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ORIGINAL RESEARCH PAPER

Fungi inhabiting the aboveground organs of downy willow (*Salix lapponum* L.) and swamp willow (*Salix myrtilloides* L.)

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Abstract
The species of downy willow (*Salix lapponum*) and swamp willow (*Salix myrtilloides*) are extremely rare relicts among all members of the genus *Salix*. In Poland, they occur only in the eastern part of the Karkonosze Mountains, Masurian Lake District, and Polesie Lubelskie area. A huge threat to both species is the drainage of peat bogs and their excessive exploitation as well as diseases caused by bacteria and fungi. The present study aimed identify fungal species colonizing the aboveground organs of plants with disease symptoms and to indicate which species may pose a threat towards *S. lapponum* and *S. myrtilloides* growing under natural conditions. This research was conducted in Polesie National Park as a part of a research project by the Ministry of Science and Higher Education. Plant health assessments were carried out in natural habitats, while aboveground organs with disease symptoms were collected for laboratory mycological analysis. *Colletotrichum salicis* (teleomorph *Glomerella salicis*), *Coniothyrium concentricum*, *Cylindrosporium salicinum*, *Phomopsis* spp., *Truncatella angustata*, and *Cytospora chrysosperma* (teleomorph *Valsa sordida*) were the fungal species most frequently isolated from leaves and shoots of both willow species. The above-mentioned fungi are dangerous plant pathogens from the family Salicaceae. There were no obligatory parasites or monophagous pathogens on the aboveground organs of downy or swamp willow. Obligatory parasites may indicate a markedly low population of host plants in Polish conditions.

Keywords
Salix; mycological analysis; pathogenic fungi

Introduction
Downy willow (*Salix lapponum*) and swamp willow (*Salix myrtilloides*) are extremely rare plant species belonging to the family Salicaceae. The presence of these species has been noted in the mountainous regions of Europe, Asia, the Scandinavian Peninsula, eastern Siberia, and within the Arctic Circle. In Poland, these species occur only in the eastern part of the Karkonosze Mountains, Masurian Lake District, and Polesie Lubelskie area. These species are strongly associated with specific habitat conditions in peat ecosystems. *S. lapponum* and *S. myrtilloides*, as glacial relicts, are under strict legal protection in Poland. Since 2014, downy willow has been designated a critically endangered plant (CR), while swamp willow (EN) is considered a species in danger of extinction [1–7].

The decreases in their populations as well as their decline in Poland (recorded since the 1950's) is associated primarily with dynamic changes in habitat conditions, mainly under the influence of anthropopressure in peat ecosystems. Natural biocenoses are subject to processes of ecological succession, manifesting mainly as the entry of wetland species of woody and expansive plants. For boreal willow, this is a particularly unfavorable, phenomenon mainly because it induces interspecies competition for environmental resources, resulting in the displacement of species with a narrow range of ecological tolerance. The increase in the population of common species from the genus *Salix* favors increases in the pressure of phytopathogenic microorganisms specifically toward this group of plants. Moreover, there is an increased risk of hybridization, which is one of the most profound threats to the survival of studied species of plants [8,9].

Additionally, diseases caused by various infectious agents, including bacteria and fungi, are highly harmful for plants of different species of the genus *Salix* [10–16].

The most harmful bacterial diseases include bacterial wilting of willow caused by *Erwinia salicis* (Day) Chester and root tuberosity caused by *Agrobacterium tumefaciens* (Smith et Townsend) Conn. [14,17,18].

Important diseases caused by fungi include: powdery mildew caused by *Phyllactinia* spp. and *Uncinula aduncta*, rust caused by *Melampsora* spp., tarry willow spot (willow melanoma) caused by *Rhytisma salicinum* (Pers.) Fries, stem scab caused by *Venturia chlorospora* and *Physalospora miyabeana*, scab of *Salix* caused by *Venturia chlorospora* and *Venturia saliciperda*, and anthracnose of *Salix* caused by *Drepanopeziza sphaerioides* and *Glomerella miyabeana* [10–21].

By covering the surfaces of leaves and stems, especially the thin layer of epidermis, with mycelium and conidia as well as by taking up nutrients, powdery mildew causes weakening of the plant and the plant organs gradually die-off. Wintering of the pathogen in the form of chasmothecia on infested plants increases the amount of the infectious material annually, which favors infections in subsequent vegetation periods [10,14,19].

Fungi of the genus *Melampsora* cause rust, one of the most important diseases of the willow plant. *Melampsora epitea* and *M. capreum* belong to the most common rust species in various geographical regions [10,21]. The above mentioned fungi, through development in leaf tissues and not-related stems, damage the epidermis and the parenchyma, which disturbs the transpiration and assimilation processes of plants and contributes to significant plant weakening and dying [10,14,21,22]. Rusting off of the plant is particularly dangerous for willow species cultivated for energy purposes because defoliation and dying of shoots causes significant economic losses [10,14,21,22].

Leaf blotches, especially brown spots on leaves and stems and black (tarnished) leaf spots, are one of the most harmful diseases of leaves for many species of *Salix* plants. These diseases are caused by *Drepanopeziza sphaerioides* (Pers.) Höhn and *Rhytisma salicinum* (Pers.) Fr., respectively. These diseases are difficult to distinguish in the spring. However, in the case of brown spots, the leaves die prematurely, and on the shoots, in favorable conditions, spots and cracking of the bark are visible. In contrast, during infection by *R. salicinum*, the brown spots on the leaves are covered by a characteristic black coating of conidiophores and conidia of fungus in the summer [11,14,17,20]. Scab of willow stems, stem anthracnose, as well as willow cancer are very harmful diseases for the aboveground parts of willows. These diseases are very similar to each other in terms of symptoms but they are caused by different species of fungi of the *Cytospora*, *Glomerella*, and *Venturia* genera. Black spots, necrosis, deepening anthracnose, distortions, and cancers are formed on the infected stems, which causes the stems and even whole plants to die-off [11,14,16,20,23–25].

No mycological information was found in Polish literature on downy and swamp willows. The occurrence of *S. lapponum* and *S. myrtilloides* in phytocenoses of peat ecosystems in Polesie National Park inspired this research. The aim of this research was to determine the species of fungi inhabiting the aboveground parts of downy willow and swamp willow and indicate which of them may pose a threat to plants in natural conditions. Such studies had not been conducted until 2014 in the eastern region of Poland. In contrast, observations during 2014–2015 on the health status of willows carried out in the Karkonosze Mountains did not show that the fungi infected the aboveground organs of downy willow [26].

Material and methods

The field experiments were conducted in 2013 in eastern Poland in the Pojezierze Łęczyńsko-Włodawskie region. The research material consisted of *S. lapponum* shrubs from the populations growing on peat of the lakes, Bikcze, Długie, Karaśne, and Moszne, and *S. myrtilloides* shrubs growing on peat of the lakes, Bikcze, Moszne, and in the middle forest position of peat of the Dekowina Lake (in Sobiborski Landscape Park). The health of plants was determined based on that of 10 randomly chosen shrubs of each willow species in natural conditions. The health of the plants was estimated on the basis of disease symptoms and etiological signs occurring on the leaves and stems, as well as on the basis of microscopic observations during the following two periods: flowering, i.e., the end of April, and ripening of fruits, i.e., mid-June. The mineral medium was composed of 38 g saccharose, 0.7 g ammonium nitrate (NH_4NO_3), 0.3 g potassium dihydrogen phosphate (KH_2PO_4), 0.3 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and trace amounts of the following: ferrous chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), copper sulfate heptahydrate ($CuSO_4 \cdot 7H_2O$), and manganese sulfate pentahydrate ($MnSO_4 \cdot 5H_2O$) and 20 g agar. The leaves and stems with symptoms of necrosis were collected for further laboratory tests. Ten pieces of each organ from each habitat and plant species were procured on two dates. The plant material for mycological analysis was rinsed for 20 minutes under running water and superficially disinfected in a 10% solution of sodium hypochlorite for 1.5 minutes. Next, the material was rinsed three times for 3 minutes in sterile distilled water. Inoculum of 3-mm diameter was prepared from the disinfected plant material and then placed on the artificial culture medium (mineral medium solidified in Petri dishes) to isolate the fungi. One hundred inoculums were prepared from each organ. The material prepared was incubated at a temperature of 22°C in dark for 6 days. The colonies of fungi obtained were then transferred onto slants with potato-glucose agar medium PDA (Difco) [27]. The obtained fungal cultures were segregated on the basis of their macroscopic features after 2 weeks of growth. The genera and species of pure cultures of fungi were classified on PDA medium or on standard media, i.e., on malt agar medium (Difco) and Czapek–Dox agar medium (Difco). The obtained name of the fungal species and their author's information were provided in accordance with the binding taxonomic principles, based on Index Fungorum 2018 data (http://www.indexfungorum.org/names/names.asp).

Results

Evaluation of plant health

During the first period of observation at the end of April 2013, disease symptoms were found on all tested downy willow shrubs at the Bikcze Lake region, including progressive necrosis of tops of the stems, and in two cases, death of the whole plant. On some young and drying stems, cancers were observed with red bulges and spores of fungi belonging to the genera *Colletotrichum*, *Valsa*, and *Venturia*.

In the populations of *S. lapponum* in the Karaśne and Moszne lakes, on one of the studied plants, at both locations, two individual stems were dried. Numerous clusters and ostioles of fruiting bodies (perithecia and acervuli) with spores typical of the genera *Valsa*, *Venturia*, *Colletotrichum*, and *Phomopsis*, as well as *Fusicladium saliciperdum* (Allesch. et Tubeuf) Lind and the conidial stage of the teleomorph *Venturia saliciperda* Nüesch were observed.

During the second period of observation, the number of shrubs with disease symptoms did not increase in any of the above-mentioned locations, but necrosis of stems was observed on one of the studied plants in Długie village. Similar to the first period of observation, numerous clusters of spores were observed on the surfaces of the infected organs. These were spores produced by fungi of the genera *Colletotrichum*, *Valsa*, and *Phomopsis* and *Fusicladium saliciperdum*.

The evaluation of swamp shrubs was carried out in natural conditions in sites along the Bikcze, Moszne, and Dekowina lakes in peatland showed the drying of apical shoots

in two, two, and four shrubs, respectively, during both the observation periods. Microscopic studies of leaves and stems revealed the presence of fungal spores belonging to the genera *Alternaria*, *Coniothyrium*, *Colletotrichum*, *Phomopsis*, and *Valsa*.

No strict pathogens causing downy mildew or rust were observed on *S. lapponum* and *S. myrtilloides* shrubs during the first and second periods of observation. In addition, leaf blotches caused by the fungi *Drepanopeziza sphaerioides* and *Rhytisma salicinum* were not found on the examined plants at the above mentioned sites.

Mycological analysis of *S. lapponum* leaves and stems

The study performed on leaves and shoots of the downy willow generated a total of 523 fungal isolates belonging to 16 genera. In the first period of isolation, 256 isolates were obtained, while in the second period, 267 isolates were obtained (Tab. 1). The fungi *Coniothyrium concentricum*, *Fusarium sporotrichioides*, *Cytospora chrysosperma*, *Botrytis cinerea*, *Colletotrichum salicis*, and *Phomopsis* spp. as well as the yeast fungi, *Saccharomyces* spp., were obtained most frequently from the selected leaves of downy willow obtained during the first period of isolation. During the second period, the fungi species *Alternaria alternata*, *C. concentricum*, *C. salicis*, *Epicoccum nigrum*, *F. sporotrichioides*, *Phomopsis* spp., and *Cytospora chrysosperma* were most often isolated from the leaves (Tab. 1). In addition, the fungi *Alternaria alternata*, *C. salicis*, *C. concentricum*, *C. chrysosperma*, and *F. sporotrichioides* were isolated most frequently from stems during both the first and second periods of isolation (Tab. 1).

Mycological analysis of *S. myrtilloides* leaves and stems

The study of swamp willow leaves and stems generated 465 isolates of fungi belonging to 15 genera (Tab. 2). The number of obtained fungal isolates from the examined willow organs was similar among samples from different places of origin. The species *Alternaria alternata*, *Cladosporium cladosporioides*, *Colletotrichum salicis*, *Coniothyrium concentricum*, and *Fusarium sporotrichioides* were the most-mapped fungal species during the first period of isolation and represent 16.4%, 13.4%, 16.8%, 19.3%, and 8.4% of the total number of isolates obtained from the above-mentioned willow organs. The fungi belonging to the genus *Phomopsis* were obtained from the shoots of willows collected from the Dekowina site. Isolates of this fungus constituted 29.5% of the total number of fungal isolates obtained from shoots during the first period of isolation. During the second mycological analysis of the studied organs of plants, the fungal species *A. alternata*, *F. sporotrichioides*, *C. chrysosperma*, *Truncatella angustata*, and *C. concentricum* were isolated most frequently and constitute 24.7%, 14.5%, 14.1%, 11.0%, and 5.7%, respectively, of the total number of fungi obtained during this second period of isolation (Tab. 2).

Discussion

The obtained results indicate that fungi inhabited the leaves and shoots of all examined shrubs with disease symptoms. The species that constitute potential disease threats for downy and swamp willows were among the species identified in the studied habitats. This fact is supported by the large biodiversity of the fungi species obtained from the studied willow organs as the result of mycological analysis of their leaves and stems. Many of these species are known for causing harmful diseases in willow shoots, resulting in their death and thus complete elimination from the environment [2,14–18]. It should, therefore, be assumed that in conditions favorable for pathogenic species, these pathogens can affect a significant portion of the *S. lapponum* and *S. myrtilloides* populations. The isolation of species causing anthracnoses, shoot rot, and cancers, including *Colletotrichum salicis*, *Coniothyrium concentricum*, *Cylindrosporium salicinum*, *Phomopsis* spp., *Truncatella angustata*, and *Cytospora chrysosperma* should be considered particularly alarming. These fungi belong to a group of dangerous plant

Tab. 1 Participation (%) of fungi isolated from aboveground parts of downy mildew *Salix lapponum* L. growing in various environmental conditions.

Species/genus of fungi	Number of isolates												Sum of isolates in each period of isolation; number (%)		Total fungi; number (%)
	Bikcze				Karaśne I / Długie II				Moszne						
	Leaves		Stems		Leaves		Stems		Leaves		Stems				
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	
Alternaria alternata (Fr.) Keissl.		28		2	10	28	29	34	2	35	5	5	46 (18.0)	132 (49.4)	178 (34.0)
Aureobasidium pullulans (de Bary et Löwenthal) G. Arnaud						2								2 (0.7)	2 (0.4)
Botrytis cinerea Pers.		2			4				8		6		18 (7.0)	2 (0.7)	20 (3.8)
Cladosporium cladosporioides (Fresen.) G. A. de Vries			4	2									4 (1.6)	2 (0.7)	6 (1.1)
Colletotrichum salicis (Auersw. ex Fuckel) Damm, P. F. Cannon et Crous			3	15	2		9		4		5	7	23 (9.0)	22 (8.2)	45 (8.6)
Coniothyrium concentricum (Desm.) Sacc.	31			13									31 (12.1)	13 (4.9)	44 (8.4)
Cylindrosporium salicinum (Peck) Dearn.											7		7 (2.7)		7 (1.3)
Cytospora chrysosperma (Pers.) Fr.		4	14	2	7	2				2			21 (8.2)	10 (3.7)	31 (5.9)
Epicoccum nigrum Link	12	11		2	3	10		2	4	3		2	19 (7.4)	30 (11.2)	49 (9.4)
Fusarium sporotrichioides Sherb.		2		11	9		10	3	2	3		2	21 (8.2)	21 (7.9)	42 (8.0)
Penicillium spp.				3							10	5	10 (3.9)	8 (3.0)	18 (3.4)
Phomopsis spp.					14	8		2					14 (5.5)	10 (3.7)	24 (4.6)
Saccharomyces sp.									30		2		32 (12.5)		32 (6.1)
Trichoderma harzianum Rifai								2			10	4	10 (3.9)	6 (2.2)	16 (3.1)
Truncatella angustata (Pers.) S. Hughes								7				2		9 (3.4)	9 (1.7)
Total	43	47	21	50	49	50	48	50	50	43	45	27	256 (100)	267 (100)	523 (100)

I – first observation; II – second observation.

Tab. 2 Participation (%) of fungi isolated from aboveground parts of swamp willow *Salix myrtilloides* L. growing in various environmental conditions.

Species/genus of fungi	Number of isolates												Sum of isolates in each period of isolation; number (%)		Total fungi; number (%)
	Bikcze				Moszne				Dekowina						
	Leaves		Stems		Leaves		Stems		Leaves		Stems				
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	
Alternaria alternata (Fr.) Keissl.	15	20		4		15	22	8	2	6		3	39 (16.4)	56 (24.6)	95 (20.4)
Aureobasidium pullulans (de Bary et Löwenthal) G. Arnaud										3				3 (1.3)	3 (0.6)
Botrytis cinerea Pers.												7		7 (3.1)	7 (1.5)
Cladosporium cladosporioides (Fresen.) G. A. de Vries	15	9		4	8		2		7				32 (13.4)	13 (5.7)	45 (9.7)
Colletotrichum salicis (Auersw. ex Fuckel) Damm, P. F. Cannon et Crous					14	8	23		5	2	6		48 (20.2)	10 (4.4)	58 (12.5)
Coniothyrium concentricum (Desm.) Sacc.			20			2		6	9		17	5	46 (19.3)	13 (5.7)	59 (12.7)
Cylindrosporium salicinum (Peck) Dearn.					2								2 (0.8)		2 (0.4)
Cytospora chrysosperma (Pers.) Fr.		3	10	10				12				7	10 (4.2)	32 (14.1)	42 (9.0)
Epicoccum nigrum Link	5	5		2		2	2		2	3	5		14 (5.9)	12 (5.3)	26 (5.6)
Fusarium equiseti (Corda) Sacc.									2		3		5 (2.1)		5 (1.1)
Fusarium sporotrichioides Sherb.		7	20	8				13		3		2	20 (8.4)	33 (14.5)	53 (11.4)
Penicillium sp.	2					7							2 (0.8)	7 (3.1)	9 (1.9)
Phomopsis spp.											13	3	13 (5.4)	3 (1.3)	16 (3.4)
Saccharomyces spp.		3		8	7								7 (2.9)	11 (4.8)	18 (3.9)
Trichoderma harzianum Rifai										2				2 (0.9)	2 (0.4)
Truncatella angustata (Pers.) S. Hughes				2						18		5		25 (11.0)	25 (5.4)
Total	37	47	50	38	31	34	49	36	27	37	44	32	238 (100)	227 (100)	465 (100)

I – first observation; II – second observation.

pathogens from the family Salicaceae [2,14,17,18]. The mode of sporulation and other morphological structures of these fungal species are distinct; therefore, to properly identify them, microscopic observations and mycological analyses of the aboveground organs of plants with disease symptoms are necessary.

The isolation of these species of fungi indicates the possibility of plant infections by pathogens on the neighboring species of trees and shrubs. This fact, combined with unfavorable weather conditions for *S. lapponum* and *S. myrtilloides* and the destruction of peat bogs, indicates the presence of high disease risk for the examined willow species. The dates of fungal isolation did not affect the total number of obtained fungal cultures. However, in the case of *Colletotrichum salicis*, *Coniothyrium concentricum*, and *Phomopsis* spp., a significantly higher number of isolates were obtained in the first set of isolations than in the second set. It can be assumed that colonization of the aboveground willow organs, especially shoots, by *C. salicis*, *C. concentricum*, and *Phomopsis* spp. at the beginning of the vegetation period provides these fungi the possibility of engaging in early destructive actions and causing the destruction of shoots in the same or following vegetation period. A similar effect of these fungi on shoots was found with various species of shrubs as well as forest, fruit, and orchard trees [28–32]. The presence of occasional parasites such as *Alernaria alternata*, *Botrytis cinerea*, and *Cladosporium cladosporioides* on the leaves and shoots of *S. lapponum* and *S. myrtilloides*, especially during the later vegetation season, may accelerate the death of plants first infected by other pathogens [30,33].

The decrease in downy willow populations and a lack of monophagous pathogens (compared to in original arctic conditions) demonstrates a lack of host plants and the difficulties of survival of pathogens. In addition, obtaining fungal species from the genus *Salix* that are typically characteristic of other plants indicates high disease risk for willow and the need for its special protection. Despite the lack of information in the literature on the presence of fungi on overwintering aboveground parts of swamp willows, similar conclusions can also be made for swamp willows because health observations and the results of mycological analyses of the aboveground organs of these two species of willows are similar. There were no obligatory parasites on downy or swamp willow organs in our studied habitat conditions. However, these fungi commonly infest other willow species, especially those cultivated for energy purposes, and may cause losses of up to 40% of the crop [11,13,20,22]. It is likely that the significant humidity and reduced air temperatures compared to those of neighboring ecosystems did not support the development of these pathogens. Strict parasites require long-term uptake of nutrients from the host plant leading to host plant weakening, which negatively affects host conditions and increases susceptibility to overwintering and disease [20,22]. Similarly, the lack of powdery mildew and rust on the leaves of downy willow was found in studies by Chlebicki [2] as well as Pusz and Urbaniak [26] that were conducted in the Karkonosze Mountains. The presence of rust caused by *Melampsora epitea* and *M. alpina* was observed in the same year on two other species of willows, *Salix herbaceae* and *S. silesiaca* [26].

According to Chlebicki's research [2], among rust fungi, only *Melampsora lapponum* Lindf. is a monophagic species capable of infecting the leaves of downy willows in arctic conditions. The absence of this species of fungus in Polish conditions indicates a very low population of the host plants. The reduction in the number of sites as well as the population size of the downy willow was demonstrated in studies conducted from 2001 to 2003 in the Polesie National Park [5] and from 1994 to 2005 in Knyszyńska and Białowieska primeval forests [34].

The present study confirms that for the overall assessment of the condition of *S. lapponum* and *S. myrtilloides*, monitoring of the shrub health should be included, based on field trials and laboratory mycological analyses.

Conclusions

- The leaves and stems of the tested downy and swamp willow shrubs were colonized by numerous species of fungi. The fungi may pose a potential disease risk.

- The occurrence of species causing anthracnoses, recrements, and shoot carcinomas that belong to the genera *Coniothyrium*, *Cylindrosporium*, *Glomerella* (anamorph stage *Colletotrichum*), *Phomopsis*, and *Cytospora* is considered particularly harmful.
- The isolation of numerous polyphagic species of fungi indicates the possibility of infections of various neighboring species of trees and shrubs in a given environment.
- The lack of monophagous pathogens for *S. lapponum* in Polish conditions indicates that the population of the host plant is not high.
- The decrease in the population of relict species indicates the need to protect such species and systematically monitor the health of shrubs.

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