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Morphological and ecological screening of different collections of medicinal white-rot bracket fungus *Ganoderma adspersum* (Schulzer) Donk (Agaricomycetes, Polyporales)

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Abstract

The screening of morphological and growth characteristics of mycelial collections of medicinal bracket fungus *Ganoderma adspersum* (Agaricomycetes, Polyporales) of different geographical origins (Armenia, Georgia, Iran) on malt-extract agar (MEA) and potato-dextrose agar (PDA) media for 6 days, as well as in submerged culture (malt-extract, ME, 200 rt min) for 14 days at different temperature (25, 30, 35, 38 °C) was performed. Species-specific mycelial macro- and micromorphological characteristics, such as white, cottony-felt, later chamois, leathery, creamy-lemon-yellowish fast-growing colonies, round-shaped hyphal clamps, hyaline, round-shaped, smooth chlamydospore-like swellings and brownish cuticular cells, numerous tetrahedral crystals were described in agar and submerged cultures. The formation of small, dense and smooth pellets, as well as hyphal swellings during submerged growth was observed. The favorable growth temperature for studied collections of *G. adspersum* was 25-30 °C. The revealed cultural characteristics will be used for taxonomic identification and quality control of mycelial cultures during their biotechnological cultivation.

Key words: *Ganoderma adspersum*; mycelium; morphological characteristics; growth rate; chlamydospores; cuticular cells; pellets

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Introduction

Currently, an interest towards the study of genetic resources of medicinal polyporoid fungi (class Agaricomycetes, order Polyporales), as valuable biological resources with high exploratory potential, including the production of nutraceuticals, nutriceuticals, pharmaceuticals, and cosmeceuticals is increasing (Badalyan and Gharibyan, 2015, 2016; Badalyan et al., 2015; Saltarelli et al., 2015; Taofiq et al., 2016, 2017; Gargano et al., 2017; Badalyan and Zambonelli, 2019).

The white-rot cosmopolitan polypores of the genus *Ganoderma* Karst. (family Ganodermataceae) include more than 250 species (Ryvarden, 1991). Among these fungi, *G. applanatum* (Pers.) Pat., *G. lucidum* (Curtis) P. Karst., *G. resinaceum* Boud., and *G. tsugae* Murrill [=*Polyporus tsugae* (Murrill) Overh.] are medicinally important, particularly in the Asian continent and widely used in traditional medicine for the therapy of chronic diseases for over 2000 years (Paterson, 2006). They are considered producers of several bioactive molecules (terpenoids, polysaccharides, alkaloids, phenolics, etc.) with different therapeutic effects (antimicrobial, antioxidant, antitumor, immunomodulatory, etc.) (Gao et al., 2003; Boh et al., 2004; Teplyakova and Kosogova, 2016; Gargano et al., 2017; Kües and Badalyan, 2017; Badalyan et al., 2019; Badalyan and Zambonelli, 2019).

Ganoderma spp. develop laccate and non-laccate basidiomata. They are distributed over a broad eco-geographical range and have been reported on more than 150 host species including deciduous and conifer trees (Adaskaveg and Gilbertson, 1986; Singh et al., 2016). The low sequence variation observed in the LSU ribosomal gene suggested that the *Ganoderma* genus has recently diverged (Moncalvo et al., 1995) and, due to the high range of variability of macro- and micro-morphological characteristics of basidiomata several synonymous names have been created and that makes this genus the most difficult to classify among the polypores (Moncalvo et al., 1995; Moncalvo, 2005; Tsivileva et al., 2016). Therefore, for correct identification of *Ganoderma* spp. without having to resort to molecular testing, taxonomically valuable morphological and growth characteristics of mycelia are required particularly during their biotechnological cultivation (Badalyan and Sakeyan, 2004; Badalyan et al., 2012a; Clémançon, 2012; Badalyan and Kües, 2015; Badalyan et al., 2015; Kües et al., 2016).

Cultural studies of *Ganoderma* spp. were previously conducted by several researchers. However, available scientific reports on systematic and comparative morphological observations of mycelia, asexual sporulation in the life cycle and other characteristics, as well as evaluation of their

taxonomic value are limited (Nobles, 1965; Stalpers, 1978; Hseu, 1990; Wang and Hua, 1991; Hong and Jung, 2004; Jayasinghe et al., 2008; Bukhalo et al., 2009; Jo et al., 2009; Mohanty et al., 2011).

The presence of chlamydospores or chlamydospore-like swellings is a species-specific characteristic of the *Ganoderma* species (Adaskaveg and Gilbertson, 1989; Bukhalo et al., 2009; Kües et al., 2016; Tsivileva et al., 2016). It has been shown that host relationships, production of terminal and intercalary chlamydospores, the range of average growth rate (from 2.1 mm d^{-1} to 7.8 mm d^{-1}) and thermophily (from 20 °C to 50 °C) of different *Ganoderma* spp. are useful taxonomic criteria to distinguish their mycelial cultures and reveal phylogenetic relationships among species (Adaskaveg and Gilbertson, 1986, 1989; Moncalvo et al., 1995; Badalyan et al., 2012a-b; Tsivileva et al., 2016). A remarkable correlation between mycelial characters was particularly found: fast-growing cultures are thermophilic and produce numerous ovoid chlamydospores, while slow growing cultures are not thermophilic and do not produce chlamydospores (Moncalvo et al., 1995). It was revealed that mycelial characters are less polymorphic than morphological characters of the basidiomata between recently diverged taxa (Moncalvo et al., 1995). Therefore, culture characters are useful in distinguishing between *Ganoderma* taxa.

In general, the relatively low temperature of 21°C was not appropriate for normal growth of *Ganoderma* strains, however there are several species (*G. meredithiae* Adask. & Gilb., *G. oregonense* Murrill, *G. zonatum* Murrill) which prefer an optimal temperature range from 20 °C to 25 °C (Adaskaveg and Gilbertson, 1986; Badalyan et al., 2015; Tsivileva et al., 2016; Badalyan and Gharibyan, 2017a).

The laccate *Ganoderma* species *G. lucidum* and *G. tsugae* grows on malt-extract agar (MEA) with the average growth rate of 7.8 mm d^{-1} at optimum temperature range 30-34 °C and 2.1 mm d^{-1} at 20-25 °C. *Ganoderma lucidum* formed chlamydospores, while *G. tsugae* did not (Adaskaveg and Gilbertson, 1986). There are other reports that *G. lucidum* strains grow with average growth rate 10.6 mm d^{-1} at temperature optimum 22-26 °C (Szedlay et al., 1996) or 9.1 mm d^{-1} at 26-30 °C (Wang and Hua, 1991) on MEA. Other laccate species *G. zonatum, G. meredithiae*, and *G. oregonense* prefer optimum temperature range of 20-25 °C and do not produce chlamydospores. However, formation of staghorn hyphae, thin- and thick-walled cuticular cells in *G. zonatum, G. meredithiae*, and *G. oregonense* was reported. Previously undescribed hyphal rosettes were also typical mycelial structures in *G. zonatum* (Adaskaveg and Gilbertson, 1986). Thermophilic laccate species *Ganoderma colossus* (Fr.) C.F. Baker prefers optimal growth temperature range 35-40 ^{o}C and at 37 ^{o}C showed growth

rate 15.0 ± 0.7 mm d^{-1} (Tsivileva et al., 2016). *Ganoderma colossus* grows slowly at 45 °C and survives exposure to 50 °C. It formed spherical thick-walled globular up to 20 µm in diameter rough chlamydospores (Adaskaveg and Gilbertson, 1986; Tsivileva et al., 2016).

For non-laccate species *G. applanatum* [=*G. applanatum f. australe* (Fr.) Bourdot & Galzin] the optimum temperatures ranging from 25-30 °C were found to be suitable for the mycelial growth on potato dextrose agar (PDA). It was revealed that the colonies of *G. applanatum* are denser on PDA (8.8 mm d^{-1}), compared to MEA (7.5 mm d^{-1}) (Jeong et al., 2009; Jo et al., 2009; Luangharn et al., 2017). Several studies have reported the optimal pH range for mycelial growth of various *Ganoderma* species to be pH 5-9 (Jayasinghe et al., 2008; Jo et al., 2009). The fastest mycelial growth of *G. applanatum* was observed in alkaline media of pH 7-8 (Luangharn et al., 2017).

Previous works (Badalyan et al., 2012a-b, 2015) have shown that the temperature range for optimal mycelial growth for laccate (*G. lucidum* and *G. resinaceum*) and not laccate (*G. adspersum*, *G. applanatum*) species/strains was 25-30 °C. At 35 °C, the growth of *G. adspersum* and *G. resinaceum* was suppressed and observed mainly on inocula while *G. applanatum* and *G. lucidum* did not grow at all. At 38 °C, only *G. resinaceum* showed poor growth.

Ganoderma adspersum (Schulzer) Donk (=*Ganoderma europaeum* Steyaert) form non-laccate basidiomata, similar to *G. applanatum* and *Ganoderma gibbosum* (Blume & T. Nees) Pat. with other common morphological characteristics which often lead to misidentification of these species (Steyaert, 1980; Adaskaveg and Gilbertson, 1986, 1989; Ryvarden and Gilbertson, 1993). In Armenia, *G. adspersum* was originally collected in 2002, on ash tree (*Fraxinus* sp.) in the city park of Yerevan and morphologically identified as *G. applanatum.* Using DNA barcoding, mycelium isolated from this basidioma was taxonomically verified as *G. adspersum* (Badalyan et al., 2012b). The basidiomata of *G. adspersum* were recollected in Armenia from 2005-2015, particularly in Yerevan and Hankavan, as well as in the village Berdavan on different wood hosts (*Morus alba* L., *Acacia* sp., *Fraxinus* sp., *Quercus* sp. and *Fagus* sp.) (Badalyan et al., 2012b; 2015; Badalyan and Gharibyan, 2017a). Several medicinal properties, such as antifungal activity, have been reported in the mycelia of *G. adspersum* (Badalyan et al., 2012a; Badalyan and Gharibyan, 2015, 2017b). In this paper, the study of morphological, ecological and growth characteristics of ten different genotyped mycelial collections of *G. adspersum* is presented.

Materials and Methods

Ganoderma samples were collected in Armenia during 2002–2015 on *Acacia* sp., *Fraxinus* sp., *M. alba* and *Quercus* sp. trees, in Iran on deciduous tree in 2008 and in Georgia on *Laurus camphora* L. tree in 2013 (Table 1, Fig. 1). Traditional taxonomic keys were used for morphological identification of basidiomata (Phillips, 1981; Ryvarden, 1991). Dikaryotic cultures were isolated from collected basidiomata by tissue method (Bukhalo, 1988) and maintained on MEA or in distilled water at 5 °C in tubes. Mycelial collections were genetically identified and verified using nuclear ITS–rDNA sequence data by previously reported methods (Badalyan et al., 2012b, 2015) (Table 1).

Table 1. The studied collections of *G. adspersum*

Catalogue number	Strain	Substrate	Origin and date of culture isolation	GenBank accession number*
5501	Ga-1**	*Fraxinus* sp.	Armenia, Yerevan, 2002	JN588580
5502	Ga-2-1	*Morus alba*	Armenia, Yerevan, 2005	JN588583
5503	Ga-2-2	*M. alba*	Armenia, Yerevan, 2005	JN588581
5504	Ga-2-3	*M. alba*	Armenia, Yerevan, 2005	JN588582
5506	Ga-3	*Acacia* sp.	Armenia, Yerevan, 2010	JN588585
5507	Ga-9	*Fraxinus* sp.	Armenia, Yerevan, 2011	not available
5509	Gad-6	*Quercus* sp.	Armenia, Tavush province, village Berdavan, 2013	KP941436
5517	Gad-03	*Laurus camphora*	Georgia, Batumi, 2013	KP941440
5518	Gad-VII	*L. camphora*	Georgia, Batumi, 2013	KP941441
5520	1016	Deciduous tree	Iran, Zagh Marz, Behshahr, 2008	KP941442

*Published by Badalyan et al., 2012b, 2015

**The catalogue numbers and accession numbers of strains correspond to strain names with slash published in the catalogue (Badalyan, Gharibyan, 2017a)

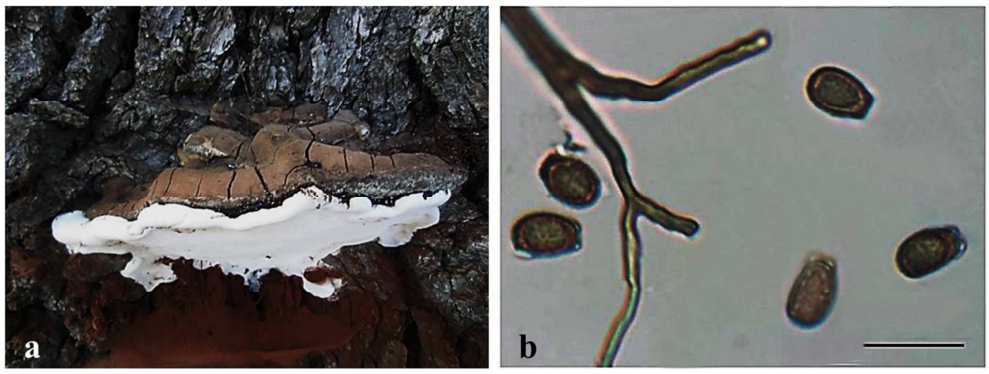

Fig. 1. Fruiting body of *G. adspersum* collected in 2002 in Yerevan (Armenia) on *Fraxinus* sp. (a); basidiospores (b)

The morphology and growth rate of mycelia were studied after inoculation of 5 mm^3 inocula (three replicates per strains) into the center of 90-mm Petri dishes at different temperatures (25, 30, 35, and 38 °C) using 1.5% MEA and PDA media (pH 6.0) for 6 days. They were also studied in submerged culture (pH 6.0, 200 rt min^{-1}, 25 °C) using Erlenmeyer flasks (250 ml, 5 inocula each) for 14 days of incubation under dark conditions. Micro- and macromorphological observation of mycelial colonies and pellets, anamorphs, as well as determination of average growth rates (GR_{avr}) were realized by previously reported methods (Badalyan et al., 2012b, 2015). The preparations were examined under the microscope Omano OM157-T Trinocular (USA) with software program (OC View 7, ver. 7.1) using 15×40 ocular/objective, as well as Axioplan-2 imaging microscope (Zeiss, Germany) using 10×40 ocular/objective. The photos were taken by OptixCam summit OCS 1.3MP and Colour View II Mega Pixel digital microscope cameras using analySIS® software (Münster, Germany).

The texture and pigmentation of colonies were described by Stalpers' scale before covering the Petri dish (Stalpers, 1978). The sizes of chlamydospore-like hyphal swellings, cuticular cells and crystals were measured by 10 replicates. The fruiting ability of mycelia was evaluated transferring the Petri dishes, after 30 days of incubation in the dark, into humid containers at room temperature 23±2 °C under day/night regime.

The statistical analysis was performed by SLOPE algorithm (Microsoft Excel; Microsoft Corp., Redmond, WA, USA) and expressed as mean ± S.D.

The cultures are preserved in the Fungal Culture Collection of the Laboratory of Fungal Biology and Biotechnology, Yerevan State University (FCC-YSU) under the catalogue numbers (Badalyan and Gharibyan,

2017a), as well as in the Culture Collection (CMI-UNIBO) of the department of Agricultural and Food Sciences, University of Bologna.

Results and discussion

Macromorphological characteristics

The colonies of *G. adspersum* were initially white, cottony-felt, later chamois, leathery, creamy-lemon-yellowish, with suppressed and smooth margin (Fig. 2). The agar is bleached but locally yellowish, in some places light brown. In submerged culture, *G. adspersum* forms small, dense, smooth and leathery pellets (Fig. 3). The development of fruiting bodies with mature basidiospores was only observed in Armenian strains Ga-9 and Ga-2-2.

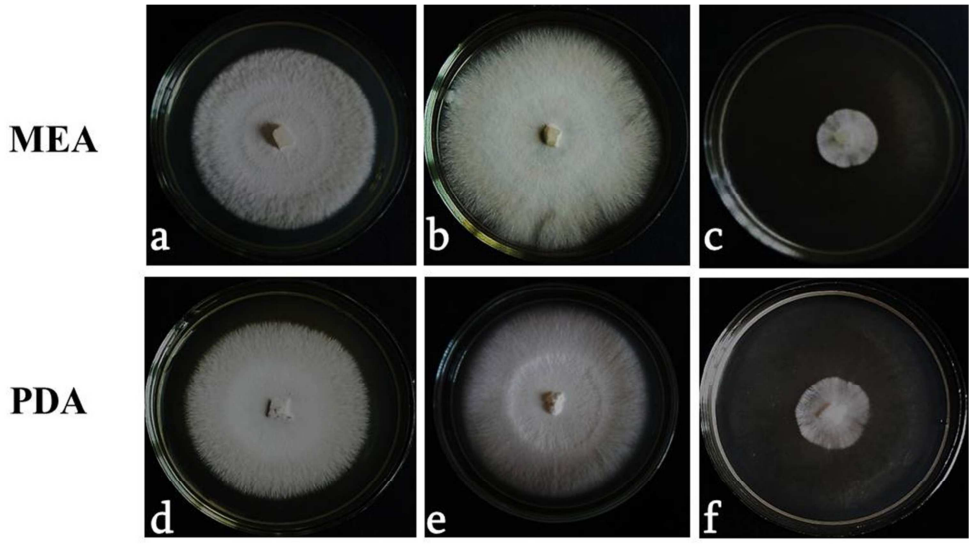

Fig. 2. Mycelial colonies of *G. adspersum* str. Ga-2-2 grown on MEA and PDA for 6 days at 25 °C (a,d), 30 °C (b,e) and 35 °C (c,f)

Mycelial growth rates

Depending on media composition and temperature, the variability of mycelial GR_{avr} indicators in tested collections was detected. However, no significant difference was found between tested media for cultivation of *G. adspersum* strains although some strains grow better on PDA (Table 2).

At 25 °C, the cultures grew relatively slower on MEA (GR_{avr} = 3.4-5.1 mm d^{-1}) than PDA (GR_{avr} = 3.5-6.0 mm d^{-1}) (Table 2; Fig. 2a,b,d,e). The GR_{avr} indicators were higher at 30 °C on MEA (4.0-6.8 mm d^{-1}), particularly

in Georgian strains Gad-03 (6.3 mm d^{-1}) and Gad-VII (6.8 mm d^{-1}), than PDA (3.0-5.5 mm d^{-1}). At 35 °C, only six strains isolated from ash, mulberry and acacia trees showed slow growth on MEA (GR_{avr} = 1.3-2.1 mm d^{-1}) and PDA (GRavr = 1.9-2.8 mm d^{-1}), while suppressed growth was observed only for on inocula of Gad-6, Gad-03, Gad-VII and 1016 strains isolated from oak, camphor and deciduous trees, respectively (Table 2; Fig. 2c,f). At 38 °C, no mycelial was observed in tested strains. Thus, optimal growth temperature range in collections of *G. adspersum* was 25-30 °C. A higher temperature above 30 °C suppressed mycelial growth of *G. adspersum*, similar to morphologically and phylogenetically close species *G. applanatum* (Moncalvo, 2000; Jargalmaa et al., 2017).

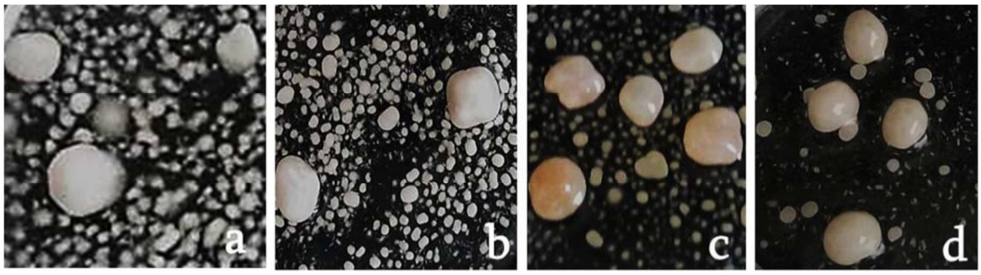

Fig. 3. Mycelial pellets formed by *G. adspersum* strains after 14 days of growth in malt-extract medium (pH 6.0, 200 rt min^{-1}) at room temperature: Ga-1 (a), Ga-2-3 (b), Gad-03 (c) and Gad-6 (d)

Micromorphological characteristics

The presence and form of hyphal clamps, asexual spores, including chlamydospores are valuable taxonomic criteria in Agaricomycetes fungi (Badalyan and Sakeyan, 2004; Badalyan et al., 2015; Badalyan and Kües, 2015; Kües et al., 2016).

Staghorn hyphae, thin- and thick-walled round-shaped hyaline or brownish cuticular cells organized from generative hyphae, hyphal rosettes and wrinkled hyphae were previously described in *Ganoderma* species (Nobles, 1965; Stalpers, 1978; Adaskaveg and Gilbertson, 1986, 1989; Bukhalo et al., 2009; Tsivileva et al., 2016).

In the advancing zone of colony, the hyphae of *G. adspersum* are hyaline with single, one-sided clamp-connections at almost each septum (Fig. 4a,b). Formation of coralloid-like hyphae was also typical for *G. adspersum* (Fig. 4c). Oidia and blastoconidia were not observed, while the round-shaped, double-walled, smooth, thick-walled hyaline chlamydospore-like swellings (3.49-5.60 x 3.13-6.22 μm) (Fig. 4e,f,g), and brownish spherical cuticular cells (4.37-8.61 x 4.31-8.96 μm) (Fig. 4j,k) were abundant in the aging agar

culture of *G. adspersum*. Cuticular cells were previously reported in *Ganoderma* cultures, including *G. adspersum* and G. *lucidum* (Nobles, 1965; Stalpers, 1978; Badalyan and Kües, 2015; Tsivileva et al., 2016).

Table 2. The average growth rate (GR_{avr}, mm d^{-1}) of *G. adspersum* strains at different culture conditions on the 6th day of growth

Strain	**Temperature**							
	25 °C		30 °C		35 °C		38 °C	
	MEA	PDA	MEA	PDA	MEA	PDA	MEA	PDA
Ga-1	5.1±0.8*	6.0±1.4	5.2±0.2	3.9±0.6	1.3±0.1	2.8±0.1	NG	NG
Ga-2-1	4.6±0.8	5.5±1.2	5.4±0.7	5.0±1.7	1.8±0.1	2.4±0.1	NG	NG
Ga-2-2	4.8±0.8	5.4±1.1	5.8±0.5	5.5±1.2	2.1±0.1	2.5±0.1	NG	NG
Ga-2-3	4.3±0.6	5.0±0.9	5.4±0.4	4.1±0.9	1.8±0.1	1.9±0.3	NG	NG
Ga-3	3.4±0.6	5.9±1.1	4.0±0.3	4.6±1.0	2.0±0.1	2.2±0.3	NG	NG
Ga-9	4.6±0.8	4.6±0.9	5.0±0.2	4.5±0.9	1.5±0.1	2.2±0.3	NG	NG
Gad-6	4.0±0.7	3.6±0.3	4.5±0.6	3.0±1.0	GI	GI	NG	NG
Gad-03	4.3±1.0	4.0±0.5	6.3±0.9	3.5±0.5	GI	GI	NG	NG
Gad-VII	4.7±0.9	3.7±0.7	6.8±1.1	3.5±0.7	GI	GI	NG	NG
1016	3.8±0.7	3.5±0.5	4.7±0.9	4.0±1.0	GI	GI	NG	NG

Notes: (MEA) - malt extract agar, (PDA) – potato-dextrose agar; (GI) – growth on inoculum; (NG) – not growth. (*) - Mean of 6 replicates.

Table 3. Sizes (μm) of cultural structures in studied collections of *G. adspersum*

Microstructure	**Length**	**Average Length**	**Wide**	**Average Wide**
Cuticular cells	4.37-8.61	6.42±1.51	4.31-8.96	6.10±1.15
Hyphal swellings in agar culture	3.49-5.60	4.69±0.82	3.13-6.22	4.91±1.09
Hyphal swellings in submerged culture	10.23-21.22	13.40±3.95	7.86-10.38	9.50±0.99
Crystals in agar culture	5.33-10.46	7.62±1.62	4.96-9.41	6.87±1.54
Crystals in submerged culture	6.31-18.39	10.38±6.94	5.72-17.28	9.64±6.62

Note: (*) - Mean of 10 replicates ± SD

The salt crystals observed in mushroom cultures are usually calcium oxalate crystals, which mainly formed on hyphae under different culture

conditions. They are a relatively stable characteristic of fungal cultures since the morphology and abundance of crystals may vary in different species/strains. Numerous tetrahedral crystals in agar (5.33-10.46 x 4.96-9.41 μm) and liquid (6.31-18.39 x 5.72-17.28 μm) cultures of *G. adspersum* were observed (Fig. 4d,i). In submerged culture vacuolated hyphae with clamps and chlamydospore-like swellings (10.23-21.22 x 7.86-10.38 μm) were abundantly observed in *G. adspersum* (Table 3; Fig. 4h,l).

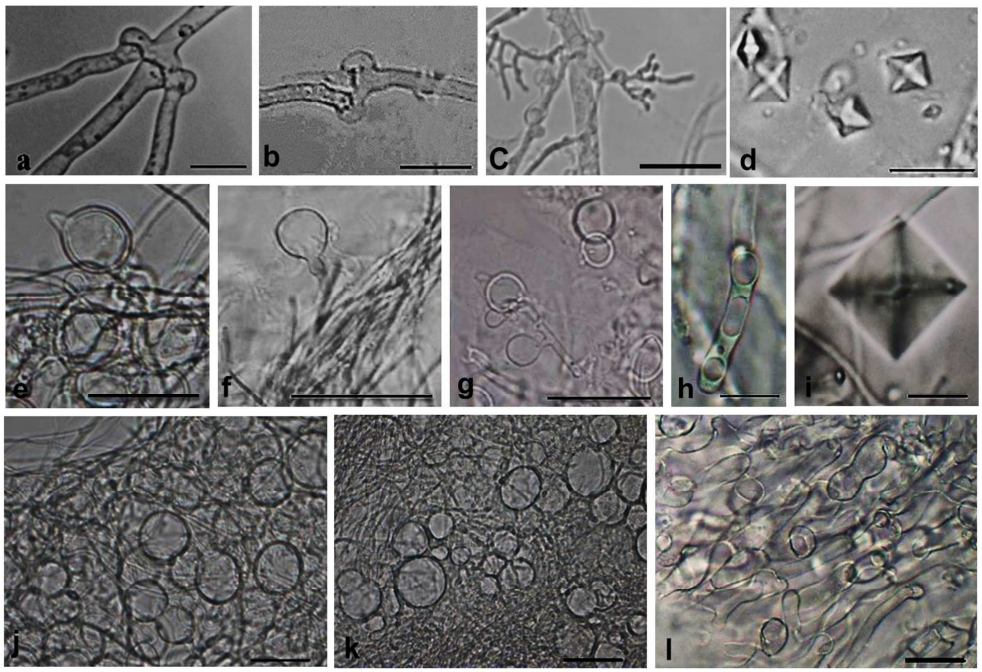

Fig. 4. Mycelial microstructures in *G. adspersum* on agar media: (a,b) hyphal clamps; (c) coralloid hyphae in str. Ga-2-3; (d) crystals in str. Ga-1; (e,f,g) chlamydospore-like swellings in str. Ga-9; (j,k) cuticular cells in str. 1016; (l) swelled and (h) vacuolated hyphae in str. Gad-6; (i) crystal in str. Ga-3 in submerged culture. Size bar 10 μm

Conclusion

The revealed taxonomically valuable mycelial characteristics of *G. adspersum* (colony texture, pigmentation, growth rate, presence and form of hyphal clamps, coralloid hyphae, chlamydospore-like swellings and/or cuticular cells, optimal growth temperature) will assist in the proper taxonomic identification of cultures, control their quality and optimal growth conditions during their biotechnological cultivation.

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