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Mycobiota of rye seeds infected with ergot fungi

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Abstract

Ascomycetes from the genus *Claviceps* are long-known pathogens of plants, including cereals. Despite of this, no effective fungicide has been developed yet. We checked whether the presence of ergot impacts the size and composition of the mycobiota in rye seeds (*Secale cereale*). The sizes of spikes and seeds were also checked. To identify endophytes, the fungal isolates were analyzed using molecular markers ITS1 and ITS2. We found nine taxa of fungal endophytes in the studied rye seeds. The most abundantly represented fungus, regardless of ergot presence, was *Alternaria infectoria*. Seeds from the spikes with and without ergot differed in the presence of two fungal species: *Microdochium nivale* occurred only in the seeds of spikes with ergot, while *Pyrenophora teres* only in the seeds of spikes without ergot. No effect of ergot presence in rye spikes on seed size was found. The lack of differences in the size and taxonomical composition of mycobiota and seed size between plants with and without ergot confirms the hypothesis on the benefits of this dangerous for humans pathogen for plants.

Keywords – ergot – fungal microbiota – molecular detection – pathogenic ascomycetes

Introduction

Each plant contains in its tissues fungal mycobiota, i.e., a co-occurring group of different fungal endophytes. These fungi accompany a plant already at the stage of seeds and are transmitted to the consecutive stages of plant's life (e.g., Bonfante et al. 2019). Mycobiota that inhabit plants provide additional benefits to their hosts. Thanks to the appropriate species composition of mycobiota, plants may improve their adaptation to environmental conditions (Bulgarelli et al. 2013, Vandenkoornhuyse et al. 2015). Fungi also supply their hosts with powerful chemical substances – alkaloids, which enable effective defence of plants against herbivores and different bacterial pathogens or viruses (e.g., Wäli et al. 2013). New taxa of endophytes are intensively searched for to control those plant pathogens for which no effective fungicides have been found yet (Gundel et al. 2013). Such pathogens are long-known ascomycetes of the genus *Claviceps*, particularly *Claviceps purpurea* (Lee 2009). These fungi occur in the majority of world regions with temperate, tropical and subtropical climates (Bandyopadhyay et al. 1998, Bové 1970, Geiger et al. 2009, Isakeit et al. 1998, Pažoutová 2002). From the economic point of view, the most important is the infection of rye (*Secale cereale* L.) that is a main crop plant in Germany, Scandinavia, Poland, Russia, Belarus and Ukraine (Geiger et al. 2009, Miedaner & Geiger 2015).

Despite substantial knowledge about *Claviceps* fungi, we are not capable of eradicating these pathogens from cereal crops. Pérez et al. (2013) showed that one species of endophytic fungi,

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commonly occurring in grasses, may significantly restrict infection with *Claviceps* fungi. Three times less spikes with ergot were found in plants inhabited by this endophyte compared to plants without it. In the discussed study, there was used an endophyte occurring in a different grass species to evaluate a fungal endophyte effect on the size of cereal infection with *Claviceps* fungi (Pérez et al. 2013). Perhaps, the obtained results might have been even better, if these authors had used an endophyte that inhabits cereals not infected with ergot fungi.

However, there have been no studies on the mycobiota composition in cereals to date. Thus, our first research aims were: (1) to detect mycobiota in the seeds of rye infected and uninfected with ergot fungi and (2) to connect the obtained results with the seed size. Two hypotheses were tested. The first one – there are essential differences in the size and taxonomical composition of mycobiota in seeds from infected plants compared to uninfected ones. We expected that mycobiota of plants infected with ergot fungi will be characterized by a lower size and taxonomical diversity. The second hypothesis – the size of seeds produced by infected plants is significantly smaller than uninfected plants. This is the first report on the presence of endophytic fungi co-occurring with ergot fungi in rye.

Materials & Methods

Samples and measurements

42 spikes with ergot and 42 without ergot were collected in a rye field in Poland, in the Kujawy-Pomerania Province (Figs 1-2) (53 N, 17 E). Samples were randomly harvested along a transect of 106 m in length. The collected spikes were placed in an envelope and transferred to the Laboratory of Department of Systematic and Environmental Botany at the Adam Mickiewicz University in Poznań. All spikes were numbered and measured using a ruler with 1 mm accuracy. Next, three seeds each were collected from each spike – 106 seeds from the spikes with ergot (C+) and 126 seeds from the spikes without ergot (C-). All ergots from the infected spikes were also sampled – 96 ergots in total. The measurements of seed length and width were conducted using the CellA software.

Cultivation and passage of fungi

The presence of fungal endophytes was checked in 30 seeds C+ and 30 seeds C-. The seeds were subjected to surface sterilization (75% ethanol 30 s, 5% NaOCl 3.5 min, 75% ethanol 15 s, with distilled water for rinsing). Next, the seeds were placed in Petri dishes with Potato Dextrose Agar (PDA) medium containing antibiotics (chloramphenicol, 100 mg/L). In total, 60 dishes for seeds (1 seed per dish) were prepared. The plates were placed in dark in an incubator at 25°C. They were observed every day, and emerging fungi were successively transplanted to new, fresh plates. To identify 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.

Molecular identification

The DNA was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol and stored at –20°C. A pair of primers, ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990), was used to amplify the ribosomal cassette, which consisted of SSU, ITS1, 5.8S, ITS2 and LSU rDNA. The PCR was conducted in a 25 µl volume containing 2.5 µl of 10X buffer, 2.5 µl of 2.5 mM dNTP mix, 0.5 µl of each primer at 10 µM, 0.5 µl of DNA Taq polymerase, 13.5 µl of nuclease-free water and 5 µl of DNA template. Amplification was conducted in a thermocycler using a programme with the following parameters: 2 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 60 s at 72°C; and 5 min at 72°C. The PCR products were purified using alkaline phosphatase and exonuclease I and sequencing was carried out at the Laboratory of Molecular Biology Techniques in the Faculty of Biology, A. Mickiewicz University, Poznań, Poland. The obtained sequences were edited using Chromas

(www.technelysium.com.au) software and submitted to GenBank. Finally, the sequences were compared to those published in the European Molecular Biology Laboratory (EMBL) nucleotide databases and in the NCBI (www.ncbi.nlm.nih.gov) databases using BLAST (Altschul et al. 1990). A positive identification of a species was confirmed if they shared ≥98% ITS region sequence identity with the reference sequence from the databases. These criteria were adopted from Canals et al. (2014).

Statistical analyses

Differentiation of spike sizes depending on the ergot presence and an effect of ergot on the seed size were tested with a nonparametric test of Mann-Whitney. Only those results that showed a probability of $\alpha \leq 0.05$ were considered statistically significant. All statistical analyses were carried out using Statistica 13.1 (StatSoft Inc. 2020).

Fig. 1 – *Claviceps* sp. in one of crop fields of rye (*Secale cereale*) in Poland. Photo T. Ordza.

Fig. 2 – Rye spikes with visible ergot. Photo T. Ordza.

Results

Spike and seed sizes

The spikes without ergot were significantly larger than the spikes with ergots (test Mann-Whitney test, $p<0,01$). The mean size of spikes C+ was 6.8 cm, while for the spikes C- 8.6 cm. No effect of ergot presence on the size of seeds was observed (Mann-Whitney test, $p>0,05$).

Table 1 Endophytic fungal species identified in the seeds of rye (*Secale cereale*)

No.	Fungal taxa	GenBank accession No.	BLAST match sequence		
			Accession No.	Similarity (%)	Coverage (%)
1	*Alternaria alternata*	MW720803	MK968044	99,5	99
2	*Alternaria infectoria*	MW720804	MG978343	99,2	100
3	*Alternaria tenuissima*	MW720805	MN589681	99,5	100
4	*Epicoccum layuense*	MW720806	MT573479	99,6	99
5	*Epicoccum nigrum*	MW720807	MK460967	99,5	100
6	*Fusarium avenaceum*	MW720808	MK907729	99,8	100
7	*Fusarium lateritium*	MW720809	KX622099	99,4	98
8	*Microdochium nivale*	MW720810	AM502266	98,4	98
9	*Pyrenophora teres*	MW720811	MN534846	98,5	99

Fungal endophytes

In total, nine taxa of fungal endophytes were found and, out of this, seven taxa occurred both in the seeds from spikes C+ and C-. The fungus *Microdochium nivale* occurred only in the seeds from spikes C+, while *Pyrenophora teres* – only in the seeds from spikes C- (Table 1). In both groups, the most abundant species was *Alternaria infectoria* – it made 63% in the pool of seeds from spikes C+ and 50% in the pool of seeds from spikes C-. Apart from *A. infectoria*, other *Alternaria* species were identified, namely, *Alternaria alternata* and *Alternaria tenuissima*. These were most abundant taxa after *A. infectoria* (Fig. 4). Among the species of the genus *Fusarium*, *Fusarium avenaceum* dominated in the seeds of spikes C+ (66.7%), while *Fusarium lateritium* in the seeds of spikes C- (66.7%) (Fig. 3). The genus *Epicoccum* was represented by *Epicoccum nigrum* and *Epicoccum layuense*. Both these fungi occurred more abundantly in the seeds of spikes C- (Figs 3-4).

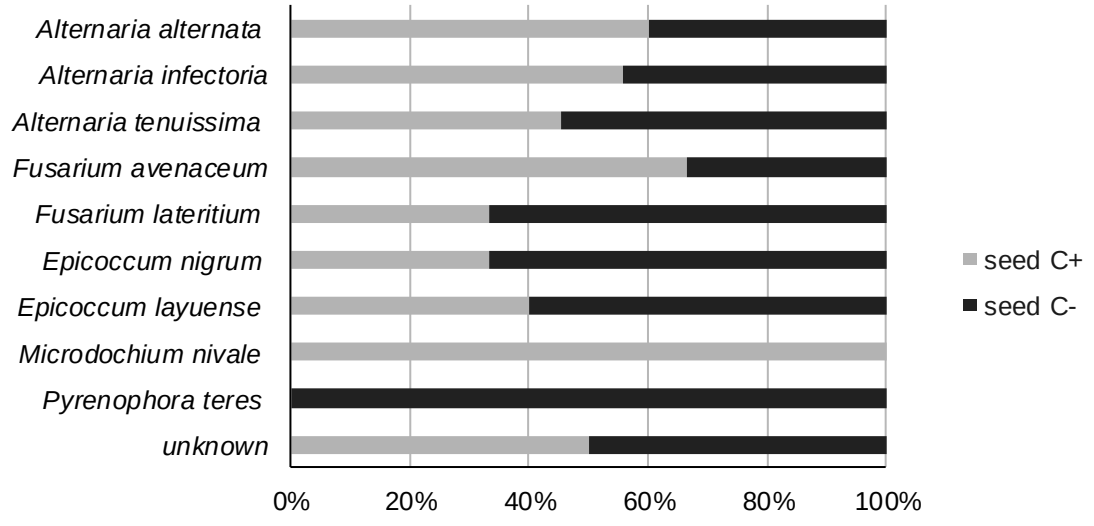

Fig. 3 – The ratio of C+ and C- seeds in which the occurrence of a given species of endophytic fungus was found.

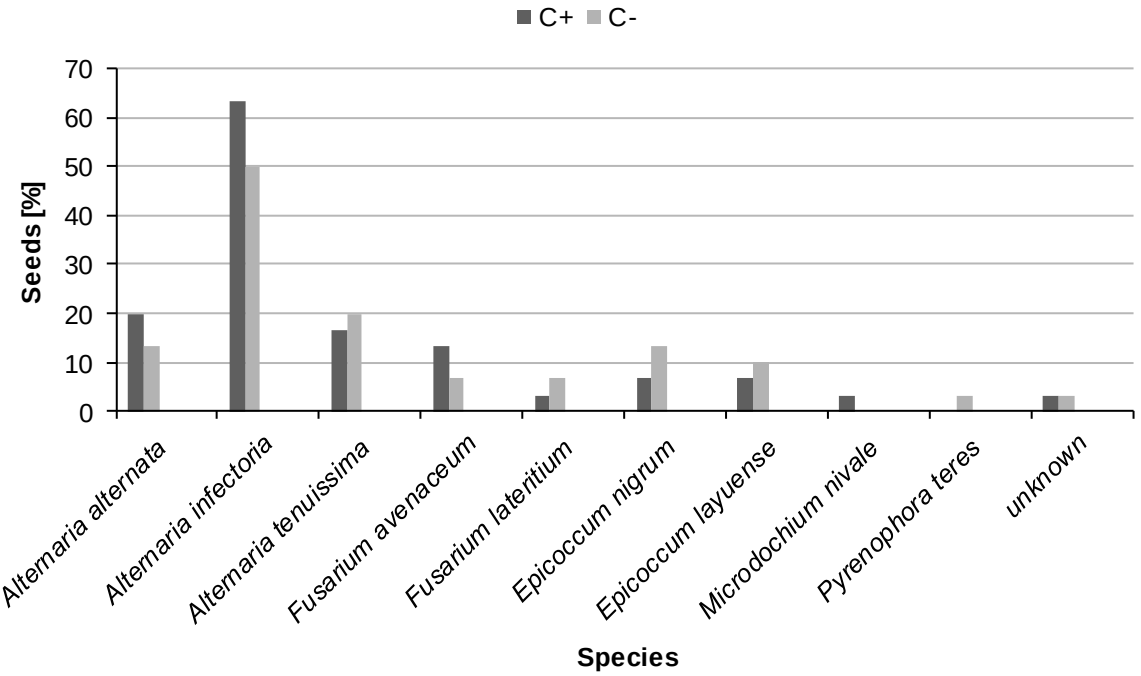

Fig. 4 – The occurrence of endophytic fungi in the seeds C+ and C-.

Discussion

The life cycle of *Claviceps* fungi and their toxic effects on animals and humans are well known (e.g., Esser & Tudzynski 1978, Miedaner & Geiger 2015). Many European countries have succeeded in stopping the spread of cereal infection with *Claviceps* fungi. However, we still do not have a cost-effective fungicide to control these pathogens (Alderman 2014). Thus, a sporadic or mass appearance of different *Claviceps* species in cereal crops is observed (Bandyopadhyay et al. 1998, Bryła et al. 2018, Isakeit et al. 1998). In 2020, we noted a mass occurrence of *Claviceps* fungi in one of rye fields in Poland (unpublished data).

We found that the spikes with ergot are significantly smaller than those without ergot. However, despite the presence of ergot in these spikes, seeds were produced and their size did not differ from the size of seeds from the spikes without ergot. This confirms the results of previous investigations indicating that although *Claviceps* fungi appear in cereal populations, they do not significantly inhibit the process of host reproduction (Wäli et al. 2013). We did not confirm the hypothesis about the differences in the size and taxonomical composition of mycobiota between the plants with and without ergot. Both groups differ only in the presence of two pathogenic species of endophytic fungi: *P. teres* and *M. nivale.*

The first species causes net blotch of barley (*Hordeum vulgare*) (Shipton et al. 1973), which is a main disease of barley crops worldwide. Infection with *P. teres* results in the decrease in the size of barley seeds (Backes et al. 2021). In our study, this fungus occurred only in the seeds of spikes without ergot. However, it did not affect the size of host seeds. *M. nivale*, the second species differentiating the plants with ergot fungi from the plants without these fungi, causes pink snow mold of grasses and grain crops (Abdelhalim et al. 2020, Ergon et al. 2003, Simpson et al. 2000).

The observed lack of substantially negative effect of *Claviceps* fungi on infected plants has been recently explained by an ecological co-evolution hypothesis between a parasite and its host. A parasite does not destroy its host totally, and a host has some profits resulting from the parasite presence. It was found that *Claviceps* fungi may deceive resistance mechanisms of their hosts (Tudzynski et al. 1995). They may use the ways of infection that are not recognized by hosts and, thus, do not initiate resistance mechanisms (Oeser et al. 2017, Tudzynski et al. 1995). *Claviceps* fungi camouflage their attack on a plant imitating a natural process of cereal pollination. After

going through pistil, they develop following the path of pollen tube outside an ovule or enter the egg cell laterally and colonize it in a few days (Tudzynski et al. 1995).

The lack of effective defence of plants against *Claviceps* fungi is advantageous for their hosts because this fungus produces alkaloids that are toxic not only for humans, but, first of all, for herbivores (e.g., Hulvova et al. 2013, Tudzynski et al. 2001). Thus, the presence of fungus protects its host against chewing by animals and, against the decrease in its sexual reproduction. Pathogenic fungi that only moderately decrease reproductive capability of their hosts, may protect these hosts against herbivores and weaken their evolutionary tendencies to develop better resistance to diseases. It has been stressed more and more often that interactions of plants with *Claviceps* fungi provide them with one more defence strategy, i.e., aposematism (Harvey & Paxton 1981, Ley-Yadun & Halpern 2007). Violet-black colours of poisonous *Claviceps* spores, which are well-visible in inflorescences of cereals and other grasses, provide a clear signal for herbivores that graze on infected plants, thus, some animals may avoid infected plants (Ley-Yadun & Halpern 2007).

In summary, the lack of differences in the size and taxonomical composition of mycobiota in plants with and without ergot and in the seed size of *Claviceps* hosts, confirms the hypothesis about the benefits of these dangerous for humans pathogens for plants. The following research step will be to check which fungal endophytes occurring in plants may effectively inhibit plant infection with ergot fungi.

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