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Endophytic fungi and latent pathogens in the sedge *Carex secalina* (Cyperaceae), a critically endangered species in Europe

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Abstract: Endophytic fungi are widespread in plants and affect the host fitness and population size. We found 12 fungal taxa in *C. secalina,* a critically endangered species in several European countries, at two study sites in Poland. The most frequently occurring fungal taxa were *Colletotrichum destructivum* and *Acremonium* sp. Both taxa were found in half of the examined tussocks. The highest number of fungal taxa was noted in the *C. secalina* plants growing in the roadside area, where 7 of the 12 identified fungal taxa occurred. These fungi, inhabiting leaf tissues, are known for their pathogenicity but no visible symptoms of any diseases were observed on *C. secalina* leaves. This suggests that these fungi are latent pathogens.

Keywords: *Colletotrichum*; fungal diseases; fungal endophytes; halophytic species

The sedge *Carex secalina* Willd. ex Wahlenb. is a halophyte with interrupted Euro-Siberian-sub-Irano-Turanian distribution (Meusel *et al.* 1965). This species is rare over its entire range, as it grows in scattered and isolated locations in Europe and Asia (Egorowa 1999). In several European countries, *C. secalina* is classified as a critically endangered or endangered species (Ludwig & Schnittler 1996; Holub & Procházka 2000). This species is thus protected by law based on the Convention on the Conservation of European Wildlife and Natural Habitats, adopted in Bern in 1979.

In Poland, at the beginning of the 20th century, *C. secalina* occurred in nine localities concentrated in the Kujawy Region (Bock 1908). *C. secalina* has been observed for the longest period in Rąbin, which is currently a housing estate in Inowrocław (Wodziczko *et al.* 1938; Urbański 1930). Post-war investigations of halophytic flora and vegetation by Wilkoń-Michalska (1963) did not confirm the occurrence of this species in these localities. For over 40 years, *C. secalina* was thus considered an extirpated taxon, both in the Kujawy Region (Wilkoń-Michalska 1963) and in Poland (Zając & Zając 1993). In 2000, its presence was confirmed again at two historical localities, and its presence was documented in a new locality (Lembicz *et al.* 2009). The extant populations of *C. secalina* occur close to each other, mainly on the regularly used pasture grounds, near small water bodies (Lembicz *et al.* 2009; Dominiak & Jakubas 2015; Sandecki 2016). Currently, this species is considered as critically endangered in Poland (Chmiel *et al.* 2014).

C. secalina reproduces sexually by seeds in the first year of its life. A single tussock consists of approximately 400 generative shoots (Bogdanowicz *et al.* 2011; Lembicz *et al.* 2011). Significant differences in generative shoot production between populations have been found, and the highest sexual reproduction

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rate has been observed in the populations occurring in man-made habitats (Lembicz *et al.* 2011). Furthermore, *C. secalina* seeds show a high germination ability (Lembicz *et al.* 2011). These features should theoretically ensure the success of this sedge in terms of habitat colonisation. However, large fluctuations in the size of the populations have been found, resulting in frequent disappearances or extirpations of this species (Lembicz *et al.* 2009). The factors contributing to population disappearances of this rare sedge in Europe are unknown.

The aim of our study was to check for the presence of endophytic fungi in the sedge *C. secalina*. 'Endophytes' are organisms, mostly bacteria and fungi, that can colonise internal plant tissues without causing apparent harm to the host (Petrini 1991); organisms that can enter the epiphytic phase and latent pathogens can be included in this group (Petrini 1991). When it was found that endophytic fungi occur in almost every plant (Petrini 1986), the taxonomical diversity of endophytic fungi and the effects of their interactions with plants began to be intensively studied (Rodriguez *et al.* 2009). One fungus may be both beneficial endophyte and pathogen depending on the circumstances. Understanding these endophytes and their effect on plants is essential for preparing a proper strategy that will minimise their negative influence (Busby *et al.* 2016). In our opinion, both the absence of fungal endophytes that positively affect plant hosts as well as the presence of disease-causing fungal endophytes may result in the disappearance of populations of endangered plants This study is the first study on the occurrence of endophytic fungi in the tissues of *C. secalina* and, to our knowledge, the second study on the presence of these fungi in the genus *Carex* (Canals *et al.* 2014).

MATERIAL AND METHODS

Study sites. The tussocks of *Carex secalina* that were selected for this study originated from two locations: Rąbin (denoted as locality R) and Wierzchosławice (denoted as localities W1 – pasture and W2 – the side of a gravel road). Both locations are situated in the Inowrocław District of Kujawy-Pomerania Province, Poland, approximately 13 km away from each other (Table 1). The studied populations of sedges were found in the natural range of this species in Poland (Lembicz *et al.* 2009). The population in locality R was small and contained 5 tussocks, while in localities W1 and W2, there were 410 tussocks in total. Localities W1 and W2 are near each other, but we treat them separately because of their habitat differences.

Table 1. Origin of sampled *Carex secalina* tussocks

Site		Location	Tussocks (pcs)
Rąbin	R	N 52°46'29.3" E 18°14'56.4"	2
Wierzchosławice	W1	N 52°52'44.6" E 18°21'00.8"	7
	W2		3

Plant sampling and isolation of fungal endophytes. Twelve tussocks of *Carex secalina* were selected as samples (Table 1); all selected tussocks were 2 years old (Figure 1). Three healthy-looking leaves without herbivore or other damage were taken from each tussock for further analysis. Relatively large fragments (approximately 2 cm long) were excised from the basal, middle and apical parts of each leaf. The leaf fragments were surface-sterilised in 75% ethanol (30 s), 4% sodium hypochlorite (1 min), 75% ethanol (15 s), and then rinsed in sterile water. After drying (5 min), all the fragments were cut into four (0.5 cm long) pieces, placed on PDA (potato dextrose agar) in Petri dishes, sealed with parafilm and stored at room temperature. The plates were observed every day, and emerging fungi were successively transplanted to new, fresh plates. The surface sterilisation procedure was carried out in a laminar hood to avoid contamination. To confirm the efficiency of the sterilisation process, 50 µl of rinse water was spread onto potato dextrose agar (PDA) and incubated at room temperature for 14 days.

Figure 1. A tussock of the sedge *Carex secalina* with generative tillers. It is a perennial, monoecious plant (Photo R. Sandecki)

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Identification. For the identification of endophytes, the fungal isolates were grouped into morphotypes, based on macroscopic characteristics, such as the appearance and colour of the mycelium. Then, isolates representative of each morphotype were analysed using molecular methods. Small parts of the mycelium from the fungal cultures were ground in liquid nitrogen in a 1.5-ml microcentrifuge tube. DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and stored at –20°C. The primer pair of ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) was used to amplify the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA. PCR was performed in a total volume of 25 µl containing 12.5 µl of 2 × PCR MasterMix (Fermentas, Lithuania), 1 µl of each primer (10 pM/µl), 5.5 µl of nuclease-free water, and 5 µl of the DNA template. The amplification cycle conditions were as follows: initial denaturation (94°C, 4 min), 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 30 s), product extension (72°C, 30 s) and a final extension (72°C, 5 min). The PCR products were purified with the Wizard PCR Kit (Promega, USA) and directly cycle sequenced with ABI BigDye Terminator v3.1 (Applied Biosystems, USA). The obtained sequences were edited using Chromas (www.technelysium.com.au) software and submitted to GenBank (Table 2). Finally, the sequences were compared to those published in European Molecular Biology Laboratory (EMBL) nucleotide database and in the NCBI (www.ncbi.nlm.nih.gov) databases using BLAST (Altschul *et al.* 1990). The positive identification of a species was confirmed if it shared ≥ 98% of the ITS region sequence identity with the reference sequence from the databases; if the similarity was between 97.9 and 95%, only the genus was accepted. These criteria were adopted from Canals *et al.* (2014).

Analyses. The means of fungal taxa per tussock and per site were calculated as well as the incidence of individual endophyte taxon in the *Carex secalina* tussocks, expressed as the percentage of tussocks with a given taxon. Isolates we were unable to identify to the species level were treated as separate species if they did not show sequential similarity to other isolates.

RESULTS

Thirty-six fungal isolates were obtained. The isolates were grouped into 12 morphotypes that, after identification, turned out to be different taxa. Nine of them were identified to the species level but for the other three sequence-based matches were too low so they were identified to the genus level only (Table 2). The most frequent was *Colletotrichum destructivum,* followed by unidentified *Acremonium* sp. 1. Both taxa were observed in half of the examined tussocks (Figure 2). Some fungal endophytes occurred only in single tussocks (*C. circinans, Fusarium armeniacum, F. lateritium* and *Stagonospora trichophoricola*).

The mean number of fungal taxa per tussock sample was 3.0 ± 0.85 (SD). The richest site was W2 (3.67 ± 0.58), whereas the poorest was W1 (2.71 ± 0.76). All tussock samples revealed at least two endophytic taxa, and the maximum number of taxa per tus-

Table 2. Endophytic fungal isolates obtained from the leaves of *Carex secalina* used for DNA sequencing

No	Isolate name	Fungal taxa	GenBank accession No.	BLAST match sequence		
				accession No	similarity (%)	coverage (%)
1	C6_2	*Acremonium* sp. 1	MG978341	MG250388	96.3	99
2	C9_1	*Alternaria infectoria*	MG978343	AY154690	99.8	100
3	C11_1	*Colletotrichum* sp. 1	MG978346	EU400139	95.1	99
4	C5_1	*Colletotrichum circinans*	MG978340	AJ301955	98.8	99
5	C2_2	*Colletotrichum dematium*	MG978337	KC790957	100.0	97
6	C3_2	*Colletotrichum destructivum*	MG978339	HQ674658	99.3	99
7	C2_1	*Colletotrichum graminicola*	MG978336	AB233343	99.5	94
8	C3_1	*Colletotrichum* sp. 2	MG978338	KX069822	96.8	99
9	C10_2	*Fusarium acuminatum*	MG978345	MF509746	99.7	99
10	C8_2	*Fusarium armeniacum*	MG978342	KF624790	98.9	99
11	C12_2	*Fusarium lateritium*	MG978347	GU480949	99.5	99
12	C10_1	*Stagonospora trichophoricola*	MG978344	KJ869110	100.0	93

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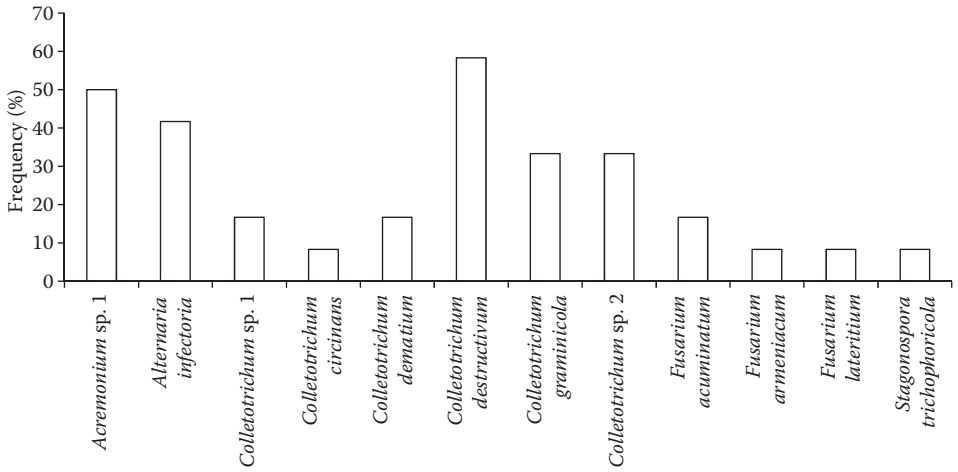

Figure 2. The incidence of individual endophyte taxa in the *Carex secalina* tussocks, expressed as the percentage of tussocks with a given taxon

sock sample was 4. Some taxa were characteristic of specific localities. These taxa showed both the high (*Alternaria infectoria*) and low frequency of occurrence, e.g., *F. armeniacum* or *C. dematium* (Table 3). The highest number of taxa was noted for locality W2, where 7 of the 12 identified fungal taxa occurred; in other localities (R and W1), this number was lower, 4 and 6, respectively. Although we analysed tissue samples from three parts of the leaves, we did not observe any differences in the endophyte presence between these parts. In other words, if an endophyte was present in a given leaf, we detected its mycelium in all three parts. Neither fungi nor bacteria were found in rinse water spread onto potato dextrose agar (PDA), which confirmed the efficiency of the sterilisation process and endophytic origin of identified isolates.

DISCUSSION

Our study showed first that *Carex secalina* was colonised by 12 taxa of endophytic fungi. Two fungal taxa, *Colletotrichum destructivum* and *Acremonium* sp. 1, occurred in half of the sedge tussocks. Thirdly, the highest number of fungal endophytes was found in the roadside site (W2).

All identified fungal taxa belonged to Ascomycota. In *C. secalina*, the most abundantly represented taxon is the genus *Colletotrichum* (Figure 2) and 11 out of 12 analysed tussocks were colonised with at least one *Colletotrichum* species. *Colletotrichum* includes species with endophytic, epiphytic, saprobic, and phytopathogenic lifestyles (Hyde *et al.* 2009). As plant pathogens, *Colletotrichum* species cause anthracnose diseases (Freeman *et al.* 1998). The genus

Table 3. Occurrence of identified fungal taxa in the studied localities and their incidence

No	Fungal taxa	Site			Frequency (%)
		R	W1	W2	
1	*Acremonium* sp. 1	–	+	+	50.00
2	*Alternaria infectoria*	–	+	–	41.67
3	*Colletotrichum* sp. 1	–	–	+	16.67
4	*Colletotrichum circinans*	–	+	–	8.33
5	*Colletotrichum dematium*	+	–	–	16.67
6	*Colletotrichum destructivum*	+	+	+	58.33
7	*Colletotrichum graminicola*	+	–	+	33.00
8	*Colletotrichum* sp. 2	–	+	–	33.33
9	*Fusarium acuminatum*	+	–	+	16.67
10	*Fusarium armeniacum*	–	+	–	8.33
11	*Fusarium lateritium*	–	–	+	8.33
12	*Stagonospora trichophoricola*	–	–	+	8.33
	Species count	4	6	7	

R – Rąbin; W1 – Wierzchosławice pasture; W2 – Wierzchosławice roadside

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was recently voted the eighth most important group of plant pathogenic fungi in the world, based on its perceived scientific and economic importance (Dean *et al.* 2012). On the other hand, studies have shown that some *Colletotrichum* species are endophytes that benefit host plants by reducing disease incidence and damage caused by other plant pathogens (Arnold *et al.* 2003; Herre *et al.* 2007). The representative of this genus, *C. destructivum*, is the most frequently observed fungal taxon in the leaves of *C. secalina*. This fungus is an agent of anthracnose disease in soybean (Manandhar *et al.* 1986), snapdragon (Tamioka *et al.* 2011) or curly dock (Liu *et al.* 2017). In its endophytic form, this fungus has been found in the leaves of the Chinese ground orchid (*Bletilla ochracea*) (Tao *et al.* 2013) and in sweet violet (*Viola odorata*) plants (Katoch *et al.* 2017), but the effects of this fungus and other species of this genus on plant hosts have not been investigated. In our case, the sedge leaves collected for this study did not show any external symptoms of fungal disease. However, the lack of external symptoms does not exclude the possibility of symptoms occurring later.

The second most frequent fungal taxon observed in *C. secalina* was *Acremonium* sp. 1 (Figure 2). Species of this genus have already been noted as soil fungi and root endophytes (e.g. Macia-Vicente *et al.* 2008a). It has been shown that in barley *Acremonium furcatum* may reduce the symptoms caused by the root pathogen *Gaeumannomyces graminis* (Macia-Vicente *et al.* 2008b). The third most frequent fungus, *Alternaria infectoria*, found in over 40% of the *C. secalina* tussocks, was also previously noted as a grass endophyte (e.g., Martin & Dombrowski 2015). This species also occurs in the bud tissues of *Malus domestica* and during flower and fruit development it transforms into its pathogenic form causing core rot of apples (Serdani *et al.* 1998). Similarly, fungi of the genus *Stagonospora* can occur either in the form of an endophyte that enhances the growth and biomass of seedlings of the reed *Phragmites australis* (Ernst *et al.* 2003) or in the form of a pathogen called *Stagonospora nodorum* leaf and glume blotch of wheat (Olivier *et al.* 2012). Interestingly, *S. trichophoricola* identified in this study in *C. secalina* was previously observed only in another representative of Cyperaceae – the sedge *Trichophorum cespitosum* (Crous *et al.* 2014).

The three species of *Fusarium* that were found in this study are mainly saprophytic fungi, but they were also noted as plant pathogens. *F. acuminatum* can cause postharvest rot of kiwi fruits (Wang *et al.* 2015) and damping-off disease in Aleppo pine (*Pinus halepensis*) (Lazreg *et al.* 2013), and *F. armeniacum* causes seed rot and root rot of soybean (*Glycine max*) (Ellis *et al.* 2012), while *F. lateritium* has been documented as a wound pathogen of tree species and as causing chlorotic leaf distortion (CLD) of sweet potato (*Ipomoea batatas*) or nut grey necrosis (NGN) of hazelnut (*Corylus avellana*) (Vitale *et al.* 2011). The mycelium of *Fusarium* fungi produces some chemical compounds that have a notable effect on human and animal health (Ferrigo *et al.* 2016.).

A previous study on the presence of fungal endophytes in *Carex brevicollis* showed the occurrence of 14 different taxa associated with this sedge species (Canals *et al.* 2014). However, only one of these taxa was identified to the species level, others were identified only to the genus level, and some were described as 'unknown Ascomycetes'. The only taxa in common with our study were the genera *Colletotrichum* and *Alternaria*; however, *Alternaria* was much more abundant in the *C. secalina* tussocks than was *C. brevicollis*. *Biscogniauxia nummularia,* the species present in 80% of plants studied by Canals *et al.* (2014), was not found in *C. secalina* tissues.

In summary, this study is the first study that shows the presence of endophytic fungi in populations of the critically endangered species *Carex secalina* in Europe. The fungi found in the leaf tissues of *Carex secalina* are known for their pathogenicity but we found them in endophytic form, showing no visible signs of their presence. Thus, they may be considered to be latent pathogens. Initial infection does not result in disease. Later, disease may be induced by environmental factors (Rodriguez *et al.* 2009). One of the most important latent pathogens of plants is *Colletotrichum destructivum*. The effects of its endophytic form on a host are unknown, but in its epiphytic form, it causes anthracnose disease (Damm *et al.* 2014). All pathogenic fungal endosymbionts we identified may affect fluctuations in the *C. secalina* population size. The sedge may also be threatened by the rust fungi *Puccinia dioicae*, which has been previously noted in *C. secalina* (Scheuer 2010), but not in our three sample sites. Since foliar microbiome transplants confer disease resistance in a critically endangered plant (Zahn & Amend 2017), the next step will be to check for the presence of these endosymbionts over the distribution of the studied sedge.

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