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CHARACTERISTICS OF *Aureobasidium pullulans* (de Bary et Löwenthal) G. Arnaud ISOLATED FROM APPLES AND PEARS WITH SYMPTOMS OF SOOTY BLOTCH IN POLAND

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Abstract. Sooty blotch is a disease of apple and pear caused by a complex of fungi that blemish the fruit surface. Results of molecular studies indicated approximately 30 different fungi species associated with this disease. Apples and pears with symptoms of sooty blotch were collected in summer and early autumn 2006–2010 from trees grown in fungicide non-treated orchards and small gardens located in various regions of Poland. Fungi causing sooty blotch were isolated from fruits and the isolates were divided into six groups, according to their morphological characters. Growth of the fungi colonies were tested on different agar media (PDA, CMA, MEA and Czapek). The ITS region of rDNA from 16 isolates from the first group was amplified by PCR technique and one representative sequence of this isolates was used to alignment in Gene Bank. This isolate was identified as *Aureobasidium pullulans* and isolates from this group were compared with it on the base of morphological features.

Key words: identification, sooty blotch, PCR, *Gloeodes pomigena*

INTRODUCTION

Sooty blotch is one of the most common diseases of apples (*Malus* × *domestica* Borkh) growing in ecological orchards in many countries [Williamson and Sutton 2000]. The term "sooty blotch" denotes fungi which form a dark mycelial mat with or without sclerotium-like bodies. Colonies of these fungi create a diverse range of mycelial types on fruit surface e.g. ramose, punctate, fuliginious, ridged honeycomb etc. [Batzer et al. 2005].

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Until 1997, this disease was thought to be caused only by the fungus *Gloeodes pomigena* (Schwein) Colby [Baines and Gardner 1932, Groves 1933, Brown and Sutton 1993, Wilcox 1994]. Later studies conducted in the USA showed that sooty blotch can be caused by a complex of fungi species: *Leptodonthium elatius* (G. Mangenot) de Hoog, *Peltaster fructicola* Johnson, Sutton et Hodges and *Geastrumia polystigmatis* Batista et M.L. Farr [Johnson et al. 1996, 1997, Williamson and Sutton 2000, Williamson et al. 2004].

Observations in the European countries revealed that disease can be caused by many causal agents as well. In Germany besides *Peltaster fructicola,* other fungi, i.e.: *Phialophora sessilis* de Hoog, *Tripospermum myrti* (Lind) S. Hughes and *Tripospermum camelopardus* Ingold, Dann et P.J. McDougall were the cause of this disease [Noga et al. 2000, Lohrer 2004]. Grabowski [2004, 2007] and Wrona and Grabowski [2004] found four species identified in Germany and additional fungi such as *Tripospermum acerinum* (P. Syd.), *Aureobasidium pullulans* (de Bary et Löwenthal) G. Arnaud, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Torula herbarum* (Pers.) Link and fungi of genera S*eptoria*, *Ramularia*, *Pseudocercosporella* when they examined isolates collected from southern part of Poland. Molecular studies carried out by Batzer et al. [2005] in USA indicated that about thirty different fungi were associated with sooty blotch. Identity of many of them is still to be determined.

The aim of this study was to investigate a fungus *Aureobasidium pullulans*, which was isolated from surface of fruits with symptoms of sooty blotch.

MATERIALS AND METHODS

Apples and pears with symptoms of sooty blotch were collected from trees growing in orchards and small gardens in various regions of Poland (voivodships: lubelskie, łódzkie, mazowieckie, podlaskie and pomorskie) in summer and early autumn 2006–2010. None of the orchards was chemically protected against fungal diseases. Fungi were isolated from the spots on fruits surface. Before isolation the examined fruits were rinsed one hour under running tap water. Fungi were isolated from colonies visible as spots on the fruit surface. Individual colonies on apple and pear were labelled under stereoscopic microscope (SZ11 Olympus) and photographed. Fragments of mycelia were placed with sterile preparation needle or scalpel into Petri dishes with potato-dextrose agar (PDA). Isolates were divided into 6 groups according to their morphological characters, i.e. colour and texture of mycelium, shape and size of spores, type of conidioma and conidiogenesis in accordance to the descriptions published by Deighton [1973], Braun [1995, 2000], Kirk et al. [2008]. Isolates from the first group (identified as *Aureobasidium pullulans*) were the only analysed in detail. For each isolate spore length and width (100 spores per sample) were measured under the light microscope (BX50 Olympus). Sixteen isolates representative for this 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, 5.8S rDNA gene, ITS2) [Larena et al. 1999, Goodwin and Zismann 2001]. Total DNA was extracted from five days isolates using Wizard Genomic DNA

Purification Kit (Promega Corporation), according to manufacturer's protocol. The examined isolates were representatives of the first group and the rest of isolates from this group were compared with them on the basis of morphological feature. 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 et al. [2005], with temperature of annealing modified: initial denaturation: 94ºC, 95 sec., denaturation: 94ºC, 35 sec., annealing: 57ºC (instead of 52ºC), for 60 sec., extension: 72ºC, 2 min, and final extension: 10 min, 72ºC, for 30 cycles (Applied Biosystems Veriti 96 Wel Thermal Cycler). Amplified fragments were separated electrophoretically in 1.2% agarose/TBE gels in the presence of ethidium bromide. Nucleotide sequences were analysed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) software and compared with sequences collected in NCBI Gene Bank databases with the use of BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Comparative analysis of nucleotide sequences of isolates of *A. pullulans* and dendrogram were performed using MEGA5 (the Neighbor-Joining method). All positions containing gaps and missing data were eliminated. Bootstrap values greater than 50% are denoted above branches.

Some isolates of this group were checked for their growth on different agar media: PDA, CMA (corn meal agar), MEA (malt extract agar) and Czapek (Czapek solution agar).

Compliance to Koch's postulates was verified by inoculation of fruits of apple cv. 'Golden Delicious' with spores suspended in water (5×10^5 infection units per 1 ml). Before inoculation fruits were washed under tap water and surface-sterilized with 70% ethanol. Inoculated fruits were placed in foil bags and incubated at room temperature for five weeks. If after this time disease symptoms appeared, the fungi were re-isolated from infected fruits in order to verify their pathogenicity.

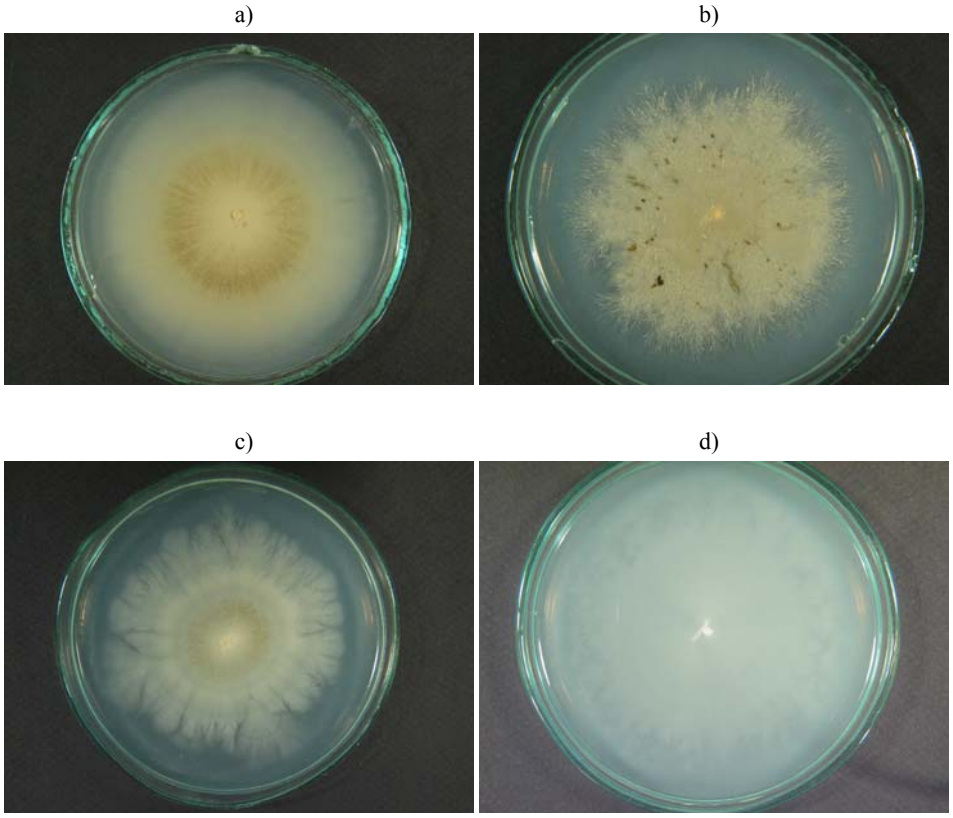
RESULTS

Characteristic symptoms of sooty blotch, visible as sooty smudges or olive-green spots on the surface of infected fruits were observed on the apple and pear fruits sampled in various localities. Fungal colonies varied in shape, from nearly circular with distinct margins, to rather large, shapeless blotches with diffuse margins. The macroscopic observations of symptoms of this disease indicated on diversity in their mycelial types.

Approximately 300 single-spore isolates were obtained from pome fruits with visible symptoms of sooty blotch. Isolates of fungi grown on PDA were divided into six groups, based on their morphological characters. First group was represented by the isolates with pale pink mycelium, second one with yellow-olive one, and the rest of groups by isolates with dark mycelium.

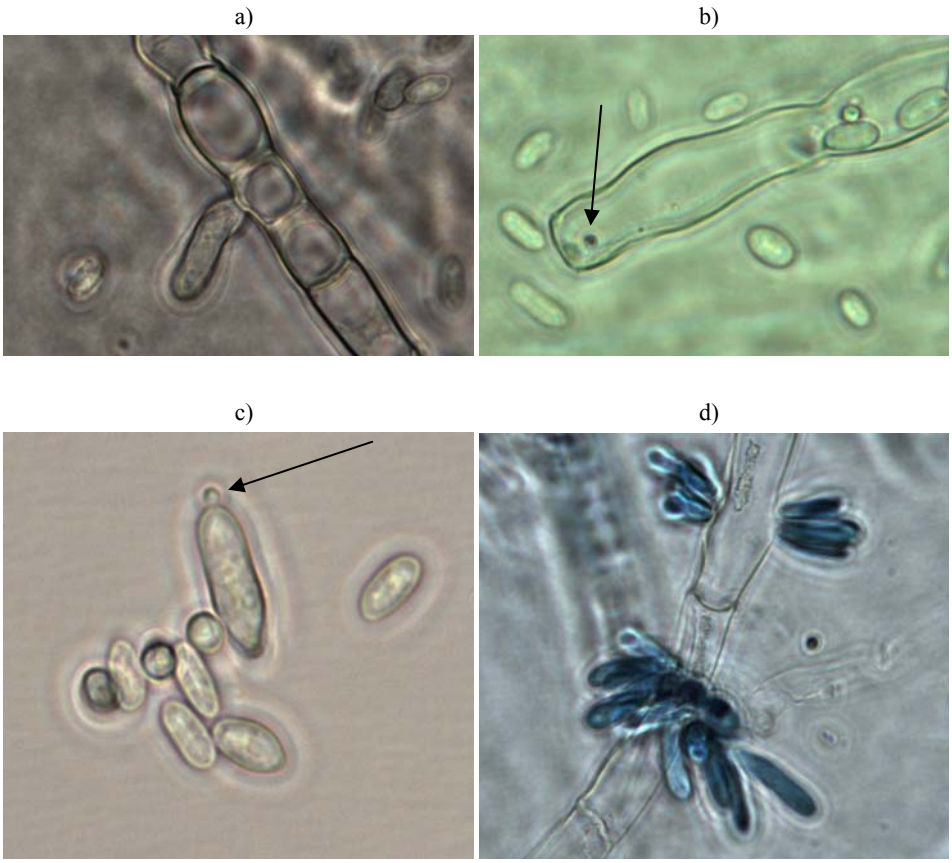
The first group of fungi contained 77 isolates. The fastest linear colony growth and sporulation of these isolates was observed on PDA medium (8.4 × 8.4 cm). The slowest growth of mycelium was noted on Czapek (4.7 × 4.4 cm). Growth on MEA and CMA was moderately fast (7.7 × 7.3 and 7.8 × 7.8, respectively) (phot. 1a, b, c, d). Colonies were flat, smooth, yeast-like, shiny and leathery in appearance, covered with slimy

masses of conidia. The surface was white, pale pink at the beginning, while later brown to black and velvety with a grayish fringe with age. Reverse side was pale (creamy) with darkened margins developing with the age.



Phot. 1. *Aureobasidium pululans* growth on various media: a – PDA, b – Czapek, c – MEA and d – CMA (photo. E. Mirzwa-Mróz)

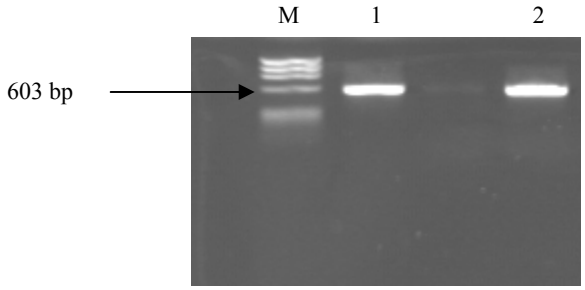
Hyphe were approximately of 2.60 µm width, septate (every 11.71 µm on average) with thickening, hyaline at the beginning and darkened with age (phot. 2a). Conidiogenous cells were intercalary or located terminally in the hyphae. Conidia were produced directly from the hyphe or at the ends of many colony branches. Conidia (6.86 × 2.87 µm in size on average) were hyaline, smooth-walled, one-celled and ellipsoidal or oval in shape (phot. 2b). Sometimes conidia multiplied by budding and produce blastoconidies (phot. 2c). Development of blastoconidia in tufts was typical (phot. 2d).



Phot. 2. Characteristic of *Aureobasidium pullulans* (isolate U-7844/1-1): a – hyphae with septa; b – conidia and hyphae with and conidiogenous cells (arrow); c – budding conidium (arrow); d – conidia developing in tuffs (photo E. Mirzwa-Mróz)

Electrophoresis of PCR amplification products revealed a distinct band of approximately 600bp (phot. 3) for all the 16 fungi isolates grouped into group 1, and no differences (100% sequence identity) was confirmed after subsequent analysis. Therefore, only one of these amplicons was used in further studies as representative for the entire group.

Amplified fragment showed 100% identity through the whole length (550 nucleotides) with approximately 20 sequences of *Aureobasidium pullulans* already collected in the Gene Bank, including the first sequence-JX462673 (isolated in the USA) submitted by Peterson et al. in 2013.



Phot. 3. Elektrophorogram of PCR products amplified with ITS1F and ITS4 primers set: M – marker ΦX174 DNA Hae III Digest, Lane 1 – DNA isolated from fungus (isolate Wbar4 p2), Lane 2 – positive control

Comparison of reference sequence obtained in this work with sequences deposited in Gene Bank shows fig. 1.

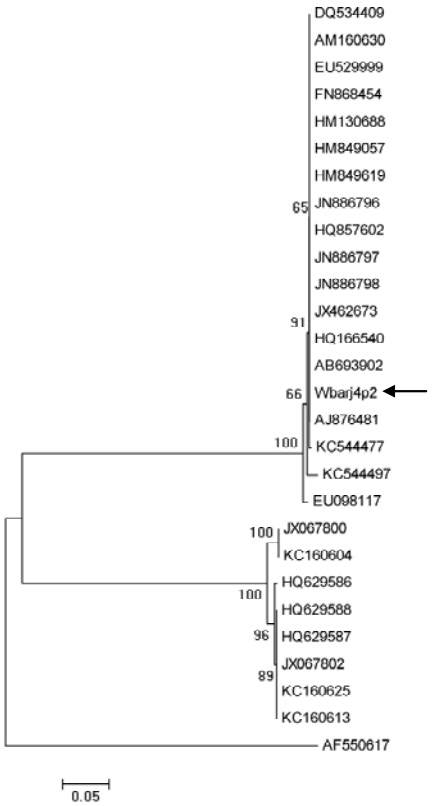


Fig. 1. Comparative analysis of nucleotide sequences (ITS region of rDNA) from *Aureobasidium pullulans.* The tree is rooted to *Chondrostereum purpureum* (AF550617)

Phot. 4. Sooty blotch symptoms on apple fruits inoculated with spore suspension of *Aureobasidium pullulans* (photo E. Mirzwa-Mróz)

Results of classical mycological analysis, i.e. measurements of conidia and hyphae, were essentially similar in case of both the isolates which DNA was sequenced and the remaining isolates from group 1. Koch's postulates for these fungi have been satisfied (phot. 4).

DISCUSSION

A. pullulans belongs to the phylum *Ascomycota*, class *Euascomycetes*, order *Dothideales* family *Dothioraceae*. Teleomorf of *A. pullulans* is *Discosphaerina fulvida* (F.R. Sanderson) Sivanesan (Kirk et al. 2008, www.indexfungorum).

A few years ago, *Aureobasidium pullulans* was included to the sooty blotch fungi complex [Grabowski 2007, Sun et al. 2008]. In southern Poland this fungus was 16.45% of all sooty blotch isolates [Grabowski 2007]. In China, *A. pullulans* was classified as sooty blotch fungus with fuliginious mycelial type (mycelial mat without sclerotium-like bodies) [Sun et al. 2008].

Search for typical sooty blotch symptoms on fruits, from which *A. pullulans* isolates were obtained, gave negative results. *A. pullulans* was often simultaneously isolated with other sooty blotch complex fungi of various mycelial types. On the inoculated apples, it developed a type of mycelium similar to observed by Sun et al. [2008], but in contrast to this observations, mycelia did not tightly adhere to the fruit surface. *A. pullulans* mycelium locked as loosely bound spots which might be washed out under running tap water [Mirzwa-Mróz and Wińska-Krysiak 2011]. It is classified as sooty mould fungus, which blemish cuticle similarly to sooty blotch fungi.

Sooty mould fungi are a non-parasitic, superficially growing on plant surface covered with honeydew [Nelson 2008]; sooty mould is usually classified together with sooty blotch as one complex. We isolated *A. pullulans* from plants without honeydew, similarly as Grabowski [2007]. Presence of honeydew does not seem to be necessary for

the development of this fungus because ripening fruits with sugar content above 9% are sites for developing mycelium of sooty mould fungi [Grabowski 2007].

In our studies shape and sizes of conidia of *Aureobasidium pullulans* were very variable, but generally similar to those described by de Hoog [2000], Takesako et al. [1991] and others [Gniewosz 2003]. In our investigations, hyphae frequently became dark and formed chains thick-walled, darkly pigmented chlamydospores as described by Pechak and Crang [1977], Salkin et al. [1986], Takesako et al. [1991], Bell and Wheeler 1986 cited by Gniewosz [2003].

Up to date, none of the *A. pullulans* stored in Gene Bank originated from fruits with sooty blotch symptoms. This fungus commonly occurs in environment [Salkin et al. 1986, Takesako et al. 1991, Sun et al. 2008], therefore sequences deposited in Gene Bank used for alignment (fig. 1) were derived from *A. pullulans* from isolates obtained from apple leaves, apple juice, insects, floral nectar of mediterrnean plants, aromatic plants, marine environment etc. Such a variability of sources was also reflected in results of phylogenetic analysis, where two subgroups were found. Representative sequence obtained in this work, Wbarj4 p2 (HQ267772), was in one of the subgroups, together with 16 sequences of *A. pullulans* originated from different hosts (DQ534409, AM160630, EU529999, FN868454, HM130688, HM849057, HM849619, JN886796, HQ857602, JN886797, JN886798, JX462673, HQ166540, AB693902, AJ876481 and KC544477). The other subgroup were sequences originated from floral nectar of mediterrnean plants (JX067800, JX067802), aromatic plants (KC160604, KC160613, KC160625) and from South American tree *Nothofagus nervosa* (HQ629586-HQ 629588). *A. pullulans* occurs commonly on leaves and fruits of apple trees [Matteson Heidenreich et al. 1997], but sooty blotch symptoms on ripening fruits develop after prolonged rain period [Grabowski 2007]. In the present study dark spots caused by this fungus were observed only on inoculated fruits. *A. pullulans* is noted as a causal agent of apple russet as well [Goffinet et al. 2002].

CONCLUSIONS

1. *A. pullulans* was frequently isolated together with sooty blotch fungi
2. The same DNA sequences of *A. pullulans* were obtained from isolates originated from various localities in Poland
3. Fungus develops dark spots only on inoculated fruits.

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CHARAKTERYSTYKA GRZYBA *Aureobasidium pullulans* (de Bary et Löwenthal) G. Arnaud WYIZOLOWANEGO Z JABŁEK I GRUSZEK Z OBJAWAMI BRUDNEJ PLAMISTOŚCI W POLSCE

Streszczenie. Przyczyną brudnej plamistości jabłek i gruszek jest kompleks grzybów powodujących oszpecenie powierzchni skórki owoców. Wyniki badań molekularnych wskazują, że z tą chorobą wiąże się ponad 30 różnych gatunków grzybów. Jabłka i gruszki z widocznymi objawami brudnej plamistości jabłek zbierano z niechronionych chemicznie sadów i ogródków działkowych w różnych regionach Polski. Owoce były zbierane latem i wczesną jesienią w latach 2006–2010. Z plam znajdujących się na owocach wyizolowano sprawców brudnej plamistości jabłek i na podstawie charakterystyki cech morfologicznych uzyskanych izolatów grzybów podzielono je na sześć grup. Badano również wzrost i wygląd kolonii grzybów na różnych podłożach (PDA, CMA, MEA i Czapek). Wyizolowano, a następnie zamplifikowano za pomocą techniki PCR z wykorzystaniem starterów ITS1F i ITS4, genomowe DNA 16 izolatów należących do pierwszej grupy. Grzyb ten został zidentyfikowany jako gatunek *Aureobasidium pullulans*, a jego zsekwencjonowane izolaty stanowiły wzorzec przy identyfikacji pozostałych izolatów zaklasyfikowanych do tej grupy.

Słowa kluczowe: identyfikacja, brudna plamistość jabłek, PCR, *Gloeodes pomigena*

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