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Morphological and growth characteristics of *Clathrus ruber* P. Micheli ex Pers. vegetative mycelium *in vitro* condition

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Abstract

Clathrus ruber is a saprotroph species with a large distribution area in some Central European countries. The fungus is assessed for the Red List of the International Union for Conservation of Nature, this species is also included in the Red Data Book of Ukraine. The features of *C. ruber* mycelium growth in pure culture were studied. The modified Melin-Norcrans nutrient agar was the most optimal medium for fungal growth *in vitro*. The *C. ruber* colony forms a large number of mycelial strands and aerial hyphae. Investigation of hyphae microstructure showed the presence of anastomosis, clamp connections and intercalary cells with ribbed inclusions. Specific morphology of the last-mentioned cells is the distinguishing feature of this species among other fungi.

Keywords – fungal conservation – hyphae – mycelial growth – pure culture

Introduction

Clathrus ruber P. Micheli ex Pers belongs to the family *Phallaceae* Basidiomycota division, also known as a stinkhorn fungus (Yakar et al. 2019). The mushroom was named after the shape of a fruiting body that resembles a round or oval hollow sphere with intertwined or lattice branches. The French authors describe it as "fenetretreillisee" (window with bars). English and American mycologists call it lattice – stinkhorn (Stijve 1997). The first image of this mushroom appeared in the large work about fungal biodiversity of Austria/Hungary "Fungus in Pannoni is observatorum brevis historia (1601)" of the 16th -century mycologist Charles de l'Ecclus (Stijve 1997). However, the Italian scientist Per Antonio Micheli made the first description of this fungus in 1729 (Stijve 1997).

Since the mushroom was first described by an Italian researcher, the habitat distribution is considered southern and central parts of Europe. The occurrence of *C. ruber* in Ukraine was reported from south Ukraine – Crimean steppe and southern coast (Didukh 2009, Dudka 2015), as well as from western part – Ivano-Frankivsk region (Dudka 2015, Helyuta & Zykova 2019). It can also be found in Australia (Cunningham 1931), North and South America (Toledo 1995, Burk 1979), and China's southern provinces (Zhishu et al. 1993).

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The species has a disjunctive distribution area and is considered invasive in most countries of the world. It is a saprotrophic species that grows on wood mulch, leaf litter, and grassy lawns of gardens and parks (Didukh 2009). Although the species has a wide distribution in some Central European countries, it is assessed for the Red List of the International Union for Conservation of Nature (IUCN Red List of Threatened Species). According to the lists of the European Council for the Conservation of Fungi, *C. ruber* has Vulnerable (VU) or Near Threatened (NT) IUCN status in countries such as Albania, Bulgaria (Uzunov et al. 2014), Romania (Tănase & Pop 2005) and Slovakia (Lizon 2001). This species is also included in the Red Data Books of Ukraine (Didukh 2009) and the Russian Federation (Agafonov 2011).

However, *C. ruber* has a very low presence in pure culture collections. According to the WFCC database, only three strains are known, two of which were received in Germany (GFCC 6894, 6895) and one from France (MUCL 51920) (http://gcm.wfcc.info/strains.jsp?strain_number=&strain). There is no information about conservation and growth characteristics of this species *in vitro*.

Therefore, the purpose of our work was to determine the optimal storage conditions and growth characteristics of this species *in vitro*.

Materials & Methods

Mycological material was collected by M.M. Sukhomlyn in the Montpellier Zoo territory of FRANCE: 43°38′22.95″N, 3°52′25.79″W, 92 m.a.s.l. (29 Dec. 2018). The matured fruit bodies were growing on the ground with the litter of deciduous trees and looked like a large red sphere with a mesh receptacle (Fig. 1).

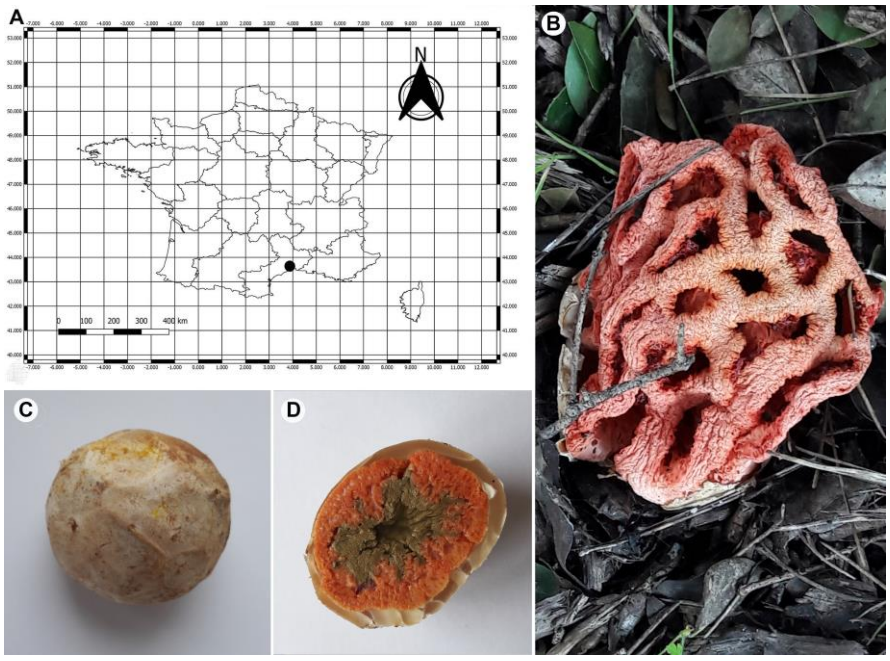

Fig. 1 – Location and general appearance of the fruiting body of *Clathrus ruber*. A Point of collection in France. B General view of a mature fruiting body. C Young fruiting body at the egg stage. D "Egg" in section.

The young fungus samples of *C. ruber* were kindly transferred to the Mycology Laboratory of the Department of Plant Biology at the Educational and Scientific Centre "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, to determine the growth characteristics of this species *in vitro*.

The culture was segregated from the egg stage by the Bukhalo method (Bukhalo 1988). Morphological observation of mycelial colonies and growth rate (GR) calculation were performed on three different media:

potato-glucose agar medium (PGA) pH 6, included glucose (10 g), potato (200 g), agar (10 g), water 1 liter;

malt extract agar medium (MEA) pH 6, included malt extract (80 g), agar (10 g), water 1 liter;

modified Melin-Norcrans agar (MMN) medium pH 5, included malt extract (3 g), glucose (10 g), NH_4NO_3 (0.3 g), KH_2PO_4 (0.5 g), K_2HPO_4 (0.33 g), $MgSO_4$ (0.15 g), 1% $CaCl_2$ (5 ml), 1% $FeCl_3$ (1.2 ml), 1% NaCl (2.5 ml), Thiamine Chloride (0.1 mg), agar (15 g), distilled water 1 liter.

Media were sterilized by autoclaving at 1.1 atm for 15 min and after cooling inoculated with 5 mm^3 inocula into the center of 90 mm Petri dishes (20 mL medium in each) and incubated in the dark at 24±1°C for 36 days.

Mycelial GR was measured every two days and calculated according to the formula:

$$GR = \Delta d / \Delta t,$$

where Δd is the difference between diameters of colonies (mm) during Δt time (days). The average growth rate (GRavr) was calculated from GR data obtained during 36 days of growth (Badalyan et al. 2019). Statistical analysis and data visualization were performed in OriginPro 2017.

The colonies were described using Bukhalo method (Bukhalo 1988, Bukhalo et al. 2009). Vegetative mycelial structures were examined using a light PrimoStar microscope Zeiss and a Scanning Electron Microscope (SEM) Jeol JSM-6060 LA (Japan). Samples of vegetative mycelium for SEM were prepared by a modified method of Quattlebaum & Carner (1980). During the inoculation of the mycelium, several sterile pieces of 4 x 4-mm covering glasses were placed at different distances from the inoculum on Petri dishes with MEA medium. When the pieces of the covering glasses were completely overgrown with mycelium, they were cut from the surface of the nutrient agar medium and transferred to microscope slides. The microscope slides were then placed into sealed glass vessel for fix the mycelium with osmium tetroxide vapor (1% solution) for 6 hours. After fixation, the slides with mycelium samples transfer to an empty Petri dish to dry for 72 h. Then dry samples were covered with gold a vacuum spray gun JII-4X with rotation and were observed using SEM JSM-35C and Jeol JSM-6060 LA (Japan) by the Bukhalo method (Bukhalo & Didukh 2005). The images graphs of mycelial structures from the light microscope were taken with a Science Lab DCM 520 digital camera and Axiovision 4.3.7 image processing software.

Results

C. ruber is a slow-growing fungus. The average growth rate of the mycelium on MMN medium was 1.2 mm / day, on PGA medium – 0.7 mm / day and on MEA medium – 1 mm / day (Fig. 2).

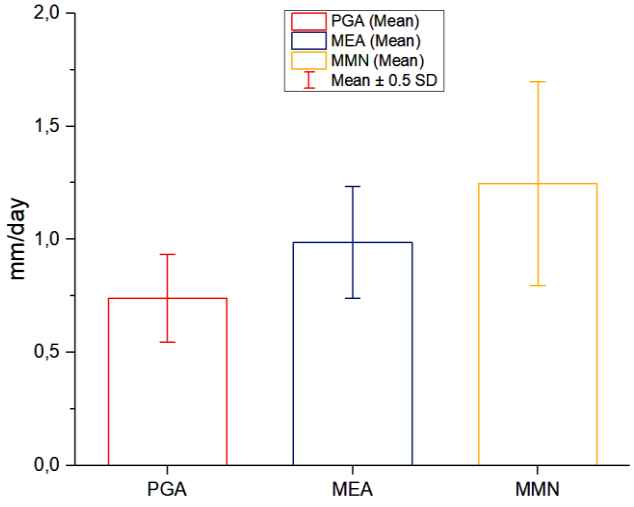

Fig. 2 – Mycelium speed growing on different medium

According to these data, modified Melin-Norcrans nutrient agar was the more suitable medium for culture growth *in vitro*. Including the information of the Global Catalogue of Microorganisms (http://gcm.wdcm.org/), it is recommended to grow *C. ruber* on a modified MMN medium. When we used this medium in our experiments, we managed to get the formation of a mycelial colony much faster than on other mediums.

C. ruber forms white colonies with a large number of unevenly spaced branching mycelial cords and well-developed aerial hyphae on the MEA medium. Also, colonies have silky concentric zones and unevenly growth. The edges are sprawling and overtop the substrate. The centre of the colony tinged with yellow in the course of time. The colony reverse is cream coloured with clearly visible mycelial branching cords and intense orange-red colour in the centre (Fig. 3A, B).

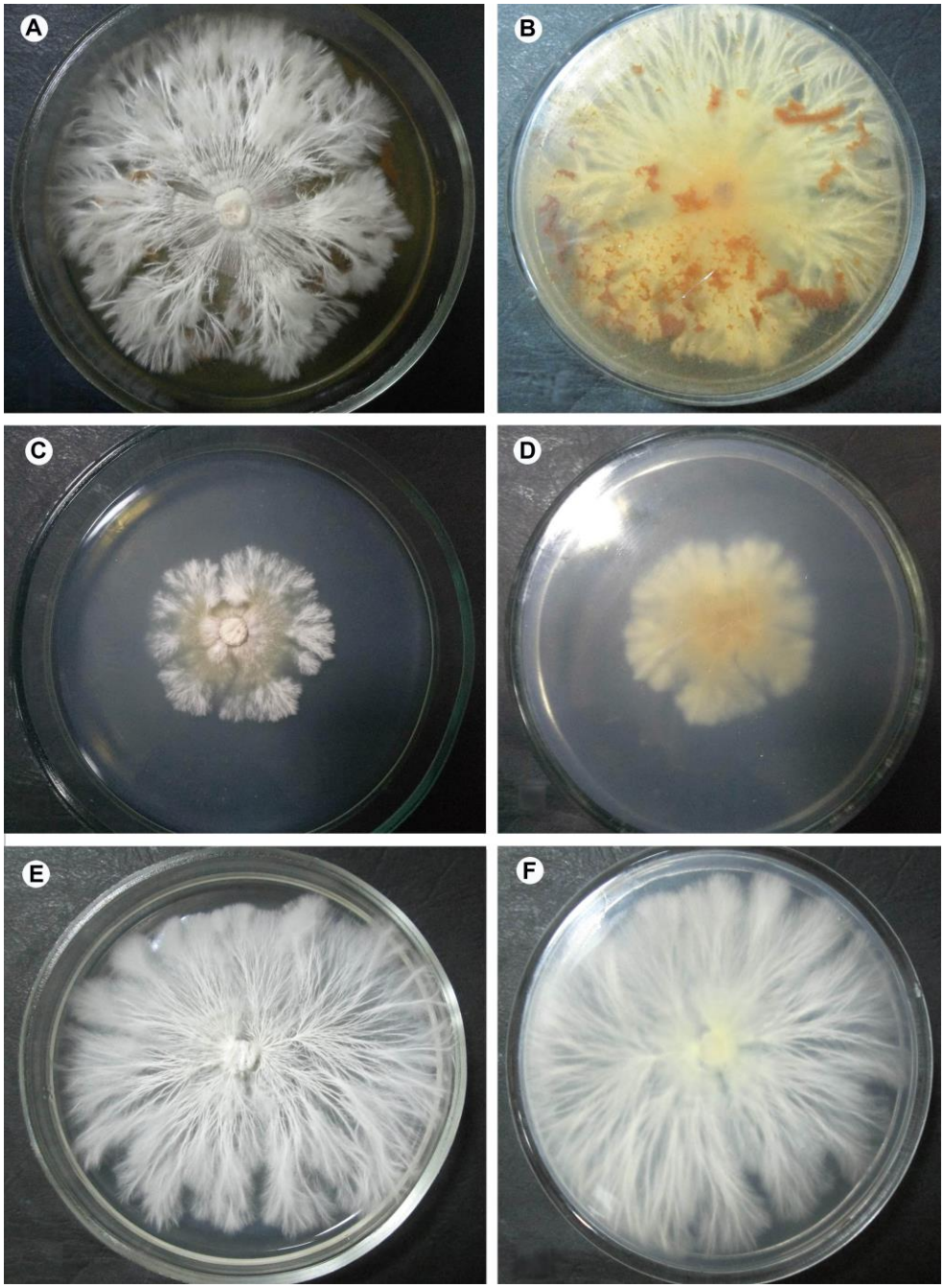

Fig. 3 – General view of colonies and reverse of *Clathrus ruber* on different nutrient media for 36 days of incubation. A Colony on MEA medium. B A reverse colony on MEA medium. C A colony on PGA medium. D A reverse colony on PGA medium. E A colony on MMN medium. F A reverse colony on MMN medium.

The colony growing on PGA medium is white with a yellow shade in the center, has distinct mycelium cords and a small number of aerial hyphae. Concentric zones of the colony are barely visible: only the inner zone and the outer zone are clearly distinguished. Mycelium growth is uneven. The colony reverse is yellow with a brown tint in the centre (Fig. 3C, D).

The colony growing on the MMN medium is white with well-developed branched mycelium cords and aerial hyphae. Concentric zones of the colony are indistinct. Mycelium growth is patchy. The reverse of the colony is white, with a yellow tinge in the center and noticeable mycelium cords (Fig. 3E, F).

The light microscopy showed thin-walled, branched, septate mycelial hyphae with 2.3 – 3.8 μm diameter (Fig. 4A). The hyphae form anastomosis (Fig. 4B) and a single clamp connection (Fig. 4C). The colony also has crystal incrustation located on the surfaces of hyphae (Fig. 4D). The quantities of such hyphae increase during the time.

A large number of hyphae comprises intercalary cells. Inside of these cells, we found dense, rounded inclusions with ribbed surface (Fig. 5A, B, C).

The size and shape of these structures and intercalary cells changes with time. At 14th day of incubation**,** the cells have an elongated shape**,** which becomes rounder and range from 12.42 x 22.85 to 21.55 x 23.16 μm in size. The inclusions that located inside of these cells have a circular shape and diameter from 8.05 to 14.95 μm. In addition, the number of these inclusions and intercalary cells increases with time. However, the intercalary globular cells without these inclusions are observed in colonies incubated over two months (Fig. 5D). The presence of such cells may indicate a decrease in the amount of substance that compound these inclusions.

Scanning electron microscopy made it possible to detect the formation of pellicle spots on vegetative mycelium (Fig. 6A). Sometimes they reach sizes 38,89 x 72,22 μm. In addition, it is confirmed that the rounded inclusions are located directly inside of the mycelial hyphae (Fig. 6B).

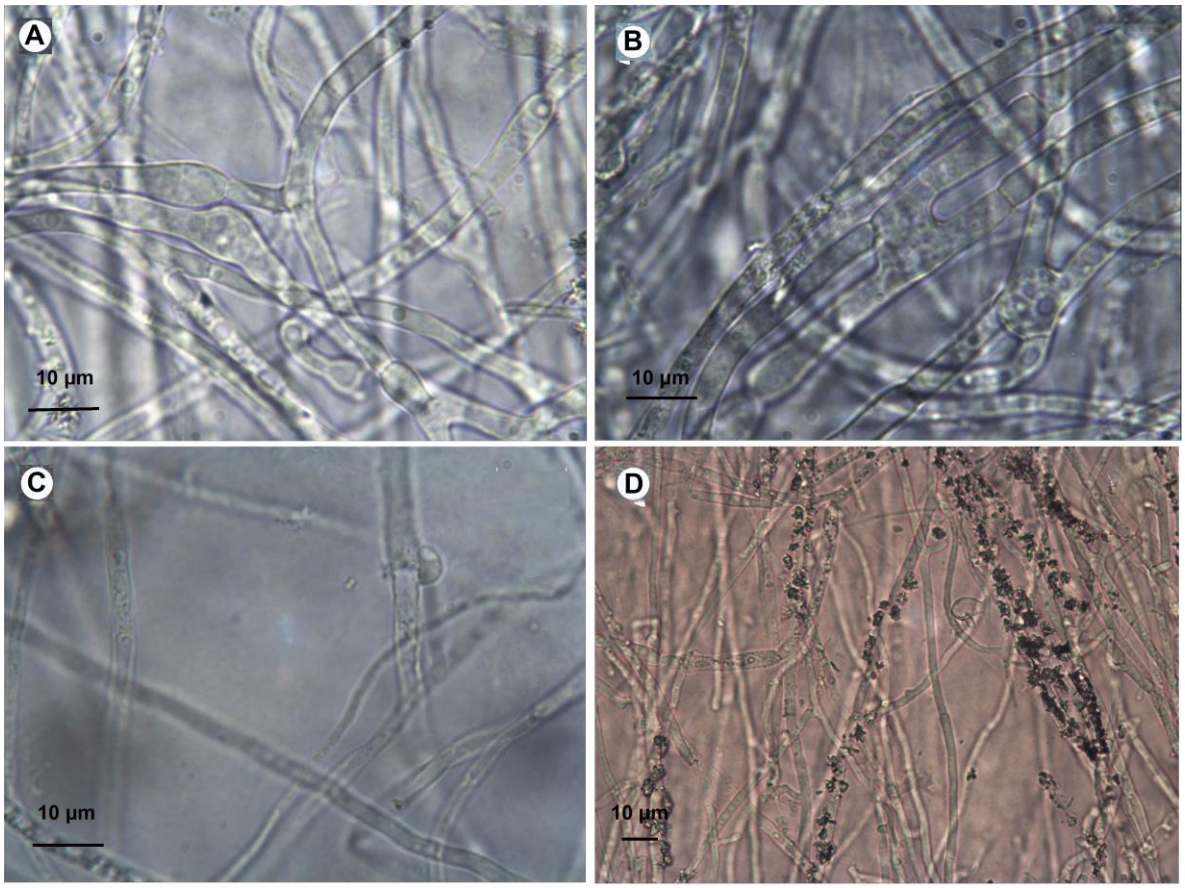

Fig. 4 – Hyphae microstructures of mycelium *Clathrus ruber*. A Vegetative hyphae (100x). B Anastomoses (100x). C Clamp connection (100x). D Incrustation hyphae by crystals (40x).

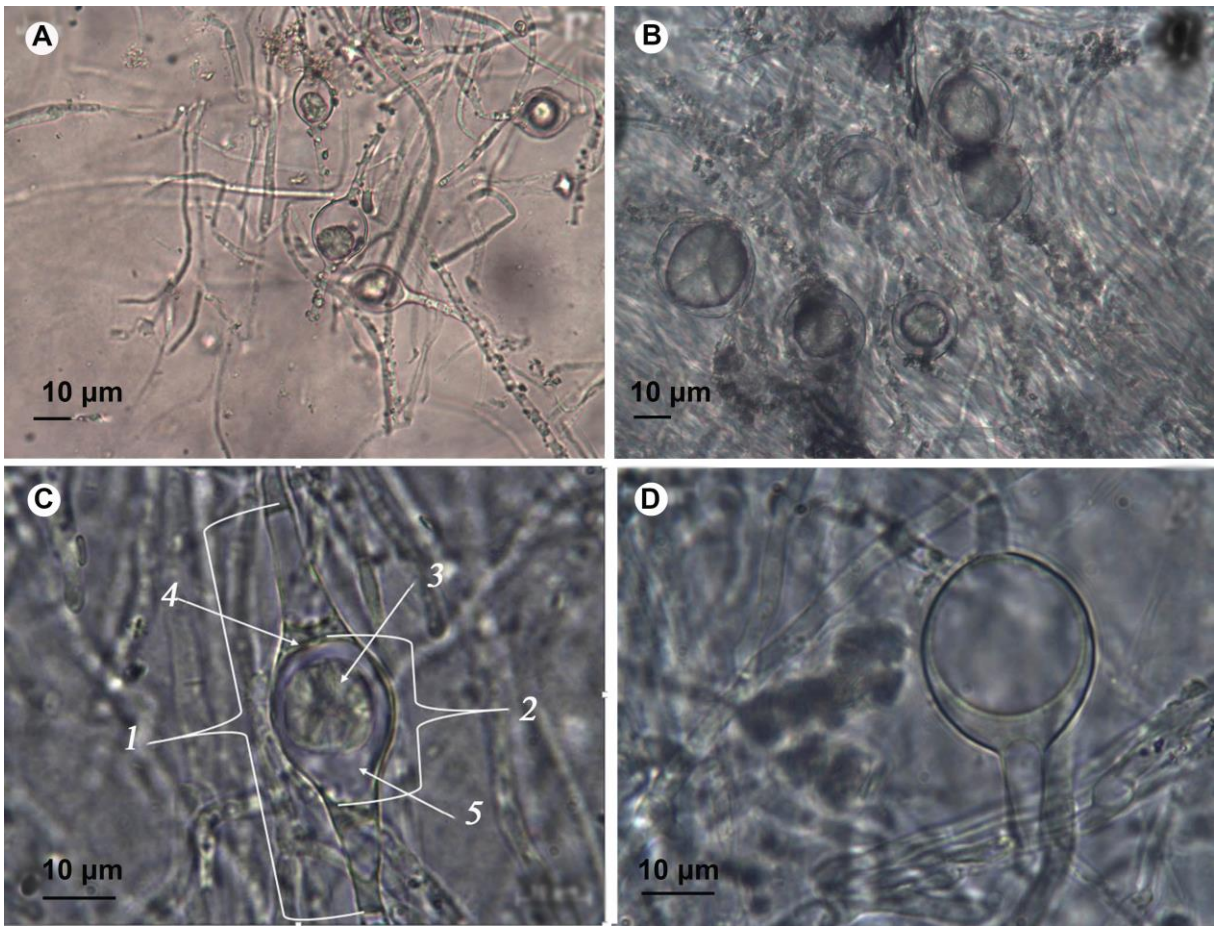

Fig. 5 – Intercalary cells with crystalline inclusions on hyphae of mycelium *Clathrus ruber*. A Intercalary cells of young mycelium (40x). B Intercalary mycelial cells of culture at the age of 36 days (40x). C The image of the intercalary cell with crystalline inclusions (100x): 1 – hyphae cell, 2 – intercalary cell, 3 – crystal inclusion, 4 – cell wall of intercalary cell, 5 – cytoplasm of intercalary cell. D Mycelium intercalary cells without crystalline inclusions in the colony of more than 36 days old (100x).

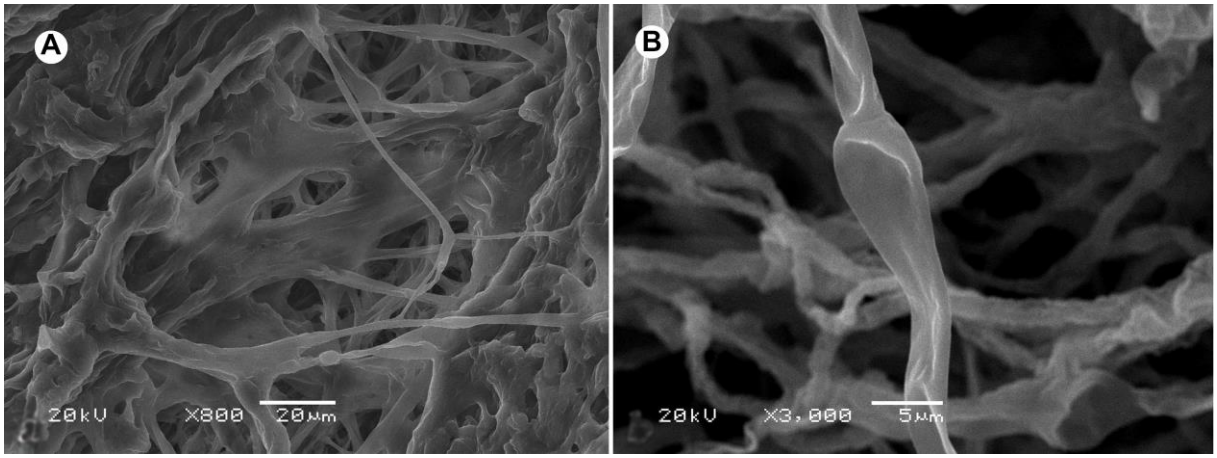

Fig. 6 – SEM mycelium *Clathrus ruber*. A Pellicle spots on vegetative mycelium. B Young intercalary cells.

Discussion

In this work, we first described the characteristic features of *C. ruber* mycelium in pure culture. The study of hyphae microstructure in this fungus showed the presence of anastomosis and

clamp connections. Also, we found intercalary cells with ribbed inclusions which is characteristic for other species of the family *Phallaceae* (Bukhalo 1988).

The more suitable medium for growth of this species was MMN nutrient agar at pH 5. The *C. ruber* colony presented a large number of mycelial strands and aerial hyphae. Also, one of the distinguishing features of this species was the presence of the intercalary cells with ribbed inclusions. These structures were first found in *Phallus impudicus* L. and *Clathrus archeri* (Berk.) Dring where have been noted as intercalary crystalline inclusions (Bukhalo 1988, Bukhalo et al. 2009).

The nature of their origin and function has not yet been established. It is suggested that inclusions are crystals of calcium carbonate ($CaCO_3$), because its structure looks like crystals of calcium carbonate in urine (https://laboratoryinfo.com/types-of-crystals-in-urine). It is known that calcium ions take a place in stabilization of the gelatinous layer which protects the embryonal carpophore during the egg's growth (Stijve 1997). Perhaps fungus stored calcium ions as a crystal inside the hyphal cells.

On the other side, Ca^{2+} is important for the apical growth of fungal hyphae. The calcium concentration within metabolically active fungal cells is under strict control: Ca^{2+} must be concentrate at the apex and instantly decreased in subapical regions (Bindschedler et al. 2016). Average concentrations of free cytoplasmic Ca^{2+} range from 100 to 350 nM, whereas at the tip, concentrations up to 2600 nM are observed. In order to keep such calcium ions, graduation needs effective regulation. Ca^{2+} can enter the cytoplasm both actively and passively, so Ca^{2+} concentration in the cytoplasm is maintained at low levels by actively pumping it either out the cell, or by sequestration in organelles, or by binding it onto cytoplasmic proteins and within the cell wall. Actually, exorbitant calcium concentration is a trigger for $CaCO_3$ formation and mineralization (Bindschedler et al. 2016). Taking note of this statement, we suppose that function of intercalary cells of *Phallaceae* fungi is an accumulation of Ca^{2+} in the crystal forms. These formations can provide intracellular protection against excess Ca^{2+} and as it an additional source.

The results of this work are for the first time show the image of hyphae intercalary cells for *C. ruber* and provide additional information on the nature of growth and development of *C. ruber* mycelium *in vitro*. This characteristic makes it possible to distinguish this culture from other cultures of gasteroid fungi.

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