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First report of the occurrence of *Sphaeropsis visci* on Mistletoe (*Viscum album* L.) in Poland

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Abstract

Mistletoe (*Viscum album*) is a semi-parasite of many forest trees. During water scarcity, its presence can be particularly dangerous for the host plant. In Poland, the subspecies *V. album* subsp. *austriacum* inhabits Scots pine massively. Therefore, an attempt was made to search for a natural barrier in limiting its development. For this purpose, in spring 2019 in the southwestern part of Poland, mistletoe shoots inhabiting Scots pine were collected. A fungus was isolated from the collected plant fragments, which was then identified by Koch's postulates and DNA analysis of the given sequences. The data presented here confirmed that the pathogen responsible for the disease was *Sphaeropsis visci*. This is the first report on the occurrence of this mistletoe pathogen in Poland.

Keywords: *Sphaeropsis visci*, *Viscum album* subsp. *austriacum*, New pathogen, Scots pine, Mistletoe

Introduction

Mistletoe is a semi-parasitic plant that is widely distributed throughout Europe and southwest Asia (Barney et al. 1998). It can be a serious threat to host plants, especially during periods of water shortage. During periods of drought, the tree closes the stomata to reduce water transpiration, while the mistletoe stomata remain open at this time (Zuber 2004). In addition to water and mineral salts, mistletoe also takes photosynthesis products from the host plant. Richter and Popp (1992) showed that between 23 and 45% of carbon in mistletoe comes from the trees on which these plants grow.

Within the *Viscum album* species, there are several subspecies that are very difficult to recognize based on morphological traits, it is easier to distinguish them based on the host plant (Zuber 2004, Plagnat 1950, Böhling et al. 2002). *V. album* subsp. *album* is usually found on deciduous trees, *V. album* subsp. *austriacum* is observed on species of the genus *Pinus* and *Picea*, *V. album* subsp. *abietis* strikes firs and *V. album* subsp. *creticum* has been observed only on Calabrian pine (*Pinus brutia*) in Crete. Over the last few years *V. album* subsp. *austriacum* is gaining importance as a tree pest in the Polish forests (Szmidla et al. 2019). In the context of the observed climate changes and water scarcity, Scots pine (*Pinus sylvestris*) is inhabited by mistletoe. This phenomenon further enhances the weakening of the tree and increases its predisposition to damage caused by fungi and insects (Stypinski 1997). Therefore, it became necessary to search for methods to reduce the number of mistletoes in pine stands. According to Zuber (2004), mistletoe is infected by fungal pathogens, including *Plectophomella visci, Septoria visci, Sphaeropsis visci, Colletotrichum gloeosporoides, Botryosphaeria dothidea, Gibberidea visci, Botryosphaeria visci, Botryosphaerostroma visci, Alternaria alternata* and *Acremonium kiliense*. These organisms can be a natural barrier to mistletoe spreading to more and more trees. So far, there are no reports of the occurrence of mistletoe pathogens in Poland.

The aims of the work are to confirm the occurrence of *S. visci* infecting *V. album* subsp. *austriacum* in Poland and to investigate in vitro its growing capacity at different temperatures.

Materials and methodology

Samples were collected in spring 2019 in the Legnica Forest District (51° 17'40.0" N 16° 10'09.4" E), in pine stands, where mistletoe appearance was massively observed. From five felled trees with developed mistletoe bushes, five shoots were taken with visible black pycnidia. Then, plant fragments were placed in a moist chamber and allowed to incubate for seven days. After this time, hyphae appeared on the shoot surfaces and were transplanted onto Malt Extract Agar (MEA) medium. The isolates were incubated in darkness at room temperature. A total of 4 pure cultures were selected for molecular identification, whereas 7 fungal isolates were discarded, because based on morphological traits a role as causal agent of the symptoms was considered unlikely for these fungi.

DNA was extracted from fresh mycelia harvested from MEA plates using the Plant DNA Mini Kit (Syngen, Poland), following the manufacturer's instructions. The polymerase chain reaction (PCR) was performed using the universal primers ITS1F and ITS4, which amplify the above-mentioned sequence (White et al. 1990, Gardes and Bruns 1993). The PCR reactions (25 µl) contained 1 × PCR Buffer (Taq PCR Core Kit, QIAGEN), 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer, 1 U of Taq polymerase and 10–20 ng of template DNA. The PCR thermal protocol consisted of an initial 5 min denaturation step at 95°C, 35 cycles of amplification cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 50 s, and a final extension step of 72°C for 10 min. Amplicons were analysed by electrophoresis, visualized in a 1% agarose gel stained with the GelRed® dye (Biotium, USA), purified using CleanUp Kit (A&A Biotechnology, Poland) and sequenced on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific™, USA). The nucleotide Basic Local Alignment Search Tool (blastn) was used to compare obtained sequences with the National Center for Biotechnology Information database (NCBI 2019).

The next step was to determine the growth rate of mycelium at temperatures between 5 and 30°C. To this end, the obtained mycelium was subcultivated onto MEA-type substrates, and then left to incubate at room temperature. Three days later, three Petri dishes with transplanted cultures were placed in incubators with temperatures of 5°C, 10°C, 15°C, 20°C, 25°C and 30°C, followed by mycelium measurements at 24 h intervals. This operation was repeated until the pan was completely overgrown.

To meet Koch's postulates, the next stage of the work was to perform pathogenicity tests for the isolated pathogen. For this purpose, shoots were collected *V. album* subsp. *austriacum* and laid out on wet gases in sterile Petri glass dishes. Control plant fragments were left wrapped in moist gauze to maintain their constant humidity. Nine leaves and five branches were inoculated with the resulting isolates and then incubated at 20°C for 20 days. After this time, each shoot was carefully examined under a stereoscope and fragments were taken from symptomatic tissues for inoculation onto MEA-type substrates.

Results

Based on the analysis of the sequence of the ITS region and microscopic features of mycelium obtained from mistletoe shoots, the culprit of leaf and shoot was identified as *Sphaeropsis visci* (teleomorph: *Phaeobotryosphaeria visci*).

A curve illustrating the growth rate at individual temperatures was drawn up for the studies related to pathogen physiology (Figure 1). The pathogen developed the fastest at 20°C and 25°C, after a week the mycelium covered the entire dish. A slowdown in growth was observed at 30°C, while at 5°C the pathogen did not grow at all.

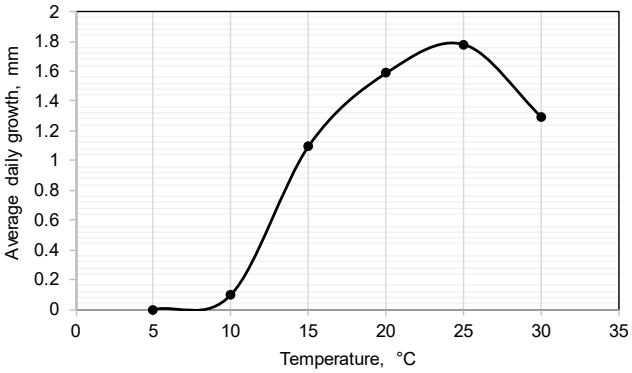

Figure 1. Average daily growth of *S. visci* colonies depending on ambient temperature

The pathogenicity tests carried out confirm that this pathogen can successfully infect *V. album* subsp. *austriacu*. On infected shoots after 20 days, mycelial growth was observed on 66% of inoculated leaves and on 80% of inoculated shoots. Mycelia could be reisolated by implanting on MEA substrates, thus fulfilling Koch's postulates. The isolates obtained were identified as *Spheropsis visci*.

Discussion

The research presented in this work allowed identifying the pathogen *V. album* subsp. *austriacum* as *Spheropsis visci*. This fungus is a pathogen that infects both the berries and the mistletoe shoots and leaves themselves (Zlatkovic et al. 2016). Infection causes chlorosis, impaired growth and, in favourable conditions, necrosis of colonized mistletoe tissues. This pathogen infects not only all *V. album* subspecies but also *V. coloratum*. Chen et al. (2008) reports that the disease caused by *S. visci* is able to completely destroy Asian mistletoe bushes. To date, information on this fungus comes from Austria, the Czech Republic, Egypt (Phillips et al. 2013), Romania (Sutton 1980), Ukraine (Phillips et al. 2008), Hungary (Poczai et

al. 2015), Serbia, Luxemburg (Zlatkovic et al. 2016) and China (Chen et al. 2018). The research presented here is the first report on the occurrence of this organism in Poland. From information collected from employees of the State Forests from areas of south-western Poland, where the number of mistletoes is the largest, it follows that the phenomena of *V. album* subsp. *austriacum* bush decay is more and more often observed, which may be associated with pathogen activity. During the conducted research, teleomorphs could not be found, which is in accordance with previous investigations (Varga et al. 2014, Chen et al. 2018).

The conducted studies related to the rate of pathogen development at various temperatures indicate that this organism is particularly active at a temperature in the range of 20–25°C. This may indicate that it prefers warmer climate, as prevails in the Balkans. However, extreme temperatures, as they occurred in recent years in Poland and other Central European countries, are increasingly observed (Kotlarski et al. 2014, Szmidla et al. 2019). Climate change is conducive to shifting the natural ranges of individual organisms. Forecast changes for the following years indicate that *S. visci* in Polish conditions can develop without major obstacles by finding optimal thermal conditions.

Observations made in the field, isolation of the pathogen and the positive result of the pathogenicity tests carried out confirm the point that this pathogen is able, under favourable conditions, to infect and kill common mistletoe. This information may help to develop an efficient method to reduce the number of *V. album* subsp. *austrian* in pine stands in Poland.

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