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Geographical distribution and effects of choke disease caused by *Epichloë* typhina in populations of the grass Puccinellia distans in Poland

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Choke disease caused by *E. typhina* was found in 5 out of 24 studied populations of the nonagricultural, Euro-Siberian grass *Puccinellia distans*. The infected populations occurred in man-made habitats and were affected by salty waste from the nearby Soda Producing Plant. The incidence of fungal infection ranged from 24.4 % to 91.2 % of the plants. A three-year field experiment showed that choke disease significantly reduced the number of reproductive tillers of P. distans during the three years of their life. Moreover, the length of tillers and size of seeds of these individuals were significantly smaller in comparison to the individuals without choke disease. Fungal stromata appeared twice a year – in spring and late summer – and the number of stroma-bearing tillers increased with the tussock age in both periods. The late summer stromata were always white and did not produce perithecia. Hence, the additional stromata of *E. typhina* in late summer did not provide any benefit to the fungus. Their presence might be an indication that the association of E. typhina with P. distans in man-made habitats is relatively new on an evolutionary scale and might be subject in the future to modification by natural selection.

Keywords: fungal infection, wild grass, reproductive tillers, size of seeds, phylogenetic analysis.

'Choke' is the name of a disease caused by fungi of the genus *Epichloë,* Clavicipitaceae, Ascomycota (Western & Cavett 1959, Kohlmeyer & Kohlmeyer 1974, Siegel *et al.* 1987, White 1987). In the asexual stage, the fungi grow between the host cells without causing visible signs of disease, while in the sexual stage they produce stromata on all or some tillers of an infected plant. Unfertilized stromata are white and become yellow when perithecia are produced. Perithecia with asci and ascospores, capable of infecting other plants, are formed as a result of cross-fertilization of the fungus. This is made possible thanks to

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anthomyiid flies of genus *Botanophila* (Bultman & White 1988, Bultman *et al*. 1998), which are specifically attracted by volatiles produced by the fungus (Schiestl *et al*. 2006, Steinebrunner *et al*. 2008a, Steinebrunner *et al*. 2008b) and carry and spread spermatia from different fungal stromata. After fertilization, flies lay eggs on the fungal stromata which are used as food source by the emerging larvae. Stromata produced by *Epichloë* totally or partially prevent host grasses from flowering and thus reproduction by seed. Relations between the sexual form of *Epichloë* fungi and their hosts are considered as parasitic (Kirby 1961, Raynal 1991, Lembicz 1996, Chung & Schardl 1997, Pfender & Alderman 1999, Pfender & Alderman 2006).

Choke disease has been known for a long time and is found on many grasses, especially in Europe (Sampson 1933, Eriksson 1967, Koponen & Mäkelä 1976, Pshedetskaya 1984, Eckblad & Torkelsen 1989, White 1993, Wennström 1996, Clay & Brown 1997, Leuchtmann & Schardl 1998, Leuchtmann *et al*. 1994, Groppe *et al*. 1999, Leyronas & Raynal 2001, Meijer & Leuchtmann 2000, Romo Vaquero *et al*. 2003, Spooner & Kemp 2005, Zabalgogeazcoa *et al*. 2008). More recently it has also been recorded in Japan (Tsukiboshi *et al*. 2002, Yanagida *et al*. 2005) and China (Li *et al*. 2009).

In Poland, three species of *Epichloë* (*E. bromicola, E. clarkii, E. typhina*) have been found so far, with *E. typhina* most frequently recorded. The fungus has been reported in several grasses species (Schröter 1908, Mułenko *et al*. 2009), but occurrence in populations of these grasses was rare. By contrast, in populations of *Puccinellia distans* (L.) Parl. (weeping alkaligrass), a high incidence of symptoms characteristic of choke disease has been observed (Lembicz 1996, 1998). *Puccinellia distans* is a perennial Euro-Siberian halophyte occurring in coastal and inland salines (Hughes & Hallidays 1980). Since the 1960s, *P. distans* has been rapidly colonizing anthropogenic habitats in Central Europe (Dettmar 1993, Jackowiak 1995). It was observed in highly disturbed habitats, including municipal waste grounds, roadsides and railway verges and the vicinity of industrial plants. In 1992, we observed the first symptoms of choke disease in a single population of *P. distans* in central Poland. The frequency of infected individuals that produced stromata increased from 7.5 % in 1992 to 67.2 % in 1996 (Lembicz 1998). In 2000, the systematic search for *E. typhina*-related choke disease in other populations of *P. distans* was initiated.

In this article we report on (i) the incidence of choke disease in Polish populations of *P. distans* (ii) the identity and phylogenetic position of the fungus and (iii) the effect of choke disease on the reproduction of host plants. This is the first study in Poland of the distribution and host effects of the sexual stage of *E. typhina* in populations of the non-agricultural grass *P. distans*.

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Materials and Methods

Searching for choke disease – the selection of populations

The 24 Polish populations of *P. distans* were screened for the presence of choke disease (Tab. 1). These populations were classified into two groups according to origin of habitat salinity, i.e., natural *versus* anthropogenic. The first group comprised natural populations occurring in the coastal and inland salines. The second one consisted of populations growing in man-made saline habitats. In the first group, 7 populations were examined (4 coastal and 3 inland), while the second group included 17 populations. At each locality, two areas of 25 m^2 each and with different densities of *P. distans* individuals were selected. Below 25 % of the area covered by the grass was considered low density and above to almost 100 % was considered high density. In the last week of May, all grass tussocks (except seedlings) growing within designated plots of 1 m^2 of each area were counted and disease status recorded. The infection rate at each locality was determined as the percentage of tussocks with choke disease by combining counts from plots of both areas.

Molecular detection and identification

For the identification of the fungus, nucleotide sequences of the beta-tubulin gene, tubB, were used. Total genomic DNA was prepared from 100 mg of fresh leaves using a miniprep extraction kit (Qiagen), according to the manufacture's instructions. DNA concentrations were determined using fluorimeter DyNA Quant 200 and Hoechst 33258 (Amersham Biosciences, GE Healthcare). The quality of each DNA sample was checked on 0.8 % agarose gels. The isolation procedure yielded usually ± 4 µg DNA per 100 mg of plant tissue. The DNA of the endophyte was detected by the polymerase chain reaction (PCR) using two specific fungal primers designed for *tubB* of *Epichloë* and *Neotyphodium* species (Doss *et al.* 1998). The primer IS-1 (5'-GGTGTTGAGCCCCCCTGATTT-3') was complementary to a fragment of intron 1 and the primer IS-3 (5'-GTCTCATCTCCGGGGCGGTAT-3') complementary to a fragment of intron 3. Amplifications were performed on a DNA thermal cycler (PTC-200, MJ Research) programmed for the following parameters: 95 °C for 3 min, then 35 cycles with 94 °C for 15 s and 60 °C for 1 min, followed by 72 °C for 10 min. The PCR products of 350 bp were separated by electrophoresis in 2 % agarose gels, and visualized by ethidium bromide staining. To confirm DNA identity, the product of the PCR reaction was purified and sequenced (CEQ 2000 XL, Beckman Coultier). Sequences of the amplified DNA fragments confirmed their origin from the *E. typhina* genome (GenBank Accession No. DQ267692)

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Tab. 1. – The status of infection with *Epichloë typhina* in 25 populations of *Puccinellia distans* occurring in natural and man-made habitats in the different geographical regions of Poland. Populations infected with the sexual and/or asexual stage of the fungus are marked with '+' and the uninfected ones with '–'. Populations used in field experiment are: Karsibór (NO) and Janikowo (A0, A1).

Localities of populations	Location (GPS)	Infection	
		Sexual stage	Asexual stage
Natural habitats			
Coastal salines			
Karsibór	52°51.171'N, 14°19.476'E	–	–
Chrząszczewo	53°57.521'N, 14°44.446'E	–	–
Międzywodzie	54°00.256'N, 14°41.790'E	–	–
Kołobrzeg	54°10.495'N, 15°34.821'E	–	–
Inland salines			
Ciechocinek	52°52.943'N, 18°47.528'E	–	–
Aleksandrów Kujawski	52°52.438'N, 18°42.000'E	–	–
Owczary	50°27.100'N, 20°45.203'E	–	–
Salinated man-made habitats			
Meadows and pastures			
Pakość	52°47.531'N, 18°06.118'E	+	+
Dulsk	52°45.161'N, 18° 21.000'E	–	–
Turzany	52°47.295'N, 18°20.445'E	–	–
Bąbolin	52°52.646'N, 18°23.790'E	–	–
Skotniki	52°41.555'N, 18°28.365'E	–	–
Jacewo	52°48.000'N, 18°17.738'E	–	–
Węgierce	52°45.493'N, 18°08.276'E	+	+
Roadsides			
Poznań	52°24.380'N, 16°55.510'E	–	–
Kraków	50°03.875'N, 19°56.705'E	–	–
Warszawa	52°13.780'N, 21°00.733'E	–	–
Świnoujście	53°54.588'N, 14°14.865'E	–	–
Kłodzko	50°26.000'N, 16°39.671'E	–	–
Wrocław	51°06.473'N, 17°02.311'E	–	–
Inowrocław	52°47.901'N, 18°15.271'E	–	–
Wastelands formerly arable			
Janikowo	52°46.384'N, 18°08.032'E	+	+
Giebnia	52°46.544'N, 18°06.190'E	+	+
Sikorowo	52°41.655'N, 18°18.788'E	+	+

Phylogenetic analysis

Preparation of genomic DNA, amplification and sequencing followed methods outlined in Brem & Leuchtmann (2003). A fragment containing the first three introns of the β-tubulin gene (*tubB*) was amplified using primers described by Craven *et al.* (2001). Maximum parsimony (MP) analysis of aligned sequences were performed by branch-and-bound search in PAUP* 4.0b10 (Swofford 2003). Character changes were unweighted and gaps treated as missing information. Trees were built by 100 iterations of random stepwise taxon addition using tree bisection-reconnection (TBR) branch swapping resulting in a sin-

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gle most parsimonious tree. Confidence levels of individual clades were assessed by 1000 bootstrap replications.

New sequences obtained from Polish isolates in this study are deposited in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) under the accession numbers HM007550-HM007553 (infecting *P. distans*), HM007554-HM007560 (infecting *D. glomerata*), and HM007561 (infecting *H. lanatus*).

Field experiment

In the three year experiment conducted in the years 2000 to 2003, 90 individuals of *P. distans* were monitored. These individuals were divided into three experimental groups, with 30 individuals each. The first group (N0) consisted of uninfected individuals, entirely free from the fungus (neither sexual nor asexual infection) over the whole period of the experiment. The individuals in this group originated from a natural population inhabiting a coastal marsh (53°51.563'N, 14°19.947'E). The second group (A0) was also uninfected like group N0, but its individuals originated from a population growing in a man-made habitat (52°46.384'N, 18°08.032'E). The third group (A1) included individuals infected by the fungus and showing the symptoms of choke disease. Plants of this group occurred in the same man-made habitat as those of group A0.

At the beginning of the experiment, all individuals were in the tillering stage in the first year of their life. All plants were tested for the presence of asexual infections of *E. typhina* using the molecular technique described above. In groups N0 and A0, the test was repeated each year to confirm that plants remained uninfected until the end of the experiment. Every individual included in the study was marked with a wooden stake and labeled with the name of its group. Twice a year, in spring and late summer, all reproductive tillers (including flowering tillers and choked tillers) of each individual were counted. Next, all the reproductive tillers that were free of choke disease and produced inflorescences and seeds were cut down. These tillers together with the seeds from all individuals of a given group were placed in paper bags and transported to the laboratory. The measured length of each reproductive tiller was the sum of the stem and panicle length. Then, 50 seeds from each group were selected at random and the average seed volume was calculated as seed length x width x width. As freshly emerged stromata within studied groups were also found in late summer, we decided to repeat procedures described above at the beginning of October.

Statistical analyses

The statistical analyses were conducted using the general linear model from the SPSS package, version 11.0 (SPSS Inc., Chicago, USA).

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Because the same individuals were measured in consecutive years, a repeated measures ANOVA was used with age defined as a within-subject factor. In cases where the interaction between population and age was significant, repeated measures ANOVA was applied to each population separately and one-way ANOVA followed by a Tukey test was used within age categories.

Results

Incidence of infections in *P. distans*

In natural populations of *P. distans* none of the examined individuals was infected by *E. typhina*, neither as asexual endophyte nor in the choke forming stage (Tab. 1). In contrast, choke disease occurred in 5 of the 17 populations examined in man-made habitats. These 5 populations were located in habitats under strong anthropopressure, affected by leaking brine, salty water and emissions of calcium dust and carbon dioxide related to the production process at the nearby Soda Producing Plant (Tab. 2). In the meadow population (Pakość) of *P. distans*, 91.2 % of the tussocks showed choke disease, which was the largest percentage observed. The pasture population (Węgierce) with 89.3 % had a similar high rate of choked plants, while in formerly arable saline wastelands the percentage of diseased tussocks was somewhat lower: 81.0 % (Giebnia), 74.0 % (Janikowo) and 24.4 % (Sikorowo).

Tab. 2. – Description of five man-made habitats in which choke disease was recorded. All habitats had a high level of salinity, ranging from 3.11 mS/cm to 7.61 mS/cm (Lembicz & Olejniczak 2009).

	Location				
	Sikorowo	**Janikowo**	**Giebnia**	**Węgierce**	**Pakość**
Habitat	saline wastelands, formerly arable			pasture	meadow
Habitat factors	leaking brine	salt water leaking, emission of calcareous dust and CO_2		leaking brine, pasturage and grazing, periodical ploughing	pasturage, grazing and mowing

Identity and phylogenetic position of the fungus

From the infected populations in Janikowo, Giebnia, Węgierce and Pakość between 5 and 20 isolates (40 in total) were sequenced, and all isolates had identical sequences at a 646 bp portion of the β-tubulin gene (*tubB*) including the first three introns. Phylogenetic analysis placed these isolates in a separate subclade close to *E. typhina* from

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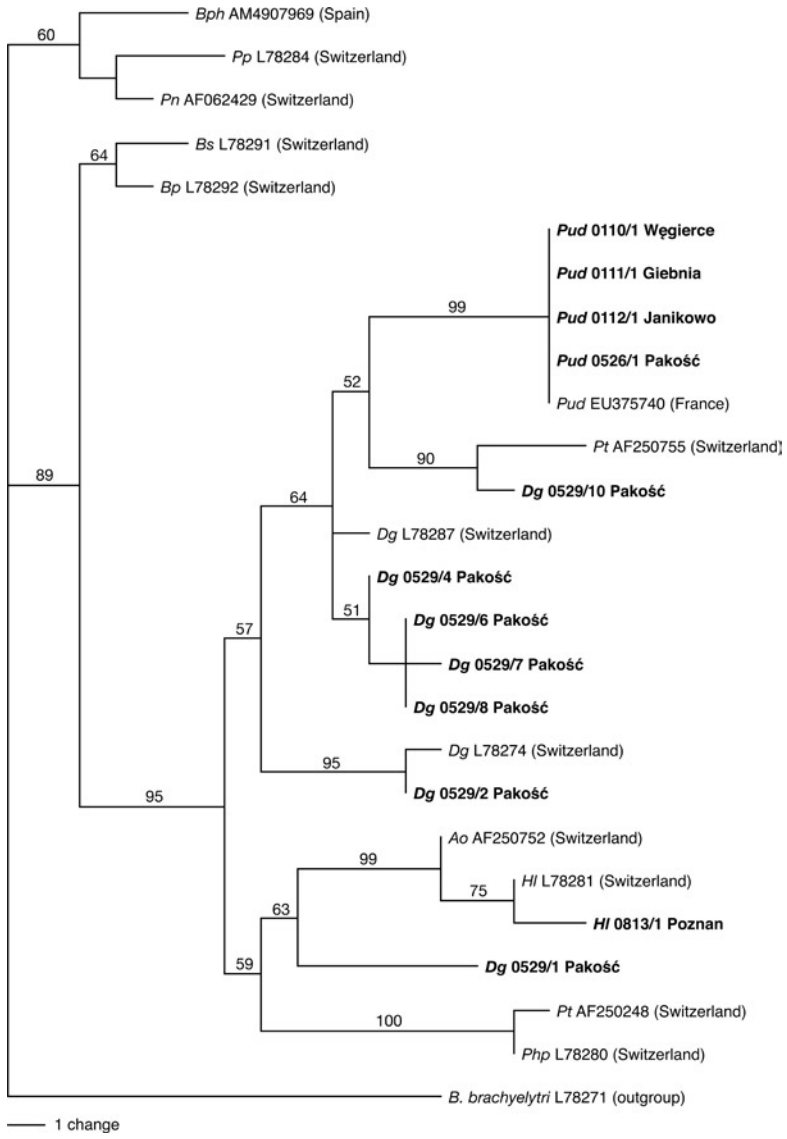

Fig. 1. – Maximum parsimony (MP) phylogram of Polish *Epichloë* isolates (in bold) and selected reference strains of the *E. typhina* complex (ETC) derived from partial nucleotide sequences of *tubB* including introns 1–3. The single MP tree was obtained by branch-and-bound search in PAUP* 4.0b10 and bootstrap values indicated on branches are based on 1000 replications. The tree is rooted with the outgroup taxon *E. brachyelytri*. Isolate coding: first letters refer to host species (**Ao,** *Anthoxanthum odoratum;* **Bp,** *Brachypodium pinnatum;* **Bph,** *B. phoenicoides;* **Bs,** *B. sylvaticum;* **Dg,** *Dactylis glomerata;* **Hl,** *Holcus lanatus;* **Pn,** *Poa nemoralis;* **Pt,** *P. trivialis;* **Php,** *Phleum pratense;* **Pud,** *Puccinellia distans*), followed by strain number (Polish isolates) or GenBank accession number, and location in Poland or country of origin.

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Dactylis glomerata L. and *Poa trivialis* L. within members of the *E. typhina* complex (ETC) (Fig. 1). An additional isolate from *P. distans* Jacq. collected in France had a 2 bp insertion at position 37, but was otherwise identical. Sequences of isolates of *E. typhina* from *D. glomerata* collected at the edge of the *P. distans* population in Pakość showed considerable variation that covered the range observed in *E. typhina* isolates from this and other hosts in Switzerland, but these were clearly distinct from the Polish *P. distans* isolates. The sequence of a single isolate of *E. clarkii* from *Holcus lanatus* L. in Poznan, Poland, was similar to a *H. lanatus* isolate from Switzerland.

Effects of choke disease

Reproductive tillers without choke disease

The largest number of tillers was found in the individuals free of choke disease from group A0 with averages of 17.4, 18.8, and 20.0, respectively, during the three years of the experiment (Fig. 2). Tiller numbers without choke disease of infected individuals of group A1 was significantly lower in each of the three years averaging 10.6, 7.9, and 5.3 (Tukey's test, $p < 0.001$), while tiller numbers of group N0 were significantly different only in the second and third year (Tukey's test, $p < 0.001$ in both cases). The number of non-diseased tillers in the infected individuals of group A0 decreased significantly over the three years of observation (repeated measures ANOVA, Huynh-Feldt; df = 1.699, F = 15.93, p <0,001).

The length of tillers in individuals with choke disease was significantly smaller compared to those without choke disease (Fig. 3, repeated measures ANOVA, Huynh-Feldt; df = 1.699, F = 15.93, $p < 0.001$). The length of flowering tillers did not significantly change during the 3-year observation period, both in individuals with (A1) and without (A0 and N0) choke disease (repeated measures ANOVA, Huynh-Feldt; df = 1.895, F = 1.519, not significant).

The panicle lengths of flowering tillers in the infected individuals of group A1 did not significantly differ from lengths measured in the non-diseased group N0 (Fig. 4, Tukey's test, not significant), while panicles in group A0 from the natural population were significantly longer compared to those of group A1 and N0 (Tukey's test, $p < 0.001$ for each year).

Size of seeds

The seeds of flowering tillers from individuals with choke disease (A1) were significantly smaller than seeds from plants without choke disease (group N0 and A0) (Tukey's test, $p < 0.001$ for each year). The average volume of seeds (in mm^3) in seeds from the infected group was 0.135, 0.132, and 0.081 at harvest in the three consecutive years, while

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volumes of seeds from the uninfected group A0 was 0.598, 0.382, and 0.551 (Fig.5). The largest seeds (0.829 mm^3) were recorded in three-year-old individuals originating from the natural population (N0).

Reproductive tillers with choke

The choke forming sexual stage of *E. typhina* on *P. distans* plants has been previously observed only in spring. Thus, infection resulting in reduced fertility of the grass affected only the early shoots. In 2000, we noted a second appearance of the sexual stage in late summer. However, the frequency of stromata formed by tillers of infected plants

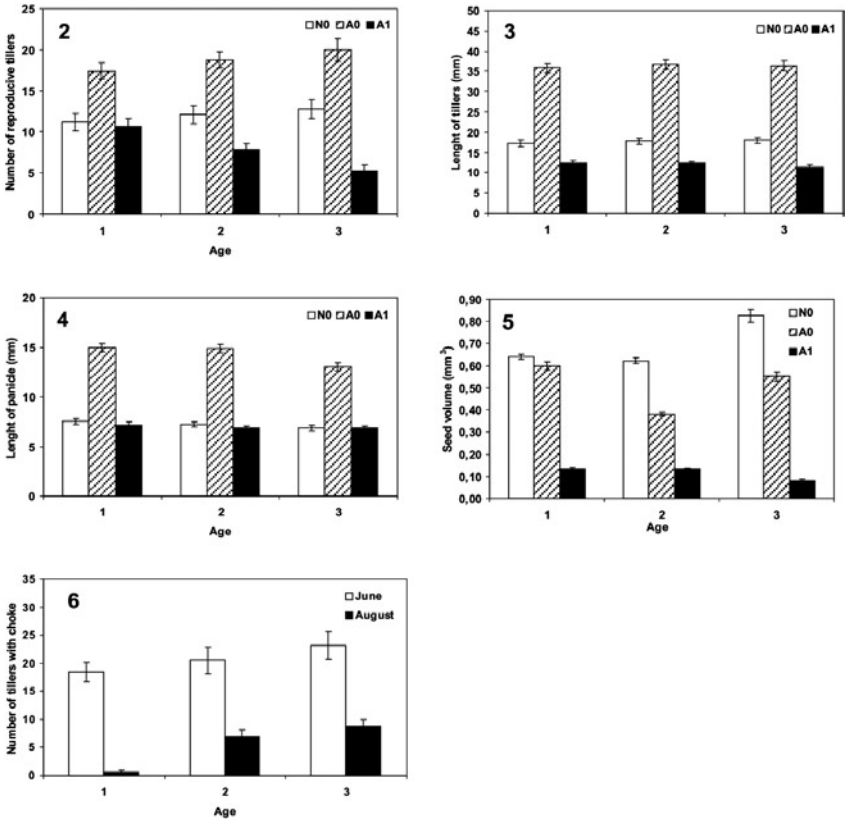

Fig. 2–6. – Effects of choke disease in the populations of *Puccinellia distans* in the three-year field experiment. The study involved three groups of individuals: N0 – individuals from a natural population, A0 – non-infected individuals from a population in a man-made habitat, and A1 – infected individuals from a population in a man made habitat. The figure shows the means and standard deviations (bars) of **(2)** the number of reproductive tillers produced in spring, **(3)** length of reproductive tillers, **(4)** average length of panicle, **(5)** average seed volume calculated as seed length x width x width, **(6)** the number of tillers with choke disease in spring and late summer.

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in June was higher than on those formed by late shoots in August (Fig. 6). Frequencies of stromata of both early and late shoots increased significantly with age of the plant (Fig. 6, repeated measures ANOVA, Huynh-Feldt; df = 1.895, F = 5.061, $p < 0.05$ for early shoots, and df = 1.698, F = 30.221, $p < 0.05$ for late shoots).

Discussion

The results of this study show that (i) choke disease caused by *E. typhina* occurs only in populations of *P. distans* that grow in man-made habitats, (ii) fungal infection significantly reduces the number and length of reproductive tillers and seed size of the host grass, (iii) stromata are produced twice a year – in spring and late summer, and (iv) the number of tillers bearing stromata increases with the tussock age, both in spring and late summer.

We explain the occurrence of choke forming *E. typhina* in weeping alkaligrass in Poland by habitat expansion of its host into new man-made habitats. To our knowledge no cases of choke disease have been noted in the populations inhabiting natural coastal and inland salines in Poland. We assume that the fungus appeared after weeping alkaligrass started to colonize the polluted and highly disturbed area around industrial plants. Presumably, colonization was accomplished by a small group of individuals with features particularly suited for long distance dispersal. Such colonization events resulted in low genetic diversity within grass populations of the new habitat and marked differences in life history traits between individuals of man-made and natural habitats (Lembicz 1998). The infection by *E. typhina* might have spread from the neighboring infected populations of other grasses. Field observations confirmed the occurrence of *E. typhina*-related choke disease in a *Dactylis glomerata* population, growing in the immediate vicinity of the infected populations of weeping alkaligrass. Alternatively, the fungus may have been already present in the founder plants, either in its asymptomatic stage or at such low frequency that it was not detected. The distinct and rather uniform fungal genotypes of *E. typhina* associated with *P. distans* in Poland and France seem to support the scenario of a pre-existing infection in the founder plants (Fig. 1). The absence of choke forming *E. typhina* in natural populations in Poland could be explained by, firstly, the prevalence of host genotypes that are incompatible with *E. typhina*, or if latently infected, prevent disease expression. A correlation between plant genotype and disease expression has also been found in *Brachypodium sylvaticum* (Huds.) P. Beauv. infected by *E. sylvatica* (Meijer & Leuchtmann 2000). By contrast, man-made habitats appear to be colonized by a distinct genotype which is prone to infection with *Epichloë* (Lembicz & Olejniczak 2009). Secondly, disease expression of the fungus could be stimulated by high concentration of CO_2 (Groppe *et al.* 1999, Meijer

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& Leuchtmann 2000). Such conditions occur in habitats colonized by weeping alkaligrass where choke disease was observed (Lembicz 1998).

Choke disease reduced reproduction in infected individuals of weeping alkaligrass. The amount of reproduction, measured as the number of reproductive tillers produced per year, decreased significantly over the three year of the plant's life. A different result has been obtained for *Brachypodium phoenicoides* (L.) Roem. & Schult. (Zabalgogeazcoa *et al*. 2008). With this host, also in a three-year experiment, infected plants produced significantly more reproductive tillers than those without choke disease. The authors have explained the observed increase in the number of reproductive tillers as a response of the plant to the arrest of tiller development caused by the disease, which may be similar to clipping (Skinner and Nelson 1994, Hamilton *et al*. 1998). Alternatively, an increase in the number of reproductive tillers in plants with choke disease could have been the result of chemical regulation by substances produced by the fungus and/or a plant in response to infection (Pan & Clay 2002). In our experiment, we found a significant increase in the number of tillers with stromata in diseased populations during the three years, which is also different from *B. phoenicoides* where a decrease has been observed (Zabalgogeazcoa *et al*. 2008).

The infected individuals of *P. distans* growing in man-made habitats appear to counteract the negative influence of choke disease on their fertility through production of additional reproductive tillers in late summer. In populations inhabiting natural salines late summer tillers do not appear (Lembicz 1998). Fungal stromata developed also on these late tillers, although in lower frequency, but numbers were increasing with the plant's age. The appearance of *E. typhina* twice a year in populations of *P. distans* and, according to our knowledge, also on other grass species has not been observed before. Late summer stromata are always of white color and do not produce perithecia and ascospores. The reason for this may be the absence of adult *Botanophila* flies, necessary for cross-fertilization (Bultman *et al*. 1998). *Botanophila* flies appear only at the beginning of the spring season closely matching formation of spring fungal stromata (Górzyńska *et al*., in press). Thus, the production of fungal reproductive structures in late summer does not provide any benefits to *E. typhina*. Their presence may be another indication that the association of *E. typhina* with *P. distans* in man-made habitats is relatively new on an evolutionary scale and will be subject in future to further modification by natural selection.

Additional studies regarding the behavior of *E. typhina* in the populations of *P. distans* are needed. In particular, the effects of choke-forming *E. typhina* on genotypes of weeping alkaligrass from natural habitats should be tested. It would be interesting to see if they behave

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similar to those from anthropogenic habitats or like the infected plants of *B. phoenicoides* (Zabalgogeazcoa *et al.* 2008).

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