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# **PLANT ENDOPHYTE IN FOOD CHAIN – FRIEND OR FOE?**

by

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This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

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- II** Saari, S., Helander, M., Lehtonen, P., Wallius, E. and Saikkonen, K. Fungal endophytes reduce regrowth and affect competitiveness of meadow fescue in early succession of pastures. Submitted manuscript.
- III** Saari, S., Helander, M., Faeth, S.H. and Saikkonen, K. The effects of endophytes on seed production and seed predation of *Schedonorus pratensis* and *Schedonorus phoenix*. Submitted manuscript.
- IV** Saari, S., Sundell, J., Huitu, O., Helander, M., Ketoja, E., Ylönen, H. and Saikkonen, K. Bottom-up regulates top-down: grass endophytes in diet protect voles from predators? Submitted manuscript.
- V** Saikkonen, K., Saari, S. and Helander, M. 2010. Defensive mutualism between plants and endophytic fungi? *Fungal Diversity*, in press.

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# 1. INTRODUCTION

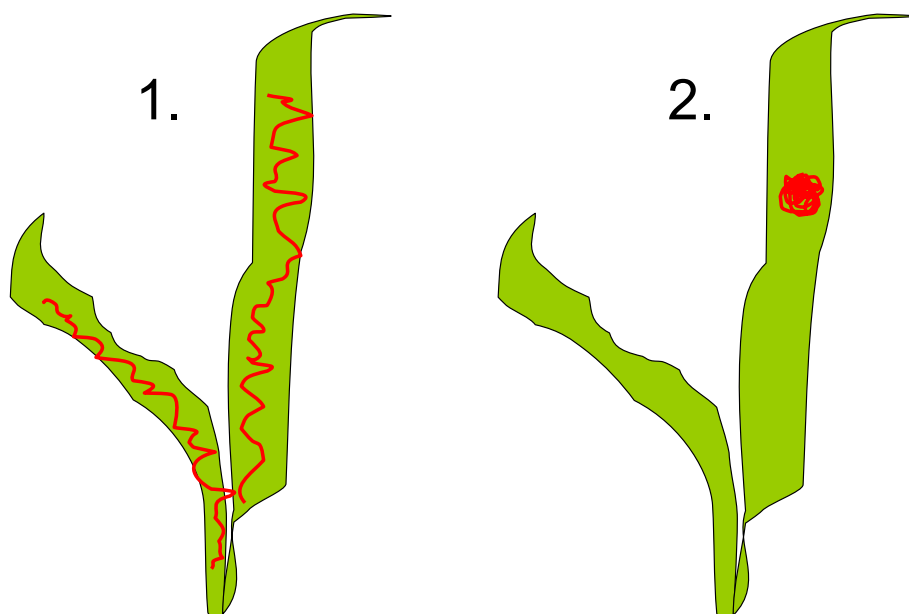
Natural communities involve complex interactions among a number of species. The term ‘symbiosis’, Greek for “life together”, is commonly defined as a close and often long-term interaction between different species. Since its first use by de Bary (1887), the term has been applied to a wide range of biological interactions; these can be categorized as mutualistic (benefiting both participants), parasitic (benefiting one species, harmful to the other), or commensal (benefiting one species while the other is unaffected) (e.g. Begon et al. 1996). The type of interaction within a symbiotic association, however, is not always stable but may vary over time and space from parasitism to mutualism (e.g. Bronstein 1994, Saikkonen et al. 1998, Neuhauser and Fargione 2004).

Symbiotic relationships may be either obligate, i.e. necessary for the survival of at least one of the organisms involved, or facultative, where the relationship is beneficial but not essential for the survival of the interacting organisms (Sapp 1994). An endosymbiotic association, in which one organism lives within the tissues of another, is often obligate in nature (Sapp 1994). Ecological theory predicts that an obligate symbiont, without the capacity for horizontal transmission, will be lost from the host population if the net effect of the symbiont is detrimental to the host (Fine 1975, Lipsitch et al. 1995, Frank 1997). Thus the evolution of obligate endosymbiosis has been closely linked with mutualism. Recent evidence, however, suggests that obligate endosymbionts incapable of horizontal transmission may also have an antagonistic relationship with the host (Saikkonen et al. 1998), raising the need for further studies to test the reliability and inclusiveness of the ecological theory.

In this thesis I examine obligate endosymbiosis and the nature of the relationship between the symbiont and the host, using fungal shoot endophyte-plant symbiosis as a model system. I deal with multitrophic study systems, variable conditions, cultivated and wild plants, and discuss the applied potential of endophyte-plant associations in agricultural management. I also review the current state of knowledge of endophyte-plant-herbivore-predator interactions.

## 1.1. Endophytic fungi-host plant symbiosis

Endophytic fungi of plants grow intercellularly within the host shoot tissue and cause asymptomatic infections in the host plant. The growth form of the fungi can be non-systemic or systemic (Fig. 1). Non-systemic endophytes cause single spore origin infections, each spore infecting a small fraction of the host (Stone 1987). Systemic endophytes grow intercellularly within the host tissues, infecting both vegetative and reproductive plant tissues. Endophytes may be transmitted either horizontally via spores or vertically by seed production and clonal growth of the host plant. (Fig. 2). Some

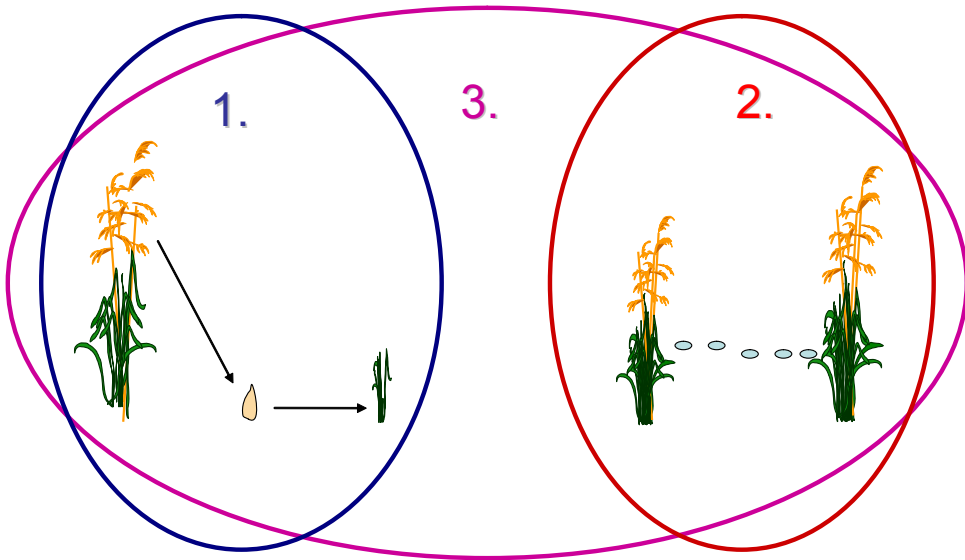


**Figure 1.** Two growth forms of endophytes. Systemic endophytes cause asymptomatic infections throughout the aerial parts of the host plant (1); non-systemic endophytes cause single-spore origin infections, each individual infecting a small spot in a single leaf (2).

endophytes have the capacity for transmission both horizontally by spores and vertically by seeds. Systemic, vertically transmitted plant endophytes have only been found to infect grasses, while non-systemic, horizontally transmitted endophytes infect all major groups of plants. The experimental studies of this thesis concern systemic and vertically transmitted *Neotyphodium* (formerly *Acremonium*) grass endophytes (Clay 1990). The review article includes a survey of the literature dealing with vertically and horizontally transmitted, systemic and non-systemic plant endophytes.

### ***1.1.1. Endophyte-grass symbiosis***

Systemic endophytes infect approximately 20% - 30% of all grass species, including a number of significant agricultural species (Leuchtman 1992). In natural environments the frequencies of infected plants vary among grass species and among populations within species, depending on local selection pressures; the infection levels of cultivars, on the other hand, are variable but usually either very high or very low (see e.g. Clay 1996, Lewis et al. 1997, Saikkonen et al. 2000, Wäli et al. 2007). High frequencies of endophyte-infected plants in cultivars are suggested to result from selective breeding if the endophyte improves the agronomic characteristics of the host (Saikkonen et al. 2000). Further, selective breeding is not necessarily associated with endophyte infection;



**Figure 2.** Three reproductive categories of plant endophytes. Vertically transmitted endophytes are transmitted only by seeds, from parental plant to offspring (1); Horizontally transmitted endophytes reproduce only by spores (2); The third category consists of endophytes that reproduce both vertically and horizontally (3).

alternatively, it may eliminate endophyte-infected plant individuals if the endophyte adversely affects desirable characters of the host (Saikkonen et al. 2000).

As *Neotyphodium* grass endophytes are considered to be obligate endosymbionts without the capacity for horizontal transmission, the ecological theory suggests that *Neotyphodium* grass-endophyte symbiosis is mutualistic. The viewpoint that fungi benefit the host plant has been supported by studies demonstrating increased growth, reproduction, tolerance of drought and flooding, mycotoxin-based herbivore and pathogen resistance, and enhanced competitive abilities of the host grass (Clay 1990, Elbersen and West 1996, Clay and Holah 1999). There is, however, accumulating evidence suggesting that the relationship between grasses and endophytes may vary from antagonism to mutualism, depending on such factors as the availability of nutrients in the soil and the complexity of the food web (Lehtonen et al. 2005b, Saikkonen et al. 2006).

### 1.1.2. Endophyte-woody plant symbiosis

Most studies of horizontally transmitted plant endophytes have been conducted using endophyte-woody plant study systems. Unlike seed-transmitted grass endophytes, horizontally transmitted endophytes are more likely to form a neutral or antagonistic relationship with the host plant (Saikkonen 2007). This is because horizontally transmitted endophytes are less dependent on host survival and form less tight a relationship with the host than seed-borne endophytes. Tree leaves are free of endophytes at leaf-burst

but soon become infected by fungal endophyte spores, with the frequency of infection increasing toward the end of the growing season. In evergreen plants endophyte infection frequencies may increase in time until an ecological niche is fully booked (Helander et al. 1993, Faeth and Hammon 1997). Due to the horizontal mode of transmission an infection is likely to be affected by such factors as the surrounding vegetation, tree density, weather conditions and the phase of growing season (Saikkonen et al. 1996, Saikkonen 2007).

## **1.2. Endophyte-host plant-herbivore interactions**

### ***1.2.1. Grass-endophyte-herbivore interactions***

The primary driving force behind selection for endophyte-grass mutualism appears to be defence against herbivory (Saikkonen et al. 2006, Clay 2009). Systemic endophytes have attracted increasing attention since observations of toxicosis on livestock grazing fungal-infected tall fescue [*Schedonorus phoenix* (Scop.) Holub.] pastures in USA and New Zealand in the mid-20th century and the majority of research on grass endophytes has focused on endophyte-mediated resistance to herbivory (Saikkonen et al. 2004a). Negative effects of endophytes on herbivores result mainly from endophyte origin mycotoxins (Bacon et al. 1977, Miller 1986, Clay 1988, Saikkonen et al. 2004a). Endophytes may also change nutritional quality of plants to herbivores (Smith et al. 1985, Rasmussen et al. 2009) or induce responses in plants that may turn the host plant less palatable or unattractive to herbivores (Clay 1993). Increased herbivore resistance has been suggested to make infected plants competitively superior compared to endophyte-free plants which may lead to increased infection frequencies in plant communities (Cheplick and Clay 1988, Clay 1989, Schardl et al. 2004).

### ***1.2.2. Woody host-endophyte-herbivore interactions***

The effects of horizontally transmitted plant endophytes on herbivores have been studied considerably less widely than interactions between grass endophytes and herbivores. Existing studies demonstrate that the effects of tree endophytes on herbivores are more variable than those of grass endophytes (see e.g. Minter 1981, Lappalainen and Helander 1997, Ahlholm et al. 2002a). This variability has been suggested to result from the horizontal transmission mode, the localized nature of infections and the length of the latency period of fungi (Saikkonen et al. 2004a, b, Saikkonen 2007). In woody plants, the effects of microfungi on herbivores are often neutral and indirect rather than direct (Hatcher 1995, Saikkonen et al. 2001). For example, horizontally transmitted endophytes may alter plant quality for herbivores by using leaf nutrients (Smith et al. 1985) or by changing the physical structure of the plant (Hatcher et al. 1995). In addition, the

temporal and spatial distribution and the abundance of partners affect the probability of encounters and the opportunity for species to interact with each other (Saikkonen et al. 1996, Ahlholm et al. 2002b).

### **1.3. Effects of grass endophytes on multitrophic interactions**

There is accumulating evidence suggesting that grass endophytes may have negative indirect effects on predators at higher trophic levels, mediated through herbivores (see e.g. Bultman et al. 1997, Omacini et al. 2001, Härrri et al. 2009). For example, endophytes have been shown to negatively affect the fecundity, reproductive capacity and survival of invertebrate predators (de Sassi et al. 2006, Bultman et al. 2009, Härrri et al. 2009). Taken together, these findings illustrate a trophic cascade in which the microbial symbiont alters host plant chemistry and palatability, thereby affecting herbivores and their predators. However, experimental studies on endophytes and their effects on higher trophic levels are still scarce.

### **1.4. Effects of grass endophytes on plant performance**

Some studies have found endophyte infection to increase plant growth, seed production and the ability to tolerate stressful conditions such as drought or flooding (Latch et al. 1985, Clay 1987, Elbersen and West 1996). The effects on plant performance, however, have been partly inconsistent, and the results have been suggested to be conditional on the availability of nutrients in the soil, the plant species studied, and the fungal strains in question (Cheplick et al. 1989, Cheplick 1998, Ahlholm et al. 2002b, Hunt et al. 2005). The mechanisms of the improved tolerance by endophyte-infected grasses of stressful conditions are still mainly unclear, but have been suggested to be mainly physiological and morphological in character (Clay and Schardl 2002). Increased drought tolerance may result from altered stomatal or endophyte metabolic activities, which may change the osmotic adjustment of the host plant (Siegel and Bush 1997). Endophytes have also been suggested to reduce the feedback inhibition of photosynthesis and to enhance biomass production in plants (Clay and Schardl 2002, Spiering et al. 2006).

### **1.5. Aims of the thesis**

Although endophyte-grass symbiosis is seemingly well researched, certain aspects have received relatively little attention. For instance, endophyte studies have mainly been conducted using only two grass species – tall fescue and perennial ryegrass; endophyte-grass-herbivore interactions are mainly limited to invertebrate herbivores; the effects of endophytes on higher trophic levels are still poorly known. In particular, no study has as yet examined the effects of endophytes at the higher trophic levels of vertebrate food chains.

In this thesis I focus on these less known aspects of the endophyte-grass relationship, mainly using the *Neotyphodium* endophyte-meadow fescue study system. The effects of endophytes on meadow fescue [*Schedonorus pratensis* (ex *Lolium pratense* (Huds.) Darbysh)] are still poorly known, even though meadow fescue is widely cultivated grass species in the Nordic countries; thus knowledge of the effects of endophytes could be valuable in agricultural management. The aim of the thesis was to find answers to the following questions:

1) How common are endophytes in cultivated and wild grasses?

Frequencies of infection by endophytes were studied in 1) seven commercially-available Scandinavian meadow fescue cultivars (**I**), 2) 13 commercially-available European tall fescue cultivars (**III**), 3) 4, 5, 7 and 21 years old meadow fescue pastures grazed by livestock compared to ungrazed fringe-areas (**II**) and 4) wild tall fescue in Åland, Gotland, Södermanland and Estonia (**III**). Based on previous studies of frequencies of endophyte infection in commercially available grass cultivars (Latch et al. 1987, Saikkonen et al. 2000, Canals et al. 2008), endophytes were expected to be common in cultivars but frequencies of infection were expected to be either very high or low. It was predicted that frequencies of endophyte-infected plants are higher in grazed areas of pastures because endophytes are assumed to increase herbivore resistance of the host which in turn has been suggested to lead to increased infection frequencies in plant communities (Cheplick and Clay 1988). In natural environments the frequencies of infected plants were suggested to vary among grass populations, depending on local selection pressures (see e.g. Saikkonen et al. 2000, Wäli et al. 2007).

2) Does endophyte infection affect plant-herbivore interactions?

I studied the effect of *Neotyphodium* endophytes on 1) the number of aphids (**I**), 2) seed predation by moths (**III**), and 3) body mass, population size and mobility in voles (**IV**). In addition, based on the available literature, a meta-analysis concerning the effects of endophytes on grass-herbivore and woody plant-herbivore interactions was carried out (**V**). Since earlier studies suggest a negative effect of grass endophytes on several invertebrate and vertebrate herbivores (Siegel 1990, Saikkonen et al. 2006, Huitu et al. 2008), I predicted that endophytes would increase the resistance of grasses to the herbivorous species studied.

3) Do grass endophytes affect higher trophic levels?

I studied the effect of endophytes on preferences with regard to vole prey in the least weasel (*Mustela nivalis nivalis* L.) (**IV**). As the least weasel is known to be an olfactory hunter (Ylönen et al. 2003), I also examined whether least weasels are able to distinguish between the olfactory cues of voles fed on an endophyte-infected and endophyte-free diet (**IV**). A meta-analysis of the effects of endophytes on plant-herbivore-predator

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interactions was also conducted (**V**). Based on previous findings of negative effects of endophytes on invertebrate food chains (see e.g. Bultman et al. 1997, Omacini et al. 2001, Härrilä et al. 2009), I predicted that feeding on endophytes would have a positive indirect top-down effect on the host through the increased vulnerability of herbivores to predation. Furthermore, if an endophyte-containing diet affects the chemical composition of vole urine, as demonstrated by Huitu et al. (2008), I predicted that olfactory-hunting least weasels might be able to discriminate and prefer the scent of the weaker and more vulnerable voles fed on endophyte-infected grass as against those fed on endophyte-free grass (**IV**).

#### 4) Do endophytes affect the performance of the host grass?

I examined the effect of endophytes and nutrient availability on meadow fescue regrowth (**II**) and the effect of endophytes on seed production by the host plant (**III**). Based on previous studies demonstrating that under high nutritional conditions endophyte infection increases plant growth and seed production (Clay 1987, Stovall and Clay 1988, Sullivan et al. 2007), I predicted that endophytes would increase the regrowth of meadow fescue when nutrients were abundantly available and would improve seed production by the host grass.

## 2. MATERIALS AND METHODS

### 2.1. Meadow fescue and the endophyte

Meadow fescue [*Schedonorus pratensis* (ex *Lolium pratense* (Huds.) Darbysh)] is a perennial forage grass widely used in Finland. In the southern part of the country it also grows partly naturalized in meadows, wastelands and roadsides (Hämet-Ahti et al. 1998). Grasslands are cultivated across Finland. Domestic seed production farms produce most of the seeds used in forage cultivation in Finland. Meadow fescue seed producing farms receive the seeds from stock seed producers. Seeds are sown in early summer with nurse crops. Harvesting is conducted annually in the following three to four autumns until the restocking of the field. Annually harvested seeds of one seed producing farm constitute a seed lot. The fields for seed cultivation are commonly fertilized twice a year: in autumn after harvest (ca. 20 kg N/ha) and in spring (60-80 kg N/ha). Seeds are stored from one to nine months in grain dryers in +5 ° C –20 ° C, until collected by a supplier.

Both agricultural and wild populations of meadow fescue are commonly infected with *Neotyphodium uncinatum* (W. Gams, Petrini and Schmidt) endophyte (Gams et al. 1990, Saikkonen et al. 2000). *N. uncinatum* is a hybrid species, strictly asexual and transmitted vertically via host seeds (Leuchtman 1994, Craven et al. 2001). *N. uncinatum* produces saturated amino pyrrolizidine alkaloids within the tissues of the host. These alkaloids include loline and its derivatives, N-formylloline (NFL), N-acetylloline (NAL), N-acetylnorloline (NNL) and N-methyloline (NML) (Justus et al. 1997, Ball and Tapper 1999, Blankenship et al. 2001). NFL and NAL are highly toxic to insect herbivores and lolines cause variable responses in vertebrates, but no negative effects on livestock have been reported (Siegel and Bush 1997, Clay and Schardl 2002).

### 2.2. Tall fescue and the endophyte

Tall fescue [*Schedonorus phoenix* (Scop.) Holub.] is a close relative of meadow fescue and a native European grass species. It is an important forage grass in the USA and New Zealand, and is becoming more popular in agricultural use in Finland and Scandinavia. Wild populations of tall fescue are common in central Europe; in the north distribution is limited to southwestern Finland. Both cultivated and wild tall fescues are commonly infected by the systemic, seed-transmitted endophyte *Neotyphodium coenophialum* (Morgan- Jones and Gams) Glenn, Bacon and Hanlin (Saikkonen et al. 2000). *N. coenophialum* produces ergot alkaloids, lolines and peramines (Bush et al. 1997). Ergot alkaloids (ergovaline and related ergopeptines) have been identified as the cause of the “fescue toxicosis” and “fescue foot” syndromes suffered by livestock grazing on *N. coenophialum*-infected grass (Bacon et al. 1977, Read and Camp 1986, Ball et al.

1993). Several studies have reported negative effects of endophyte-infected tall fescue on invertebrate grazers (Saikkonen et al. 2006)

## 2.3. Animal species

### 2.3.1. Bird cherry oat aphid

Bird cherry oat aphid (*Rhopalosiphum padi* L.) is a holocyclic aphid species common in Finland. The aphid shows host plant alternation, using bird cherry (*Prunus padus* L.) as a primary host, and grasses – including meadow fescue and cereals – as secondary hosts. Bird cherry oat aphids obtain their nutrition by sucking sap from plants, and feeding may result in the transmission of plant viruses (Lehtonen et al. 2005b). Female aphids reproduce parthenogenetically on secondary hosts throughout the summer (Leather and Lehti 1982); males emerge only in the autumn, when sexual reproduction takes place on the primary host. The eggs overwinter on *P. padus* and hatch, under Finnish conditions, in May.

### 2.3.2. Cocksfoot moth

Cocksfoot moth (*Glyphipterix simplicella* Stephens) is a common seed predator of *Festuca* species in southern and central Finland and elsewhere in Europe. It may cause a serious reduction in seed production of the host grass. The larvae feed on the florets of the host plant, hollowing out the kernels of the seeds. Each larva consumes about ten seeds before becoming full grown. The full-grown moth moves down and bores a hole into the stem, where it spins a cocoon in which it overwinters. Adult cocksfoot moths hatch in the summer and lay their eggs in the florets of the host plant. The larvae hatch in 10-15 days. (Carter 1984)

### 2.3.3. Sibling vole

The sibling vole (*Microtus levis* Miller ex *M. rossiaemeridionalis* Ognev) inhabits the southern and western parts of Finland, and shows cyclic population fluctuations with a period of three years (Huitu et al. 2004, Korpimäki et al. 2005). The sibling vole lives mainly in meadows and agricultural environments, such as cultivated fields; it feeds almost exclusively on grasses. It produces 5-6 young and a total of 35-40 offspring during the summer (Norrdahl and Korpimäki 2002). Voles of boreal habitats actively use scent marks to mark territories and advertise mating quality (Viitala and Hoffmeyer 1985).

### 2.3.4. Least weasel

The least weasel (*Mustela nivalis nivalis* L.) is a small predator on the *Microtus* vole species (Korpimäki et al. 1991). Because of its specialization on voles the survival of the

least weasel is greatly dependent on vole availability, especially during the winter, when alternative prey is scarce. The least weasel density follows the densities of its main prey with a time lag (Korpimäki et al. 1991, Tapper 1979, Norrdahl 1995). Weasels use the scent signals of voles for orientation and searching and as cues for the most profitable prey (Ylönen et al. 2003).

## 2.4. Study system

### 2.4.1. Endophyte infection frequencies

Frequencies of infection by endophytes were studied 1) in seven commercially available meadow fescue cultivars (**I**); 2) in meadow fescue pastures aged 4, 5, 7 and 21 years grazed by livestock, and in their ungrazed fringe-areas (**II**); and 3) in 13 commercially available tall fescue cultivars and in wild tall fescue in Åland, Gotland, Södermanland and Estonia (**III**). Endophyte infection frequencies were detected in all three studies by staining seeds and by microscopic examination of the status of the plants (Saha et al. 1988).

*Frequencies in meadow fescue cultivars.* Infection frequencies were studied in the following meadow fescue cultivars: ‘Antti’, ‘Fure’, ‘Ilmari’, ‘Inkeri’, ‘Kalevi’, ‘Kasper’ and ‘Salten’. From these seven cultivars, 109 seed lots were examined in total. The seeds were obtained from seed-producing farms and from the Plant Production Inspection Centre, Seed Testing Department, Loimaa, Finland. Infection frequencies were studied from 25 individual plants on each farm, and in 50 seeds per seed lot received from the Plant Production Centre.

*Frequencies in tall fescue cultivars.* To determine frequencies of endophyte infections in tall fescue cultivars, ~100 seeds of 13 cultivars (‘Arminda’, ‘Barbitzon’, ‘Barcel’, ‘Barfelix’, ‘Bariane’, ‘Bonnet’, ‘Cochise’, ‘Elfina’, ‘Kora’, ‘Kord’, ‘Max’, ‘Retu’ and ‘Wrangler’) were examined for their infection status. The seeds were obtained from the Plant Production Inspection Centre, Seed Testing Department, Loimaa, Finland.

*Frequencies in pastures.* The effect of 4, 5, 7 and 21 years of grazing by dairy cows on endophyte frequencies in meadow fescue was studied. The study was accomplished in four pastures. One year after establishment of the fields, light electrical fences were built around each pasture and the cattle was introduced into the enclosures. The pastures were grazed by 15-20 dairy cows annually throughout the growing season from May to October. In August 2000 meadow fescue seeds (three seeds per plant), from 18-42 plants from the grazed and ungrazed sides of the fences of each pasture were collected, in order to estimate the endophyte frequencies of the populations.

*Frequencies in natural grass populations.* In order to study endophyte infection frequencies of natural tall fescue populations, seeds were collected from four locations

around the Baltic Sea – Åland, Gotland, Södermanland and Estonia – in the summer of 2003. Seeds were collected from five to 22 populations per location and from two to 64 plant individuals per population. Three seeds per parental plant were examined to detect the endophyte status of the host.

#### 2.4.2. Effects of endophytes on herbivores and predators

I studied the effect of *Neotyphodium* endophytes on 1) the number of aphids (**I**); 2) seed predation by moths (**III**); 3) body mass, population size and mobility in voles (**IV**); 4) least weasel preference with regard to vole prey (**IV**); and 5) olfactory cues of voles (**IV**). In addition, using the available literature, a meta-analysis of the effects of endophytes on grass-herbivore-predator and woody plant-herbivore interactions was carried out (**V**).

*Aphids.* To determine endophyte-mediated within- and among-cultivar resistance to aphids, the reproduction of *R. padi* was examined on endophyte-infected and endophyte-free plants of four seed lots of the cultivar ‘Kasper’ and two of the cultivar ‘Salten’ in a greenhouse study in 2002. The total number of plants in the experiment was 120. The plants were randomly assigned to five blocks, with four plants (two endophyte-infected and two uninfected) per seed lot (four Kasper and two Salten seed lots) in each block. When the grasses were *c.* three months old, they were placed in plastic boxes and one aphid was introduced onto one leaf of each individual plant. After two weeks the above-ground plant parts with aphids were cut, placed in plastic bags and frozen. The number of aphids on individual plants was counted and the plants were dried in an oven at *c.* 60° C.

*Moths (tall fescue).* The associations between endophytes and seed predation in tall fescue were studied in natural grass populations. The study comprised three to five tall fescue populations in Åland, Gotland and Södermanland, with 8 to 25 plant individuals in each population. Seeds were collected from the study plants and the endophyte status of each plant individual was determined from the seeds using microscopy (Saha et al. 1988). All the seeds collected from each area were combined in seed bulks of endophyte-infected and endophyte-free seeds, and 10 × 100 seeds per bulk were subjected to further analysis. The number of predated seeds was counted.

*Moths (meadow fescue).* The effect of endophytes on seed predation of meadow fescue was studied in a field experiment. The field was established in 2002 by sowing endophyte-infected and endophyte-free seeds separately in ten (five endophyte-infected and five endophyte-free) right-angle-shaped areas in a randomized complete block design. Each of the ten areas was 15 × 20 m<sup>2</sup> in size. In July 2007 seeds were collected from 5-8 meadow fescue individuals per area. The number of predated seeds was counted separately for each parental plant.

*Vole body mass.* In order to observe the effect of endophytes on individual sibling voles, I recorded the body masses of 72 voles feeding on endophyte-infected and

endophyte-free grass in a laboratory experiment. At the beginning of the experiment the voles were arranged in pairs by weight and sex. Within the pairs the voles were then randomized between endophyte-free and endophyte-infected diets. The body mass of the voles was recorded at the beginning and end of the experiment after seven days of the diet.

*Vole populations.* In order to examine the effect of endophytes on the population size of voles, voles were introduced into an experimental field and the population size was recorded after a period of four and a half months. The experiment comprised 50 male and 50 female voles. The experimental field contained endophyte-infected and endophyte-free meadow fescue in a random block design of five replicates. Each plot ( $25 \times 39 \text{ m}^2$  in size) was surrounded with a sheet-metal fence, to keep the experimental voles inside and voles of natural populations out of the experimental areas. I measured vole body mass and allocated the voles to same-sex pairs according to their body mass. Individuals within pairs were randomly assigned to either endophyte-infected or endophyte-free treatments. The voles were then released into five enclosure pairs, with each enclosure including five male and five female voles. At the end of the experiment vole population sizes were estimated using live capture traps.

*Weasel predation.* The purpose of the predation experiment was to find out whether the endophyte status of the diet affects the vulnerability of voles to least weasel predation. The experiment was conducted using 24 vole pairs, with one vole in each pair fed on endophyte-infected grass, the other on endophyte-free grass. The predation experiment was conducted in an experimental enclosure ( $10 \times 10 \text{ m}^2$ ). The enclosure was divided into six sectors, separated by short-grass belts ca. 30 cm in width and cut 2 cm above the ground surface to allow monitoring of vole mobility. A single experimental trial consisted of exposing one vole fed on endophyte-infected and one on endophyte-free grass to least weasel predation in the enclosure. I observed and recorded the number of belt crossings by the voles within the enclosure until one of the voles was captured by the least weasel (seven trials were terminated for external reasons).

*Vole excrement odour.* The purpose of the vole odour experiment was to determine whether least weasels prefer the olfactory cues of voles fed on endophyte-infected grass to those fed on endophyte-free grass. As a source of odour I used urine-soaked vole bedding material from the cages of voles that had been feeding on the experimental diet for the weasel predation experiments (see above). The experiment was carried out in an Y-maze arena (see Ylönen et al. 2003), consisting of three transparent plastic tubes 80 mm in inside diameter and 80, 60 and 60 cm in length, forming a Y. The weasel entered the 80 cm long tube and came to a bifurcation of  $\sim 60^\circ$ . Here it had to choose to continue into one of the two 60 cm tubes until it reached a target “nest box” containing vole odour at the end of the tube. Prior to each trial I randomized bedding from the same vole feeding pair between the two ends of the Y-maze. Altogether 21 weasels were tested.

### 2.4.3. *Effect of endophytes on plant performance*

I examined the effect of endophytes and nutrient availability on meadow fescue regrowth (II) and that of endophytes on seed production of the host plant (III).

*Regrowth in the field.* The effect of clipping and nutrient availability on the regrowth of endophyte-infected and endophyte-free grasses was studied in two fields established in 2002 and 2006. Five pairs of patches were seeded with endophyte-infected and endophyte-free meadow fescue seeds in a random block design in the two fields. In July 2007, four plots ( $62 \times 62$  cm<sup>2</sup>) in each of the patches were randomly assigned to the following treatments: control, clipping, fertilization, or clipping + fertilization. At the end of August, all above-ground grass parts were cut in each experimental plot, dried in an oven at 60 °C and weighed.

*Regrowth in the greenhouse.* In 2007, a greenhouse experiment was performed to determine the effect on grass regrowth of endophyte infection, nutrient availability in soils, plant density, genetic differences among plants, and their interactions. Following a randomized block design, 10 half-sib families of endophyte-infected and endophyte-free plants were assigned to the following treatments in seven replications: high and low in nutrients and high and low sowing densities (80 pots/replicate, a total of 560 pots). The number of tillers was counted and the plants were clipped with scissors three months after sowing. At the final harvest six months after sowing, the number of tillers was counted and above-ground plant parts were dried in an oven at 60 °C and weighed.

*Tall fescue seed production.* In August 2004, a total of 90 seedlings were grown in a greenhouse in a random block design: 10 seedlings each of endophyte-free and endophyte-infected plants of three wild origins (Åland, Gotland and Södermanland), ten each of endophyte-free and infected plants of the 'Kentucky-31' cultivar, and 10 of the 'Retu' cultivar (endophyte-free only). The seedlings were assigned to a field experiment in a randomized complete block design with 10 replicates. At the end of August 2007 the seeds of the plants were collected and weighed separately for each plant.

*Meadow fescue seed production.* The effect of endophytes on seed production by the host grass was studied using the same plants as in the seed predation experiments (see paragraphs on 'Moths' above). The effect was estimated by combining the amounts of filled and predated seeds separately for each host plant.

### 3. RESULTS

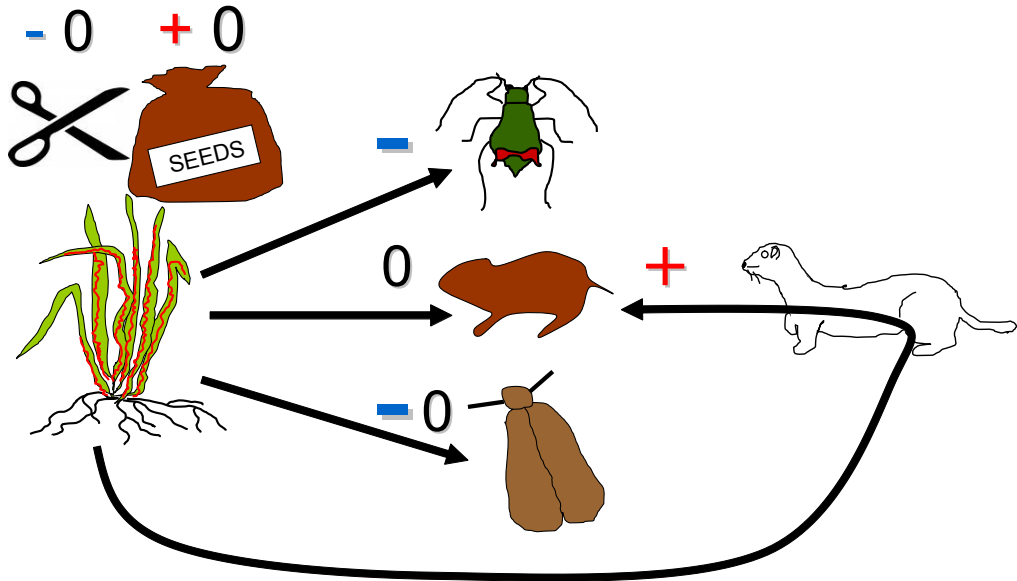
#### 3.1. Endophyte infection frequencies

Endophyte infection frequencies were variable both among and within meadow fescue cultivars (**I**). The mean frequencies of infection of the three cultivars remained below 0.10. The ‘Fure’ cultivar was uninfected, while all three seed lots of ‘Inkeri’ harboured infections exceeding 0.92. The ‘Kasper’ and ‘Salten’ cultivars showed substantial variation among seed lots in *Neotyphodium* infection rates, ranging from 0 to 0.96 and from 0.40 to 1.00 respectively. A closer examination of the ‘Kasper’ cultivar demonstrated that even plants grown from the same parental seed lot exhibited substantially different endophyte frequencies. Endophyte-infected seeds were detected in only one cultivar (‘Wrangler’) out of the total of 13 tall fescue cultivars examined, and in this cultivar the infection level was < 10% (**III**). Study **II** also demonstrated that endophyte infection frequencies and species composition may be affected by grazing: infection frequencies were found to be significantly lower in the grazed parts of pastures. In natural tall fescue populations endophyte infection frequencies were generally high and varied only slightly within and among the study locations of Åland, Gotland and Södermanland. Among the study populations of Estonia, on the other hand, infection frequencies varied from 0 to 1.0. Populations with low infection frequencies were found only inland, while all populations close to the coast were highly infected.

#### 3.2. Effects of endophytes on herbivores and predators

The studies of this thesis showed neutral, negative and positive effects of endophytes on herbivores (Fig. 3). Endophytes increased the resistance of meadow fescue to *R. padi*, overriding the effect of both seed lot and cultivar (**I**). While endophyte infection did not affect cocksfoot moth predation on meadow fescue seeds, it was negatively associated with cocksfoot moth seed predation on tall fescue in Gotland and Södermanland but not in Åland (**III**). Endophyte-infected meadow fescue reduced the mobility of voles, while no effect was detected on vole weight or population size (**IV**). Although least weasels preferentially preyed on voles fed on endophyte-free grass, they did not prefer the odour of either type of vole excrement.

According to the meta-analysis, past studies have generally suggested that grass endophytes have a positive effect on host herbivore resistance but that the effect on the herbivore is not transmitted to predators. Endophyte infection did not appear to affect the plant-herbivore interactions of woody plants (**V**).



**Figure 3.** Effect of endophytes on herbivores (bird cherry oat aphids, sibling voles and cocksfoot moths) and plant performance (regrowth and seed production). Scissors represent regrowth; seed sack represents seed production. + indicates positive, - negative and 0 neutral effect of endophytes.

### 3.3. Effect of endophytes on plant performance

Endophyte infection reduced the relative regrowth and biomass of clipped plants in the one-year-old field and greenhouse experiments, but not in the five-year-old field (**II**). Neither nutrient availability nor plant density interacted with the effect of endophytes, and the number of tillers was not affected by infection. Before clipping, the half-sib family variance in dry biomass was significantly higher in endophyte-infected grasses than in endophyte-free ones under low nutrient conditions when a couple of statistically influential outliers were excluded from the analysis. No effect of endophyte infection on half-sib family variance was detected in the final harvest.

Study **III** indicates that endophyte infection may increase the seed production of meadow fescue but does not affect that of tall fescue. There was no interaction between seed origin and endophyte infection in the biomass of tall fescue seeds.

## 4. DISCUSSION

### 4.1. Endophyte infection frequencies of wild and cultivated grasses

The results of this thesis suggest that endophyte infection frequencies may vary considerably among and within Scandinavian meadow fescue cultivars and that European tall fescue cultivars are practically free of endophytes (**I**, **III**). Endophyte infection frequency was found to be high in natural tall fescue populations (**III**). The results also suggest that grazing by cattle may affect infection frequencies in pastures (**II**).

Grass endophytes are largely ignored in European plant breeding programs. However, breeders may select for or against the endophyte infection even if they are unaware of presence of endophytes, if endophyte infection affects the host characters in which the plant breeder is interested. Indirect selection for or against endophyte-mediated plant characters can thus explain the infection levels found in meadow fescue and tall fescue cultivars. Selective breeding, however, is not necessarily associated with endophyte infection; alternatively, it may eliminate endophyte-infected plant individuals if the endophyte adversely affects desirable host characters (Saikkonen et al. 2000). Breeding programmes may also operate on plant traits that are not linked to endophyte infection, in which case the level of infection of the new cultivar depends on the infection status of the germ plasm. This could explain the differences in levels of infection among meadow fescue cultivars, but fails to explain the differences within them.

Intermediately-infected cultivars challenge previous findings suggesting either a high or a low rate of endophyte infections in cultivars of *S. pratensis* (see e.g. Saikkonen et al. 2000). Loss of infection during a long storage period may change cultivar characteristics in unexpected ways. During long or unfavourable periods of storage endophytes are known to lose their viability (Welty and Azevedo, 1985, Rolston et al. 1986, Wheatley et al. 2007); even under optimal storage conditions they appear to retain their viability for only a few years (Wheatley et al. 2007). Thus the probability of losing the infection should increase over time, and higher levels of variation in infection among seed lots should be observed in older cultivars. Endophyte infections were detected in cultivars of all ages, but the variation in frequencies of endophyte infection within infected cultivars was higher in older ones. All three seed lots of the youngest cultivar Inkeri, registered in 2003, were highly infected, whereas the frequencies of infection of the older cultivars, ‘Salten’ and ‘Kasper’, registered in 1974 and 1989 respectively, ranged among seed lots between 0.40 and 1.00 and between 0 and 0.96 respectively.

Unlike agricultural pastures and cultivars, in natural environments the frequencies of infected plants are suggested to vary according to grass species and among populations within species, depending on local selection pressures (see e.g. Saikkonen et al. 2000, Wäli et al. 2007). Study **III** demonstrated that endophyte infection frequencies of tall

fescue were high in Åland, Gotland and Södermanland. This suggests that endophyte infection may confer a selective advantage on the host close to the northernmost limit of the distribution range of some grass species. Some infection-free populations, on the other hand, were found in inland Estonia. These infection-free populations are probably the descendants of agronomical tall fescue cultivars; endophyte-free tall fescue is used in agricultural management in Europe, and the seed samples were collected close to areas under grass cultivation.

The significantly lower infection frequencies in grazed areas of pastures demonstrate that the intensity of grazing by cattle may act as major selective force affecting the endophyte infection frequencies of meadow fescue. Information as to initial infection frequencies is crucial before definite conclusions can be drawn as to the direction of the effect of grazing on infection levels. The lower infection levels found in grazed areas, however, support some previous studies indicating that dairy cattle can tolerate the alkaloids produced by meadow fescue (Siegel and Bush 1997, Clay and Schardl 2002), that they are unable to discriminate against endophyte-infected grasses, or even that they prefer feeding on endophyte-infected grasses.

## 4.2. Endophyte-herbivore interactions

In the studies of this thesis grass endophytes were found to have variable effects on plant-herbivore interactions, although most previous studies report negative effects of endophytes on herbivores (Saikkonen et al. 2006). In study **III** endophyte infection was negatively associated with seed predation on tall fescue in two of the three locations investigated, while in the case of meadow fescue no effect on seed predation was observed. Endophytes increased the resistance of meadow fescue to *R. padi*, overriding the effects of seed lot and cultivar (**I**). Furthermore, although endophyte-infected meadow fescue reduced mobility in voles, no effect on vole body mass or population size was observed (**IV**).

Although endophyte-infected grasses are commonly thought to be chemically protected against herbivores (Saikkonen et al. 2006), the following factors may account for the variable effects of endophytes on herbivores observed in my experiments. First, the mycotoxin profile varies considerably among fungal species and strains and among the genotypic combinations of fungus and host plant (Siegel and Bush 1997). In wild grasses with high genetic variation, endophyte-induced alkaloid production has been suggested to be more variable than in genetically more uniform cultivars (Faeth and Fagan 2002). Tall fescue and meadow fescue are infected with different endophyte species, which in turn produce different mycotoxins. Insects have been found to be sensitive to all of these endophyte-origin alkaloids (Schardl et al. 2004), although this sensitivity varies greatly among species (Prestidge and Ball 2002). The *Neotyphodium* endophyte of the meadow fescue studied is known to produce lolines (Lehtonen et al. 2005b); these are

known to be highly insecticidal (Siegel and Bush 1997, Lehtonen et al. 2005a, b), but only occasionally detrimental to vertebrates (see e.g. Siegel and Bush 1997, Huitu et al. 2008). Secondly, mycotoxin production is conditional on environmental conditions; negative effects of endophytes on vertebrates, for instance, have commonly been detected following extended dry and hot periods (Hemken et al. 1979, Saikkonen et al. 2006).

In grasses, seed-borne endophytes are often found to increase the herbivore resistance of the host, while the endophytes of woody plants appear to have more variable effects on plant-herbivore interactions (Saikkonen et al. 1996, Saikkonen et al. 2006, Saikkonen 2007). The results of the meta-analysis (V) support this. One should be cautious in drawing conclusions related to grass-herbivore interactions, however, since the endophyte literature appears to be biased in many ways. First, studies of grass endophytes have mostly been conducted using only two grass species, tall fescue and perennial ryegrass (*Lolium perenne* L.), and only two invertebrate herbivores, the fall armyworm (*Spodoptera frugiperda* L.) and the bird cherry oat aphid. Secondly, the literature is strongly biased toward short-term laboratory and greenhouse experiments rather than long-term field experiments, in which endophytes do not seem to affect the herbivore resistance of the host grass. The current literature, based on the study of agricultural grass cultivars under high nutrient conditions, is insufficient to capture the breadth of variability inherent in wild grass-endophyte symbiosis.

### 4.3. Effects of endophytes on multitrophic interactions

Study IV is the first to demonstrate that plant-associated microbial symbionts may affect tritrophic interactions in vertebrate communities. In the experiment, the consumption of endophyte-infected grass was associated with a reduced risk of least weasel predation in voles. While earlier studies have demonstrated that both mobility and olfactory cues predispose the prey to least weasel predation (Norrdahl and Korpimäki 1998, Ylönen et al. 2003, Banks et al. 2000), both variables failed to explain the apparent preference of weasels for voles that were fed on endophyte-free grass, regardless of the fact that endophyte consumption independently reduced mobility in voles. During the experiment, voles fed on endophyte-infected grass were often observed to freeze as an antipredator tactic (Sundell and Hyvönen 2004), while voles fed on endophyte-free grass seemed to prefer escape and ended up as prey. It is plausible that their reduced mobility was related to freezing under predation risk, thus explaining our counter-intuitive results.

All in all, studies of the effects of endophytes at higher trophic levels are still too scarce to allow any strong generalizations. The few existing studies, however, demonstrate variable effects of endophytes on invertebrate predators (V). The outcomes of grass-endophyte interactions may be conditional on the complexity of multi-trophic interactions, and it is possible that other plant properties than mycotoxins determine the quality of foliage for herbivores and their natural enemies (Wootton 1993, Abrams

1995, van Veen et al. 2006). The complex effects of endophytes on plant biochemical and nutritional properties for herbivores and their predators, for example, may vary in different environments.

#### **4.4. Effects of endophytes on the host grass performance**

In general, endophytes have been found to have variable effects on host performance. Positive effects have been pronounced particularly in environments with plentiful nutrients, while under low nutrient conditions the plant may suffer from an endophyte which consumes its nutrients (Saikkonen et al. 2006). Contrary to predictions, the results of study **II** imply that when the grass is clipped or grazed the costs of endophytes can outweigh their benefits, irrespective of nutrient availability. Endophytes significantly reduced relative regrowth and biomass in the one-year-old field and under high nutrient conditions in the short-term greenhouse experiments respectively, while no effects were detected in the five-year-old field or under low nutrient conditions in the greenhouse. These results suggest that the effects of endophytes may be linked to the ontogeny of the host. On the other hand, some positive effects of endophytes were observed in terms of higher seed production rates in meadow fescue (**III**). However, no effects were detected on seed production in tall fescue. These contradictory results underline the complexity and context-dependency of endophyte-symbiont interaction. According to the life-history theory, under some conditions competition among different plant functions may result in negative correlations among these functions (Cody 1966, Reekie and Bazzaz 1992). Previous studies suggest that the energetic requirements of systemic grass endophytes may be high enough to affect plant-internal resource allocation (Ahlholm et al. 2002b, Faeth and Fagan 2002). Increased seed production, for example, may reduce vegetative growth (Ahlholm et al. 2002b).

## 5. CONCLUSIONS

While some scientists continue to maintain the conception of grass endophytes as strong plant mutualists (Clay 2009), there is accumulating evidence to suggest that the effects of vertically transmitted endophytes on the host may vary from mutualism to antagonism (e.g. Saikkonen et al. 1998). The results arrived at in this thesis, demonstrating neutral, negative and positive effects, support the view of the endophyte-host plant relationship as complex and contradictory. The direction and magnitude of the effects were found to depend on such features as the response variable in question and the complexity of the food web. These variable results are not necessarily inconsistent with the ecological theory, which predicts that obligate endosymbionts without horizontal transmission will form mutualistic associations with the host. This occurs when selective forces in grass populations have changed over a relatively brief period and the grass populations, as long-lived plants, have not had time to respond to the prevailing pressures; thus endophyte infections may mirror previous rather than present selective forces (Saikkonen et al. 2004b, Cheplick and Faeth 2009). Alternatively, the endophyte-host grass relationship may be antagonistic if *Neotyphodium* endophytes are transmitted horizontally. Some studies have supported this by demonstrating cues of possible horizontal transmission of *Neotyphodium* endophytes, although horizontal transmission itself remains to be verified (White et al. 1996, Moy et al. 2000, Faeth et al. 2007, Cheplick and Faeth 2009).

The results of this thesis are valuable from an agronomical viewpoint because endophytes were found to affect agriculturally important characteristics of the host. In this thesis I demonstrate that endophytes may increase plant resistance to insect pests, increase seed production and reduce regrowth of the host (Fig. 3). All the effects of endophytes should be summed together to determine the realized net effect of endophyte infection for the host grass. Evaluation of the net effect is difficult because endophytes may have surprising indirect effects when higher trophic levels are included, as demonstrated in study IV. So far, endophytic fungi have been largely ignored in European agroecosystems, even though many of the pasture grasses used in the northern hemisphere are infected with endophytes. I argue that official variety trials, such as those conducted in Finland, fail to adequately capture agriculturally important variation within and among cultivars by ignoring systemic fungal endophytes.

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