Xanthoria parietina (L). TH. FR. MYCOBIONT ISOLATION BY ASCOSPORE DISCHARGE, GERMINATION AND DEVELOPMENT IN "IN VITRO" CULTURE

CRISTIAN Diana, BREZEANU Aurelia

Abstract. The article is focused on fungal partner isolation from the *Xanthoria parietina* (Teloschistaceae) lichen body by ascospore discharge from golden disk-like fruits - ascoma, followed by germination and subsequent development on liquid nutrient medium Malt-Yeast extract (AHMADJIAN, 1967a) under different temperature and light/dark regime conditions. The morphology of the mycobiont and the inner structure were characterized by stereomicroscope Stemi 2000 C, light microscope Scope. A1, Zeiss and by the JEOL - JSM - 6610LV Scanning Electron Microscope.

Keywords: mycobiont, ascospore isolation, lichen culture.

Rezumat. Izolarea micobiontului de *Xanthoria parietina* (L.) TH. FR. prin descărcarea sporilor, germinarea şi dezvoltarea în cultură „in vitro". Acest articol se axează pe izolarea partenerului fungal din talul de *X. parietina* (Teloschistaceae), prin eliberarea ascosporilor din apoteciile disciforme aurii, urmată de germinarea şi dezvoltarea pe mediu nutritiv lichid Malt-Yeast extract (AHMADJIAN, 1967a) la diferite temperaturi şi sub un regim diferit de lumină/întuneric. Morfologia micobiontului şi structura sa internă au fost caracterizate la stereomicroscop Stemi 2000 C, la microscopul optic Scope. A1, Zeiss şi la microscopul electronic scanning JEOL - JSM - 6610LV.

Cuvinte cheie: micobiont, izolarea ascosporilor, cultura lichenică.

INTRODUCTION

Lichens are a product of symbiotic association of two unrelated organisms, a primary producer (photobiont) - cyanobacteria or algae - and a primary consumer, a type of fungi (mycobiont), forming a new biological entity, with no resemblance to its individual components, due to non-structural, biochemical changes and physiological essentials for morphological differentiation, interaction and stability of the association. High degree of integration is reflected by the appearance of structures (heteromerous or homeomerous thallus, soredia, isidia), intracellular or intramembranar haustors, etc.) and metabolites ("lichen substances"), never produced by individual components (ZARNEA & POPESCU, 2011).

In terms of mycobiont culture history, AHMADJIAN (1961, 1993), CRITTENDEN et al. (1995), YOSHIMURA et al. (2002), STOCKER-WÖRGÖTTER & HAGER (2008) were noted in this research field.

TÖBLER (1909) and THOMAS (1939) have been mentioned by SANGVICHIEN et al., 2012 like pioneers in the field of lichen - forming fungi isolation although TÖBLER was primarily interested in the resynthesis of lichens from their individual symbionts (TURBIN, 1936). However, WERNER (1927) innovatively examined the effect of different media and additions on the growth of selected mycobionts from a range of lichens.

CRITTENDEN et al. (1995) were the first to attempt the isolation of a wide range of fungal partners of lichens and also lichenicolous fungi.

Our interest for axenic culture of the lichen fungi study was stimulated by their capacity to produce a wide range of secondary metabolites (VICENTE, 1991) many of which are unique to lichens and exhibit antimicrobial activity (FILHO et al., 1990) or have other biological applications (HIGUCHI et al., 1993).

Previous studies revealed that lichen-forming fungi grow more slowly than free-living fungi and have complex nutrient requirements (STOCKER - WÖRGÖTTER, 1995).

Lichen-forming fungi belongs to Ascomycetes family and present heterotrophic nutrition. *X. parietina* was chosen by us as an experimental system model to represent lichen-forming fungi because it is one of the most commonly studied lichenized fungi, is amenable to axenic cultivation and it has a wide distribution; it can occur on a variety of substrates including rock, tree bark.

Our studies were carried out to testing the efficacy of culture method for *X. parietina* mycobiont as well as to make observations regarding culture morphology and anatomy and factors affecting germination and subsequent development on a liquid growth medium namely Malt-Yeast extract.

MATERIALS AND METHODS

Collection of the sample

The *X. parietina* thalli were collected from Pustnicu Forest, near Bucharest, in November 2012.

The taxonomic identity was confirmed on the basis of their morphology and anatomy using a stereo microscope CL 1500 Eco and spot tests by Ioana Vicol (Department of Ecology, Institute of Biology).

The samples were air-dried at room temperature (25° C) for two days and cleaned free of any other materials.

Then, they were transferred to paper envelopes with identification labels and stored in a refrigerator until the performing of the *in vitro* culture protocol.

Source of mycobiont

For laboratory cultures, the most usual method of isolating mycobionts is to start from discharged spores, primarily ascospores (YOSHIMURA et al., 2002).

For this reason, we chose the lichen *X. parietina* containing spore-bearing structures, named apothecia.

Clean and freeze material was allowed to equilibrate with the environment a few hours.

The apothecia were removed from the thallus and placed into dishes containing distilled water.

In subsequent stages, the ascoma were passed through a series of sterilizing solutions such as: ethanol 70% (5 s), sodium hypochlorite 4% (90 s) and sterile water (10 s) according to SURYANARAYANAN et al. (2005). The excess water was removed with filter paper.

These structures were aseptically fixed on a half of a Petri dish containing 4% distilled water - agar medium.

In the other half of the Petri dish it was distributed a liquid variant of Malt-Yeast extract (AHMADJIAN, 1967a) medium.

Malt extract is a source of carbohydrates and it is used at a level of 0.5 - 1g/l (GEORGE et al., 2008).

Yeast extract was also added as a source of aminoacids and vitamins, especially inositol and thiamine (Vitamin B1) (BONNER & ADDICOTT, 1937). Normally this enhances growth in media containing relatively low concentrations of nitrogen, or where vitamins lack.

We used two types of the apothecia: mature and younger.

Some Petri dishes were stored to 19 º C and others to 23 º C. Some of them were covered with aluminium foil.

Development and colony growth were made under static conditions.

We conducted subcultures every two months. The method promoted by AHMADJIAN (1993) was used to maintain the cultures.

The development of mycobiont was observed with binocular-stereo and transmission light microscopes and recorded by macro - and microphotography.

For cytology studies, squash preparations of mycobiont culture were analysed by phase contrast microscopy or contrasted with lactophenol - bleu - cotton solution prior examination by light microscopy. Lactophenol was used as the mounting medium.

For light microscopy, we used Scope. A1, model Zeiss, equipped with differential interference contrast optics.

For SEM electron microscopy samples were processed by standard protocols of SEM laboratory techniques (POSTEK et al., 1980; HALL & HAWES, 1991; FOWKE, 1995) and analysed to the JEOL - JSM - 6610LV Scanning Electron Microscope.

RESULTS AND DISCUSSIONS

It is well known that the fungi prefer moisture conditions. In this context, we consider the liquid state of the MY culture medium to be the best for fungus development (germination and culture).

The ascospores from the apothecia fell into the fluid medium below and their route from top to bottom generated a thin white column of hyphae in three days (see black arrow) (Fig. 1a), which rested on a small disc formation (see white arrow) developed in the culture medium. After another three days, the column became thicker (Fig. 1b).

Because of the pressure, the white column of hyphae suffered a rupture but this fact allowed the circular remaining formation to rise in diameter (Fig. 1c; Fig. 2).

In three weeks, the circular structure developed in a formation with many lobes arising from the surface (Fig. 3).

Mycobionts can be stored for long periods (about one year) (YOSHIMURA et al., 2002), but it was necessary to make subcultures (Fig. 4 a, b, c) to avoid the degenerative phenomena, every two weeks.

Mycelium analysis in phase contrast microscopy revealed their morphology peculiarities. These consist of septate, pluricellular, branched hyphae (Fig. 5). The cells have an eukaryotic - parasitic - heterotrophic organization.

The hyphae network appearance is illustrated by the phase contrast mycobiont analysis (Fig. 6).

We can observe the external morphology of the mycobiont plechtenchyma by means of an important tool, namely scanning electron microscopy (Figs. 7; 8a, b).

In accordance with SMITH & BERRY (1974) HAMADA & MIYAGAWA (1995); HUBER et al., 1994; MOLINA et al., 1997a, generally, free living fungi spores require organic carbon during germination in order to generate a three dimensional structure. Both yeast and malt extract of medium composition meet this requirement.

X. parietina ascospores germinated best on media with pH 5-7. These results are consistent with those of CHRISMAS (1980), OSTROFSKY & DENISON (1980). In our experimental conditions, the value of the pH of 5.5 allowed us to obtain good results.

The mycobiont prefers a higher temperature for the development. Therefore, to 23ºC the germination was faster.

The good evolution of the cultures covered by aluminium sheets suggests that mycobiont needs dark condition. This confirm the claims of YOSHIMURA et al. claims (2002) that light is not necessary for maintaining mycobiont cultures.

CONCLUSIONS

X. parietina is a responsive species to *in vitro* conditions regarding mycobiont isolation.

The mycobiont growth depends on the age of the ascoma, physical medium state, composition and the incubation conditions like temperature and light/dark regime.

The mature ascoma were more efficient than the younger ones for the discharge rate and germination.

The MY liquid medium confers a wet environment for the fungal hyphae and so offers a satisfactory condition for *in vitro* culture beside the rich organic composition of the medium.

The higher temperature (23^{o}C instead of 19^{o}C) favoured both germination and subsequent culture.

The darkness allowed the development of the mycelium in good condition.

Our investigations could encourage more experimental work on the isolation, germination and development in pure culture of fungal partners of the other lichen species of biotechnological value, namely secondary synthesized metabolites.

In the same time, these studies could also represent an important step for fundamental studies on the factors affecting the reproductive biology of lichens, generally, as well as for lichen body resynthesis processes.

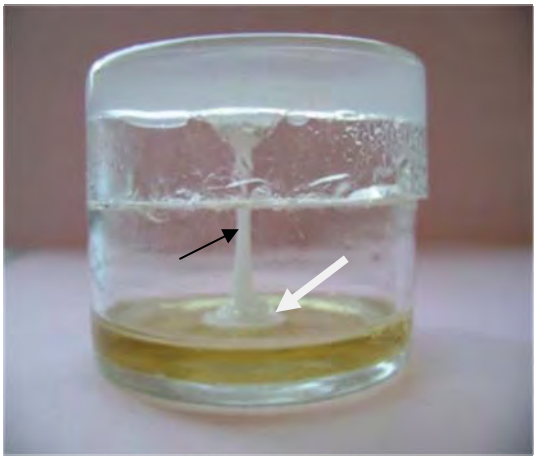


Figure 1a. The white column of the hyphae after 3 days (original photo Cristian Diana).

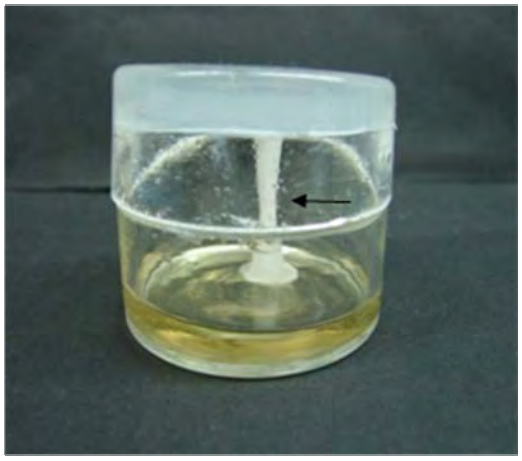


Figure 1b. The white column of the hyphae after 6 days (original photo Cristian Diana).

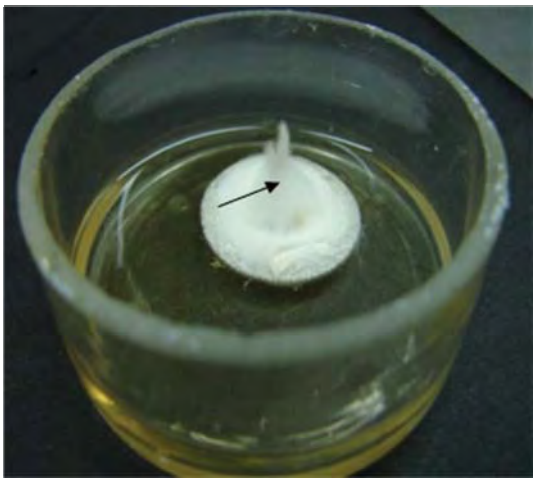


Figure 1c. Circular white formation after the rupture of the column (original photo Cristian Diana).

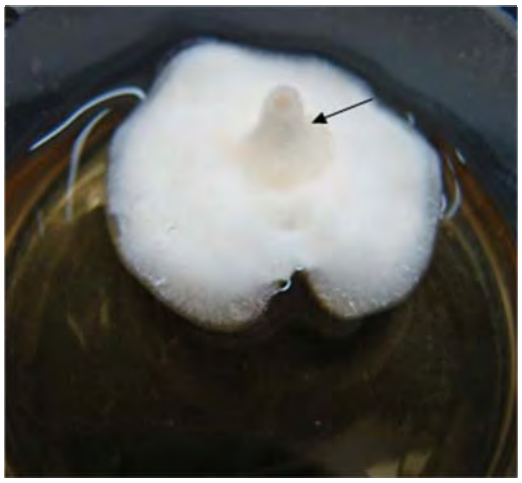


Figure 2. Nine days old formation (original photo Cristian Diana).

Figure 3. Mycobiont with lobe - like formations - three weeks old (original photo Cristian Diana).

Figure 4a. Prick - out cultures (original photo Cristian Diana).

Figure 4b. Subsequent mycobiont cultures in different developmental Stages (original photo Cristian Diana).

Figure 4c. Final stages with formations sclerotiform - like formations to the periphery of the mycelium (original photo Cristian Diana).

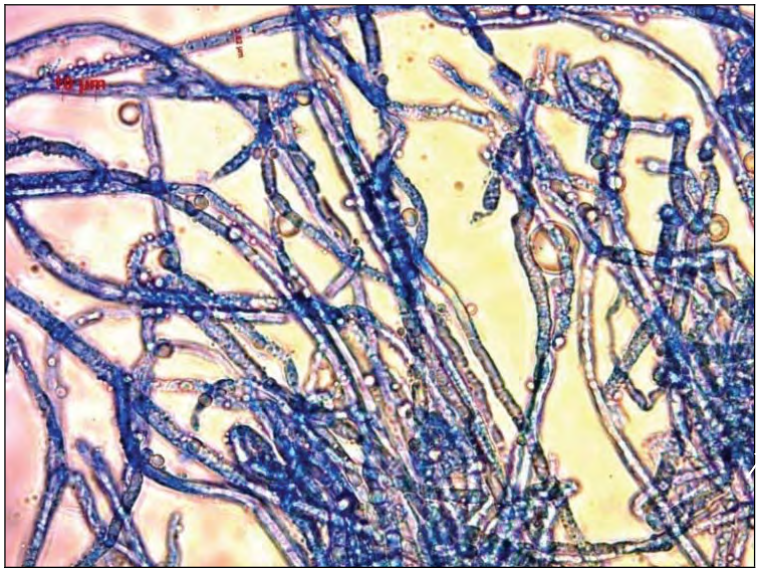


Figure 5. Lactophenol - bleu - cotton coloured mycelium (x40) (original photo Cristian Diana).

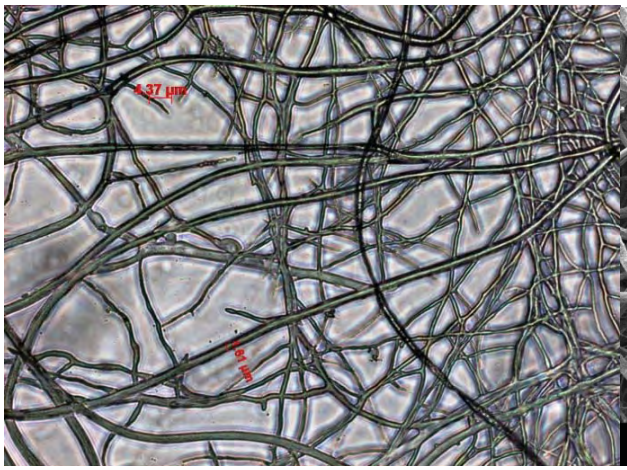


Figure 6. Phase contrast detail of the hyphae network. (x40) (original photo Cristian Diana).

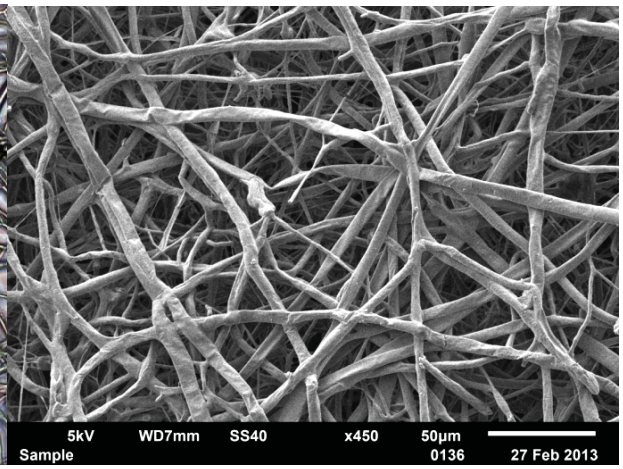
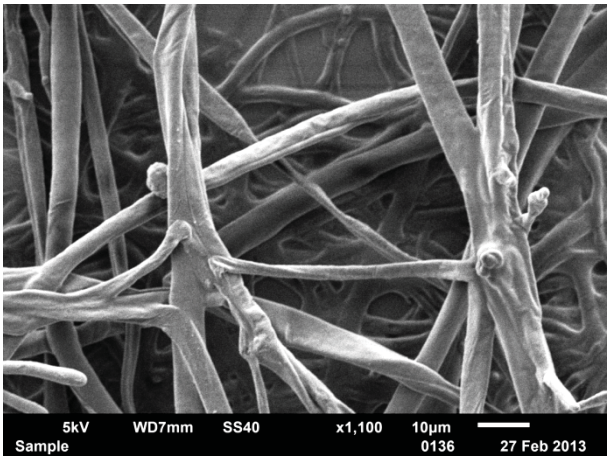


Figure 7. Hyphae ensemble - scanning microscope image. (original photo Alexandru Brânzan).



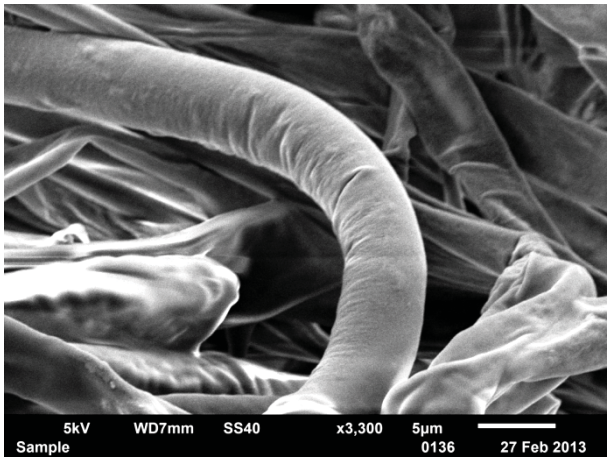


Figure 8 a, b. Details of the hyphae (original photos Alexandru Brânzan).

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Cristian Diana
Romanian Academy
Institute of Biology Bucharest
Splaiul Independentei 296,
060031, Romania.
E-mail: dimarciflori@yahoo.com

Brezeanu Aurelia
Romanian Academy
Institute of Biology Bucharest
Splaiul Independentei 296,
060031, Romania.
E-mail: aurelia.brezeanu@ibiol.ro

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