

Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*

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Summary. Many grasses are infected by endophytic fungi that grow intercellularly in leaves, stems, and flowers and are transmitted maternally by hyphal growth into ovules and seeds. The seed biology and seedling growth of endophyte-infected and uninfected perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*) were investigated under controlled environmental conditions. The percentage of filled seeds produced by infected tall fescue was over twice of uninfected tall fescue; infected and uninfected perennial ryegrass had similar percentages. Weights of seeds from infected and uninfected plants were similar in both species. Seeds from infected plants of both species exhibited a higher rate of germination than seeds from uninfected plants. Shoot growth in the greenhouse was compared by making three sequential harvests of above-ground plant parts from infected and uninfected plants of both species. Infected perennial ryegrass plants produced significantly more biomass and tillers than uninfected plants after 6 and 10 weeks of growth and significantly more biomass after 14 weeks of growth. Infected tall fescue plants produced significantly more biomass and tillers than uninfected plants after 10 and 14 weeks of growth. The physiological mechanism of enhancement of growth is not known. The results of this study suggest that infected plants may have a selective advantage in populations with uninfected members.

Key words: *Lolium perenne* – *Festuca arundinacea* – Leaf endophytes – Seed endophytes – Germination – Growth

A common theme in the evolution of higher plants has been the formation of symbiotic associations with heterotrophic microorganisms. Plants infected by nitrogen-fixing bacteria or mycorrhizal fungi frequently exhibit increased ability to acquire limiting nutrients or tolerate physical stresses (Lewis 1973). These associations arose long ago and are essentially obligate in many plant groups, precluding experimental attempts to quantify the ecological advantages and disadvantages of symbiosis. The ecological advantages of symbiosis between plants and microorganisms are best studied when the interaction is facultative and exists as a polymorphism in host plant populations.

Many grasses are infected by clavicipitaceous fungi that occur perennially as intercellular endophytes (Clay 1986a). While some grass species appear to be ubiquitously infected, others are not known to serve as hosts; many other species

fall between these extremes (Diehl 1950; Clay 1984, 1986a; White and Cole 1985, 1986). The widespread grasses *Lolium perenne* (perennial ryegrass) and *Festuca arundinacea* (tall fescue) often are infected by the imperfect fungi *Acremonium loliae* and *A. coenophialum*, respectively (Morgan-Jones and Gams 1982; Latch et al. 1984). These fungi closely resemble the asexual (anamorphic) state of *Epichloe typhina* (Ascomycetes, Clavicipitaceae) and probably are derived from it (Sampson 1933; Bacon et al. 1977; White 1987). *Epichloe* and closely allied genera in the tribe Balansiae are related to the genus *Claviceps* (Diehl 1950). While *Claviceps* species are transient and localized ovarian parasites of grasses and sedges, the Balansiae fungi are systemic and perennial. Intercellular hyphae of *A. loliae* and *A. coenophialum* can be observed in the meristematic region, in the mesophyll of leaves, in the pith of flowering culms, and in the scutellum and aleuron layer of seeds (Neill 1940, 1941; Bacon et al. 1977; Clark et al. 1983). Fungal hyphae do not occur intracellularly or within roots.

The endophytes of perennial ryegrass and tall fescue do not produce spores in their hosts (although they can in pure culture) and do not spread contagiously; instead, they are transmitted from maternal plant to progeny by vegetative growth of hyphae into the developing ovule or seed (Sampson 1933; Neill 1941; Bacon et al. 1977; Clay 1986a). Therefore, the persistence of the infection in natural populations depends upon the effect of the fungi on the survival, growth, and reproduction (i.e., fitness) of their hosts, compared to uninfected plants. Previous research has shown that infected plants of perennial ryegrass and tall fescue exhibit greater resistance to some insect herbivores and greater vigor in pastures compare to uninfected plants (Bradshaw 1959; Funk et al. 1983; Clay 1986a; Clay et al. 1985). Here I compare the seed and seedling biology of infected and uninfected plants of perennial ryegrass and tall fescue as an initial step in estimating the effect of endophyte-infection on host plant fitness. Experiments were conducted in a controlled environment without herbivores to distinguish direct effects of endophyte-infection on shoot growth from indirect effects on growth due to differential herbivory.

Materials and methods

Seeds of perennial ryegrass (*Lolium perenne* L.) were obtained from a commercial seed company (Loft's Seed Co., Bound Brook, NJ, USA). Seeds of the cultivar "Repell"

have a high frequency of infection by the endophyte *Acremonium loliae* Latch, Christensen & Samuels while seeds of the cultivar "Yorktown" have a low frequency of infection. I determined the frequency of infected plants by microscopically examining leaf sheaths of 25 plants raised from seed for each cultivar. The two cultivars are advanced generation selections from the same parental stock and are genetically similar (pers. comm., R. Hurley, Loft's Seed Co.). Seeds of tall fescue (*Festuca arundinacea* Schreb.) were bulk collected from a stand containing a high frequency of infected plants and from a stand containing a low frequency of infected plants located in a garden on the Louisiana State University campus (Baton Rouge, LA, USA). The stands of tall fescue were originally planted with lots of seeds of cultivar "KY-31" differing in infection level of *Acremonium coenophialum* Morgan-Jones and Gams (provided by Dr. Malcolm Siegel, University of Kentucky). To confirm levels of infection I examined leaf sheaths of 25 plants raised from seed collected from each stand.

Abundant hyphae of *A. loliae* were observed in the leaf sheaths of 23 of 25 (92%) Repell perennial ryegrass plants; no hyphae were observed in the leaf sheaths of Yorktown perennial ryegrass plants. The leaf sheaths of all 25 tall fescue plants collected as seed from the infected stand contained hyphae of *A. coenophialum*; 3 of 25 (12%) plants collected as seed from the uninfected stand were found to contain hyphae in the leaf sheaths. Hereafter I refer to the seeds and plants originating from the different seed lots as either infected or uninfected, recognizing that low levels of contamination may be present. Individual seeds and plants used in experiments were not examined for infection but were presumed to resemble the frequencies found in the above subsamples. Statistical tests comparing infected and uninfected plants are therefore conservative.

The percentage of filled seeds was estimated by taking a random sample of 200 from each of the four categories: infected and uninfected perennial ryegrass, and infected and uninfected tall fescue. Each seed was pressed individually on a glass plate with the fingertip to detect a caryopsis within the lemma and palea. A sample of approximately 100 filled seeds (caryopsis, lemma, and palea) in each category were weighed individually on an electronic balance to the nearest 0.01 mg.

Germination was compared by planting 100 seeds of infected ryegrass (ten seeds each in ten pots) and 100 seeds of uninfected ryegrass. Each 10 cm² pot was filled with a soil mixture (one part sterile potting soil, one part peat, one part perlite, one part sand). The pots were placed in an environmental growth chamber with a diurnal light/temperature cycle (14 h light/24 C, 10 h dark/20 C) and the soil was kept constantly moist. Emergent seedlings were counted every day until new seedlings ceased to appear. Germination tests of tall fescue were conducted in a similar manner except there were 250 seeds each from infected and uninfected plants (25 per pot). The higher sample size was used because it was felt that germination levels might be lower than in commercial cultivars of perennial ryegrass.

Growth comparisons were made using seedlings from seeds germinated in plastic trays. Individual seedlings were washed free of soil, blotted dry, and weighed for subsequent use as a covariate in statistical analyses. Each seedling was planted in sterile soil into individual cavities of "Speedling Trays" (Speedling, Inc., Sun City, FL, USA), styrofoam trays with 72 inverted pyramidal cavities 5 cm² at the open-

Table 1. Percentage seed set (sample of 200) and seed weights (mg) of infected and uninfected perennial ryegrass and tall fescue. Asterisk indicates a significant difference ($P < 0.05$) based on a chi-square test. Mean seed weights \pm one standard error are presented (sample sizes in parentheses)

Species	Type	% Filled Seeds	Mean Seed Weight
Perennial Ryegrass	Infected	88%	1.90 \pm 0.05 (98)
	Uninfected	90%	1.86 \pm 0.05 (102)
Tall Fescue	Infected	44% *	2.13 \pm 0.05 (98)
	Uninfected	19%	2.13 \pm 0.06 (102)

ing, 7.5 cm deep, and approximately 62 cm³ in volume. Each tray was divided into two blocks of 36 cavities each and either seedlings from infected or uninfected seeds were planted in each block. A total of eight blocks were planted with infected seedlings and eight with uninfected seedlings, for each grass species. The trays were randomly arranged on benches in the greenhouse and rotated biweekly. All plants were watered daily and fertilized weekly with a dilute liquid fertilizer (Peters 20-20-20, 1 g per l); insecticidal soap (Safer, 10 ml per l) was sprayed on all plants biweekly to prevent colonization by aphids. Dead plants were not replaced. Two blocks of infected plants and two of uninfected plants, for both species, were harvested after six weeks by clipping at the soil surface. The number of tillers were counted and dried at 80 C for 48 h and then weighed. Another harvest was conducted at 10 weeks by sampling two additional blocks from each category in the same manner. The remaining four blocks in each category were harvested at 14 weeks.

Non-parametric chi-square tests were utilized to compare the percentages of filled seeds and germination of infected and uninfected seeds of each species (Siegel 1956). One-way analysis of variance was used to compare seed weights and analysis of covariance was used to compare tiller numbers and weights of harvested plants, between infected and uninfected plants of the same species. Infection status of the plants was considered a fixed main effect while original seedling weight was considered as a covariate. Untransformed and log-transformed weights and tiller numbers were analyzed, and because transformation of dependent variables did not increase the amount of variation explained by the model (and in some cases decreased it), only the results from untransformed variables are presented here.

Results

Seeds from infected and uninfected perennial ryegrass were similar with respect to the percentage of filled seeds and seed weight (Table 1). In contrast, the percentage of filled tall fescue seeds from infected plants was over twice that from uninfected plants, a highly significant difference ($X^2 = 20.8$, $P < 0.0005$). However, the weights of filled seeds were equal (Table 1).

The germination of seeds of both species followed a similar course. There was a flush of germination over a

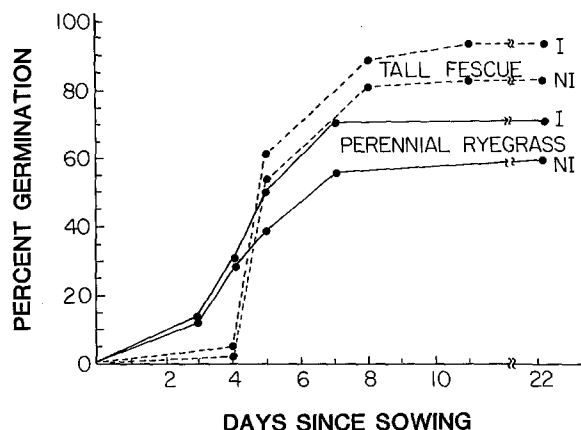


Fig. 1. Percent germination of endophyte-infected (I) and uninfected NI seeds of perennial ryegrass and tall fescue

three or four day period after which few additional seeds germinated (Fig. 1). For both species there was a consistent trend for seeds from infected plants, which themselves were endophyte-infected, to germinate at a higher frequency at each sample point and in total. Approximately 10% more infected seeds than uninfected seeds germinated for both perennial ryegrass and tall fescue (Fig. 1). Given the sample size, a chi-square test for differences in total seed germination was not significant for eight species. However, infected seeds of both species had higher germination than uninfected seeds at each of five sample dates ($P < 0.05$, sign test).

For both species, the number of tillers and above-ground biomass produced by infected and uninfected individuals grown in the greenhouse differed significantly in most cases (Table 2). In perennial ryegrass there was a trend for the differences in tiller number and dry weight to be greatest earlier and decrease later. This trend was paralleled by the decreasing effect over time of the covariate (seedling weight) and the amount of variation explained by the ANCOVA model (Table 2). In contrast, infected and uninfected plants of tall fescue exhibited no significant differences at the first harvest but highly significant differences in tiller number and dry weight at the second and final harvests. These results were paralleled by an increase in the amount of variation explained by the ANCOVA model over time (Table 2). The covariate was significant only once for dry weight at week 10.

Tiller numbers of infected perennial ryegrass were 50% higher than those of uninfected perennial ryegrass at week 6 and 10 but were slightly lower at week 14 (Table 3). Above-ground biomass was significantly higher for infected perennial ryegrass plants at all three harvest dates; the greatest proportional difference in dry weight occurred at week 6. While dry weights of both infected and uninfected perennial ryegrass increased from week 6 to 14, tiller numbers of infected perennial ryegrass first increased then declined; tiller numbers of uninfected perennial ryegrass increased from week 6 to 14 (Table 3).

Tiller numbers of infected and uninfected tall fescue were identical at week 6, but infected plants had produced 18% and 42% more tillers than uninfected plants at week

Table 2. Summary of analysis of covariance. Tiller number (TL) and dry weight (DW) were analyzed separately for each species and harvest date. The infection status of the plant was analyzed as a fixed main effect and initial seedling weight was analyzed as a covariate. Transforming the data did not increase the explained variance so only the results of untransformed data are presented. One, two, and three asterisks signify $P < 0.05$, 0.005, and 0.0005, respectively

	DF	Perennial Ryegrass						Tall Fescue					
		Week 6		Week 10		Week 14		Week 6		Week 10		Week 14	
		TL	DW	TL	DW	TL	DW	TL	DW	TL	DW	TL	DW
Model	2	***	***	***	**	NS	***	NS	NS	*	**	***	***
Inf	1	***	***	***	***	NS	***	NS	NS	*	*	***	***
Cov	1	**	***	NS	*	NS	*	NS	NS	NS	*	NS	NS
R^2		0.32	0.49	0.32	0.10	0.01	0.08	0.04	0.00	0.05	0.14	0.22	0.45

Table 3. Numbers of tillers and above-ground biomass of infected and uninfected perennial ryegrass and tall fescue raised in the greenhouse. Means \pm one standard error are presented (sample sizes in parentheses). Weights are in milligrams

	Week 6		Week 10		Week 14	
	Tillers	Dry Weight	Tillers	Dry Weight	Tillers	Dry Weight
Perennial Ryegrass						
Infected	14.5 \pm 0.6 (58)	502.9 \pm 19.8	19.1 \pm 0.7 (59)	544.9 \pm 19.2	16.7 \pm 0.5 (111)	710.3 \pm 19.5
Uninfected	9.6 \pm 0.5 (50)	291.0 \pm 16.0	12.3 \pm 0.6 (56)	478.8 \pm 23.8	17.6 \pm 0.9 (96)	607.5 \pm 23.6
Tall Fescue						
Infected	4.6 \pm 0.2 (49)	700.0 \pm 30.8	4.7 \pm 0.3 (46)	1066.7 \pm 55.8	5.1 \pm 0.2 (94)	1547.2 \pm 33.5
Uninfected	4.6 \pm 0.2 (50)	786.0 \pm 29.2	4.0 \pm 0.2 (37)	862.4 \pm 48.3	3.6 \pm 0.1 (89)	979.8 \pm 33.0

10 and 14, respectively (Table 3). Above-ground biomass of infected plants was lower than that of uninfected plants at the first harvest but was 24% greater at week 10 and 58% greater at week 14. As in perennial ryegrass, dry weight increased from week 6 to 14 for both infected and uninfected tall fescue. Tiller numbers of infected tall fescue increased from week 6 to 14 but declined in uninfected tall fescue over the same period.

Discussion

The results of this and other studies generally support the idea that the relationship between tall fescue and perennial ryegrass and their endophytes is mutualistic. Increased survival and growth of other grasses and sedges infected by related fungi has been reported previously (Bradshaw 1959; Clay 1984, 1986b; Antonovics et al. 1987; Stovall and Clay unpublished work). The experimental technique of comparing identical or closely related host genotypes with and without the endophyte allows a direct assessment of the effect of the fungus on its host. Latch et al. (1985) compared the growth of infected and uninfected clones of perennial ryegrass (cultivar "Grasslands Ruanui"). Endophyte-infected clones produced 38% more biomass than uninfected clones after eight weeks in a controlled-environment room, a significant difference. Gaynor and Hunt (1983) and Mortimer and di Menna (1983) reported increased forage yields of endophyte-infected perennial ryegrass pastures but it is not clear whether the differences resulted from increased growth or decreased herbivory of infected plants. However, Neill (1941, 1952) observed no differences between infected and uninfected ryegrass. Read and Camp (1986) have recently reported that infected tall fescue pastures yielded more forage than uninfected pastures in the absence of grazing and exhibited greater persistence under drought conditions. In contrast, Siegel et al. (1984) observed no differences in forage production between infected and uninfected tall fescue. Differences reported here in seed set (in tall fescue) and germination have not been noted previously. The increased vigor of infected plants also appears to be expressed during flowering and seed set of maternal plants and during seed germination.

One possible basis for greater growth of infected plants in nature is their increased resistance to herbivory. Several studies have demonstrated that endophyte-infected grasses are discriminated against by herbivorous insects and if they are consumed, insects suffer reduced survival and growth compared to those reared on uninfected conspecifics (Funk et al. 1983; Clay et al. 1985; Johnson et al. 1985; Stewart 1985). The results of this study and those of Latch et al. (1985) cannot be ascribed to differential herbivory because plants were grown in herbivore-free environments; the fungi appear to enhance growth of their hosts directly.

The physiological basis for the increased growth of endophyte-infected perennial ryegrass and tall fescue is unknown. Porter et al. (1985) have recently demonstrated that *Balansia epichloe* produces auxin in vitro. The sedge *Cyperus virens* often produces viviparous plantlets when infected by *B. cyperi*, a phenomenon often associated with hormonal imbalances (Clay 1986b). The concentration of fungal hyphae around meristematic regions of their hosts might facilitate hormonal regulation of cell division and differentiation. Possible hormonal differences between infected and

uninfected perennial ryegrass and tall fescue should be investigated in relation to their observed growth differences.

Endophyte infection has obvious implications for the ecology of host populations and grassland communities. While this study was conducted in an artificial environment and over a short time span, the results suggest infected plants may possess advantages over uninfected plants in seed set, germination, and seedling growth. Insect herbivory was shown to significantly decrease the survival of uninfected perennial ryegrass seedlings compared to infected seedlings, which suffered little herbivory (Stewart 1985). Differences in plant growth and size are frequently correlated with fecundity differences (Harper 1977). Infected plants may also exhibit greater interspecific competitive ability, as shown in the grass *Danthonia spicata* infected by a related *Balansiae* fungus (Kelley and Clay 1987).

In many areas endophyte-infected perennial ryegrass and tall fescue plants comprise the majority of the population (Neill 1940; Funk et al. 1983; Rycyk and Sharpe 1984; Siegel et al. 1985). Recent studies have revealed the presence of endophytic fungi resembling those of perennial ryegrass and tall fescue in many additional grasses (White and Cole 1985; White 1987; Latch 1987). Thus, the symbiotic association between grasses and fungal endophytes appears to be widespread. Because these fungi are not known to spread contagiously from infected to uninfected plants, alternative hypotheses explaining the high frequency of infected plants are required. The results of this study suggest that endophyte-infection may enhance host plant fitness, thereby increasing the frequency of infected plants in the next generation.

The question arises why uninfected plants still exist. Populations consisting of uninfected plants will remain so because infection is maternally inherited, barring immigration of infected seeds. Within mixed populations uninfected plants may persist if there is little or no selection against them. For example, the disadvantage of being uninfected may be minimal in low density populations lacking herbivory. Infected plants may also lose the endophyte. While adult plants are not known to become uninfected if previously infected, the same is not true of seeds. Endophyte viability decreases more rapidly than seed viability so prolonged seed dormancy may result in seeds without viable endophyte (Neill 1940; Welty and Azevedo 1985; Latch 1987). However, the importance of regeneration from seed banks is not known for many wild grasses. Perennial ryegrass and tall fescue are native to Europe but have been disseminated worldwide by man. Latch (1987) surveyed collections of *Lolium* and *Festuca* species from natural habitats and long established pastures in Europe and found that the frequency of endophyte-infection was much higher than in commercial cultivars. He suggested that endophyte-free plants have become common only following the development of modern agriculture involving seed harvesting and storage.

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