

Pathogenicity and Ultrastructural Studies of the Mode of Penetration by *Phoma strasseri* in Peppermint Stems and Rhizomes

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Abstract

Pathogenicity and ultrastructural investigation of the inoculation of peppermint stems and rhizomes with *Phoma strasseri* conidia was undertaken using scanning and transmission electron microscopy to examine the host-parasite relationship. Pathogenicity experiments demonstrated that all tested *P. strasseri* isolates had infected the stems and rhizomes of peppermint. Of all inoculation methods, direct placement of colonized agar plugs on damaged epidermis and soaking stems and rhizomes in conidial suspension were the most effective. The behavior of the conidia deposited on the stems and rhizomes was investigated at different time intervals after inoculation: 6, 16, 24, 36 and 48 h. Conidia produced an appressorium directly at the end of a short germ tube. Appressoria were formed over the cuticle, but never over stomata. Direct penetration to host tissue through the cuticle was observed. The spore and hyphae were covered with a mucilaginous sheath.

Key words: *Mentha piperita*, black stem and rhizomes rot, infection process, SEM, TEM

Introduction

Black stem and rhizomes rot of peppermint (*Mentha piperita* L.), also called phomosis of mint, is caused by *Phoma strasseri* Moesz (Boerema *et al.*, 2004). The occurrence of this disease has been so far found in the United States (Horner, 1971; Farr *et al.*, 1995), in Japan (De Gruyter *et al.*, 2002), in India (Kalra *et al.*, 2004), in Hungary (Paizs and Naggy, 1975) and in Poland (Zimowska and Machowicz-Stefaniak, 2005; Zimowska, 2007). The disease symptoms on the plants of peppermint cultivated in the field are visible on the stems, first in the form of necrotic, slightly hollow spots enfolding the stem around. With time, the tissue in the place of the spots gets rotten. Such symptoms are most often formed just under the ground surface to the height up to 10 cm from the base. The secondary symptom is the reddening or reduction of the leaf blades. Very young stems usually die out without any secondary symptoms of the disease (Horner, 1971; Zimowska, 2007). The rot proceeds very fast on the rhizomes. Young rhizomes rot away wholly, while the bark layer often comes off on older ones (Horner, 1971; Zimowska, 2007). The symptoms on peppermint cultivated in a glasshouse are similar to those that are observed in the field, with no secondary symptoms in the form of the reddening and reduction of the leaves, however (Horner, 1971). The yield losses as a result of plant infection can even reach 90% (Horner,

1971). Etiological signs in the form of pycnidia, including the conidia of *P. strasseri*, occur on the stems and rhizomes with the symptoms of black rot (Zimowska and Machowicz-Stefaniak, 2005; Zimowska, 2007).

The accessible literature provides information on disease symptoms caused by *P. strasseri* (Horner, 1971; Paizs and Naggy, 1975; Zimowska, 2007), biotic interactions of *P. strasseri* with the fungi colonizing the phyllosphere of peppermint stems and rhizomes as well as the effect of the thermal conditions on the formation of the infection material by the fungus (Zimowska, 2011a). The histopathological and ultrastructural aspects of the infection of peppermint by *P. strasseri* has not been so far documented. Hence, the present research undertakes studies on the ultrastructure of the inoculated stems and rhizomes of peppermint with the aim of explaining the relation between *P. strasseri* and the host plant.

Experimental

Material and Methods

Fungal isolates. The studies used one-spore cultures of three isolates of *P. strasseri* obtained from the naturally infected plants of peppermint with the signs of black stem and rhizomes rot from the production plantations situated in the south-eastern part of Poland (Zimowska, 2007) and isolate CBS. 126.93

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obtained from Centraalbureau voor Schimmelcultures, Utrecht Netherlands.

Inoculation techniques. Isolates of *P. strasseri* were incubated on a maltose agar MA throughout the first week at the temperature of 22°C without any light access, and then for 13 hours in ultraviolet light (UV). After that, the isolates were incubated in the same conditions as in the first week of the culture (De Gruyter and Noordelos, 1992). Three methods of inoculation were used to prove the pathogenicity. The first method used plugs of the colonized agar (5 mm diameter) cut out from 2-week-old cultures of each isolate. Those plugs were placed at the stem and rhizome fragments which were disinfected on the surface by being immersed for 60 seconds in 10% sodium hypochlorite. In the second method, colonized plugs were placed at disinfected fragments of stems and rhizomes, together with the epidermis injured with a needle puncture (Zimowska, 2004). The third method used a suspension of conidia with the density of 10^6 conidia per 1 ml. It was obtained by rinsing the surface of the cultures of particular isolates with sterile distilled water. The disinfected fragments of stems and rhizomes were soaked in the suspension for 5 minutes (Horner, 1971). Each method was tested in humidity chambers. Those were 9 cm diameter Petri dishes, laid with three layers of cellulose tissue and one layer of filter paper moistened with 4 ml of distilled sterile water (Zimowska, 2004). For each method 120 fragments of stems and rhizomes were used. Control fragments of stems and rhizomes were inoculated with sterile agar plugs (methods I and II) or sterile water (method III). The experiment was conducted twice. Humidity chambers were kept in a thermostat at the temperature of 22°C for 12 days. During that time, observations were made every 3 days on the development of disease symptoms. After 12 days, the infection index was calculated on the basis of the disease scale. Next, all fragments of stems and rhizomes were analyzed for the presence of fungus according to Koch's postulates. The results obtained from the experiment were statistically analyzed using a two-factor variance analysis (Anova) according to SAS program (Snedecor and Cochran, 1982).

Sample preparation for scanning electron microscope (SEM). Fragments of stems and rhizomes inoculated by conidial suspension were cut into 2–3 mm sections. Next, the specimens 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 CO₂ by using a BAL-TEC CPD 030 Critical Point Dryer, and finally gold sputter-coated. Observations of six samples were

carried out at different time intervals after inoculations: 6, 16, 24, 36 and 48 hours. Micrographs were obtained using a Vega 2, Tescan scanning electron microscope.

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 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 G² microscope, operating at an acceleration voltage of 100 kV.

Results

Pathogenicity of isolates to stems and rhizomes of peppermint and inoculation techniques. All studied isolates of *P. strasseri* caused signs in the form of necrosis and then rot on the inoculated fragments of stems and rhizomes. As early as already 3 days after the inoculation, symptoms were observed on peppermint parts inoculated according to method II. Those were necrotic spots, 3 to 10 mm long, around the infection site. After 6 days, the necrosis grew and covered from 25% to 30% of the area of the inoculated parts. After that time, the softening of the tissues in the place of the necrosis could be seen. After 9 days, the rot covered from 40% to 70% of the surface of the stems and rhizomes, and after 12 days – from 90% to 100% of the surface of the

Table I
Pathogenicity of *Phoma strasseri* to stems and rhizomes of peppermint using various inoculation methods
(means for 4 isolates)

Inoculation method	Infection index (%) after 12 days ^x	
	stems	rhizomes
Colonized plugs placed at non-injured epidermis	87.92 a	84.38 a
Untreated	0	0
Colonized plugs placed at injured epidermis	96.67 b	96.67 b
Untreated	0	0
Stems and rhizomes soaked in conidial suspension (1×10 ⁶ conidia/ml)	94.79 b	95.00 b
Untreated	0	0
	HSD = 3.0537	HSD = 3.1825

^x Infection index evaluated on the basis of 5 – degree disease scale: 0° – lack of disease symptoms; 1° – sign of necrosis visible only around the inoculation point; 2° – 25% to 50% surface of inoculated organs showed disease symptoms; 3° – 51% to 75% surface of inoculated organs showed disease symptoms; 4° – 76% to 100% surface of inoculated organs showed disease symptoms

Values marked with the same letter do not differ significantly
HSD – Honest Significant Difference

inoculated parts of peppermint covered with the rot. The development of disease symptoms on peppermint stems and rhizomes inoculated according to method III was similar. In the combination with inoculation through the undamaged epidermis (method I), a trace of necrosis was seen after 6 days around the inoculation site. After 6 days, necrosis accompanied by the softening of the tissues covered from 15 to 20% of the surface of the inoculated parts. After 12 days, from 70% to 90% of the stem and rhizome surface of the inoculated parts was covered with the rot. The symptoms were similar to those observed on the plants of peppermint in the conditions of field cultivation. The most effective methods of inoculation proved to be methods II and III. Values of the infection index were, respectively, 96.67% and 94.79% for the stems and 96.67% and 95.00% for the rhizomes and they were significantly different from values of the index obtained for method I (Table I). The highest values of the infection index in all inoculation

methods among the tested isolates were for isolate M 743 (Tables II, III, IV). Generally, those values did not significantly differ from the values of the index obtained for the other isolates, except isolates M 289 and M 126 tested according to method II (Table III), the same isolates and isolate CBS. 126 93, which were

Table III
Effect of inoculation of *Phoma strasseri* isolates on occurrence of black stem and rhizomes rot – method II (mean of 6 replications)

Isolate	Infection index (%) after 12 days ^x		Reisolation (%) ^y	
	stems	rhizomes	stems	rhizomes
M 126	95.00 ab	90.33 a	100	100
M 289	90.50 a	95.83 ab	100	100
CBS. 126.93	99.17 bc	98.33 ab	100	100
M 743	100.00 b	100.00 b	100	100
Untreated	0	0	0	0
	HSD = 8.6408	HSD = 9.0047		

Note: see tables I and II

Table II
Effect of inoculation of *Phoma strasseri* isolates on occurrence of black stem and rhizomes rot – method I (mean of 6 replications)

Isolate	Infection index (%) after 12 days ^x		Reisolation (%) ^y	
	stems	rhizomes	stems	rhizomes
M 126	88.33 ab	84.17 a	100	100
M 289	82.50 a	77.50 a	100	100
CBS.126.93	88.33 ab	81.67 a	100	100
M 743	92.50 b	94.17 b	100	100
Untreated	0	0	0	0
	HSD = 8.7309	HSD = 9.1322		

Note: see table I.

^y For stems and rhizomes showing black rot symptoms, percent isolations that resulted in *P. strasseri* colonies.

Table IV
Effect of inoculation of *Phoma strasseri* isolates on occurrence of black stem and rhizomes rot – method III (mean of 6 replications)

Isolate	Infection index (%) after 12 days ^x		Reisolation (%) ^y	
	stems	rhizomes	stems	rhizomes
M 126	91.67 ab	92.50 a	100	100
M 289	90.83 a	92.50 a	100	100
CBS. 126.93	97.50 ab	96].67 a	100	100
M 743	99.50 b	98.33 a	100	100
Untreated	0	0	0	0
	HSD = 8.7408	HSD = 9.8573		

Note: see tables I and II

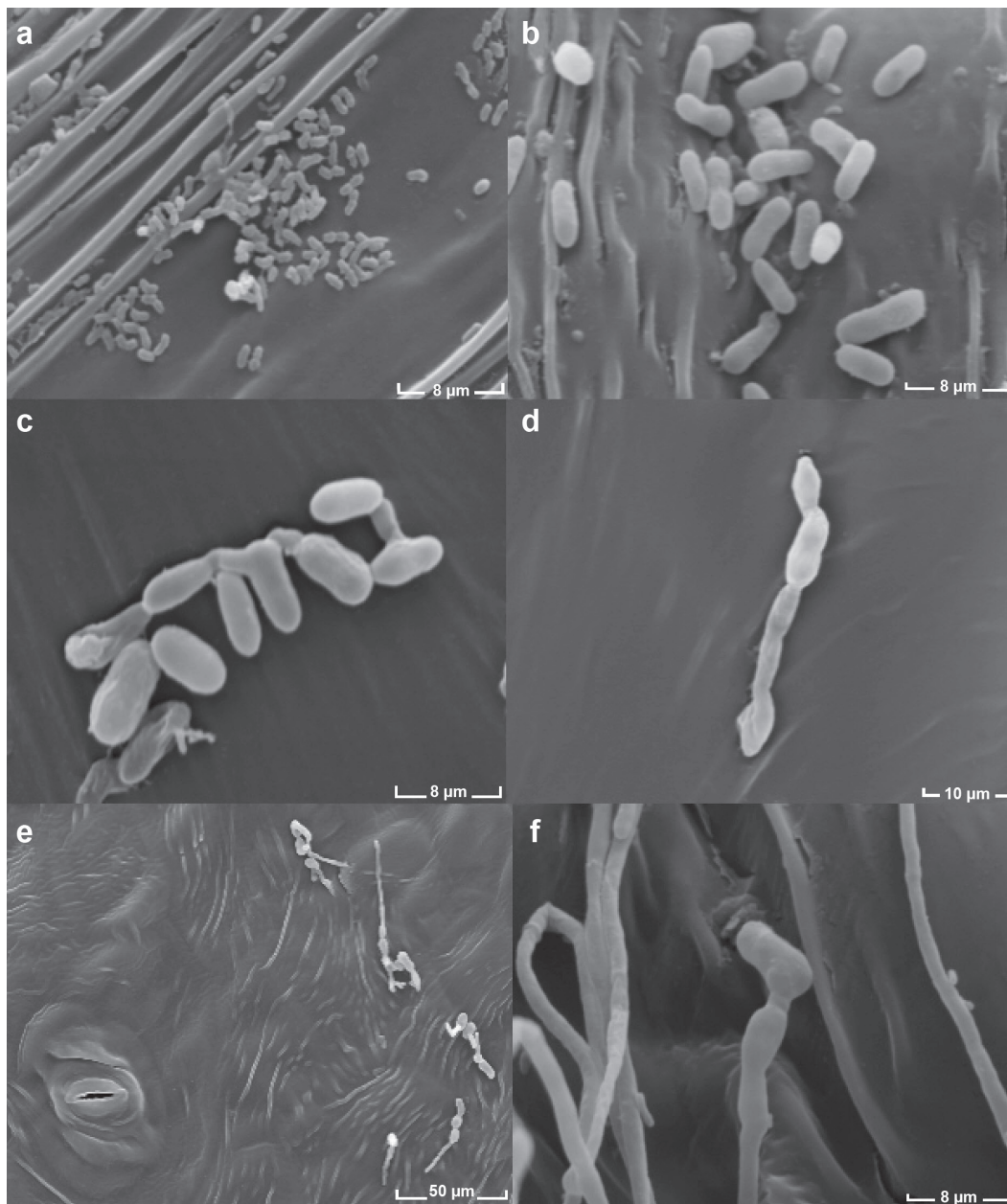


Fig. 1. Scanning electron micrographs of *Phoma strasseri* on peppermint stems and rhizomes.

(a, b), view of pycniospores 6 h after inoculation (scale bar = 8 μ m). (c), germinated spores 16 h after inoculation (scale bar = 8 μ m). (d), adhesion structure in the form of appressorium on the end of germ tube 24 h after inoculation (scale bar = 10 μ m). (e), development of appressoria and germinating of spores were present in some distance of the stomata (scale bar = 50 μ m). (f), development of hyphae at the surface of cuticle 36 h after inoculation (scale bar = 8 μ m). (Photo. M. Wróbel)

used to inoculate the rhizomes tested according to method I (Table II) and isolate M 289, used to inoculate the stems in method III (Table IV). *P. strasseri* cultures were reisolated from all inoculated organs, for all methods (Tables II, III, IV). Morphological features of reisolated cultures corresponded to the features of cultures considered in the research. Uninoculated con-

trols remained symptomless, and results from the two experiments were similar.

Scanning electron microscope. Six hours after inoculation, conidia of *P. strasseri* were visible on the surface of peppermint stems and rhizomes (Fig. 1 a, b). After 16 hours, single conidia formed germ tubes of length not exceeding 5 μ m (Fig. 1c). Twenty four hours

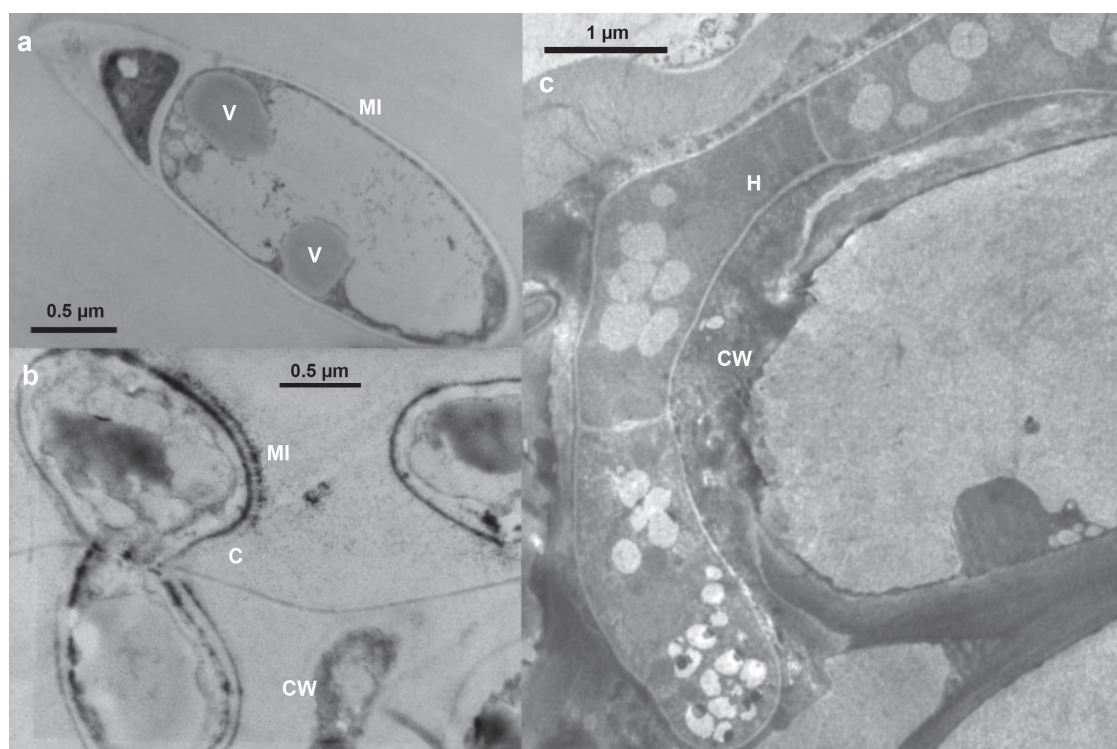


Fig. 2. Transmission electron micrographs of *Phoma strasseri* on peppermint stems and rhizomes.

(a), germinating and highly vacuolated (V) *Phoma strasseri* spore with the mucilaginous layer (MI) present on the wall (scale bar = 1 μm). (b), direct penetration through the cuticle (C) (scale bar = 1 μm). (c), expanding septate hyphae (H) within the cell wall (CW) (scale bar = 1 μm). (Photo. M. Rudaś)

after inoculation, an adhesive structure in the form of an appressorium was seen at the end of the germ tube (Fig. 1d). Conidia germination and appressorium formation always took place at a certain distance from the stomata (Fig. 1e). After 36 hours, unbranched hyphae were observed on the surface of the cuticle of peppermint stems and rhizomes (Fig. 1f).

Transmission electron microscope. The conidia of *P. strasseri* germinating on the surface of the cuticle had big vacuoles (Fig. 2a). A layer of a mucilaginous sheath not greater than 0.3 μm thick was visible on the surface of the cell wall of the conidia (Fig. 2a). Direct penetration of the pathogen by the cuticle of the host plant was observed between 36 and 48 hours after inoculation. A sheath of a mucilaginous substance μm was present on the surface of the wall of the hyphae (Fig. 2b). After 48 hours, septate hyphae was visible in epidermis cells (Fig. 2c).

Discussion

Pathogenicity studies showed that all tested isolates of *P. strasseri* caused infection of the inoculated stems and rhizomes of peppermint. This is testified to by high

values of infection indexes and the fulfillment of Koch's postulates. Of all methods of inoculation, the most effective proved to be the one consisting in placing plugs of colonized agar on the injured tissue of the stems and rhizomes, and the method considering soaking of the fragments in a conidial suspension. These results are consistent with information from literature, according to which the enumerated inoculation methods also proved the most effective for other facultative pathogens such as *Phoma linguam*, (Sock and Hoppe, 1999), *P. exigua* (Koike *et al.*, 2006), *P. exigua* var. *foveata* (Giebel and Dopierała, 2004) and *P. multirostrata* (Garibaldi *et al.*, 2010). The fact that the disease symptoms on the inoculated peppermint parts are similar to those that are observed in the conditions of field cultivation is certainly related to the production of pectolytic enzymes, especially polygalacturonase and maceration enzymes, by *P. strasseri* (Melouk and Horner, 1972a). It follows from studies conducted by Melouk and Horner (1972b) that enzymes of *P. strasseri* show the greatest activity 5 days after infection. Studies confirm the thesis posed by American researchers because already after 6 days, the symptoms of tissue softening were visible on inoculated fragments of stems and rhizomes.

Studies of the ultrastructure of the inoculated stems and rhizomes of peppermint with an conidial suspension pointed to the formation of an adhesive structure at the end of the germ tube in the form of an appressorium and to the direct infection of the pathogen by the cuticle. The ability of fungi for active infection is connected with the fact that they form special structures by means of which they first get attached to the host plant, after which they penetrate its tissues. This is an important condition of successful infection and next the development of a disease (Kulik, 1988; Maurin, 1993). It follows from the present studies that the germinating conidia of *P. strasserii* were fixed to the surface of peppermint stems and rhizomes by means of the appressorium. It has been known for long that in the majority of fungi the formation of the appressorium at the end of the germ tube takes place as a result of a mechanical contact of the fungus with the substrate (Büsgen, 1893; Kerchung and Hoch, 1995). The formation of the appressorium was found in the species closely related to genus *Phoma*, i.e. *P. exigua* var. *linicola* (Roustaee *et al.*, 2000), *Ascochyta pisi* (Heath and Wood, 1969), *A. fabae* (Maurin *et al.*, 1993) and *A. rabiei* (Pandey *et al.*, 1987). In *P. strasserii*, the germinating conidia were also attached to the surface of stems and rhizomes by means of a mucilaginous sheath that covered the wall of the conidia and the hyphae. The presence of the mucilaginous sheath was observed during the studies on conidiogenesis of *P. strasserii* (Zimowska, 2011b). It is formed at the last stage of differentiation of the conidia wall to its final structure (Boerema and Bollen, 1975). Many fungi species that perform the infection directly through the cuticle form this type of mucilaginous exudates. It occurs, for example in *Phyllosticta amplicida* (Kerchung and Hoch, 1995), *Phomopsis phaseoli* (Kulik, 1988) and *Phoma macdonaldii* (Roustaee *et al.*, 2000). Its role is to strengthen the contact with the host plant. Besides, it seals up the site where the infection hyphae penetrates and it protects the appressorium from drying out and from unfavourable atmospheric conditions (Roustaee *et al.*, 2000). The studies pointed to a direct penetration of *P. strasserii* by the cuticle of peppermint stems and rhizomes omitting the stomata. The majority of fungi penetrate into the tissues of their hosts directly through the cuticle. This model of infection is usually accompanied by the formation of the appressorium (Kulik, 1988). Direct penetration was observed for example in *Phomopsis scabra* (Ammon and Vann, 1994), *Colletotrichum lagenarium* (Bonnen and Hammerschmidt, 1989) and in *C. gloeosporioides* (Dickman *et al.*, 1982). It is known that direct penetration of pathogenic fungi occurs as a result of the joint action of two factors, namely the mechanical pressure of a fast growing infection hyphae and the enzymes decomposing cutin and then pectin compounds and cellulose making the composition of

the cell wall (Isaac, 1992). In the case of *P. strasserii*, pectolytic and maceration enzymes produced by the pathogen certainly take part in the process of active penetration. The involvement of pectolytic and hemicellulolytic enzymes has been reported for *P. linguam* (Hammond *et al.*, 1985). The presence of *P. strasserii* hyphae in epidermis cells as early as after 48 hours can point to a short period of the pathogen incubation.

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