Structural aspects regarding formation and emission of *Diaporthe* (*Phomopsis*) *helianthi* ascospores

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ABSTRACT

Ascospores are the main infection source of Phomopsis in sunflower. However, the concept of their structure is still being contradicted and is not complete enough. The objective of this work was to study the structural organization of the ascospore formation and emission for the sunflower *Phomopsis* pathogen. Perithecia of the fungus both in their intact and crushed condition were studied using light microscopes, digital photography and computer-aided analysis. It was determined that 16 ascospores were produced within an ascus and that was twice as many as those considered earlier. The ascospores are pressed out of the water-swollen perithecium wall in the form of a colorless sphere, whose membrane has a granular structure. The infection source contained within the sphere is transmitted by wind.

Key words: ascospore – fungus – perithecium – Phomopsis – structure – sunflower.

INTRODUCTION

Severe infection of sunflower stems caused by Phomopsis (*Diaporthe helianthi* Munt.-Cvet. et al.) was first observed in Yugoslavia (Mihalicevic et al., 1980) and quickly spread in other countries. The severe damage caused by this disease prompted general interest in the Phomopsis pathogen biology. It was determined that the fungus penetrated into the plant through the leaves, spread along the vessels and then entered the stem (Petrov et al., 1981; Bertrand and Tourvieille, 1987). Pathohistological bases of the fungus penetration and resulting stem tissue changes (Muntanola-Cvetkovic et al., 1989) were studied. Nowadays, it is recognized everywhere that ascospores developing in perithecia are a main infection source. Perithecia, of an irregular round shape and 150-430 x 180-850 μm in size, are produced on overwintering plant residues and contain elongated clavate asci (44-67.5 x 4.5-12.0 μm in size), each of which includes 8 ascospores (Assemat and Fayret, 1988; Yakutkin, 1988). The ascospores, which are colorless, with one partition, of an elongated elliptic shape, 15-17 x 5-7.5 μm in size, slightly pressed in the middle beside the partition, have two equal cells containing generally two fat drops per cell and, as some researchers consider, have constricted ends (Maric et al., 1982). According to other scientists' observations they have rounded ends (Assemat and Fayret, 1988) and may reach 7.5 μm in length (Yakutkin, 1988). It is accepted that the ascospores are thrown out from the perithecium to the height of about 3 mm above the plant residue surface. Sometimes, a mucoid drop or a white mass containing ascospores form on the rostrum apex (Maric et al., 1982; Yakutkin, 1988).

The structure of fruit bodies, conidia and ascospores serves as a basis for fungus identification. However, until now their understanding has been incomplete and rather contradictory.

The objective of this work was to study the structural organization of the ascospore formation and emission for the sunflower Phomopsis pathogen.

MATERIALS AND METHODS

For two years, the fungal fruit body formation was observed in the Phomopsis-affected seeds and stems of the sunflower cultivars Rodnik and Berezansky, the hybrids Fly (Monsanto) and Melody (Syngenta), as well as the stems of *Sonchus oleraceus* (L.) Scop. by keeping them under natural or storage or stationary (refrigerator) conditions.

The stem fragments of the above plants and sunflower seeds with the Phomopsis infection symptoms were washed with water, disinfected with ethyl alcohol; afterwards, the stems were flamed with a gas-stove burner and the seeds were washed with sterile water and placed on sterile wet filter paper inside Petri dishes, which were incubated in the environmental chamber (Sanyo), at 25°C, 16-hour photoperiod

(3000 lux) and 80% of humidity. A part of the experiment was conducted under the same conditions but at the night temperature of 12-15^{o}C.

Intact fruit bodies of Phomopsis forming on the stem or on seeds were examined with a stereozoom trinocular microscope (MLS) and perithecia isolated from the plant substrate and crushed – with a laboratory (ML2300) microscope and trinocular microscopes (Meiji, Japan). Their typical structures were photographed with the digital cameras Canon Digital (Canon) and Cyber-short (Sony) and CCD Digital Microscope camera with software. The pictures were analyzed and processed on a computer.

RESULTS

It was determined that the content of the clavate ascus transformed from an undifferentiated condition to the ascospore formation. The ascospore formation happened stepwise. First, 8 colorless structures with an elongated elliptic shape and slightly deppressed beside the partition were produced within the asci. Each of these structures had two equal cells containing generally two fat drops or, although seldom, one fat drop, and constricted ends (Fig. 1a). Their size exceeded 10 µm, but did not reach 20 µm. They did not leave the asci, which were still so firm that they did not collapse while the preparation was produced by crushing the perithecium on the microscope slide. Being in the ascus, the structures started to change: their membranes becoming thinner and their ends rounded (Fig. 1b). Further, each cell of these structures transformed to a bicellular colorless structure of an elongated elliptic shape, depressed in the middle near the partition, with two rounded fat drops – one at each of the opposite ends (Fig. 1a). These structures were identified by us as ascospores, and the initial ones were called biascospores. Two bicellular ascospores, whose length did not exceed 10 µm, were generated by each biascospore. The biascospore cell that had one fat drop turned into a unicellular ascospore with one fat drop in its center (Fig. 1c).

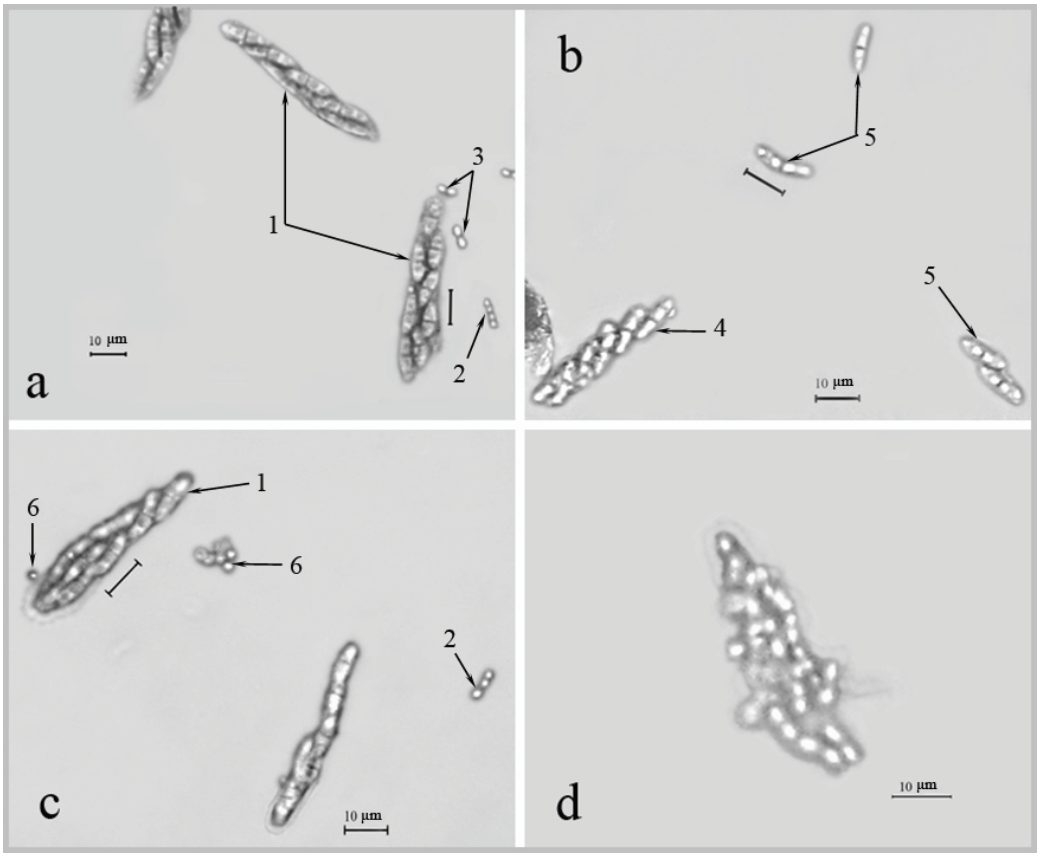

Fig. 1. Structures emerging from a crushed perithecium at their different developmental stages: 1- intact asci with biascospores; 2- ascospores still forming a pair and connected by a thin layer of a common membrane; 3 – bicellular ascospores; 4- a collapsed ascus with biascospores that already have rounded ends; 5- biascospores with rounded ends; 6- unicellular ascospores; d- a collapsing ascus with ascospores (ML2300) x 400.

The transformation of biascospores to ascospores did not happen within an ascus simultaneously, but sequentially: it started at the narrow end and finished at the broad part (from bottom to top). At that period the asci, which developed irregularly within the same perithecium, became fragile because of the thinning of their membranes especially at the sites where the ascospores had already been produced. Thus, the following structures emerged from the crushed perithecium at that period: intact asci with biascospores (Fig. 1a); collapsed asci with biascospores already having rounded ends; ascospores keeping to the arrangement in the form of the ascus already collapsed (Fig. 1d); ascospores still united into a pair by a thin layer of a common membrane (Fig. 1a; Fig. 1c); separate ascospores, generally bicellular and rarely unicellular. 16 ascospores developed in each ascus. No paraphyses were identified. As the last biascospore turned into two ascospores, the ascus membrane collapsed (Fig. 1d). From the preparation generated from a crushed ripe perithecium only free ascospores emerged (Fig. 2). Most of them were bicellular. The quantity of unicellular ascospores is, as a rule, not high and depends on the perithecium formation conditions. For example, during a drought, the number of unicellular ascospores increases.

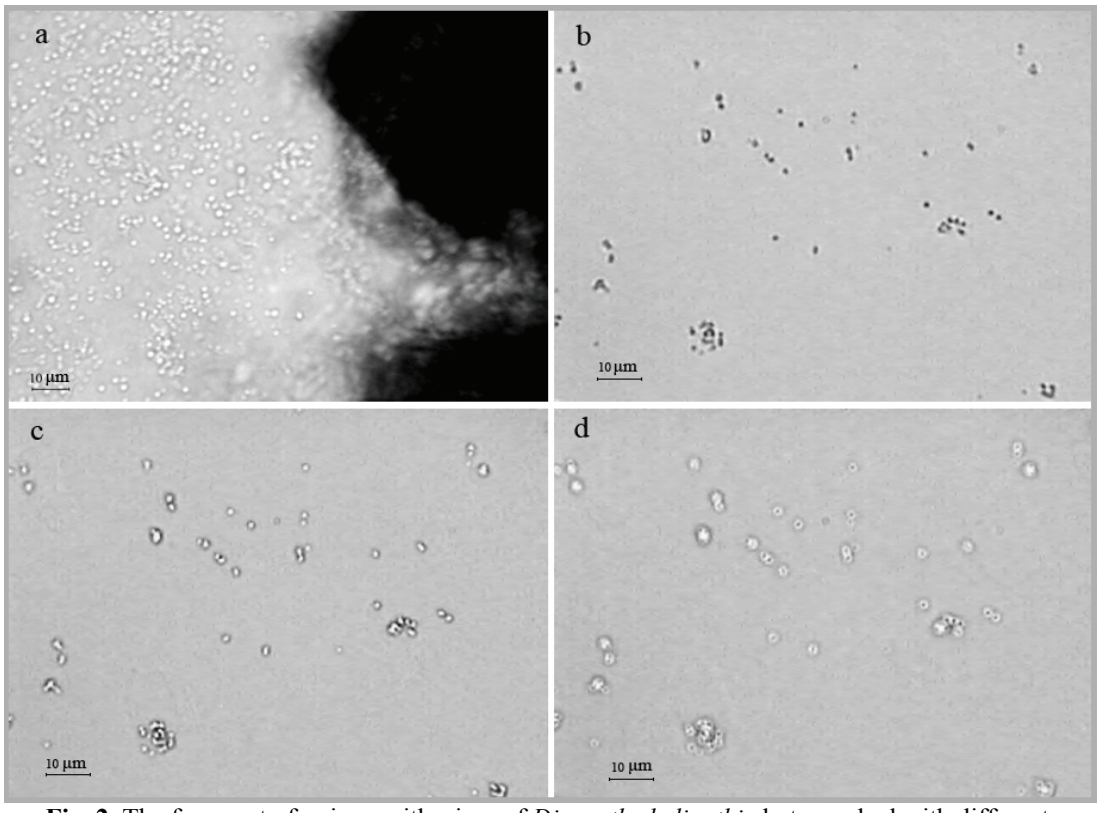

Fig. 2. The fragment of a ripe perithecium of *Diaporthe helianthi* photographed with different sharpnesses of the microscope: a- ascospores emerging from the crushed perithecium, (ML2300) x 400; b- on its surface; c- inside a cell; d- with optical border effect on the membrane and partition, CCD camera x 400.

Before the ascospore emission, a colorless transient sphere (Fig. 3a) with a granular structure of its membrane and smooth surface (Fig. 3b) appeared at the rostrum apex under the pressure of the perithecium walls swollen with water. The ascospores (Fig. 3c) started to be pressed into this sphere. The size of the sphere was from 150 to 200 µm. The color of the filled sphere changed from beige to bright yellow and depended on color and size of fat drops in ascospores, the larger the drops the yellower the sphere (Fig. 3d). The filled spheres became detached from the rostrums and fell into the humid chamber (Fig. 4). Under natural conditions they are caught by ascending air currents and borne by the wind.

Thus, it was determined that 16 ascospores developed in the perithecium ascus of *D. helianthi* which was twice as many as had been considered earlier. Bicellular, or more rarely unicellular, ascospores developed as pairs, one pair per each cell of a bicellular biascospore, and each ascus contained 8 biascospores. The ascospores were pressed by the water-swollen perithecium walls into a colorless sphere, whose membrane had a granular structure and whose surface was not smooth. The infection was transmitted by wind together with the sphere.

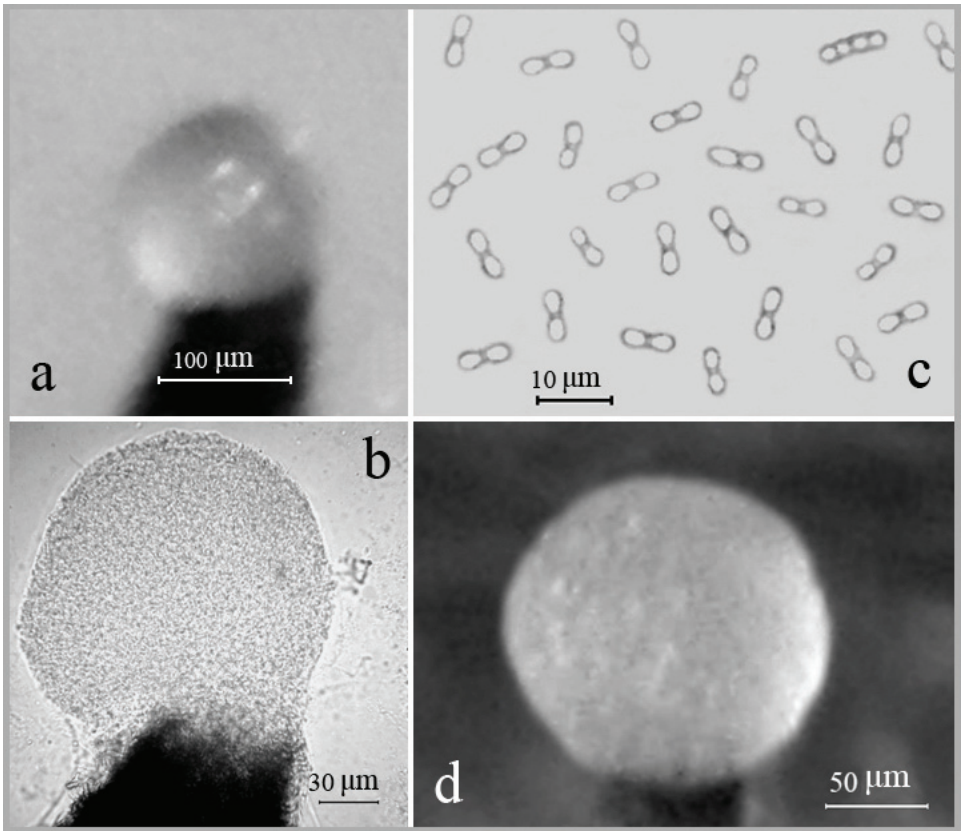

Fig. 3. Apexes of rostrums with spheres: a- an empty sphere under the stereozoom microscope. MLS x 12; b- membrane of an empty sphere. (ML2300) x 400; c- ascospores. (ML2300) x 400; d- a sphere filled with ascospores. MLS x 42.

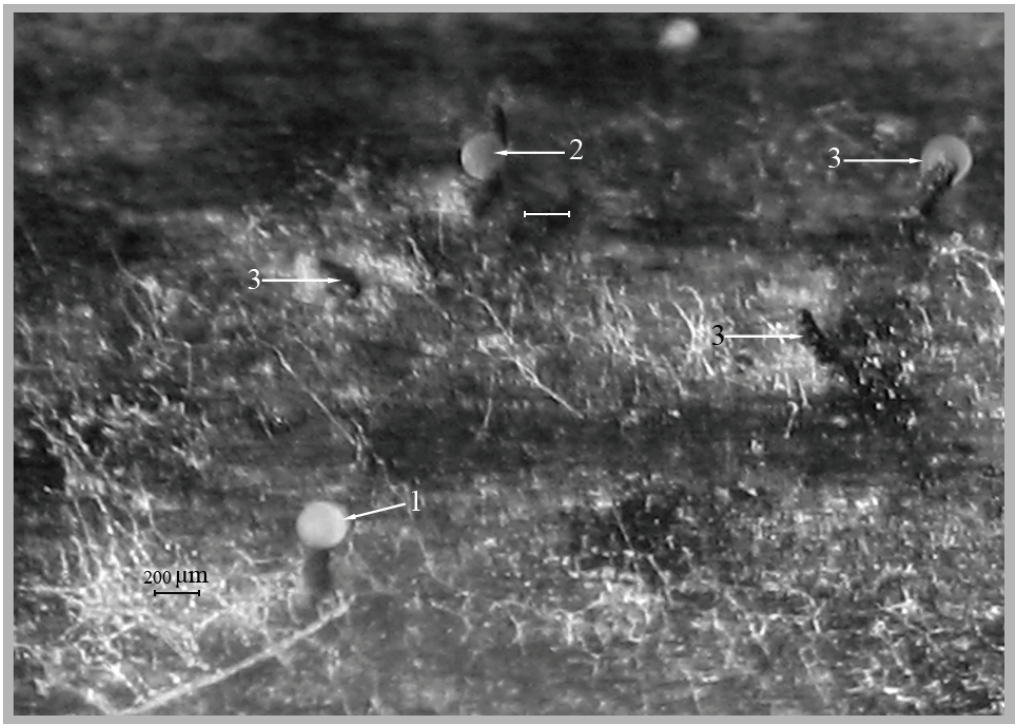

Fig. 4. Appearance of the *Diaporthe helianthi* infection source from perithecia. MLS x 12: 1- a sphere filled with ascospores; 2 – a sphere detached from the rostrum; 3- rostrum.

DISCUSSION

Earlier unknown phenomena have been demonstrated: spreading the infection source by spheres and a stepwise formation of ascopspores including their development within the larger structures (biascospores) formed earlier in an ascus. According to their structure the biascospores correspond to spores, and depending on their function – to additional asci or capsules as they are not spread from the perithecium and do not affect plants.

The results presented differ from the already known concepts regarding the structure and formation of the *D. helianthi* ascospores. However, they solve the contradictions described in the introduction to this paper. Now there are no doubts that bicellular ascospores have rounded ends, which contain one fat drop and reach about 10 μm in length. It should be noted that the formation of ascospores happens stepwise. This fact was unknown earlier and conventional approaches were used here: some researchers took larger biascospores for ascospores, others – bicellular ascospores with rounded ends. These contradictions resulted in indicating significantly different sizes and forms of the ascospore ends in the world literature.

It is not clear what the reason for this stepwise formation of ascospores within the *D. helianthi* asci is. Why isn't the fungus spread by biascospores that have a thicker membrane (i.e., are better protected)? Evidently, the answer to this question is concealed in the perfect stage characteristics of the fungus.

The infection distribution by spheres does not exclude the emission of ascospores from the perithecium as described above. It is possible that they coincide. The spheres could be easily taken for mucoid drops mentioned in earlier publications. Moreover, the filled spheres subjected to internal pressure easily collapse at the slightest pressure from outside and it is impossible to detect their membranes. However, some empty and full drop-shaped structures were noticed on the rostrum apexes. But a drop cannot be empty – then, it is nothing but a membrane. This phenomenon has been shown by us. The formation of spheres on the rostrum apexes often occurs under nightly temperature drop conditions. It should be noted that the spheres are a very convenient means of transporting ascospores because the infection source is well protected inside them. Due to the spheres there may be a high concentration of spores on the leaves of infected plants. Though the sphere is large and contains a great number of ascospores it cannot land for a long time, as its surface is not even.

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