

# Responses of endophyte-bearing and endophyte-free varieties of *Lolium perenne* L. to fungicide treatment and simulated herbivory

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## Abstract

The effects of the presence of fungal endophytes, treatment with a systemic fungicide, and simulated herbivory on growth and biomass allocation were investigated in 2 varieties of perennial ryegrass (*Lolium perenne*): 'Repell', an endophyte-bearing variety, and 'Pennfine', a low-endophyte variety. In the absence of herbivory or fungicide there were no significant differences in the growth or pattern of biomass allocation between varieties. Treatment with the systemic fungicide benomyl reduced growth of both varieties by approximately 50% and reduced root growth more than shoot growth; fungicide effects were similar in the 2 varieties. Simulated herbivory reduced root growth more in endophyte-bearing Repell plants than in endophyte-free Pennfine plants, and root:shoot ratios of Repell plants were significantly lower than those of Pennfine plants following either moderate or severe herbivory. Statistically significant interactions between fungicide treatment and simulated herbivory were frequent in Repell plants but absent in Pennfine plants, suggesting that the fungicide had both direct phytotoxic effects and indirect effects mediated through the loss of endophytes by the Repell plants. While the proximate cost to seedlings bearing endophyte seemed small, the presence of the endophytes altered the allocation pattern of biomass following herbivory in such a way as to increase the probability of mortality.

**Key Words:** endophytes, fungicide, ryegrass

Many prairie and pasture grasses are infected by fungal endophytes that ramify throughout leaf and stem tissues (White 1987, Clay 1988). For example, perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinaceae* Schreb.) are infected with the imperfect fungi *Acremonium lolii* and *A. coenophialum* respectively (Latch et al. 1984, White and Cole 1985). These fungi do not produce spores with great dispersal and infection capability; rather the fungi are transmitted from one generation of grasses to another by vegetative growth of hyphae into developing ovules or seeds (Bacon et al. 1977, White and Cole 1986). Thus the persistence of this relationship in natural communities is dependent on the fitness of the host plant.

A number of studies have recently established the toxicity of *Acremonium* in *Festuca* and *Lolium* spp. to a variety of animals (Hoveland et al. 1983, review by Clay 1988). Cattle, sheep, and deer grazing on infected ryegrass or tall fescue have experienced reduced mass gain, tremors, staggers, and even death (Mackintosh et al. 1982, Fletcher 1983). The tendency of some mammals to avoid at least some infected grasses protects both the host grass and the fungus.

Alkaloids produced by fungal endophytes of grasses have also been shown to reduce feeding rates and oviposition and increase mortality in a variety of insects (Clay 1988). Though these chemical

defenses are currently effective in reducing insect herbivory, thereby increasing plant yield and fitness, a wide range of studies suggest that insects often become tolerant or resistant to trace compound defenses more rapidly than they evolve *de novo* in plants (Hodkinson and Hughes 1982). This then raises the question of what costs and/or benefits having the fungal endophyte will confer on the host grass once insect herbivores become resistant to the fungal alkaloids.

Little information is currently available on the proximate effects of fungal endophytes on plant growth or biomass allocation (Latch et al. 1985). Two approaches to investigating fungal effects on growth are available: (1) comparisons of endophyte-bearing and endophyte-free varieties of a given species of grass, and (2) comparisons of endophyte-bearing individuals with ones in which the fungus has been killed either by fungicides or by lengthy seed storage (Latch et al. 1985). In this study, we compare those 2 approaches as means of determining how endophyte-bearing and endophyte-free plants will recover from simulated herbivory. Our specific objectives were to: (1) compare the growth of endophyte-bearing and endophyte-free varieties of *Lolium perenne* under controlled conditions, (2) compare the growth of endophyte-bearing *Lolium perenne* plants to plants of the same variety in which the endophytes have been removed by fungicide treatment, and (3) compare the effects of simulated herbivory on an endophyte-bearing variety, an endophyte-free variety, and plants of an endophyte-bearing variety in which the endophytes have been removed by fungicide treatment.

## Methods

Cohorts of 2 varieties of *Lolium perenne*: 'Repell', a variety with 100% of its seed infected with *Acremonium lolii*, and 'Pennfine', a variety with less than 25% of its seed infected (Halisky and Funk 1984) were established in flats under an intermittent mister in a greenhouse. Single culms were transplanted to 10-cm pots of 80:20 sand:perlite at 3 wk of age; half the plants of each variety were treated every other day for 2 weeks with a 1.2 g/l solution of benomyl (methyl 1-butylcarbamoyl, 2-benzimidazolecarbamate), systemic fungicide shown to be effective against fungal endophytes of grasses (Clay, Personal communication). This fungicide inhibits mitosis in all fungi except Oomycetes by binding to tubulin and preventing spindle formation (Griffin 1981). All plants were fed weekly with 100 ml of Peters' 20-20-20 complete fertilizer and randomized weekly to minimize bench position effects.

After 4 wk of growth in the greenhouse at ambient light (maximum 800  $\mu\text{E m}^{-2} \text{sec}^{-1}$ ) and temperature (range 10–29° C), each variety-by-fungicide combination was divided into 3 groups: (1) control, (2) moderate simulated herbivory: clipping of each culm to 3 cm height, and (3) severe simulated herbivory: clipping to 1 cm height. Each variety-by-fungicide-by-herbivory combination was replicated 6 times. The clipped portions of each plant were dried at 70° C for 24 hrs and dry mass determined to the nearest 0.1 mg. After 2 wk of post-clipping growth (9 wk total growth) all plants

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were harvested, divided into root and shoot, and dried and weighed. Leaves from extra fungicided and control plants were cleared, stained, and examined for the presence of fungal endophytes using the trypan blue method of Phillips and Hayman (1970). No endophytes were present in fungicided Repell plants or Pennfine (fungicided or control); all examined nonfungicided Repell plants had endophytes present.

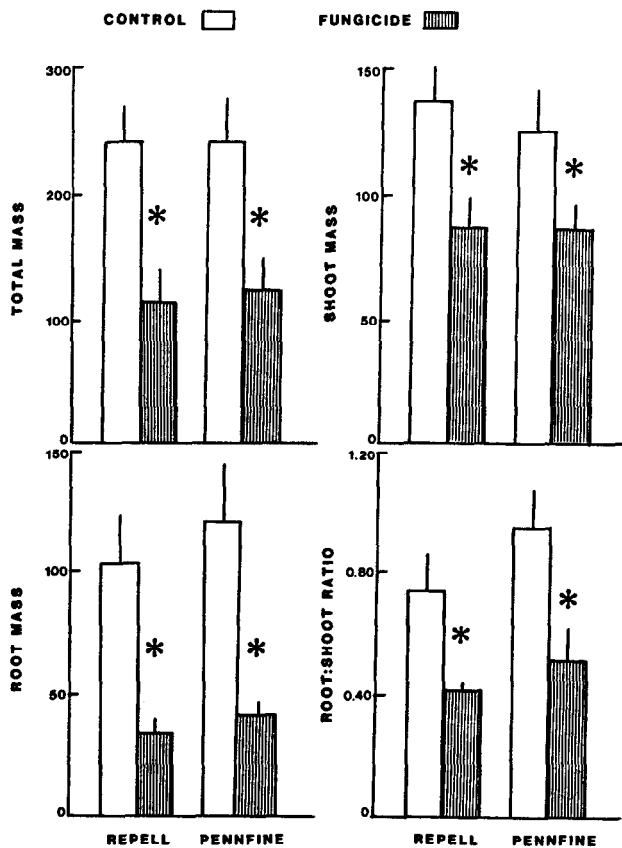
Differences among treatment groups in total plant mass (shoot + root at harvest), total plant yield (mass at harvest + clipped biomass), root mass, shoot mass and yield, and root:shoot ratio were analyzed by analysis of variance and Tukey's Studentized Range Test (S.A.S. 1986) using the 0.05 significance level unless otherwise noted.

## Results

In the absence of either herbivory or fungicide, there were no significant differences in the growth or yield of the 2 *Lolium* varieties (Table 1). Growth of both varieties were reduced by

**Table 1. Comparison of biomass (mg dry mass) and root:shoot ratio of Repell and Pennfine varieties of *Lolium perenne* after 9 weeks of growth. Standard errors of the means are given in parentheses.**

Parameter	var. Repell	var. Pennfine
Total Plant Mass	240.4 (29.3)	243.3 (33.2)
Shoot Mass	137.5 (13.1)	124.7 (14.6)
Root Mass	102.9 (18.2)	118.5 (20.5)
Root:Shoot Ratio	0.737 (0.102)	0.936 (0.122)



**Fig. 1. Total, shoot, and root mass (mg dry mass) at harvest and root:shoot ratio of Repell and Pennfine plants, with and without fungicide treatment. Means and one standard error are given. Significant differences at  $p = 0.05$  from analysis of variance are indicated by an asterisk.**

fungicide treatment by an average of 49% in total plant mass, 34% in shoot mass, and 66% in root mass (Fig. 1). As a result of the greater effect of the fungicide on roots than shoots, the root:shoot ratio decreased by an average of 82% following the fungicide treatment (Fig. 1). There were no significant differences between varieties in the effect of the fungicide treatment.

As there were no significant differences in the growth of the 2 varieties in the absence of fungicide treatment, we chose to assess the effect of the presence of endophytes on recovery from simulated herbivory by comparing recovery in non-fungicided Repell and Pennfine plants. Total plant mass, total yield (total mass at harvest + clipped biomass), shoot mass, and shoot yield all decreased with increasing simulated herbivory, but with no differences between varieties (Table 2). Root mass also decreased significantly with increasing simulated herbivory; the root mass of Repell

**Table 2. Total and shoot growth (mg dry mass) of 2 *Lolium perenne* varieties exposed to moderate and severe simulated herbivory. Within a row, means followed by different lower case letters were different at  $p = 0.05$  following analysis of variance and Tukey's Studentized Range test. Standard errors of the means are given in parentheses.**

Parameter/-variety	Moderate simulated herbivory		Severe simulated herbivory	
	Control	Control	Control	Control
<b>Total Mass at Harvest</b>				
var. Repell	240.4a (29.3)	133.7b (20.4)	82.9c (7.5)	
var. Pennfine	243.3a (33.2)	191.5b (32.1)	78.3c (12.6)	
Combined	241.8a (21.1)	162.6b (20.1)	80.6c (7.0)	
<b>Total Yield</b>				
var. Repell	240.4a (29.3)	140.2b (21.3)	92.6c (8.5)	
var. Pennfine	243.3a (33.2)	196.3b (31.9)	85.7c (13.9)	
Combined	241.8a (21.1)	168.2b (20.2)	89.1c (7.9)	
<b>Shoot Mass at Harvest</b>				
var. Repell	137.5a (13.1)	94.6b (15.6)	58.0c (6.2)	
var. Pennfine	124.7a (14.6)	107.5b (13.7)	48.1c (6.1)	
Combined	131.1a (9.5)	101.1b (13.6)	53.1c (4.4)	
<b>Total Shoot Yield</b>				
var. Repell	137.5a (13.1)	101.1a (16.4)	67.7b (7.1)	
var. Pennfine	124.7a (14.6)	112.3a (13.7)	55.5b (7.5)	
Combined	131.1a (9.5)	106.7a (10.3)	61.6b (5.2)	
<b>Root Mass at Harvest<sup>1,2</sup></b>				
var. Repell	102.9a (18.2)	39.1b (5.2)	24.9c (2.0)	
var. Pennfine	118.6a (20.5)	83.9b (21.2)	30.2c (6.7)	
<b>Root:Shoot Ratio<sup>2,3</sup></b>				
var. Repell	0.737a (0.102)	0.430b (0.036)	0.446b (0.041)	
var. Pennfine	0.936a (0.122)	0.750b (0.162)	0.603b (0.057)	

<sup>1</sup>difference between varieties significant at  $p = 0.074$ .

<sup>2</sup>no results for combined varieties are given because of significant or marginally significant differences between varieties.

<sup>3</sup>difference between varieties significant at  $p = 0.008$ .

plants was lower than that of Pennfine plants, though the difference was only significant at the 0.074 level (Table 2). Overall, root:shoot ratio decreased by an average of 29.5% and 37.3% following moderate and severe clipping, respectively. However, the root:shoot ratios of Repell plants were significantly lower than those of Pennfine plants at both levels of herbivory (Table 2).

Fungicide treatment reduced the recovery from simulated herbivory in both varieties. For Pennfine, this effect was presumably due only to direct toxic effects of the fungicide since we observed no endophytes in this variety. Total plant mass at harvest, total yield, shoot mass, and shoot yield of Pennfine plants were all significantly lower in heavily clipped plants than in moderately clipped or unclipped plants, regardless of fungicide treatment (Fig. 2). The root mass of heavily clipped Pennfine plants was significantly lower than that of unclipped plants in both fungicided and control groups, but the moderately clipped plants root mass did

PENNFINE

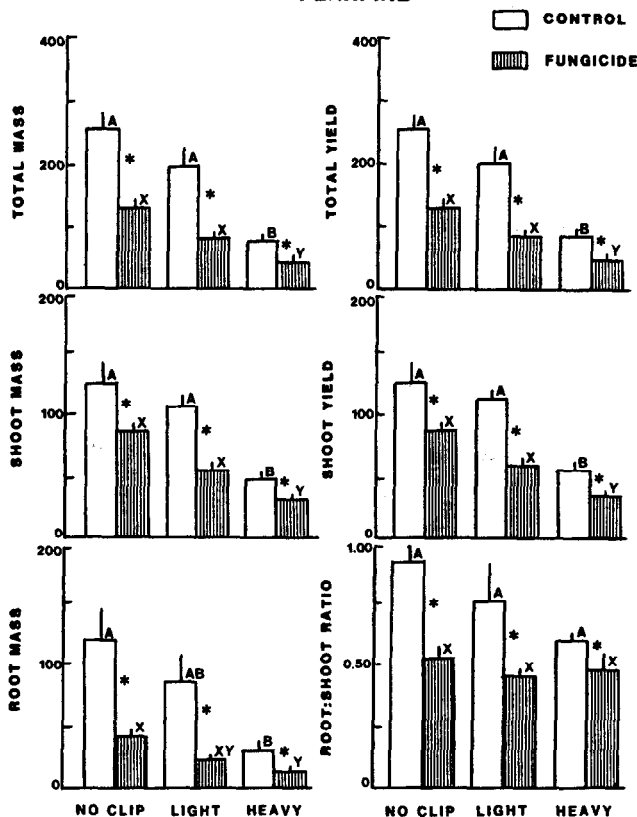


Fig. 2. Growth of Pennfine plants in relation to fungicide treatment and clipping. Significant differences between fungicided and control plants within a clipping treatment are indicated by an asterisk. Means labelled with the different upper case letters (A, B, for control plants; X, Y for fungicided plants) were significantly different. Standard errors of the means are plotted; all significant differences were at  $p \leq 0.05$ .

not differ significantly from that of the other 2 groups (Fig. 2). The root:shoot ratios of Pennfine plants were lower in fungicided plants than controls, but did not change significantly as a function of simulated herbivory. Simulated herbivory and fungicide treatments did not display a significant interaction for any growth parameters in the Pennfine variety.

In contrast, statistically significant interactions between fungicides and simulated herbivory were frequent in the Repell variety, suggesting that the fungicide had both direct phytotoxic effects and indirect effects mediated through the loss of the endophyte. Total mass at harvest and total yield decreased with increasing clipping intensity in control Repell plants, whereas only the severe clipping affected these parameters in fungicided plants (Fig. 3). Shoot mass and shoot yield also decreased with increasing clipping intensity in both control and fungicided plants, but only the heavily clipped plants were significantly smaller than the unclipped plants (Fig. 3). Both moderate and severe clipping resulted in lower root mass and root:shoot ratio in control plants but not in fungicided plants (Fig. 3). Thus, 2 wk was insufficient for even moderately clipped plants to recover in biomass or yield, and the effects on belowground tissues were greater on the Repell (endophyte-bearing) plants than on the Pennfine (endophyte-free) plants.

Of the 4 fungicide-variety combinations, only the Repell-control combination had fungal endophytes. In that combination, simulated herbivory caused a decrease in relative biomass allocation to roots compared to shoots (Fig. 3). In all 3 endophyte-free treatment combinations, the pattern of biomass allocation (as mea-

REPELL

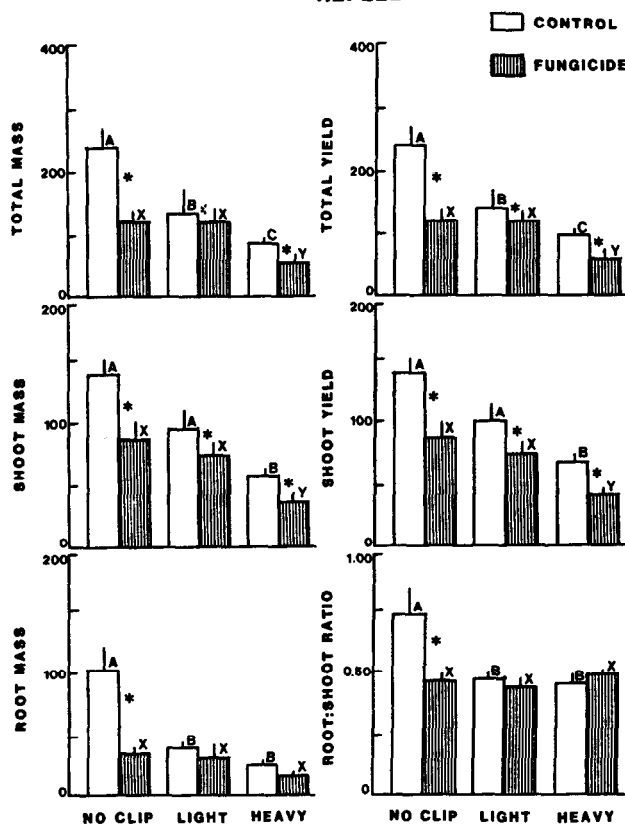


Fig. 3. Growth of Repell plants in relation to fungicide treatment and clipping. Symbols follow Figure 2.

sured by root:shoot ratio) remained constant over the recovery period no matter what the level of simulated herbivory (Figs. 2 and 3).

Discussion

Some studies of the effect of fungal endophytes on host growth have demonstrated higher growth rates in endophyte-bearing hosts than in endophyte-free plants (Gaynor and Hunt 1983, Clay 1987); in contrast, other studies have found no difference in growth related to the presence of endophytes (Neill 1941, 1952; Siegel et al. 1984). Greater growth in endophyte-bearing hosts has been related to reduced herbivory and to possible production of phytohormones or phytohormone analogues by the fungus (Porter et al. 1985). We have demonstrated that the method used to remove endophytes may induce toxic effects producing differences between endophyte-bearing and endophyte-free plants.

Assessing the cost of the endophyte to the host is a difficult problem because unlike mycorrhizae, which colonize plants after germination, these fungal endophytes are present in the seed even before dispersal. Two methods for removing the endophytes have become available: removal of the endophyte by fungicide application or longterm seed storage and comparative studies of naturally endophyte-bearing and endophyte-free varieties of a species. Our data demonstrate that the fungicide benomyl may be phytotoxic. Benomyl application to a variety of *L. perenne* which lacked endophytes produced a growth decrease of approximately 50% relative to non-fungicided plants, and this growth difference persisted over at least 8 weeks. Others using this method to remove endophytes have resorted to holding the plants for a length period of time after fungiciding before beginning experiments; for exam-

ple, Latch et al. (1985) held their plants for over 12 months after fungiciding. Though this method seems to help obviate the phytotoxic effects of the benomyl it does constrain studies of the physiology and ecology of endophytes to those with mature, overwintered plants with a perennial life history. At the very least, our results demonstrate that using benomyl to remove endophytes from young grasses has effects beyond the simple removal of the fungus.

One alternative is to store ungerminated seed for a lengthy period of time. As endophyte viability decreases more rapidly than seed viability, longterm storage reduces endophyte infection (Siegel et al. 1985). This process will also reduce seed mass as the seed's carbohydrate reserve is respired. It is clear from a number of studies that seedlings originating from seeds with larger mass are more competitive than those from smaller seeds, at least over the first few weeks of growth (e.g., Black 1958, Stanton 1984). No data are available comparing growth of seedlings rendered free of endophytes by longterm storage with endophyte-bearing seedlings from fresh seed.

A third possibility exists for exploring the costs and benefits of these endophytes: comparative studies of endophyte-bearing and endophyte-free varieties of a given species. We assume this approach has been less popular because it becomes impossible to separate effects due to endophytes from those caused by the genetic differences among cultivated varieties. Clay (1987) reported that aboveground growth of Repell plants was greater than that of plants of Yorktown, another endophyte-free variety of *L. perenne*, though those differences were restricted to aboveground tissues and decreased over time. In contrast, our data suggest that the growth rate and biomass allocation pattern of *Lolium perenne* var Pennfine (a low-endophyte or endophyte-free variety) was indistinguishable from that of the endophyte-bearing var Repell, at least over the initial 8 wk of growth, despite any genetic differences which might exist between cultivars. If such similarities in growth continue throughout the life of the plant, this experimental approach to assessing the effects of endophytes may be superior to the more common ones discussed above.

In the absence of direct enhancement of growth by, for example, phytohormone production (e.g., Porter et al. 1985), an increase in yield and fitness of endophyte-bearing plants will likely result from decreased herbivory. For this to be a longterm advantage to the plant, the losses of carbon and other nutrients to fungal feeding must be small compared to the potential losses to herbivory. In plants which synthesize their own herbivore-inhibiting chemicals, heavily defended plants often produce fewer leaves and are less competitive than plants with lower levels of defensive compounds in the absence of herbivory (Windle and Franz 1979, Coley 1986). When subjected to insect herbivores, however, the growth advantage shifts to the more heavily defended plants. Thus the benefit of synthesizing defensive compounds only outweighs the cost when herbivores are present. Insect herbivores, however, have a tendency to become tolerant or resistant to chemical deterrents, both natural and man-made. Once this occurs with the alkaloids the endophytes produce, the question becomes: what cost will the fungus have to the plant in the absence of any potential benefit?

In 3 of our 4 variety-fungicide treatment combinations, the level of simulated herbivory we applied did not change the relative allocation of photosynthate to root vs shoot; only in endophyte-bearing plants did we observe a decrease in the allocation of biomass to the root system. Though in established grasslands grazing may result in a shift of allocation to leaves and leaf meristems (e.g., Ryle and Powell 1975, Detling et al. 1979), such a change may not be beneficial to a seedling. As seedlings of perennial grasses often allocate a large proportion of their first year carbon gain to developing an effective root system, the combina-

tion of a reduction in total plant mass by herbivory and a reduced allocation to roots may decrease the likelihood of that individual successfully overwintering after that first growing season. Whether this endophyte-related change in allocation is due to phytohormone production by the fungus or to utilization of photosynthate before that photosynthate could reach the roots cannot be determined from our experiment. It is clear, however, that the presence of the endophyte does alter the biomass allocation pattern of clipped *Lolium perenne* seedlings and may affect plant fitness even if the total cost in photosynthate to support the fungus is small.

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