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Original Article

Morphological and Ultrastructural Characteristics of *Dumontinia tuberosa* (Bull.) L.M. Kohn from *Ranunculus ficaria* L.

Cristina MIRCEA[1], Marcel PÂRVU[1]*, Septimiu-Cassian TRIPON[2], Oana ROȘCA-CASIAN[3]

[1]*Babeș-Bolyai University, Faculty of Biology and Geology, 42 Republicii Street, 400015 Cluj-Napoca, Romania;* mircea.i.cristina@gmail.com*;* marcel.parvu.biolog@gmail.com *(*corresponding author)*

[2]*Babeș-Bolyai University, Faculty of Biology and Geology, Electron Microscopy Center, 3-5 Clinicilor Street, 400004 Cluj-Napoca, Romania;* tripon_septimiu@yahoo.com

[3]*Babeș-Bolyai University, Alexandru-Borza Botanical Garden, 42 Republicii Street, 400015 Cluj-Napoca, Romania;* casioana@yahoo.com

Abstract

Dumontinia tuberosa fungus was observed in different stages of development growing on the roots of *Ranunculus ficaria* in Cluj-Napoca (Romania), over the course of several years. It is not frequently encountered, therefore, we decided that will be interesting to cultivate it on nutritive medium, and to use the morphological data and the colony's characteristics to identify the fungus. The identity of the fungus was reaffirmed through a molecular analysis of sclerotium DNA sequences. A new favourable nutritive medium for the growth and development of the fungus is proposed. The morphology and ultrastructure of the fungus were examined by light and electron microscopy, and micrographs of the relevant details are provided. Differences between the ultrastructural characteristics of sclerotium, apothecium stipe, ascospores, asci and paraphyses are presented and discussed. Intercellular spaces were identified in sclerotium and apothecium stipe and the paraphyses. Asci and ascospores were found to be covered by a sticky mucilage. The ultrastructural characteristics of the aforementioned tissues are presented for different developmental stages. Additionally, the main food stores of the fungus, represented by glycogen, lipids and proteins, are located and described along with other characteristic compounds, such as melanin pigment and a glucan extracellular matrix. The present study complements prior information on the worldwide distribution of this rare species in nature and contributes new findings regarding its morphological and ultrastructural characteristics.

Keywords: asci, ascospores, DNA sequences, microscopy, nutritive medium, paraphyses

Introduction

The sclerotium-forming fungi are ecologically diverse and phylogenetically dispersed among 85 genera in 20 orders of Basidiomycota and Ascomycota. Many unrelated fungi with diverse trophic modes may form sclerotia, but these structures have been overlooked (Smith *et al*., 2015). Sclerotia are highly variable in their morphology and help fungi survive challenging conditions, such as freezing, desiccation, microbial attack or the absence of a host (Smith *et al*., 2015). Sclerotial development is a complex, multistage process that is accompanied by both morphological and biochemical changes. The developmental stages are affected by numerous factors, including photoperiod, temperature, oxygen concentration, mechanical factors and nutrients (Chet and Henis, 1975). The sclerotium-forming fungi have an extremely wide trophic diversity, with some species being plant pathogens and others being ectomycorrhizal or saprotrophic (Smith *et al*., 2015). Because of this, they contain a veritable treasure trove of interesting secondary metabolites.

Dumontinia tuberosa (Bull.) L.M. Kohn is a sclerotium-forming fungus, and it is the only species of the genus *Dumontinia* from the Sclerotiniaceae family (Kohn, 1979; Kirk *et al*., 2008). This species was described in 1979 for the first time (Kohn, 1979). *D. tuberosa* sclerotia are born superficially on plant rhizomes and are irregularly shaped to globose, with a black outer rind and white inner cortex. Apothecia are black, stipitate and cupulate and consist of a stipe originating from a sclerotium and a receptacle with asci and paraphyses. The asci are cylindrical and contain eight ascospores, the paraphyses are hyaline and filiform and the microconidial state presents globose and hyaline microconidia, produced superficially on the hymenium surface or culture medium (Kohn, 1979).

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The fungus *D. tuberosa* is pathogenic on rhizomes of *Anemone* (Kohn, 1979; Kim *et al*., 2009), *Hepatica nobilis* Schreb. var. *japonica* Nakai f. *magna* (M. Hiroe) Kitam. (Uzuhashi *et al*., 2010) and occasionally on *Ranunculus* plants (Phillips, 2006). This species is rare (Phillips, 2006) and is associated with black rot disease (Pepin, 1980; Uzuhashi *et al*., 2010).

The aims of this study were to uncover new information regarding the morphological and ultrastructural characteristics of the species *Dumontinia tuberosa* (Bull.) L.M. Kohn from *R. ficaria*; to test a new nutritive medium favourable for *in vitro* development of the fungus and suitable for its thorough examination; and last, but not least, to complete the information regarding its geographic distribution.

Materials and Methods

Fungal samples

The fungus *Dumontinia tuberosa* (Bull.) L.M. Kohn was found on roots of *Ranunculus ficaria* L. plants for the first time in the spring of 2004, in the Mikó Garden of Cluj-Napoca (46°45′ 53.5″ N; 23°34′ 51.2″ E), Romania. Every year from 2004-2016, between the end of March and April, for three weeks, we observed different stages of growth and development for the fungus in nature at the same location. This fungus was identified by Dr. M. Pârvu and a voucher specimen (CL 656075) was deposited at the Herbarium of Babeș-Bolyai University of Cluj-Napoca. *D. tuberosa* colonies were obtained after inoculating the centre of Petri dishes (70 mm in diameter) containing Czapek-agar (Atlas, 2010) and 2% soluble starch Sigma-Aldrich with ascospores isolated from young apothecium (10 mm in diameter) and incubating at 22 °C for 20 days. Mycelia and sclerotia characteristics were examined in colonies at 10 and 20 days post inoculation, respectively.

Microscopic studies

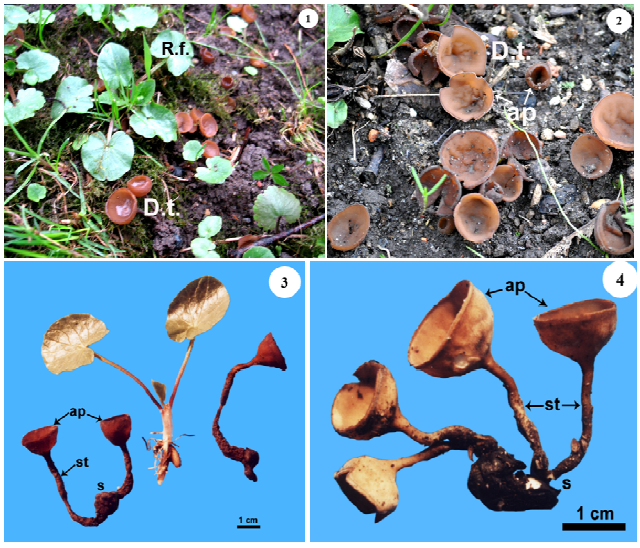
D. tuberosa sclerotium, ascospores, paraphyses, asci and stipe situated 1 cm below the apothecium were examined using light microscopy (LM) and electron microscopy (EM). The samples were fixed in 2.7% glutaraldehyde (in phosphate-buffered saline for 90 min). For scanning electron microscopy (SEM), the samples were critical-point dried in liquid CO_2 mounted on sticky carbon tabs and sputter-coated with gold (10 nm). For transmission electron microscopy (TEM), the fixed and dried samples were infiltrated with resin, deposited onto colloidal-carbon-coated copper grids and negatively stained with lead citrate and uranyl acetate. The grids were examined by SEM with a JEOL JSM 5510 LV electron microscope (Vánky, 1994) and by TEM with a JEOL JEM 1010 electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) (Hayat, 2000). For electron microscopy samples, the chemicals used were glutaraldehyde, resin (Epon 812), lead citrate, uranyl acetate, bismuth subnitrate (Electron Microscopy Sciences, Fort Washington, USA); sticky carbon tabs, colloidal carbon coated grids (Agar Scientific, Cambridge, England).

Molecular analysis

Mature sclerotium were used for *D. tuberosa* DNA analysis. DNA was extracted using the Animal and Fungi DNA Preparation Kit (Jena Bioscience) according to the manufacturers' instructions. The extracted DNA was quantified using the Nano Drop ND 1000 Spectrophotometer (NanoDrop Products) and had a concentration of 228.8 ng/µL. The universal barcode for fungi, ITS (Internal Transcribed Spacer), was used for the molecular analysis. The primers for polymerase chain reaction were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') targeting the ITS1 region and ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') targeting the ITS2 region (White *et al*., 1990). The 18S rRNA gene (SSU- Ribosomal small subunit) was also used for the molecular identification. The primers used for amplification were FR1 (5'-AICCATTCAATCGGTAIT-3') and FF390 (5'-CGATAACGAACGAGACCT-3') (Prévost-Bouré *et al*., 2011). For all PCR amplifications the following mixture was used in a 25 µL final volume: 5 µL of 5X MangoTaq Colored Reaction Buffer (Bioline), 1.25 µL of 50 mM $MgCl_2$ (Bioline), 0.5 µL of 10 mM dNTP (Bioline), 1.25 µL of each primer (Macrogen Sequencing Service, Korea), 0.25 µL of 5 U/µL MangoTaq (Bioline) and 2 µL of DNA. Negative control samples were used for each pair of primers. The amplification series consisted of 35 cycles of the following: 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s.

Results

The *D. tuberosa* fungus forms tuberoid sclerotia, of 0.5-1.5(-2) × 0.5-0.8 cm, that develop on the roots of *Ranunculus ficaria* and do not incorporate into the host tissues (Figs. 1, 2, 3, 4). These sclerotia are irregularly shaped to globose, have a black outer rind, have a white inner cortex and develop 1-4 apothecia. In nature, the young apothecia of the fungus occurring at the soil surface are 3-4 mm in diameter, and after two-three weeks of development, these apothecia reach maturity and have a diameter of up to 20-25 mm. The mature apothecium has a black-brown stipe of up to 8-10 cm in length and 1-2 mm in



Figs. 1-4. Macroscopic images of *Dumontinia tuberosa* and *Ranunculus ficaria* in nature. 1. The fungus and host plant leaves. 2. Fungus apothecia. 3. Sclerotia with one and two apothecia and the host plant. 4. Sclerotium with four apothecia. Legend: ap. apothecium; *Dumontinia tuberosa* (D.t.), *Ranunculus ficaria* (R.f.), s. sclerotium; st. stipe

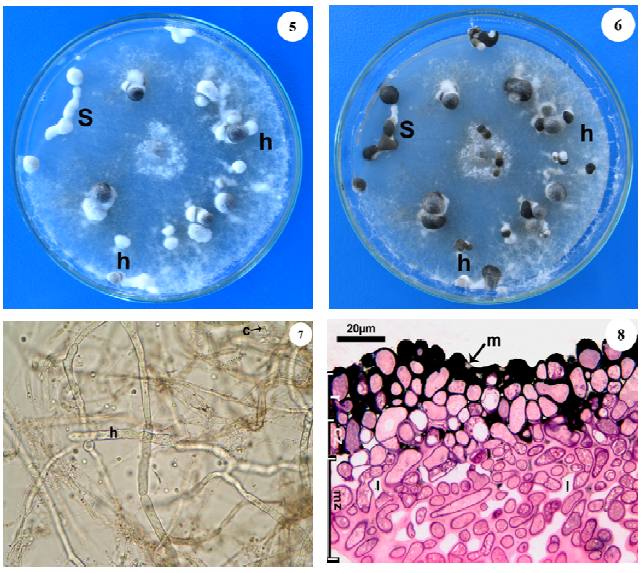
diameter (Figs. 3 and 4).

On Czapek-agar with starch, *D. tuberosa* colonies formed: whitish to greyish mycelium with branched and multicellular hyphae (Figs. 5 and 6); globose, hyaline and unicellular microconidia, 2-3 µm in diameter (Fig. 7); and 30-40 black or grey sclerotia of approximately 0.5-1.0 cm × 0.3-0.5 cm (Fig. 6). The black sclerotia obtained on nutritive medium were examined in different sections after histochemical staining with epoxy tissue stain (E.T.S.). All of the cells of the sclerotium have thicker walls than those of vegetative hyphae, and cross sections showed three sclerotial layers of different thicknesses: rind, cortex and medulla (Fig. 8).

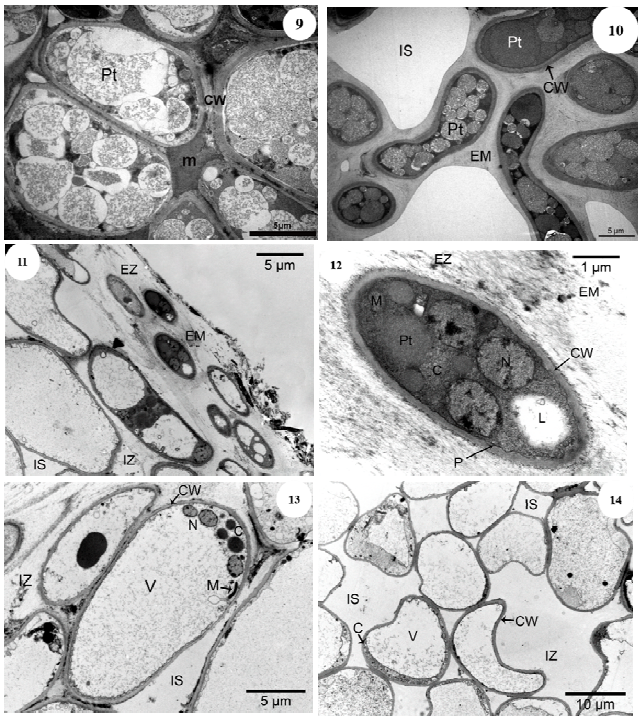
Rind cells have two to four layers, are oval shaped, have dark brown melanised walls and form a dense 4-5 µm thick crust (Figs. 8 and 9). Moreover, between these cells there is an extracellular matrix of melanin pigment. Small lacunae can be observed between cortical cells, which are two to four layers, oval shaped, coated with an intercellular matrix and formed by medullary cells (Fig. 8). The medulla is formed by numerous cells of different shape, targeted in different directions, an extracellular glucan matrix and large lacunae (Fig. 10). Cells from the medullary zone have storage vacuoles with heterogeneous content and different electron density. Some of these vacuoles seem to contain proteins and are more electron dense than the protein containing large vacuoles from the rind and cortex (Figs. 9 and 10).

The external zone of the mature apothecium stipe contains several cells stacked in the intercellular matrix while the inner zone is formed from cells with different shapes and sizes and intercellular spaces (Figs. 11, 12, 13, 14). The cells of the external zone have intense metabolic activity, divide and generate cells of the inner zone which are bigger and have large storage vacuoles and very little cytoplasm (Figs. 13 and 14). The inner zone cells have different shapes, are more numerous than those from the external zone and are in various stages of development. These cells have low metabolic activity, vacuoles of various sizes and less peripheral cytoplasm. They become senescent and surround a gelatinous mass, which is replaced by the channel situated in the centre of the stipe (Figs. 13 and 14). The internal and external zones of the stipe have cells with several nuclei, and the major cytoplasmic storage reserve of these cells is represented by proteins (Fig. 12).

The *D. tuberosa* apothecia are black, stipitate and cupulate (Figs. 3 and 4) and have a receptacle with a hymenial layer with rows of asci and paraphyses (Figs. 15 and 16). *D. tuberosa* asci and ascospores isolated from mature apothecia are found in different stages of development (young, mature and senescent). The asci are cylindrical (120-170 × 8-10 µm), possess a lateral wall with one layer and with low electron density and contain eight unicellular and ellipsoid ascospores situated in the upper half (Figs. 15, 17, 18, 19). Between the asci, in the apothecium there are hyaline, filiform, unbranched and septate paraphyses, of 1.5-2 µm width, that are slightly dilated at the top (Figs. 15, 16, 20). The paraphyses have a one-layer cell wall adherent to the plasmalemma, contain four nuclei and in cross-sections have a circular shape. On the external wall surface, there is a thin layer of melanin pigment and the mature paraphyses are coloured (Figs. 19 and 20). The *D. tuberosa* ascospores have a smooth and elastic cell wall covered by a sticky mucilage containing different nutrients (Figs. 21 and 22). The young ascospores are hyaline, but mature and senescent ascospores



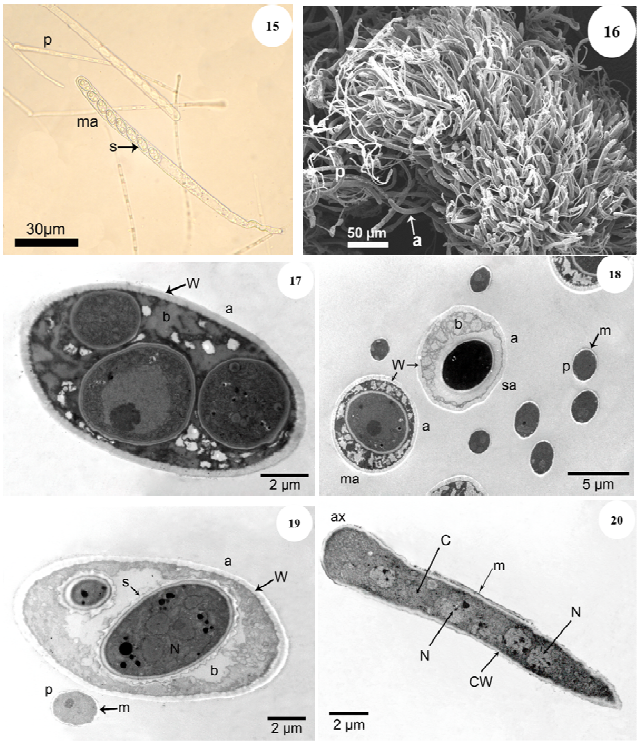
Figs. 5-8. Macroscopic and microscopic characteristics of *D. tuberosa* colonies obtained on Czapek-agar with starch: 1. Colony after 10 days of incubation. 2. The same colony after 20 days of incubation. 3. Branched and multicellular hyphae (h) and microconidium (c) isolated from nutritive medium. 4. Sclerotium in cross section, epoxy staining. Legend: c. microconidium; h. hyphae; l. lacunae; cortical zone (cz); medullary zone (mz); r. rind; s. sclerotium



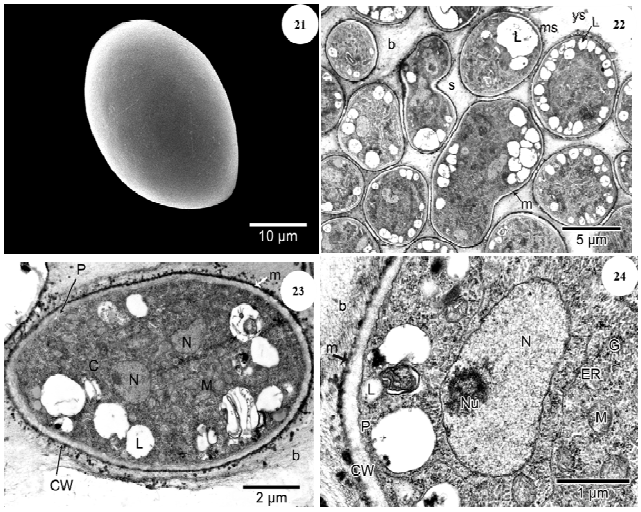
Figs. 9-14. Transmission electron micrographs showing ultrastructural characteristics of *Dumontinia tuberosa* sclerotium (9, 10) and stipe (11, 12, 13, 14). 1. Cells from the sclerotial rind. 2. Cells from the medullary zone of sclerotium. 3. Oblique section of the stipe, with external zone and internal zone. 4. A cell with some nuclei from the external zone of the stipe (detailed). 5. An oblique section of stipe, with the internal zone formed and intercellular spaces. 6. A cross section of the stipe, with internal zone and intercellular spaces. Legend: C. cytoplasm; CW. cell wall; EM. extracellular matrix; EZ. external zone; IS. intercellular space; IZ. internal zone; L. lipids; m. melanin; M. mitochondrion; N. nucleus; P. plasmalemma; Pt. proteins; V. vacuole

become pigmented and possess a thin layer of dark brown melanin pigment on the external wall surface. Each ascospore has 2-4 nuclei, the plasmalemma is attached to the internal face of the cell wall and the cytoplasm contains mitochondria, endoplasmic reticulum, storage reserves, etc. The young ascospore cell wall is 200-250 nm thick and is composed of two layers, while the mature and senescent ascospores has a one-layer cell wall and is covered by melanin pigment (Figs. 19, 22, 23, 24). The young ascospores have intense metabolic activity and contain many mitochondria and small lipids located peripherally in the cytoplasm, while mature ascospores have medium metabolic activity and fewer mitochondria. In the case of the senescent ascospores, the structure of the cytoplasm is very different from that of young and mature ascospores due to the absence of organelles (Figs. 19, 22, 23, 24). The major cytoplasmic storage reserve of *D. tuberosa* ascospores is composed of glycogen and lipid droplets of different sizes, depending on the developmental stage of the ascospores (Figs. 22, 23, 24).

The DNA sequences were used as a query in a BLAST search against the NCBI nucleotide databases (Altschul *et al*., 1997). High scored matches were chosen. For ITS1, the query



Figs. 15-20. *Dumontinia tuberosa* asci and paraphyse in light microscopy (15), scanning (16) and transmission electron microscopy (17, 18, 19, 20): 1. Septate paraphyses and mature asci with eight biguttulate ascospores. 2. Asci with ascospores and paraphyses. 3. Young ascus obliquely sectioned, showing three ascospores covered by sticky mucilage. 4. A cross section through two asci in different stages of development (mature and senescent) and paraphyses. 5. A cross section of a paraphyse and a senescent ascus showing two ascospores covered by sticky mucilage. 6. A longitudinal section of a paraphyse. Legend: a. ascus; ax. apex; b. sticky mucilage; C. cytoplasm; CW. cell wall; m. melanin; ma. mature ascus; N. nucleus; p. paraphyse; s. ascospore; sa. senescent ascus; W. wall



Figs. 21-24. Scanning (21) and transmission electron micrographs (22, 23, 24) showing ultrastructural characteristics of *Dumontinia tuberosa* ascospores: 1. The smooth surface of an ascospore. 2. Ascospores in different sections and covered by sticky mucilage (b). L. lipids; m. melanin; ys. young ascospores with numerous small lipid droplets; ms. mature ascospore with large lipid droplets formed by fusion. 3. Ascospore with small lipid droplets, in oblique section, covered by sticky mucilage. 4. Longitudinal section of a mature ascospore, covered by sticky mucilage. Legend: b. sticky mucilage; C. cytoplasm; CW. cell wall; ER. endoplasmic reticulum; G. glycogen; L. lipids; m. melanin; M. mitochondrion; ms. mature ascospore; N. nucleus; Nu. nucleolus; P. plasmalemma; s. ascospore; ys. young ascospores

sequence was highly similar (98%) with *Dumontinia tuberosa* (GenBank accession number: KJ941073) (Galan-Marquez *et al*., 2015). For ITS2, the sequence was identical with *Dumontinia tuberosa* (GenBank accession number: KJ524536) (Hilszczanska *et al*., 2014). The sequence obtained for SSU matched *Sclerotinia sclerotiorum* (GenBank accession number: L37541, similarity 100%) (Gargas and Taylor, 1995) or *Botrytis cinerea* (GenBank accession number: CP009808, identity 100%) (van Kan and Scalliet, 2015) belonging to the Sclerotiniaceae family.

Discussion

D. tuberosa sclerotia help the fungus survive challenging conditions and are highly variable in their morphology having different sizes and shapes depending on the specimen, the host plant and the environmental conditions. Thus, *D. tuberosa* sclerotia that develop on *Anemone raddeana* are 6-35 × 3-22 mm, on *Hepatica nobilis* Schreb. var. *japonica* Nakai f. *magna* (M. Hiroe) Kitam 8-15 × 10-13 mm (Uzuhashi *et al*., 2010) and on *R. ficaria* 0.5-1.5(-2) × 0.5-0.8 cm. Moreover, *D. tuberosa* that grow naturally on *R. ficaria* have black and tuberoid sclerotia that do not incorporate into host tissues (Kohn, 1979).

D. tuberosa isolates may be cultivated on PDA nutritive medium (Kim *et al*., 2009; Uzuhashi *et al*., 2010; Pârvu and Pârvu, 2014). Our results proved, for the first time, that on

Czapek-agar medium with starch, *D. tuberosa* colonies form numerous sclerotia compared to similar colonies grown on PDA (Kim *et al*., 2009; Uzuhashi *et al*., 2010); branched and multicellular hyphae (Kohn, 1979); and globose and unicellular microconidia, which have an unknown biological function (Bolton *et al*., 2006). The Czapek-agar medium with starch appears to be favourable for the *in vitro* cultivation of *D. tuberosa* because most of the formed sclerotia matured and had a dark, hard surface which accumulated different amounts of melanin (Li and Rollins, 2009).

Our results confirmed that the *in vitro* development of sclerotia is comprised of different stages depending on biotic and environmental factors. *S. sclerotiorum* colonies have three (Erental *et al*., 2007; Xiao *et al*., 2014) to six developmental stages (Li and Rollins, 2009), and the last stage of *in vitro* sclerotial development is maturation (Erental *et al*., 2007; Li and Rollins, 2009; Xiao *et al*., 2014). In the normal sclerotial development of the plant-pathogen *S. sclerotiorum* an important role is played by the protein Ss-Caf1 (Xiao *et al*., 2014) several genes (Erental *et al*., 2007; Jurick and Rollins, 2007; Li *et al*., 2012) and by the production of oxalic acid (Sharma *et al*., 2015).

Many fungi with diverse trophic modes may form sclerotia (Smith *et al*., 2015), but the structural characteristics of sclerotia cannot be used to distinguish these taxa (Kohn and Grenville, 1989). However, structural characteristics of the Sclerotiniaceae fungi proved that there are two types of sclerotia, either possessing or lacking lacunae in the cortex and medulla (Kohn and Grenville, 1989). *D. tuberosa* belongs to the first type because it has lacunae in the cortical and medullary extracellular matrix of its sclerotia, similar to *Sclerotinia sclerotiorum*, *S. trifoliorum* and *S. minor*. The second type is represented by *Botrytis cinerea*, *B. porri* and *Monilinia fructicola* sclerotia, which show a continuous extracellular matrix without lacunae in the cortex and medulla (Kohn and Grenville, 1989).

For demarcating the sclerotial zones (rind, cortex and medulla) of *D. tuberosa* and other Sclerotiniaceae (*Sclerotinia sclerotiorum*, *S. trifoliorum, S. minor, Sclerotium cepivorum, Botrytis cinerea, B. porri, Ciborinia erythronii, Myriosclerotinia dennisii, M. borealis, Monilinia fructicola* and *Stromatinia gladioli*), histochemical staining with toluidine blue O was successfully used (Kohn and Grenville, 1989). Our results proved that epoxy tissue staining may be efficiently used for demarcating the sclerotial zones of *D. tuberosa* (Fig. 8) and that the sclerotial rind contains melanin pigments which are highly resistant to degradation (Sharma *et al*., 2015).

D. tuberosa isolated from *R. ficaria* produces sclerotia which help the fungus survive in challenging conditions (Smith *et al*., 2015). All of the cells of the sclerotia have thicker cell walls than those of vegetative hyphae (Kohn, 1979; Kohn and Grenville, 1989; Webster and Weber, 2007) and contain major intrahyphal storage reserves (Blackhouse and Willetts, 1985).

Our results showed that the *D. tuberosa* sclerotia are comprised of fungal cells rich in β-glucans and proteins similar to *S. sclerotiorum* sclerotia (Sharma *et al*., 2015) and other Sclerotiniaceae fungi (Kohn and Grenville, 1989). The sclerotial proteins are usually the major cytoplasmic storage reserve (Kohn and Grenville, 1989). On the other hand, *D. tuberosa* sclerotia are pseudoparenchymatous aggregations of hyphae embedded in a copious extracellular glucan matrix (Willetts and Bullock, 1992), which may be used as a carbohydrate source during sclerotial germination (Blackhouse and Willetts, 1985).

We proved, for the first time, that there are structural characteristics of the apothecium stipe which are similar to those of *D. tuberosa* sclerotia. Hence, the stipe grown by the sclerotium displayed lacunae as intercellular spaces and a channel and electron-dense proteins, which are the major cytoplasmic storage reserve, also mentioned by other authors (Kohn and Grenville, 1989; Webster and Weber, 2007).

The *D. tuberosa* fungus forms one to several apothecia, which arise from one sclerotium and are usually dark at the base of the stipe (Kohn, 1979; Pepin, 1980). Thus, *D. tuberosa* isolates from *Anemone raddeana* produce apothecia in groups of one to eight (Kim *et al*., 2009), while those formed on *Hepatica nobilis* var. *japonica* f. *magna* differentiate one to four apothecia (Uzuhashi *et al*., 2010). We recognize that the number and size of apothecia formed in nature by sclerotia are different according to the *D. tuberosa* isolate and the developmental stage.

The fungus *D. tuberosa* has cylindrical asci with eight unicellular and ellipsoid ascospores (Kohn, 1979). The asci and ascospores studied in this paper were obtained from mature apothecia collected at different developmental stages. Our results are consistent with other authors' results, which show that a mature apothecium of *S. sclerotiorum* contains asci in different developmental stages: immature, mature and empty (Wu *et al*., 2007).

The *D. tuberosa* ascus apex is inoperculate and contains a thickened dome of wall material with a central canal. As the ascus explodes, the apical apparatus is everted (Verkely, 1993). The ascus wall material includes chitin, polysaccharides and proteins, but there is no evidence of lipids. The contraction of the elastic ascus wall provides the force for ascospore discharge (Webster and Weber, 2007).

The *D. tuberosa* asci are described in the literature as having an elastic wall with two layers of different thicknesses and the two layers are not separated from each other and the ascus is non-fissitunicate (Verkely, 1993). We proved that the lateral wall of *D. tuberosa* asci is elastic and this is formed only of one layer. These results can be explained by the fact that the ascus wall was shown to have one or more layers, depending on the fungus species and the stage of development within the same species (Hyde *et al*., 1999; Lee *et al*., 2004).

The ascus/ascospore maturation process is complex and might involve different factors such as temperature, light, ventilation, etc. In the case of *S. sclerotiorum,* temperature is an important factor affecting ascus maturation, and the optimum temperature is approximately 21 □. The germination of ascospores is another measure of the maturation process (Wu *et al*., 2007).

In the *D. tuberosa* apothecium there are hyaline and filiform paraphyses, septate, simple or sparsely branched, of 1.5-2 μm width (Kohn, 1979) or unbranched, aseptate and filiform paraphyses (Uzuhashi *et al*., 2010). Our results showed, for the first time, that *D. tuberosa* paraphyses are tetra-nucleate, have a single-layer cell wall and become pigmented because they are covered by a thin layer of melanin (Figs. 19 and 20).

We showed that *D. tuberosa* ascospores are surrounded by a sticky mucilage and we suspect that this contains nutrients that

aid in the growth and development of the ascospores in the ascus and their germination in nature, depending on environmental conditions (Clarkson *et al*., 2003). Our results are consistent with other authors' results showing that the ascospores of some species are covered by a mucilaginous sheath (Clarkson *et al*., 2003; Lee *et al*., 2004; Ferer *et al*., 2011). Among the Sclerotiniaceae fungi, *S. sclerotiorum* ascospores are covered by a sticky mucilage that could be either residual liquid from asci or part of the cell wall (Sharma *et al*., 2015). This mucilage aids in the adhesion of *S. sclerotiorum* ascospores to substrate, and these spores can survive on plant tissue for approximately 2 weeks, depending on the environmental conditions (Clarkson *et al*., 2003). Ascospores can germinate on the surface of healthy tissue but cannot infect the plant without an exogenous nutrient source and a film of water. Therefore, senescent or necrotic tissues generally serve as the nutrient source to initiate ascospore germination, giving rise to mycelial infection of the host plant (Abawi and Grogan, 1979; Lumsden, 1979).

The morphological and ultrastructural characteristics showed that *D. tuberosa* ascospores are smooth (Kohn, 1979; Uzuhashi *et al*., 2010), situated in the upper half of asci (Kohn, 1979), and similar to other fungi, they have a dynamic and complex structure depending on the developmental stage (Bowman and Free, 2006). The fungal ascospores have a cell wall that may be thin or thick, hyaline or coloured, smooth or rough, and it may have a mucilaginous outer layer (Lu *et al*., 1998; Webster and Weber, 2007).

The wall surrounding the protoplast of ascospores is formed from one to several layers, depending on the species (Webster and Weber, 2007). The fungal cell wall is composed of chitin, glucans, mannans and glycoproteins and is a dynamic structure that protects the cell from changes in osmotic pressure and maintains the cell shape (Bowman and Free, 2006).

Our findings showed that the pigmentation of *D. tuberosa* ascospores generally results from a late metabolic process, is independent of the wall-layered structure and the melanin pigment is deposited in a special sublayer of the ascospore wall (Bellemère, 1994). Moreover, melanins are black or brown macromolecules that occur in all kingdoms of life and which protect hyphae and spores from UV light, free radicals and desiccation (Bell and Wheeler, 1986; Butler and Day, 1998).

Our results confirmed that the ascospores from *D. tuberosa* are bi-nucleate to tetra-nucleate, which is consistent with prior descriptions (Kohn, 1979; Uzuhashi *et al*., 2010).

In many fungi, the ascospores are resting structures which survive adverse conditions and they may have extensive food reserves (Webster and Weber, 2007). Different ascomycetes have one or more oil drops in the ascospore cytoplasm (Phillips, 2006). For example, *Helvella corium* ascospores contain one large oil drop; *D. tuberosa*, *Peziza badia* and *Otidea onotica* ascospores have two oil drops; and *Sarcoscypha coccinea* ascospores have several small oil drops (Phillips, 2006). We observed that *D. tuberosa* ascospores become biguttulate at maturity (Fig. 15) and have two oil drops formed by fusion apically located in the cytoplasmic matrix, which is consistent with prior descriptions in the literature (Kohn, 1979; Phillips, 2006; Uzuhashi *et al*., 2010).

The growth and development of *D. tuberosa* is a continuous, dynamic and complex processes accompanied by various morphological, structural, biochemical and physiological changes, occurring during the life cycle (Pârvu and Pârvu, 2014). However, these changes are influenced by environmental conditions and thus are difficult to quantify (Bellemère, 1994). The morphological and structural characteristics are necessary for the identification of species, but the molecular analysis confirmed that the fungal samples isolated from *R. ficaria* roots are *D. tuberosa*. The DNA sequences obtained were submitted to NCBI and assigned the following GenBank accession numbers: KU291978 (for the ITS1 fragment), KU291979 (for the ITS2 fragment) and KU291980 (for the SSU fragment) (Benson *et al*., 2012). Because the primers FR1 and FF390 were designed for quantifying fungal communities, they target a conserved region from the 18S rRNA gene in contrast to primers designed for the ITS regions (Bellemain *et al*., 2010). The conserved nature of the SSU target region limits identification to only the Sclerotiniaceae family level.

The fungus *D. tuberosa* occurs commonly on *Ranunculus ficaria* plants, but further work is needed to confirm the pathogenicity of this species (Kim *et al*., 2009) and to study its unique enzymatic activities (Moț *et al*., 2012; Coman *et al*., 2013; Sharma *et al*., 2015).

Conclusions

Our study brings new data regarding morphological and ultrastructural characteristics of *D. tuberosa* asci, ascospores, paraphyses, stipe and sclerotium and contributes with new information about the sclerotium-forming fungi, including a nutritive medium (Czapek-agar with starch) favourable for the growth of mycelium and sclerotia of *D. tuberosa* isolates.

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