

Endophytic fungi in *Festuca pratensis* grown in Swedish agricultural grasslands with different managements

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Abstract

Cool-season forage grasses infected with asexual endophytic fungi (*Neotyphodium* spp.) often have advantages over uninfected grasses, including increased herbivore resistance. Since these fungi reproduce by growing into the developing seeds of the host, hyphal loading (amount of fungal hyphae in the plant) gives important information about the balance between host and endophyte in the symbiosis. This work provides insights into how ecological factors external to the symbiosis may influence the symbiosis. By repeated sampling of the same infected plants, we investigated how hyphal loading changes in *Festuca pratensis* through the growing season and how it is affected by fertilization and ley age in northern Sweden. Infection frequency in *F. pratensis* ranged from 25–65%. Hyphal loading, based on standard curves of immunoblot assay imprint intensity versus counts of hyphal density across grass leaf sheaths, increased significantly during the growing season and was correlated with cumulative degree days. There were no clear effects of fertilization treatment or ley age on hyphal loading. The symbiosis deserves further study with respect to plant physiology, ecology, crop production and forage quality.

Keywords: Cultivar, endophytes, *Festuca pratensis*, grazing, hyphal loading, immunoblot technique, infection frequency, leys, *Neotyphodium*, seasonal variation

1. Introduction

Grass fungal endophyte associations are ecologically common symbioses involving the largest, and arguably most economically important, family of plants (Poaceae). They also provide important model systems for understanding the evolution of symbiosis. Asexual fungal endophytes reproduce by the growth of intercellular hyphae into the developing seeds of the host grass and inhabit above-ground tissue (Carroll, 1988). From an ecological point of view, grasses infected with these fungi have many advantages compared with uninfected grasses, as observed primarily in two non-native agricultural grass species, *Festuca arundinacea* and *Lolium perenne* (Saikonen et al.,

2006). Observed benefits of endophyte infection include enhanced drought resistance (Elmi and West, 1995), enhanced competitive abilities (Clay and Holah, 1999), increased resistance to pathogens (Yue et al., 2000) and increased resistance to herbivory due to endophyte-produced toxic alkaloids (Clay, 1988; Hoveland, 1993; Bazely et al., 1997).

While endophyte abundance has been widely investigated in North American grasslands, only a limited number of studies have been undertaken on grass-endophyte symbioses in Europe. This could be attributed to the relatively few reported cases of acute toxicosis in livestock (Emile et al., 2000). Nonetheless, the presence of various endophyte-infected grass species has been documented in agricultural and natural settings throughout Europe (Bazely et al., 1997; Zabalgoitia et al., 1999; Saikonen et al., 2000; Jensen and Roulund, 2004; Koh et

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al., 2006). In northern areas, forage grasses are exposed to both cattle grazing and repeated cuts for silage during intense, short summers, while also experiencing harsh and long winters. Variations in environmental conditions and in the management of the grasses are likely to affect the balance between the grass and the fungus in the symbiosis.

Grass endophytes are vertically transmitted from maternal plants to seeds, and concentrations of the endophyte within the host may influence the success of transmission to the next generation. Therefore, persistence of the symbiosis could be directly affected by external factors that alter the rate of vertical transmission. However, the importance of plant age and external factors, such as cutting and fertilization, for the symbiosis is poorly known. We have, therefore, examined the seasonal pattern in the amount of fungal hyphae in the plant (hyphal loading) in grasses subjected to different agricultural managements and conditions.

Our work focused on *F. pratensis* and its fungal endophyte, *Neotyphodium uncinatum* Gams, Petrini and Schmidt (Glenn et al., 1996). We addressed the following questions: 1) What is the infection frequency of cultivated *F. pratensis*? 2) How does hyphal loading change through the growing season of the host grass? 3) How is hyphal loading affected by fertilization and ley age through the growing season? This work was carried out in cut and grazed grass-clover leys in Northern Sweden, with a ley in Southern Sweden included for comparison. We also sampled a variety trial with *F. pratensis* and *F. arundinacea* for endophyte infection.

2. Materials and Methods

Field site

Experimental plots were located at The Swedish University of Agricultural Sciences (SLU) in Umeå, Sweden (63°45'N, 20°17'E). The site is situated in the boreal zone with long days during short growing seasons, while winters are long with frozen topsoil and a snow cover. Monthly mean temperature during the study period in 2005 (June–August) ranged from 6.7–14.5°C. Daily mean temperatures, measured on site, were used to calculate degree days with 5°C and 10°C as base temperature.

Cut leys

Ley plots (blocks) sampled, 15 × 5 m, included *Festuca pratensis* L. cv. Kasper, *Phleum pratense* L. cv. Grindstad and *Trifolium pratense* L. cv. Betty. The first year ley, ley 1, was established as an under-sowing in barley in 2004, ley 2 in 2003 and ley 4 in 2001. All leys were cut twice in the growing season to about 5 cm height. Three ley plots of

each age were included in the study, and one plot of each age received one of three N-P-K chemical fertilizer treatments twice in the season: 1) a single dose, 75-15-90 kg ha⁻¹; 2) double dose, 150-30-180 kg ha⁻¹; 3) single dose, 65-5-45 kg ha⁻¹ plus 10 tons ha⁻¹ of cattle manure at (20% dry matter) added to treatment 3 in the preceding autumn. Fertilizer treatments were not randomized within each age block (ley) and there was no replication of blocks. In each of the 9 plots, 10–20 plants of *F. pratensis* were tagged at 1 m intervals along two transects 3 m apart. A single tiller from each plant was collected on June 8th or 14th and screened for endophyte infection. The first cut of the season took place on July 2nd, followed by a second sampling of infected individuals on July 22nd and the second cut on August 15th was followed by a third sampling of infected individuals on August 23rd.

Cattle-grazed leys

Two paddocks A (2.7 ha) and B (2.8 ha) were established in 2002 as a mixture of *F. pratensis* cv. Kasper, *P. pratense* cv. Jonatan and *T. repens* cv. Lena. Paddocks were grazed equally and cut twice during the season. Individual *F. pratensis* plants were randomly sampled throughout the paddocks on July 25th; 15 from paddock A and 20 from paddock B.

A ley established in 1997 in Ljungsbro, Sweden (58°51'N, 15°15'E) was sampled on July 11th, 2005. This ley contained *F. pratensis* of an unknown variety, *P. pratense*, *L. perenne* and *T. pratense*. The ley was cut once per year, cattle were allowed to graze freely in it and it was fertilized with cattle manure every second year.

Festuca varieties

Varieties of *F. pratensis* from Sweden, Norway, Finland, and Russia and one variety of *F. arundinacea* from Finland from trials previously set up by SLU in 2004 were screened for endophyte infection. The plots were cut twice in the season. Ten individuals per plot (14 × 1.5 m) were sampled on July 23rd at 1 m intervals along a transect.

Hyphal loading and immunoblot assay

Tillers collected were screened for endophyte infection with Phytoscreen Field Tiller Endophyte Detection Kits (Agrinostics Ltd. Co., Watkinsville, GA, USA). The presence of *Neotyphodium* spp. in the sample is indicated by a red imprint on the immunoblot card, while uninfected samples leave no imprint. Hyphal loading for all samples in the same immunoblot card can be predicted from a linear relationship between the visually counted numbers of hyphae mm⁻¹ and the red to green pixel values (RGB scale) of the imprint left by the infected individual. Further details are given by Koh et al. (2006) who also showed that the

method works for *F. pratensis*. DIMAGE viewer v2.3.3 (Konica Minolta Inc.) was used in this study to obtain the RGB values.

Data analysis

All data analyses were carried out using R v2.2.0 (R Development Core Team, 2005). The effect of seasonality on hyphal loading was examined using a linear mixed effect model in which "month" was a fixed continuous effect and sampled individuals were designated as a random effect. In order to test for the effects of ley age and fertilization on hyphal loading, a one-way ANOVA was undertaken for each effect per month. A *post-hoc* analysis of significant effects was done using Tukey's HSD test. Assumptions of normality were checked using the defaults diagnostic plots of R. For the grazing paddocks and the different *Festuca* varieties, comparisons among mean hyphal loading values were carried out with multiple Wilcoxon rank sum tests.

3. Results

Frequency of infected plants

Endophyte infection frequency in the sampled ley plots ranged from 25% to 65% for *F. pratensis* (Table 1). Mean infection frequency in the grazing paddocks A and B was

Table 1. Endophyte infection frequency in *Festuca pratensis* individuals sampled in ley plots during June 2005.

Ley age (years)	Fertilization treatment	Sample size	Infected plants (%)
1	1	20	40
1	2	20	50
1	3	20	60
2	1	10	60
2	2	20	40
2	3	12	25
4	1	20	65
4	2	20	45
4	3	20	35

Table 2. Infection frequency and hyphal loading (number of hyphae mm⁻¹) in varieties of *Festuca pratensis* and *F. arundinacea*. Ten individuals were sampled from each variety.

<i>Festuca</i> species	Variety	Origin	Infection (%)	Hyphal loading (Mean±s.d.)	Seed source
<i>F. pratensis</i>	Inkeri	Finland	90	26.0±2.2	Boreal Plant Breeding Ltd, Jokioinen, Finland
<i>F. pratensis</i>	Norild	Norway	60	22.3±1.7	Graminor AS, Ilseng, Norway
<i>F. pratensis</i>	Hibinskaja	Russia	0	0	Polar Research Station, Apatity, Murmansk, Russia
<i>F. pratensis</i>	Kasper	Sweden	30	23.0±0.9	Svalöf Weibull AB, Svalöv, Sweden
<i>F. arundinacea</i>	Retu	Finland	0	0	Boreal Plant Breeding Ltd, Jokioinen, Finland

33% and 40%, respectively. The ley in Southern Sweden had a mean infection rate of 50%. In contrast, there was a large variation in infection rate in the variety trials, from 0 to 90% (Table 2). The highest infection was found in the Finnish variety (90%) while the Swedish variety Kasper had 30% infection frequency.

Hyphal loading in leys, *Festuca* varieties and grazed leys

Monthly sampling of infected *F. pratensis* individuals in the leys showed an increase in hyphal loading through the growing season (Fig. 1). The linear mixed effect model confirmed that the effect of seasonality was highly significant ($F_{1,149}=332.28$, $p<0.0001$). Cumulative degree days from the beginning of the season until sampling was 162 in June, 626 in July and 968 in August when 5°C was used as the base. For 10°C as the base the corresponding values were 25 in June, 289 in July and 454 in August. Hyphal loading (Fig. 1) was significantly correlated with cumulative degree days both with 5°C and with 10°C as the base (Spearman rank correlation coefficient, $r_s=0.95$, $p<0.01$ in both cases). For the grazing paddocks, mean hyphal loading of infected individuals was similar for individuals in paddock B (29.9±8.3, mean±s.d.) and in paddock A (28.4±5.5) (Wilcoxon rank sum test, $W=20$, $p=1$). For the *F. pratensis* varieties, hyphal loading in infected individuals was highest in the Finnish variety Inkeri (Table 2). Hyphal loading was significantly higher in the Finnish variety compared with the Norwegian variety Norild (Wilcoxon rank sum test, $W=48$, $p=0.01$), but similar to the Swedish variety Kasper.

Fertilization and ley age effects on hyphal loading

Fertilization treatment had a significant effect on hyphal loading in *F. pratensis* only in June (One-way ANOVA, $MS=41.17$, $df=2$ $F=6.076$, $p=0.036$). Pairwise comparisons indicated that only hyphal loading in fertilizer treatment 3 was significantly lower than in fertilizer treatment 1 (Tukey's HSD test, fertilizer 3 vs. fertilizer 1, $p=0.037$). However, sample sizes were low so results should be interpreted with caution. In July and August there were no significant effects of fertilization or ley age on hyphal loading.

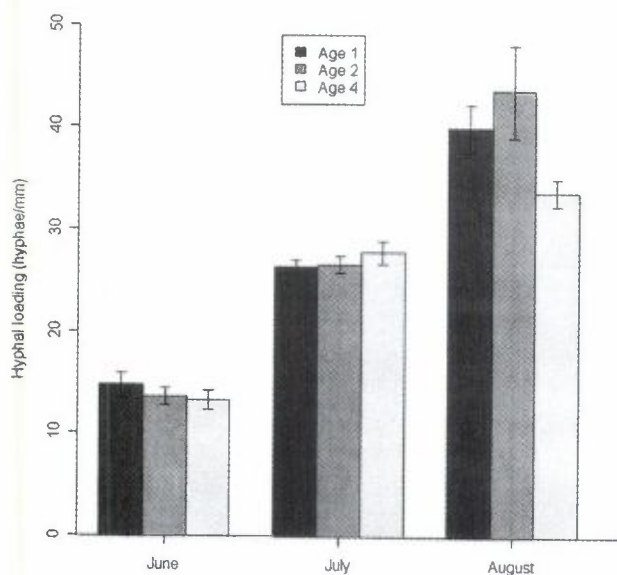


Figure 1. Mean hyphal loading (number of hyphae mm^{-1}) in infected *Festuca pratensis* individuals for each ley age category through the growing season of 2005. (Mean \pm s.e.).

4. Discussion

This study has shown that endophyte infection frequencies in *F. pratensis* sampled in ley plots from Sweden ranged 25–65%. The increase observed in hyphal loading during the growing season of the host *F. pratensis* (Fig. 1) is the first report for this grass species. A similar trend has been observed for *L. perenne* in New Zealand and Denmark (Di Menna and Waller, 1986; Jensen, 2005). In the Danish study, this correlated with an increase in lolitrem B production from spring to late summer, with levels peaking in July. However, in contrast to our study, Jensen (2005) did not follow the same infected individuals through the growing season.

Ju et al. (2006) have shown that endophyte infection frequency and endophyte concentration *in planta* are correlated with monthly mean temperature throughout the year in *F. arundinacea*, with both frequency and concentration being higher in the summer months. From growth experiments under controlled conditions they found the cardinal minimum temperature for growth of *F. arundinacea* to be 5°C while the cardinal minimum temperature for its endophyte was higher, about 10°C. Corresponding values for *F. pratensis* and its endophyte in our study are not known, but it is interesting to note that hyphal loading (Fig. 1) was significantly correlated to cumulative degree days calculated both for 5°C and 10°C as the base. At high latitudes temperature is, in general, a

crucial factor for plant growth and development. Physiological and morphological changes occurring in the plant, during the vegetative and inflorescence development stages, could affect effective transmission of the fungus and thus, the balance between the two partners in the symbiosis. Cutting the grass could also have an effect on concentrations of the fungus *in planta*. In line with the findings of Bazely et al. (1997), that grazed patches of *F. rubra* had greater loading of endophytic hyphae than ungrazed patches, we can not exclude that our results on hyphal loading could be due to cutting during the growing season. With respect to the levels of within-plant variation in hyphal loading, while few studies have examined this, Koh et al. (2006) found that among-plant variation in hyphal loading was significantly greater than within-plant variation in loading at the tiller level in *F. rubra*. This supports our findings that the within-season increase in hyphal loading is occurring. However, it is important to note that in our study, seasonal and cutting effects may have been confounded, and a separation of the two would require repeated sampling throughout the season of both controls and cut individuals of the same age and genotype and with the same fertilization. Nonetheless, our results suggest that further examination of cutting effects on hyphal loading is worthwhile.

Overall, the different fertilization treatments and ley age categories had no clear effect on hyphal loading in *F. pratensis*. Nevertheless, in June, the lower fertilizer regime (treatment 3 compared with treatment 1) produced less hyphal loading. It has been suggested that the presence of the endophyte could cause competition with the host for nutrients when these are in short supply (Cheplick et al., 1989). Saikkonen et al. (1998; 2006) have proposed that only at high nutrient conditions can the endophyte be considered a mutualist and that under lower nutrient conditions, it could be a parasite. However, it has been shown that the endophyte can enhance nutrient acquisition as well (Malinowski and Belesky, 1999). In our study, it seems that the difference in amounts of fertilizer among treatments were not large enough to result in any sustained difference in hyphal loading.

We know of no previous studies that have examined hyphal loading in grass leys of different ages as we did. On the other hand, age effects have been observed on endophyte infection frequencies over longer periods of time. An initial infection frequency of 79% in Australian *L. perenne* pastures increased to 100% infection after only 4 years (Cunningham et al., 1993), and in the USA, an average increase of 4% per year in infection was calculated for *F. arundinacea* in a 12-yr study (Shelby and Dalrymple, 1993). It is important to remember that due to the vertical spreading of the fungus an increase in infection frequency would depend on faster elimination of uninfected than infected individuals in a population. This could occur by means of increased competitive abilities of infected plants

in the grass population. Hyphal loading, on the other hand, is a matter of the interactions between host and endophyte within a grass individual.

The Swedish variety of *F. pratensis*, Kasper, had a mean infection frequency of 30% in the variety trials, which was in the range found in experimental ley plots (25–65%). Koh et al. (2006) sampled *F. pratensis* in other leys planted at the field site in Umeå and found infection frequency to be 50%. The variety Kasper has also been examined by Saikkonen et al. (2000) in Finland, where they found an average of 41% infection frequency. In natural populations of *F. pratensis* Saikkonen et al. (2000) found a very broad range of infection frequency, 10–100%; this was similar in the trial of different grass varieties in our study (0–90%). While *F. pratensis* is a common forage grass in Sweden, alkaloid-induced toxicosis in cattle has not been specifically documented. However, this does not preclude the possibility that other sub-lethal effects may be occurring given the fairly high infection frequencies in our study.

Concluding remarks

Through our approach of studying hyphal loading repeatedly in the same individuals, we were able to examine *in planta* changes in the concentration of the fungus through most of the growing season of the host grass *F. pratensis*. It is clear that sampling time in the field is crucial for conclusions to be made about the balance between plant and fungus in this symbiosis. It is proposed that several external factors such as temperature and management of grasslands have important effects on the endophyte-*F. pratensis* symbiotic relationship.

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