Incidence, Identification, and Mycoparasitic Activity of *Clonostachys epichloë*, a Hyperparasite of the Fungal Endophyte *Epichloë typhina*

Karolina Górzyńska[†] and Magdalena Ślachetka, Department of Plant Taxonomy, Adam Mickiewicz University in Poznań, Poland; Przemysław Ryszka and Katarzyna Turnau, Institute of Environmental Sciences, Jagiellonian University in Kraków, Poland; Bartosz J. Płachno, Department of Plant Cytology and Embryology, Institute of Botany, Jagiellonian University in Kraków, Poland; and Marlena Lembicz, Department of Plant Taxonomy, Adam Mickiewicz University in Poznań, Poland

Abstract

Fungi of the genus *Epichloë* (Clavicipitaceae, Ascomycota) cause choke disease in many grass species. The disease manifests itself as fungal stromata that form around developing inflorescences, thereby suppressing their maturation. Economic losses in agricultural production due to choke have long been known in the U.S.A. and France, but attempts to control choke disease have not been successful. The interaction between *Epichloë typhina* (Pers.) Tul. & C. Tul. and its naturally occurring fungal hyperparasite, *Clonostachys epichloë* (Speg.) Schroers (sexual morph *Bionectria epichloë*) was investigated in populations of the grass *Puccinellia distans* (L.) Parl. Fungal hyperparasites occur widely in nature, and

many are successfully used commercially as biological control agents against plant pathogenic fungi. Microscopy of *Epichloë* stromata infected with *C. epichloë* revealed a lack of asci with ascospores in perithecia and damage to mycelia at sites colonized by *C. epichloë*. The ability of *C. epichloë* to colonize *E. typhina* was confirmed via two in vitro experiments. The percent inhibition of growth of *E. typhina* strains by *C. epichloë* varied from 18.40 to 46.50%, and the mycoparasite colonized up to 100% of *Epichloë* mycelia in a precolonization experiment. We discuss the possibility of using *C. epichloë* to control choke disease caused by *E. typhina* in grass populations.

Choke disease is a condition of grass in which the development of inflorescences is suppressed by mycelial stromata (Western and Cavett 1959). This disease is caused by fungi of the genus *Epichloë* (Clavicipitaceae, Ascomycota), which form symbiotic associations with cool-season grasses. During the vegetative phase of the host grass, fungal hyphae grow in plant tissues without causing visible signs of infection (Schardl 1996). In spring, some *Epichloë* species may form external stromata that form around the developing plant inflorescences, thereby suppressing their maturation and seed production. Fungal mating takes place on stromata, upon which ascospores capable of infecting new grass plants are produced (Chung and Schardl 1997). Thirty-five *Epichloë* species have been described, and 11 of them produce stromata (Leuchtmann et al. 2014).

Economic losses in agricultural production due to *Epichloë* have been recognized. For example, fungal infection was estimated to cause serious losses (more than \$820,000 per year) in orchardgrass (*Dactylis glomerata*) yields in the Willamette Valley (Oregon, U.S.A.), where 90% of fields were infected with *E. typhina* (Pfender and Alderman 2006). The incidence of choke commonly reaches 30% in orchardgrass fields in France by the fourth year of seed production, making the stand unprofitable (Raynal 1991). Direct losses due to choke disease were estimated at more than \$100,000 in the Czech Republic in 2008 (Cagaš and Macháč 2012). Previous attempts to control the spread of choke disease have not yielded the desired results. Chemical fungicides such as propiconazole and azoxystrobin

(Pfender and Alderman 2003) and the copper fungicides Kocide 3000 and Copper-Count N (Alderman et al. 2008) have failed to control the disease. The only method that achieves satisfactory results is the frequent rotation of crops, resulting in the rapid liquidation of unprofitable crops and the establishment of new cultivated areas in other locations, which can be costly (Pfender and Alderman 2003). An effective but high-cost approach is to burn plant residues; during this process, the above-ground parts of plants are destroyed along with endophytes, reducing the level of disease the following year. Unfortunately, this type of action, apart from the obvious devastation to the ecosystem, is difficult to control over larger cultivated areas.

It has recently been suggested that other fungi found on *Epichloë* stromata are potentially effective biocontrol agents against choke disease. Alderman et al. (2010) found a fungal hyperparasite, *Dicyma pulvinata*, on *E. typhina* stromata in orchardgrass fields in the United States. Infected stromata had fewer perithecia and were stunted and pale gray, in contrast to the orange-colored noninfected stromata with mature perithecia. These results suggest that naturally occurring fungi on *Epichloë* stromata may be useful biocontrol agents in reducing dissemination of ascospores. Although further studies of *D. pulvinata* against choke disease have been abandoned, the application of fungal hyperparasites seem promising because some of them have already been commercially used, e.g., a biofungicide with active *Trichoderma harzianum* spores sold under the trade name Trichodex WP (Makhteshim Chemical Works, LTD) controls *Botrytis cinerea* (gray mold) in greenhouse crops and vineyards, *Sclerotinia sclerotiorum* (white mold) in various greenhouse and field crops, *Cladosporium fulvum* (leaf mold) in tomato, and the powdery mildew *Sphaerotheca fusca* in cucurbits (Elad 2000). Mycoparasitism is widespread in nature (Jeffries 1995; Jeffries and Young 1994), and has been intensely studied as a potential biocontrol measure in fungal diseases. For instance, the mycoparasite *Sphaerellopsis filum* decreases the winter survival of *Puccinia graminis* in perennial ryegrass (Gordon and Pfender 2012), *Clonostachys rosea* has potential as a biocontrol agent for the control of alfalfa blossom blight caused by *B. cinerea* (Li et al. 2004), while *Sphaerodes retispora* may play a similar role against watermelon wilt caused by *Fusarium oxysporum* (Harveson et al. 2002).

The number of grass species reported to be infected with *Epichloë* fungi in Poland continues to increase (Lembicz et al. 2010; Żurek

[†]Corresponding author: K. Górzyńska; E-mail: karjan@amu.edu.pl

Funding: This work was funded by National Science Centre, grant number UMO-2014/13/D/NZ8/02420 (to K. Górzyńska). Part of this research (i.e. anatomical part) was supported financially by the Ministry of Science and Higher Education of Poland as part of the statutory activities of the Department of Plant Cytology and Embryology (B. J. Płachno), Institute of Botany at the Jagiellonian University in Kraków. The publication costs of this article were supported by the KNOW RNA Research Centre in Poznań (No. 01/KNOW2/2014).

Accepted for publication 11 April 2018.

et al. 2012). In some populations of *Puccinellia distans*, a grass species that serves as a model for fungus-plant interaction studies, as many as 90% of individuals were infected with *Epichloë typhina* (Lembicz et al. 2011; Lembicz and Olejniczak 2009). We observed dark green spots on the stomata of *E. typhina* infecting the grass species *P. distans* in 2010. Preliminary analyses showed that these spots were caused by a hyperparasite. The present study is aimed at (i) identification of the hyperparasite, (ii) determine its incidence in the *E. typhina*-*P. distans* association, (iii) assessing the effects of its presence on the sexual reproduction of *E. typhina*, and (iv) assessing its mycoparasitic ability in vitro.

Materials and Methods

Study system. The fungus *E. typhina* (Pers.) Tul. & C. Tul. grows endophytically in tissues of aerial parts of host plants, forming

systemic and asymptomatic associations (Schardl 1996). Fungi of this genus are transmitted vertically in seeds from plants to their offspring. The endophytic stage of *Epichloë* may increase the growth, reproduction, and antiherbivore protection of their hosts (Brem and Leuchtman 2001; Gundel et al. 2006; Novas et al. 2003). The latter is possible thanks to the production of antiherbivore alkaloids produced by the fungus (e.g., Schardl et al. 2007). However, positive fungal effects depend on many factors such as the particular host and endophyte genotype combinations, environmental conditions, and host age (Hesse et al. 2005; Malinowski et al. 2000; Olejniczak and Lembicz 2007). Additionally, the mycotoxins produced by these fungi cause many adverse symptoms in farm animals that feed on the infected grasses (Guerre 2016; Hoveland 1993) and in other herbivores living in grassland ecosystems (Conover 2003; Czarnoleski et al. 2012; Meister et al. 2006). In spring, some *Epichloë* species

Table 1. Origin of *Epichloë typhina* and fungi found on its stomata, sampling sites, and GenBank accession nos. of sequences obtained from the isolates corresponding to each studied locality

Site	Habitat	Location	Fungus	Strain symbol	GenBank accession no.	Species identified
Giebnia	Grassland in the vicinity of the soda production plant	52°46.544' N	<i>Epichloë typhina</i>	EpiG	MG280831	<i>Epichloë typhina</i>
		18°06.190' E	hyperparasite	CloG	KX812527	<i>Clonostachys epichloë</i>
Janikowo	Grassland on former farmland	52°46.384' N	<i>Epichloë typhina</i>	EpiJ	MG280830	<i>Epichloë typhina</i>
		18°08.032' E	hyperparasite	CloJ	KT948055	<i>Clonostachys epichloë</i>
Pakość	Pasture with extensive grazing	52°47.293' N	<i>Epichloë typhina</i>	EpiP	MG280832	<i>Epichloë typhina</i>
		18°06.721' E	hyperparasite	CloP	KX812528	<i>Clonostachys epichloë</i>

Fig. 1. *Clonostachys epichloë* infecting the stomata of *Epichloë typhina* on the grass species *Puccinellia distans*. **A**, green conidial masses produced by *C. epichloë* on the orange stroma of *E. typhina*. **B**, divergently branched conidiophores (from cultures on PDA), bar = 10 μ m. **C**, transverse section through an uninfected part of *E. typhina* stroma showing perithecia with asci, bar = 200 μ m. **D**, transverse section through an infected part of stroma directly beneath the mycoparasite: 1, *C. epichloë*; 2, an empty perithecium, where asci development appeared to be suppressed; 3, hyphae on the plant surface; 4, 6, and 8, leaf sheaths; 5 and 7, layers of hyphae; 9, hyphae inside the stroma and surrounding the stem; and 10, stem (inflorescence). Bar = 200 μ m. **E**, part of a transverse section through a leaf trapped within the stroma; the red arrow indicates a sample of hyphae between the cells: 1, xylem (vessel); 2, phloem; 3, sclerenchyma; 4, mesophyll; and 5, epidermis. Bar = 50 μ m.

enter a sexual life cycle and produce external white stromata fully covered with conidia. Stromata enclose grass shoots, preventing their further development and consequently restricting the flowering and seed production of infected plants (Chung and Scharl 1997). This condition is known as 'choke disease' (Western and Cavett 1959). This fungus is heterothallic, and its sexual reproduction depends on the transport of conidia (spermatia) to an individual of the opposite mating type. The usual vectors are *Botanophila* flies (Bultman et al. 1998), which are specifically attracted by volatile compounds of the fungus (Steinebrunner et al. 2008c). Following mating, perithecia containing asci with ascospores develop, and stromata turn from white to orange. Mature ascospores are ejected from perithecia and initiate new infections of neighboring plants (horizontal transmission) (Chung and Scharl 1997).

P. distans (L.) Parl. is a perennial Euro-Siberian halophyte occurring on marine and inland salines (Hughes and Halliday 1980). The first symptoms of choke disease caused by *E. typhina* were observed in 1992, in a single population of *P. distans* in central Poland (Lembicz 1996). The frequency of infected individuals in this population increased from 7.5% in 1992 to 91.2% in 2003 (Lembicz 1996; Lembicz et al. 2011). *E. typhina* has been recorded in five populations of *P. distans*, with all growing in anthropogenic habitats (Lembicz and Olejniczak 2009). The association between *E. typhina* and *P. distans* has already been investigated in several studies (Czarnecki et al. 2013; Górzynska et al. 2011, 2017; Lembicz et al. 2011, 2013; Olejniczak and Lembicz 2007); we reported a previously unrecorded hyperparasite appearing as dark green spots on *E. typhina* stromata in 2010 (M. Lembicz and K. Górzynska, annual report, unpublished).

Identification and incidence. The identification and distribution of the hyperparasitic fungus were studied at three localities of *P. distans*-*E. typhina* associations in the vicinity of Inowrocław, Poland (Table 1). To evaluate the distribution of the hyperparasite, we collected 130 stromata of *E. typhina* at each locality at the end of June. At each of three localities, a plot of 130 m² was delimited and subdivided into subplots of 1 m². One stroma from an infected *P. distans* individual was collected within each subplot. Stromata were analyzed for the presence of the hyperparasite, and the proportion

of stroma covered by its mycelium was evaluated visually (<25% of the area, 25 to 50%, or >50%).

The stromata of *E. typhina* gathered at each site were analyzed using a dissecting microscope (Olympus SZ61). Isolates of the hyperparasite were collected from stromata by touching a drop of sterile water to the sporulating surface and spreading the conidia that adhered to the drop over the surface of potato dextrose agar (PDA) plates with a glass rod. Mycelia were grown in an incubator at 25°C. The fungus was identified based on its microscopic characteristics, which were compared with information provided by Schroers (2001) and Kirschner (2006). These colonies were also used for molecular identification and dual-culture experiments.

Five isolates from the stromata of each of the three different origins (for a total of 15; Table 1) were additionally identified using molecular methods. To isolate DNA, we ground mycelia of the hyperparasitic fungus separately in liquid nitrogen in 1.5 ml microcentrifuge tubes. DNA was isolated using a DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and then stored at -20°C. A pair of primers, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), was employed to amplify the ribosomal cassette, which consisted of SSU (partial), ITS1, 5.8S, ITS2, and LSU (partial) 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 pmol/µl), 5.5 µl of nuclease-free water, and 5 µl of DNA template. The amplification cycling conditions were as follows: initial denaturation (94°C, 4 min); 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 30 s), and product extension (72°C, 30 s); and a final extension (72°C, 5 min). The PCR products were then cycle-sequenced with an ABI BigDye Terminator ver. 3.1 (Applied Biosystems, U.S.A.). The analyses were conducted at the Institute of Environmental Sciences, Jagiellonian University (IES UJ), Cracow, Poland, and in the Plant Taxonomy Department, A. Mickiewicz University, Poznan, Poland. The obtained sequences were submitted to GenBank (Table 1).

Obtained sequences and sequences of best BLAST matches (99% for sequences from Janikowo and Pakość and 100% for those from Giebnia) along with other sequences available in GenBank were selected for further analysis. Sequences were aligned using MAFFT

Fig. 2. Phylogenetic relationships among obtained sequences. A maximum likelihood tree based on the internal transcribed spacer (ITS) region sequences is shown. Values at the nodes indicate the tree topology support: maximum likelihood bootstrap values (5,000 replicates) are followed by Bayesian posterior probabilities (7,000,000 generations, two runs and four chains) and neighbor-joining bootstrap values (50,000 replicates). Sequences obtained during this study are given in bold italics.

(Kato et al. 2002; Kato and Standley 2013). The phylogenetic relationships between sequences were inferred using the maximum likelihood method (PhyML – Guindon et al. 2010). Bayesian posterior probabilities of the tree topology were calculated using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003). Analysis with the neighbor-joining method as implemented in PAUP* ver. 4.0 (Swofford 1998) was also included. All analyses were performed with the evolutionary model set to the generalized time-reversible (GTR) model with gamma-distributed rate variation across sites and a proportion of invariable sites.

Effects on the sexual reproduction of *E. typhina*. The hyperparasitic fungus in the structures of *Epichloë typhina* stromata. Three infected plants from each site were collected, and from each plant, four stromata were chosen for sectioning (36 altogether). The samples were prepared as described previously (Plachno and Świątek 2009, 2010). Briefly, the material was fixed in 2.5% formaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.0) for 2 days. The material was then postfixed in 1% OsO₄ in cacodylate buffer for 24 h at ~4°C, rinsed in the same buffer, treated with 1% uranyl acetate in distilled water for 1 h, dehydrated with acetone, and embedded in Epoxy Embedding Medium (Fluka). Semithin sections (0.8 µm thick) were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained with methylene blue and examined under an Olympus BX60 microscope. Some fixed material was hand-sectioned and examined under an Olympus BX60 microscope with a DP12 digital camera (Olympus, Tokyo, Japan) and AnalySIS 3.2 software (Olympus Soft Imaging Solutions, Münster, Germany). For each infected stroma, both infected and noninfected parts were sectioned. When distinguishing mycelia of the two species, we took into account the staining grade of mycelia together with their position – the hyperparasite was outside of the plant tissues.

Artificial infection. The ability of the hyperparasite to infect *E. typhina* stromata at various developmental stages was determined in growth chamber. *P. distans* grass was collected from the field

(Giebnia, 52°46.544'N, 18°06.190'E) in late summer of 2013 and stored outside during the winter. This site was chosen because it had the highest incidence of choke in 2013.

For conidial production, we used a strain of the hyperparasite that had been previously isolated from *E. typhina* stromata collected at the Giebnia site (Table 1). After conidia were incubated for 1.5 months on oatmeal agar (OA) medium, they were collected in sterile distilled water and transferred to sterile tubes. Suspensions were adjusted to three different concentrations (10³, 10⁴, and 10⁵ spores ml⁻¹) using a Thoma hemocytometer.

Thirty grass individuals were grown in a chamber under constant temperature and humidity (20/15°C, 40% humidity, 16/8 h light/dark photoperiod). Emerging stromata were successively divided into two groups: the first group (unfertilized stromata, U) was treated on the day of appearance; the second (fertilized stromata, F) was treated at 1, 2, 10, and 20 days after fertilization. We used a total of 15 unfertilized and 60 fertilized stromata. Fertilized stromata were obtained by rubbing stromata of the opposite mating type on the stroma surface of the plants (Bacon and White 1994). We assumed that fertilization had taken place when the first indications of perithecial development appeared. Every stroma was sprayed with 0.5 ml of the *C. epichloë* spore suspension at three different concentrations. Control stromata (75) were sprayed with sterile water. To verify spore viability in prepared suspensions, we inoculated PDA and corn meal agar (CMA) media supplemented with chloramphenicol (100 mg/liter) with 0.5 ml of every suspension. Fungal stromata that developed on *P. distans* tillers were examined daily.

Mycoparasitic ability in vitro. Two experiments were designed to evaluate the antagonistic activity of the hyperparasite against *E. typhina* in vitro. Three strains of *E. typhina* and three strains of the hyperparasitic fungus, each originating from one of three sites (Table 1), were used in both experiments. *E. typhina* strains were obtained by placing sterilized *P. distans* leaf segments on PDA medium in Petri dishes. Leaves were collected from the same

Fig. 3. The presence of *Clonostachys epichloë* on *Epichloë typhina* stromata: **A**, percentage of infected stromata and **B**, coverage of stromata by *C. epichloë*. Sites: G, Giebnia; J, Janikowo; and P, Pakość; *n* = 130 (at each site).

Table 2. Results of repeated measures ANOVAs. Effects of *Clonostachys epichloë* strain (CloG, CloJ, CloP), time, and their interactions on mean weekly mycelial growth of three *Epichloë typhina* strains (EpiG, EpiJ, EpiP).

Source of variation	EpiG				EpiJ				EpiP			
	df ^a	F ^b	MS ^c	P	df	F	MS	P	df	F	MS	P
Clo strain	3	24.32	77.46	<0.01	3	22.38	148.76	<0.01	3	12.91	179.01	<0.01
Error	16		3.19		16		6.65		16		13.86	
Time	4	65.97	128.89	<0.01	4	17.99	144.14	<0.01	4	23.77	202.31	<0.01
Clo strain × time	12	11.31	22.09	<0.01	12	5.29	42.38	<0.01	12	5.33	45.38	<0.01
Error	64		1.95		64		8.01		64		8.51	

^a Degrees of freedom.

^b F-statistic.

^c Mean squares.

individuals of *P. distans* from which stromata infected with the hyperparasite were previously collected. Morphological and molecular identification confirmed the identity of the obtained strains as *E. typhina* (Table 1). All available combinations of hyperparasitic fungus and *E. typhina* strains were tested.

Experiment 1. An antagonism assay was performed on PDA in Petri dishes using a dual-culture method. Two 5 mm diameter agar plugs, one fully covered with the hyperparasite and one with *E. typhina* mycelium, were placed at opposite ends of PDA plates (90 mm), 1 cm from the edge. Dishes inoculated with only *E. typhina* served as controls. Plates were incubated at 25°C, and five replicates of both the paired cultures and controls were performed.

Measurements of the growth of dual cultures and controls were performed at weekly intervals starting 1 week after inoculation. The percent growth inhibition (PGI) was calculated using the following formula: $PGI (\%) = (R - R1) / R \times 100$, where R represents the distance (measured in mm) from the point of inoculation to the

colony margin on the control dishes, and R1 represents the distance of fungal growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist (Korsten et al. 1995). These measurements were also used to estimate mean weekly mycelial growth on dual and control plates.

Additionally, 15 glass slides covered with a thin layer of PDA were inoculated with the hyperparasitic fungus and *E. typhina* 1.5 cm apart. Slides were subsequently placed in a Petri dish containing moist filter paper. The sealed Petri dishes served as humid chambers and were incubated at 25°C in the dark. The slides were checked daily under an Olympus BX53 microscope to observe typical mycoparasitic hyphal interactions.

Experiment 2. The precolonized plate method was used as described in Evans et al. (2003). An agar plug of 5 mm diameter, covered with a colony of the hyperparasite, was placed at one edge of a colony of *E. typhina* on a PDA plate (90 mm). Colonies of *E. typhina* were 12 weeks old and ranged from 47 to 68 mm in diameter. After 5 weeks of incubation at 25°C in the dark, six to nine samples were removed with a 7 mm cork borer, starting at the hyperparasite inoculum. The number of samples that were taken varied because of the variable diameters of *E. typhina* colonies on precolonized plates. Samples were plated on OA medium, incubated at 25°C in the dark, and observed after 4 weeks to detect the hyperparasite. Percentage of colonization (number of samples with *Clonostachys*/total number of samples $\times$ 100) was determined, and comparisons were made between each strain's combinations.

Statistical analysis. A χ^2 test was used in intergroup comparisons of the hyperparasite from different sites. We used repeated-measures ANOVA and a subsequent post hoc Tukey's test to determine whether the hyperparasite strain, the time the measurements were taken (after 1, 2, 3, 4, and 5 weeks) and their interactions had a significant effect on the mycelial growth of *Epichloë* strains. Factorial ANOVA was used for determining the effect of fungal strains on the PGI and percentage of *Clonostachys* colonization on plates inoculated with *Epichloë*. The assumptions of the tests were validated before the analyses were conducted. The Shapiro-Wilk test was performed to test for normal distributions, while Levene's test was used to confirm the homogeneity of variance in groups. The assumption of sphericity was tested with Mauchly's test. Variables expressed in proportion were arcsine-transformed prior to analysis. Probability values lower than 0.05 were considered to indicate statistically significant differences. All statistical analyses were conducted using Statistica 12 software (StatSoft, Poland).

Results

Identification and incidence. Dark green sporodochia observed on *E. typhina* stromata (Fig. 1A) were covered with densely packed, branched conidiophores bearing cylindrical conidia. The colonies on PDA reached up to 22 mm diameter in 7 days at 25°C and produced divergently branched conidiophores with conidia 5.9 to 7.1 μ m long and 2.1 to 2.8 μ m wide ($n = 100$) at the broadest part (Fig. 1B), corresponding well to the description of *C. epichloë* (Speg.) Schroers (sexual morph *Bionectria epichloë*) provided by Schroers (2001) and Kirschner (2006). Obtained sequences and sequences of best

Fig. 4. Growth patterns of *Epichloë typhina* mycelia in strains EpiG (A), EpiJ (B), and EpiP (C) in the presence of three different strains of *Clonostachys epichloë* (CloJ, CloP, CloG) and without *Clonostachys* (Clo-). Values represent means $\pm$ CI. Different letters indicate significant differences between treatments at each time point (repeated measure analysis was performed using ANOVA and a subsequent Tukey's HSD test; $P < 0.05$).

Table 3. Percentage growth inhibition (PGI) of *Epichloë typhina* growth in dual cultures with *Clonostachys epichloë* and percentage of *E. typhina* colonization by *C. epichloë*, for all *E. typhina*-*C. epichloë* strain combinations

<i>Epichloë</i> strain	<i>Clonostachys</i> strain	PGI (%)			Percentage of colonization (%)
		Week 3	Week 4	Week 5	
EpiG	CloG	8.17	23.64	38.14	68.33
	CloJ	8.00	10.74	18.40	5.00
	CloP	5.29	20.25	35.48	100
EpiJ	CloG	24.25	28.67	42.58	76.51
	CloJ	24.22	35.98	43.59	2.22
	CloP	5.41	21.93	38.96	87.70
EpiP	CloG	16.93	32.38	46.51	85.28
	CloJ	10.72	23.44	37.70	75.00
	CloP	4.06	24.36	41.48	75.00

BLAST matches were 99% for sequences from Janikowo and Pakość and 100% for those from Giebnia. Phylogenetic analysis of DNA sequences supported the positioning of the fungus within the *Clonostachys/Bionectria* group (Fig. 2).

The presence of *C. epichloë* on *E. typhina* stromata was recorded for samples in the studied localities. The percentage of infected stromata differed significantly between sites ($\chi^2_2 = 8.92$; $P < 0.05$) and ranged from 4.1 to 13.8% depending on locality (Fig. 3A). Overall, *C. epichloë* was present on 8.5% of all collected *Epichloë* stromata. In over 70% of the *Clonostachys*-infected stromata, less than 25% of the area was covered by *Clonostachys* mycelia, while the coverage exceeded 50% in only 5% of these stromata (Fig. 3B). This situation occurred in all localities, although stroma coverage percentages differed among populations.

Effects on the sexual reproduction of *E. typhina*. *C. epichloë* in the structures of *Epichloë* stromata. *C. epichloë* were present both on the surface of and within stromata. In semi-thin sections of the material observed under a microscope, no asci of *E. typhina* were observed within the perithecia of the parts of stromata colonized by *C. epichloë* whereas uninfected parts of *E. typhina* had fully developed perithecia with asci (Fig. 1C and D). Results were consistent for all 36 stromata. In addition, a portion of the stroma was destroyed, resulting in only compacted, empty mycelial walls being visible. Some loose mycelia, presumably from the hyperparasite, were present inside the perithecia. *Epichloë* hyphae were clearly visible between the cells of various plant tissues; even the sclerenchyma and xylem were infected (Fig. 1E). While the presence of *E. typhina* within plant tissues was clear, we could not exclude the possibility that *C. epichloë* was also present there. The same results were obtained for all prepared sections.

Artificial infection. None of the *E. typhina* stromata treated with spore suspensions of *C. epichloë* and none of the control stromata, sprayed with water, showed signs of *C. epichloë* mycelia. The spore suspensions contained living spores of *C. epichloë*, which was confirmed by inoculation of PDA and CMA media.

Mycoparasitic ability in vitro. *Experiment 1.* All three strains of *C. epichloë* inhibited growth of each of the three *Epichloë* strains (Table 2), and all *Clonostachys* strains similarly impacted the mean weekly growth of individual *Epichloë* strains (Tukey HSD, NS). The only exception was *Clonostachys* strain CloJ, which restricted the growth of the *Epichloë* EpiG strain less than the other two *Clonostachys* strains (Tukey HSD; $P < 0.05$). Inhibition of *Epichloë* growth by *Clonostachys* was first recorded in the third week of the experiment, for the CloJ-EpiJ combination (Fig. 4B). This *Clonostachys* strain inhibited the growth of two other *Epichloë* strains only in the fourth (EpiP strain, Fig. 4C) or fifth week (EpiG, Fig. 4A). The remaining *Clonostachys* strains—CloG and CloP—inhibited the growth of *Epichloë* in the fourth week (CloP affected all three *Epichloë* strains) and in the fourth (CloG-EpiP, CloG-EpiG) or fifth week (CloG-EpiJ).

The PGI of *E. typhina* strains by all three *C. epichloë* strains was calculated. Inhibition increased consistently in all combinations of *Epichloë* and *Clonostachys* associations and, in the fifth week, varied from 18.40% for EpiG-CloJ to 46.50% for EpiP-CloG associations (Table 3). Analysis of the PGI values showed no differences in

inhibition between individual *Clonostachys* strains (Table 4), but two *Epichloë* strains—EpiJ and EpiP—were more inhibited by *Clonostachys* (Table 4, Fig. 5A).

Mycelia of *C. epichloë* and *E. typhina* occur together on slides with PDA; nonetheless, no typical mycoparasitic hyphal interactions (e.g., hyphal coiling or penetration resulting in the ultimate collapse of the host cells) were observed.

Experiment 2. The percentage of *Clonostachys* colonization on plates inoculated with *Epichloë* was determined using the precolonized method. The use of OA medium enabled easy recognition of *Clonostachys* because of the dark green sporodochia that it forms on this medium. The percentage of colonization ranged from 2.22 to 100% (Table 3). The percentage of colonization by the mycoparasite did not differ between the three *Epichloë* strains, but differed between the three *Clonostachys* strains (Table 4); the CloJ strain colonized *E. typhina* significantly less than the other strains (Fig. 5B). Total colonization of *E. typhina*, resulting in the complete suppression of growth, was achieved with only one *C. epichloë* strain, CloP, which overgrew *Epichloë* strain EpiG. Only one *Epichloë* strain—EpiP—was more than 70% colonized by all three *Clonostachys* strains. Low percentages of colonization were observed when the two remaining strains, EpiG and EpiJ, were inoculated with *Clonostachys* strain CloJ (Table 3).

Discussion

Results of our study demonstrate that (1) stromata of *E. typhina* on *P. distans* are colonized by the hyperparasitic fungus, *C. epichloë*; (2) *C. epichloë* invades the stromata and restricts the sexual reproduction of *E. typhina*; and (3) *C. epichloë* inhibits the growth of *E. typhina* mycelia under in vitro conditions. These findings support the parasitic relationship between these two fungal species.

C. epichloë was previously found on dead rachises of ferns (*Pteridium aquilinum*) and grasses (e.g., *Sasa* sp.) (Schroers 2001). It was also found on *Epichloë* stromata in America, Asia (Schroers 2001), and Europe (Kirschner 2006; Steinebrunner et al. 2008a). In both cases, this fungus was described as a mycoparasite, but its incidence, distribution, and effect on developing *Epichloë* stromata were not investigated. This study is therefore the first that investigates the ecology of *Epichloë-Clonostachys* interaction.

The presence of *C. epichloë* in *E. typhina* stromata, which was observed at all of the studied localities, results in an abortion of the sexual reproduction of the latter. Ascospore development was completely suppressed by *C. epichloë*, and only empty perithecia without asci and ascospores were observed. *C. epichloë* can thus restrict the potential number of ascospores and decrease the horizontal spread of *E. typhina*. In vitro experiments also support these findings, although no typical mycoparasitic hyphal interactions between *E. typhina* and *C. epichloë* mycelia were observed. *C. epichloë* restricted *E. typhina* growth, with PGI values over 30% in the fifth week of the experiment. The precolonized plate method also indicated the negative impact of *C. epichloë* on *Epichloë*. After this

Table 4. Results of ANOVA showing effects of fungal strains on the percentage growth inhibition (PGI) and colonization ability of *Clonostachys epichloë* (as measured by the precolonized method) toward *Epichloë typhina*

Source of variation	PGI				Colonization ability			
	df ^a	F ^b	MS ^c	P	df	F	MS	P
Epi strain	2	5.14	0.06	<0.01	2	0.10	0.01	NS ^d
Clo strain	2	2.65	0.03	NS	2	66.53	3.16	<0.01
Epi strain × Clo strain	4	1.58	0.02	NS	4	1.16	0.05	NS
Error	36		0.01		36		0.05	

^a Degrees of freedom.

^b F-statistic.

^c Mean squares.

^d Not significant.

Fig. 5. A, Percentage growth inhibition (PGI) of three *Epichloë typhina* strains (EpiG, EpiJ, EpiP) in dual cultures with *Clonostachys epichloë* (due to lack of differences in inhibition ability between *Clonostachys* strains [Table 4], they were treated as a whole in the figure) and B, colonization ability of *C. epichloë* (as measured by the precolonized method) toward *E. typhina*. Values represent means $\pm$ SE. Means with different letters are significantly different (Tukey's HSD, $P < 0.05$).

fungus was transferred to a plate colonized with *Epichloë*, it quickly spread and covered up to 100% of the area. In both experiments, the CloJ strain showed a clearly lower effectiveness, e.g., when the pre-colonized plate method was used, CloJ colonized just 2.22% of EpiJ and 5% of EpiG (Table 3). This result suggested that different strains of a particular pathogenic fungal species may respond differently in terms of inhibition of a particular antagonist. *C. epichloë* inhibited *E. typhina* from 18.40 to 46.51% in dual culture, and its ability to colonize *E. typhina* in the pre-colonized experiments ranged from 2.2 to 100%. These results suggest that *C. epichloë* strains exhibit broad spectrum antagonistic activity that is important for biocontrol.

The mycoparasitic abilities of *C. epichloë* on *E. typhina* enable this fungus to potentially function as a biocontrol agent against choke disease. Prevention and eradication of choke disease is difficult. Chemical fungicides are ineffective (Alderman et al. 2008; Fermaud 1986; Leyronas and Raynal 2008; Pfender and Alderman 2003), and other more effective methods (e.g., burning plant residues) are presently abandoned due to their negative impact on ecosystems. For these reasons, the use of biological agents based on natural enemies of the pathogen may be a promising alternative.

Although *Epichloë* is rarely infected by hyperparasites in nature, several mycoparasites have been found on its stromata: *Dicyma pulvinata* (Alderman et al. 2010), *Cladosporium epichloës* (Heuchert et al. 2005; Ruszkiewicz-Michalska 2010), *Ramichloridium epichloës* (Arzanlou et al. 2007), *Phyllosticta epichloës* (van der Aa and Vanev 2002), *Alternaria* sp. (Mułenko et al. 2008), and *Nodulisporium* sp. (Steinebrunner et al. 2008a), in addition to *C. epichloë*. However, these reports only note the occurrence of these fungi on stromata. Significant reduction in *Epichloë* perithecial development as the results of mycoparasitism has only been reported for *D. pulvinata* (Alderman et al. 2010). In the U.S.A., *D. pulvinata* was recognized as a potential biocontrol agent of leaf spot disease of peanut caused by *Cercosporidium personatum* (Mitchell and Taber 1986). Further studies on the usefulness of *D. pulvinata* for choke disease prevention have not been conducted.

C. epichloë was present at all studied *P. distans* sites, including two other *P. distans* sites (M. Lembicz, and K. Górzyska, unpublished). The incidence of *C. epichloë* was rather low—it was found in less than 10% of the collection, which is comparable to the incidence of *D. pulvinata* on *Epichloë* stromata in the U.S.A. (Alderman et al. 2010). Moreover, the area covered by mycelia was below 25% in more than 70% of all the *Clonostachys*-infected stromata. These results are not spectacular when we consider the potential application of *C. epichloë* as a biocontrol agent, but they may be explained in two ways: the incidence of occurrence, and colonization of *E. typhina* by *C. epichloë* reflects the natural occurrence under the current environmental conditions of the study; also, it represented only one year. Environmental factors influence spore germination, infection, and the destructive activity of mycoparasites (e.g., Partridge et al. 2006). During our study, the weather conditions were unfavorable for both fungi (very high temperature and low humidity).

The studies of the hyperparasite-*E. typhina* interaction involving the artificial infection were not successful. We had hoped to demonstrate the most appropriate time to inoculate with a biocontrol agent, during initial stromatal development or after fertilization of the *E. epichloë*. The failure of *Clonostachys* inocula to form hyperparasitic colonies on *E. epichloë* in our experiments is difficult to explain. Temperature and humidity conditions as well as concentrations of conidial suspensions were confirmed on control plates with *Clonostachys* spores. A possible explanation is that volatile organic compounds (VOCs) that are emitted by *Epichloë* stromata prevented conidium germination. These compounds attract *Botanophila* flies, a vector of spermatia that enables *Epichloë* to cross-fertilize and sexually reproduce (Steinebrunner et al. 2008c). The VOCs also exhibit antimicrobial properties toward the fungal hyperparasites, including *Clonostachys* (Steinebrunner et al. 2008a). The presence of VOCs was also investigated in *E. typhina* on *P. distans* grass, and methyl 2,4,8-trimethylundecanoate (MTE) was the main compound involved in this association (Steinebrunner et al. 2008b). MTE was not produced under in vitro conditions and it has been suggested that

precursory substances of plant origin are needed for its production (Steinebrunner et al. 2008a). These findings may explain the ease with which *C. epichloë* spores germinate on PDA plates and the difficulties with its germination on stomata on grass culms. Although the antifungal activity of MTE has not been proved (Steinebrunner et al. 2008a), its high production in *P. distans*-associated stromata and lack of in vitro production are consistent with our results. Further studies are needed to test the factors mentioned above.

In summary, *C. epichloë* is a significant hyperparasite of *E. typhina*, an important parasite of many grass species, including *P. distans*. *C. epichloë* is potentially a viable candidate as a biocontrol agent, but additional studies are needed to determine the optimum conditions for infection.

Literature Cited

- Alderman, S. C., Rao, S., and Martin, R. C. 2010. First report of *Dicyma pulvinata* on *Epichloë typhina* and its potential for *E. typhina* control. Plant Health Prog. doi.org/10.1094/PHP-2010-0216-01-RS
- Alderman, S. C., Rao, S., Spinney, R. L., Boren, P. K., and Cacka, J. F. 2008. Summary of choke control studies. Pages 19-25 in: Seed Production Research at Oregon State University. W. C. Young III, ed. Ohio State University Extension Service, Columbus, OH.
- Arzanlou, M., Groenewald, J. Z., Gams, W., Braun, U., Shih, D., and Crous, P. W. 2007. Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera. Stud. Mycol. 58:57-93.
- Bacon, C. W., and White, J. F., Jr. 1994. Biotechnology of endophytic fungi of grasses. CRC Press, Boca Raton, FL.
- Brem, D., and Leuchtmann, A. 2001. *Epichloë* grass endophytes increase herbivore resistance in woodland grass *Brachypodium sylvaticum*. Oecologia 126:522-530.
- Bultman, T. L., White, J. F., Jr., Bowdish, T. I., and Welch, A. M. 1998. A new kind of mutualism between fungi and insects. Mycol. Res. 102:235-238.
- Cagaš, B., and Macháč, R. 2012. Effect of some factors on the incidence of choke (*Epichloë typhina*) in grass seed stands in Czech Republic. Plant Prot. Sci. 48: 10-16.
- Chung, K. R., and Schardl, C. L. 1997. Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. Mycol. Res. 101:295-301.
- Conover, M. R. 2003. Impact of the consumption of endophyte-infected perennial ryegrass by meadow voles. Agric. Ecosyst. Environ. 97:199-203.
- Czarnoleski, M., Olejniczak, P., Górzyska, K., Kozłowski, J., and Lembicz, M. 2013. Altered allocation to roots and shoots in the endophyte-infected seedlings of *Puccinellia distans* (Poaceae). Plant Biol. 15:264-273.
- Czarnoleski, M., Pawlik, K., Olejniczak, P., Kozłowski, J., and Lembicz, M. 2012. An endophytic fungus reduces herbivory in its recently colonized grass host: a food-choice experiment on common voles, weeping alkaligrass and *Epichloë typhina*. Plant Ecol. 213:1049-1053.
- Elad, Y. 2000. *Trichoderma harzianum* T39 preparation for biocontrol of plant diseases - control of *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Cladosporium fulvum*. Biocontrol Sci. Technol. 10:499-507.
- Evans, H. C., Holmes, K. A., and Thomas, S. E. 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycol. Prog. 2:149-160.
- Fermaud, M. 1986. Epidémiologie de la quenouille du dactyle porte-graine due à *Epichloë typhina* (Pers. ex Fr.) Tulasne. Mémoire de fin d'étude pour l'obtention du DAA. Ecole Nationale Supérieure d'Agronomie, Montpellier, France.
- Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.
- Gordon, T. C., and Pfender, W. F. 2012. Effects of the mycoparasite *Sphaerellopsis filum* on overwintering survival of stem rust in perennial ryegrass. Plant Dis. 96:1471-1481.
- Górzyska, K., Lembicz, M., Olszanowski, Z., and Leuchtmann, A. 2011. *Botanophila-Epichloë* interaction in a wild grass, *Puccinellia distans*, lacks dependence on the fly vector. Ann. Entomol. Soc. Am. 104:841-846.
- Górzyska, K., Rysza, P., Anielska, T., Turnau, K., and Lembicz, M. 2017. Effect of *Epichloë typhina* fungal endophyte on the diversity and incidence of other fungi in *Puccinellia distans* wild grass seeds. Flora 228:60-64.
- Guerre, P. 2016. Lolitrem B and indole diterpene alkaloids produced by endophytic fungi of the genus *Epichloë* and their toxic effects in livestock. Toxins (Basel) 8:47.
- Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59:307-321.
- Gundel, P. E., Maseda, P. H., Vila-Aiub, M. M., Ghersa, C. M., and Benech-Arnold, R. 2006. Effects of *Neotyphodium* fungi on *Lolium multiflorum* seed germination in relation to water availability. Ann. Bot. 97:571-577.
- Harveson, R. M., Kimbrough, J. W., and Hopkins, D. L. 2002. Novel use of a pyrenomycetous mycoparasite for management of Fusarium wilt of watermelon. Plant Dis. 86:1025-1030.

- Hesse, U., Schöberlein, W., Wittenmayer, L., Forster, K., Warnstorff, K., Diepenbrock, W., and Merbach, W. 2005. Influence of water supply and endophyte infection (*Neotyphodium* spp.) on vegetative and reproductive growth of two *Lolium perenne* L. genotypes. *Eur. J. Agron.* 22:45-54.
- Heuchert, B., Braun, W., and Schubert, K. 2005. Morphotaxonomic revision of fungicolous *Cladosporium* species (hyphomycetes). *Schlechtendalia* (Halle) 13:1-78.
- Hoveland, C. S. 1993. Importance and economic significance of *Acremonium* endophytes on performance of animals and grass plant. *Agric. Ecosyst. Environ.* 44:3-12.
- Hughes, W. E., and Halliday, S. 1980. *Puccinellia* Parl. Pages 167-169 in: *Flora europaea* 5. Alismataceae to Orchidaceae (Monocotyledones). T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, O. H. Valentine, S. M. Walter, and D. A. Webb, eds. Cambridge University Press, U.K.
- Jeffries, P. 1995. Biology and ecology of mycoparasitism. *Can. J. Plant Pathol.* 73: 1284-1290.
- Jeffries, P., and Young, T. W. K. 1994. *Interfungal Parasitic Relationship*. CAB International, Wallingford, U.K.
- Katoh, K., Misawa, K., Kuma, K., and Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30:3059-3066.
- Katoh, K., and Standley, D. M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30:772-780.
- Kirschner, R. 2006. New records of *Clonostachys epichloë*, a mycoparasitic fungus on the grass parasitic ascomycete *Epichloë typhina* for Europe. *Feddes Repert.* 117:307-311.
- Korsten, L., De Jager, E. S., De Villiers, E. E., Lourens, A., Kotze, J. M., and Wehner, F. C. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado postharvest diseases. *Plant Dis.* 79:1149-1156.
- Lembicz, M. 1996. Modification of the life cycle and reproductivity of *Puccinellia distans* (Poaceae) under the effect of parasitic fungus *Epichloë typhina*. *Fragm. Flor. Geobot.* 41:591-592.
- Lembicz, M., Górzynska, K., and Leuchtman, A. 2010. Choke disease, caused by *Epichloë bromicola*, in the grass *Agropyron repens* in Poland. *Plant Dis.* 94: 1372.
- Lembicz, M., Górzynska, K., Olejniczak, P., and Leuchtman, A. 2011. Geographical distribution and effects of choke disease caused by *Epichloë typhina* in populations of the grass *Puccinellia distans* in Poland. *Sydowia* 63:35-48.
- Lembicz, M., Górzynska, K., Olszanowski, Z., Michelsen, V., and Leuchtman, A. 2013. The occurrence and preference of *Botanophila* flies (Diptera: Anthomyiidae) for particular species of *Epichloë* fungi infecting wild grasses. *Eur. J. Entomol.* 110:129-134.
- Lembicz, M., and Olejniczak, P. 2009. The fungus *Epichloë typhina* in populations of halophyte *Puccinellia distans*: salinity as a possible inhibitor of infection. *Acta Soc. Bot. Pol.* 78:81-86.
- Leuchtman, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* 106:202-215.
- Leyronas, C., and Raynal, G. 2008. Role of fungal ascospores in infection of orchardgrass (*Dactylis glomerata*) by *Epichloë typhina* agent of choke disease. *J. Plant Pathol.* 90:15-21.
- Li, G. Q., Huang, H. C., Acharya, S. N., and Erickson, R. S. 2004. Biological control of blossom blight of alfalfa caused by *Botrytis cinerea* under environmentally controlled and field conditions. *Plant Dis.* 88:1246-1251.
- Malinowski, D. P., Alloush, G. A., and Belesky, D. P. 2000. Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. *Plant Soil* 227:115-126.
- Meister, B., Krauss, J., Häri, S. A., Schneider, M. V., and Müller, C. B. 2006. Fungal endosymbionts affects aphid population size by reduction of adult life span and fecundity. *Basic Appl. Ecol.* 7:244-252.
- Mitchell, J. K., and Taber, R. A. 1986. Factors affecting the biological control of *Cercosporidium* leaf spot of peanuts by *Dicyma pulvinata*. *Phytopathology* 76: 990-994.
- Mulencko, W., Majewski, T., and Ruskiewicz-Michalska, M. 2008. A preliminary checklist of micromycetes in Poland. *Szafer Institute of Botany, Polish Academy of Sciences, Krakow, Poland*.
- Novas, M. V., Gentile, A., and Cabral, D. 2003. Comparative study of growth parameters on diaspores and seedlings between populations of *Bromus setifolius* from Patagonia, differing in *Neotyphodium* endophyte infection. *Flora* 198:421-426.
- Olejniczak, P., and Lembicz, M. 2007. Age-specific response of the grass *Puccinellia distans* to the presence of a fungal endophyte. *Oecologia* 152: 485-494.
- Partridge, D. E., Sutton, T. B., and Jordan, D. L. 2006. Effect of environmental factors and pesticides on mycoparasitism of *Sclerotinia minor* by *Coniothyrium minitans*. *Plant Dis.* 90:1407-1412.
- Pfender, W. F., and Alderman, S. C. 2003. Evaluation of postharvest burning and fungicides to reduce the polyetic rate of increase of choke disease in orchardgrass seed production. *Plant Dis.* 87:375-379.
- Pfender, W. F., and Alderman, S. C. 2006. Regional development of orchardgrass choke and estimation of seed yield loss. *Plant Dis.* 90:240-244.
- Plachno, B. J., and Świątek, P. 2009. Functional anatomy of the ovule in *Genlisea* with remarks on ovule evolution in *Lentibulariaceae*. *Protoplasma* 236:39-48.
- Plachno, B. J., and Świątek, P. 2010. Unusual embryo structure in viviparous *Utricularia nelumbifolia* with remarks on embryo evolution in genus *Utricularia*. *Protoplasma* 239:69-80.
- Raynal, G. 1991. Libération des ascospores d'*Epichloë typhina*, agent de la quenouille du dactyle. Conséquences pour l'épidémiologie et la lutte. *Fourrages* (Versailles) 127:345-358.
- Ronquist, F., and Huelsenbeck, J. P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572-1574.
- Ruskiewicz-Michalska, M. 2010. *Cladosporium epichloë*, a rare European fungus, with notes on other fungicolous species. *Pol. Bot. J.* 55:359-371.
- Schardl, C. L. 1996. *Epichloë* species: fungal symbionts of grasses. *Annu. Rev. Phytopathol.* 34:109-130.
- Schardl, C. L., Grossman, R. B., Nagabhyru, P., Faulkner, J. R., and Mallik, U. P. 2007. Loline alkaloids: currencies of mutualism. *Phytochemistry* 68:980-996.
- Schroers, H. J. 2001. A monograph of *Bionectria* (Ascomycota, Hypocreales, Bionectriaceae) and its *Clonostachys* anamorphs. *Stud. Mycol.* 46:1-214.
- Steinebrunner, F., Schiestl, F. P., and Leuchtman, A. 2008a. Ecological role of volatiles produced by *Epichloë*: differences in antifungal toxicity. *FEMS Microbiol. Ecol.* 64:307-316.
- Steinebrunner, F., Schiestl, F. P., and Leuchtman, A. 2008b. Variation of insect attracting odor in endophytic *Epichloë* fungi: phylogenetic constraints versus host influence. *J. Chem. Ecol.* 34:772-782.
- Steinebrunner, F., Twele, R., Francke, W., Leuchtman, A., and Schiestl, F. P. 2008c. Role of odour compounds in the attraction of gamete vectors in endophytic *Epichloë* fungi. *New Phytol.* 178:401-411.
- Swofford, D. L. 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.
- van der Aa, H. A., and Vanev, S. 2002. A revision of the species described in *Phyllosticta*. *Centraalbureau voor Schimmelcultures, Utrecht, Netherlands*.
- Western, J. H., and Cavett, J. J. 1959. The choke disease of cocksfoot (*Dactylis glomerata*) caused by *Epichloë typhina* (Fr). *Tul. Trans Br. Mycol. Soc.* 42: 298-307.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Shinsky, and T. J. White, eds. Academic Press, San Diego.
- Żurek, M., Wiewióra, B., Żurek, G., and Prończuk, M. 2012. Occurrence of endophyte fungi on grasses in Poland – Review. *Fungal Ecol.* 5:353-356.