Short running title: Influence of endophyte on meadow fescue

Influence of the fungal endophyte *Neotyphodium uncinatum* on the persistency and competitive ability of meadow fescue (*Festuca pratensis* Huds.)

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Abstract

Influence of fungal endophyte (*Neotyphodium uncinatum*) on persistency and competitive ability of two cultivars of meadow fescue (MF; *Festuca pratensis* Huds.) was investigated in a 3-year field trial under simulated intensive grazing. The two MF cultivars were 'Harusakae' and 'Tomosakae'. Each MF cultivar was grown as a monoculture or a mixture with white clover (two cultivars), orchardgrass or perennial ryegrass, with (E+) or without (E–) infection by the endophyte. In the monocultures and the mixtures with white clover or orchardgrass, endophyte infection (E+) resulted in higher proportions of MF than non-infection (E–) mainly in the second and third years of the experiment, though the effect was not always significant. On the other hand, endophyte infection always had no effect on the proportion of MF mixed with perennial ryegrass. Annual herbage yield of the 'Harusakae' monoculture was significantly higher in E+ than in E– in one year out of two, whereas that of the 'Tomosakae' monoculture did not vary with endophyte infection for two years. The results show that endophyte infection can increase persistency and competitive ability of MF plants in both monocultures and mixtures, though the effect in mixtures depends on the companion species.

Keywords

Competitive ability; endophyte; *Festuca pratensis*; *Neotyphodium uncinatum*; persistency.

Introduction

Perennial ryegrass (*Lolium perenne* L.) is the most widely used cool-season forage grass for intensive grazing in Japan because of its high suitability to grazing (e.g. productivity, palatability and persistency). However, this grass cannot be cultivated in eastern Hokkaido due to severe cold winters. Although timothy (*Phleum pretense* L.) is grown as a major forage grass in the region, this grass is not suited for intensive grazing, showing low regrowth after frequent defoliation. Meadow fescue (MF; *Festuca pratensis* Huds.) is a cool-season forage grass widely adapted to the lowlands of central and northern Europe. This grass is highly palatable and cold resistant, and thus has a potential as a leading grass for intensive grazing in eastern Hokkaido. At the same time, MF has a problem in that it shows lower competitive ability and herbage yield than perennial ryegrass under intensive grazing management (Crocker and Martin 1964).

MF plants are often infected with the fungal endophyte *Neotyphodium uncinatum* (Gams, Petrini & Schmidt) Glenn, Bacon, Price & Hanlin (Gams *et al.* 1990; Glenn *et al.* 1996). It has been reported that endophyte influences host grasses in such aspects as herbage yield, plant survival, aphid deterrence (Schmidt 1993), competitive ability (Malinowski *et al.* 1997b) and adaptation to drought stress (Malinowski *et al.* 1997a). Therefore, there is a possibility that the problem with MF under intensive grazing can be overcome by endophyte infection. However, relatively little is known about the endophyte effects on the performance of MF grown in the field.

In the study reported here, we investigated persistency and competitive ability of two cultivars of MF grown as monocultures or mixtures under simulated grazing management, using endophyte-infected and non-infected plants. The objectives of the study were to evaluate the effects of endophyte on the performance of the grass and to obtain some information on the possibility of using endophyte for intensive grazing of MF in the cold regions like eastern Hokkaido.

Materials and methods

Two cultivars of MF, 'Harusakae' and 'Tomosakae' (Takai *et al.* 2001), infected with their naturally occurring fungal endophyte, *Neotyphodium uncinatum* (E+) and corresponding non-infected seeds (E–) isolines, were used. Seeds of the E– lines were produced from cross pollination of endophyte free plants derived from the first synthetic generation seeds treated with dry heating at 70°C for 5 days using a modified procedure from Sato *et al.* (1995). Seeds of the E+ lines were the cross progeny of non-treated seeds with the same lot (first synthetic generation). To confirm the endophyte infection status, eighty seeds from the second synthetic generation of each line were examined for the presence or absence of mycelium of the endophytic fungus by light microscopy using the method of Clark *et al.* (1983).

The field experiment was carried out on a volcanic ash soil at the National Agricultural Research Center for the Hokkaido Region, Sapporo, Japan (43°00'N, 141°24'E). The experimental design was a randomized complete block with a factorial arrangement of 2 levels of endophyte-infection states (E+ and E–) × 2 MF cultivars ('Harusakae' and 'Tomosakae') with two replications. The effects of the treatments on the performance of MF were evaluated for five sward types, a monoculture and four mixtures. The mixtures were MF + middle-leaved white clover (cv. 'Sonja'), MF + small-leaved white clover (cv. 'Grasslands Tahora'), MF + orchardgrass (cv. 'Okamidori') and MF + perennial ryegrass (cv. 'Frend', forage type, endophyte free). The plots (10 × 1.2 m each) received basal dressing of 40–55–40 kg $N–P_2O_5–K_2O$, 1,000 kg magnesium-calcium and 1,000 kg fused magnesium phosphate per ha before sowing. Seeds of the grasses and white clovers were hand-broadcast on to the plots on 9 August 2000 at rates of 40 and 5 kg ha^{-1}, respectively, and roller-packed after surface harrowing.

After sowing, the swards were grown and maintained by cutting and fertilizer application until 2002. To simulate intensive grazing, the plots were harvested to a 5 cm height above the ground level when the plant height reached about 30 cm, using a forage harvester (HEGE Co., HEGE212). In total, the plots were cut twice, six and five times in 2000, 2001 and 2002, respectively. As topdressing, the plots were fertilized with compound fertilizer on 12 September 2000, 15 April 2001, 2 July 2001 and 15 April 2002. The fertilizer rates as kg N–P_2O_5–K_2O per ha were 30–23–30, 40–55–40, 20–28–20 and 40–55–40, respectively.

Sward composition was measured for all plots on 17 October 2000, 2 May and 25 October in 2001, 15 October in 2002 and 1 May in 2003, by recording vegetation status (sown species, weeds or bare ground) in a 1 cm^2 area at 10 cm intervals on a 10-m line transect positioned to cross the center of each plot. Herbage yield (for all species including weeds) was measured only for the MF monoculture plots, by sampling herbage from two 0.5 × 0.5 m quadrats randomly placed in each plot prior to each harvest in 2001 and 2002.

Herbage samples from the monocultures were dried at 70°C for 48 h for dry weight determination. Ground samples were measured for loline alkaloids by capillary gas chromatography analysis (GC) using a modified procedure from Yates *et al.* (1990). The ground material (0.2 g dry wt) was extracted with 3 ml of CH_2Cl_2/MeOH (75:25) for 1 h. After centrifugation the supernatants were combined, then an eluant of 0.5 ml was evaporated using N_2 gas, and the residue was redissolved in 1 ml MeOH. GC was performed with a Shimadzu 14A (Shimazu Co., Kyoto, Japan) with a flame thermionic detector (FTD). The oven temperature was maintained at 50°C for 2 min and then programmed to 230°C at 10°C min^{-1}; the injector temperature was 230°C, and the detector temperature was 250°C. Wide-bore (0.53

mm) DB-1 columns (15 m) with a film thickness of 1.5 μm were used. A N-formylloline reference sample from the Forage Crop Research Institute of Japan Grassland Farming and Forage Seed Association (Nasushiobara, Tochigi, Japan) was used to obtain a calibration curve, and its retention time was measured for standardizing the analytical procedure.

The effects of endophyte infection, MF cultivar and their interaction on the proportion of MF in each sward type on each measurement date were evaluated by two-way ANOVA, and the means were separated by Student's t-test. The effect of endophyte infection on the annual herbage yield was evaluated by Student's t-test within each MF monoculture.

Results

The initial infection frequency of the E+ and E– isolines observed in seeds was respectively 74 and 0% in 'Harusakae' and 70 and 0% in 'Tomosakae'.

The effects of endophyte on the proportion of MF in the swards were not significant until the spring of 2001, but became significant thereafter (Table 1). After the fall of 2002 onward, endophyte had significant effects on the MF proportion in all swards except for the MF + perennial ryegrass. By contrast, the effects of cultivar were always nonsignificant except the two occasions (fall of 2000 and 2001) in the MF monoculture. The endophyte × cultivar interaction was always nonsignificant throughout the study.

The MF monocultures and mixtures with white clover received considerable invasion by weeds in 2000, but included almost no weeds after the fall of 2001 onward due to the increased proportions of MF and white clover (Figure 1). The major weed species were annual bluegrass (*Poa annual* L.), shepherds purse (*Capsella bursa-pastoris* (L.) Medic) and chickweed (*Stellaria media* (L.) Villars). In the monoculture of 'Harusakae', the E+ plot showed higher proportions of MF than the E– throughout the experiment, although the difference was significant only in the fall of 2002. In the monoculture of 'Tomosakae', significant differences between the E+ and E– plots were detected in 2002 and 2003. In the mixtures with white clover 'Sonja', the E+ plots of both MF cultivars showed significantly higher proportions of MF than the E– plots in 2002. In the mixed swards with white clover 'Tahora', the E+ plots showed significantly higher proportions of MF than the E– plots from the fall of 2001 to 2003 for 'Harusakae' and from 2002 to 2003 for 'Tomosakae'. In the mixtures with orchardgrass, the E+ plots showed significantly higher proportions of MF than the E– plots in the fall of 2001 and 2003 for 'Harusakae' and from 2002 to 2003 for 'Tomosakae'. In the mixed swards with perennial ryegrass, the proportion of MF did not differ between the E+ and E– plots throughout the study in any of the MF cultivars. The mixtures with white clovers showed highest proportions of MF, followed by those with orchardgrass and with perennial ryegrass.

Annual herbage yield of the 'Harusakae' monoculture was significantly higher in the E+ plot than in the E– in 2001 and for the average over 2001 and 2002 (Figure 2). On the other hand, annual herbage yield of the 'Tomosakae' monoculture did not differ between the E+ and E– plots in 2001 or 2002, or for their average.

Plants grown in the E+ plots always contained N-formylloline, showing average contents of 61 and 62 ppm in the 'Harusakae' and 'Tomosakae' monocultures, respectively (Table 2). The N-formylloline content remained high even in 2002 (up to 83 ppm), indicating stable association of the endophyte with the MF plants throughout the study. By contrast, N-formylloline was rarely detected in plants grown in the E– plots.

Discussion

The results from the present study show that infection with the *Neotyphodium* endophyte could affect the persistency, competitive ability and herbage yield of MF. In the monocultures and the mixtures with white clover or orchardgrass, endophyte infection resulted in higher proportions of MF than non-infection mainly in the second and third years of the experiment, though the effect was not always significant (Figure 1). Annual herbage yield of the 'Harusakae' monoculture was significantly higher in E+ than in E– in one year out of two, whereas that of the 'Tomosakae' monoculture did not vary with endophyte infection for two years (Figure 2). Although our study did not use animals, the results agree with those by Fletcher *et al.* (2000) who found beneficial effects of endophyte infection on herbage production and competitiveness in MF grazed by lambs.

The endophyte infection enhanced the competitive ability of MF with the white clover cultivars 'Sonja' and 'Tahora' (Figure 1). Clay and Holah (1999) and Fletcher *et al.* (2000) showed a lower proportion of unsown clover in endophyte-infected plots. The number of white clover plants declined in pastures dominated by endophyte-infected perennial ryegrass (Percival and Duder 1983; Sutherland and Hoglund 1989). The enhanced competitive ability of the host plants over clover can be attributed to the following two mechanisms. First, endophyte infection is known to produce allelopathic properties in host plants. Seed extracts from endophyte-infected tall fescue inhibited the germination of *Trifolium* spp. (Springer 1996). Loline alkaloids have been listed as an endophyte-related secondary metabolite with allelopathic properties (Bush *et al*. 1997; Malinowski *et al*. 1999). The detection of N-formylloline in the endophyte infected plants (Table 2) suggests that the allelopathic properties of the endophyte could be the cause of the higher competitive ability of the infected MF. The N-formylloline concentrations in our plants, 14–121 ppm, were equal to those reported for leaf blades and leaf sheathes in MF (Justus *et al.* 1997; Kasai *et al.* 2006), though they were lower than those reported for seeds or seed heads. We harvested the plots when the plant height was about 30 cm and the herbage was composed mainly of leaf blades and leaf sheathes. Second, loline alkaloids are known to enhance resistance of host plants to insects, including bird-cherry-oat aphid, *Rhopalosiphum padi* (L.) (Siegel *et al*. 1990), greenbug aphid, *Schizaphis graminum* (Rondani) (Siegel *et al*. 1990), larvae of the horn fly, *Haematobia irritans* (L.) (Dougherty *et al*. 1998), larvae of the Japanese beetle, *Popillia japonica* Newman (Patterson *et al*. 1991) and the rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae) (Shiba and Sugawara 2009). In a membrane-feeding test using *T. caelestialium*, purified N-formylloline significantly reduced the survival of the larvae of the insect at a concentration of as low as 50 ppm (Shiba and Sugawara 2009). Further studies are needed to investigate the allelopathic effects of loline alkaloids on herbivores and clarify the mechanisms behind the enhanced competitive ability caused by the endophyte.

Malinowski *et al.* (1997b) evaluated the influence of *N. uncinatum* on the competitive ability of MF in a growth chamber using one set of infected and uninfected clones. Infected plants had greater root and shoot dry weight than non-infected plants when grown in an E–/E+ binary mixture and in mixtures with orchardgrass under optimal water supply and also under water stress. This indicated that the greater competitive ability of the E+ plants was related to improved shoot and root growth and thus enhanced drought resistance. The higher proportion of MF on E+ than on E– in mixed swards with orchardgrass (Figure 1) is in agreement with the results in Malinowski *et al.* (1997b).

The absence of the endophyte-mediated enhancement in the competitive ability of MF in the mixtures with perennial ryegrass (Figure 1) may reflect the lower proportion of MF in the swards. Crocker and Martin (1964) found that the size of MF plants grown with perennial ryegrass was half that of MF plants in a pure stand 9 months growth after sowing. Under the severe pressure from the ryegrass, the effect from the endophyte in MF may be masked.

The endophyte effect on the annual herbage yield of the MF monocultures varied with the MF cultivars (positive and nil effect in 'Harusakae' and 'Tomosakae', respectively; Figure 2) despite the similar infection rates in the E+ seeds (74 and 70%, respectively) and similar N-formylloline contents in the herbage (61 and 62 ppm on average, respectively; Table 2). Easton (2007) has also reported different responses of herbage production among two cultivars of perennial ryegrass infected with two endophyte strains, i.e. a significant effect of plant genotype × endophyte strain interaction on herbage yield. On the other hand, the endophyte had a positive effect on the proportion of both MF cultivars in the monocultures (Figure 1). This coincides with the finding by Clay and Holah (1999) that endophyte infection increased dominance of tall fescue in a monoculture despite no effect on the overall productivity (aboveground biomass).

In conclusion, the present study shows that infection of fungal endophyte, *N. uncinatum* can increase persistency and competitive ability of MF, even in multispecies grasslands. Breeding and introducing E+ MF into grasslands may therefore increase the persistency and productivity of MF in Hokkaido, Japan.

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Table 1 Summary of ANOVA (*F*-values) for meadow fescue proportion in sward

Sward type	Source of variation	Oct 2000	May 2001	Oct 2001	Oct 2002	May 2003
Meadow fescue (MF)	Endophyte (A)	0.81	7.98	22.20**	21.66**	11.99*
	Cultivar of MF (B)	11.21*	5.13	15.03*	0.01	2.37
	A × B	2.14	0.17	0.18	1.06	1.09
MF + white clover cv. 'Sonja'	Endophyte (A)	6.11	0.77	4.95	22.57**	10.63*
	Cultivar of MF (B)	6.62	2.20	0.96	0.49	0.01
	A × B	0.04	0.57	0.01	0.30	0.01
MF + white clover cv. 'Grasslands Tahora'	Endophyte (A)	6.14	4.31	12.85*	72.54**	44.10**
	Cultivar of MF (B)	5.14	0.60	0.09	0.90	0.50
	A × B	3.57	3.82	7.38	3.09	0.00
MF + orchardgrass	Endophyte (A)	5.97	0.13	6.13	17.21*	53.57**
	Cultivar of MF (B)	5.20	1.07	1.02	0.03	3.26
	A × B	0.64	0.03	0.00	0.08	0.01
MF + perennial ryegrass	Endophyte (A)	0.07	1.73	0.56	0.26	0.27
	Cultivar of MF (B)	0.73	2.28	0.06	2.24	4.73
	A × B	0.11	0.33	0.01	1.13	0.16

*$P < 0.05$, **$P < 0.01$.

Table 2 Influence of endophyte on N-formylloine content (ppm, mean ± SD, n = 2) in herbage from monocultures of two meadow fescue cultivars

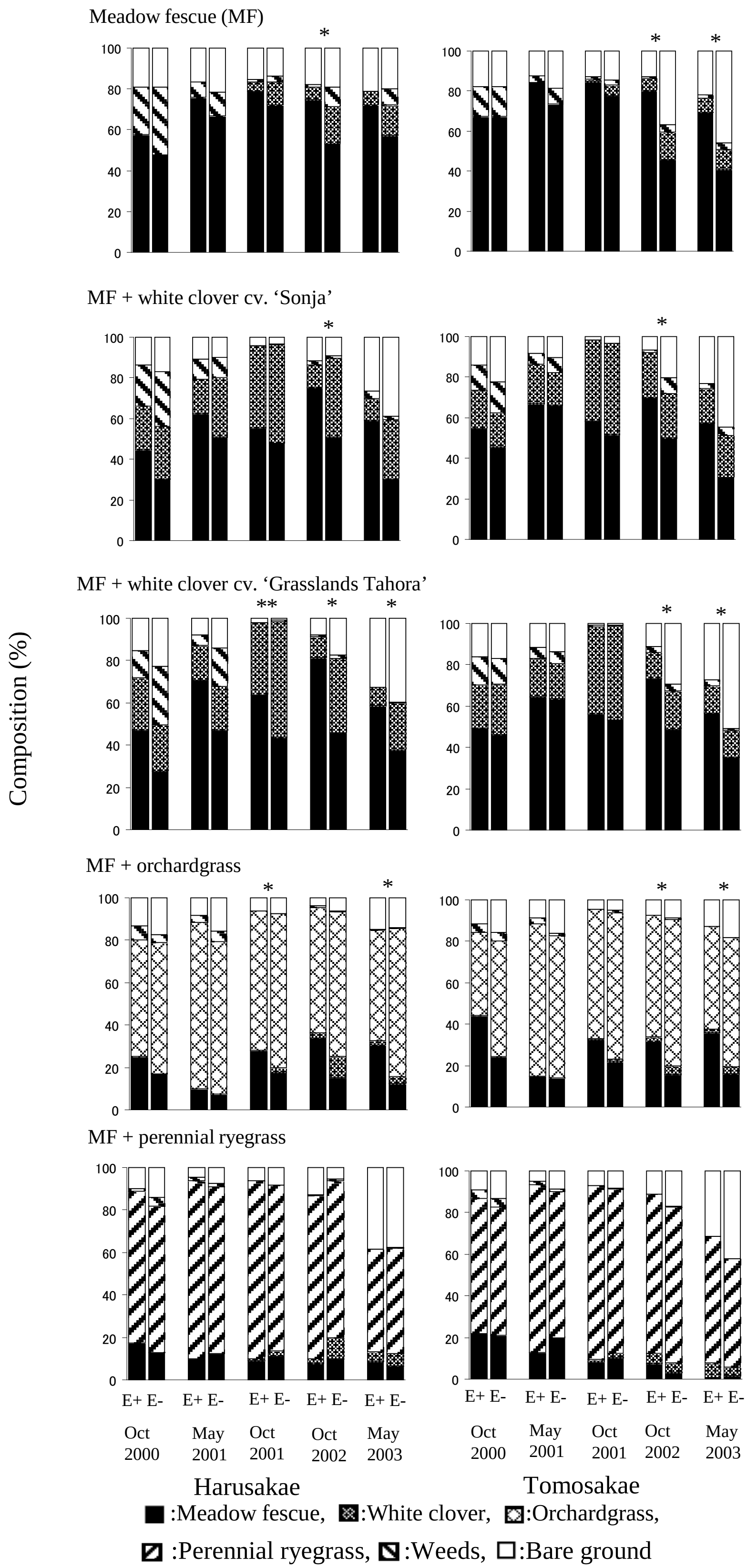
Cultivar	Endophyte-infection status†	17 May 2001	4 Jun 2001	16 May 2002	4 Oct 2002
Harusakae	E+	14 ± 2.5	121 ± 13.7	66 ± 11.7	44 ± 8.0
	E–	0	0	0	3 ± 1.3
Tomosakae	E+	16 ± 1.5	80 ± 5.3	68 ± 15.7	83±19.1
	E–	0	0	0	0

†E+, endophyte-infected; E–, endophyte not infected.

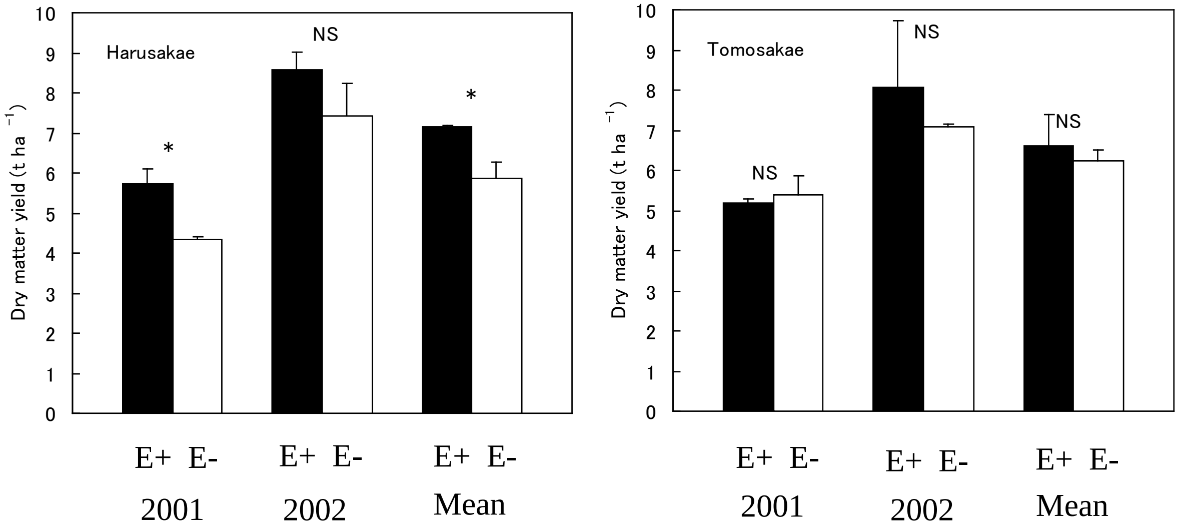
Figure captions

Figure 1 Influence of endophyte (E+, infected; E–, non-infected) on composition of swards with two meadow fescue cultivars as a monoculture or a mixture component. *, $P < 0.05$; **, $P < 0.01$ for meadow fescue proportion.

Figure 2 Influence of endophyte (E+, infected; E–, non-infected) on annual herbage yield of monocultures of two meadow fescue cultivars. Vertical bars indicate standard error ($n = 2$). *, $P < 0.05$.



Takai et al. Figure 1



Takai et al. Figure 2