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PATHOGENICITY AND ULTRASTRUCTURAL STUDIES OF THE MODE OF PENETRATION BY *Phyllosticta plantaginis* IN RIBWORT LEAVES

Beata Zimowska
University of Life Sciences in Lublin

Abstract. Pathogenicity and ultrastructural investigation of the ribwort leaves inoculated with *Phyllosticta plantaginis* conidia was undertaken using scanning electron microscopy to examine the host-parasite relationship. Pathogenicity experiments demonstrated that all tested *P. plantaginis* isolates had infected leaves of ribwort. Of all inoculation methods, direct placement of colonized agar plugs on damaged epidermis and soaking leaves in conidial suspension were the most effective. The behavior of the conidia deposited on the leaves was investigated at different time intervals after inoculation: 7, 18, 25, 48 and 72 h. An appressorium appeared directly at the end of a short germ tube grown from conidia. Appressoria were formed over the cuticle in some distance from the stomata. Penetration through the stomata was observed.

Key words: *Plantago lanceolata,* herbs, infection process, SEM

INTRODUCTION

The genus *Phyllosticta* Pers. ex Desm., including the teleomorphic stages belonging to *Guignardia* Viala & Ravaz, constitute an economically important group of fungi with a wide range of host plants [van der Aa 1973, van der Aa and Vanev 2002]. They comprise saprobes, e.g. *Phyllosticta cocoicola* (Bat.) Sivan., a species colonizing the leaves of palm trees in Europe [Taylor and Hyde 2003], or *P. capitalensis* Henn., isolated from the leaves of *Magnolia lilifera* in Thailand [Okane et al. 2003]. The second group includes endophytes, e.g. *P. bifinariae* O.L. Pereira, obtained from the leaves of plants of family *Orchideacea* in Brazil, which do not show disease symptoms [Glienke et al. 2011], or *P. ilicina* Sacc., which colonizes the leaves of *Quercus ilex* in Switzerland without any symptoms [Collado et al. 1996]. The biggest group is made up, however, of phytopathogens, including, e.g, *P. sphaeropsoidea* Ellis & Everh., causing spots on horse-chestnut in Europe and North America [Hudson 1987], or *P. citricarpa* (McAl-

Corresponding author: Beata Zimowska, Department of Plant Pathology and Mycology, University of Life Sciences in Lublin, Leszczyńskiego 7, 20-069 Lublin, Poland, e-mail: beata.zimowska@up.lublin.pl

pine) van der Aa, causing spots on grapefruit leaves [Wulandari et al. 2009]. *P. plantaginis* Sacc. is described in literature as a pathogen causing spots on ribwort plantain (*Plantago lanceolata* L.) and great plantain (*Plantago major* L.) [Saccardo 1878, Farr et al. 1995, Zimowska 2012a]. Those plants occur in natural state and in cultivation and they have a big importance in herbal and pharmaceutical industries. The qualitative features of those herbs are lowered by pathogenic fungi [Zimowska 2012a, 2013]. Disease symptoms on the leaves of ribwort plantain caused by *P. plantaginis* are small, regular necrotic spots with the diameter of 2–4 mm. With the passage of time, the tissue around the spots gets yellow, and then it dies out [Zimowska 2013]. Crop losses due to disease caused by *P. plantaginis* range from 10 to 25% (personal communication).

The accessible literature provides information on disease symptoms caused by *P. plantaginis* [Saccardo 1878, Farr et al. 1995, Zimowska 2012a], the effect of thermal conditions and culture media on the growth and formation of morphological structures [Zimowska 2012a], pycnidia formation and the manner of releasing conidiospores [Zimowska 2012a]. On the other hand, the ultrastructural aspect of infection of ribwort leaves by *P. plantaginis* has not been documented yet. Therefore, the present work undertook studies on the fulfillment of Koch's postulates for the Polish isolates of *P. plantaginis* and the ultrastructure of the inoculated ribwort leaves to elucidate the host-parasite relationship.

MATERIAL AND METHODS

Fungal isolates. A single conidial 5 isolates of *P. plantaginis* isolated in 2009–2011 from naturally infected plants of ribwort collected from the production plantations situated in the south-eastern Poland were used [Zimowska 2013]. The proportion of the fungus isolates in successive years of the studies was, respectively, 33.99, 44.86 and 33.89%. The morphological characteristic of this isolates was performed on malt agar medium (MA; Difco Laboratories, Detroit, USA), according to the descriptions given by Saccardo [1878] and van der Aa and Vanev [2002].

Pathogenicity of isolates to leaves of ribwort and inoculation techniques. Inoculum of five randomly selected isolates of *P. plantaginis* was prepared by growing each isolate on malt medium for two weeks at the temperature of 22ºC, without any light access. Three methods of inoculation were used to prove the pathogenicity. In the first method disks (5 mm diameter) cut out from 2-week-old agar cultures of each isolate were used. Those plugs were placed at six places on the leaf blades which were surface – sterilized by soaking in 10% blench (0.525% sodium hypochlorite) solution for 60 seconds [Zimowska 2012b]. In the second method, the disks were placed at six places on the leaf blades injured with a needle puncture. Methods I and II included for six discs of medium overgrown by mycelium placing on the top surface of leaves. In the third method a suspension containing 10^6 of conidia per 1ml was used. It was obtained by rinsing the surface of the cultures of particular isolates with sterile distilled water [Roustaee et al. 2000]. The disinfected, non – injured leaf blades were soaked in the suspension for 5 minutes [Zimowska 2012b]. All inoculated leaves were placed in Petri dishes with the diameter of 14 cm, laid with three layers of cellulose tissue and one layer of filter paper moistened with 5 ml of distilled sterile water [Zimowska 2004]. For

each method 150 leaves were used (5 isolates × 30 leaves). Control leaf blades were inoculated with sterile agar disks (methods I and II) or sterile water (method III). The experiment was conducted twice with one week interval. Petri dishes with inoculated leaves were kept in a thermostat at the temperature of 22ºC 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 [Marcinkowska and de Gruyter 1996]. Next, all leaf blades 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 leaf blades inoculated with the 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 5ºC. 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 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. Observations of six samples were carried out at different time intervals after inoculations: 7, 18, 25, 48 and 72 hours. Micrographs were obtained using a Vega 2, Tescan scanning electron microscope.

RESULTS

Fungal isolates. All isolates of *P. plantaginis* were obtained from the leaves of ribwort with the symptoms of small, regular, necrotic spots of 3 to 5 mm (phot. 1). On the surface of such spots, etiological signs in the form of pycnidia, including conidia typical for the genus *Phyllosticta* were observed. All isolates were morphologically similar and they were identified as *P. plantaginis* on the basis of the growth of the colonies on malt agar medium (phot. 2) as well as pycnidia and conidia characteristics (phot. 3).

Pathogenicity of isolates to leaves of ribwort. All studied isolates of *P. plantaginis* caused characteristic symptoms on the inoculated leaves of ribwort. As early as 4 days after the inoculation, symptoms were observed on the leaves inoculated according to methods II and III. After 6 days, the symptoms were seen on leaves inoculated according method I. These symptoms were similar to those observed on the leaves of ribwort in the conditions of field cultivation. Methods II and III proved to be the most effective for inoculation. Values of the infection index were, respectively, 94.49 and 90.17% and they were significantly higher than obtained for method I (tab. 1). The infection values of P 938/2011 isolate were significantly higher than the values obtained for the isolate P 743 tested according to method I (tab. 2), isolate P 201 in method II (tab. 3), as well as isolates P 395/2010 and P 743 which were tested according to method III (tab. 4). *P. plantaginis* cultures were reisolated from all inoculated leaves, for all methods (tab. 2, 3, 4). Morphological features of reisolated cultures corresponded to the features of cultures used for inoculation. Control leaves remained symptomless, and the results from the two experiments were similar.

Phot. 1. Necrotic, regular spots from which *Phyllosticta plantaginis* was isolated. Photo B. Zimowska

Photo 2. 14-day-old colony of *Phyllosticta plantaginis* on malt agar MA. Photo E. Zalewska

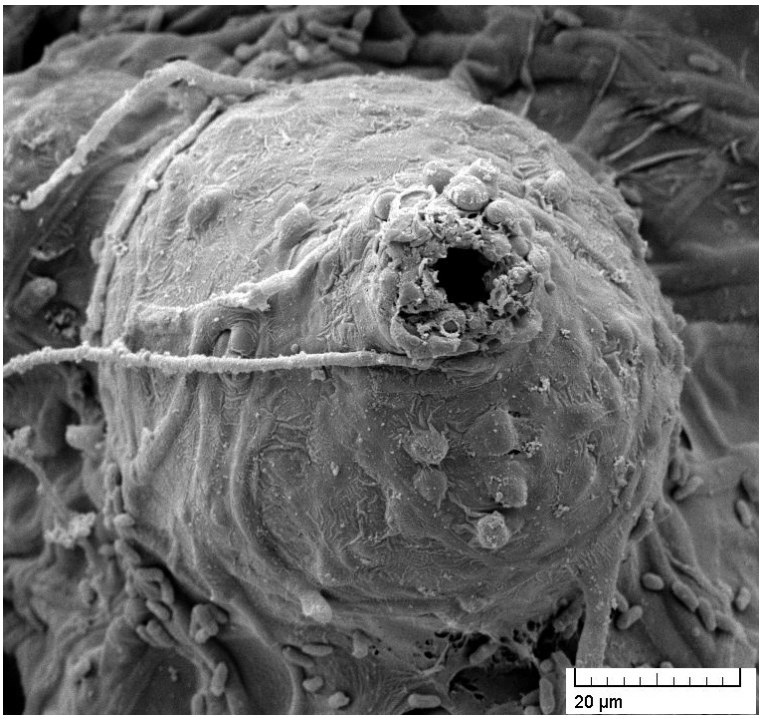

Photo 3. Typical pycnidium and conidia of *Phyllosticta plantaginis* from malt agar MA. Photo M. Wróbel

Table 1. Pathogenicity of *Phyllosticta plantaginis* to leaves of ribwort using various inoculation methods (means for 5 isolates)

Inoculation method	Infection index (%) after 12 days [x]
Colonized medium placed at non – injured epidermis – method I	83.38 a
Untreated	0
Colonized medium placed at injured epidermis – method II	94.49 b
Untreated	0
Leaves soaked in conidial suspension (1×10^6 conidia/ ml) – method III	90.17 b
Untreated	0

HSD = 5.0238

[X] Infection index for I and II method evaluated on the basis of 3-degree disease scale :0º – lack of disease symptoms; 1º – up to 50% inoculated places on the leaf showed disease symptoms; 2º – 50 to 100% inoculated places on the leaf showed disease symptoms

[X] Infection index for III method evaluated on the basis of 5-degree disease scale: 0º – lack of disease symptoms; 1º – single necrotic spots visible on up to 25% surface of the leaf; 2º – 25 to 50% surface of inoculated leaf showed disease symptoms; 3º – 51 to 75% surface of inoculated leaf showed disease symptoms; 4º – 76 to 100% surface of inoculated leaf showed disease symptoms

Values marked with the same letter do not differ significantly

HSD – Honest Significant Difference

Table 2. Effect of ribwort inoculation with method I by *Phyllosticta plantaginis* isolates on occurrence leaf spot (mean of 6 replication)

Isolate	Infection index (%) after 12 days[x]	Reisolation (%)[y]
P 201	84.25 ab	100
P 289	82.38 ab	100
P 395 / 2010	81.33 ab	100
P 743	80.25 a	100
P 938 / 2011	88.67 b	100
Untreated	0	0

HSD = 7.9433
Note: see table 1
[y] For leaves showing spot symptoms, percent isolations that resulted in *P. plantaginis* colonies

Table 3. Effect of ribwort inoculation with method II by *Phyllosticta plantaginis* isolates on occurrence leaf spot (mean of 6 replication)

Isolate	Infection index (%) after 12 days[x]	Reisolation (%)[y]
P 201	90.55 a	100
P 289	91.33 b	100
P 395 / 2010	97.25 ab	100
P 743	94.67 ab	100
P 938 / 2011	98.67 b	100
Untreated	0	0

HSD = 8.0215
Note: see table 1 and 2

Table 4. Effect of ribwort inoculation with method III by *Phyllosticta plantaginis* isolates on occurrence leaf spot (mean of 6 replication)

Isolate	Infection index (%) after 12 days[x]	Reisolation (%)[y]
P 201	92.25 ab	100
P 289	89.21 ab	100
P 395 / 2010	86.38 a	100
P 743	85.55 a	100
P 938 / 2011	97.45 b	100
Untreated	0	0

HSD = 8.9433
Note: see table 1
[y] For leaves showing spot symptoms, percent isolations that resulted in *P. plantaginis* colonies

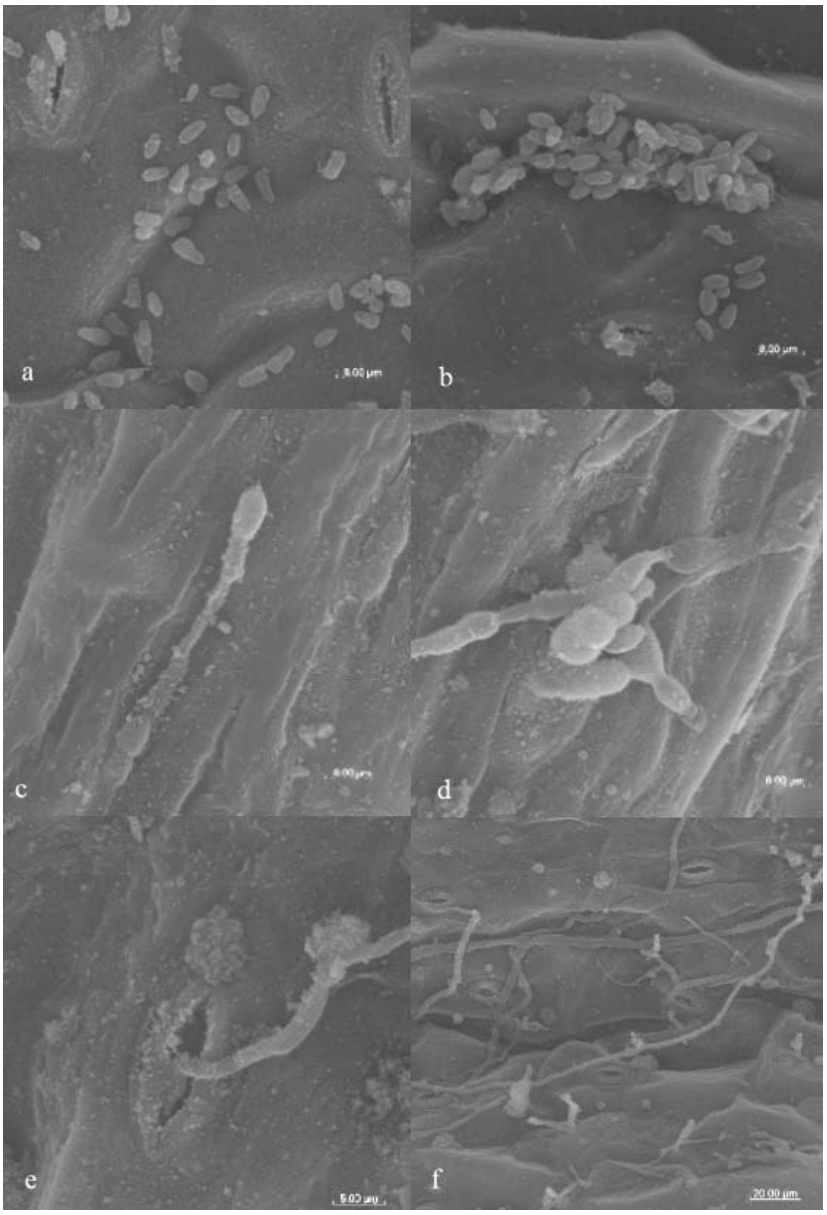

Photo 4. Scaning electron micrographs of *Phyllosticta plantaginis* on ribwort leaves. (a) view of conidia 7 h after inoculation; (b) germinated conidia 18 h after inoculation; (c) adhesion structure in the form of appressorium on the end of germ tube 25 h after inoculation; (d) development of appressoria were present in some distance of the stomata; (e) penetration peg from the appresorium entered the leaf through the stomatal opening after 48 h after inoculation; (f) development of branched hyphae at the surface of cuticle 72 h after inoculation. Photo M. Wróbel

Scanning electron microscope. Seven hours after inoculation, conidia of *P. plantaginis* were visible on the surface of ribwort leaves (phot. 4a). After 18 hours, single conidia formed germ tubes of the length not exceeding 5 μm (phot. 4b). Twenty five hours after inoculation, an adhesive structure in the form of an appressorium was seen at the end of the germ tube (phot. 4c). Appressoria were mostly found on cuticle, at a certain distance from the stomata (phot. 4d). After 48 hours, a penetration peg from the appresorium entered the leaf through the stomatal opening (phot. 4e). After 72 hours, branched hyphae of *P. plantaginis* were observed on the surface of the cuticle of ribwort leaves (phot. 4f).

DISCUSSION

Knowledge of disease symptoms caused by *Phyllosticta* genus fungi, including *P. plantaginis*, is of importance both for taxonomists and phytopathologists. It allows to find out the appearance of disease in natural conditions, and next to identify the pathogen. Characteristic symptoms, like those that occurred on naturally infected plants, in the form of 2–4 mm regular, necrotic spots observed in the present studies in field conditions fulfil one of Koch's postulates. Pathogenicity studies showed that all tested isolates of *P. plantaginis* caused infection on the inoculated ribwort leaves, which is shown in the high values of the infection index, although differentiated pathogenicity was observed within the tested isolates. Besides, the fulfillment of Koch's other postulates, that is a 100% reisolation of the pathogen and the consistence of morphological features of the reisolated cultures with the features of the isolates used in the studies, testifies to the pathogenic character of the tested isolates of *P. plantaginis*.

Among the three inoculation methods considered in the studies, the most effective proved to be the one consisting in placing disks of medium overgrown by mycelium on the injured leaf tissue, and the method in which the leaves were soaked in the infection suspension. Those results agree with the literature information, according to which the above mentioned methods also turned out the most effective for other pathogens closely related to genus *Phyllosticta* [van der Aa and Kestereen 1971, van der Aa and Vanev 2002] i.e.: *Phoma lingam* [Sock and Hoppe 1999], *P. nigrificans* [Marcinkowska and de Gruyter 1996], *P. multirostrata* [Garibaldi et al. 2010] and *Boeremia strasseri* comb. nov. (*Phoma strasseri*) [Zimowska 2012b].

The ultrastructural investigation of the artificial inoculation of ribwort leaves with *P. plantaginis* conidia showed penetration through the stomata with appressorium formation. The natural openings enabling pathogens to penetrate into the plants are spiracles, hydatodes, secretory channels in nectaries and stomata. The movement of spores towards the stomata and their germination is simulated by different chemical substances secreted by the stoma. *P. plantaginis* conidia germinated and formed appressoria not directly at the wall of the stoma cell but at a certain distance from it. A similar manner of germination was observed on soybean seedlings inoculated with *Phomopsis phaseoli* [Kulik 1988] and on the leaves of mulberry that were infected with *Colletotrichum dematium* [Babu et al. 2008]. According to Wood [1967], fungi anamorphs usually infect leaves through the stomata, which was confirmed in the present studies. This way

of infection was also documented for other leaf pathogens, e.g. *Leptosphaeria maculans* (*Phoma lingam*) [Hammond et al. 1985], *Ascochyta pisi* [Heath and Wood 1969], *Septoria tritici* [Cohen and Eyal 1993] and *S. apicola* [Donovan et al. 1990]. The formation of appressorium by fungi is known to depend on such factors as the presence of the *epicuticular wax and the stiffness and hardness of the substrate* [Höhl et al. 1990]. The formation of this structure, important in the process of infection, in the place where appressorium is formed is stimulated by means of protein kinases in the hyphae, which next leads to the accumulation of sugars and lipids necessary for its functioning, [Hames and Hooper 2000]. Appressorium formation was earlier observed in a few species of fungi connected with genus *Phyllosticta* i.e. *Phyllosticta ampelicida* [Kuo and Hoch 1995], *Phomopsis helianthi* [Muntanola-Cvetkovic 1989], *Boeremia strasseri* comb. nov. [Zimowska 2012b], *Ascochyta pisi* [Heath and Wood 1969] and *A. rabiei* [Höhl et al. 1990].

Further histopathological studies are necessary to examine the process of colonization of ribwort plantain leaves by *P. plantaginis,* and to explain the role of metabolites, i.e. phyllostine and phyllotoxin [Tuzi et al. 2010] in the infection process and in pathogenesis.

CONCLUSIONS

1. *P. plantaginis* were found to be occasional pathogen of ribwort.
2. The infection process of *P. plantaginis* takes place with appressorium formation.
3. The leaves of ribwort are invaded by *P. plantaginis* through the stomata.

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PATOGENICZNOŚĆ I ULTRASTRUKTURALNE BADANIA NAD MODELEM INFEKCJI LIŚCI BABKI LANCETOWATEJ PRZEZ *Phyllosticta plantaginis*

Streszczenie. W pracy podjęto badania nad spełnieniem postulatów Kocha dla pięciu izolatów *Phyllosticta plantaginis* oraz ultrastrukturą inokulowanych liści babki w celu wyjaśnienia powinowactwa pomiędzy *P. plantaginis* a rośliną żywicielską. Badania wykazały, że wszystkie testowane izolaty spowodowały infekcję na inokulowanych liściach babki. Z trzech uwzględnionych w badaniach metod inokulacji, najbardziej efektywnymi okazały się metoda polegająca na umieszczaniu krążków pożywki przerośniętej przez grzybnię na zranionej tkance liścia oraz metoda uwzględniająca zanurzenie liści w zawiesinie zarodników konidialnych. Obserwacje ultrastruktury inokulowanych liści babki prowadzono w mikroskopie skaningowym po 7, 18, 25, 48 oraz 72 godzinach. Wykazały one tworzenie się appresorium na końcu strzępki kiełkowej oraz wnikanie patogenu przez aparaty szparkowe.

Słowa kluczowe: *Plantago lanceolata*, herbs, infection process

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