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Fungal endophytes protect grass seedlings against herbivory and allow economical seed production

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ABSTRACT

Background: When infected with seed-transmitted fungal endophytes, some grasses produce a larger quantity of seeds, although these seeds are smaller than normal. The principles of life-history theory suggest that this size reduction could evolve if the infection reduces predation pressure.

Question: Does the endophyte protect grass seedlings against herbivory?

Hypothesis: Herbivores consume more seedlings that develop from endophyte-free grass seeds than from endophyte-infected grass seeds.

Organisms: *Epichloë typhina* is the seed-transmitted endophytic fungus, the weeping alkaligrass (*Puccinellia distans*) seedlings are the hosts, and the grove snail (*Cepaea nemoralis*) is the herbivore.

Methods: Our experiment consisted of two 66-h food-choice tests. The tests were performed consecutively and on the same individual snails. We offered snails freshly harvested weeping alkaligrass seedlings with and without the endophyte.

Results: Starved snails consumed approximately 20% less biomass of infected seedlings than uninfected seedlings. When the same snails were tested again shortly after their first contact with the endophyte, they exhibited an approximately 40% reduction in seedling consumption, and food discrimination became undetectable.

Conclusions: Toxins produced by endophytic fungi reduce the vulnerability of grass seedlings to snail herbivory

Keywords: fungal endophytes, herbivory, life history, resource allocation, seedlings, seed mass, seed quality, snails.

INTRODUCTION

Endophytic fungi can inhabit plant tissues and are particularly widespread in C3 grasses (Saikkonen *et al.*, 2004; Cheplick and Faeth, 2009). They are postulated to be mutualistic symbionts that stimulate a larger size of host plant (West *et al.*, 1993; Groppe *et al.*, 1999; Malinowski *et al.*, 1999), increase

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production of generative shoots and seeds (Clay, 1987; Petroski *et al.*, 1990), and enhance resistance to infections and herbivory (Read and Camp, 1986; Siegel and Bush, 1996; Jones *et al.*, 2000; Brem and Leuchtmann, 2001; Vázquez de Aldana *et al.*, 2001; Clay, 2004; Wang *et al.*, 2004; Jensen, 2005; Spiering *et al.*, 2005). Complementary evidence shows that endophytes have adverse effects on plant enemies by retarding the development of insect larvae (Jallow *et al.*, 2004) and decreasing fecundity and survivorship of herbivores (Tibbets and Faeth, 1999; Brem and Leuchtmann, 2001; Jallow *et al.*, 2004; Meister *et al.*, 2006). The protective effects of fungi, primarily from the genus *Epichloë* / *Neotyphodium*, are attributed to the production of mycotoxins, which consist mainly of alkaloids such as ergopeptines (e.g. ergovaline), lolitrems (e.g. lolitrem B), pyrrolizidines (e.g. loline), and pyrrolopyrazines (e.g. peramine) (Vázquez de Aldana *et al.*, 2003, 2007; Zhang *et al.*, 2009).

Given the well-established evidence on the beneficial effects of endophytic fungi, it is surprising to observe that the *Epichloë typhina* endophyte stimulates the host, weeping alkaligrass (*Puccinellia distans*), to produce seeds that are several times smaller than the seeds developed by uninfected individuals (Olejniczak and Lembicz, 2007). Seed mass is regarded as a major contributor to seedling fitness because the amount of storage inside seeds influences germination success as well as seedling growth and competitiveness (Fenner and Thompson, 2006). Thus, the infected small-seeded individuals of *P. distans* appear to be handicapped rather than advantaged by the endophyte. But is this really the case?

The aim of this study was to explore why weeping alkaligrass reduces its seed size when infected with the *Epichloë* endophyte. Given size–number trade-offs in offspring production (Smith and Fretwell, 1974; Stearns, 1992), this reduction can manifest a shift in optimal resource allocation of a grass that increases production of seeds. However, if an increase in the overall number of seeds at the cost of their quality was adaptive, then *P. distans* would benefit from this allocation strategy either with or without an endophyte. Apparently, a drop in seed quality associated with smaller seeds makes such a strategy unbeneficial for individuals without the fungus, but it may be rewarding for individuals hosting the endophyte. We hypothesized that seedlings of the endophyte-infected individuals would have superior anti-herbivore resistance compared with seedlings originating from the endophyte-free grasses. We examined this expectation in a food-choice experiment using the *Cepaea nemoralis* grove snail and *P. distans* seedlings that were either uninfected or infected with the fungal endophyte *E. typhina* (hereafter referred to as E− and E+ seedlings). If the endophyte reduces snail herbivory in seedlings, our experimental snails should consume more biomass of E− than E+ seedlings. Our field observations indicate that the *C. nemoralis* snail may be an important invertebrate grazer in the grasslands studied by Olejniczak and Lembicz (2007). Gastropods are also a good model for herbivores that feed preferentially on young grasses (Motheral and Orrock, 2010).

MATERIALS AND METHODS

Snails and grass seedlings

We housed study organisms and conducted our experiment in an air-conditioned growth chamber (22°C, 70% humidity, 17/7 h light/dark photoperiod) at the Institute of Environmental Sciences at Jagiellonian University in Kraków, Poland.

In summer 2006, we collected the seeds of *P. distans* from nine locations in central Poland. Based on earlier research (Lembicz, 1998; Olejniczak and Lembicz, 2007), we classified our populations as either infected or uninfected by *E. typhina*: infected (Pakość 52°47.531N, 18°06.118E; Węgierce 52°45.493N, 18°08.276E; Janikowo 52°46.384N, 18°08.032E; Giebnia 52°46.544N, 18°06.190E; and Kobylniki 52°41.655N, 18°18.788) and uninfected (Dulsk 52°45.329N, 18°20.518E; Turzany 52°47.348N, 18°20.276E; Jacewo 52°48.048N, 18°17.846E; and Bąbolin 52°52.631N, 18°23.783E). In the *Epichloë*-infected locations, we collected seeds only from the tufts of grass with visible fungal stromata. We mixed seeds from different locations within each infection category and divided each group into two approximately equal seed pools (hereafter referred to as pool one and pool two) to provide a backup for seedling cultivation. To induce germination, we froze the seeds at −20°C for one week and then sowed them in 40 flowerpots (10 per infection group and seed pool) filled with universal soil ('Casto' Hortipack, Poznań, Poland). Sowing from pool two was done a week after sowing from pool one. We individually enclosed the flowerpots in sun bags (Sigma-Aldrich, Poland) and exposed them to plant-growing light (Flora, Osram 30 W). The bags were transparent and equipped with a ventilation system for flora cultivation (0.02 μm pore diameter).

In autumn 2006, we collected *C. nemoralis* snails (body mass 6.2–8.6 g) in a park area of Kraków, Poland, that was not inhabited by *P. distans*. We housed the snails in plastic containers, regularly sprayed them with dechlorinated tap water, and fed them lettuce and carrots.

Detection of endophytes

We used a molecular method to determine the presence of the fungal endophyte *E. typhina* both in maternal *P. distans* tufts from which seeds were collected for laboratory cultivation and in laboratory-cultivated seedlings (50 seedlings per studied population). We prepared total genomic DNA from 100 mg of fresh leaves using a miniprep extraction kit (Qiagen) according to the manufacturer's instructions. We determined DNA concentrations using DyNA Quant 200 and Hoechst 33258 fluorimeters (Amersham Biosciences, GE Healthcare) and checked the quality of DNA samples by electrophoresis in 0.8% agarose gels. The isolation procedure yielded approximately 4 μg of DNA per 100 mg of plant tissue. We detected endophyte DNA with the polymerase chain reaction (PCR) using two specific fungal primers designed for the beta-tubulin gene of *Epichloë* endophytes (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′) was complementary to a fragment of intron 3. We performed amplifications on a DNA thermal cycler (PTC-200, MJ Research) programmed with the following parameters: 95°C for 3 min, then 35 cycles at 94°C for 15 s and 60°C for 1 min, followed by 72°C for 10 min. We separated PCR products of approximately 350 bp in length by electrophoresis in 2% agarose gels and visualized them with ethidium bromide staining. To confirm DNA identity, we purified the product of the PCR reaction and sequenced it (CEQ 2000 XL, Beckman Coulter). The sequencing results for the amplified DNA fragments confirmed that they originated from the *E. typhina* genome (GenBank Accession No. DQ267692).

Experiment

Our experiment comprised two 66-h food-choice tests. The tests were performed consecutively on the same individual snails. We offered snails freshly harvested weeping alkaligrass seedlings that were either hosting (E+) or not hosting (E−) the fungus *E. typhina*.

Before the experiment, we deprived 40 snails of food for one week. On the fifth day of starvation, we marked the snails with a permanent marker and placed randomly assigned pairs in 20 plastic boxes (2.7 dm^3) for 3 days of acclimation. Hanley *et al.* (2003) demonstrated that experimental units of at least two snails exhibit less variable eating habits than those of individual snails. Immediately before each test trial, we harvested leaves from E+ and E− seedlings. We used ten flowerpots per infection group: five flowerpots with seedlings from pool one and five flowerpots with seedlings from pool two. Harvest for the first test occurred on the 32nd (pool one) and 25th (pool two) day after germination. We mixed the harvested leaves within each infection category and grouped them in approximately equal bunches. We weighed these bunches (fresh weight) to the nearest 0.0001 g on an electronic balance and fastened the leaves of each bunch together with a paperclip. We placed a bunch of E+ and E− seedlings on the bottom of each box on two opposite sides. After 66 h, we removed seedling remains for further analysis and repeated a food-choice test on the same snails using new bunches of seedlings. We collected the seedling remains after each test, dried them for 24 h at 60°C in a ventilated oven, and determined dry weight. We calculated the amount of consumed dry biomass of leaves during each test trial. To convert fresh biomass to dry mass, we calculated a conversion coefficient (average dry-to-wet biomass ratio) from five reference samples harvested together with test samples. We used coefficients calculated separately for each test trial and for the E+ and E− samples.

Statistical analysis

To determine whether endophyte infestation influenced snail food choice, we analysed data on food consumption with a General Linear Model (GLM, Statistica 9.0, StatSoft) that considered the following: two fixed factors, which were infection status (E+ vs. E−) and food-choice test (first vs. second); one random factor, which was the effects of 20 experimental boxes; and the 'infection × choice test' interaction. We checked for normality and homogeneity.

RESULTS

The results of the food-choice experiment (Table 1, Fig. 1) show that the effect of the endophyte on snail consumption of grass seedlings depended on the food-choice test trial (significant infection × choice test interaction, $P = 0.042$); food consumption also differed between experimental boxes ($P = 0.000001$). In the first test trial, snails showed an aversion to *Epichloë*-infected seedlings, and consumed approximately 20% less dry biomass of E+ than E− leaves. When we offered both types of food again to the same snails, they were generally less interested in feeding regardless of the presence or absence of the endophyte. Consumption in the second test dropped on average to 64% with respect to the first test ($P = 0.000001$); deterrence of snails by the endophyte in food was undetectable in the second test.

DISCUSSION

Grasses are evolutionarily well equipped to tolerate and resist herbivory. The location of their meristematic tissue and the vegetative mode of reproduction allow regrowth after grazing, and deposition of silica reduces the palatability of grass tissues (Kotanen and Rosenthal,

Table 1. GLM results of food-choice experiments with the *Cepaea nemoralis* snails

Factor	Effect	d.f.	*F*	*P*
Experimental box	Random	19	6.04	0.000001
Infection	Fixed	1	3.41	0.07
Choice test	Fixed	1	51.51	0.000001
Infection × choice test	Fixed	1	4.34	0.042
Error		57		

Note: *Puccinellia distans* grass seedlings that were either infected or uninfected by the *Epichloë typhina* endophytic fungus were offered to the snails paired in experimental boxes in two consecutive food-choice tests. The snails were naïve and long-starved in the first test. In the second test, the snails had already experienced the endophytic toxins ingested during the first test trial.

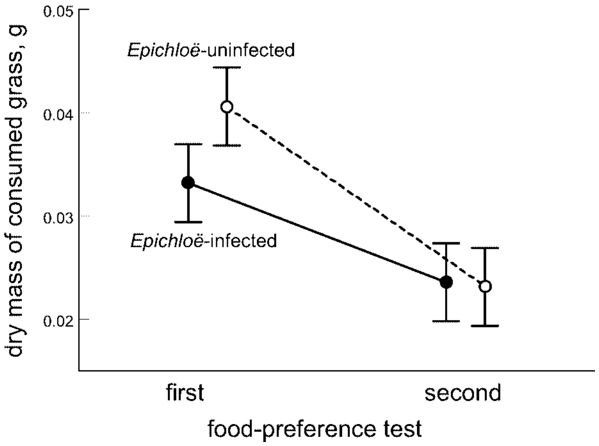

Fig. 1. Results of food-choice tests with the *Cepaea nemoralis* snails offered *Puccinellia distans* grass seedlings that were either infected or uninfected with the *Epichloë typhina* endophytic fungus (least squares means ±CI). Snails were naïve and long-starved in the first test. In the second test, the snails had already experienced the endophytic toxins ingested during the first test trial.

2000; Hawkes and Sullivan, 2001; Massey *et al.*, 2007). Grasses can gain additional anti-herbivore protection from toxins produced by endophytic fungi. Although fungal endophytes are known to decrease herbivory in adult grasses (Conover, 2003), their toxins should be especially beneficial for grasses early in life at the seed and seedling stages, in which innate anti-herbivore properties are not yet efficient. There is ample evidence to support this view. Zhang *et al.* (2009) demonstrated that mock herbivory (clipping) increased production of endophyte-related alkaloids in meadow fescue, mainly in younger leaf blades. Food-choice experiments have shown that grass seeds infected with endophytic fungi deter birds and rodents (Varney *et al.*, 1991; Wolock-Madej and Clay, 1991). Our results suggest that fungal endophytes that grow from maternal grasses into seeds may reduce herbivore pressure during development of seedlings. Our food-choice experiment demonstrated that long-starved, naïve *Cepaea nemoralis* snails consumed 20% less biomass of *Epichloë*-infected than *Epichloë*-uninfected seedlings. When we examined food preferences of the same individuals shortly after the first test, we found that the snails were averse to any type of food, irrespective of the presence or absence of the endophyte; the snails consumed approximately 40% less food in the second test than in the first test. Although we cannot rule out the possibility that the snails became satiated during the second test, the reduction in feeding rate through time suggests that prior contact with endophyte toxins decreased the snails' consumption of seedlings (note that all experimental snails tasted E+ food during the first test trial). A similar reduction in feeding with concomitant drops in activity, weight gain, and reproductive output was observed in rodents and cattle on an endophyte-rich diet (Osborn *et al.*, 1992; Durham and Tannenbaum, 1998; Spiers *et al.*, 2005). We do not know which alkaloids caused the feeding responses of snails in our experiment. The type and amount of chemicals produced by endophytic fungi change during a season, differ between grass and endophyte species, and vary across grass life stages (Leuchtmann *et al.*, 2000; Schardl, 2001; Vázquez de Aldana *et al.*, 2001, 2003, 2007; Zhang *et al.*, 2009). Nonetheless, our results demonstrate that naïve snails discriminated between E− and E+ seedlings and that the feeding rate decreased following the first contact with the fungal endophyte. This decrease suggests that the chemicals in *Epichloë*-infected weeping alkaligrass affect *C. nemoralis* snails both by contact and through metabolic toxins (Bush *et al.*, 1997; Leuchtmann *et al.*, 2000).

Fungal endophytes are typically classified as symbionts that increase the fitness of their plant hosts. This mutualistic view is supported by studies that have focused on short-term direct effects of endophytes (West *et al.*, 1993; Groppe *et al.*, 1999; Malinowski *et al.*, 1999), but emerging evidence demonstrates the long-term fitness costs of hosting endophytic fungi (Olejniczak and Lembicz, 2007; Faeth, 2009). Our results suggest yet another perspective. Production of protective toxins by fungal endophytes must alter the evolutionary pressure exerted by herbivores on host plants. Life-history theory posits that organisms have limited access to resources, and the opportunity and potential to convert these resources to offspring production depend on physiological constraints and selection factors (Kozłowski, 1992; Stearns, 1992). Therefore, organisms that maximize lifetime fitness are expected to adjust resource allocation to shifts in selection. Olejniczak and Lembicz (2007) demonstrated that *Epichloë*-infected weeping alkaligrass produces seeds that are several times smaller than the *Epichloë*-uninfected conspecifics. Parental investment in offspring quality at the expense of offspring number is a vital component of allocation strategies (Smith and Fretwell, 1974; Stearns, 1992). In plants, such investment is largely reflected by seed size, which is an important determinant of the future of seedlings (Fenner and Thompson, 2006). We propose that weeping alkaligrass harbouring the fungal endophyte supplies embryos with smaller food reserves because the survival prospects of their progeny are already secured by the endophyte growing into the seeds. The remaining resources can be spent on the increase in seed production. The production of small seeds may not be beneficial for the uninfected plants because such 'economical' offspring would be numerous, but of low value. Endophyte-free seedlings growing from small seeds would have lower survival and competitive abilities under grazing. Evidence shows that seedlings germinating from small seeds have low defoliation tolerance because their regrowth ability is limited by the amount of resources in seeds (Armstrong and Westoby, 1993). Seedlings originating from small seeds may have an absolute size disadvantage compared with the seedlings germinating from large seeds (Fenner and Thompson, 2006). Such size-effects of seed size were recently observed in the initial developmental stages of weeping alkaligrass (M. Lembicz *et al.*, unpublished), and they are likely to affect the potential of seedlings to build-up belowground stores for regrowth after defoliation. Indeed, the biomass of belowground parts is closely related to the biomass of photosynthetic tissue in the seedlings of weeping alkaligrass (M. Lembicz *et al.*, unpublished).

On the basis of these integrated findings on the relationship between fungal endophytes and their hosts, we can try and offer some conclusions. Our explanation as to why endophyte-infected weeping alkaligrass develops small seeds is that it produces seedlings with two types of anti-herbivore phenotypes: the grazing-tolerant seedlings originating from large endophyte-free seeds and the herbivore-resistant seedlings originating from small endophyte-infected seeds. The importance of anti-herbivore value of grass seedlings implies that they are commonly subject to herbivory in nature. This notion is reinforced by evidence on the significance of snail herbivory on seedlings of different grass species (Motheral and Orrock, 2010). Production of small seeds is adaptive only if it enables the endophyte-protected grasses to produce more seeds, and this seems to be the case. Olejniczak and Lembicz (2007) observed that weeping alkaligrass infected with *Epichloë* endophyte produced more seeds over the course of the first 3 years of life. Stimulated production of seeds and generative and vegetative shoots has also been reported in many other plants infected with fungal endophytes (Clay, 1987; Petroski *et al.*, 1990; West *et al.*, 1993; Groppe *et al.*, 1999; Malinowski *et al.*, 1999).

Distinct resource allocation to offspring quality and number in plants with endophytic fungi requires further scientific attention. If this phenomenon were ubiquitous in nature, it might help to form a cohesive view of the relationships between these endophytes and their hosts. Overlooking the unequal prospects for infected and uninfected plants may also lead to unrealistic assumptions. Olejniczak and Lembicz (2007) predicted a decrease in lifetime seed production in the endophyte-infected weeping alkaligrass, but they assumed no difference between infected and uninfected plants with respect to their mortality and seed quality. From a broader perspective, studies on the dependence of seed quality on endophytic fungi may have important implications for improving our understanding of the evolution of seed banks and plant phenologies. Recent theoretical studies highlight the importance of the influence of environmental forces on the future of offspring for the evolution of phenology of resource allocation to growth and reproduction (Ejsmond *et al.*, 2010).

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