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ORIGINAL ARTICLE

Influence of the fungal hyperparasite *Trichoderma harzianum* on the growth of *Epichloë typhina,* an agent of choke disease in grasses

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Abstract
In its sexual stage, the fungus *Epichloë typhina* (Ascomycetes: Clavicipitaceae) is a pathogen that causes choke disease in many grass species. It forms stromata on developing inflorescences, resulting in reductions in flowering and seed production in the infected plants. As a result of fungal infection, economic losses in meadows and pastures have been reported. Unfortunately, there are no effective methods for reducing the spread of *E. typhina*. One potential solution is the use of hyperparasites as biocontrol agents, for example, the fungus *Trichoderma harzianum,* known for its ability to parasitize other parasites. We investigated the effects of *T. harzianum* on *E. typhina* (strain CBS 122147) in Petri dishes containing PDA medium using two methods: a dual-culture method and a precolonized plate method. Three strains of *E. typhina* of different origins were used. These experiments showed that: (1) *E. typhina* mycelium grew slower in the presence of *T. harzianum* and could be overgrown by hyperparasite mycelium, and (2) all strains of *E. typhina* responded similarly to *T. harzianum.* The results indicate that the presence of *T. harzianum* has an inhibitory effect on the growth of mycelium in *E. typhina*. Therefore, this hyperparasite can be used to develop safe methods of limiting the spread of choke disease.

Keywords *Epichloë typhina* · Biocontrol · Choke disease · Hyperparasite · *Puccinellia distans* · *Trichoderma harzianum*

Introduction

Hyperparasitism takes place when a parasite lives at the expense of another parasite, either on or inside it (Ulloa and Hanlin 2012). This type of dependence is very common in the world of fungi (Jeffries and Young 1994). Hyperparasitism is often used in biological plant protection (Brožova 2004) as an alternative to chemical methods. It is estimated that 90% of all fungi used in plant protection products belong to the genus *Trichoderma* (Benitez et al. 2004). These products reduce the development of diseases, stimulate plant growth, and increase their resistance to stress. According to studies by Bettiol and Morandi (2009), the costs of cultivation are significantly reduced by using fungi of this type. In Brazil, the use of products with *Trichoderma* has increased significantly during the last years, mainly because of its profitability—the average cost of treatment against, e.g., bean white mold with *Trichoderma* is $54.00/ha, while with fungicides is about $92.00/ha. In addition, studies conducted by Monte (2001) have shown that their use together with reduced doses of fungicides in integrated agriculture increases the health of plants, comparable to the level of protection provided by the use of full doses of fungicides. Currently available on the market are biological agents based on fungi of this genus such as Trichodex, which is successfully used against various phytopathogenic fungi, such as *Rhizoctonia solani*, *Botrytis cinerea*, and *Sclerotium rolfsii* (Omann and Zeilinger 2010).

Hyperparasitism has recently been introduced to control "choke disease" of grasses (Alderman et al. 2010; Górzyńska et al. 2018). This disease is caused by fungi of the genus *Epichloë* (Ascomycetes: Clavicipitaceae) which during the vegetative phase of the host grass, grow in plant tissues without causing visible signs of infection (Schardl 1996). In this stage, they are transmitted vertically in seeds

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from plants to their offspring and may increase the growth, reproduction, and anti-herbivore 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 anti-herbivore alkaloids produced by the fungus (e.g., Schardl et al. 2007). In spring, some *Epichloe* species may form external stromata that surround the leaf sheath, and the development of inflorescences is overwhelmed by the rapid growth of the fungus (Western and Cavett 1959). Fungal mating takes place on stromata, upon which ascospores capable of infecting new grass plants are produced (horizontal transmission) (Chung and Schardl 1997). Thirty-five *Epichloë* species have been described, and 11 of them produce stromata (Leuchtmann et al. 2014).

One of the *Epichloë* species capable of vertical and horizontal transmission is the fungus *Epichloë typhina* (Pers.) Tul. & C. Tul., which has been noted in populations of *Puccinellia distans* since 1996 (Lembicz 1996). *P. distans* L. (Parl.) (weeping alkali grass) is a perennial Euro-Siberian halophyte that is found on marine and inland saline environments (Hughes and Hallidays 1980). Due to human activity, it is currently noted on saline grasslands (Lembicz 1998). Although not cultivated, its chemical composition indicates the possibility of its use as fodder grass (Kozłowski et al. 2004). In Poland, it is commonly found in meadows and pastures where livestock is grazing (Lembicz et al. 2011).

It has been shown that the endophytic stage of *E. typhina* has a positive effect on the growth (Czarnoleski et al. 2013) and reproduction (Olejniczak and Lembicz 2007) of *P. distans*, whereas the sexual stage leads to the partial or complete sterilization of the host (Lembicz et al. 2011). *E. typhina* is the only species of the genus *Epichloë* reported so far in connection with *P. distance* (unpublished). In the case of other grass species, significant economic losses occur as a result of *Epichloë typhina* in the yields of grasses used as feed in meadows—in Oregon, USA, losses due to choke disease were estimated at more than $820,000 per year (Pfender and Alderman 2006), whereas at more than $100,000 in the Czech Republic in 2008 (Cagaš and Macháč 2012).

In Poland, *Epichloë* species are widespread. Żurek et al. (2012) revealed their presence in more than 70% of semi-natural communities dominated by grass species and in 37% of communities of grass species alone. In populations of *P. distans*, *E. typhina* were recorded for at least 20 years, and the size of the infection grows year by year (Lembicz 1998; Lembicz et al. 2011).

Unfortunately, to date, no effective method has been developed to reduce the occurrence of *Epichloë* fungi. Previous attempts to control choke disease by using the chemical fungicides Kocide 3000 and Copper-Count N (Alderman et al. 2008) have failed. Frequent rotation of crops, which results in the rapid liquidation of infected plants, is a satisfactory but costly method (Pfender and Alderman 2003). It has been recently shown that the use of hyperparasites naturally occurring on *Epichloë* stromata such as *Dicyma pulvinata* (Alderman et al. 2010) and *Clonostachys epichloë* (Górzyńska et al. 2018) has decreased the development of the fungi causing choke disease, which confirms that the direction of research was well chosen.

However, the influences of known and currently used species of fungal hyperparasites have not been investigated. Thus, the present study aimed to determine the influence of the fungus *Trichoderma harzianum*, a hyperparasite already widely used in biocontrol, on the growth of the fungus *E. typhina*, which is responsible for choke disease.

Materials and methods

Fungal isolates and identification

Plant material was collected at three localities of *P. distans–E. typhina* association in the vicinity of Inowrocław, Poland (Table 1). All three localities are characterized by high fungal infection rates (Table 1, Lembicz et al. 2011). *E. typhina* isolates were obtained by placing surface-sterilized *P. distans* leaf segments on PDA medium supplemented with an antibiotic (chloramphenicol, 100 mg/L) in Petri dishes. Emerging mycelia were subsequently transferred onto new plates and grown in an incubator at 25 °C. For the fungus *T. harzianum,* we choose well-characterized, open-access isolate (CBS 122147) obtained from the CBS-KNAW collection (Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands).

Isolates were identified based on their microscopic characteristics, which were evaluated based on information provided by Alderman et al. (2010) and St-Germain and Summerbell (2011), as well as with molecular methods. To isolate DNA, we ground mycelia of *T. harzianum* and of three fungal isolates originating from three different *P. distans* sites 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. The PCR was conducted in 25 µl volume containing 2.5 µl of 10X buffer, 2.5 µl of 2.5 mM dNTP mix, 0.5 µl of each primer at 10 µM, 0.5 µl of DNA Taq polymerase, 13.5 µl of nuclease-free water, and 5 µl of

Table 1 Origins of *Epichloë typhina* and *Trichoderma harzianum* strains used in the study; for *E. typhina*, infection rates are given for three *Puccinellia distans* sites according to Lembicz et al. (2011)

Grass species	Site	Locality	Infection rate (%)	Strain symbol	Fungal species	Accession no. of closest relative and % identity
Puccinellia distans	Giebnia	N 52°46.544′ E 18°06.190′	81	EpiG	*Epichloë typhina*	AB105952.1 97%
	Janikowo	N 52°46.384′ E 18°08.032′	74	EpiJ	*Epichloë typhina*	AB105953.1 98%
	Pakość	N 52°47.293′ E 18°06.721′	91.2	EpiP	*Epichloë typhina*	KU710348.1 99%
	CBS 122147 (CBS-KNAW)			*Trichoderma harzianum*		KJ755187.1 99%

DNA template. Amplification was conducted in a thermocycler using a program with the following parameters: 2 min at 95 °C; 37 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; and 5 min at 72 °C. The PCR products were purified using alkaline phosphatase and exonuclease I, and sequencing was carried out at the Laboratory of Molecular Biology Techniques in the Faculty of Biology, A. Mickiewicz University, Poznań, Poland. The obtained sequences were compared to those published in the NCBI (www.ncbi.nlm.nih.gov) databases using BLAST (Altschul et al. 1990).

Morphological and molecular identification confirmed the identities of the obtained strains as *E. typhina* and *T. harzianum* (Table 1). Three strains of *E. typhina*, each originating from one of three sites, were used in in vitro experiments.

Dual-culture method

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 *E. typhina* and one with a *T. harzianum* mycelium, were placed at opposite ends of each PDA plate (90 mm) at 1 cm from the edge. Dishes inoculated with only *E. typhina* served as controls. A pilot study revealed a significant difference in growth rate between the two fungal species, with *T. harzianum* colonizing the entire surface of the plate before *E. typhina* began to grow. For this reason, the plugs with *T. harzianum* were added one month after the plugs with *E. typhina* were added. Plates were incubated at 25 °C, and five replicates of the paired cultures and controls were performed. Measurements of the growth of the paired cultures and controls were performed once a day for 6 days. These measurements were used to estimate mean daily mycelium growth (mm day^{-1}) (e.g., growth increase) on both the dual-culture and control plates.

Precolonized plate method

The colonization ability of *T. harzianum* toward the three *E. typhina* strains was analyzed via the precolonized plate method described in Evans et al. (2003). An agar plug of 5 mm diameter, covered with a colony of *T. harzianum,* was placed at one edge of a colony of *E. typhina* on a PDA plate (90 mm). After incubation at 25 °C in the dark, 6–9 samples (depending on diameters of *E. typhina* colonies on each precolonized plate) were removed with a 7-mm cork borer, starting at the inoculum. Samples were plated on PDA medium, incubated at 25 °C in the dark and observed after few days to detect hyperparasite mycelium. The recognition of *T. harzianum* was easy due to the characteristic dark-green conidia that it forms. The percentage colonization (number of samples with *Trichoderma*/total number of samples × 100) was determined, and comparisons were made among *Epichloë* strains. Three variants of the experiment were performed: (A) plugs with *T. harzianum* were added after 2 months of *E. typhina* growth, and measurements were made after 5.5 weeks; (B) plufgs with *T. harzianum* were added after 4 months of *E. typhina* growth, and measurements were made after 5.5 weeks; and (C) plugs with *T. harzianum* were added after 2.5 months of *E. typhina* growth, and measurements were made after 5 months.

Additionally, five 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

Table 2 Results of repeated-measures ANOVAs

Source of variation	*df*	*F*	MS	*P*
Trichoderma (p/a)	1	107.80	51.09	**< 0.001**
Epichloë strain	2	0.53	0.25	0.60
Trichoderma (p/a) × *Epichloë* strain	2	0.32	0.15	0.73
Error	24		0.47	
Time	5	14.62	5.68	**< 0.001**
Trichoderma (p/a) × time	5	22.52	8.74	**< 0.001**
Epichloë strain × time	10	0.91	0.35	0.53
Trichoderma (p/a) × *Epichloë* strain × time	10	1.38	0.53	0.20
Error	120		0.39	

Effects of *Trichoderma harzianum* presence, *Epichloë typhina* strain, time and their interactions on the mean daily mycelial growth of *E. typhina*

df degrees of freedom, *F* statistic F, *MS* mean square, *P* p value

Significant *P* values < 0.05 in bold

Olympus BX53 microscope to observe typical mycoparasitic hyphal interactions.

Statistical analysis

Repeated-measures ANOVA and a subsequent post hoc Tukey's test were used to determine whether the *T. harzianum* mycelium, the *E. typhina* strain, the measuring time (after 1, 2, 3, 4, 5 and 6 days), and their interactions had significant effects on the mycelial growth of *E. typhina.* Factorial ANOVA was used for determining the effect of *E. typhina* strain on the colonization ability of *T. harzianum* toward *E. typhina.* The assumptions of the tests were validated before the analyses were conducted with the Shapiro–Wilk test for distribution normality and Levene's test for homogeneity of variance within groups. Variables expressed in proportions were arc-sine-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

In the dual-culture experiment, the presence of the *T. harzianum* hyperparasite negatively impacted the mean daily growth of *Epichloë* mycelium regardless of strain (Table 2). In all *Epichloë* strains, inhibition of growth was observed on the fourth day of the experiment and continued until the end of the experiment (Fig. 1). On day 4, the growth of *Epichloë* mycelium was two times higher in the absence of the hyperparasite than in its presence. Mycelia of *T. harzianum* and *E. typhina* not only occur together on slides with PDA, coiling was observed as well, which is typical initial mycoparasitic hyphal interaction.

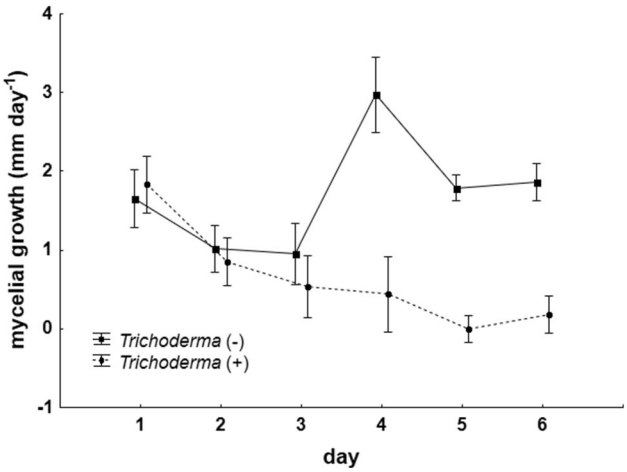

Fig. 1 Effects of *T. harzianum* on the daily mycelial growth of *E. typhina.* Due to a lack of difference in growth inhibition among *E. typhina* strains (Table 2), the strain data were pooled for the figure. Values represent means ± CI

The extent of *T. harzianum* colonization on the plates colonized with *Epichloë* determined using the precolonization method ranged from 25 to 100% depending on *Epichloë* strain and variant of the method (Table 3). Total colonization of *E. typhina*, resulting in the complete suppression of growth was achieved only in variant C with one *Epichloë* strain—EpiP. Overall, the degree of *T. harzianum* colonization did not differ among the three *Epichloë* strains (Table 4) but did differ among the variants of the precolonization method (Fig. 2, Table 4). The most effective was variant C, in which *T. harzianum* was added after 2.5 months of *E. typhina* growth and hyperparasite colonization was determined after 5 months. There was no difference in colonization extent among *Epichloë* strains within variants (Tukey HSD, NS), and none of the variants

Table 3 Average extent of *Trichoderma harzianum* colonization on plates colonized with *Epichloë* by *Epichloë* strain and methodological variant

	Colonization (%)				
		Variant A	Variant B	Variant C	Mean for *Epichloë* strain
Colonization (%)	EpiJ	25.0	57.5	64.5	49.0
	EpiP	55.0	50.0	100	68.0
	EpiG	40.0	30.0	66.7	46.0
	Mean for variant	40.0	46.0	77.0	

Table 4 Results of ANOVA showing the effects of *Epichloë typhina* strain and variant of precolonization method on mycoparasitic activity of *Trichoderma harzianum* (as measured by percentage colonization) toward *E. typhina*

Source of variation	*df*	*F*	MS	*P*
Epichloë strain	2	3.52	2293.0	0.06
variant (*A, B, C*)	2	8.99	5863.2	**< 0.001**
Epichloë strain × variant (*A, B, C*)	4	1.43	993.6	0.24
Error	36		651.9	

df degrees of freedom, *F* statistic F, *MS* mean square, *P* p value
Significant *P* values < 0.05 in bold

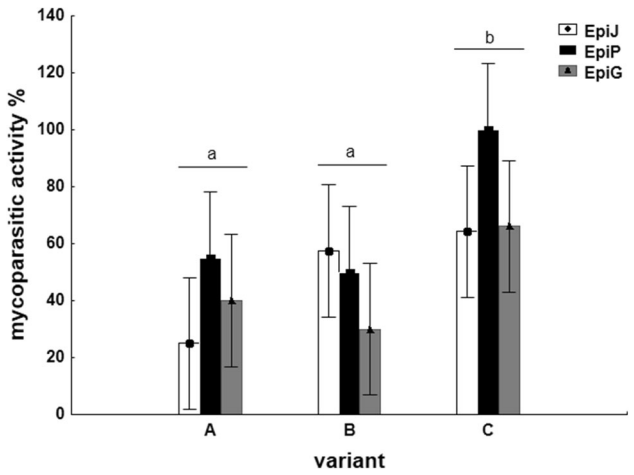

Fig. 2 Colonization ability of *T. harzianum* (as measured by the precolonization method) toward three *E. typhina* strains obtained with different variants of the method. Values represent means ± CI. Different letters indicate significant differences between means of the groups (variants)

was more effective for a particular *Epichloë* strain (Tukey HSD, NS).

Discussion

The hyperparasitic abilities of *T. harzianum* against *E. typhina* were confirmed in two experiments. In the first experiment, it was assumed that the *T. harzianum* fungus limits the growth of *E. typhina* mycelium in vitro, and in the second experiment, it was assumed that the *T. harzianum* fungus is capable of parasitizing the already grown mycelium of *E. typhina.* The same results were obtained across the three strains of *E. typhina*. Differences in colonization ability were observed among the methodological variants in the second experiment, which differed in the age of *E. typhina* at the time of application of the fungus *T. harzianum* and duration of coexistence of both fungi on plates. The variant with younger *E. typhina* mycelium (variant A) was found to be less effective compared with older mycelium (variant B), at the same interaction time. However, with increasing exposure time (variant C), the hyperparasite is able to colonize the young mycelium, achieving colonization level as high as 100%. Microscopic observation of the hyphal interaction of both fungi on the plates, confirmed parasitic activity of *T. harzianum* toward *E. typhina.*

To date, two species of naturally occurring hyperparasites have been tested with respect to their usefulness for controlling choke disease. The first species, *D. pulvinata* (Alderman et al. 2010), appeared on the stromata of *E. typhina* in orchardgrass fields in the USA. The infected stromata had fewer perithecia and stunted relative to uninfected stromata, which had an orange color and mature perithecia. The second fungal parasite found in natural conditions occurred on *E. typhina* stromata and was *C. epichloë* (Górzyńska et al. 2018). Microscopy of *Epichloë* stromata infected with *C. epichloë* revealed a lack of ascospores in the perithecia and damage to mycelia at sites colonized by *C. epichloë*. In that study, three strains of *E. typhina* were used (the same strains as in the present study), and similar to *T. harzianum*, *C. epichloë* inhibited the growth of each *Epichloë* strain. One difference was that the effects of three different *C. epichloë* strains were examined. The percentage of colonization ranged from 2.22 to 100%. Only one *Epichloë* strain—EpiP—was more than 70% colonized by all of three *Clonostachys* strains. Interestingly, this is the same strain that was the most colonized in the present study. These results show that the use of fungal hyperparasites, including *T. harzianum*, to control choke disease seems promising. However, additional tests are necessary in the greenhouse or under field conditions because environmental factors influence spore germination,

infection, and the destructive activity of mycoparasites (e.g., Partridge et al. 2006).

An important factor affecting the experiments conducted here was the different growth rates of the two species of fungi. In the previously conducted pilot studies, while the mycelium of *T. harzianum* overgrew the whole plate, the mycelium of each *E. typhina* strain grew only to approximately 15 mm in length. Thus, the design of the first experiment had to be modified accordingly. In the second experiment, when the mycelium of *E. typhina* covered the entire plate, *T. harzianum* required much more time to perform effectively as a hyperparasite. These results are important because they show a key role of timing during the use of a given choke disease protection agent.

The use of *T. harzianum* to control *E. typhina* in grass populations could be of two types. First, the hyperparasite could be used to control the endophytic form of *E. typhina*. Some *Trichoderma* strains establish long-lasting colonization of plant roots and penetrate into the epidermis (Harman et al. 2004). Additionally, some research shows that *T. harzianum* is capable of reducing symptoms of *Alternaria solani* on tomato leaves to 80%, when it is present only on roots (Seaman 2003). The use of a *T. harzianum* in the form of a water solution applied to the soil may affect not only endophytic growth of *E. typhina* in the grasses, but also, if given at the right time, will not allow the development of sexual forms and the appearance of choke disease. Early spring seems to be the best moment—grass seeds germinate and grasses begin their next life cycle. Mycelium of *E. typhina* is still young then and there is a chance that this fungal pathogen will be killed, before it enters the stage of sexual reproduction and stromata production. The second method would be to apply *T. harzianum* to already developed stromata, similarly as for *D. pulvinata* or *B. epichloë*. In this case, however, it would be necessary to carry out additional studies that will confirm a parasitic activity of *T. harzianum* against the sexual stage of *E. typhina*.

In summary, the *T. harzianum* fungus exhibits parasitic properties against the *E. typhina* fungus; however, to be used in biocontrol, it should be tested under natural conditions. Tests carried out on dishes under laboratory conditions do not account for all of the factors that occur in the natural environment, which could affect the fungus *T. harzianum*.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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