

THE FUNGUS *EPICHLÖË TYPHINA*
IN POPULATIONS OF A HALOPHYTE *PUCCINELLIA DISTANS*:
SALINITY AS A POSSIBLE INHIBITOR OF INFECTION

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(Received: January 9, 2009. Accepted: February 4, 2009)

ABSTRACT

Puccinellia distans is a non-agricultural halophytic grass that has become another host plant for *Epichloë typhina*, hitherto not reported from Poland. In 1992 we noticed the first symptoms of choke disease in a single population of *P. distans* in central Poland. Since then we have observed choke disease in 5 populations of *P. distans* only in man-made habitats. These habitats are strongly anthropogenically salinated but they exhibit the pattern of species composition characteristic of natural salines. In this paper we test the hypothesis that the level of salinity affects the infection of *P. distans* by the fungus *E. typhina*. Seven plots were established in the field and each plot was divided into 25 subplots. Within each plot the level of infection in a spring generation of shoots was negatively correlated with salinity (common regression within the plots, $\beta = -0.674$, $df = 117$, $p < 0.001$). Negative correlation was also found in an autumn generation within the plots ($\beta = -0.682$, $df = 94$, $p < 0.001$) after excluding plot P in which the frequency of infected individuals was the lowest and equal only to 0.05. The proportion of individuals infected by the endophytic stage of the fungus in the populations was assessed using diagnostic polymerase chain reaction. The greatest percentage (98.3%) of infected individuals was found in the population growing in the habitat of the lowest salinity. The high salinity reduces the chance of *P. distans* to become infected, but may promote the stroma formation of *E. typhina* twice in the season. Disease expression in autumn clearly represents a misadaptation which could be explained by the fact that the species interactions described here appeared relatively recently as a result of human activity. This hypothesis requires further experimental verification.

KEY WORDS: choke disease, diagnostic polymerase chain reaction (PCR), field experiment, fungal endophyte, frequency of infection, man-made habitats, *Puccinellia distans*, salinity.

INTRODUCTION

Epichloë typhina (Pers.) Tul. (Clavicipitaceae, Ascomycota) is an endophytic fungus infecting many grass species (Sampson 1933; Bradshaw 1959; Bacon et al. 1977; White 1987; Eckblad and Torkelsen 1989; Wennström 1996; Pfender and Alderman 1999; Schardl and Leuchtman 1999). In the sexual stage, *E. typhina*, like most other species of the genus, is a pathogen causing choke disease of grasses. Stromata develop on infected tillers and by suppressing floescence plants remain partly or totally sterile. Perithecia with sacks containing ascospores capable of infecting other plants are formed as a result of cross-fertilisation. The process of cross-fertilisation requires visitation of an anthomyiid fly of genus *Botanophila* carrying spermatia in faeces; these flies also deposit eggs on stromal tissues

upon which larvae feed (Kohlmeyer and Kohlmeyer 1974; Bultman et al. 1998; Bultman and Leuchtman 2003). The asexual stage of *Epichloë* (referred to as *Neotyphodium* (Glenn et al. 1996)) does not produce visible symptoms in plants. Its mycelium is intercellular in the grass tissue and absorbs nutrients from the plant released into the apoplast. *Neotyphodium* and some species of *Epichloë* are seed-transmitted growing into ovules and seeds, and thus are vertically transmitted to the next generation (White et al. 1993).

Epichloë typhina has been found in several host plants from the subfamily Pooideae of the *Poaceae*. Because in many grass species *E. typhina* has never been detected and in the infected populations there are usually individuals that are non-infected, the question arises as to the reasons for the observed variation. The answer requires investiga-

tion of the grass genotype predisposition for getting infected on the one hand, while on the other identification of environmental factors acting as inhibitors or promoters of infection.

The factors that can initiate plants infection with this fungus have not been fully recognised yet. Such authors as Marks and Clay 1990, Wennström 1996, Groppe et al. 1999, Meijer and Leuchtman 2000, Groppe et al. 2001, on the basis of experimental study indicate the importance of different environmental factors. It has been suggested that infection of host plants with *E. typhina* is a result of many factors. Their recognition requires investigation of *E. typhina* infections of natural non-agricultural grass popula-

tions as the majority of the hitherto studies have concerned the grass of agricultural use infected with *E. typhina*. It is expected that a comparative study of the number, distribution and behaviour of *E. typhina* and other fungi species of this genus in the natural systems and agricultural situations will help identify the factors favouring the infection.

Puccinellia distans is a non-agricultural halophytic grass that has become another host plant for *E. typhina*, hitherto not reported from Poland (Fig. 1). 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 forming stromata increased from 7.5% in 1992 to 67.2% in 1996 (Lembicz 1998). However, the actual frequ-



Fig. 1. *Puccinellia distans* population in the field in Janikowo (a); a tuft of the grass infected with the sexual stage of the fungus (b); stroma-sexual stage of *Epichloë typhina* infecting *Puccinellia distans* grass (c) (photo K. Jankowska).

ency of the fungus may have been even higher because grass individuals with the asexual stage of *E. typhina* were indistinguishable from uninfected plants.

It seems that this fungus is a new event in the life history of *P. distans* and is a result of this halophyte expansion onto new habitats (Lembicz 1998). No choke disease has been noted in the populations established for a long time in the natural marine and inland salines. These habitats are strongly anthropogenically salinated but showing the pattern of species combination characteristic of natural salines and its distribution according to the level of salinity. In these habitats we have observed a different number of *P. distans* individuals infected with *E. typhina* and a different pattern of the fungus behaviour, hitherto not reported in literature for this species.

Prompted by the above observations we have decided to check in a field experiment whether the degree of salinity – being a key factor determining the species composition and its distribution in space – is a significant inhibitor or promoter of the choke disease in *P. distans* populations. In addition, the proportion of the host individuals infested by the asexual (endophyte) stage of *E. typhina* in these *P. distans* populations was studied.

MATERIAL AND METHODS

The host

The grass *Puccinellia distans* (L.) Parl. is a perennial Euro-Siberian halophyte occurring on marine marshes and inland salines (Hughes and Holliday 1980). Inflorescences are formed in late spring and after wind pollination small wind-dispersed seeds are produced. The grass is often grazed by herbivores and then in autumn additional shoots with inflorescences are produced (Lembicz 1998). Since the 1960s *P. distans* has rapidly colonised anthropogenic habitats in Central Europe (Dettmar 1993; Jackowiak 1996). It was observed in highly disturbed habitats including municipal waste grounds, along roads and railroads and in the vicinity of industrial plants.

Field experiment

The study was performed on five populations: Janikowo (J), Pakość (P), Węgiecie (W), Giebnia (G), Sikorowo (S) of *P. distans* in northern and central Poland, where *E. typhina* has been detected earlier (Table 1). In each of the five populations a permanent plot of 25 m² size was delimited and subdivided into subplots of 1 m². The plots covered fragments of vegetation with different species concurrent with *P. distans*. In one of the populations (J) three plots were established (J_a, J_b, J_c) due to substantial zonal variation in vegetation. These plots differed in the relative proportion of *P. distans*: a – domination of the grass (nearly 80% cover); b – co-domination of *P. distans* and *Spergularia salina*; c – domination of *Salicornia herbacea* (nearly 50% cover). In all other plots *P. distans* was the dominant species. Within the core subplots each of the permanent plots the number of infected and non-infected *P. distans* tufts was determined in years 2000 and 2001 twice in the season (last decade of June and September). The tufts were classified as infected when showing stromata, and the number of unfertilized stromata and fertilized stromata producing ascospores were noted. The degree of salinity was measured twice per year in 25 subplots of each permanent plot in soil samples collected at the depth of 10 cm. The soil samples were transported to the laboratory and the measurements were performed by the conductometric method.

Statistical analyses

Correlation analysis was used to study the relationship between the mean frequency of infection and mean salinity in different permanent plots for values determined in spring and in summer. The correlations within a given plot and between subplots were tested by the analysis of covariance with frequency of infection as a dependent variable and salinity as a covariate. Congruence of the regression lines describing the relation between infection and salinity within particular plots or subplots was tested (test for interaction of groups); if no differences were found, significance of the common slope coefficient beta was checked for all groups combined.

TABLE 1. Characterisation of *Puccinellia distans* populations infected with *Epichloë typhina* studied in the field with locations, degree of salinity (as measured by soil conductivity) and mean frequency of infected individuals.

Population	Symbol	Location	Conductivity (mS/cm)	Mean frequency of infected plants	
				Spring	Autumn
Pakość	P	N 52°47.531' E 18°06.118'	3.11±0.18	0.84	0.04
Węgiecie	W	N 52°45.493' E 18°08.276'	3.10±0.18	0.76	0.08
Janikowo	J _a		5.06±0.45	0.46	0.34
	J _b	N 52°46.384'	6.00±0.48	0.20	0.08
	J _c	E 18°08.032'	7.52±0.15	0.20	0.24
Giebnia	G	N 52°46.544' E 18°06.190'	7.61±0.18	0.20	0.15
Sikorowo	S	N 52°41.655' E 18°18.788'	3.29±0.23	0.19	0.17

Molecular detection

Presence of asymptomatic infections (asexual stage) in populations of *P. distans* was analysed based on molecular markers. From each of the five populations 60 randomly chosen two-year old individuals without disease symptoms were sampled. Total genomic DNA was prepared from 100 mg fresh leaves using a miniprep extraction kit (Qiagen) according to the manufacturer'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 $\pm 4 \mu\text{g}$ 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 the beta-tubulin gene of Neotyphodium endophytes (Doss et al. 1998). The primer IS-1 (5'-GGTGTGAGCCCCCT-GATTT-3') was complementary to a fragment of intron 1 and the primer IS-3 (5'-GTCTCATCTCCGGGGCGG-TAT-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 sec. and 60°C for 1 min., followed by 72°C for 10 min. The PCR products of a length 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 Coulter).

RESULTS

Infection with the asexual stage of *E. typhina*

In four of the five populations studied infections by *E. typhina* were detected in asymptomatic, normally flowering plants based on molecular markers (Fig. 2). The percentage of these infections varied between populations with the greatest number found in population P, where 98.3% of the asymptomatic individuals analysed were infected by the asexual stage of *E. typhina*. In the other populations or subpopulations the rates of infection were: 95.0% in W, 70% in J_a, 68.3% in J_b, 13.3% in J_c and 3.3%

in G. In population S no individual was found to be infected by the asexual stage of *E. typhina*.

Infection with the sexual stage of *E. typhina*

Across populations the frequency of infected individuals showing stromata in spring was not significantly correlated with the degree of salinity ($r = -0,684$, $N = 7$; NS). However, within individual plots the level of infection was negatively correlated with salinity (common regression within the groups, $\beta = -0,674$, $df = 117$, $p < 0.001$). Despite the substantial variation in the degree of salinity among the plots, the slopes of the regression lines describing the relations between the infection and salinity were very similar in all plots as indicated by ANCOVA ($F_{4,113} = 1,875$; NS) (Fig. 3a).

Similarly, the level of stroma formation on shoots produced in autumn was not significantly correlated with the degree of salinity when tested across all plots ($r = 0,378$, $N = 7$; NS). While a negative correlation between these parameters was evident within particular plots and subplots (regression within groups, $\beta = -0,682$, $df = 94$; $p < 0.001$) with the exception of plot P in which the frequency of stroma-forming individuals was very low (5%) (Fig. 3b). The slope of the regression lines describing the frequency of infection versus salinity did not differ among plots (ANCOVA, $F_{3,91} = 1,433$; NS with plot P excluded, and $F_{4,113} = 2,630$; $p < 0.05$ with plot P included) (Fig. 3b).

DISCUSSION

Results of the field study and the experimental data suggest that salinity of the soil may influence the abundance and reproductive success of *Epichloë typhina* on the host grass *Puccinellia distans*. Other studies have documented previously that environmental factors are important for the distribution of *Epichloë* fungi in populations of different host grasses. According to Wennström (1996) who studied *E. typhina* in the natural populations of *Calamagrostis purpurea* in Sweden, infected plants occurred only in wet habitats where nutrient levels were high, whereas in dry and nutrient poor habitats the fungus was absent. Other authors have shown that carbon dioxide enhanced the production of fungal stromata in infected plants but simultaneously in-

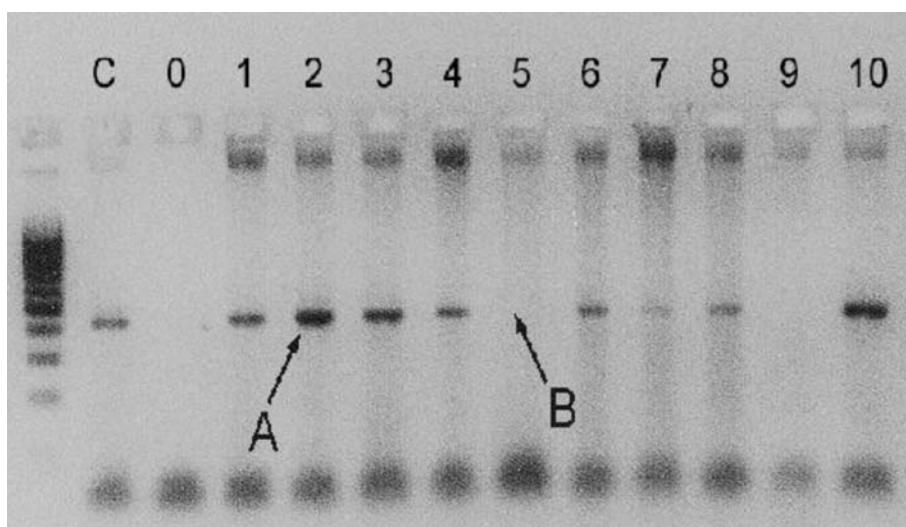


Fig. 2. Molecular detection of the asexual stage of the fungus *Epichloë typhina* in *Puccinellia distans* plants of one population – Pakość (P) in man-made habitats. The product of the PCR reaction of 350 bp length was separated in a 2% agarose gel. The numbers from 1 to 10 represent different individuals. A, infected individual; B, non-infected individual; C and 0, controls.

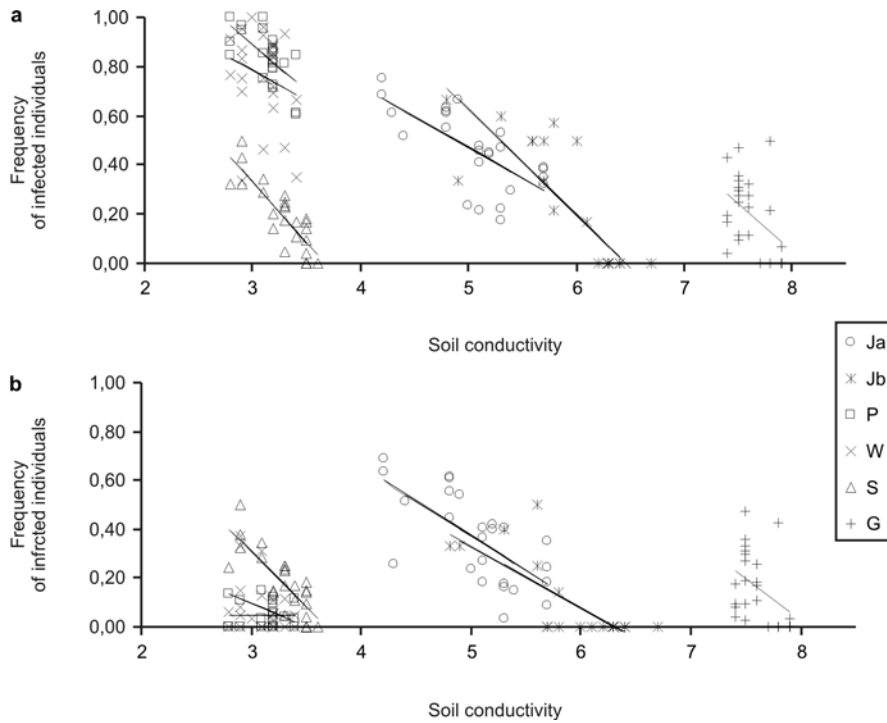


Fig. 3. Relationship between the level of salinity and the frequency of stroma-forming individuals of *Puccinellia distans* observed in spring (a) and in autumn (b) shoots at each of the studied plots (J_a, J_b, P, W, S, G). Plot J_c is not included in the graph due to very low density of *P. distans*.

hibited production of inflorescence in uninfected plants (Marks and Clay 1990; Groppe et al. 1999; Meijer and Leuchtmann 2000). It has been reported that the small-scale habitat fragmentation can increase the intensity of infections with *Epichloë* in populations of *Bromus erectus* (Groppe et al. 2001). Also, plant species diversity of plant communities has been found to affect the growth and reproduction of both, the host *B. erectus* and the fungus *E. bromicola* (Groppe et al. 1999). The numbers of vegetative and potentially reproductive tillers of *B. erectus* and stromata of *E. bromicola* were greater in plant communities with medium and high, rather than low plant species diversities (Groppe et al. 1999). However, in most cases of choke disease in grass populations the factors controlling development of the disease and its spread in space are not known.

The choke disease in populations of *P. distans*, unknown on this host in Poland before 1996, occurs only in man-made habitats. The habitats are strongly salinated as a result of pollution from the plants producing soda, carbon dioxide and sodium chloride. They support plant communities typical of natural inland salines. On the basis of the field experiment we have shown that the level of infection by stroma-forming *E. typhina* is related to the level of salinity. In spring and autumn the frequency of stroma-forming plants decreases with increasing salinity within individual study plots. This tendency was consistent in all study plots with the exception of one population in which the frequency of infected individuals in summer was the lowest. There was no significant relation between the mean level of infection and the mean salinity when analysed across all study plots, both in spring and autumn, although the trend was clearly negative. The population that diverted from the general pattern of higher infections rates at low degrees of salinity was localised in the habitat most disturbed by man and used to be arable land each year subjected to intense agrotechnological procedures. This population

was composed of young individuals of *P. distans* and has not yet been infected by the fungus.

There are two important questions we are not yet able to answer: (1) when and in which way was *P. distans* grass first infected with *E. typhina* and (2) why is there a negative correlation between the degree of salinity and the level of disease expression within individual plots in spring, but no such correlation among plots over a range of different salinities? There are no records on how long the choke disease has occurred in populations of *P. distans* in Poland. It is supposed that infection appeared in the time when *P. distans* started migration from natural salines into man-made habitats. Migration of *P. distans* and successful colonisation of man-made habitats in Europe and in Poland was first observed in the beginning of the 60s of the last century (Dettmar 1993; Jackowiak 1996). Supposedly, after colonisation of the new habitats, some *P. distans* individuals became infected by *Epichloë* fungi originating from neighbouring infected populations of *Dactylis glomerata*. The *P. distans* populations with the highest level of infection are those closest to a population of *D. glomerata* infected with *E. typhina*. Alternatively, the fungus may have been already present in the founder plants but at such a low frequency that it was not detected. The fungus, proved to be genetically uniform in all infected populations on the basis of 12 allozymes (data not shown) and could have spread in the population either by seeds, or contagiously by ascospores. The relatively short time since the presumed colonisation of the new habitat did not allow any detectable genetic differentiation of the endophyte.

As to the second question, according to the results of our study the degree of salinity does not appear to be a key factor determining the level of disease expression in populations of *P. distans*, although within particular populations it did matter and the highest infection levels were observed in plots with the lowest salinities. It is interesting to note that *P. distans* plants in our populations produced generati-

ve shoots twice a year, and thus could partly compensate the negative effect of the fungus on its fertility by the additional summer-autumn shoots. However, the number of these additional shoots was significantly lower than that of the shoots produced in spring (data not shown). On shoots of the second reproduction effort in summer fungal stromata started to appear as well, but in lower frequency. From the fungal point of view, appearance of the stromata in late summer is useless, because female *Botanophila* flies, which serve as vectors of fungal spermatia, are not present at that time. Stromata remain unfertilised and do not form ascospores capable of infecting new plants. Disease expression in autumn clearly represents a misadaptation which may be explained by the fact that the interaction of *P. distans* with *Epichloë* described here appeared relatively recently as a result of human activity.

In conclusion, the negative correlation between the salinity and the level of disease expression of *E. typhina* within plots and the observation that the highest frequency of stroma-forming *P. distans* individuals occurred in populations with the lowest salinity may suggest that salinity can inhibit disease expression. Salinity may also promote the formation of reproductive shoots of *P. distans* twice in the season and thus disturb the life cycle of *E. typhina* by generating stromata that are left unfertilized. This hypothesis requires further experimental verification.

ACKNOWLEDGEMENTS

This work was supported by State Committee for Scientific Research in Poland grant No. 2P04F 015 30. We thank A. Leuchtmann (Plant Ecological Genetics, Institute of Integrative Biology, Zürich, Switzerland) and Z. Olszanowski and Karolina Jankowska (A. Mickiewicz University, Poznań) for comments on the manuscript and linguistic corrections. We thank also A. Jarmołowski (Department of Gene Expression, A. Mickiewicz University) for help in molecular detection and A. Łapa for laboratory assistance.

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