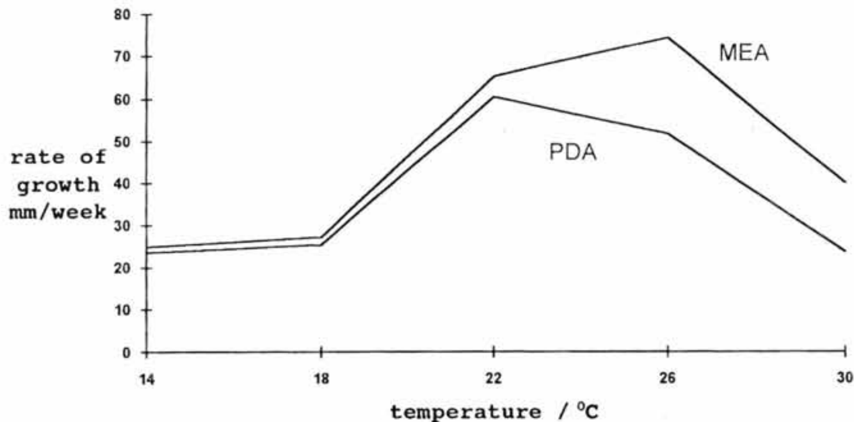


Fig. 1. The average growth curve of the one-week-old mycelial cultures grown on PDA and MEA

Table 2. The growth characteristics of isolates depending on the temperature. Comparison of our results with some data of literature. (1 – own results; 2 – AASKAVEG & GILBERTSON (1986); 3 – WANG & HUA (1991)

		1	2	3
Growth rate (mm/day)	MEA	10.6	7.8	9.1
	PDA	8.6	no data	7.8
Temperature optimum (°C)		22–26	30–34	26–30
Growth interval (°C)		10–30	<10–42	14–38

strains grew consequently only in an interval of 20 °C. The average optimal growth temperature of our strains was 26 °C on MEA and 22 °C on PDA. Speed of mycelial growth was 10.6 mm/day and 8.6 mm/day, respectively. This differs significantly from the results of ADASKAVEG & GILBERTSON (1986) and WANG & HUA (1991).

Macroscopic morphology of the mycelium cultures

In order to characterize the strains a test list of 19 characteristics had been established. The following features were examined:

1. Homogeneity
   - a – homogeneous
   - b – the central part is different
   - c – the central part forms concentric circles
2. The macroscopic structure of the dominating part of the mycelium surface
   - a – velvety
   - b – velvety-cottony
   - c – cottony
   - d – filamentous
   - e – velvety-filamentous
3. The macroscopic structure of the central part, if any exists
   - a – velvety
   - b – thick, concentric mycelial rings
   - c – excreted drops in concentric rings
   - d – chamois-like
   - e – brown
   - f – submers
4. The colour of the dominating part of the mycelium
   - a – white
   - b – brown
5. Presence of submers hyphae
6. Presence of small hyphal tufts

These characteristics were suitable for describing strains of different age and growing on different media. There were only few exceptions, mainly among the six-week-old

cultures growing on MEA, which could not be described precisely because of the variability of these features within the two experiments or repetitions. This set of characters is much more suitable and much simpler than the one used before although it has been established for one species only.

The macroscopical picture is rarely homogeneous. In the case of two-week-old cultures the mycelium mainly consists of several concentric circles of different structure. By the age of 6 weeks a coherent central region and a different peripherial part is usually formed. A visible brown colouration appears only after the second week. In the six-week-old cultures grown on PDA the coloured region is larger than in the MEA cultures. The presence of submers mycelium is commonly characteristic. In the case of six-week-old cultures it is weakly visible as it is covered by a surface hyphal layer. Initiative forms of fruit body formation is not characteristic of the isolates. Only one of the strains produced it regularly. The most characteristic of all is the picture of the two-week-old cultures on PDA, the data of which can be seen on Table 3.

Microscopical morphology of the cultures

Five features suitable for characterizing the differentiation rate of the cultures have been found. These are:

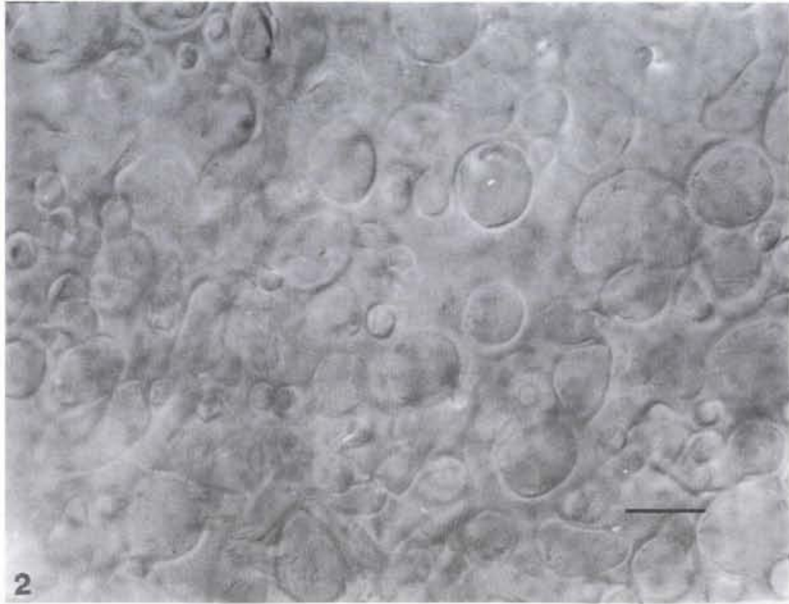
1. the chlamydospores;
2. the thick generative hyphae;
3. the cuticular cells (thin- (Fig. 2) or thick-walled (Fig. 3), hyaline or brownish spherical cells originated from the generative hyphae);
4. the staghorn hyphae (Fig. 6) (cluster of frequently branched, thin walled, thin hyphae);
5. the presence of yellow pigment.

It is generally true that in the two-week-old cultures on MEA no more than one of these characteristics appears. There were only two exceptions out of fifteen cases. In the two-week-old cultures on PDA differentiation is much more definite, especially in the central region. In the case of the two third of these cultures at least two of the differentia-

Table 3. Characteristics of some 2-week-old mycelial cultures on PDA (explanation see in the text)

Strains	1	2	3	4	5	6
C17	c	e	e	a	+	–
C64	c	e	a	a	–	–
C114	b	a	a, f	a	+	–
C115	b	a	a, e	a	+	–
C116	c	b, c	a, f	a	+	–
C118	c	a	a	a	+	–
C119	c	a	a, e, f	a	+	–

Fig. 2. The thin-walled spherical cuticular cells appear during the differentiation of the mycelial culture (Nomarski optics). Bar 10 µm



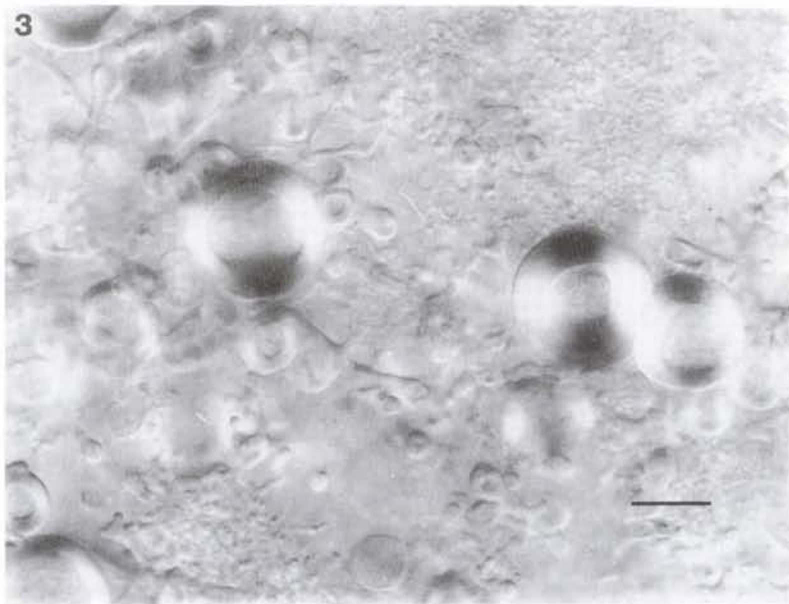
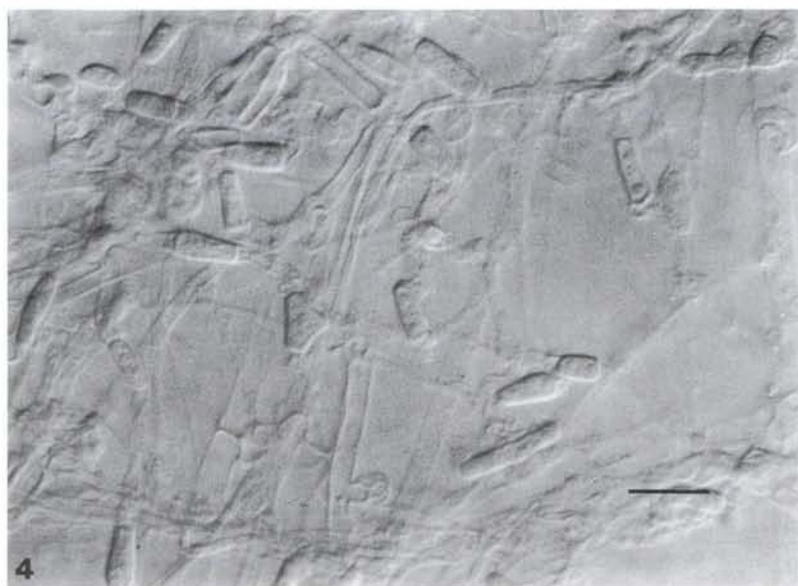


Fig. 3. In the central part of the six-week-old mycelium cultures cuticular cells become thick-walled and yellow pigmented (Nomarski optics). Bar 10 µm

Fig. 4. In some mycelium cultures characteristic arthrospores are formed in the hyphae (Nomarski optics). Bar 10 µm



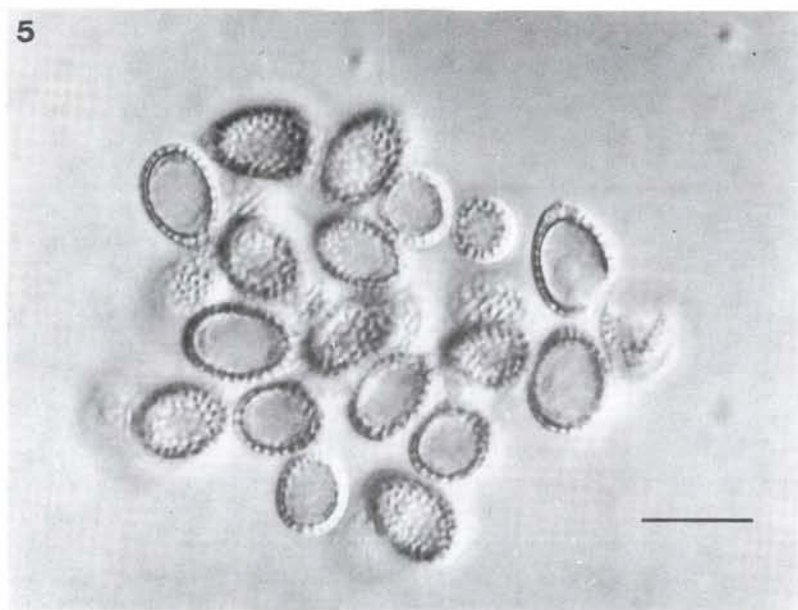


Fig. 5. Basidiospores under Nomarski-microscope. The smooth outer layer of the spore wall and the interwall pillars can be observed clearly (Nomarski optics). Bar 10 µm

tion markers appear. One third of the six-week-old MEA cultures did not show any differentiation, except for hyphae of different thickness form the structure. Among the other strains investigated differentiation markers appear only in the central region. Within the six-week-old PDA cultures, except for two, cuticular cells, staghorn hyphae and the presence of yellow pigment can be detected regularly. It has been noticed that the yellow colouration appears only in the thick-walled forms of the cuticular cells, so it can be concluded that the pigment is incorporated into the cell wall. Chlamydospores were not detected, arthrospores (Fig. 4) in one case only.

The morphology of basidiospores

Basidiospores (Fig. 5) are brown, ovate with truncated apex. The surface of the spores is slightly dimpled, uneven. They have a double wall with inter-wall pillars. The size of the basidiospores is 7.7–(9.5)–10.8 × 5.5–(6.2)–6.7 µm which is significantly smaller, than 11.5 × 7 µm measured by ADASKAVEG & GILBERTSON (1986) and PEGLER & YOUNG (1973) but corresponds with the values of the lower range measured by IGMÁNDY (1991) on strains isolated from Hungary. ADASKAVEG & GILBERTSON (1986) did not determine exact values for the density of interwall pillars but comparing their photos with ours it can be concluded that according to the pillar density our strains have intermediate values between the species groups of *Ganoderma lucidum* and *G. tsugae*.

The morphology of pilocystidia

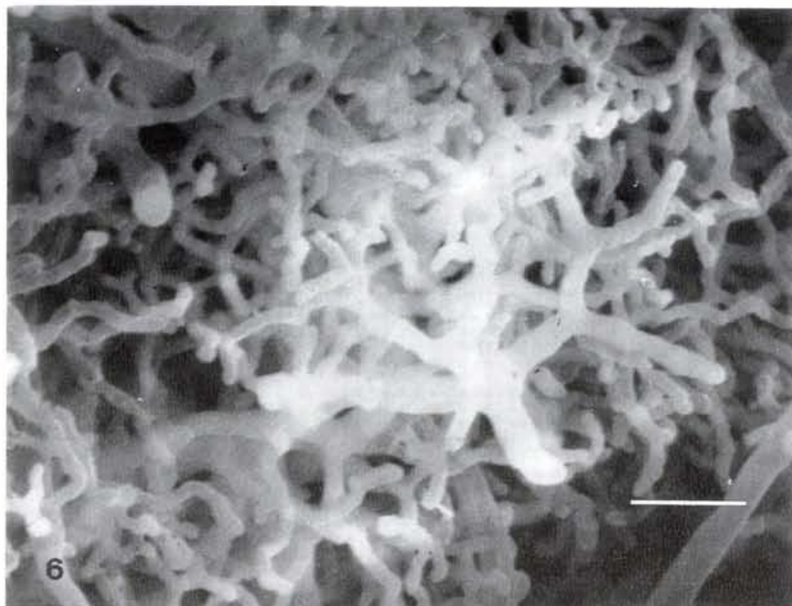
Pilocystidia (Fig. 7) are clavate and unbranched. The apical part is spherical with smooth surface. The stalk part is narrow, rarely branched. No other cells take part in forming the structure of the surface layer.

DISCUSSION

Macroscopically basidiocarps show a high degree of consistency but microscopical and cultural characters are different within the species *Ganoderma lucidum* depending on geographical distribution as CORNER (1983) established. The present investigations seem to verify this statement. The temperature requirements of strains isolated from distinct regions are different. There are differences in the temperature optimum, in the speed of growth and also in the temperature range suitable for growth. The isolates investigated here have a lower optimal growth temperature than others known from literature (ADASKAVEG & GILBERTSON 1986, WANG & HUA 1991) and on the other hand they grow faster. We also observed that optimal temperature of growth is different in almost every strain on the two media investigated. This is evidently caused by the varying effect of limitating factors influencing growth differently on different media. This calls attention to the fact that the optimal growth temperature determined in laboratory do not correspond with that of the mycelium growing in natural conditions.

Our isolates turned out to be quite homogeneous in the macroscopical morphology of the mycelium culture. The strains could be described well using the set of characteristics established by us.

Fig. 6. Young forms of densely branching staghorn hyphae (SEM). Bar 2 µm



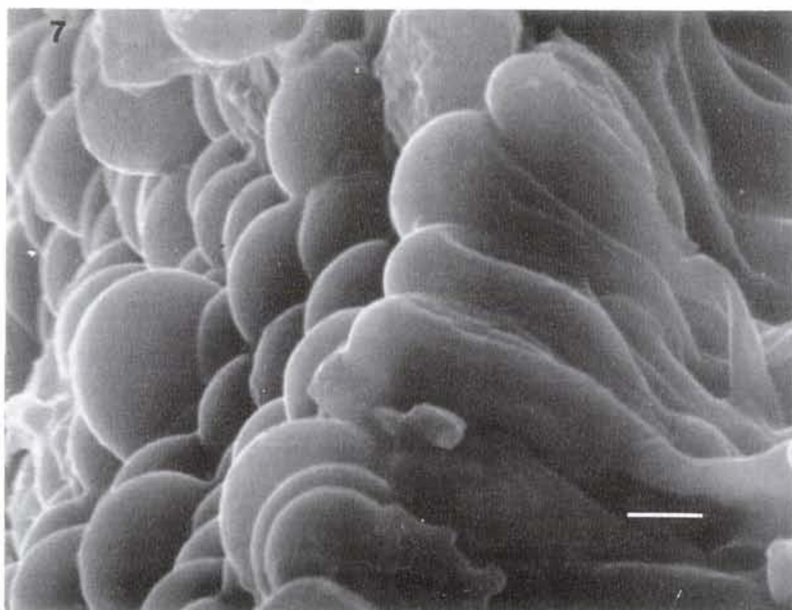


Fig. 7. The surface of the pileus built up by pilocystida after dissolving the laccate layer with acetone (SEM). Bar 2 µm

More differences have been found in microscopical features as compared to other authors. According to ADASKAVEG & GILBERTSON (1986) chlamydospore formation is regular and characterize *G. lucidum* in two-week-old cultures on MEA. In contrary, the results of WANG & HUA (1991) show that only the half of the strains form chlamydospores within two weeks, some of them only within six weeks and others not at all. Our isolates did not form any even within six weeks. According to ADASKAVEG & GILBERTSON (1986) this absence of chlamydospores is characteristic of the *G. tsugae* group. In one isolate we could observe a great amount of arthrospores. This had been also mentioned by WANG & HUA (1991) in the case of other species.

The macroscopical and also the microscopical investigations proved that differentiation of the cultures is faster on PDA than on MEA. That may be because of the lower concentration of some substances in PDA.

STEYAERT (1972) suggested the influence of temperature on spore size. Spore size is increasing with geographical latitude or elevation. The spore size measured by us was smaller than determined by ADASKAVEG & GILBERTSON (1986) and PEGLER & YOUNG (1973).

ADASKAVEG & GILBERTSON (1988) claimed that the surface tissue of basidiocarps in *G. lucidum* is composed of a dense palisade of clavate pilocystidia intermixed with branching non-swollen hyphae. The apical projections on pilocistidia are common. Tapering shafts of pilocystidia are occasionally branched. We observed slightly different pilocystidia. Hypha-like cells are not intermixed with pilocystidia. The pilocystidial cells are smooth, knob-like projections are not seen. These properties are characteristic to the *G. tsugae* complex but the shape of these cells is similar to *G. lucidum* complex according to ADASKAVEG & GILBERTSON (1988).

Based on the characteristics investigated also by us the *G. lucidum* species complex is divided into two groups in literature nowadays: to the group *G. tsugae* and to *G. lucidum*. Our results call the attention to the fact that these characteristics can appear in intermediate forms also within one strain, so they cannot be regarded as features characterizing the species complex. The investigation of our strains will be continued with the analysis of their biologically active substances. An answer to the question if morphologically homogeneous strains show differences in producing active substances or not is looked for.

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