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Research Article

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Occurrence and Characterization of *Phyllosticta plantaginis*

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Abstract

During 2009 to 2011 in southeastern Poland the species *Phyllosticta plantaginis* was isolated from leaves of ribwort (*Plantago lanceolata* L.) showing the symptoms of small, regular, necrotic spots. Six isolates were randomly chosen from the fungus population and their morphology and conditions for growth and sporulation were studied .In addition, the growth of isolates was compared on seven different agar media at 24°C. The temperature range of 16°C to 28°C was considered optimal for the growth of this fungus; 20°C to 28°C was the optimal for the formation of pycnidia and conidia. Considering morphology and quantity of typical macro- and microscopic conidiophores, the malt agar medium and the media made with an extract from ribwort leaves are recommended for use in characteristic observations of this pathogen. Ultra structural observations of morphological structures were undertaken using scanning electron microscopy. The presence of a characteristic cap of compact hyphae which closed the ostiole was visible. The ostiole was filled with round pores. When the conidia were released from the pycnidia, the cap came off and the pore network broke.

Keywords: *Plantago lanceolata*; Ribwort; Morphology; Pycnidia

Introduction

In the taxonomic system presented by Saccardo in the middle of the 19th century, and based mainly on the appearance of fungi in vitro, the genus *Phyllosticta* which encompasses about 2,000 species, was characterized as a family of fungi that contained single-cell, hyaline conidia that formed in pycnidia on the leaves [1]. This system, however, proved to be too simple and vague since both single-cell and multi-cell spores can be found in the pycnidia formed on the leaves. The consequence of using this system lead to a problem that similar species were described under different names, hence, they had synonyms in a few morphologically similar genera [2]. Taxonomic research conducted in the 1970's, which also considered in vitro observation, reported that 46 species belonged to genus *Phyllosticta* Pers. *Sensustricto* [3]. Those fungi within the genus *Phyllosticta,* included telemorphic stages of *Guignardia that* cause spots, not only on leaves, but also on fruit and young shoots, thus constituting an economically important group of fungi [4-6]. *Phyllosticta plantaginis* Sacc. is described in the literature as a pathogen causing spots of the leaves of ribwort (*Plantago lanceolata* L.) and broadleaf (*Plantago major* L.).

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In recent years, in southeastern Poland different species of spice and medicinal plants, including *Plantago lanceolata* L., have been often cultivated. The Interest in the cultivation of ribwort results from the demand for the raw material of *Plantaginis lanceolatae folium,* which contains valuable iridoid glucosides, especially aucubin and catalpol. Grouping of the herbaceous plants in this region causes their frequent cultivation to the same fields, which has led to the accumulation of fungi decreasing the quality and quantity of the yield [7]. Crop losses due to disease causes by *P. plantaginis* range from 10% to 25% (personal communication).

Research conducted in 2009-2011 on production plantations of ribwort growing in south-eastern Poland demonstrated colonization of ribwort leaves by *P. plantaginis,* which had not been recorded in Poland. Therefore, in this paper we characterized Polish isolates *P. plantaginis* morphologically and biologically.

Materials and Methods

Isolation and identification of *Phyllosticta plantaginis*

The research material consisted of isolates of *P.plantaginis* Sacc obtained from the leaves of one – year-old plants of ribwort plantain grown on three plantations in the Lublin province in 2009-2011. The fore crops on those plantations were usually other herbs, e.g. lemon balm, common thyme and motherwort. Each year the percentage of plants with disease symptoms was established twice during the vegetation period, directly on the plantation. From 2009 to 2011, sets of twenty symptomatic ribwort plants were collected each year from three plantations. For mycological analysis, from each plant five symptomatic leaves were taken. Selected leaf blades were surface-sterilized by soaking in a 10% bleach (0.525% sodium hypochlorite) solution for 3 min and then rinsing three times with sterile distilled water. Small (approximately 3 x 3 mm) section of tissue were aseptically excised and placed into 90 mm diameter petri plates (10 pieces/plate) containing mineral medium (0.7 g NH_4NO_3, 0.3 g KH_2PO_4, 0.3 g $MgSO_4$ x $7H_2O$, 0.01 g $FeCL_3$ x $6H_2O$, 0.01 g $ZnSO_4$ x $7H_2O$, 0.01 g $CuSO_4$ x $7H_2O$, 0.01 g $MnSO_4$ x $5H_2O$ + 38 g saccharose + 20 g agar + 1000 ml H_2O) [7]. Within 3 days of incubation in the dark at 24°C, small parts of colonies growing around inocula were transferred into malt agar medium (MA; Difco Laboratories, Detroit, USA) slants [8]. After 10 days, the obtained isolates were segregated and identified according to the descriptions given by Saccardo [9] and van der Aa and Vanev [6].

Effect of temperature and culture medium on growth and conidia production

Six isolates of the fungus 110, P 184, P 198, P 201, P 254 and P 415, were randomly chosen from the collection of single-spore cultures for further studies. Each isolate was cultured at the temperatures of - 6°C, 5°C, 10°C, 16°C, 20°C, 24°C, 28°C,and 32°C on malt agar medium. The growth of isolates was observed on the following media at 24°C: malt agar (MA), oatmeal agar (OA; 20 g oat-flakes + 1000ml H_2O + 20 g agar), cherry agar (CA; 100 ml juice of 500 g cherries + 900 ml H_2O + 20 g agar),potato dextrose agar (PDA; Difco Laboratories, Detroit, USA), Czapek-Dox Broth (Difco, Becton, Dickinson and Company

Sparks, USA), malt agar with an addition of ribwort fragments (50g/ 2-3 mm leaf fragments/ 1l water + 20 g agar) [10] and malt agar with a water extract from ribwort leaves (50 g leaves/ 1l water + 20 g agar) [8]. The inoculation material consisted of rings of sporulating mycelium with the diameter of 5 mm cut from 14 – day-old mother cultures growing on the malt medium at 24°C. Four repetitions were used for each isolate. Observations of the radial growth were made for 14 days, and the formation of morphological structures was assessed for up to 30 days [11]. The colony diameter was measured every second day. The results obtained from the experiment were subjected to statistical analysis using a two-factor variance analysis ANOVA – according to SAS program. Simultaneously, the macroscopic features of the colonies were observed. The color of the obverse and the reverse, the character of the growth of the colony's edge and the structure of the aerial mycelium were determined. Colony color was determined using the color charts of Rayner [12]. In addition, the shape of the pycnidia, the character of the ostiole, the color of the conidial exudates and the structure of the pycnidial wall were studied. One hundred twenty pycnidia (6 isolates x 20 pycnidia) and 240 conidia (6 isolates x 40 conidia) were measured. Photographic documentation was made using light and scanning electron microscopy (Vega, Tescan).

Results

Isolation and identification of *Phyllosticta plantaginis*

Isolates of *P. plantaginis* were obtained from ribwort leaves with small, regular, necrotic spots of 2 to 4 mm diameter (Figure 1), where pycnidia and conidia were observed that were typical of the genus *Phyllosticta* (Figure 2). The percentage of plants with disease symptoms ranged from 10% to 20% at the beginning of vegetation and from 15% to 30% at full vegetation. The proportion of the fungus isolates in successive years of the studies was, respectively, 33. 99%, 44.86% and 33.89%

In total, 161 isolates of *P. plantaginis* were obtained during the 3 years of studies, which constitutes 32.99% of all fungi obtained from the analyzed leaves of ribwort plantain (Table 1).

Effect of temperature and culture medium on growth and conidia production

The studies conducted in vitro indicated differentiation in the growth of *P. plantaginis* colonies grown at different temperatures. At -6°C none of the examined isolates formed any aerial mycelium until the 14th day of the observation (Figure 3). The cultures were then transferred from -6°C to 24°C. After 4 days, *P. plantaginis* isolates formed aerial mycelium, while after 8 days; pycnidia appeared with conidia in them. At 32°C, the colony growth was observed already after 2 days of incubation. The aerial mycelium was of white cream color and had a compact structure. After 6 days, numerous round swellings were visible on the hyphae (Figure 4b). At 32°C, none of the studied isolates formed pycnidia until the last day of the observation. At temperatures ranging from 16°C to 28°C, a dynamic growth of the colonies was observed after 2 days of culturing. All isolates formed olive grey aerial mycelium with a fluffy structure and a white margin of paraphysae. The edge of the colonies was regular. The color of the reverse was similar to that of the obverse (Figure 3). After 4 days at 5°C and 10°C, loose, delicate, white cream hyphae of the aerial mycelium were observed. On the successive days of the observations the color and structure of the mycelium did not change. The averse and the reverse of the colonies were white cream color (Figure 3). At the temperatures ranging from 20°C to 28°C the formation of single pycnidia was observed as early as 4 days of culturing. On the following days the number of pycnidia increased. They were formed on the whole area of the colony or, sometimes, in the sectors. After

Figure 1: Necrotic, regular spots from which *Phyllosticta plantaginis* was isolated (photo B. Zimowska).

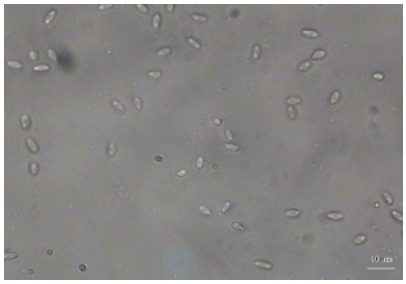

Figure 2: Typical hyaline and aseptate conidia of *Phyllosticta plantaginis* (photo E. Zalewska).

Table 1: Participation of *Phyllosticta plantaginis* isolates in fungal communities obtained from the diseased leaves of ribwort in 2009-2011.

Years	Number(and percent) of isolates			
	2009	2010	2011	total
Phyllosticta plantaginis	52 (33,99)	48 (44,86)	61 (33,89)	161 (32,99)
Otherspecies of fungi	101	107	119	327
Total	153	155	180	488

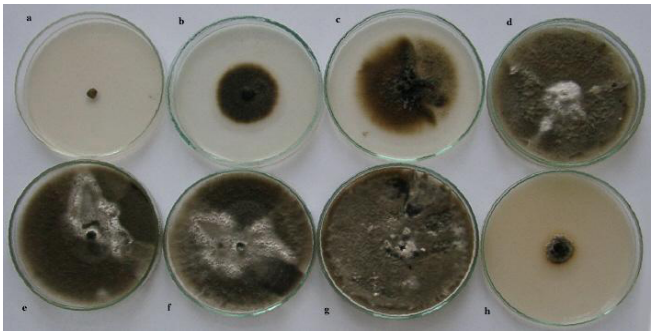

Figure 3: 14 – day – old colonies of *Phyllosticta plantaginis* on malt agar MA at different temperature: - 6°C (a); 5°C (b); 10°C (c); 16°C (d); 20°C (e); 24°C (f); 28°C (g); 32°C(h).Each temperature was replicated four times with each petriplate considered a replicate (photo E. Zalewska).

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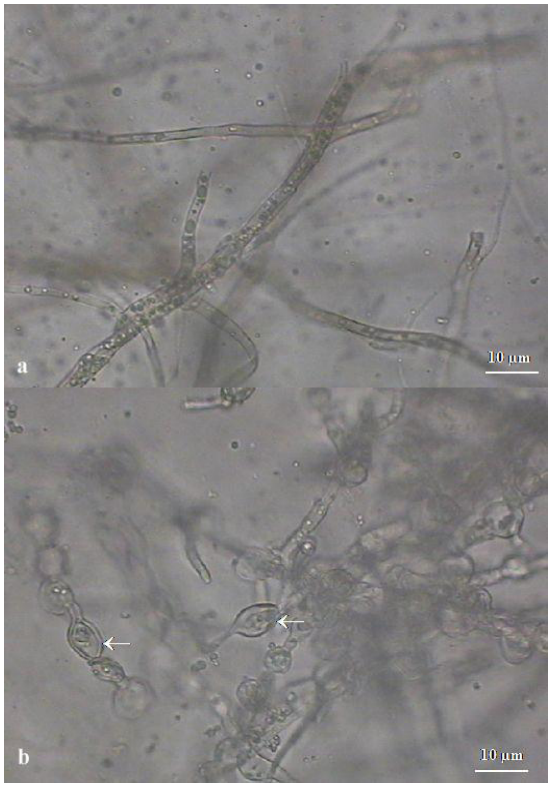

Figure 4: Hyphae without swellings at the temperature 24°C (a); round swellings on the hyphae of *Phyllosticta plantaginis* incubated at the temperature of 32°C (b - arrows) (photo E. Zalewska).

8 days, beige-salmon pink drops of conidial exudate were also visible (Figure 5). At 16°C, the first pycnidia were visible after 6 days of culture incubation, while at 10°C the pycnidia were visible after 8 days. At 5°C none of the studied isolates formed pycnidia until the 30th day of observations.

The diameter of the colonies of *P. plantaginis* isolates growing at the same temperature did not differ significantly except isolate P 110 growing at the temperature of 32°C (Table 2). The biggest, maximal diameter was achieved by the fungus isolates at the temperatures of 16°C,20°C,24°C and 28°C which differed significantly from isolates incubated at5°C,10°C and32°C (Table 2).

The studies on the effect of culture media on the growth and formation of *P. plantaginis* did not show any significant differences in the colony diameter (Table 3). On all media, the diameter of the colonies of the analyzed isolates after 14 days reached the maximum value, with an exception of Czapek-Dox, where only isolate P 198 reached the maximum diameter (Table 3). On the majority of the tested media, *P. plantaginis* isolates formed an olive grey air mycelium of a fluffy structure typical of this species (Figure 6). It was only on Czapek-Dox medium that the colonies were of cream pink color and the hyphae of the air mycelium formed quite a compact structure (Figure 6). No pycnidia were formed on Czapek-Dox until the 30 day of the observations. Isolates of *P. plantaginis* began to sporulate earliest on malt agar with the leaf extract of ribwort and leaf fragments. Pycnidia, with numerous conidia in them, were formed as soon as 2 days after culturing. The number of pycnidia grew in the following observations. They were formed on the whole area of the colony or in the sectors (Figure 7). After 6 days, a conidial exudate

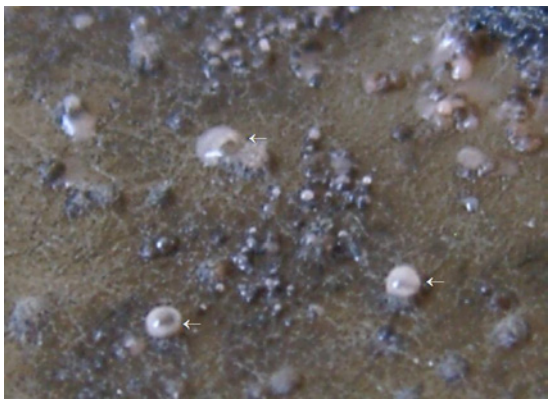

Figure 5: Beige-salmon pink drops of the conidial exudate of *Phyllosticta plantaginis* (arrows) (photo E. Zalewska).

Table 2: The effect of temperature on the diameter of 14-day-old colonies of *Phyllosticta plantaginis* on malt agar (MA).

Temp.	32°C			28°C			24°C			20°C			16°C			10°C			5°C			-6°C		
Isolates	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2
Pp 110	12,7	d	B	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	61,0	b	A	45,5	c	A	5,0	e	A
Pp 184	21,0	d	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	65,0	b	A	43,5	c	A	5,0	e	A
Pp 198	22,2	d	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	66,5	b	A	47,8	c	A	5,0	e	A
Pp 201	21,0	d	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	65,0	b	A	44,3	c	A	5,0	e	A
Pp 254	19,3	d	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	64,5	b	A	42,6	c	A	5,0	e	A
Pp 415	23,0	d	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	64,5	b	A	40,2	c	A	5,0	e	A

x- diameter of colonies in mm; p_1- differences depending on temperature for isolate- small letters; p_2- differences between isolates at given temperature-capital letters. The means differ in a significant way if they are not marked with the same letter at α=0,05 (Tukey's test)

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Table 3: The effect of culture media on the diameter of 14-day-old colonies of *Phyllosticta plantaginis* at 24°C.

Agar media Isolates	PDA			MA			OA			CA			Czapek-Dox			Malt agar with a water extract from ribwort leaves			Malt agar with an addition of leaf fragments		
	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2	x	P_1	P_2
Pp 110	88,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A
Pp 184	90,0	a	A	89,2	a	A	87,2	a	A	90,0	a	A	85,7	a	A	86,5	a	A	90,0	a	A
Pp 198	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A
Pp 201	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	85,0	a	A	89,5	a	A	90,0	a	A
Pp 254	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	87,0	a	A	90,0	a	A	90,0	a	A
Pp 415	90,0	a	A	90,0	a	A	90,0	a	A	90,0	a	A	87,7	a	A	90,0	a	A	90,0	a	A

x- diameter of colonies in mm; p_1- differences depending on culture medium for isolate- small letters; p_2- differences between isolates at given culture medium-capital letters. The means differ in a significant way if they are not marked with the same letter at α=0,05 (Tukey's test)

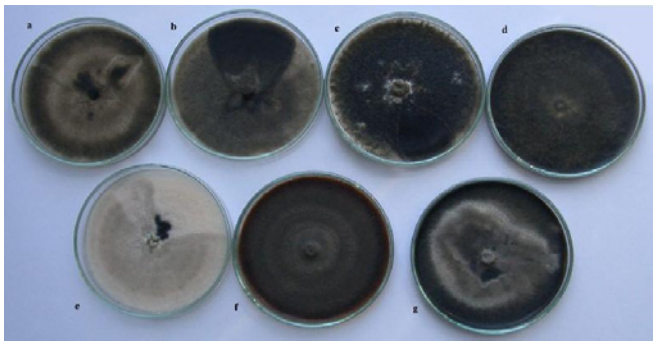

Figure 6: 14 – day-old colonies of *Phyllosticta plantaginis* on different culture media: PDA (a); MA (b); OA (c); CA (d); Czapek-Dox (e); malt agar with a water extract from ribwort leaves (f); malt agar with an addition of leaf fragments (g). Each medium was replicated four times with each petriplate considered a replicate (photo E. Zalewska).

of beige-salmon pink color was observed on the surface. Structures of conidial sporulation appeared after 4 days on PDA, MA, CA and OA. Conidia formed numerously throughout the area, while beige-salmon pink drops of the conidial exudate flowed from the ostioles (Table 4).

The pycnidia formed on MA medium were round or slightly flattened and brown, with a thick wall. The wall of pycnidia was covered with soft mycelial outgrowths, which were also found on the ostioles (Figure 8a and 8b). They appeared singly or in small clusters (Figure 8a). One or two verrucoseostioles were found at the top of the pycnidia using SEM (Figure 8b). Initially, the ostioles were closed with a characteristic cap built of compact hyphae (Figure 8c). After some time, the cap opened. The ostiole itself was filled with round pores (Figure 8d). After the conidia were released from the pycnidia and the cap came off, the pore network broke apart (Figure 8e). The sizes of the pycnidia were, on average, 63-82 µm (Table 4). The conidia were hyaline, single-cell, oval to ellipsoidal, with the average length ranging from 5.2 µm to 5.5 µm and the width from 1.8 µm to 2.2 µm (Table 4, Figure 8f).

Discussion

Phyllosticta plantaginis was isolated from the leaves of ribwort showing small, regular, necrotic spots and pycnidia with conidia typical for *Phyllosticta* sp. The positive results of pathogenicity tests of the fungus isolates conducted on ribwort plantain leaves in *invitro* conditions (oral communication), verified that *Phyllosticta* caused the symptoms. The limited information on *Phyllosticta* includes a short description of disease symptoms and morphological structures [6,9,13]. Nothing has been reported on the biological requirements of this pathogen. In vitro studies provided the thermal requirements of *P. plantaginis* and the media for its growth and the development. The temperature range of 16°C to 28°C was optimum for the growth of the fungus, while the thermal optimum for the formation of pycnidia and conidia was 20°C to 28°C. The average temperature in Poland during vegetation period ranges from 13°C to17°C in May and April and from 18°C to 29°C in June, July and August. It follows from the present studies that the thermal conditions in Poland during the growing season of ribwort are conducive for the growth and formation of *P. plantaginis* conidia, which can result in dynamic development of disease symptoms on ribwort leaves.

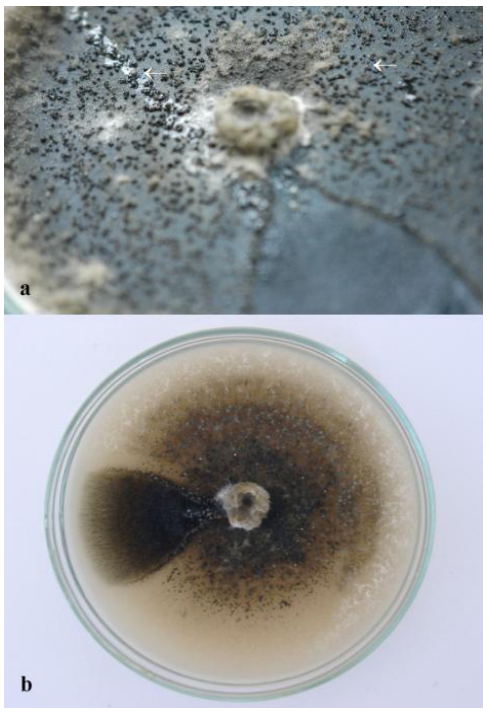

Figure 7: Pycnidia in sectors of *Phyllosticta plantaginis* on malt agar MA (a - arrows) (photo E. Zalewska).

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Table 4: Morphology and the size (µm) of pycnidia and conidia of *Phyllosticta plantaginis* on malt agar MA (mean for 6 isolates).

Author	The shape of pycnidia	Characterof pycnidiaostiole	Colour and the structure of the pycnidial wall surface	The size of pycnidia (µm)	Colour of the conidial exudates	The shape of conidia	The size of conidia(µm)
Own data	Round or slightly flattened	One or two verrucoseostioles which opened the characteristic cap. The ostiole is filled with round pores	Pycnidia brown with thin wall	63 x 82	Beige-salmon pink	Oval to ellipsoidal, hyaline, aseptate	5,2-5,5 x 1,8-2,2
Saccardo 1887	Round or slightly flattened	The ostiole is filled with round pores	Pycnidia brown with thin wall	60 x 80		Oval to ellipsoidal, hyaline, aseptate	5 x 2
Van der Aa &Vanev 2002						Oval to ellipsoidal, hyaline, aseptate	5 x 2

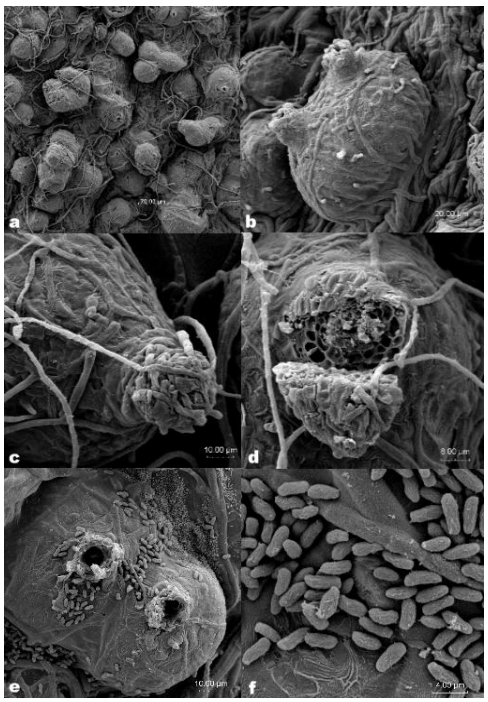

Figure 8: Scanning electron micrographs of *Phyllosticta plantaginis*: groups of pycnidia (a); verrucoseostioles of pycnidia (b); characteristic cap on the top of the pycnidialostiole(c); round poresfilles the ostiole of pycnidium (d);broken pore network in the pycnidialostiole (e); conidia of *Phyllosticta plantaginis* (f) (photo M. Wróbel).

P. plantaginis can therefore be included within the group of eurythermic organisms, which can live and sporulate in a wide range of temperatures [14,15]. The thermal requirements of *P. plantaginis* are similar to those of other species from genus *Phyllosticta*, i.e. *P. vaccini* [4], *P. beaumorisis* sp. nov. [16] or *P. zingiberi* [17]. American researchers proved that the most dynamic development of disease symptoms on the leaves and fruit of Northern high bush blueberry inoculated with an infection suspension of *P. vaccine* takes place at 28°C [4]. The majority of species of *Phyllosticta* spp. Described in the literature infect thermophilic plants growing in the tropical or sub-tropical climates [18,19], which can testify to high thermal requirements of those fungi, and is also confirmed by the present studies. Although the temperature of -6°C can be considered negative for the growth of *P. plantaginis,* it was not fatal for the fungus, since after transferring the cultures to the temperature of 24°C the isolates resumed the growth, even beginning to sporulate. This suggests that pathogen's mycelium might live on the residues of the infected leaves of ribwort plantain. In spring, when the temperature begins to increase, pycnidia, with propagation spores that cause the primary infection of the leaves, probably begin forming in the overwintering mycelium. That has led to the recommendation to deep plough in autumn to bury the infected plant residues deeper to minimize the source of the primary infection. At 32°C, the studied isolates of *P. plantaginis* formed round dark swellings on the hyphae demonstrating the negative effect of this temperature on the development of the fungus. The formation of this type of morphological structure was also observed in other cultured fungi in stress conditions [8,11].

The present studies showed that all the tested culture media, with an exception of Czapek-Dox, were adequate for the growth and formation of *P. plantaginis* conidia. However, due to the formation of the typical macro- and microscopic features and the most intensive sporulation, the malt agar and the media made with an extract from ribwort leaves and fragments of leaves should be recommended for the culture and identification of the pathogen. The stimulating effect of the two media on the sporulation of *P. plantaginis* likely results from the presence of specific substances in the ribwort plantain leaves. Incorporation of plant material into media has improved growth of other species of pathogens such as *Colletotrichum dematium* [10] and *Seimatosporium hypericinum* [20].

The present studies presented information on the morphological structures of *P. plantaginis*, which filled the gap in the literature. A taxonomic feature that should be considered significant, but is little discussed in mycological papers, is the characteristic structure of the pycnidia ostiole in the form of porous openings through which conidia escape. The second feature which deserves attention is the opening of the ostiole of pycnidia with a characteristic cap built of compact hyphae. Our electron – microscopic observations on the pycnidia structure and the conidia extraction in *P. plantaginis* have furnished more precise information on the essential features of this species. Studying the aforementioned elements of the morphology of *P. plantaginis* pycnidia was possible through the use of scanning microscopy. Therefore, in taxonomic research the use of electron microscopy to define the correct taxa within the genus *Phyllosticta* is very important.

The fact that *P. plantaginis* isolates were obtained for the first time in Poland from the leaves of ribwort showing specific diseases symptoms indicates that the studied species can have a negative effect on the quality of the material of *Plantaginis lanceolatae folium.*

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Phyllosticta sp. Can form toxic metabolites [21], which could lead to accumulating undesirable substances in ribwort leaves and a negative effect on health.

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