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Fungal endophytes help prevent weed invasions

Kari Saikkonen[a,*], Kalle Ruokolainen[b], Otso Huitu[c], Pedro E. Gundel[a,d], Tiina Piltti[b], Cyd E. Hamilton[a], Marjo Helander[b]

[a] *Agrifood Research Finland, Plant Protection, FI-31600 Jokioinen, Finland*
[b] *Section of Ecology, Department of Biology, University of Turku, FI-20014, Finland*
[c] *Finnish Forest Research Institute, Suonenjoki Research Unit, Juntintie 154, FI-77600, Suonenjoki, Finland*
[d] *IFEVA-CONICET – Faculty of Agronomy, Buenos Aires University (UBA), Buenos Aires, Argentina*

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ABSTRACT

This study tested whether the endophyte-promoted competitive superiority of forage grass can be used in biological weed control. Feasibility of endophytes in weed control was tested by manipulating endophyte colonization of meadow fescue (*Scherodonus pratensis* ex. *Lolium pratense* and *Festuca pratensis*) in three experiments. First, species richness, the cover percentages and biomass of detected species were estimated in replicated field plots seeded with endophyte-free (E−) or endophyte colonized (E+) meadow fescue. Four years after establishment of the grass monocultures, weed species richness and coverage was higher in E− plots compared to E+ plots. The cover percentages of meadow fescue decreased with weed invasions being 75% and 98% in E− and E+ plots, respectively. Similarly, the proportion of weeds in the total biomass was over 45% higher in E− plots compared to E+ plots at the end of the six years study. Half of the plots were subjected to herbivory by voles, but the effect of endophyte overrode the effect of herbivory. Second, the survival of individually grown E+ meadow fescues was higher and the plants were 50% larger and produced 54% more inflorescences than E− plants in a common garden experiment. Third, a seed germination test demonstrated that recruitment of new meadow fescues was not mediated by substances inhibiting seed germination potentially released by E+ plants. These results demonstrated that endophyte promoted competitive superiority of grass cultivars can hinder weed invasions.

1. Introduction

The species assemblage of plant communities is primarily governed by the physical environment, resources and biological interactions (Tilman et al., 1997; Shea and Chesson, 2002). In addition to traditionally emphasized interactions in community ecology such as competition and plant–herbivore interactions, the importance of interactions involving microbes is increasingly recognized. For example, mycorrhizae facilitate water and nutrient acquisition, pathogens can be detrimental, and some grass fungal endophytes can increase plant tolerance to stressful abiotic environmental conditions and mediate virtually any type of plant-plant, plant-herbivore and plant-pathogen interaction (Saikkonen et al., 2006; Clay and Holah, 1999; Wäli et al., 2006; Saari et al., 2010a,b; Rudgers et al., 2010). Thus, microbes can mediate adaptive radiation, and invasion and competitive success of plants in successional plant communities (Tilman et al., 1997; van der Heijden et al., 1998; Clay and Holah, 1999; Saikkonen, 2000; Rudgers et al., 2005, 2007; Callaway and Maron, 2006; Aschehoug et al., 2012).

Neotyphodium endophytes [type species *N. coenophialum* (ex *Acremonium*) Clavicipitaceae, Hypocreales, Ascomycota] and their sexual antecedents in genus *Epichloë* are estimated to infect 20–30% of grass species (Leuchtmann, 1992) but are often ignored because they form asymptomatic infections in the host (Wilson, 1993). However, recent evidence suggests that these fungi can strongly affect grassland plant community productivity and composition in both nature and agro-environments (Hoveland, 1993; Clay and Holah, 1999; Rudgers et al., 2010). Endophyte colonization can increase plant growth, reproduction and resistance to various biotic and abiotic stress factors (Clay, 1990) thus promoting the invasiveness of the host plant into new grassland communities (Rudgers et al., 2005). *Neotyphodium* endophytes form systemic infections throughout the aerial parts of the host plant including the inflorescence and developing seeds, and are therefore vertically transmitted in mother plant lineages (Saikkonen et al., 2004). Compared to other inherited grass traits, endophyte-driven traits are based on the outcome of mutual exploitation between the two

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[*] Corresponding author. Tel.: +358 29 5317714; fax: +358 20 772 040.
E-mail addresses: kari.saikkonen@mtt.fi (K. Saikkonen), kalle.ruokolainen@utu.fi (K. Ruokolainen), otso.huitu@metla.fi (O. Huitu), pedro.gundel@mtt.fi (P.E. Gundel), tiipilt@utu.fi (T. Piltti), phyllium@gmail.com (C.E. Hamilton), helander@utu.fi (M. Helander).

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interacting species. Furthermore, selection may operate on the phenotypes of host-fungal units together or separately on the fungus and/or the host plant (Saikkonen et al., 2004). Thus, conflicting selective forces can destabilize the interaction leading to loss of the endophytic fungal partner (Saikkonen et al., 2004, 2010b) and associated changes in plant traits. Thus, the implications of systemic and vertically transmitted grass-endophytes should be acknowledged in turf and forage production, and grassland conservation and restoration.

In two long term field experiments we studied firstly if endophyte (*N. uncinatum*) colonization promotes meadow fescue (*S. pratensis* ex. *L. pratense* and *F. pratensis*) survival, growth and reproduction and competitive dominance, thus slowing weed invasion into meadow fescue monocultures. Secondly, because competitive dominance is suggested to be mediated in part by herbivory (see e.g. Saikkonen et al., 2006, 2010a; Takai et al., 2010), we subjected half of the plots to herbivory by sibling voles (*Microtus levis* ex *M. rossiaemeridionalis*). Thirdly, potential allelopathic effects of E+ plants via soil and litter to seedling recruitment in meadow fescue populations were examined in a greenhouse experiment (Springer, 1996; Orr et al., 2005; Vázquez de Aldana et al., 2011, 2012). We predicted (1) endophyte colonization increases host plant growth and reproduction, (2) the maintenance of high frequencies of E+ plants is also promoted by endophyte-origin substances inhibiting seed germination of E− plants, and (3) endophyte colonization promoting competitive dominance of meadow fescue can suppress weed invasions.

2. Materials and methods

Meadow fescue is a native grass species in Europe, one of the most important forage grasses in Nordic countries occurring also commonly outside of agronomic use in meadows, roadsides and wastelands (Hämet-Ahti et al., 1988). Several meadow fescue cultivars are commonly colonized by systemic *N. uncinatum* endophyte but the frequency of colonization varies substantially within and among cultivars (Saari et al., 2009). *N. uncinatum* produces lolines which appear to be non-toxic to large mammal herbivores (Clay and Schardl, 2002) but can be noxious to invertebrates and small vertebrates (Conover, 2003; Saikkonen et al., 2006; Huitu et al., 2008). We used 'Kasper', a common meadow fescue cultivar in Nordic countries, which was registered and commercialized in 1989 (Saari et al., 2009).

2.1. *Testing the effects of endophytes and herbivory on weed invasions*

The study plots were established (20 plots, 25 m × 39 m) in May 2006 at the MTT Agrifood Research Finland Experimental fields in Jokioinen. The site was tilled, fertilized with cow manure (30,000 kg/ha) and seeded with E− (0% endophyte frequency) or E+ (79% infection frequency) seed lots of meadow fescue cultivar 'Kasper' at a rate of 20 kg ha^{-1}. E+ and E− treatments were randomly assigned in 10 plot pairs. Seed lots were obtained from seed production farms via the Finnish Food Safety Authority (EVIRA), Seed Certification Unit, Loimaa, Finland. Initially the experiment was designed to study the importance of endophytes on the population development of sibling voles (*M. levis* ex *M. rossiaemeridionalis*) (Saari et al., 2010b). Thus each plot was surrounded with a sheet metal fence (embedded 60 cm below ground while 60 cm remained above ground) in order to keep the experimental voles inside and voles of natural populations and small mammal predators out of the experimental areas. In June 2007, plots were fertilized again with a commercial fertilizer [16:9:22 (N:P:K) with micronutrients, Kemira, product number: 0647334]. The population development of sibling voles was then studied by introducing five male and five female voles into five enclosure pairs and recording population size during a four and a half month period which approximately equates the annual length of the reproductive period of voles in Finland (Saari et al., 2010b). All procedures were carried out in accordance with Act on the Use of Animals for Experimental Purposes established by Ministry of Agriculture and Forestry, Finland. The study was approved and supervised by the Animal Experiment Committee of Finland (License number for ethics approval: STH393A).

To quantify weed invasion the cover (%) of all detected plant species in 24 consecutive 1 m^2 squares along the 21 m transect in the middle of each plot was recorded in August 2010. Because species often overlap, cover percentages for all plant species were separately determined by vertical projections. The outermost 2 m of each plot, alongside the fence perimeter, was not included into the survey to avoid edge effect. In this method the spatial configuration of the response variable and the treatment, or independent variable, were considered as two superimposed layers. First, the strength of the relationship between the observed response and independent variable was calculated to obtain the original value of the test statistics. Next, the two layers were convoluted to make two superimposed torus surfaces. Thereafter one of the torus surfaces was moved in relation to the other surface and after each movement the test statistic was recalculated. The original values of the test statistics were finally compared to values obtained after translocations in order to obtain an estimation of the error probability of the observed relationship between the dependent and independent variable. Our sampling configuration produced 420 possible positions between the two torus surfaces. Because of the modest number of possible positions, observed test statistics were compared to all possible values.

To quantify total productivity and the proportion of meadow fescue in the total biomass, five 1 m^2 quadrats along the transect in the middle of each plot were harvested in August 2012. In the laboratory, samples were sorted into meadow fescues or weeds, dried (48 h 60 °C) and weighed. The data was analyzed statistically by using SAS 9.1 (Enterprise Guide 4.0) with the Mixed procedure.

2.2. *Testing endophyte mediated plant performance*

Seeds of known endophyte status of meadow fescue cultivar 'Kasper' were germinated and pre-grown in a greenhouse for two weeks in April 2009. The seedlings (160 in total) were randomly planted to 16 tilled field plots of University of Turku Ruissalo Botanical Garden. The distance between individual seedlings was ca. 25 cm. No fertilizers, pesticides or fungicides were used during the experiment. The individual plots were hand-weeded during the experiment. The endophyte status of each grass individual (78 E− and 82 E+ individuals) was verified by microscopic examination of three seeds of each plant (Saha et al., 1988). At the end of the second growing season (August 2010) survival, growth and reproduction of the plants were recorded. The vegetative growth of the plants was estimated using the cylinder volume of the plants (measuring height and diameter of the plant) and reproductive investment measured by counting the flowering tillers of each individual. The data were analyzed statistically by using SAS 9.1 (Enterprise Guide 4.0) with the GLM procedure.

2.3. *Testing allelopathic effects of E+ plants via soil*

In 2010, ripe meadow fescue cultivar 'Kasper' seeds were collected from 50 flower heads separated by at least 1 m, from every plot of the field experiment. After threshing, seeds from every plot were pooled and stored in dry and temperate conditions. Four seed samples of 100 seeds each were separated from every plot (E− or E+), resulting in 8 seed samples per block. In spring 2011, four layers

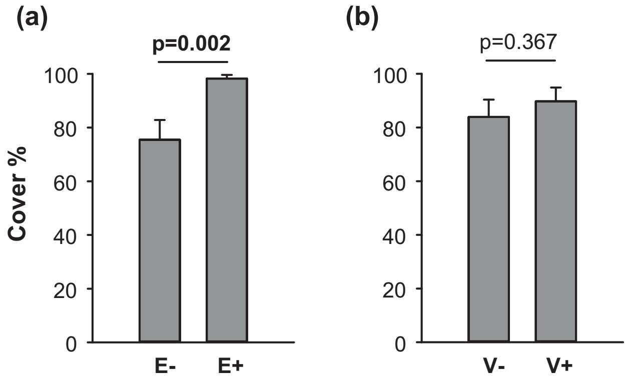

Fig. 1. The effects of (a) endophyte colonization (E− = endophyte-free; E+ = endophyte colonized) and (b) vole grazing (V− = ungrazing; V+ = grazed) on the percentage cover of meadow fescue four years after the establishment of the field experiment.

(4 cm) of topsoil (14.3 cm × 9.6 cm) maintaining the soil structure were taken from each plot within block, and put it into plastic trays. At the same time, 4 litter samples were collected from every plot; the collected litter was dead biomass from meadow fescue produced the year before (or older). A 1 cm thick litter mattress was established on trays. The experimental design results from the full combination of soil type (E+ and E−), litter type (E+ and E−) and seed type (E+ and E−) repeated in 10 blocks (80 trays in total). The sowing was done by spreading the 100 seeds over the litter and then gently shaking the litter allowing the seeds to reach the soil. All the trays were watered to maintain the soil moisture levels throughout the experiment. Seedling emergence was periodically recorded for two months. At the end of the experiment, all the seedlings were harvested, counted, and weighed (after oven drying at 70 °C for two days). Percentage of emerged seedlings (seedling number/sown seeds) and aboveground biomass per seedling were statistically analyzed using mixed model ANOVA, considering block as random and soil, litter and seed as fixed effects. Percentage of emerged seedlings was transformed (arcsin(sqrt(prop))) to meet the statistical test assumptions.

3. Results

Four years after establishment of the meadow fescue monocultures, the percentage cover of E+ and E− meadow fescue monocultures were 98% and 75%, respectively (Fig. 1a). Similarly, the proportion of meadow fescue in the total biomass was markedly higher in E+ plots compared to E− plots two years later (Fig. 2; df = 1, $F = 32.35$, $p < 0.0001$). Total productivity was marginally higher in E− plots (Fig. 2; df = 1, $F = 3.74$, $p = 0.0563$). In the E− plots, weeds had readily displaced meadow fescue (Fig. 2 (df = 1, $F = 34.51$, $p < 0.0001$) and Fig. 3a). This suggests endophyte colonization can promote the stability of meadow fescue monocultures.

In total, 21 and 13 weed species were recorded in E− and E+ plots, respectively. The most common weed species were couch grass (*Elymus repens*), creeping thistle (*Cirsium arvense*), timothy (*Phleum pratense*) and dandelion (*Taraxacum officinale*) reaching 8%, 6% 2% and 2% mean cover during the study, respectively. In addition, *Fragaria × ananassa*, *Tripleurospermum inodorum*, *C. vulgare*, *Poa pratensis*, *Lathyrus pratensis*, *Galium spurium*, *Deschampsia caespitosa*, *Trifolium pratense*, *Galeopsis speciosa*, *T. repens*, *Artemisia vulgaris*, *Sonchus arvensis*, *Prunella vulgaris*, *Cerastium fontanum*, *Myosotis arvensis*, *Polygonum aviculare*, *G. uliginosum*, *Urtica dioica* and *Ranunculus repens* were detected, but the mean cover of these species remained lower than 1%.

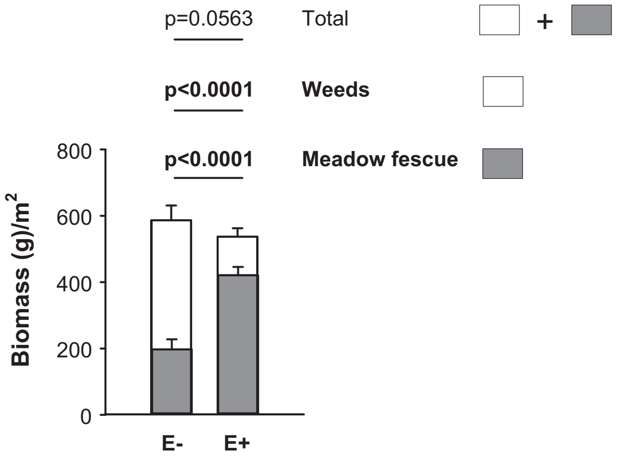

Fig. 2. The effects of endophyte colonization (E− = endophyte-free; E+ = endophyte colonized) on total productivity and the proportion of meadow fescue and weeds in the total biomass at the end of the six year field experiment.

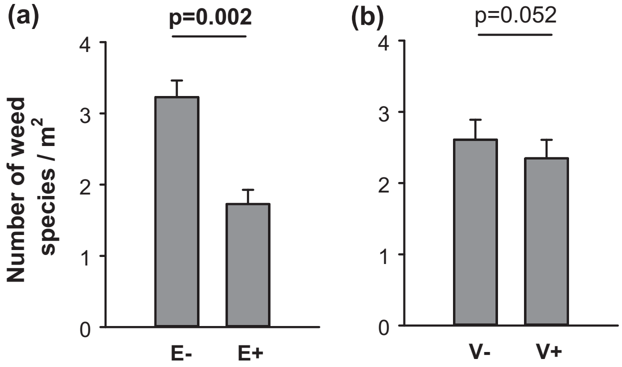

Fig. 3. The effects of endophyte colonization (a) and vole grazing (V− = ungrazing; V+ = grazed), (b) on the number of weed species per square meter four year after the establishment of the field experiment.

Vole grazing did not affect the percentage cover of meadow fescue (Fig. 1b). However, grazing tended to suppress weed invasion in the study plots, but not quite statistically significantly ($p = 0.052$) (Fig. 3b). Endophyte colonization status did not bear on the effects of vole grazing (the results of the torus-translation test for interactive effect of endophyte colonization and vole grazing: cover of meadow fescue, $p = 0.4333$; cover of couch grass, $p = 0.6214$; cover of creeping thistle, $p = 0.3095$; cover of timothy, $p = 0.3857$; cover of dandelion, $p = 0.9881$; number of species/m^2, $p = 0.8333$). Total productivity (df = 1, $F = 0.14$, $p = 0.707$), and the proportion of meadow fescue (df = 1, $F = 1.05$, $p = 0.3080$) and weeds (df = 1, $F = 1.39$, $p = 0.2421$) in the total biomass were not affected by vole grazing.

The survival of individually grown E+ meadow fescues was higher and the plants were 50% larger, producing 54% more inflorescences than E− plants in the common garden experiment (Fig. 4; survival, df = 1, $F = 15.07$, $p < 0.0002$; plant volume, df = 1, $F = 14.14$, $p < 0.002$; number of inflorescence, df = 1, $F = 27.08$, $p < 0.001$).

Neither the seed germination rate nor the shoot biomass of E+ and E− meadow fescue seedlings were affected by soil, litter or the endophyte colonization (all *p*-values > 0.2).

4. Discussion

This study clearly demonstrated that endophyte colonization promoted competitive dominance of meadow fescue in experimental monocultures and can retard weed invasions. Over the six year

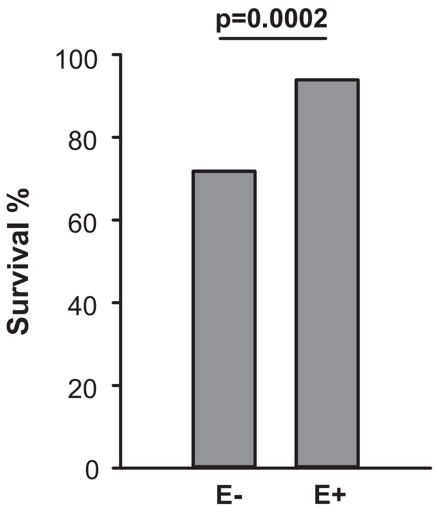

Fig. 4. The effects of endophyte colonization (E− = endophyte-free; E+ = endophyte colonized) on survival of the plants after two growing seasons in a common garden experiment.

study period, the percentage cover and biomass of E− meadow fescue monocultures was reduced more than 20% and 45% compared to E+ monocultures. The maintenance of high frequencies and productivity of E+ plants was a result of higher survival, growth and reproduction of the plants, and not mediated by endophyte-origin substances inhibiting seed germination of E− plants. Weed species invasion was negatively related to the cover and productivity of meadow fescue. Species richness, percentage cover and biomass of weed species were markedly higher in E− plots.

These results emphasize the importance of individual keystone species (Ernest and Brown, 2001) and functional diversity (Tilman et al., 1997; Hulot et al., 2000; Thébault and Fontaine, 2010) in stabilizing community dynamics. In this study, a mutualistic endophytic fungus markedly increased grass resistance to weed invasions, and promoted the stability of meadow fescue monoculture. Over a six-year study period, weed species richness, cover and biomass increased in both E− and E+ plots. However, the increase of weed species richness, weed cover and the proportional biomass of weeds were 38%, five times and over 45% higher in E− plots, respectively. Vole grazing potentially facilitated competitive dominance of E+ meadow fescues by slightly decreasing the percentage cover of weeds (see e.g. Conover, 2003; Saikkonen et al., 2010a), but endophyte had a greater impact on weed invasion into the community than did herbivory.

Instead of viewing them as community stabilizing plant mutualists, grass endophytes are commonly regarded, in a negative manner, to promote the ability of the host plant to expand its distribution range and threaten native plant diversity and associated food webs (Ball et al., 1993; Hoveland, 1993; Clay and Holah, 1999; Saikkonen, 2000; Rudgers et al., 2005, 2010). This concept has largely been based on studies of two Eurasian grass species, tall fescue and perennial ryegrass, in North America (Saikkonen et al., 2004, 2006, 2010a; Cheplick and Faeth, 2009). In contrast, these and other cool season grass species have never caused comparable disorder either in nature or agriculture in Europe (Nösberger, 1993; Zabalgogeazcoa and Bony, 2005; Saari et al., 2009, 2010a). For example, native European study species, meadow fescue, appears to be a relatively poor competitor and invader into nature irrespective of its endophyte colonization status (Nösberger, 1993; Saari et al., 2009, 2010a).

4.1. Endophytes in European meadow fescues

In theory, vertical transmission should align the interests of partners so that the fungus–host association should evolve toward mutualism, and tightly linked fitness of the fungus and its host plants should increase the frequency of infected grasses over time in plant populations if endophyte increases the fitness of the host (e.g. Leuchtmann and Clay, 1997; Saikkonen et al., 2004). However, although both naturalized and cultivated plants are commonly infected by *N. uncinatum* endophyte, frequencies of endophyte colonization are demonstrated to be highly variable among and within populations and varieties (Hamilton et al., 2009; Saari et al., 2009). This suggests that the symbiosis between the *N. uncinatum* endophyte and meadow fescue ranges from antagonistic to mutualistic depending on the genetic match of the fungus and the host grass, and environmental conditions (Ahlholm et al., 2002; Saikkonen et al., 2004, 2010b). In the case of naturalized meadow fescue, the cost of endophyte colonization is detected in nutrient limited environments in terms of decreased growth and reproduction (Ahlholm et al., 2002) which are negatively correlated with the competitive ability of plants. Colonization frequencies may also decrease due to the loss of infection from seeds or vegetative tillers (Saikkonen et al., 2010b).

Selective plant breeding and agricultural practices appear to direct endophyte colonization frequencies in cultivars toward the extremes of very low and very high (Saari et al., 2009). Breeding programs may operate on endophyte infection if the original seed collection hosts endophytes and they affect agriculturally significant plant characteristics such as winter-hardiness, yield, nutritive value, resistance to pests and pathogens, irrespective of whether the breeder is aware of the presence of endophytes. The loss of endophyte infection during long or unfavorable periods of storage (Rolston et al., 1986; Wheatley et al., 2007; Gundel et al., 2009) can explain the variation detected among cultivars and seed lots within cultivars (Saari et al., 2009). Saari et al. (2009) recently suggested that the probability of losing an infection should increase over time, and higher levels of variation in infection among seed lots should be detected in older cultivars.

4.2. Implementation in agriculture

Systemic grass-endophytes should be acknowledged in plant breeding, agricultural practices and environmental management strategies in Europe. This study emphasizes economically measurable consequences and implementation in sustainable forage productivity. For example, the loss of plant growth promoting endophyte infection can lead to a rapid decrease in variety productivity (Bouton et al., 2001), and fungal strains which do not produce mycotoxins but increase plant growth, seed production, seed germination rate and stress tolerance (e.g. drought, low temperatures), can be used to increase forage productivity when introduced to cultivars used as forage. Most importantly, these results demonstrate that the presence of the endophyte in meadow fescue slows weed invasion in a set-aside field.

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