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Fungi associated with dieback of *Abies alba* seedlings in naturally regenerating forest ecosystems

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ABSTRACT

Dieback of young *Abies alba* seedlings was identified as an important limitation for natural regeneration of this species in the mountain regions of Central Europe. Approximately 2–4 weeks after emergence, during cool and wet weather, necrotic spots developed on seedling cotyledons. Later they expanded and merged to finally cover all above-ground tissues. Symptomatic seedlings of *A. alba* were sampled at 16 stands, and fungi were isolated from the cotyledons, stems, and roots. Morphological and molecular methods based on ITS rDNA sequencing were then used for isolate identification. In total, 105 distinct taxa were identified, with Ascomycota dominating, representing 97.4% of the isolates. The most frequently isolated taxa were *Allantophomopsis lycopodina*, *Gyoerffyella rotula*, *Leptosphaeria* sp., *Phialocephala fortinii*, *Pseudaegerita* sp., *Scirrhia aspidiorum*, *Sydowia polyspora*, *Peyronellaea* sp., *Phomopsis* sp., and *Varicosporium elodeae*. The frequency of colonization by individual fungal species differed among the three plant organs and among forest types. In symptomatic cotyledons, the dominant species were *G. rotula*, *S. polyspora*, and *Pseudaegerita* sp. whose pathogenicity, together with that of *Gyoerffyella* sp., *V. elodeae*, and *Hormonema carpetanum*, was tested on young *A. alba* seedlings. Only *G. rotula* and *Gyoerffyella* sp. caused serious dieback of above-ground seedling tissues and necrotic symptoms on cotyledons. This study is one of the few comprehensive reports showing that aquatic hyphomycetes commonly occur in forests beyond their preferred habitat.

1. Introduction

Many microbes, including soil-borne fungal and oomycete pathogens, are known to kill seedlings in natural and managed forests. The most destructive disease affecting seeds, germinants, and young seedlings is damping-off, usually characterized by root decay, wilting, collapsing, and rapid death (Butin, 1995). One of the most common diseases causing seedling loss in Polish forest nurseries is damping-off (Mańka, 1993) caused by pathogens belonging to the genera *Fusarium*, *Ilyonectria*, *Neonectria* (Ascomycota), *Pythium*, *Phytophthora* (Oomycota), and *Rhizoctonia* (Basidiomycota) (Mańka, 1993; Kwaśna and Bateman, 2009).

In comparison with forest nurseries, the diseases of seedlings under natural conditions have been little studied, although several soil-borne fungi have been reported to induce damping-off in seedling populations on the forest floor (Mańka et al., 1968; Kowalski, 1980, 1982; Mwanza and Kellas, 1987; Sahashi et al., 1995; Axelrood et al., 1998; Taniguchi et al., 2008; Hirooka et al., 2012; Jankowiak et al., 2016). A noteworthy case of associations between fungal pathogens and dynamics of forest ecosystems has been documented by studies on natural regeneration in silver fir (*Abies alba*) stands. Silver fir is a shade-tolerant species, which on mesic to Eutric sites of montane regions in Europe forms mixed stands with *Fagus sylvatica* and *Picea abies*. *A. alba* also occurs in mono-specific stands that are mostly degraded forms of natural mixed forests. In mono-specific stands, *A. alba* accumulates slowly through natural regeneration, and several authors have reported better performance of *A. alba* regeneration in mixed stands than in mono-specific stands (Jaworski, 1973; Dobrowolska and Veblen, 2008; Paluch and Jastrzębski, 2013). Analysis of the spatial pattern of regeneration occurrence has further revealed a negative

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association with inter-crown openings and a positive association with under-crown zones of mature trees (Paluch, 2005a,b).

The lower abundance of *A. alba* regeneration in gaps and inter-crown zones than in under-crown microsites has been linked experimentally to higher mortality of young seedlings caused by fungal pathogen attacks (Paluch and Stępniewska, 2012). These disease symptoms appeared to be associated with heavy rainfall in May and June and usually occurred within 2 weeks after seedling emergence. At first, brownish discoloration appeared on the cotyledons, and the plant growth became stunted. Later, the necrosis extended to the stem, and the infected seedling died rapidly. Contrary to the report by Kowalski (1980, 1982) of infection in 1- to 6-year-old *A. alba* seedlings by the soil-borne pathogen *Cylindrocarpon destructans*, the diseased seedlings generally showed no disease symptoms in their root system. Moreover, when *A. alba* seeds were sown on topsoil samples taken from microsites with contrasting seedling abundance in a nursery and subjected to different watering regimes, the germination and juvenile survival of the seedlings did not differ considerably. Overall, these findings suggest that disease incidences are restricted to the forest interior with variation between forest types and are linked with infection through aboveground parts of young *A. alba* seedlings.

Nevertheless, very little is known about the fungi involved in the dieback of *A. alba* seedlings under natural conditions. Therefore, the aims of this present study are: to identify the fungi and oomycetes associated with diseased seedlings; to compare their occurrence in different forest types (mono-specific and mixed forests) and plant organs (cotyledons, stems, and roots); and to investigate the pathogenicity of these fungi on *A. alba* seedlings.

2. Material and methods

2.1. Study area

The study was conducted in the Western Carpathians (southern Poland) between 18.794°E and 22.722°E longitude and 49.133°N and 49.784°N latitude, in stands located in the lower montane belt (between 600 and 920 m a.s.l.). In that region, depending on altitude and location, the growth period (with a mean daily temperature above 5 °C) is ca 180–200 d, the average annual temperature is 5–7 °C, and the annual precipitation is 900–1450 mm with a maximum in June–July (Paszyński and Niedźwiedź, 1999). In the Polish part of the Western Carpathians, *A. alba* occupies 26.1% of the total forested area, grows on Eutric loamy Cambisols developed on flysch material, and commonly builds mixed forests with *F. sylvatica* and/or *P. abies* classified as *Dentario glandulosae – Fagetum* (Matuszkiewicz, 2001).

Diseased *A. alba* seedlings were collected from 16 stands representing: (1) mono-specific *A. alba* forests (six stands, hereafter fir forests), (2) mixed stands of *A. alba* and *F. sylvatica* (four stands, hereafter beech-fir forests), and (3) mixed stands of *A. alba* and *P. abies* (six stands, hereafter fir-spruce forests). Each species composition variant was represented by one to three stands in the eastern, middle, or western part of the Western Carpathians massif (Fig. 1). The number of beech-fir forests surveyed was lower than the number of fir and fir-spruce forests because seedlings with disease symptoms commonly occurred in these latter forest types, whereas in the beech-fir forests they were rather difficult to find. Two beech-fir stands preliminarily selected for this study had to be excluded because seedlings with infection symptoms were not found. In addition to species composition, the criteria for stand selection included the occurrence of naturally established *A. alba* regeneration growing among multi-species herbal vegetation of a loose and discontinuous cover. In the case of stands with a lower proportion of *A. alba*, attention was also paid to the possible uniform distribution of that species throughout the stand.

According to field measurements, the stand volume ranged between 390 and 710 $m^3\ ha^{-1}$ (mean = 567 $m^3\ ha^{-1}$) in the fir and fir-spruce forests and between 430 and 730 $m^3\ ha^{-1}$ (mean = 523 $m^3\ ha^{-1}$) in the beech-fir forests. In the fir stands, the partition of *A. alba* trees in the stand volume was above 95%, in the beech-fir stands it ranged between 16 and 52% and in fir-spruce stands it was between 62 and 81%.

2.2. Disease symptoms and plant material

In the selected stands, the seedlings were monitored from the time of germination. The first symptoms developed on seedling cotyledons shortly after emergence and heavy rainfall in May/June and had the appearance of irregular necrotic spots. These spots later expanded into brown blotches. Similar symptoms also occurred on seedling stems. As the disease progressed, the entirely discoloured cotyledons often hung downward along the seedling stems. The majority of the infected seedlings died before formation of the first needles (Fig. 2). Following excavation, the symptoms of root dieback were only observed occasionally. Seedlings with less extensive infection (e.g. with only a single cotyledon infected) were able to develop their first needles and survive at least until the end of the growing period. In the fir and fir-spruce stands, the majority of the seedlings showed distinct disease symptoms, whereas in the beech-fir stands, seedlings with disease symptoms were difficult to find and occurred almost exclusively in microsites with bare mineral soil.

In each of the 16 stands, 40–50 diseased *A. alba* seedlings with root systems were excavated in June 2014 (i.e., about 6 weeks after germination) from at least seven locations evenly scattered over the stand's area. The excavated seedlings were packed individually in plastic bags and transported to the laboratory, where they were stored for a maximum of 12 h in a cool room at 4 °C.

2.3. Isolation and fungal identification

The seedlings were washed in running tap water and divided into cotyledons, stems, and roots. From each stand, 30 cotyledons with discoloration, 14 stems, and 14 roots were selected randomly for isolation of fungi. Additionally, from each stand, 10 randomly selected root systems were excised for isolation of oomycetes. Tissue fragments for fungal isolation were surface sterilized in 96% ethanol (10 s) and in 4% sodium hypochlorite (3 min). After rinsing in sterile water (three times for 3 min) and drying on sterile filter paper, each cotyledon with chlorotic spots was divided into three sections, and was placed in a Petri dish containing malt extract agar [MEA: 20 g Biocorp malt extract (Biocorp Polska Sp. z.o.o., Poland), 20 g Biocorp agar (Biocorp Polska Sp. z.o.o., Poland), 1000 mL sterile water and 50 mg L^{-1} tetracycline (Polfa S.A., Poland)]. After surface sterilization and drying, each stem and root was cut into three sections (5–10 mm long) and placed on a medium. For oomycete isolation, roots were surface sterilized in 0.5% sodium hypochlorite (3 min). After rinsing in sterile water (three times for 3 min) and drying on sterile filter paper, each root was cut into three sections and placed on P5ARP [17 g L^{-1} Corn Meal Agar (CMA, Sigma-Aldrich Corp., USA), 5 mg L^{-1} pimaricin (Pimafucin, Yamanouchi, The Netherlands), 250 mg L^{-1} ampicillin (Amplicillin, Polfa, Poland), 10 mg L^{-1} rifampicin (Rifampicin, Polfa, Poland), 100 mg L^{-1} PCNB (Sigma-Aldrich Corp., USA)], and P5ARPH [P5ARP + 50 mg L^{-1} hymexazol (Tachigaren 50WP, Sankyo co. Ltd., Japan)] selective media for the isolation of *Pythium* spp. and *Phytophthora* spp., respectively (Jeffers and Martin, 1986).

Overall, a total of 1132 sections from cotyledons, stems, and roots were collected from 406 *A. alba* seedlings. Hereof, 480 were