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DIVERSITY OF SOOTY BLOTCH FUNGI IN POLAND

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Abstract. Sooty blotch is one of the most common disease of apples in organic orchards in many countries. Results of molecular studies performed in USA indicated approximately 30 different fungi species associated with this disease. Fungi species causing sooty blotch in Northern, Central and Eastern Poland were identified on the basis of morphology and nucleotide sequence of the rDNA internal transcribed spacer region (ITS). A total 245 isolates were collected in spring and early summer in the years 2006–2009 from fruits with visible symptoms of the disease. Isolates were grown on PDA medium and identified on the basis of morphological characters. DNA was extracted from representative isolates and used as matrices for PCR amplification with ITS1F and ITS4 primers. Fragments of amplified rDNA ITS were sequenced. It was found that 66.53% of all isolates causing sooty blotch were species from genera *Microcyclosporella,* followed by *Aureobasidium pullulans* – 22.86%, *Microcyclospora* sp. – 6.12%, *Phialophora sessilis* – 3.67%, *Peltaster* sp. and *P. fructicola* – 0.41%.

Key words: apple, Microcyclosporella sp., Microcyclospora sp., Peltaster fructicola

INTRODUCTION

Sooty blotch is one of the most common disease of apples (*Malus* × *domestica* Borkh) in organic orchards in many countries. Reports published before 1997 indicated that this disease was caused by *Gloeodes pomigena* (Schwein) Colby [Baines and Gardner 1932, Brown and Sutton 1993, Groves 1933, Wilcox 1994]. Further studies shown that etiology of sooty blotch was complex and at least three species: *Leptodontidium elatius* (F. Mangenot) de Hoog, *Peltaster fructicola* Eric M. Johnson, T. B. Sutton et Hodges and *Geastrumia polystigmatis* Batista et M. L. Farr [Johnson et al. 1996, 1997, Tarnowski et al. 2003, Williamson et al. 2004, Williamson and Sutton 2000] were considered as causal agents. Subsequently, additional species were identified as causing sooty blotch: *Stomiopeltis uniloculata* and *Stomiopeltis multiloculata* [Abad et al. 2005], *Phialophora sessilis* de Hoog, *Tripospermum myrti* (Lind) S. Hughes and *Tripo-*

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spermum camelopardus Ingold, Dann et P. J. McDougall [Lohrer 2004]. Results of molecular studies performed by Batzer [2005] and Frank et al. [2010] indicated approximately 30 different fungi species associated with this disease in USA, Slovenia and Germany.

Eight species of fungi and three genera being the causal agents in southern Poland were identified, with most important: *Tripospermum myrti* (Lind) S. Hughes, *Aureobasidium pullulans* (de Bary et Löwenthal) G. Arnaud, *Cladosporium cladosporioides* (Fres.) de Vries and *Tripospermum camelopardus* Ingold, Dann & P.J. McDougall [Grabowski 2007].

The aim of this study was to identify fungi causing sooty blotch in Northern, Central and Eastern Poland based on the internal transcribed spacer region (ITS) of the rDNA and morphology of isolates.

MATERIALS AND METHODS

The experiments were carried out in 2006–2009. Apples with symptoms of sooty blotch were collected in summer and early autumn from orchards and gardens located in Northern, Central, Eastern, Northeastern and Southeastern regions of Poland (a total of 23 localisations) (tab. 1).

Fruits were rinsed for one hour under running tap water. Fungi were isolated from colonies visible as spots on the fruit surface. Individual colonies on apple were labeled under stereoscopic microscope (SZ11 Olympus) and photographed. Fragments of mycelia were picked up with sterile preparation needle or scalpel and placed on Petri dishes on Potato Dextrose Agar (PDA). All isolates were purified by multiple transfering and stored on PDA slants at 4°C.

Isolates were preliminary identified and classified to groups based on morphological characters: colour and texture of mycelia, shape and size of spores, type of conidioma and conidiogenesis, in accordance to the descriptions published by Deighton [1973], Braun [1995, 2000], Marcinkowska [2004], Kirk et al. [2008] and Frank et al. [2010]. Isolates representative for each group (50–100% of all isolates per group) were selected for further identification with molecular techniques.

Genetical identification was based on the differences in the nucleotide sequences of the PCR-amplified fragments of ITS region of rDNA (ITS1, 5.8 S rDNA gene, ITS2).

Total DNA was extracted from 10 days' isolates of the fungus grown on PDA medium with Wizard Genomic DNA Purification Kit (Promega Corporation) according to manufacturer's protocol.

ITS fragments were amplified with two sets of primers ITS1F [Gardes and Bruns 1993] and ITS4 [White et al. 1990]. PCR amplification were performed according to Batzer at al. [2005], with temperature of annealing modified: initial denaturation: 94°C, 95 sec., denaturation: 94°C, 35 sec., annealing: 57°C (instead of 52°C), for 60 sec., extension: 72°C, 2 min, and final extension: 10 min, 72°C, for 30 cycles (Applied Biosystems Veriti 96 Wel Thermal Cycler). Amplified fragments were separated electrophoreticaly in 1.2% agarose /TBE gels in the presence of ethidium bromide. PCR amplicons were sequenced (Institute of Biochemistry and Biophysics Polish

Academy of Sciences). Nucleotide sequences were analysed using ClustalW software [http://clustalw.genome.ad.jp] and compared with sequences collected in Gene Bank records with BLAST software [http://www.ncbi.nlm.nih.gov/BLAST/].

Comparative analysis of nucleotide sequences of isolates of fungi was performed using CLC Sequence Viewer 6.4. Typical sequences of representative isolates were presented in a form of dendrogram.

Isolates with identity confirmed by molecular analysis were considered to be references for the whole group and morphology of the all other isolates from these groups were compared to them. For selected isolates with the same kind of sequence spore sizes (100 spores per isolate) were measured under the light microscope.

Compliance to Koch's postulates was verified by inoculation of fruits cv. 'Golden Delicious' with spores suspended in water (5×10^5 infection units per 1 ml). Fruits were washed under tap water and surface sterilized with 70% ethanol prior to the inoculation. Inoculated fruits were placed in foil bags and incubated at room temperature for five weeks. After development of disease symptoms, fungi were reisolated and compared with isolates used for inoculation.

RESULTS AND DISCUSSION

Symptoms of sooty blotch observed as smudges or olive-green spots on the surface of infected fruits were confirmed in fruit specimens collected from various localisations (fig. 1).

Fig. 1. Sooty blotch symptoms on apple fruit
Ryc. 1. Objawy brudnej plamistości na jabłku

A total number of 245 fungi causing sooty blotch on apples were isolated. Isolates grown on PDA medium were divided into 6 groups according to their morphological character: mycelium colour (pale pink, or different shade of dark, from olive to black) and texture. As much as 130 out of 245 isolates were sequenced.

Microcyclosporella sp. (66.53% of all fungi species) were commonly found in nearly all orchards except one (Grzymkowice). Also *Aureobasidium pullulans* (22.86% occurred in majority of orchards. *Ph. sessilis* was present occasionally (3.67%) in 5 localizations (Wilanów, Popówek, Piaseczno, Zalesie and Błażejowice). Single isolates (0.41% each) of *Peltaster* sp. (Włodawa) and *P. fructicola* (Wilanów) were also obtained. (tab. 1, fig. 2).

Table 1. Origins of isolates
Tabela 1. Pochodzenie uzyskanych izolatów grzybów

Localization Miejscowość	Coordinates Współrzędne	Województwo Province	*Microcyclosporella* sp.	*Microcyclospora* sp.	*Peltaster fructicola*	*Peltaster* sp.	*Phialophora sessilis*	*Aureobasidium pullulans*
Dobryń Mały near Terespol	52°3'8"N, 23°24'5"E	lubelskie (E)	+					
Dołha near Biała Podlaska	52°0'13"N, 22°55'19"E		+					
Michałki near Biała Podlaska	52°5'37"N, 23°18'30"E		+					
Piotrawin near Opole Lubelskie	51°6'20"N, 21°47'57"E		+					
Sawin near Chełm	51°16'20"N, 23°25'58"E		+					
Włodawa near Chełm	51°32'38"N, 23°33'17"E		+			+		+
Błażejowice near Rawa Mazowiecka	51°50'20"N, 20°29'37"E	łódzkie (C)	+				+	+
Grzymkowice near Rawa Mazowiecka	51°51'31"N, 20°31'25"E							+
Rogów near Łódź	51°48'56"N, 19°53'4"E		+					+
Ciszyca near Warsaw	52°6'28"N, 21°11'1"E	mazowieckie (C)	+					+
Ojrzanów near Tarczyn	52°0'29"N, 20°44'9"E		+					
Piaseczno near Warsaw	52°4'4"N, 21°0'56"E		+				+	
Popówek near Pruszków	52°7'29"N, 20°47'21"E		+				+	+
Warsaw Gocław	52°13'48"N, 21°4'51"E		+					
Warsaw Wilanów	52°9'26"N, 21°6'19"E		+	+	+		+	+
Zalesie Dolne near Warsaw	52°3'40"N, 21°0'58"E		+				+	
Mostki near Zwoleń	51°20'55"N, 21°31'58"E		+					+
Jarosław	50°1'7"N, 22°41'0"E	podkarpackie (SE)	+					
Drohiczyn near Siemiatycze	52°23'49"N, 22°39'32"E	podlaskie (NE)	+					+
Gdańsk	54°20'55"N, 18°39'13"E	pomorskie (N)	+					+
Kopalino near Łeba	54°47'21"N, 17°51'3"E		+					+
Pruszcz Gdański	54°15'26"N, 18°39'1"E		+					+
Suchedniów near Skarżysko Kamienna	51°2'51"N, 20°49'46"E	świętokrzyskie (S)	+					

E – Eastern, C – Central, N – Northern, NE – Northeastern, SE – Southeastern regions of Poland
E – wschodni, C – centralny, N – północny, NE – północno-wschodni, SE – południowo-wschodni region Polski

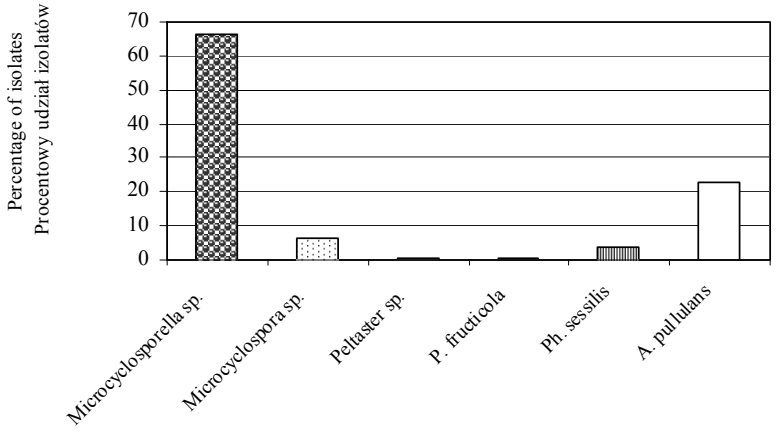

Fig. 2. The structure of population of sooty blotch and sooty mould fungi in years 2006–2009
Rys. 2. Struktura populacji grzybów powodujących brudną i sadzowatą plamistość jabłek w latach 2006–2009

PCR amplification of fungi isolates DNA preparation with ITS1F and ITS4 primers resulted in a distinct band of approximately 480–1000 bp for template (fig. 3).

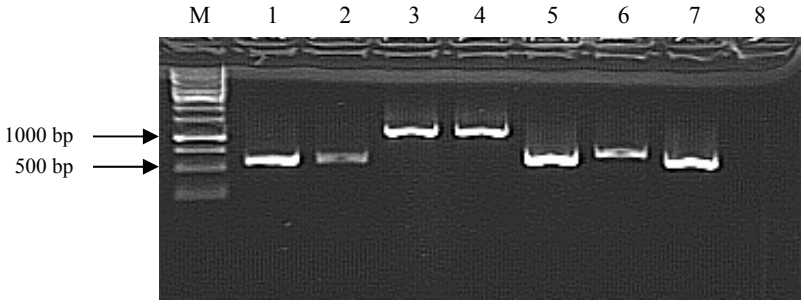

Fig. 3. Elektrophorogram of PCR products amplified with ITS1F and ITS4 primers set: M – marker GeneRuller 1kb DNA Ladder (Fermentas), 1 – *Microcyclosporella* sp., 2 – *Microcyclospora* sp., 3 – *Peltaster* sp., 4 – *Peltaster fructicola,* 5 – *Aureobasidium pullulans*, 6 – *Phialophora sessilis*, 7 – positive control, 8 – negative control
Rys. 3. Elektroforegram produktów reakcji PCR otrzymanych z wykorzystaniem starterów ITS1F i ITS4: M – marker GeneRuller 1kb DNA Ladder (Fermentas), 1 – *Microcyclosporella* sp., 2 – *Microcyclospora* sp., 3 – *Peltaster* sp., 4 – *Peltaster fructicola,* 5 – *Aureobasidium pullulans*, 6 – *Phialophora sessilis*, 7 – kontrola pozytywna, 8 – kontrola negatywna

The BLAST analysis of fungal DNA sequences obtained from apple isolates has shown high homology (98–100%) to sequences of fungi causing sooty blotch in other countries deposited in Gene Bank (noteworthy, *A. pullulans* as deposited in the Gene Bank was not considered as a casual agents of sooty blotch). Koch's postulates for these fungi have been satisfied.

A consequtive step was evaluation of dendrogram of fungi of sooty blotch (fig. 4).

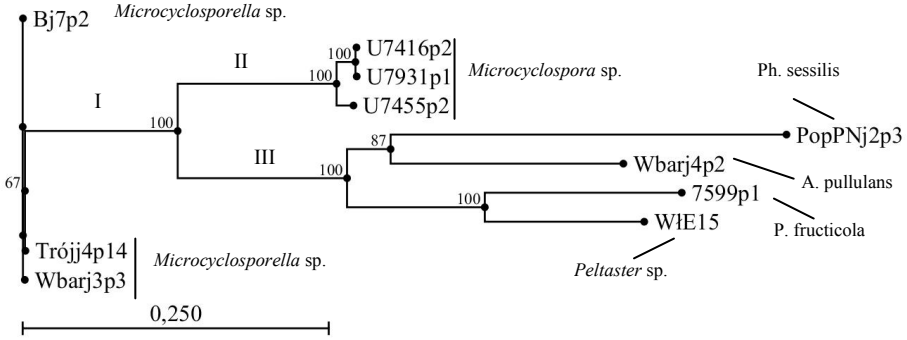

Fig. 4. Comparative analysis of nucleotide sequences of isolates of fungi causing sooty blotch on apple: I, II, III – groups of isolates

Rys. 4. Analiza porównawcza sekwencji nukleotydowej izolatów grzybów powodujących brudną plamistość jabłek: I, II, III – grupy izolatów

Previous identification was confirmed by identification based on the analysis of DNA sequences. Isolates of fungi of classified as *Microcyclosporella* genus, were matching NCBI Gene Bank sequences in 99%. Differences between our isolates were limited to single nucleotides. *Microcyclospora* species were slightly more diversified. While U7416p2 and U7931p1 shown only single nucleotide differences, in the case of U7455p2 differences were observed in more than one position, but still matching Gene Bank sequences in up to 99%.

Not surprisingly, most diversified was the third group, containing isolates from genera *Peltaster*, *A. pullulans* and *Ph. sessilis*. However, these species show more similarities to each other than to fungi from genera *Microcyclosporella* and *Microcyclospora*.

Results of this research complies with studies conducted in southern Poland in 2004 [Grabowski 2004, Wrona and Grabowski 2004] and 2007 [Grabowski 2007] and the results of our study also suggest that the causal agents of sooty blotch on apples should be a few species of fungi.

Microcyclosporella sp. (previously assigned as *Pseudocercosporella* [Frank et al. 2010]) were dominant fungi in this study, but also in the USA and Serbia (up to 78%) [Ivanović at al. 2010]. Probably they are anamorphs of *Mycosphaerella* genus [Batzer et al. 2005].

However, Grabowski [2007] provides that in southern Poland *Microcyclosporella sp.* are rather uncommon and their occurrence in his study did not exceed 1.31% of all isolates. In his work, most frequent sooty blotch fungus was *Tripospermum myrti* (25.97%), and then *T. camelopardus* and *T. acerinum* (Syd.), (14.98 and 4.95%, respectively) which were not found during our studies. Noteworthy, both *T. myrti* and *T. camelopardus* were considered to cause sooty blotch in Germany [Noga et al. 2000]. The lack of these fungi in our collection was possibly a consequence of our metod of sampling. In this work, isolates were derived only from spots typical for sooty blotch, that is mycelia tightly adhering to the fruit surface. Sooty mould fungi, which mycelia forms a loosely bound spots and might be washed out under tap water were not examinated in this study except *A. pullulans*, commonly isolated also from compact, spots typical for sooty blotch and due to its frequent occurrence was also investigated.

Presence of *A. pullulans* (22.86%) was confirmed in nearly all localisations. Formally, *A. pullulans* is a sooty mould fungus, which blemish cuticle similarly to sooty blotch fungi. Sooty mould fungi are a non-parasitic, superficial growth on plant surface covered with honeydew [Nelson 2008]; sooty mould is usually classified together with sooty blotch as one complex. Presence of honeydew does not seems to be necessary for the development of this fungus, we isolated it from plants without honeydew, similarily as Grabowski [2007] who noted that *A. pullulans* was 16.45% of all sooty blotch isolates.

Genus *Microcyclospora* (formerly *Pseudocercospora* [Frank et al. 2010]) was the third group of sooty blotch fungi, but it developed disease symptoms in one localization only. This is the first report on the occurrence of *Microcyclospora* sp. as sooty blotch agent in Poland, while it is known as sooty blotch agent in the USA, Serbia, Slovenia and Germany [Batzer et al. 2005, Díaz Arias et al. 2010, Frank et al. 2010, Ivanović et al. 2010]

Frequency of *Phialophora sessilis, Peltaster fructicola* and *Peltaster* sp. described in this work is comparable to these found by Grabowski [2007] in southern Poland. It is interesting, that *P. fructicola* which was quite common in southern Poland in 2004 [Grabowski 2004], recently was found incidentally [Grabowski 2007, this work]. In the USA *P. fructicola* is widely spreaded, but occurence of *Peltaster* sp. was limited to certain regions [Diaz Arias 2010].

Ph. sessilis, caused sooty blotch in Poland, but also in the Eastern and Midwestern United States [Díaz Arias 2010] and in Germany [Noga et al. 2000].

Gloeodes pomigena which for many years have been noted as a causal agent of sooty blotch on apples only [Brown and Sutton 1993] was not found in this study.

In our study, as in previous reports of Batzer et al. [2005], Díaz Arias et al. [2010], Frank et al. [2010], Ivanović et al. [2010], Grabowski [2005], macroscopic observations of symptoms caused by sooty blotch complex fungi indicated diversity in mycelial types. Fungal colonies growing on varied in shape from nearly circular with distinct margins to rather large, shapeless blotches with diffuse margins.

Fungi of sooty blotch colonize cuticle of apple fruits [Belding et al. 2000]. However symptoms of sooty blotch, visible as sooty smudges or olive green spots on the surface of infected fruits causes only reduction in fruit quality [Williamson and Sutton 2000]. Up to date, they were not considered as important pathogenes due to the advanced

chemical disease control in high productivity orchards, however they needs a special attention in the rapidly increasinsg organic orchards.

CONCLUSIONS

1. Most frequent sooty blotch causal agents in this study were fungi from genus *Microcyclosporella.*
2. This work is the first report that fungi of genus *Microcyclospora* are causal agents of sooty blotch in Poland.
3. *Peltaster* sp. and *Peltaser fructicola* were only incidentally isolated from infected fruits
4. Presence of *Gloeodes pomigena* was not observed in this study.

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RÓŻNORODNOŚĆ GRZYBÓW POWODUJĄCYCH BRUDNĄ PLAMISTOŚĆ JABŁEK W POLSCE

Streszczenie. Brudna plamistość jabłek jest powszechnie występującą chorobą owoców w sadach ekologicznych w wielu krajach. Wyniki badań molekularnych przeprowadzone w USA wykazały, że sprawcami tej choroby może być około 30 różnych gatunków grzybów. Celem pracy było określenie składu populacji grzybów powodujących brudną plamistość jabłek w centralnej, wschodniej i północnej Polsce z wykorzystaniem techniki PCR i tradycyjnych metod. Latem i wczesną wiosną w latach 2006–2009 z owoców z widocznymi objawami choroby uzyskano 245 izolatów grzybów – sprawców brudnej plamistości jabłek. Na podstawie cech morfologicznych izolaty grzybów rosnące na PDA wstępnie podzielono na 6 grup, a następnie z wybranych izolatów reprezentujących daną

grupę wyizolowano DNA i zsekwencjonowano. Amplifikację DNA przeprowadzono za pomocą techniki PCR z wykorzystaniem starterów ITS1F i ITS4. Spośród uzyskanych izolatów sprawców brudnej plamistości najliczniejszą grupę stanowiły grzyby należące do rodzaju *Microcyclosporella* (66,53%). Pozostałe grzyby to: *Aureobasidium pullulans* – 22,86%, *Microcyclospora* sp. – 6,12%, *Phialophora sessilis* – 3,67% oraz *Peltaster* sp. i *P. fructicola* – 0,41%.

Słowa kluczowe: jabłka, *Microcyclosporella* sp., *Microcyclospora* sp., *Peltaster fructicola*

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