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BASIDIOCARP AND MYCELIUM MORPHOLOGY OF *GANODERMA LUCIDUM* KARST. STRAINS ISOLATED IN HUNGARY

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Morphological, anatomical and cultural characteristics of 14 *Ganoderma lucidum* (Fr.) Karst strains isolated in Hungary have been investigated. Macroscopically the basidiocarps of the Hungarian strains are absolutely identical with those of described previously about the *Ganoderma lucidum* species-complex. Microscopic features of the fruitbodies and basidiospores showed some differences from the typical *G. lucidum* species. Pilocystidia, forming a homogenous layer on the surface of the pileus, have smooth heads without protrusions and stalks not ramifying. Cell wall pillar density and width of the basidiospores also differ from that of regarded to be characteristic to *G. lucidum*. Although according to several authors chlamydospore formation is a characteristic feature of *G. lucidum* it has not been observed in mycelial cultures of the Hungarian strains.

Antagonistic reactions between the Hungarian and Far Eastern *G. lucidum* isolates were mostly similar to the interspecific reactions between the two species *G. lucidum* and *G. applanatum* and corresponded only in a few cases to the interactions within one species.

Our results suggest that the Hungarian strains significantly differ from the Far Eastern strains. To determine the taxonomic degree of this divergence genetical examinations should be carried out.

The genus *Ganoderma* had been established by Karsten [1] with a single species called *Ganoderma lucidum*.

Murrill [2] declared first that identification of the species is impossible by morphological investigations only and a complex system of criteria is to be elaborated. This is necessary because of the heterogenity within the species and the similarity between the different species of the genus. The macromorphologically similar species have been contracted by him to the *Ganoderma lucidum* species-complex, while keeping their individual species names.

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Since this establishment a lot of investigations has been carried out to determine which properties are necessary and sufficient to distinguish the members of the genus *Ganoderma*. The most characteristic features used to identify *Ganoderma lucidum* species are the morphology of the fruit body [2], the host [2], the geographical area where the fruit body was collected [2], the morphology of pilocystidia [3], structure of the spore wall [4–7] and the properties of mycelial cultures [8–10].

Adaskaveg and Gilbertson [11] tried to synthesize the investigations on the most characteristic features known up to that time. The *Ganoderma lucidum* species-complex has been divided into two groups: to *G. lucidum* living on hardwood and *G. tsugae* living on softwood. As the most distinctive characteristics, the properties of the basidiospore and the parameters of mycelial cultures were investigated, and hybridization probes were carried out. All isolates of the *G. lucidum* group formed terminal or intercalar chlamydospores but none of those of the *G. tsugae* group. They demonstrated that the morphology of the pilocystidia and the density and size of the spore interwall pillars is specific [12]. Later Pegler and Young [4] found that all transient forms of pillars could be detected within the individual specimens. Wang and Hua [13] confirm that *G. tsugae* isolates do not form chlamydospores but they have not found them in all strains of *G. lucidum*, either.

The phenomenon of intraspecific antagonism has been known only for a few decades. Adams and Roth [14] recognized for the first time that a barrier can arise between the dikaryotic hyphae of the same species on agar plates. Antagonism is less intensive between genetically similar strains. According to Brasier the process has a polygenic regulation [15]. Examining this reaction of isolates collected from different sampling sites can help us to determine the taxonomic distance between two strains.

Adaskaveg and Gilbertson [15] carried out experiments with *G. lucidum* and *G. tsugae* strains to investigate intraspecific antagonism. Different stages of antagonism were detected. *G. lucidum* pairs always showed a strong reaction independently from being collected from spots near to or far from each other.

Ganoderma lucidum is common also in Hungary. The aim of our work was to investigate the macromorphological and anatomical features of Hungarian isolates considered to be characteristic for the species and compare them with the data known from literature.

Materials and methods

Strains investigated. Fruitbodies of *Ganoderma lucidum* have been collected from different locations of Hungary. All samples have been collected in deciduous (mainly oak) forests. Collection data are shown in Table I. Mycelial cultures have been isolated by cutting pieces of about 2×2 mm from the inner parts of the fruitbodies with a sterile scalpel and placing them on malate agar plates. The isolates were maintained on malate agar (MEA) at 26 °C. Beside our own isolates three strains from the Culture Collection and Research Centre, Taiwan (CCRC) have been studied. The strains are deposited in the collections of the Plant Anatomy Department of Eötvös Loránd University, Budapest and the Hungarian Natural History Museum.

Light microscopy (LM). Samples for light microscopy were prepared from mycelia of different age grown on malate agar (MEA) and potato-dextrose agar (PDA) or from basidiospores obtained from the basidiocarps. Mycelial samples were taken from the central (old) and marginal (young) parts of two-week-old and six-week-old cultures. Preparates were mounted in lactophenol (lactic acid : glycerol : phenol : distilled water = 2 : 4 : 2 : 2). Staining with 1% aniline blue was applied occasionally. The samples were examined by Nomarski microscope using immersion objective of ×100 magnification.

Table I

Designation and place of collection of the Ganoderma lucidum strains used in our investigations

Strain	Sampling place
C17	Budakeszi
C64	Budakeszi
C114	Bükk
C115	Pilis, Kevélynyereg
C116	Pilis, Kevélynyereg
C118	Kópháza-Nagycenk
C119	Kópháza-Nagycenk
C120	Budakeszi
C122	Budai-mountains, Normafa
C123	Mátra, Valley of Tó-réti stream
C124	Mátra, Valley of Tó-réti stream
C125	Mátra, Valley of Tó-réti stream
C126	Mátra, Valley of Tó-réti stream
C127	Budai-mountains, Farkasvölgy
C129	Budai-mountains
C130	CCRC 36111
C131	CCRC 36144
C132	CCRC 36021

Scanning electron microscopy (SEM). Mycelial cultures, basidiospores and pilocystidia were examined by Hitachi 2300-N scanning electronmicroscope.

Agar blocks of 2×2 mm size cut from the mycelial cultures were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium-tetroxide. After washing in 0.07 M phosphate buffer (pH=7.2) a stepwise dehydration in ethanol (25%, 50%, 75%, 90%, 96%, absolute) and amyl-acetate was carried out. The samples were critical point dried, followed by coating with carbon and gold layers according to the slightly modified method of Wang and Hua [13]. The spores were dehydrated in xylol before coating.

Spore size was determined based on the SEM photos. Results are given as the averages of ten spores in three repetitions.

Pilocystidia were prepared for SEM investigations by solving the covering laccate layer with acetone from the surface of 5×5 mm pieces cut from the pileus. Treatments of 24, 48 and 72 h were used

according to Adaskaveg and Gilbertson [12]. After this the samples were treated as the mycelial samples.

Density of interwall pillars were determined by counting them in a 2×2 µm quadrate of the spore surface. An average of twenty squares was calculated. As no exact density values of interwall pillars have been published in the literature we tried to compare our data to those of other authors by determining pillar density from their published photos using the same calculating method.

Estimation of optimal growth temperature. The temperature optimal for growth was determined in two culture media (MEA and PDA [13]). Agar plates of 85 mm in diameter (15 ml medium) were inoculated in the centre with small mycelial discs. The diameter of the colonies grown on seven different temperatures (14 °C, 18 °C, 22 °C, 26 °C, 30 °C, 34 ° C and 38 °C) was measured on the seventh day. The experiment was carried out three times in three repetitions. Two values were treated as identical if their difference was not greater than 3 mm, which is about the error of the measurement.

Investigation of intraspecific antagonism. To investigate intraspecific antagonism besides our own *Ganoderma lucidum* strains a *Ganoderma applanatum* (C117) and a *Trametes versicolor* (own isolates) were used. The strains were inoculated in pairs to Petri dishes of 85 mm in diameter containing MEA. Mycelia of the same strains were inoculated together for control. Cultivation was carried out on 26 °C for 8 weeks.

Results

The basidiocarps, which the strains had been isolated from, were completely identical with the descriptions of Adaskaveg and Gilbertson [11]. All fruitbodies were collected from the soil of foliage forests, mainly oakwoods. As these associations are composed of different tree species usually no host could be identified.

The pilocystidia of the basidiocarps investigated are clavate, with shafts narrow at the base, rarely branched. The apical part is spherical, with smooth surface. No other type of cells appear in the surface layer (Fig. 1).

Table II

The size of the basidiospores in Ganoderma lucidum based on data from the literature and own measurements

Spore size	Treatment of spore	Sampling place	Author
9–(11.5)–13 × 6–(7)–8 µm	10% KOH solution	Great-Britain	Pegler and Young [4]
9.5–12 × 6–6.5 µm	drying	East-England	Corner [7]
10–13 × 6.8–8 µm	KOH solution	East-England	Corner [7]
10.6–(11.5)–11.8 × 6.8–(7.4)–7.8 µm	2% KOH	North America	Adaskaveg and Gilbertson [11]
7.0–10.5 × 5.0–7.0 µm	nd	Hungary	Igmándy [19]
9–12 × 6–7 µm	SEM-fixed	North America	Mims and Seabury [20]
7.7–(9.5)–10.8 × 5.5–(6.2)–6.7 µm	drying	Hungary	own results

The average values are in brackets if published.

nd = no data

Corresponding to the characteristic form of the genus, basidiospores are ovate, with truncated apex when dried and brown in colour. The surface of the spores is slightly dimped, uneven (Fig. 2). Spore wall has two layers with inner-wall pillars. The average number of pillars in a 2×2 μm quadrate is 7.8. The size of the spores corresponds to that measured previously [4, 7, 12, 19, 20] (Table II).

The mycelial cultures of the Hungarian strains were homogenous in their growth parameters. The optimal growth temperature was different on the two media investigated (Fig. 3, Table III). The optimal temperature for the majority of the strains on PDA was 22 °C. 26 °C was better by the use of MEA although in the case of two strains growth on 22 °C and 26 °C was equal. At extreme temperatures the rate of growth was quite similar in all strains. These rates were about the half or the third of the maximal values.

Fig. 1. Pilocystidia form a homogenous surface. No other types of cells occur. The pilocystidal apices are smooth, without any projections (SEM). a = pilocystidal apices

Generative hyphae and skeletal hyphae of different thickness could be observed in the mycelial cultures (Fig. 4). Cuticular cells are characteristic to all strains. They can occur as young, thin-walled or old, thick-walled forms. Incorporation of a yellow pigment into the walls of the old cuticular cells and the densely branched staghorn hyphae (Fig. 5) is common. Arthrospores could be found in several strains (Fig. 6) but none of the Hungarian isolates produced any chlamydospores.

Fig. 2. Basidiospores of *Ganoderma lucidum* (SEM). Depressions indicating the peaks of the interwall pillars are visible. The apical part of the spore is slightly truncated. d = depression indicating the pillar, p = truncated apex

The intraspecific antagonism of 15 Hungarian and 2 Far-Eastern *Ganoderma lucidum* strains has been investigated as well as their interactions with *Trametes versicolor* and *G. applanatum*. The latter two species grew much more intensively than the *G. lucidum* isolates. Within two weeks they completely grew over the mycelium of *G. lucidum*. No special structural alteration in the mycelia of the different species was visible macroscopically. This confirms the observations of Rayner et al. [16] who detected a similar interaction between two strains of different species.

The Far-Eastern strains grew also much faster than the Hungarian ones. The Hungarian isolates inhibited the growth of the Far-Eastern ones unequally. A barrier of

mycelium emerging from the medium has been produced but the Far-Eastern strains covered this in most cases by the end of the experiment. This phenomenon corresponds to an intraspecific reaction (as a barrier arises) and also with an interspecific reaction (the mycelia of Far Eastern strains covered the Hungarian ones).

Fig. 3. Growth of Hungarian *Ganoderma lucidum* strains on PDA and MEA at different temperatures. Growth on MEA is more intensive than that on PDA

Table III

Growth of mycelial cultures of Ganoderma lucidum and G. tsugae at different temperatures (comparison of published data with own results)

Species	*Ganoderma lucidum*			*Ganoderma tsugae*	
Author	AG [11]	Wang and Hua [13]	own results	AG [11]	Wang and Hua [13]
Growth on MEA	7.8	9.1	10.6	1–3	1.8
Growth on PDA	nd	7.8	8.6	nd	nd
Optimal growth	30–34	26–30	22–26	20–25	22–26
Temperature range of growth	<10–42	14–38	10–30	10–30	14–30

nd = no data
AG = Adaskaveg and Gilbertson

Antagonistic reaction between the Hungarian isolates had fully developed by the end of the sixth week. During the first week, similarly to the result of Rayner and Todd [17], only normal anastomoses could be observed like within the mycelia of the same strain. The antagonistic reaction started in the second week. In some cases a macroscopically visible white or brown barrier emerged from the mycelium during the second or third week. This changed its colour or height later. In other cases at first a white, slightly emerging mycelial barrier arose which was decomposed between the fourth and sixth week and a macroscopically empty zone was formed. This zone was bordered by two white mycelium zones, slightly thicker than the normal mycelium. In some cases (e.g. strains C64 and C125) this zone has arisen also between two barriers. Among strains collected in Hungary no correlation between the type of antagonism and the origin of the strains could be demonstrated although isolates from distant places have been investigated. The formation of barrier was more frequent than the formation of empty zone. Thus some strains (e.g. C114, C118, C122) are rather of the latter type.

Fig. 4. Different types of hyphae from the mycelial culture of *Ganoderma lucidum*. Generative hyphae with clamps and skeletal hyphae of different thickness are visible. Nomarski IM. g = generative hypha, c = clamp, t = thin skeletal hypha, k = thick skeletal hypha, bar = 10 μm

Combining the isolates within themselves always formed normal anastomoses. The mycelial structures developing during the antagonistic interactions have been investigated also microscopically. The mycelial barrier arising between two strains mainly consisted of pseudoparenchym containing a brown pigment. Both sides of the frontier were absolutely similar to the normal lateral zone of the young mycelium.

In the other type of antagonism, which is characterized by the decomposition of the meeting hyphae, a loose, submers mycelial net is formed in the macroscopically empty zone. At the border of the submers zone and the part covered by hyphae no swollen or

densely branching cells could be seen as it had been observed by Adaskaveg and Gilbertson [15] in other *Ganoderma lucidum* samples. Only the hyphal net was denser at the frontier. The border of the submers and the superficial parts was similar to the mycelium observed at the border of Petri dishes. Submers-type antagonism caused intensive arthrospore-formation in strain C114. (This strain does not form arthrospores alone.) This phenomenon could not be observed in other strains. No chlamydospores in any of the Hungarian strains have been formed but they always could be detected in the Far Eastern strains.

Fig. 5. Densely branched, well-developed staghorn hypha. Yellow pigment is incorporated into the cell walls. Nomarski IM. s = staghorn hypha, bar = 10 µm

Discussion

Our results show significant differences in specific characteristics of *Ganoderma lucidum* previously described by other authors.

The structure of the pilocystidial layer differs from that of observed by Adaskaveg and Gilbertson [12]. They suggested that the simple structure, composed by a single cell-type, (having been found also by us) is characteristic to the group *G. tsugae* although the shape of the cells is similar to that of the *G. lucidum* group. Also pillar density of the spores, considered to be an important property, differs slightly from that of visible in the photos of Adaskaveg and Gilbertson [11], where the number of pillars in a 2×2 µm

quadrat is 5.9 in *G. tsugae* and 10.0 in *G. lucidum*. The Hungarian strains show transient values of these two characteristics.

Fig. 6. Arthrospores from the mycelial culture of *Ganoderma lucidum*

The characteristics of mycelial cultures are used for more precise identification. The optimal temperature of growth of the Hungarian isolates was lower than that of any previously published isolate (Table III). The growth of our strains on 26 °C was faster than that of investigated by Adaskaveg and Gilbertson [11] and Wang and Hua [13] and even faster at the temperatures optimal for their strains. Similarly to our results Adaskaveg and Gilbertson [11] observed significant growth also beyond 14 °C while Wang and Hua [13] could demonstrate growth at 14 °C by one out of 12 strain. 34 °C was the optimal temperature for several strains of these authors while none of ours could even grow at this temperature. The temperature range adequate for the Hungarian isolates was significantly more restricted in both media investigated than those of determined by Adaskaveg and Gilbertson [11] and Wang and Hua [13].

A basic difference has been found in chlamydospore formation, considered by Nobles [10] and Adaskaveg and Gilbertson [11] as extremely suitable for differentiation between species. None of the Hungarian isolates formed any chlamydospores. However, arthrospores mentioned also by Wang and Hua [13], had been produced also in some of the Hungarian isolates.

The interaction of *G. lucidum* strains with *Trametes versicolor* and *G. applanatum* was similar to that described by Rayner et al. [16] as interspecific reaction. However intraspecific reactions of our strains differed significantly from those of described previously. Within *G. lucidum* and *G. tsugae* [15] and within *T. versicolor* itself only [18] a reaction resulting in an empty zone had been observed. Whereas the majority of our strains produced a previously unknown reaction called by us “barrier-type” antagonism.

Taken also into consideration the microscopic structure two possible mechanism is suggested. In both cases excretion of a substance is supposed to cause the antagonism as quite a long time is needed for its development.

According to the first theory, significant part of the mycelium in the meeting zone, leaving only a loose mycelial net, will be degraded if much excreted substance of great activity is produced. Out of the zone of this effect a normal mycelium develops. If the excreted substance is few or less active a weak reaction takes place. The strains can live quite close to each other but a more differentiated structure separating the two strains arises at the border.

According to the other theory in typical barrier-forming strains a pseudo-parenchymatous barrier arises faster than in submers-type ones. The inhibiting substance, which causes the degradation of the loose, thin-walled mycelium cannot invade into the pseudoparenchymatous tissue formed by the thick-walled cells. Therefore the thin-walled mycelium will be degraded but the barrier already developed cannot degrade. In case of both antagonistic reactions outside the zone of the inhibitor the structure of the mycelium is similar to the normal type. The exact clearing up of these processes should be carried out by the methods used for examining interspecific allelopathic reactions. Confirmation or denial of these theories need further investigations and could be a possible continuation of this work.

Based upon microscopic characteristics, used generally in the literature for interspecific distinction, we may state that the Hungarian *Ganoderma lucidum* isolates form a distinct category at a species or subspecies level. This is especially verified by the absence of chlamydospores in Hungarian isolates, a characteristic to be stated as specific by all authentic authors. In addition the pilocystidal layer, the spore-wall structure and the morphological type of the intraspecific antagonistic reactions are also different from strains known from the literature.

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