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CONIDIOGENESIS OF *Phoma strasseri* THE FUNGUS RESPONSIBLE FOR BLACK STEM AND RHIZOMES ROT IN PEPPERMINT (*Mentha piperita*)

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Abstract. *Phoma strasseri* is one of the major pathogen of peppermint (*Mentha piperita*). An important criterion in the classification of *Phoma* spp. is conidiogenesis, which has never been studied in *P. strasseri* before. The analysis using a FEI Tecnai Spirit G^2 electron microscope revealed enteroblastic phialidic ontogeny as a type of conidiogenesis present in this species. Conidia are formed on small conidiogenous cells, called phialides. The tip of the conidiogenous cell is surrounded by a characteristic collarette. The surface of the conidium wall is covered with a mucilaginous sheath, which may enable conidia to attach to the plant surface.

Key words: phomosis of mint, conidiogenesis, enteroblastic phialidic ontogeny

INTRODUCTION

A majority of mycologists consider the taxonomy of various *Phoma* species problematic due to unclear morphological criteria and uncertain phylogenetic relations [Aveskamp et al. 2008]. It is often the case that some isolates look similar and are classified within the same species but differ in phylogenetic terms. Nevertheless, widespread species of different morphology may derive from a common ancestor [Aveskamp et al. 2008]. Saccardo [1884], Grimes et al. [1932], Wollenweber and Hochapfel [1936], Dennis [1946], Sutton [1964], Monte et al. [1990, 1991] and Rai and Rajak [1993] all attempted to classify numerous species of *Phoma*. Yet, it was Boerema together with his associates from the Plant Protection Service (Plantenziektenkundige Dienst), Wageningen, the Netherlands, who contributed most to examining the cultures of *Phoma* spp. [Marcinkowska 1995, Aveskamp et al. 2008]. They ceased to consider the host plant as the most important classification criterion to identify particular species. The aim of the Dutch mycologists was to define the taxa within the genus *Phoma* on the

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basis of stable morphological features observed *in vivo* and *in vitro* in cultures developing in standard conditions [Boerema 1976, Marcinkowska 1995]. Furthermore, they were the first ones to draw attention to the use of new techniques in taxonomic research, namely the use of electron microscopy to study conidiogenesis as an important criterion of fungal classification [Brewer and Boerema 1965, Boerema and Bollen 1975, Marcinkowska 1995]. Owing to their research, the distinction between the genus *Phoma* and *Ascochyta* was introduced. They often share species of the same teleomorph genus and similar conidial morphology, which is why they used to be confused with each other [Marcinkowska 1995, Aveskamp et al. 2008].

Phoma strasseri is one of the most important pathogens in the main regions of the world where peppermint (*Mentha piperita*) is grown, including Poland. It causes black stem rot, called sometimes phomosis of mint. Yield losses, reaching up to 90%, are due to quickly progressing tissue degradation of stems and rhizomes, resulting from the enzymatic decomposition of pectins by polygalacturonase and macerating enzymes produced by *P. strasseri* [Melouk and Horner 1972].

This study presents the results of ultrastructural research on conidiogenesis of *Phoma strasseri*, as this process has never been studied in this species before.

MATERIAL AND METHODS

Plant material. The studied material consisted of 10 specimens containing pycnidia, obtained from a 14-day-old colony of *P. strasseri* isolate M 326 grown on maltose medium [De Gruyter and Noordeloos 1992]. The isolate was randomly selected from my own collection of cultures obtained between 2004 and 2006 from damaged stems of peppermint [Zimowska and Machowicz-Stefaniak 2005, Zimowska 2007].

Sample preparation for scanning electron microscope (SEM). Ten specimens containing pycnidia of *P. strasseri* were fixed with 4% glutaraldehyde for 3 hours at room temperature and then, for 24 hours, at 5°C. After that time, the specimens were placed in 1% cacodylate buffer for 2 hours at room temperature [Kulik 1988]. Next, the specimens were dehydrated in an alcohol series (30%, 50%, 70%, 95% and 100%, for 15 minutes at each concentration. The specimens were then dried in liquid CO_2 by using a BAL-TEC CPD 030 Critical Point Dryer, and finally gold sputter-coated. The materials were examined by means of a TESLA BS-300 scanning electron microscope, as described by Kulik [1988].

Sample preparation for transmission electron microscope (TEM). The specimens were fixed with 4% glutaraldehyde for 2 hours at room temperature and post-fixed in 2% osmium tetroxide in the phosphate buffer for 2 hours at 20°C. The following fixation was conducted in 0.1 M cacodylate buffer at pH 7.4 for 2 hours at 4°C. Afterwards, the specimens were double-rinsed for 5 minutes in the same buffer and then for another 5 minutes in distilled water. After rinsing, the specimens were post-fixed in 0.5% uranyl orthosilicate dihydrate solution for 2 hours at room temperature [Maurin et al. 1993]. Next, they were dehydrated in an alcohol series (30%, 50%, 70%, 95% and 100%, for 15 minutes at each concentration at 4°C, followed by 90%, 95% and 100% for 15 minutes at each concentration at room temperature). Then, ethyl alcohol was

replaced with propylene oxide. After absolute alcohol, the specimens were placed in increasing concentrations of propylene oxide in alcohol: 33% for 10 min, 50% for 10 min, 67% for 10 min, and 100% twice for 10 min [Maurin et al. 1993]. Next, the specimens were hardened with increasing concentrations of Spurr Low Viscosity resin in propylene oxide: 33% for 1 hour, 50% for 1 hour, 67% for 1 hour, and 100% for 1 hour. Next, the specimens were placed in polyethylene capsules (filled with resin) and left for 12 hours at 70°C in order to polymerize. After the polymerization, the specimens were cut into ultra-thin 85-nm sections by using a Reichert Ultracut S microtome. Next, they were dyed with 8% uranyl acetate solution in 0.5% acetic acid for 45 min. Finally, they were compounded with lead citrate for 10 min [Maurin et al. 1993].

The materials were examined by means of a FEI Tecnai Spirit G^2 microscope, operating at an acceleration voltage of 100 kV.

RESULTS

The pycnidia of *P. strasseri* used in the study demonstrated the features typical of this species (photo 1a). Most frequently they were round and had 1–2 verrucose ostioles. The wall of the pycnidia was covered with soft mycelial outgrowths, which were also found on the ostioles (photo 1b). Pycnidia formed usually small groups of 2 to 10 (photo 1c). Conidia were formed on small bottle-shaped cells, i.e. phialides, 0.68–1.21 µm long and 1.3–1.89 µm wide (photo 2a), located on the inner wall of the pycnidia. The tip of the conidiogenous cell was surrounded by a characteristic collarette, which was 0.2 µm thick (photo 2b). Due to fixation and staining of the specimens with uranyl acetate and then with lead citrate, the large vacuoles became visible in the electron-dense cytoplasm (photo 2b).

Moreover, apart from the young conidia in pycnidia, there were also mature ones, which were detached from the conidiogenous cells (photo 2c). Conidia were surrounded by a 0.3 µm thick cell wall. The surface of the wall was covered with a mucilaginous sheath of variable thickness, ranging from 0.2 µm to 0.5 µm (photo 2c). In the examined *specimens*, the single-celled conidia predominated. Only in one *specimen,* a conidium with a secondary septum was observed (photo 2d). The septum was 0.3 µm thick (i.e. visibly not thicker than a conidium wall without a septum) and had a small indentation (photo 2d).

DISCUSSION

The TEM images revealed enteroblastic phialidic ontogeny [Boerema and Bollen 1975] as a type of conidiogenesis present in *P. srasseri*. The characteristic feature of this process, as shown in the present study, is the formation of conidia on small flask-shaped cells called phialides. The development of the first conidium is initiated by papilliform, pronounced thickening in the wall at the top of the conidiogenous cell, with a more or less electron-dense middle layer of the wall, which functions as an "opener"

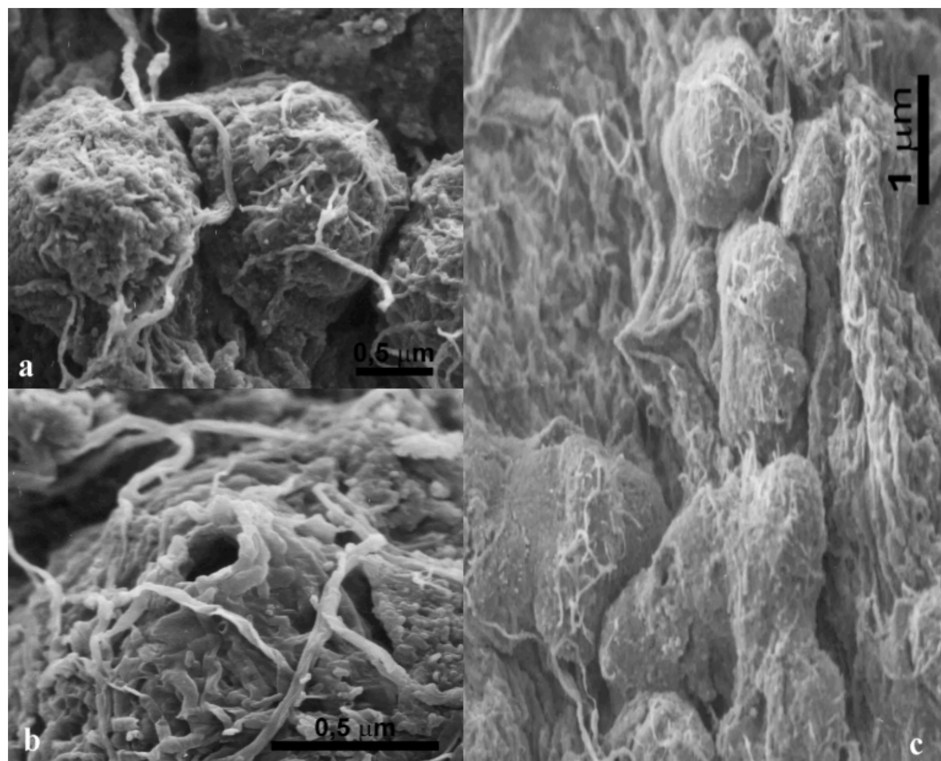

Photo 1. Scanning electron micrographs of *Phoma strasseri*: a – pycnidia with mycelial outgrowths on the wall. SEM × 900, b – verrucose ostiole of a pycnidium. SEM × 1500, c – group of pycnidia. SEM × 600; phot. M. Wróbel

Fot. 1. Mikrofotografie *Phoma strasseri* wykonane przy użyciu skaningowego mikroskopu elektronowego: a – piknidia ze strukturami strzępkowymi na powierzchni ściany. SEM × 900, b – brodawkowate ujście piknidium. SEM × 1500, c – grupa pikindiów. SEM × 600; fot. M. Wróbel

of the fixed conidiogenous locus [Boerema and Bollen 1975]. An additional feature of the enteroblastic phialidic ontogeny is the fact that mucilage production takes place during the differentiation of the conidium wall until it reaches the final structure. The mucilage, together with dissolved wall material (resulting from releasing of the first conidium and detaching of the successive conidia), creates mucilaginous substance surrounding conidia in *Phoma* spp. [Boerema and Bollen 1975]. Such a mucilaginous substance was observed on the surface of conidia of *P. strasseri* during this study. The substance, most frequently, consists of polysaccharide, glycoproteins, and xylan. Mucilaginous exudate is created by various species of fungi, such as: *Phoma macdonaldii, Ascochyta rabiei, Phyllosticta amplicida* and *Phomopsis phaseoli* [Kulik 1988, Höhl et al. 1990, Kerchung and Hoch 1995, Rustaee et al. 2000]. According to Gold and Mendgen [1984], the mucilaginous substance surrounding conidia has various func-

tions. It is responsible for attaching the spore to a plant, sealing the site of penetration of infectious hyphae, and protecting appresoria from drying and unfavourable atmospheric conditions. In *Colletotrichum graminicola* [Pascholati et al. 1992], *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici*, it is the enzymes degrading the cell wall that make up the substance [Bishop and Cooper 1983]. In the case of *Erysiphe graminis*, the mucilaginous exudates surrounding conidia include substances that make it possible for the pathogen to distinguish an adequate host plant [Pascholati et al. 1992]. So far, the role of the mucilaginous substance that covers conidia *P. strasseri* has not been found. Its function may resemble that in *P. macdonaldii* [Rustaee et al. 2000], i.e. enabling conidia to attach to the surface of stems and rhizomes of peppermint.

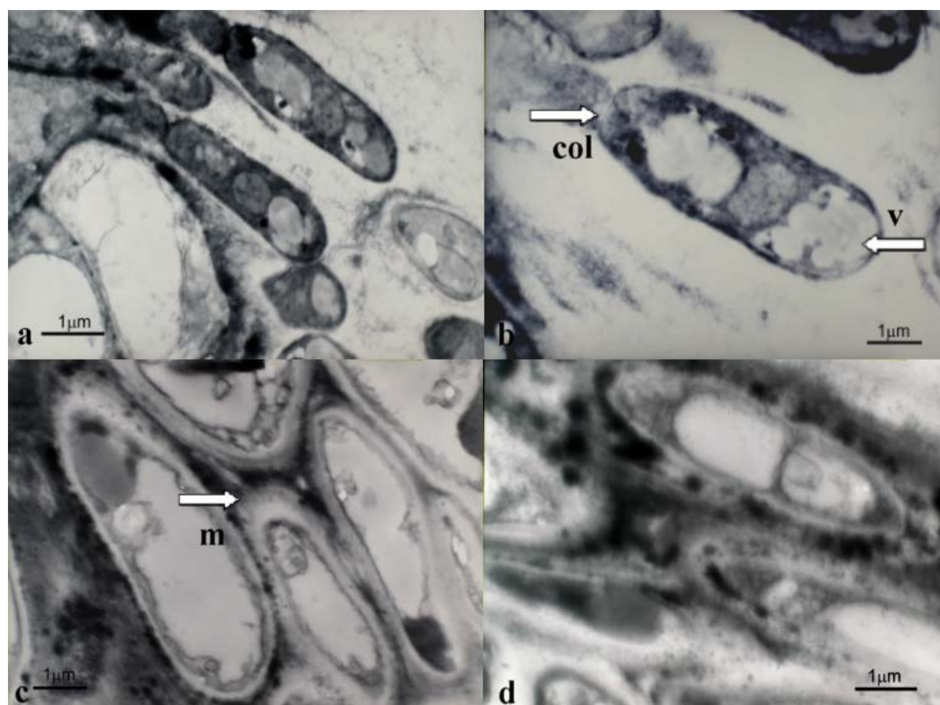

Photo 2. Transmission electron micrographs of *Phoma strasseri*: a – development of conidia on conidiogenous cells. TEM × 13 000, b – characteristic collarette (col.) on the tip of the conidiogenous cell and visible vacuoles (v). TEM × 15 000, c – mucilaginous substance (m) on conidia. TEM × 15 000, d – conidium with a secondary septum. TEM × 15 000; Phot. M. Rudaś

Fot. 2. Mikrofotografie *Phoma strasseri* wykonane przy użyciu transmisyjnego mikroskopu elektronowego: a – tworzące się na komórkach konidiotwórczych konidia. TEM × 13 000, b – charakterystyczny kołnierzyk (col.) na szczycie komórki konidiotwórczej oraz widoczne wakuole (v). TEM × 15 000, c – substancja śluzowata (m) pokrywająca konidium. TEM × 15 000, d – konidium z wtórną przegrodą. TEM × 15 000. Fot. M. Rudaś

The formation of the characteristic collarette on the tip of a conidiogenous cell of *P. strasseri* is typical of enteroblastic phialidic ontogeny [Boerema and Bollen 1975, Marcinkowska 1995]. It is formed after detachment of the first conidium and consists of undissolved papilla wall remains. The collarette surrounds the fixed conidiogenous locus, from which both the second and the successive conidia arise [Boerema and Bollen 1975]. The development of the successively produced conidia looks the same as that of the first conidium. They are formed as outgrowths from the wall that closes the conidiogenous cell after detachment of the previous conidium and are always covered with some mucilaginous substance [Boerema and Bollen 1975].

The conidial wall in the observed process of conidiogenesis reaches its full size almost immediately. Moreover, in case of two-celled conidia, the septum wall is clearly not thicker than the unseparated conidium wall [Boerema and Bollen 1975], which was also observed in the present study. The formation of two-celled conidia in *Phoma* spp. is very rare, especially *in vitro* [Boerema and Bollen 1975, Zimowska and Machowicz--Stefaniak 2005]. Conidial septation proceeds very quickly. It is initiated by the formation of a very thin, highly electron-transparent layer called a septal-plate, growing vertically from the lateral wall. At the same time, on both sides of the septal-plate, electron-dense wall layers occur [Boerema and Bollen 1975].

The presence of large vacuoles, in the forming *P. strasseri* conidia, indicates the important role of these organelles from the very beginning of conidia, which was also pointed out by other authors [Rustaee et al. 2000, Veses et al. 2008]. The diversity of functions for which vacuoles are responsible, i.e. hydrolysis and glycoprotein transmission, storage of Ca^{+2} ions, appropriate osmoregulation in cells, and transportation of nutrients in hyphae, indicate the vital role of these organelles for the life of fungi [Veses et al. 2008]. Moreover, in conidial fungi, vacuoles have a special relevance in appresorium morphogenesis and the formation of infectious hyphae [Veses et al. 2008].

This study showed how conidia of *P. strasseri* were formed by means of enteroblastic phialidic ontogeny. Furthermore, the ultrastructure of selected parts of the pathogen's spores was shown.

Taking into consideration the problem of precise identification of taxa in *Phoma* spp., currently discussed in many scientific publications [Aveskamp et al. 2008], it appears that the hitherto used methods, including electron microscope studies, have been insufficient.

The ongoing debate on the limits of the *Phoma* species populations comes down to finding out about phylogenetic relations between *Phoma* species. Owing to that, the need for studying the genetic material of the most common species and the related genera as well as strains of reference was pointed out [Aveskamp et al. 2008]. Moreover, to allow fast and precise taxon identification and to create a new database based on the "barcode" concept, it is necessary to elaborate a method aiming at finding the unique nucleotide sequence of the hitherto described species [Hebert et al. 2002, Armstrong and Ball 2005].

Taking the above into account, the need for conducting molecular analysis aimed at finding the nucleotide sequence in *P. strasseri* should be reflected upon.

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KONIDIOGENEZA *Phoma strasseri*, PATOGENA POWODUJĄCEGO CZARNĄ ZGNILIZNĘ ŁODYG I ROZŁOGÓW MIĘTY PIEPRZOWEJ (*Mentha piperita*)

Streszczenie: *Phoma strasseri* jest jednym z najgroźniejszych patogenów mięty pieprzowej (*Mentha piperita* L.). Jednym z ważniejszych kryteriów w taksonomii grzybów z rodzaju *Phoma* są badania procesu konidiogenezy. Przeprowadzone badania przy użyciu elktronowego mikroskopu FEI Tecnai Spirit G^2 wskazały na fialidową enteroblastyczną ontogenezę jako typ konidiogenezy występujący u *P. strasseri.* Konidia tworzą się na krótkich komórkach macierzystych o kształcie fiolki, zwanych fialidami. Szczyt komórki konidiotwórczej otoczony jest charakterystycznym kołnierzykiem, a powierzchnię ściany konidiów pokrywa warstwa substancji śluzowej.

Słowa kluczowe: fomoza mięty, konidiogeneza, fialidowa enteroblastyczna ontogeneza

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