

Effects of systemic fungal endophytes on the performance of meadow fescue and tall fescue in mixtures with red clover

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Abstract

A symbiosis between grasses and systemic fungal endophytes exists in both natural and agricultural grassland communities. Our objective was to examine the effects of systemic endophytes on the competitive ability of two agronomically important grass species: meadow fescue [*Festuca pratensis* (Huds.) syn. *Schedonorus pratensis* (Huds.) P. Beauv] and tall fescue [*Festuca arundinacea* (Schreb.) syn. *Schedonorus phoenix* (Scop.)]. Plants of meadow and tall fescue were grown for 48 days in replacement series of interspecific mixture with a legume (red clover, *Trifolium pratense* L.) in different nutrient environments in a greenhouse. Neither of the grass species gained endophyte-promoted competitive advantage over red clover in grass–clover mixtures. Endophyte infection increased the growth of meadow fescue monocultures by 89% compared to endophyte-free monocultures in high-nutrient soils, but plant competition or the cost of endophyte infection to the meadow fescue decreased the yield in resource-limited conditions. On average, endophyte-infected and endophyte-free meadow fescues produced 0.15 and 0.17 g, and 0.14 and 0.14 g dry biomass per plant in mixtures with red clover in high- and low-nutrient soils respectively. In contrast to meadow fescue, endophyte-promoted growth of tall fescue monocultures was not detected. Endophyte-infected and endophyte-free tall fescue monocultures produced 0.76 and 0.95 g biomass per pot, respectively, in the high-nutrient environment. Endophyte infection can increase the

performance of the host grass, but the positive effects depend on the host species, the species composition and soil nutrient availability.

Keywords: fungal endophytes, grass–legume mixtures, plant competition, meadow fescue, tall fescue, red clover

Introduction

Systemic fungal endophytes are prevalent symbionts in 20–30% of Pooidae grass species, including several important agricultural forage species (Leuchtman, 1992). In the symbiosis, the fungus grows intercellularly and asymptotically into green tissues of the host plant (Wilson, 1995). While growing into the developing inflorescences, these endophytes are vertically transmitted via the seeds from a plant to its offspring. Systemic grass endophytes are generally considered to be plant mutualists because of benefits to both partners whose fitness is tightly linked (Law, 1985; Ewald, 1994; Frank, 1994; Wilkinson and Schardl, 1997); the fungus subsists entirely on the host plant, particularly in the case of strictly asexual *Neotyphodium* endophytes. The fungus can increase host growth and reproduction, as well as resistance and tolerance to abiotic and biotic stressors such as drought, flooding, herbivores and pathogens (Clay, 1990). Because of endophyte-enhanced plant fitness, endophyte-infected grasses are suggested to be competitively superior to uninfected conspecifics and other interacting species in grassland communities (Marks *et al.*, 1991; Clay and Holah, 1999; Saikkonen, 2000; Rudgers *et al.*, 2005; Vázquez de Aldana *et al.*, 2012; Saikkonen *et al.*, 2013). Evidence has, however, revealed that grass–endophyte interactions are variable and range from parasitic to mutualistic (Saikkonen

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et al., 1998, 2004, 2006; Müller and Krauss, 2005; Cheplick and Faeth, 2009).

Plant competition is one of the major driving forces of plant communities (Grace and Tilman, 1990). The outcome of competition is, however, conditional and depends on both biotic and abiotic factors. For example, systemic grass endophytes increase the competitive ability of the host grasses either by increasing the host plant growth and reproduction rates (Hill *et al.*, 1991; Marks *et al.*, 1991; Malinowski *et al.*, 1997; Clay and Holah, 1999; Brem and Leuchtman, 2002; Rudgers *et al.*, 2005) or through negative allelopathic effects on competitors (Hoveland *et al.*, 1999; Malinowski *et al.*, 1999; Vázquez de Aldana *et al.*, 2012). On the other hand, several studies have demonstrated conflicting results that show neutral to negative effects of endophytes on the competitive ability of the host grass (Hoveland *et al.*, 1997; Brem and Leuchtman, 2002; Faeth *et al.*, 2004; Saari *et al.*, 2010). Endophyte-promoted competitive superiority of host plants may be most pronounced in bred grass cultivars and in nutrient-rich agro-ecosystems (Clay and Holah, 1999; Saikkonen, 2000; Saikkonen *et al.*, 2006, 2013; Gundel *et al.*, 2013a,b).

Endophyte-mediated grass–legume competition in different nutrient environments and its consequences on biomass production remain unsettled. In most agronomic situations, grass–legume mixtures are suggested to have advantages over monocultures; for example, in terms of higher forage yield due to nutritional complementation, forage quality and economic profitability (Ledgard, 1991; Bulson *et al.*, 1997; Gooding *et al.*, 2007; Malézieux *et al.*, 2009). In conjunction with optimized nitrogen fertilization, mixing plant species with a legume may reduce the adverse environmental impacts of accumulated nutrients, and the leaching of residual soil nutrients (Malézieux *et al.*, 2009). Similarly, systemic grass endophytes can be incorporated into breeding programmes and sustainable grass-cropping systems because, by producing mycotoxins, they can adversely affect the quality of forage supplied to animals (Gundel *et al.*, 2013c) and also improve both grass yield and resistance to pests, pathogens and weeds (Gundel *et al.*, 2013c; Saikkonen *et al.*, 2013). There is an additional value associated with using grass–legume mixtures as forage because the presence of the legume reduces the proportion of grass–endophyte-produced alkaloids in the animal diet (Tekeli and Ateş, 2005; Gundel *et al.*, 2013c).

Here, we examined the effects of systemic endophytes on the competitive ability of two agronomically important grass species with a legume in different nutrient environments. The objective of this study was to compare the yield of grass monocultures with grass–legume mixtures in the presence or absence of

endophyte infection in high- and low-nutrient environments. As model species, we selected two important forage grass species of temperate grassland: meadow fescue [*Festuca pratensis* (Huds.) syn. *Schedonorus pratensis* (Huds.) P. Beauv. syn. *Lolium pratense* (Huds.) Darbysh], and tall fescue [*Festuca arundinacea* (Schreb.) syn. *Schedonorus phoenix* (Scop.)], naturally colonized (E+) or uncolonized (E–) by fungal endophytes [*Epichloë coenophiala* (Morgan-Jones & W. Gams) C. W. Bacon & Schardl, comb. nov. and *Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtman & Schardl, comb. nov. respectively] (Leuchtman *et al.*, 2014). Both grass species were grown in replacement series of interspecific mixtures (Radosevich *et al.*, 2007) with red clover (*Trifolium pratense* L.) in different nutrient environments. Although the different vertical profile of grass and red clover leaves minimize their aboveground competition for light (Mooso and Wedin, 1990; Lantinga *et al.*, 1999), the proportion of red clover in the mixture naturally declines in the field over time (Mela, 2003). We predict that an optimized proportion of endophyte-infected and/or endophyte-free grass seeds with red clover can increase the productivity of the mixture compared to grass monocultures.

Materials and methods

Plant materials

Seeds of meadow fescue cv. Kasper, tall fescue cv. Kentucky-31 and red clover cv. Global were obtained from the Finnish Food Safety Authority (EVIRA), the University of Kentucky, Lexington, USA, and Riista Siemen Company (Sweden) respectively. Because successful growth of red clover requires microbial symbiont, we inoculated its seeds with the recommended dose of *Rhizobium leguminosorum* biovar *trifolii* (Elomestari Typpi Ympäri; 1/10 peat of bacterium per seeds) before sowing.

Experimental design and growth conditions

Competition experiments of meadow and tall fescues with red clover followed identical experimental designs. Both endophyte-infected (E+) and endophyte-free (E–) grasses were grown in a separate replacement series of interspecific mixture (Radosevich *et al.*, 2007) with the legume in pots [four plants (4 grasses, 1 legume + 3 grasses, 2 legumes + 2 grasses, 3 legumes + 1 grass, 4 legumes) per pot (8 × 8 × 8.5 cm in size)] assigned either to low- or high-nutrient soils [low: 500 mg N kg⁻¹, 90 mg P kg⁻¹ and 800 mg K kg⁻¹ (Kekkilä-Viljely Seos, light sphagnum turf, sand and sandy clay, dolo-

mite: 5.2 kg m⁻³, pH 6; <http://www.kekkila.com/>); high: 750 mg N kg⁻¹, 550 mg P kg⁻¹ and 3700 mg K kg⁻¹ (Biolan-Puutarhan Mustamulta, composted soil mixture, light plant peat, chicken manure, wood fibre, pH 6.5; <http://www.biolan.fi/english/>) with 12 replications. The total number of pots was 216, and they were organized in the greenhouse according to a randomized block design.

The experiments were conducted in a greenhouse at Ruissalo Botanical Garden (60°26 N, 22°10 E), Finland, in the summer of 2011. The plants were exposed to natural long-day light cycle, but the daytime air temperature in the greenhouse was adjusted to 25°C and the night-time temperature to 20°C. The plants were watered when needed. The pots assigned to high-nutrient soil were fertilized [N:P:K 11:5:18, 250 mg N kg⁻¹ soil (YaraMila Puutarhan Y3)] at 20 days after sowing. After 48 days, the number of fescue tillers and plant height were recorded, and the aboveground biomass of single plants of fescue and red clover were harvested separately, oven-dried (60°C, 48 h) and weighed. For the meadow fescue experiment, there was no germination in seven pots, so 249 were taken into account, while for the tall fescue experiment, all 216 pots were analysed.

Statistical analysis

First, we presented the total dry biomass of fescues and clover separately for endophyte infection, nutrition status and interspecific mixture of the pot (0, 25, 50, 75 and 100% of clover) using only pots with four surviving plants. In a more detailed analysis, the proportion was calculated by including all the pots with surviving plants (varying from one to four) in the analyses as a continuous variable. Besides dry biomass and plant height of both fescue species and red clover, we also analysed factors associated with tillering (presence/absence of extra tillers) of fescues. We used a SAS mixed procedure except in the case of the tillering, where SAS genmod procedure with binomial distribution and logit link was applied. Model assumptions, normality and homogeneity were checked graphically, and when necessary (dry matter of meadow and tall fescue, dry matter of red clover when it was grown with meadow fescue), response variables were transformed to meet assumptions of statistical tests.

While nutrient level of the soil (low and high) and endophyte infection status of the fescue (E+ and E-) were considered to be categorical fixed factors, the proportion of red clover per pot was considered as a continuous fixed factor, and the block was included as a random factor in the models. Interactions between factors were estimated, but three-level interactions

were reported only when they were statistically significant.

Models were illustrated by plotting the estimated model-based response variables (biomass or tillering) on the proportion of red clover in the pot. This generated four lines per graph, as responses were presented separately as endophyte (+/-) and nutrient (high, low) status lines. Note that 100% red clover means that no fescues were present in the pot (i.e. extrapolation of fescues was not fully extended).

Results

Total yield

Fertilization positively affected the total yield of plants in pots (Table 1; Figure 1). Red clover comprised, on average, 0.76 and 0.79 of the total yield from the pots for meadow fescue and tall fescue mixtures respectively (Figure 1). Thus, the total yield increased from fescue monoculture to red clover monoculture primarily with the number of red clover in the replacement series of mixture for both soil nutrient levels (Figure 1). On average, compared to meadow and tall fescue monocultures, red clover monocultures produced more than fourfold and nearly threefold higher biomass respectively (Figure 1).

Endophyte infection and the performance of meadow fescue

Endophyte infection affected meadow fescue biomass interactively with nutrient availability in soils and interspecific competition with red clover. On average, the total biomass of E+ monocultures was 89% higher than E- monocultures in high-nutrient conditions, but the difference levelled in the presence of red clover (Figure 1). The benefit from endophyte infection was detected only in high-nutrient conditions (Figure 1), suggesting that endophytes do not benefit the host plant in resource-limited environments. In the two lower levels of mixtures with red clover, interspecific competition with red clover appears not to constrain the growth of meadow fescue; i.e., the biomass production of meadow fescue remained the same compared to monocultures in both low- and high-nutrient soils (Figure 1). In contrast, biomass production of meadow fescue was decreased markedly in an interspecific mixture with 75% of red clover in both low- and high-nutrient soils (Figure 1).

To gain a better understanding on the effect of endophyte and nutrient status on individual plants of meadow fescue, we applied statistical models (Table 1). Increased supply of nutrients made meadow fescue taller [$F = 26.6$, $df = (1,359)$, $P < 0.001$], with

Table 1 Mixed ANOVA results for dry matter (DM) of meadow and tall fescues and red clover, and GENMOD procedure results of tillering for meadow and tall fescues.

| Sources | DM meadow fescue | | DM tall fescue | | Tillering meadow fescue | | Tillering tall fescue | |
|---|------------------|--------|----------------|------------|-------------------------|-----------|-----------------------|------------|
| | F (df) | P | F (df) | P | X ² (df,N) | P | X ² (df,N) | P |
| Endophyte | 3.15 (1,352) | 0.077† | 0.36 (1,414) | 0.5480 | 1.43 (1,369) | 0.2319 | 1.33 (1,431) | 0.2486 |
| Proportion of mixture | 0.00 (1,354) | 0.986 | 6.16 (1,414) | 0.0134* | 1.94 (1,369) | 0.1636 | 3.77 (1,431) | 0.0523† |
| Nutrient level of soil | 4.13 (1,360) | 0.043* | 30.59 (1,415) | <0.0001*** | 11.31 (1,369) | 0.0008*** | 28.79 (1,431) | <0.0001*** |
| Endophyte × Proportion | 3.55 (1,352) | 0.061† | 2.19 (1,414) | 0.1399 | 0.34 (1,369) | 0.5594 | 0.91 (1,431) | 0.3401 |
| Endophyte × Nutrient level of soil | 8.46 (1,353) | 0.004* | 1.05 (1,414) | 0.3057 | 0.81 (1,369) | 0.3694 | 5.26 (1,431) | 0.0218* |
| Proportion × Nutrient level of soil | 1.04 (1,357) | 0.309 | 2.81 (1,414) | 0.0947† | 0.47 (1,369) | 0.4937 | 2.82 (1,431) | 0.0930† |
| Endophyte × Proportion × Nutrient level of soil | 4.45 (1,353) | 0.036* | NS | NS | NS | NS | NS | NS |

| Sources | DM of red clover with meadow fescue | | DM of red clover with tall fescue | |
|---|-------------------------------------|-----------|-----------------------------------|-----------|
| | F (df) | P | F (df) | P |
| Endophyte | 3.91 (1,362) | 0.0486* | 0.18 (1,365) | 0.6693 |
| Proportion of mixture | 1.30 (1,363) | 0.2555 | 17.24 (1,365) | <.0001*** |
| Nutrient level of soil | 17.42 (1,363) | <.0001*** | 36.05 (1,365) | <.0001*** |
| Endophyte × Proportion | 2.00 (1,363) | 0.1584 | 0.11 (1,365) | 0.7419 |
| Endophyte × Nutrient level of soil | 4.89 (1,363) | 0.0277* | 0.00 (1,364) | 0.9641 |
| Proportion × Nutrient level of soil | 0.00 (1,363) | 0.9882 | 3.8 (1,365) | 0.0521† |
| Endophyte × Proportion × Nutrient level of soil | 5.22 (1,363) | 0.0229* | NS | NS |

NS, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, $0.05 < P < 0.1$.

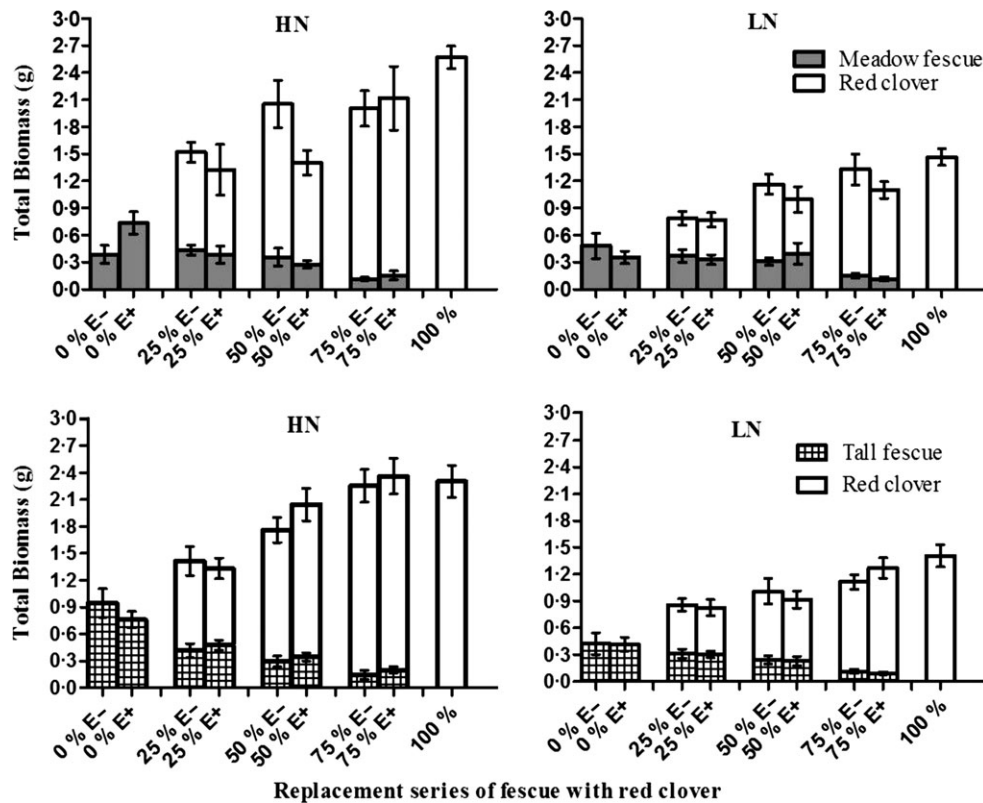


Figure 1 Total biomass of endophyte-infected (E+) and endophyte-free (E-) tall fescue-red clover and meadow fescue-red clover mixtures grown in replacement series and in high and low soil nutrient conditions. The percentage values on the X axis show the proportion of red clover in mixture. HN: High nutrient level of soil and LN: Low nutrient level of soil. Bars and error bars show means and standard errors respectively.

more tillers ($X^2 = 11.3$, $df = 1$, $P < 0.001$, Table 1), but the effect on height was also dependent on the endophyte status [$F = 8.12$, $df = (1,352)$, $P = 0.005$]. The probability of tiller production of meadow fescue was higher in high-nutrient conditions (Table 1; Figure 2).

The nutrient level was important for meadow fescue biomass, but the effect was not straightforward, as the interactions endophyte \times nutrient and endophyte \times proportion \times nutrient were statistically significant (Table 1; Figure 3). The estimates of this model show that in the high-nutrient environment, endophyte-promoted plant growth decreased rapidly with an increasing proportion of clover (decreasing continuous line); when the proportion of red clover reached 0.45, E+ and E- meadow fescues produced the same biomass (Figure 3a). The E- plants appeared to be unaffected (or even benefitting) by the presence of red clover. When nutrients were low, endophyte status and presence of clover plants did not have any effect on meadow fescue biomass (Figure 3a).

Endophyte infection and the performance of tall fescue

In tall fescue monocultures, E- plants produced equally high biomass to that of E+ plants, but their productivity gradually decreased with an increasing proportion of red clover (Figure 1).

Endophyte infection did not affect the total biomass, height or tillering of tall fescue alone or interactively with nutrient conditions (Table 1). Young seedlings of tall fescue in high-nutrient conditions seem to be prone to competition as, especially in E- plants, biomass decreased rapidly with an increasing number of clover individuals. Effects of competition were not seen in a low-nutrient environment (Figure 3b). The same effect was observed in plant height [nutrient $F = 77.8$, $df = (1,415)$, $P < 0.001$]. High-nutrient conditions resulted in more tillering, but the effect decreased, especially in E- plants, with increasing competition. Competition did not affect fescue tillering in poor nutrient conditions (Figure 2, Table 1).

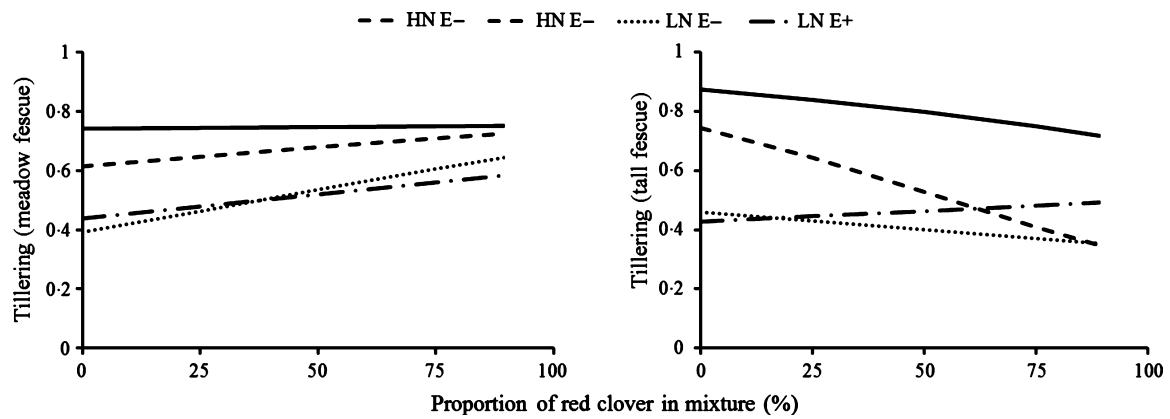


Figure 2 Model-based estimates for mean tillering (presence of extra tiller) of meadow fescue and tall fescue with different proportions of red clover in mixture. Estimated means are back transformed. HN: High nutrient level of soil and LN: Low nutrient level of soil.

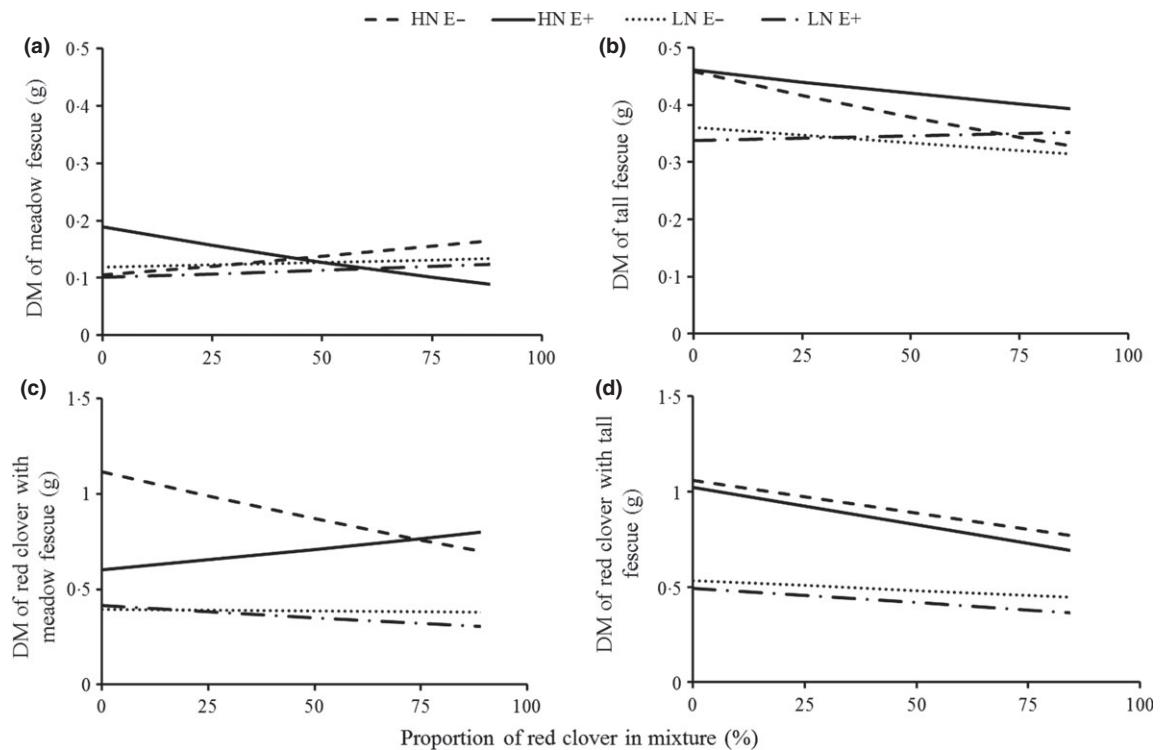


Figure 3 Model-based estimates for dry matter (g) of individual meadow fescue, tall fescue and red clover plants in different proportions in mixture. Means are back transformed. DM: Dry matter, HN: High nutrient level of soil and LN: Low nutrient level of soil.

Competitive ability of red clover

Clover responded to nutrients, and produced 75% more biomass in high-nutrient monocultures compared to low-nutrient monocultures (Figure 1).

If red clover were unaffected by competition, its biomass per plant should not depend on the number of other clover plants in the pot. However, models (Table 1, Figure 3c and d) demonstrate that, especially

in high-nutrient conditions and with tall fescue, red clover biomass decreases with increasing number of clover plants in the same pot (Figure 3d). However, clover benefitted from the presence of endophyte-infected meadow fescue plants in high-nutrient conditions, but suffered when growing with E⁻ meadow fescue in high nutrition (Figure 3c). Clover biomass remained unchanged when grown in a low-nutrient status environment.

Discussion

Our results suggest that during the early phase of establishment, neither meadow fescue nor tall fescue gain an instant endophyte-promoted competitive advantage over red clover when sown together. The production from red clover was more than fourfold and nearly threefold that of monocultures of meadow fescue and tall fescue, respectively, and it contributed most of the total yield in plant mixtures. Consistent with many other studies, however, we detected that endophyte infection can increase the performance of grass monoculture (Saikkonen *et al.*, 2013), but the positive effects of endophytes depend on host species and on soil nutrient availability (Ahlholm *et al.*, 2002; Saikkonen *et al.*, 2004, 2006; Lehtonen *et al.*, 2005a; Cheplick and Faeth, 2009). Endophyte colonization promoted the biomass production of meadow fescue in experimental monocultures, but only when the plants were fertilized. E⁺ fertilized meadow fescue suffered from competition with red clover, as its per-plant calculated biomass decreased clearly with increasing number of clover plants. Interestingly, this phenomenon was not detected in low fertilization conditions, and fertilized E⁻ meadow fescues probably even benefitted from clover. Fertilization promoted tillering and induced higher growth in meadow fescue, but an increasing number of clover plants decreased tillering especially in fertilized E⁻ plants. We acknowledge that the low- and high-nutrient soil treatments are not comparable to field conditions. However, our aim was to adjust the levels to cause measurable differences in plant growth.

When sown together with red clover, the biomass production of tall fescue was markedly lower compared to that of tall fescue monocultures, whereas meadow fescue performed equally in both monocultures and plant mixtures except in the case of particularly vigorous endophyte-infected plants in high-nutrient soils. Contrasting starkly with the majority of studies that demonstrate endophyte-promoted tall fescue growth, reproduction and competitive ability (Hill *et al.*, 1991; Marks *et al.*, 1991; Clay and Holah, 1999; Saikkonen *et al.*, 2006; Gundel *et al.*, 2013a,b), we found that the total yield of endophyte-free tall fescue

plants was comparable to endophyte-infected ones. Tall fescue monocultures appeared to gain a greater advantage through fertilization compared to meadow fescue monocultures in terms of yield, but this effect disappeared when it competed with clover. Per-plant yield decreased faster with increasing number of clover plants for fertilized E⁻, but this effect is also clear for E⁺. Thus, young seedlings of tall fescue seem to suffer from competition with clover in fertilized conditions, but not in low-nutrition situations. Tall fescue was a weaker competitor than meadow fescue, regardless of endophyte infection, in these experimental conditions. Whether this holds true in natural and agricultural environments, including under diverse but relevant stress factors such as drought (Nagabyru *et al.*, 2013), remains to be tested in future studies.

Red clover gained advantage of nutrient supply, but it also seemed to suffer from within-species or between-species competition. In contrast to other conditions and species, in high-nutrient soils, the biomass of red clover decreased with increased competition with E⁺ meadow fescue. This suggests that red clover may suffer from competition in high-nutrition conditions.

We acknowledge that our study fails to forecast the outcome of long-term competition. Our experiment also failed to capture the potentially improved yield of the companion grass resulting from the additional nutritional complementation arising from the atmospheric nitrogen-fixing ability of red clover. Advantages of a grass-clover mixture due to the fertilization effect from nitrogen fixation will be gained after a delay. Thus, higher yield and tillering of endophyte-infected meadow fescue and tall fescue, respectively, may provide them with a competitive advantage in field conditions in the long term. Although red clover appears to be competitive, at least in low-nutrition soils during the establishment phase, it is known to be a species that usually fails to persist in plant mixtures for more than a few years and is regarded as a relatively short-lived crop (Mela, 2003).

In short, among the other recent studies (Lehtonen *et al.*, 2005a,b; Saikkonen *et al.*, 2006; Cheplick and Faeth, 2009), our results demonstrate that the positive effects of endophyte infection on the performance of the host grass are conditional depending on the host species, the species composition and soil nutrient availability.

If these results are applicable under field conditions, as shown by appropriate field-based studies, then tall fescue, as larger grass species with higher yield potential, will be an effective option for conventional farming and other environments with high nutrition. However, meadow fescue could be an alternative to tall fescue, especially under conditions of

low-input sustainable agriculture because both species appear to grow equally well in low-nutrient soils.

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