Analele Ştiinţifice ale Universităţii „Al. I. Cuza” Iaşi
s. II a. Biologie vegetală, 2012, **58**, 2: 19-29
http://www.bio.uaic.ro/publicatii/anale_vegetala/anale_veg_index.html
ISSN: 1223-6578, E-ISSN: 2247-2711

DESCRIPTION OF *IN VITRO* CULTURES FOR SOME SPONTANEOUS LIGNICOLOUS BASIDIOMYCETES SPECIES

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Abstract: 13 species of lignicolous basidiomycetes from 7 taxonomic families and 4 orders, Class Agaricomycetes, Phyllum Basidiomycota, have been studied. The mycelium grown *in vitro* was analysed and the culture description of these isolates was presented. The dikaryotic mycelium from the context of the fruit bodies was used as source of inoculum in the isolation processes. Petri dishes of 9 cm diameter filled with malt extract-agar media (malt extract 2 %) were used for the culturing purpose. After the inoculation, the plates were incubated at 25 °C, in the dark, for 6 weeks. Observations made directly and using a Nikon stereomicroscope were employed in order to measure the growth rhythm and to assess the changes of the mycelium: edge of colony, surface, reverse, shape, colour, smell, presence or absence of the exudates. Microscopic slides were made after 6 weeks from the inoculation for investigations concerning the types of hyphae, the colour and the structure of the mycelium. Particular elements, such as: cuticular cells, chlamydospores, arthroconidia, conidia, basidia and crystals were also noticed. We observed similar characters for our isolates but also significant differences between them. The growth rhythm varied strongly, the slowest growth rhythm being recorded for isolates of *Ganoderma applanatum* and *Xylobolus frustulatus*.
Keywords: lignicolous basidiomycetes, fungal growth, culture description

Introduction

Lignicolous basidiomycetes represent a heterogeneous taxonomic group involved in the degradation of the wood. Due to their action, these organisms can cause economical losses but have, also, a very important positive role in the recycling of the organic matter. In the course of evolution, lignicolous basidiomycetes species developed different strategies to degrade the wood and to use the resulted products in their own metabolism. Among different enzymes involved in the processes, ligninolytic enzymes play a key role in the degradation of lignin but also in the degradation of many other chemical compounds that present structural analogies, as xenobiotics frequently are.

A great variety in terms of morphological features of *in vitro* grown mycelium is caused by the ecological adaptations of lignicolous basidiomycetes and by their different taxonomical position. Because of this variety, it is possible to distinguish between different species and even to recognize them, observing the mycelium's features. Fruit bodies or specialised structures for asexual reproduction and other particular elements may be present in the culture, but their presence is not prerequisite for all the species/isolates. Clamp connections are characteristic to basidiomycetes and they are formed from the dikaryotic cells to avoid the septum and connect with the proximal cell (Tănase and Şesan, 2006). However, not all the basidiomycetes present clamp connection.

The analysis of the macroscopical and microscopical characters of the mycelium and the culture of the pure isolates offer additional information in the taxonomic studies and can

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make possible the identification of the species when the fruit bodies are missing or are being deteriorated. Not the last, the studies concerning the *in vitro* development of different categories of fungi are very important in the elaboration of mycoremediation strategies and the optimization of culture conditions.

Although there are studies which describe the pure cultures of different lignicolous basidiomycetes isolates (Nakasone, 1990; Nobles, 1948; Stalpers, 1978, 1993), few of them treated other species than those formerly included in the order Aphyllophorales (Buchalo et al., 2011; Otieno et al., 2003). Moreover, even the formerly group of Aphyllophorales is not treated exhaustive. Some of the species presented in this paper have been poorly studied till the present. Isolates from the same species may present different characters. For this reason, it is necessary to analyse more isolates from the same species in order to characterize it.

Materials and methods

Context mycelium of fruit bodies collected from deciduous woods found in forest habitats in north-eastern Romania were used for the isolation purposes. The pure isolates have been maintained by subculturing them onto malt-extract media and stored refrigeration for further uses. Classical macroscopic and microscopic methods (Bernicchia, 2005; Borgarino and Hurtado, 2001; Eriksson and Ryvarden, 1976; Hansen and Knudsen, 1992, 1997; Jülich and Stalpers 1980; Roux, 2006; Ryvarden and Gilbertson, 1993; Sălăgeanu and Sălăgeanu, 1985) have been used for the identification of the species. The names of the studied species and the corresponding herbarium voucher are listed in Table 1, and the used nomenclature is according to The Species Fungorum database (www.speciesfungorum.org, accessed from 20th January 2012 to 25th March 2012).

The collected specimens were deposited in the Faculty of Biology Herbarium, Alexandru Ioan Cuza University of Iasi, Romania, after proper preservation through lyophilisation (UniEquip lyophilizator, UNICRYO MIC 4 L model, Planegg, Germany) or dehydration (using a dryer, Ezidri Ultra 1000 FD).

In order to describe the cultures, the method established by Stalpers (1978) has been used. Consequently, the 9 cm diameter Petri dishes were filled with 25 ml neutralized and sterilized (by autoclaving at 120 ºC) malt extract-agar media (malt extract 2 %). Small plugs of mycelium were placed near the edge of Petri dishes and the plates thus obtained were incubated in the dark at 25 ºC, for 14 days.

The colony growth rhythm was measured weekly. During this time, macroscopic changes of the colony were observed with the naked eye and with a stereomicroscope at 15-30 x magnification (stereomicroscope with phototube SZM2 Optika). After 6 weeks from the inoculation, microscopic observations were made, using a trinocular microscope (Optika), and the microscopic structures were measured at a magnification of 1000x. Hyphal system from the advancing zone, the submerged or aerial mycelium was studied. A special attention was paid to: the types of the hyphae, their colour and aspect; presence/absence of the crystals on the hyphal surface; the diameters of hyphae; the presence, form and dimension of arthroconidia, chlamydospores, cuticular cells, clamp connections; the formation *in vitro* of fruit bodies and their characters; the presence of other particular structures, of exudates etc. A solution of KOH 5% was used to verify whether some hyphal structures change the colour or swell.

Results and discussion

The culture descriptions of thirteen isolates of lignicolous basidiomycetes from seven families and four orders (Table 1), included in Class Agaricomycetes, Subclass Agaricomycotina, Phyllum Basidiomycota were presented in this paper. The growth rhythm differed from one isolate to another, thus a fast development was recorded for isolates of *Flammulina velutipes*, *Ganoderma resinaceum* and *Schizophyllum commune* while for isolates from Auriculariaceae family and for *Xylobolus frustulatus* a slow mycelium expansion was noticed.

Table 1. The tested fungal isolates, their taxonomic position and the voucher of the specimen in the Herbarium

ORDER	FAMILY	SPECIES	HERBARIUM VOUCHER
Agaricales	Agaricaceae	*Cyathus striatus* (Huds.) Willd.	[I 137369]
	Physalacriaceae	*Flammulina velutipes* (Curtis) Singer	[I 137378]
	Schizophyllaceae	*Schizophyllum commune* Fr.	[I 137382]
Auriculariales	Auriculariaceae	*Auricularia auricula-judae* (Bull.) Quél.	[I 137388]
		Auricularia mesenterica (Dicks.) Pers.	[I 137389]
Polyporales	Ganodermataceae	*Ganoderma adspersum* (Schulzer) Donk	[I 137366]
		Ganoderma applanatum (Pers.) Pat.	[I 137379]
		Ganoderma lucidum (Curtis) P. Karst.	[I 137380]
		Ganoderma resinaceum Boud.	[I 137367]
Russulales	Peniophoraceae	*Peniophora incarnata* (Pers.) P. Karst.	[I 137361]
		Peniophora quercina (Pers.) Cooke	[I 137375]
	Stereaceae	*Stereum hirsutum* (Willd.) Pers.	[I 137402]
		Xylobolus frustulatus (Pers.) Boidin	[I 137403]

A limited number of basidiomycetes species have been characterized in culture until now, especially those species with economical importance. Our research provides additional knowledge to the field, some of the species presented in this paper being poorly studied. The obtained results are in accordance with those reported by other authors. However, some of the analysed isolates behaved differently from isolates reported in the literature. As far as we know, this is the first report of arthroconidia production in culture by *Peniophora incarnata*.

The fungal isolates presented in this paper differed by the presence of asexual reproduction structures (arthroconidia, chlamydospores) and by the colour of mycelium, the general aspect of the colony, the hyphal system and the presence/absence of cuticular cells, the crystals and clamp connections (Table 2). Some of the characters mentioned above proved to be similar for all the isolates from a genus.

The species from Ganodermataceae family distinguished by the presence of numerous chlamydospores while the isolates from Peniophoraceae and Stereaceae presented a coloured mycelium and other particular structures.

It is important to understand the mechanisms involved in fungal growth on artificial media and also to know how these fungi develop and react. This information is important in elaborating mycoremediation strategies.

Macroscopic aspects and microscopic characters of mycelium grown on nutritive media

***Auricularia auricula-judae* (Bull.) Quél.** Mycelium is smooth, homogeneous, appressed with less abundant hyphae, whitish. Around the point of inoculation tree-like branched hyphae are observed, forming protrusions fluffy white that gradually increase and become cream or yellow, then gray-black colour, some remain white. The mycelium is then felty or felty-soft, whitish or ochre, uneven, sometimes with rare hyphae or appressed, white or greyish white, with denser white areas (Plate IA). Submerged mycelium and from the advancing zone presents generative hyphae, thin to moderately thick, of 1-5 μm diameter, with simple septa, thick, tree-like branched at the top, sometimes with short lateral branches, straight, hyaline, with vacuolated cytoplasm. Aerial mycelium presents generative hyphae, less branched, in a lax network. Pigmented hyphae (brown to black) form agglomerations. These hyphae are unbranched or sparsely branched, simple septate, often swollen, thick. The isolate analysed in this study showed no clamp connections or chlamydospores, although other authors (Buchalo et al., 2011) revealed the presence of simple clamps and chlamydospores in *Auricularia auricula-judae* cultures.

***Auricularia mesenterica* (Dicks.) Pers.** Initially, aerial mycelium is velvety, grouped in cords and arranged radially. Near the point of inoculation a mycelial network is formed, like a thick, fluffy wall. The mycelium is white later becomes zonate, forming an aerial hyphal network, fluffy, very thick, with concentric rings, thicker, prominent and cream. The interzones are thinner and compact, felty, white. Around the point of inoculation emerge thick hyphae, cream to reddish-brown, erect, elevated and from here fascicular radial cords are formed, fluffy darker than the rest of the colony (Plate IB). Colony edge is straight. Hyphal system is monomitic. Submerged mycelium and from the advancing zone presents generative hyphae, thin, highly branched, of 1.5-3 μm. Aerial mycelium presents long straight hyphae, unbranched, thin of 2-3 μm diameter, often grouped in cords, nonseptate or with rare septa, without clamp connections, hyaline, which tapers towards the top. Some cords are very thick, comprising a large number of hyphae and pigmenting (yellow to orange under the microscope) and oldest hyphae are thicker, up to 3.5 μm in diameter.

***Cyathus striatus* (Huds.) Willd.** Aerial mycelium forms a loose network after six weeks, elevated, cream-yellow-brown, with aerial hyphae sometimes brown and thick appressed cords, brown, arranged radially, branching divergently (Plate IC). Colony edge is regular. Submerged mycelium and from the advancing zone presents generative hyphae, hyaline and thin at first, and then thick (up to 5 μm diameter), cream to light brown, branched, with clamp connections. Aerial mycelium presents generative hyphae, 2 to 5.5 μm diameter, frequently septate, strongly branched, with swellings, thick walled, septa often without clamps, hyaline in water, meandering, sometimes with anastomosis; skeletal hyphae, unbranched, cream to brown, nonseptate, of 1.5-2.5 μm diameter; connecting hyphae, thin, unbranched, with rare septa, simple, rarely with clamps, brown, long, often grouped in mycelial cords.

***Flammulina velutipes* (Curtis) Singer.** Mycelium is concentrically zonate. Aerial mycelium is felty or appressed near the inoculum, dense, with many globular hyphal clusters, creamy white to yellow-brown. They form a concentric ring (Plate ID). In the

opposed side, mycelium is homogeneous, soft-felty, with white hyphae, straight and white or cream-yellow veins.

Table 2. The principal macroscopic and microscopic characters of fungal isolates cultured on synthetic nutritive media

SPECIES	GROWTH RHYTHM*	EXUDATES	SMELL AND REVERSE **	REPRODUCTIVE STRUCTURES	PARTICULAR ELEMENTS
Auricularia auricula-judae	5	colourless exudates	indistinct; red-brown	chlamydospores, 20-25 x 12-20 µm, hyaline	prismatic crystals, 5 x 10 µm; swollen hyphae, 6-7 µm diameter
Auricularia mesenterica	5	no exudates	indistinct; white		
Cyathus striatus	4	brown exudates	indistinct; unchanged		hyphae with swellings, 8-9 µm diameter;
Flammulina velutipes	2	no exudates	slightly phenolic; unchanged	chlamydospores, spherical or oval	short lateral branches; swellings, 10-20 x 4,5 µm
Ganoderma adspersum	2	colourless exudates	indistinct; unchanged or slightly brown	chlamydospores, 7-10 x 10-15 (20) µm, numerous, purple-brown	cuticular cells, hyaline, very numerous, up to 30 µm diameter
Ganoderma applanatum	> 6	brown exudates	indistinct; unchanged	chlamydospores, rarely	cuticular cells, amyloid; large octahedral crystals; small irregular crystals, 2 x 5-8 µm
Ganoderma lucidum	2	no exudates	indistinct; unchanged	grouped chlamydospores	cuticular cells, numerous, amyloid, 20 x 15 µm
Ganoderma resinaceum	2	no exudates	indistinct; brown	chlamydospores, 7-10 x 10-15(20) µm, numerous, purple-golden	cuticular cells, 10–30 µm
Peniophora incarnata	3	no exudates	mud; white	arthroconidia, 3-4 x 9-12 µm, cylindrical or slightly curved	gloeocystidia, 25 x 5 µm; hyphae with swellings, terminal or intercalary, up to 12 µm diameter, 15 µm length;
Peniophora quercina	2	no exudates	mushroomy; yellow or brown		numerous crystals, cubic, prismatic or octahedral
Schizophyllum commune	2	no exudates	mushroomy, strong; unchanged	primordia; chlamydospores, numerous, hyaline, 10-15 x 4-7 µm	
Stereum hirsutum	3	no exudates	rotten wood; unchanged		
Xylobolus frustulatus	> 6	no exudates	garlic, easily; yellow or brown	thin walled chlamydospores, 11 x 13 µm	irregular crystals, large, 15 x 15 µm; octahedral crystals; oval crystals, small, 8 x 13 µm

* The needed time for covering the entire plate (in weeks)

** Only the old part of the colony is considered. The recently covered medium remain, often, unchanged

Submerged mycelium and from the advancing zone presents generative hyphae, 2.5-3 μm diameter, branched, with clamp connections at septa. Aerial mycelium presents generative hyphae, thick up to 4.5 μm in diameter, with thick walls and clamps at septa; skeletal hyphae, highly branched, hyaline in Meltzer, thick. In the older colony yellow-brown areas with generative hyphae are present. Some authors (Borhani et al., 2011; Stalpers, 1987) noticed the presence of arthroconidia but we did not find any in our cultures.

***Ganoderma adspersum* (Schulzer) Donk.** Mycelium is white, with some irregular mycelial cords, compact, thin. Aerial mycelium is felty-powdery, dense, with white veins. In the centre of the colony, mycelium is soft-felty, forming a distinct network delineated by a thick mycelial cord (like a thin wall). On the plate edges a dense dust is observed, consisting of chlamydospores (Plate IE). Near the point of inoculation, but not limited to, the mycelium is lax and translucent, or felty, powdery with cream or yellow tint. Submerged mycelium and from the advancing zone presents generative hyphae, branched, with clamps, hyaline, of 1.5 to 4 μm in diameter. Aerial mycelium presents generative hyphae, thin, wavy, branched, including short lateral branches, and skeletal hyphae, pigmented.

***Ganoderma applanatum* (Pers.) Pat.** Mycelium is appressed, lax, translucent, with slightly different zones, concentrically arranged, midpoint somewhat denser and slightly velvety. Near the point of inoculation and the wall mycelium presents few crusts, white, soft-powdery, with a red edge. Submerged mycelium and from the advancing zone presents generative hyphae, branched, with large clamps, hyaline, of 1.5 to 4 μm in diameter. Aerial mycelium presents generative hyphae, 1.5 x 4 μm thick and skeletal hyphae, irregularly thickened up to 6 μm thick. Our analysed culture presented chlamydospores, rarely, but in similar experiments (Nobles, 1948) did not found chlamydospores.

***Ganoderma lucidum* (Curtis) P. Karst.** Mycelium presents different zones, concentrically arranged, appressed and powdery near the point of inoculation, and then presents a fuzzy-felty ring and a compact mycelia cord that separates the two areas. Distal zone is ± homogeneous, felty, with small hyphal clusters, sometimes compact mycelium with hyphae yellowish-orange cream. In the rest of the colony mycelium is white (Plate IF). Hyphal system is dimitic. Submerged mycelium and from the advancing zone presents generative hyphae, branched, thin, with numerous septa and clamp connections, hyaline. Aerial mycelium presents generative hyphae; skeletal hyphae, frequently branched and cuticular cells. The hyphae that support these cells are often bold and amyloid.

***Ganoderma resinaceum* Boud.** Mycelium forms a dense, dusty-felty, with frequent aerial hyphae, short. In the centre of the colony mycelium forms a distinct network delineated by a compact mycelial cord. Near the point of inoculation, mycelium is powdery, especially near the wall plate, thin, translucent or felty, with cream or yellow colour (Plate IIA). Sometimes small mycelia cords are present, arranged irregularly, like white veins. Submerged mycelium and from the advancing zone presents generative hyphae, with only thin walls and clamps at the septa, unbranched or rarely branched, 2-6 μm in diameter. Aerial mycelium presents generative hyphae with branched ends that form a network and skeletal hyphae, without clamps. Mycelium presents cuticular cells as described by Bazzalo and Wright (1982).

***Peniophora incarnata* (Pers.) P. Karst.** Mycelium is soft-woolly, cream, forming a dense ring, fluffy and a network ± homogeneous, felty and thin to thick in the distal zone, with erect hyphae, tree-like branched and small randomly arranged clusters of hyphae. Near the plate wall are also formed fluffy clusters. In the centre of the colony small primordia are formed (Plate IIB). Colony edge is straight, but unevenly. Submerged mycelium and from the advancing zone presents generative hyphae, with septa and clamp connections, branches and thin walls. Aerial hyphae are branched, with clamps, of 1.5 to 4.5 μm in diameter, often 3-4 μm, can be highly branched, and sometimes twisted, with short lateral branches, finger-like. On the plate walls thickened and brown hyphae are formed, sometimes encrusted. In this area gloeocystidia and arthroconidia are also present. Nakasone (1990) noted the presence of gloeocystidia at the colony edges.

***Peniophora quercina* (Pers.) Cooke.** Mycelium presents different zones, concentrically arranged and the old mycelial network is darker and felty. Mycelium is denser towards the edges of the Petri dish and lax in the centre of the colony. It forms a network radially arranged, creamy and with red-brown aerial hyphae. On the plate wall, a mycelial ring is formed, felty, cream-brown (Plate IIC). On the edges of colony, the aerial hyphae are thick, fluffy-felty, braided, ± homogeneous, slightly radial. Colony edge is straight. Submerged mycelium and from the advancing zone presents generative hyphae, with clamps, hyaline, ± straight, branched, thin or thick, with vacuolated cytoplasm. Aerial mycelium presents generative hyphae, 2-4 μm in diameter; connecting hyphae and anastomosis. Some of the clamps are very large, forming a space between the clamp and the hypha (loop). Other hyphae are long, without septa or with rare septa, 4 μm thick.

***Schizophyllum commune* Fr.** Mycelium is arranged ± radially, with veins and cords. It is lax-appressed near inoculum and form mycelial cords and hyphal clusters, dense, soft and white. On the plate walls hyphal clusters are formed, felty-fluffy, compact, irregular, white or cream, sometimes soft crust (Plate IID). Mycelium reaches the upper plate. Hyphal system is dimitic. Submerged mycelium and from the advancing zone presents generative hyphae, branched, thin to moderately thick, with thick septa with loops, hyaline. Aerial mycelium presents generative hyphae and skeletal hyphae, hyaline and thin.

***Stereum hirsutum* (Willd.) Pers.** The colony has irregular edges with areas of advancement-exploration mycelium. Mycelium is distributed unevenly, with zones macroscopically different. In the centre of the colony, mycelial network is very thick, fluffy-fleece, white or cream and cream-yellow outwards to cream-brown. The two areas are separated by a mycelial cord, compact and white. In the zone opposed to the inoculums hyphal clusters are formed, fluffy, white or cream, irregularly distributed and lax-appressed mycelium areas, translucent (Plate IIE). Aerial hyphae are randomly arranged and form distinct hyphal clusters or compact structures. Submerged mycelium and from the advancing zone presents generative hyphae, moderately branched, with frequent septa, with clamps, hyaline and thin walls. The aerial mycelium presents hyphae as in the advancing zone, but also long flexuous fibre hyphae are present, with thick walls, nonseptate, rarely branched.

***Xylobolus frustulatus* (Pers.) Boidin.** Mycelium presents different zones, concentrically arranged, slightly dense. Aerial mycelium is powdery or felty-hirsute with erect hyphae, thick yellow-orange-brown near the point of inoculation. In the centre of the colony aerial mycelium presents felty-fluffy hyphae, yellow and white hyphae far distally

(Plate IIF). Colony edge is straight. Submerged mycelium and from the advancing zone presents generative hyphae, branched, wavy, with simple septa of 1.5 to 4 μm thick, sometimes inlaid. Aerial mycelium presents generative hyphae, 1.5 to 5 μm thick, sometimes anastomosed with short lateral branches and skeletal hyphae, thin, nonseptate and highly branched. Older areas have clusters of hyphae, orange, long, with encrusted surface, up to 5 mm thick, with numerous septa, anastomosed.

Conclusions

The culture descriptions of thirteen isolates of lignicolous basidiomycetes from seven families and four orders, included in Class Agaricomycetes, Subclass Agaricomycotina, Phyllum Basidiomycota were presented in this paper. Therefore, the macroscopic and microscopic characters of mycelium grown *in vitro* were analysed.

Some of the tested isolates (*Flammulina velutipes*, *Ganoderma resinaceum*) developed very fast on malt-extract media while other isolates (*Xylobolus frustulatus*) grew very slow. The formation of arthroconidia or chlamydospores, the colour of mycelium or the growth rhythm were different from one isolate to another. However, some characters, such as the presence of chlamydospores and the hyphal system seemed to be constant for all the isolates from a genus (*Ganoderma*). Our isolates from genus *Auricularia* did not present clamp connections.

The cuticular cells were present only in the isolates from the genus *Ganoderma*, while the isolates of *Ganoderma applanatum* and *Xylobolus frustulatus* had the slowest growth rhythm.

Acknowledgements

This work was supported by the European Social Fund in Romania, under the responsibility of the Managing Authority for the Sectoral Operational Programme for Human Resources Development 2007-2013 [Grant POSDRU/107/1.5/S/78342).

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Explanation of the Plates

PLATE I. General aspects of colonies after 6 weeks of incubation:

A – *Auricularia auricula-judae*;
B – *Auricularia mesenterica*;
C – *Cyathus striatus*;
D – *Flammulina velutipes*;
E – *Ganoderma adspersum*;
F – *Ganoderma lucidum*.

PLATE II. General aspects of colonies after 6 weeks of incubation:

A – *Ganoderma resinaceum*;
B – *Peniophora incarnata*;
C – *Peniophora quercina*;
D – *Schizophyllum commune*;
E – *Stereum hirsutum*;
F – *Xylobolus frustulatus*.

PLATE I

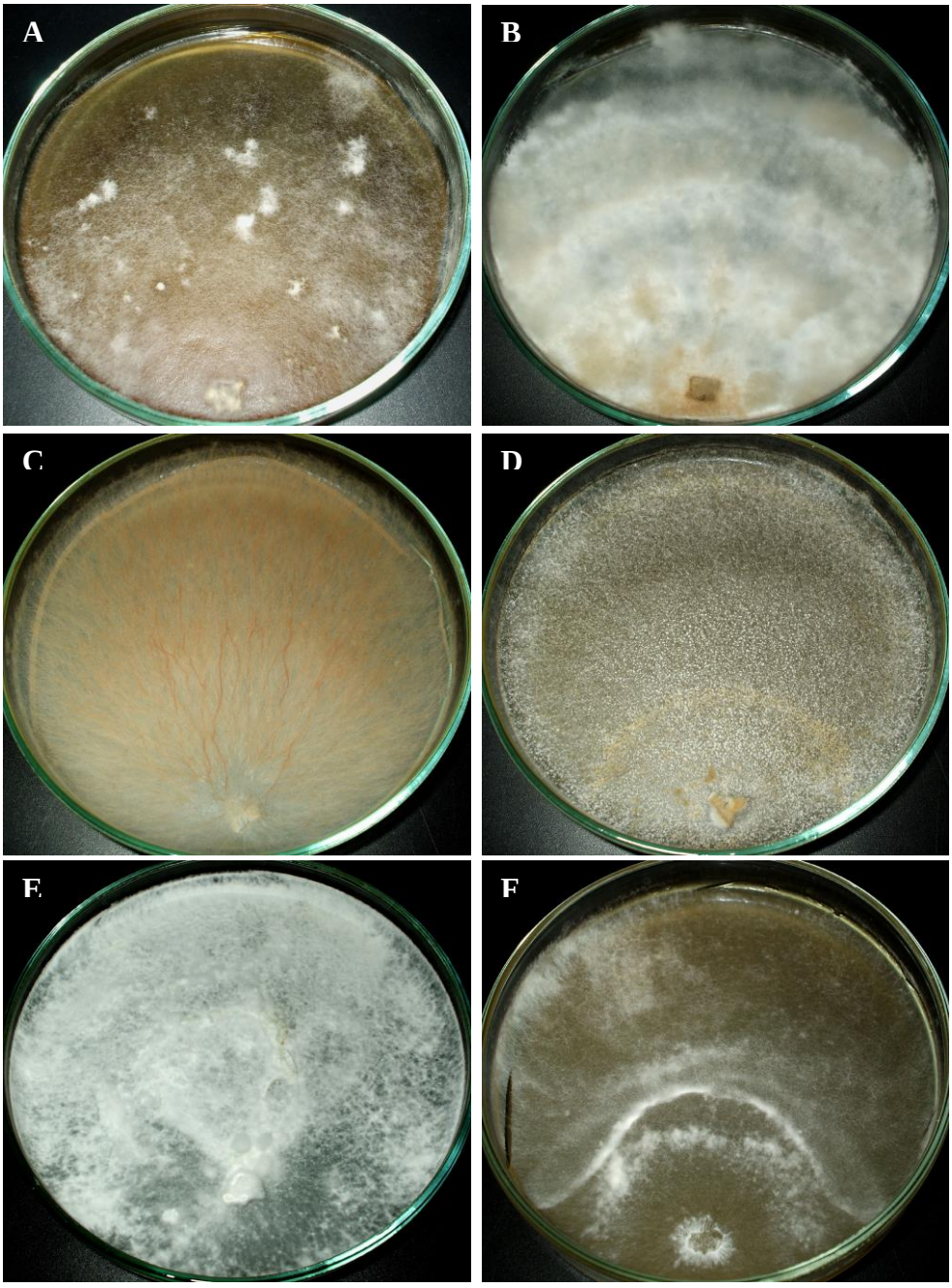

PLATE II