Endophytic Fungi in Meadow Fescue and Other Forage Grasses

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Cover: Meadow fescue (photo 1 and 2 from left) and *Epichloë uncinata* (photo 3 and 4 from left)
(photo: A. Bylin photo 1-3, S. Card photo 4)

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Abstract

Many cool-season forage grasses associate with an endophytic fungus of the genus *Epichloë*. In Sweden one of the most important forage grasses is meadow fescue (*Festuca pratensis* Huds.), which is known to associate with *Epichloë uncinata*, previously named *Neotyphodium uncinatum*.

Since the 1970´s extensive research has been done on grass/endophyte symbioses, with focus on tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) and their fungal symbionts, due to their production of toxic alkaloids. Given the importance of meadow fescue in Sweden and the Nordic countries the aim of this thesis was to study the symbiosis between meadow fescue and *E. uncinata* and to evaluate effects that *E. uncinata* may have on meadow fescue grown in Sweden. The studies focused on differences in seed germination, winter survival and plant growth between endophyte-infected and uninfected plants, as well as effects of nitrogen fertilization on loline alkaloid production.

Knowledge about optimal seed storage conditions and uniform germination behavior can generate economic profit in seed production and for farmers. Meadow fescue seed that started to germinate early had higher frequency of viable endophytes than seed starting germination later, and heavy seed had higher germination rate and germination frequency than lighter seed. During seed storage for 3.4 years in different packaging materials and environments, seed stored in paper bags in freezer (-20 °C) had the highest seed germination and endophyte viability.

In a field cultivation of meadow fescue the effect of endophyte infection on meadow fescue during two growing seasons was studied. Plants were given either a nitrogen fertilization that is normal for the area or half that amount of nitrogen. Plants fertilized with normal amount of nitrogen produced more tillers per plant. Endophyte-infected plants produced more tillers per plant than uninfected plants, but there was no difference in total biomass production or winter survival between endophyte-infected and uninfected plants. Lolines were produced by infected plants, but the production did not differ between nitrogen fertilization levels. Lolines are known to be deterrent to some insects but are not toxic to larger mammals.

Keywords: alkaloids, endophyte, *Epichloë*, loline, meadow fescue, seed germination, seed storage, tissue-print immunoblotting, winter

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Dedication

To my father

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I A. G. Bylin, S. D. Card, W. J. Mace, D. E. Hume, K. Huss-Danell. Incidence and description of *Epichloë uncinata* in Swedish meadow fescue (manuscript).

II A. G. Bylin, D. E. Hume, S. D. Card, C. M. Lloyd-West, K. Huss-Danell. Importance of seed size and storage conditions for germination and viability of *Epichloë uncinata* in meadow fescue (submitted).

III A. G. Bylin, D. E. Hume, S. D. Card, W. J. Mace, C. M. Lloyd-West, K. Huss-Danell (2014). Influence of nitrogen fertilization on growth and loline alkaloid production of meadow fescue (*Festuca pratensis*) associated with the fungal symbiont *Neotyphodium uncinatum*. *Botany* 92(5), 370-376.

Paper III is reproduced with the permission of the publisher.

The contributions of Anja Bylin to the papers included in this thesis were as follows:

I Planned the research jointly with the co-authors, performed the experiments and wrote the manuscript.

II Planned the research jointly with the co-authors, performed the experiments and wrote the manuscript.

III Planned the research jointly with the co-authors, performed the experiments and wrote the manuscript.

Abbreviations

AA	Accelerating ageing
NAL	*N*-acetylloline
NANL	*N*-acetylnorloline
NFL	*N*-formylloline
PDA	Potato dextrose agar
RH	Relative humidity
TSW	Thousand seed weight
WA	Water agar

1 Introduction

An endophyte is an organism, usually a fungus or bacterium, that lives asymptomatically in living plant tissue for part or all of its life cycle (Wilson, 1995). *Epichloë* endophytes are common fungal endophytes that are strongly associated with cool-season forage grasses (Poaceae), which are found worldwide (Schulz & Boyle, 2005). *Epichloë* belongs to the fungal family Clavicipitaceae and has been known since the 19th century (Guérin, 1898). *Epichloë* endophytes have been shown to benefit the host grass by improving its biomass production (Latch *et al.*, 1985) and drought resistance (Malinowski & Belesky, 2000), but the endophyte is also known for its production of alkaloids that are toxic to herbivores (Cross *et al.*, 1995; Bacon *et al.*, 1977).

One of the most important forage grasses in Sweden is meadow fescue (*Festuca pratensis* Huds.), which naturally associates with *Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl. The studies included in this thesis focused on the effects of *E. uncinata* on its host grass meadow fescue.

1.1 Meadow fescue

Meadow fescue (family Poaceae) is one of about 600 species in the fescue genus (Šmarda *et al.*, 2008). It is a cool-season perennial bunchgrass that can grow to heights of 120 cm and is commonly found in cool and moist environments. Meadow fescue is native to some parts of northern Europe and Central Asia and has been introduced to other parts of the world (Rognli *et al.*, 2010). Meadow fescue occurs in almost all regions of Sweden due to cultivation and spread from cultivation, and as a wild species in the country´s northern and central areas (Julén, 1997).

In Sweden meadow fescue became commonly cultivated in the 20th century (Julén, 1997). It has good persistency and adapts well to frequent cutting, which makes it suitable for grazing and the production of hay and silage (Fjellheim *et al.*, 2009).

Figure 1. Meadow fescue (photo: Anja Bylin, 2010).

There are high degrees of genetic similarity within and between Nordic and Baltic meadow fescue cultivars (Fjellheim *et al.*, 2009). This indicates that the cultivars used today were bred from a rather small gene pool. Older, long-established cultivars are commonly based on natural populations, whereas newer cultivars are bred from a restricted number of clones or previously established cultivars (Fjellheim & Rognli, 2005; Julén, 1997).

Meadow fescue is a winter-hardy forage grass (Figure 1). The Kasper cultivar, which was investigated in many of the studies included in this thesis, is highly resistant to fungi that can damage plants during winter dormancy (Julén, 1997). Most perennial cool-season grasses require a period of cool temperatures and short day length to produce flowers in the following year. In the autumn, perennial grasses produce basal buds and tiller that overwinter, and start developing into new shoots in early stages of the following spring. Seed can be sown in either spring or late summer, but requires a moist soil with a temperature of at least 4-7 ˚C (Ehlke & Undersander, 1990).

1.2 Fungal endophytes of grasses

Most of the world’s plant species are strongly associated with an endophyte. According to Cheplick & Faeth (2009) it is very unlikely that there are any plant species that are not associated with a fungal species. There are at least 80 grass genera and several hundred grass species that are known to host endophytes (Clay, 1990). In 1904, a seed-fungus of the darnel (*Lolium temulentum* L.) was reported to systemically form extensive hyphal layers within the plant´s seeds and to be transmitted in this way (Freeman, 1904). It was also suggested that this seed-fungus may be linked to the production of harmful substances within the host plant, but there was little evidence to support this hypothesis. However, about 70 years later, researchers in the USA (Bacon *et al.*, 1977) and New Zealand (Latch *et al.*, 1984) investigated tall fescue (*Festuca arundinacea* Schreb.) infected by *Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl, and perennial ryegrass (*Lolium perenne* L.) infected by *Epichloë typhina* var. *lolii* (Latch, M.J. Chr. & Samuels) C.W. Bacon & Schardl, respectively. Both research groups independently demonstrated that these endophytes were responsible for producing alkaloids that were harmful to herbivores that fed on the host plants. The seed-fungus described by Freeman (1904) is now named *Epichloë occultans* (C.D. Moon, B. Scott & M.J. Chr.) Schardl comb. nov. (Moon *et al.*, 2000). In this thesis, the term endophyte will from now on always refer to the *Epichloë* fungi.

It was no coincidence that the harmful effects of grazing on grass infected with these endophytes were discovered in the USA and New Zealand. Over the preceding decades, both countries had experienced severe problems of domestic animals showing different symptoms of poisoning after grazing on tall fescue in the USA and perennial ryegrass in New Zealand (Bacon, 1995). Both of these grass species originate from Europe but were brought to other parts of the world with European settlers during the great emigrations of the 19th and early 20th centuries. In the USA, tall fescue was recognised for its drought resistance and was therefore very widely sown and spread. Natural occurring grasslands were resowed with tall fescue, cv. Kentucky 31. In New Zealand, land were cleared from forest when European settlers arrived and grass like perennial ryegrass was sown (Williams & Cameron, 2006). When the endophyte’s alkaloid production was discovered, people initially sought to remove it from the grass (Bacon, 1995). However, this led to lower yields due to insect pests. Therefore, subsequent efforts have focused on various ways of minimizing the grasses’ contents of alkaloids that are toxic to herbivores. Due to their economic importance, most studies on the *Epichloë*/grass symbiosis have focused on endophyte-infected tall fescue in North America and perennial ryegrass in New Zealand. However, more recently there has been increasing interest in other cultivated grasses such as red fescue (*Festuca rubra* L.) and meadow fescue, and wild grasses such as Arizona fescue (*Festuca arizonica* Vasey).

1.3 Transmission of *Epichloë* endophytes

Until recently *Epichloë* spp. (Clavicipitaceae family) was divided into two separate genera, *Epichloë* and *Neotyphodium*, with different mechanisms of transmission. However, the principles governing the nomenclature of pleomorphic fungi have since been changed such that an individual species may only have one name regardless of differences in its mechanism of transmission (Leuchtmann *et al.*, 2014). The genus *Epichloë* now includes both *Epichloë* as before, with species that are transmitted both vertically (asexually) and horizontally (sexually), and also, endophytes from the previous genus *Neotyphodium*, which transmit vertically (Schardl & Philips, 1997). Vertical transmission involves infecting grass in a way that does not cause visible symptoms (Clay & Schardl, 2002).

The fungal growth is systemic throughout the aboveground tissue but is most abundant in the leaf sheaths and reproductive structures, along with a concentration of hyphae at the base of the tillers. When an endophyte-infected seed germinates, the fungus grows between the cells into the emerging leaf sheaths and follows the culm as it elongates into an inflorescence. The fungus then grows further into the ovaries and infects the seeds that will form the next grass generation. Within the plant, the fungus grows in parallel to the long axes of the plant cells and stays in the intercellular space, close to the apoplast. In this region, it can be supplied with all of the compounds it needs to survive, such as sugars and amino acids (Clay & Schardl, 2002). In mature seeds that have been dispatched but have yet to germinate, the endophyte is located between the seed coat and the aleurone cell layer. Vertically transmitted *Epichloë* has not been found outside of its grass host in its natural habitats. Horizontally transmitted *Epichloë* spp. endophytes produce fruiting bodies and stroma with spores around the developing inflorescence of the grass (Figure 2). The resulting suppression of seed production is called "choke disease" and can partially or completely sterilize the host by inhibiting or aborting the development of its reproductive organs (Scott, 2001).


Figure 2. Epichloë elymi on bottlebrush grass (*Elymus hystrix* L.) (photo: Anja Bylin, 2010).

The hyphal content of the plant tissue varies with the season (Hume & Barker, 2005), temperature and possibly plant development (Hill *et al.*, 2005). However, it has been proposed that the hyphae in the tiller are present when the leaf forms but do not grow extensively until the leaves start to develop (Christensen *et al.*, 2008; Christensen *et al.*, 2002).

The endophyte infection is often described as systemic, which would mean that the endophyte is present in every tiller of an infected grass plant and that all seed produced by the host plant will be infected. However, there have been documented cases in which only some tillers or a portion of the seed from a given plant are infected (Saikkonen *et al.*, 2010; Afkhami & Rudgers, 2008). The endophyte may thus fail to infect all tillers and seed on the host plant and sometimes it fails to infect seedlings that develop from infected seeds; this may reflect an early loss of the endophyte, or incomplete transmission.

1.4 Alkaloids

Epichloë spp. are known to produce secondary metabolites called alkaloids. Alkaloids are chemical compounds that contain one or more nitrogen atoms. They are not essential for the organism's development, growth or reproduction however *Epichloë* spp. are well known for their production of alkaloids that are deterrent or toxic to various herbivores.

Alkaloid concentrations vary with the host plant's genotype (Hill *et al.*, 1991), from season to season, and also between plant parts (Ball *et al.*, 1997) and individual plants. The highest concentrations are normally found in young leaves and in the roots during spring time, while mature seeds have high alkaloid concentrations during late summer (Justus *et al.*, 1997). Endophyte-infected plants that have been clipped are reported to contain higher levels of loline than unclipped infected plants. Bultman *et al.* (2004) showed that clipped endophyte-infected tall fescue contained higher levels of lolines than unclipped infected plants, which is a good defense against chewing insects. This has also been observed in meadow fescue infected by either *E. uncinata* or *E. siegelii* (K.D. Craven, Leuchtm. & Schardl) Leuchtm. (Craven *et al.*, 2001). Schardl *et al.* (2007) argue that this suggests a degree of communication between host plant and endophyte. Alternatively, it may occur because clipping cuts the endophyte off from its own tip, triggering the large-scale production of lolines. However, the results of a study on endophyte-infected meadow fescue under root-attack by New Zealand grass grub larvae (*Costelytra zealandica* White) suggested that the attacks caused a redistribution of the alkaloid to the targeted part of the plant rather than a general increase in alkaloid concentration (Patchett *et al.*, 2008). If this is the case, it implies that the plant can easily relocate alkaloids without communicating with the endophyte.

To date, four major types of alkaloids have been found in the *Epichloë/*grass symbiosis: ergot alkaloids, indole diterpenes, peramine and lolines.

1.4.1 Ergot alkaloids, indole diterpenes and peramine

The most common ergot alkaloid in the *Epichloë*/grass symbiosis is ergovaline, which is extremely potent and is the primary cause of fescue toxicosis (Bush *et al.*, 1997) (Figure 3).

Indole diterpenes are tremorgenic neurotoxins known to cause ryegrass staggers. The most common is lolitrem B, which has been studied extensively in New Zealand where ryegrass staggers is a major economic problem (Clay & Schardl, 2002).

Peramine is the most common alkaloid in nature (Cheplick & Faeth, 2009), followed by the ergot alkaloids (Siegel *et al.*, 1990). The primary activity of peramine is as a feeding deterrent to insects; it has no apparent activity against mammalian herbivores. Peramine, ergot alkaloids and lolitrem B can be produced by *E. typhina var. lolii* in perennial ryegrass. *E. coenophiala* in tall fescue can produce peramine, ergot alkaloids and lolines (Ball & Tapper, 1999).

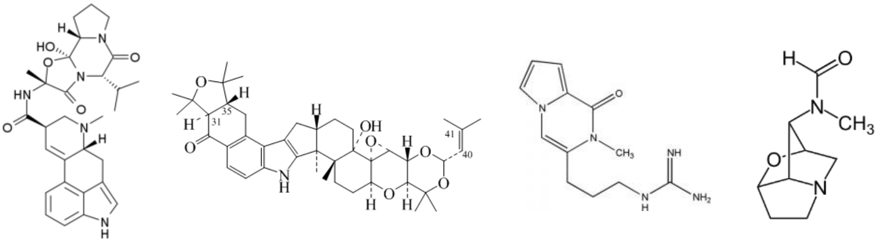

Figure 3. Molecules of ergovaline, lolitrem B, peramine and *N*-formylloline from left to right.

1.4.2 Lolines

Loline alkaloids are less widely distributed among endophyte-infected grasses than ergot alkaloids but are generally found in higher concentrations than other alkaloids (Clay & Schardl, 2002). Loline has strong anti-insect activity, with both deterrent and insecticidal (metabolic toxin) effects (Dahlman *et al.*, 1997; Bush *et al.*, 1993; Riedell *et al.*, 1991). These properties are highly desirable for pastures, in many parts of the world. Lolines are rich in nitrogen and are also often produced at high levels in uncultivated *Epichloë*/grass symbiota (Miles *et al.*, 1998; TePaske *et al.*, 1993; Siegel *et al.*, 1990) Lolines are distributed throughout the host plant, but their concentrations in the roots are usually lower than in shoot tissue (Bush *et al.*, 1993). However, even low concentrations may be sufficient to help protect against some insects (Bush *et al.*, 1997). The concentrations of lolines in endophyte-infected grasses depend on the host species and the endophyte strain (Ball & Tapper, 1999). Loline concentrations were low during winter and increased towards the flowering stage. As with the ergot alkaloids, the concentrations of the lolines vary between individual plants and tissues and also with age (Bush *et al.*, 1993).

After the initial attempts to remove all endophytes from the grass because of their alkaloid production, attention was focused on replacing endophytes that produce harmful metabolites with more desirable alternatives that are compatible with the area. For example, a beetle known as the Argentine stem weevil (*Listronotus bonariensis* Kuschel) caused severe damage to endophyte-free grasslands in New Zealand, prompting a sustained research effort to identify endophyte-infected grasses that contain insect resistance factors, but not mammalian toxins. This is achieved by identifying naturally occurring endophyte strains that produce the desired alkaloids and inoculating them into commercial grass lines.

1.5 Alkaloid effects on herbivores

Alkaloids defend the grass plant from some herbivory, which is an important defense mechanism for the plant. However, from a human perspective, this is less important than the fact that high concentrations of certain alkaloids can harm herbivores and cause economic problems in agricultural systems (Figure 4). The symptoms of cattle fed on endophyte-infected tall fescue include decreased food intake, reduced weight gain, elevated body temperature and respiration rates, very high production of saliva, poor reproductive performance (Bacon, 1995) and reduced milk production. Grazing on such grasses can also lead to fat necrosis and the loss of body parts due to restricted blood flow to extremities (Strickland *et al.*, 1996), and even death. Horses are more sensitive to alkaloids in endophyte-infected tall fescue than cattle. Common symptoms in horses from a diet containing alkaloids are increased early embryonic death rates and

Figure 4. Sheep on grassland (photo: Anja Bylin, 2003).

gestation lengths (Brendemuehl *et al.*, 1994), increased foal and thickened placentas (Porter & Thompson, 1992).

Because of these negative effects, current efforts aim to minimize or eliminate ergot alkaloids and lolitrems from commercially used grasses (Hunt & Newman, 2005) while retaining the desirable lolines and peramine. Lolines have been shown to have anti-insect activity in many different studies over the years, but it is also clear that they have no major effects on livestock and mammalian wildlife (Schardl *et al.*, 2004). Traces of lolines have been found in horse and bovine urine, indicating that the animals had digested endophyte-infected grasses. However, the loline levels in the animals' blood plasma of the same animals were too low to detect, implying that the alkaloids were cleared rapidly (TePaske *et al.*, 1993).

The most extensively studied lolines are *N*-acetylloline (NAL), *N*-acetylnorloline (NANL) and *N*-formylloline (NFL) because they tend to be the most abundant in infected grasses. NFL is usually the dominant component (Justus *et al.*, 1997). These lolines have deterrent effects on the larvae of the Japanese beetle (*Popillia japonica* Newman) at concentrations comparable to those found in tall fescue roots (about 90-300 µg g^{-1}) (Patterson *et al.*, 1991), significantly reducing larval consumption at concentrations of 100 µg g^{-1} or above. These lolines also deterred fall armyworm (*Spodoptera frugiperda* Smith) larvae and reduced larval weight gain (Riedell *et al.*, 1991). NAL also reduced weight gain in the European corn borer (*Ostrinia nubilalis* Hüber) although it was not clear whether this effect was due to toxicity, anti-feeding activity or both. There have been several studies in which the identities of the lolines responsible for the observed effects were not determined. For example, lolines have a significant negative effects on the survival of fall armyworm parasitoids (Bultman *et al.*, 1997) and are deterrent to the bird-cherry oat aphid (*Rhopalosiphum padi* L.) (Siegel *et al.*, 1990) and Argentinean stem weevil (Ball & Tapper, 1999). By increasing the host plant's resistance to these insects, its competitiveness is increased.

1.6 *Epichloë* impact on the grass host

In addition to the toxic effects of many alkaloids on herbivores, infection with asexual *Epichloë* has been shown to confer various beneficial traits on the host plant. Grass endophytes can increase the competitive ability of the host grasses by promoting plant growth given the availability of adequate resources to support both the endophyte and the host plant (Cheplick *et al.*, 1989). Ahlholm *et al.* (2002) showed that the costs and benefits of endophytes depend on the available level of resources as well as the life-history characters and resource requirements of the host and fungus. When resources were scarce, infected meadow fescue produced fewer tillers and lower root and total biomasses compared to uninfected plants. These costs and benefits were species-specific. However, these beneficial traits are subject to a high degree of variation depending on host and fungal genotype, environmental parameters, and the application of certain crop management techniques such as cutting and fertilization.

1.6.1 Growth and drought tolerance

The effects of endophytes on growth and biomass production depend on the environmental conditions. Infected meadow fescue plants have been found to exhibit greater root and shoot dry mass production than uninfected plants in several studies (Saari *et al.*, 2010; Takai *et al.*, 2010; Ahlholm *et al.*, 2002; Malinowski *et al.*, 1997a; Malinowski *et al.*, 1997b). However, in another study with endophyte-infected meadow fescue, plants produced fewer tillers and less biomass than uninfected plants in soils that were low in both nutrients and moisture but were more productive than uninfected plants in high-moisture and high-nutrient soils (Ahlholm *et al.*, 2002). The authors of the latter study suggested that the native grasses may incur appreciable costs by harboring the endophyte because their tiller, biomass and inflorescence production were all reduced under resource-limited conditions. However, in a study by Lehtonen *et al.* (2005) with different nutrient supplementations there was no difference in total plant biomass or root mass between infected and uninfected meadow fescue plants. Moreover, endophyte-infected perennial ryegrass and tall fescue produced more biomass and tillers per plant than uninfected plants (Clay, 1987). Increased root growth has been seen in perennial ryegrass and tall fescue (Schardl *et al.*, 2004; Hesse *et al.*, 2003), and endophyte-infected Italian ryegrass (*Lolium multiflorum* L.) produced more tillers, root mass and reproductive biomass than equivalent uninfected plants (Vila-Aiub *et al.*, 2005). Finally, infected meadow fescue has been found to exhibit increased persistency and competitive ability (i.e. stronger shoot and root growth, which confers enhanced drought resistance) in both monocultures and intercropping regimes (Takai *et al.*, 2010).

1.7 *Epichloë*-infected grasses in Europe

Toxicosis from tall fescue and perennial ryegrass are not widespread problems in Europe (Zabalgogeazcoa & Bony, 2005), which is quite remarkable since both of these species originate in Europe and remain widespread there. This may be due to the limited use of monocultures as opposed to species mixtures. In addition, the frequency of infection in existing European grass populations is relatively low: a seed scan covering 237 grass species collected from various places in Europe revealed that only 22 species' seeds harbored *Epichloë* endophytes (Leyronas & Raynal, 2001). These 22 species were represented by 127 samples, of which 64 were infected. Most of the infections were in ryegrass and fescue species. In Denmark, Dahl Jensen and Roulund (2004), collected perennial ryegrass from 62 locations and found endophyte infections in 48 of them. The infection frequencies at each site ranged from 4 to 82%, and more than half of the infected sites had an infection frequency of less than 20%. The sites with higher infection frequencies were generally grasslands with a history of extensive grazing by cattle or sheep. A test of 14 natural and cultivated grass species in Finland Saikkonen *et al.* (2000) revealed that 10 carried endophyte infections. Most of the infected species were fescues and hair-grasses (*Deschampsia*) spp., but infections were also observed in colonial bent (*Agrostis capillaris* L.), cock's foot (*Dactylis glomerata* L.), couch grass (*Elymus repens* L.) and timothy (*Phleum pratense* L.). In Europe the frequency of *Epichloë* spp. in meadow fescue appears to be greater in the warm, dry climates of the southern regions than in the cooler and moister northern regions (Cheplick & Faeth, 2009; Malinowski & Belesky, 2006).

1.7.1 *Epichloë* endophytes in meadow fescue

Epichloë uncinata was first described by Gams *et al*. (1990) as a loline-producing endophyte growing in the above-ground tissues of meadow fescue. Hyphae have also been found in root tips, but in very sparse quantities. *E. uncinata* is believed to be a hybrid of the sexual species *E. typhina* and *Epichloë bromicola* Leuchtm. & Schardl (Moon *et al.*, 2004; Craven *et al.*, 2001). Interestingly, although *E. uncinata* produces lolines, neither *E. typhina* nor *E. bromicola* (Leuchtmann *et al.*, 2000) produce lolines today. However, Kutil *et al.* (2007) have found indications that *E. typhina* was previously capable of loline production.

Epichloë siegelii is another fungal endophyte that can be found in meadow fescue and shares a common ancestor, *E. bromicola*, with *E. uncinata*. It has only been examined in a few studies to date, and its frequency of occurrence in meadow fescue is currently unknown. It is possible that *E. siegelii* has been mistaken for *E. uncinata* in microscopical or tissue-print immunoblot screenings because the hyphal growths of the two endophyte species are very similar. An easy way of determining which of the two *Epichloë* species is present in a given sample is to culture it and examine the conidia that are produced. Conidia of *E. uncinata* are generally “banana-shaped” (Figure 5) but can also be straight whereas those of *E. siegelii* are shaped like buns. While both *E. uncinata* and *E. siegelii* produce loline alkaloids, they produce different specific compounds in different quantities (Panaccione *et al.*, 2014; Craven *et al.*, 2001).

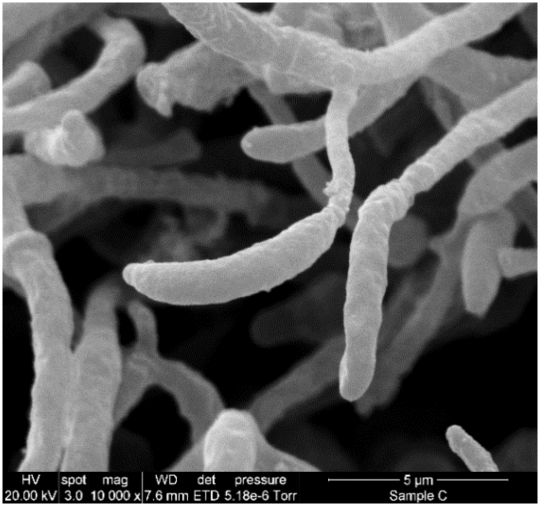

Figure 5. Conidia of *Epichloë uncinata* (photo: Stuart Card, 2011).

1.7.2 Cold temperature resistance

Large parts of Sweden experience long winters with frozen ground and heavy snow cover. Cattle, horses and sheep graze only a few months per year and are otherwise dependent on silage and hay. Lays in northern Sweden usually grow between May and September. During winter, the belowground parts of the grass plants remain alive but the leaf sheets above the soil surface die. In spring, new leaf buds then grow out from the meristem. Some plants usually die during hard winters. Because *Epichloë* spp. sometimes confers beneficial traits on its host plant, it is possible that the endophyte may help the grass host to survive the winter. Wäli *et al.* (2008) studied meadow fescue over two growing seasons in sub-arctic conditions and found that *E. uncinata* can benefit meadow fescue at this latitude, but the advantage depends on the used cultivar and environmental factors. In a plot with more nitrogen and a shorter duration of snow cover, endophyte-infected plants produced more tillers than uninfected plants. On the other hand, endophyte infection had no effect on the winter survival of tall fescue in a study performed in Wisconsin, USA (Casler & van Santen, 2008). It is important to note that the extent to which infection confers beneficial traits is very sensitive to the genotypes of the host and the fungus, as well as various environmental parameters (Wäli *et al.*, 2008; Müller & Krauss, 2005) and many studies have shown that endophyte infection can have both positive and negative effects on the host plant (Saari *et al.*, 2010; Wäli *et al.*, 2006; Hesse *et al.*, 2004; Cheplick *et al.*, 2000).

1.8 Seed harvesting and storage

The viability of fungi in mature seeds is affected by environmental conditions, particularly the temperature and relative humidity (RH). In a two year study of Italian ryegrass, Gundel *et al.* (2009) showed that high temperatures (40 °C) and RH (75%) reduced the viability of fungi during storage. However, when the seeds were stored at low temperature (5 °C) there was no significant loss of endophyte viability even at 75% RH. The same result was achieved with low RH (5%) and high temperature (40 °C). When tall fescue seed was stored at 20-30 °C for 18 months the viability of both seed and endophyte were greatly reduced (Hill & Roach, 2009), while 87% of the endophyte-infected seeds of perennial ryegrass stored at 4 °C in another study still contained viable endophytes after 12 years (Cheplick & Faeth, 2009).

The optimal time for seed harvesting is normally determined by measuring the dry matter content of the seeds, but environmental variables such as temperature, humidity and wind speed also have significant effects on seed moisture. While environmental factors have important effects on seeds, endophytes seem to respond to other factors. It has therefore been suggested that it may be better to look at the seeds' chlorophyll contents instead, since these seem to be independent of environmental conditions and correlate strongly with seed maturity (Hill *et al.*, 2005).

Many organisms experience water limitation at some point in their life cycle. LEA (late embryogenesis abundant) is a protein that is present in some plants and animals (Goyal *et al.*, 2005). LEA proteins are exceptionally hydrophilic and cause the accumulation of water during the final stage of seed maturation (Battaglia *et al.*, 2008). After harvesting, both the seed and the endophyte are affected by the stress associated with the desiccation process. Hill *et al.* (2005) suggest that both the seed and the endophyte contain LEA proteins, which protect vital organelles during periods of low water potential.

2 Objectives

The aim of this project was to gain information on the endophyte *E. uncinata* in meadow fescue and its effects on its grass hosts. An additional goal was to scan forage grasses in Sweden in order to determine the occurrence of the endophytes in the seed sold by some commercial seed distributors in Sweden. We wanted to characterize the endophyte *E. uncinata* growing in meadow fescue in terms of its morphology and alkaloid production, and to test the following hypotheses:

- Endophyte infection in meadow fescue has a positive impact on seed germination.
- Viability of *E. uncinata* decrease more rapidly than the viability of meadow fescue seed during storage.
- Infected and uninfected meadow fescue plants respond differently to nitrogen (N) fertilization.
- Endophyte infection has positive effects on meadow fescue biomass production.
- Endophyte infection has positive effect on persistence of meadow fescue during cold winters.

3 Material and methods

3.1 Grass cultivation in field and greenhouse

Two meadow fescue seed accessions of the Kasper cultivar, one Swedish and one Finnish, were sown by hand on a field at Röbäcksdalen research station at SLU in Umeå for use in the study described in Paper III. Seeds were sown in 8 rows per plot, with the rows being separated from one-another by 15 cm (Figure 6). Seeds were sown approximately 4 cm apart within each row. The plots (1.5×3 m) were fertilized with one of two nitrogen levels: 100 kg N ha^{-1} $year^{-1}$, which is the normal rate for the studied soil type and region (N-normal), and 50 kg N ha^{-1} $year^{-1}$(N-low). Plots were replicated in a row with alternating Swedish and Finnish Kasper seed accessions and the two nitrogen fertilization levels.

Figure 6. Plots sown with meadow fescue at Röbäcksdalen research station, Umeå (6A). Individual marking of meadow fescue plants (6B) (photo: Anja Bylin, 2010).

For grass accessions grown in the greenhouse, seeds were sown in trays filled with soil or a 50:50 sand:peat potting mix and watered as needed. Plants were allowed to grow until at least a two tiller stage before being tested for endophyte infection.

3.2 Methods used to detect *Epichloë* in grasses

Epichloë endophytes do not produce any symptoms in the host grass that are visible to the human eye other than the occasional production of stroma by the fungus in some grass species. However, several methods have been developed to detect the fungi. In this study we used microscopy (3.2.1), tissue-print immunoblotting (3.2.2) and isolation of *Epichloë* (3.2.4)

3.2.1 Microscopy (Papers I-III)

Microscopy is an easy way to examine plant tissue for endophyte infection. Samples were prepared by peeling off the outer leaf sheath layer and cutting the tiller into 3 mm pieces. The inner leaf sheath layers were then placed flat on a microscopy slide, stained with aniline blue solution (2 parts glycerol, 1 part lactic acid, 1 part water, and 0.06% aniline blue) and covered with a coverslip. If needed, the slides were heated quickly over a candle light, which makes the color penetrate the tissue more rapidly, before being examined using a light microscope (200-400× magnification).

In order to identify endophyte-infected seeds (Figure 7), seeds were soaked in 5% (w/v) NaOH overnight and then rinsed five times in tap water and heated to boil in Garner's solution (2 parts water, 1 part lactic acid, 0.2% aniline blue). The glomes and pericarp were removed and the seeds were crushed on a microscope slide (S. Card, personal comm. 2010).

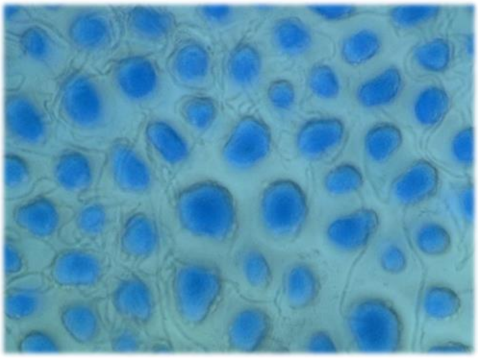

Figure 7. Epichloë uncinata between aleurone cells in a meadow fescue seed (photo: Anja Bylin, 2009).

3.2.2 Tissue-print immunoblotting (Papers I-III)

Enzyme-linked (ELISA) techniques that use antibodies to detect specific proteins became available during the 1980´s. Adapted variants of this approach known as tissue-print immunoblot assays can be used to detect endophytes (Koh *et al.*, 2006; Hiatt *et al.*, 1999). The principle of this method is that proteins from the cell walls of *Epichloë* are extracted and bound to a nitrocellulose membrane by pressing the tiller against it. The membrane is then treated with monoclonal antibodies specific to different epitopes of the *Epichloë* protein to facilitate its detection.

Two methods of immunological endophyte detection were used in the studies presented herein: the “Phytoscreen Field Tiller Endophyte Detection Kit” (Cat. #ENDO797-3) described by Hiatt *et al.* (1999) and distributed by Agrinostics Ltd. Co., Watkinsville, GA, USA (Figure 8), and a similar technique described by Simpson *et al.* (2012) that is currently used at AgResearch Ltd., New Zealand. The kit from Agrinostics Ltd. is designed for use with cross sections of fresh tall fescue tillers, however when tested on red fescue, meadow fescue, sheep´s fescue (*Festuca ovina* L.) and altai fescue (*Festuca altaica* Trin.), the results obtained using this kit were in perfect or very good agreement with those obtained by visual inspection (Koh *et al.*, 2006). The results obtained when using the kit to analyze fresh, dried and fixed tillers of red fescue were also in complete agreement with those obtained by tissue-print immunoblotting and visual inspection.

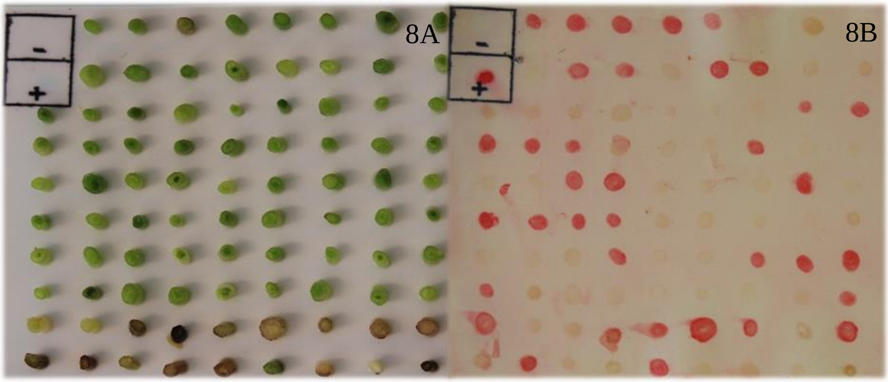

Figure 8. Meadow fescue tillers on nitrocellulose tissue-print immunoblot membrane from Agrinostics Ltd. (8A) and the same membrane after development (8B) (photo: Anja Bylin, 2010).

In this study, we placed cross-sections of tillers on a nitrocellulose membrane overnight and soaked them in a buffer solution to extract *Epichloë* proteins, which then bound to the membrane. Pooled monoclonal antibodies were then added, along with anti-monoclonal antibodies, protein-A, and an alkaline phosphatase enzyme conjugate, to catalyze chemical reactions. Finally, a red chromogen solution was added to visualize the complex. Dark pink dots developed on the membrane at spots where endophyte-infected tillers had been placed. In contrast, the AgResearch method only requires that the tiller cross-sections are pressed against the membrane for one second before being removed. Endophyte infection can be detected in seedlings that aged 7 days or more when using the Agrinostics kit (Koh *et al.*, 2006).

Tissue-print immunoblotting can also be used with seed samples, but this requires sterilization of the seed coat surface in order to reduce the risk of false positives from different epiphytes.

3.2.3 Tissue-print immunoblotting: method development

The tissue-print immunoblot cards sold by Agrinostics Ltd. for endophyte detection are designed for use with fresh grass tillers. In some countries where winters have high levels of snow cover, it is not always possible to obtain large quantities of fresh material for testing. Therefore, a preliminary study was conducted to see whether it was possible to obtain reliable results using this kit with stored meadow fescue tillers. Meadow fescue, cv. Kasper, was sown in pots of soil and placed in a greenhouse. When the plants had reached the three tiller stage, one tiller was marked and cut off close to the soil surface. The lowest cm of the tiller was then cut off and placed in a freezer at -20 °C, after which the remainder of the tiller was dried at 60 °C for 24h. After three weeks, the marked tillers had grown out again. One fresh, one frozen and one dried piece of the tiller were placed on the immunoblot card and the membrane was developed according to the manufacturer’s instructions. Identical results were achieved with fresh and frozen tillers, but lower levels of infection were observed for dried tillers.

3.2.4 Isolation and growth of fungi (Paper I)

Endophyte cultures were isolated from the basal parts of tillers. Tiller bases were harvested and surface sterilized by dipping in 95% ethanol, soaked in 10% commercial bleach for 1 minute and rinsed in sterile water three times. The stems were then placed on sterile filter paper, cut into 1 mm pieces and placed on potato dextrose agar (PDA) plates. PDA from Sigma-Aldrich was prepared according to the manufacturer’s instructions, but with the addition of 0.001 g/litre chloramphenicol to the solution

Figure 9. Epichloë uncinata growing out of meadow fescue tiller (photo: Anja Bylin, 2009).

before sterilized. The plates were then sealed with Parafilm, and incubated inverted in a dark cabinet at 21 ˚C. Plates were regularly examined for contamination and endophyte growth. When endophyte hyphae formed cultures on the PDA plate, small blocks of mycelia from the periphery of the colony were transferred to fresh PDA. Colony formation took around three weeks, but hyphae from different *Epichloë* species and genotypes grow at different speed after switching to growth outside the host plant, and hyphae could be seen after a few days of incubation (Figure 9).

For production of spores, cultures were prepared as described above but small pieces of hyphae were transferred to water agar (WA) plates. WA was prepared according to the manufacturers' instructions.

3.2.5 Alkaloid analyses (Papers I & III)

Samples from the basal parts of the tillers were freeze dried, milled and mixed with methanol/ammonium and dichloroethane. The mixtures were centrifuged and filtered into gas chromatography vials before being analysed using a gas chromatography-flame ionization detector (for details see paper III). Samples were screened for the three lolines NFL, NANL and NAL.

3.2.6 Identifying the optimal method of detection

Method selection will initially be dictated by one's available equipment. All of the methods discussed above have unique advantages and disadvantages. Microscopy and growing cultures can be time consuming, while tissue-print immunoblotting can be expensive and dependent on one's access to the necessary materials. Both tissue-print immunoblotting and microscopy can yield false positives and/or fail to detect endophyte infections. For example, Dombrowski *et al.* (2006) got 20% more positive results when studying endophyte infections in Italian ryegrass using the tissue-print immunoblotting kit from Agrinostics Ltd. than when using microscopic detection. The higher rate of endophyte infection detected using tissue-print immunoblotting may be due to false positives arising from a cross-reaction due to the contamination of the fungal protein extract with the antibodies used in the assay. In a study on the antibody specificity to *Epichloë* spp. of the antibodies in the tissue-print immunoblot kit from Agrinostics Ltd., Jensen *et al.* (2011) found that several other fungal species could give positive results. However, there is little risk of misidentifying other endophytes such as pathogenic fungi for *Epichloë* when using microscopy (Sugawara *et al.*, 2004). On the other hand, if the hyphal infection in the tiller is sparse, the endophyte infection may be overlooked when using a microscope, leading to false negatives and an underestimation of the endophyte infection frequency. To be on the safe side, it is best to combine two methods, for example by growing cultures and using microscopy, if time allows. However, Hahn *et al.* (2003), prepared antiserum with *E. uncinata* mycelium themselves and compared microscopic detection with tissue-print immunoblot on meadow fescue, tall fescue and red fescue; their results indicated that both methods provided similar results.

3.3 Seed storage (Paper II)

In order to study seed and endophyte viability after storage, meadow fescue seed, cv. Preval, distributed by Forsbecks AB, Sweden, was stored in four types of packaging (glass jar, plastic bag, paper bag and cloth bag) at three locations with different temperature and RH values (Table 1). The initial seed moisture content was 10.5% and the seed was stored for 2.2 years (26 months) or 3.4 years (41 months).

Table 1. *Temperature and relative humidity (RH) during seed storage.*

Location	Temperature (˚C)	RH (%)
Room	21	24
Refrigerator	10	34
Freezer	-20	74

After 2.2 or 3.4 years' storage, the seed was sown in soil-filled trays and placed in a greenhouse (15-20 ˚C, 50-70% RH). After 8 and 12 weeks, respectively, the tillers were tested for endophyte infection using the tissue-print immunoblotting kit developed by AgResearch Ltd.(Simpson *et al.*, 2012).

4 Summary of results

4.1 Paper I

Meadow fescue seeds originating from 28 accessions collected in Sweden and Norway were obtained from the Nordic Genetic Resource Center (NordGen) in Alnarp, Sweden. One commercial meadow fescue seed line, cv. Kasper, distributed by Forsbecks AB, Skänninge, Sweden was also included. Of the 29 meadow fescue cultivars, 12 had endophyte infections. The mean infection frequency was 43%, with a range of 2 to 98%. Nine of the endophyte-infected cultivars were tested for loline production in tillers and placed on PDA plates to study the endophytes' morphology. All of the tested plants contained the three tested loline alkaloids NFL, NANL and NAL, but their concentrations varied widely. The highest concentrations were observed for NFL (about 17,000 µg g^{-1} dry matter) and the lowest for NAL (about 1,300 µg g^{-1} dry matter).

E. uncinata isolates from nine cultivars were grown on PDA plates for four weeks and their mean culture sizes were measured. The mean radius of eight isolates ranged from 4.5 mm to 7.7 mm, while the largest culture had a mean radius of 18.8 mm. To study the conidia of each isolate, small pieces of their hyphae were transferred to WA after eight weeks' growth. Conidia were formed in seven accessions; all of them produced curved conidia that were between 4 and 7 µm long.

4.2 Paper II

In this paper we studied the viability of seeds and endophytes from endophyte-infected meadow fescue (cv. Kasper) that is sold commercially in Sweden. Both untreated seed and seed that had undergone accelerated ageing (AA) were divided into six weight classes. The study's objective was to investigate seed viability in both untreated and AA seeds of different sizes and to determine whether endophyte infection affected seed germination. The second part of this study focused on the viability of meadow fescue seed and endophytes after storage in different packaging materials and environments.

4.2.1 Seed and endophyte viability

The mean seed weights for the six classes ranged from 6.6 g to 14.3 g. Seed viability increased with seed weight. In each weight class, seed viability was lower for seeds that had undergone AA than untreated seeds (P=0.003). Untreated seeds also exhibited a stronger germination response over time than AA seeds (P<0.001) in that AA seeds germinated later than their untreated counterparts.

Microscopic analysis revealed that the total endophyte infection frequency across all accessions was 73%. There was a non-significant tendency (P=0.057) for total endophyte frequency to increase with seed weight. The viable endophyte frequency was 19% and there was a small, but statistically significant decrease in viable endophyte infection rates with increasing seed weight (P=0.026).

4.2.2 Seed storage

The initial seed viability was 92%, but decreased during storage. After 2.2 years, the average seed viability was 82% while after 3.4 years it was only 52%. There was however considerable variation between storage locations. Seed viability decreased most rapidly under room conditions while freezing or refrigeration prolonged seed viability. The different tested packaging materials had no major effect on seed viability.

Endophyte viability decreased rapidly in all storage environments, going from an average of 29% at the start of the study to 3% after 3.4 years. The endophytes were most durable in freezer storage, while seed stored in room or refrigerator conditions lost almost all viable endophytes. Storage in paper or plastic bags yielded the highest endophyte viability.

4.3 Paper III

A field study was conducted to determine whether *E. uncinata* has any impact on the survival and regrowth of meadow fescue after a cold winter. Two meadow fescue cultivars, one Swedish Kasper and one Finnish Kasper were used. To investigate the effect of nitrogen fertilization on biomass production and loline alkaloid production, two fertilization regimes were evaluated: one using a level of added nitrogen that is typical for the studied soil type and region (100 kg ha^{-1}) and a low nitrogen regime using half the normal amount of added fertilizer (50 kg ha^{-1}).

4.3.1 Biomass

The infection frequency in Swedish meadow fescue, cv. Kasper was 28% while that in the Finnish meadow fescue, cv. Kasper was 33% (P=0.086). Plants grown with a normal level of nitrogen fertilization produced more tillers than plants grown under the low nitrogen regime. Endophyte-infected plants produced slightly more tillers than uninfected plants (P=0.030).

4.3.2 Loline production

The concentrations of the lolines NFL, NAL and NANL were measured in a total of 38 endophyte-infected plants. The highest concentrations were observed for NFL and the lowest for NAL. There were no statistically significant differences between the total loline concentrations produced by plants grown under the normal and low nitrogen fertilization regimes.

4.3.3 Mixed infection in tillers

It was suspected that the meadow fescue plants might have a mixture of infected and uninfected tillers. To test this hypothesis, we performed a study to determine the extent of this so-called mixed infection, which occurs because of imperfect transmission. Meadow fescue plants of the Kasper cultivar were collected from a field at Röbäcksdalen research station in Umeå. A total of 120 plants (60 Swedish Kasper and 60 Finnish Kasper) were collected at the end of the first growing season. The tillers on each plant were counted and seven tillers per plant were selected at random and tested for endophyte infection. If a plant exhibited mixed infection, all of its tillers were tested. Swedish Kasper had an infection frequency of 42% with two plants showing mixed infection (~3%); the infection frequency for Finnish Kasper was 38%, with five plants showing mixed infection (~8%). The mean number of tillers on Swedish Kasper was 12.6 while that for Finnish Kasper was 11.6.

4.4 Swedish grasses examined for presence of endophytes

In this PhD project some additional forage grasses to the seed accessions described in paper I-III have been tested for endophyte infection (Table 2). These are both cultivars available on the Swedish market and accessions in breeding programs. The number of infected species and cultivars was relatively low: only 6 out of 82 accessions had viable endophytes.

Table 2. *Swedish forage grasses tested for endophyte infection frequency.*

Grass host	infected/tested accessions	mean infection	infection range
Meadow fescue	5/41	39	3-91
Timothy	0/4	0	0
Red fescue	0/4	0	0
Perennial ryegrass	1/18	83	83
Tall fescue	0/12	0	0
rye fescue* (*Festulolium*)	0/3	0	0

* Hybrid between Italian ryegrass and tall fescue.

While there have been relatively few observations of *Epichloë* endophytes in cultivated grass species in Sweden, the endophyte has been detected in several wild species. These include colonial bent (*Agrostis capillaris* L.) (Bazely *et al.*, 2007), Scandinavian small-red (*Calamagrostis purpurea* Trin.) (Wennström, 1996), wavy hair-grass (*Deschampsia flexuosa* L.) (Bazely *et al.*, 2007), sheep´s fescue (Granath *et al.*, 2007; Koh *et al.*, 2006), red fescue (Saona *et al.*, 2010; Bazely *et al.*, 2007; Granath *et al.*, 2007), viviparous sheep´s fescue (*Festuca vivipara* L.) (Granath *et al.*, 2007) and rough-stalked meadow-grass (*Poa trivialis* L.) (Bazely *et al.*, 2007).

5 Discussion

5.1 *Epichloë uncinata* in meadow fescue

This thesis is the first time the important forage grass meadow fescue has been studied in Sweden with respect to its association with the endophytic fungus *E. uncinata*. Paper I showed that the morphology and physiology of *E. uncinata* in the studied isolates varied widely. Even from within the same grass cultivar, the endophyte isolates occasionally had different growth patterns. Similar results have been obtained for *E. typhina* var. *lolii* (Christensen & Latch, 1991) isolated from perennial ryegrass. Different endophyte genotypes can have different effects on the host grass in different environment (Cheplick & Faeth, 2009). To truly understand the potential impact of the *Epichloë* endophyte on meadow fescue, it would be necessary to study one endophyte genotype in one meadow fescue genotype. However, because we wanted to study the infection frequency and effects of *Epichloë* on meadow fescue in situations similar to those that occur on agricultural fields, we examined commercially available meadow fescue cultivars.

Seed distributors in Sweden do not routinely test their grass seeds for endophytes. This is partly because Swedish farmers are less concerned by the presence of endophytes in seeds than are farmers in countries where monocultures of endophyte-infected grasses have caused severe suffering in livestock and economic losses. Moreover, Sweden does not suffer from problems with insect herbivores to the extent that for example New Zealand has with the Argentinean stem weevil. Therefore, Sweden has had no particular reason to establish national guidelines concerning seed storage in order to maximize seed viability and also endophyte viability in cases where the latter is desirable. In paper II we studied seed and endophyte viability after storage at three different temperatures and relative humidities. The hypothesis that the endophyte viability declined faster than meadow fescue seed viability was correct. It was very clear that storage at room temperature and high relative humidity caused endophyte viability to decline more rapidly than storage in cooler conditions. Storage at 5 °C or below has previously been recommended in order to preserve endophyte viability in perennial ryegrass seeds (Rolston *et al.*, 1986); this recommendation also seems to be applicable for meadow fescue.

Meadow fescue seed have a TSW of about 2.0g. The results presented in paper II show that heavier seeds had higher speed and frequencies of germination. Seed that germinate rapidly and seedlings that have uniform growing patterns are preferred in agriculture and breeding programs. Removing lighter seeds during commercial seed production may provide improved germination statistics but this would have to be balanced against the concomitant reduction in seed yields.

In paper II seed were treated with an accelerating aging method previously used for perennial ryegrass (Card *et al.*, 2014), but all endophytes lost viability in the meadow fescue. This study shows the importance of testing different endophyte infected grass species by themselves and not draw conclusions that all cool-season grasses infected with *Epichloë* behave in the same way. This accelerating aging method, commonly used for perennial ryegrass, was too harsh for *Epichloë uncinata* in meadow fescue. In seed not treated with the accelerating aging method, the endophyte had a positive impact on meadow fescue seed germination. A higher proportion of seed that germinating early were endophyte infected. Seed that germinates early have a competitive advantage over seed that germinates later.

Paper III, reported a study on the potential impact of *E. uncinata* on meadow fescue in cold environments. Because almost all of the nearly one thousand studied plants survived the winter, which had favourable weather for plants as the snow cover came early and lasted for a long time, it was not possible to assess the hypothesis that *E. uncinata* has positive effects on the persistence of meadow fescue during cold winters. However, winter conditions can vary from year to year, so it would be worthwhile to study winter survival over several years. It would also be interesting to include other grass species such as perennial ryegrass or tall fescue in the study. While these species are not currently capable of surviving the harsh winters of northern Sweden, they are becoming increasingly popular in other parts of the country. Because of breeding programs they can now be grown for at least two growing seasons in areas up to latitude 60°N.

In the field study described in paper III, biomass production of endophyte-infected and uninfected meadow fescue plants was evaluated in different nitrogen fertilization levels. While endophyte-infected plants produced more tillers per plant, neither fertilization level nor endophyte infection status had any statistically significant impact on the total biomass production.

As expected, the alkaloids we found in meadow fescue were lolines, the most abundant of which was NFL. However, very different mean loline concentrations were obtained in papers I and III. Given that these studies examined different host cultivars that were grown in different environments and harvested for alkaloid analysis at different stages of development in different seasons, it is not unexpected that their NFL and NANL concentrations differed. The mean loline concentration was much higher in young plants grown in greenhouses in New Zealand than in mature meadow fescue plants grown in fields in Sweden. This shows that even if we know that a plant is infected, it is not possible to reliably estimate its loline concentrations without analysis.

5.2 Alkaloids in forage grasses common in Sweden

Although the only alkaloids found in *E. uncinata*-infected meadow fescue were various lolines, which are not harmful to livestock, it is important to be aware that other endophytes and alkaloids may be introduced to Sweden along with imported perennial ryegrass, tall fescue and their hybrids.

There have recently been some reports of ill health events in Swedish horses, and suspicions that endophyte-infected grass has caused these problems (Bendroth, n/a). These events of ill health have yet to be explained, but the symptoms in many cases resemble those of fescue toxicosis (Darenius *et al.*, 2011). It may never be known whether these problems had anything to do with horses feeding on endophyte-infected fodder, but overall there have been very few incidents reported of livestock showing symptoms similar to those of endophyte toxicity in Sweden or other European countries. This may be because in Sweden forage grasses are seldom grown in monocultures. The mixtures of different grasses and legumes used in Sweden and other countries may “dilute” toxic alkaloids to less harmful concentrations.

Infected native grasses grown in their natural habitat have proven to be less toxic to herbivores than cultivated tall fescue and perennial ryegrass. This may be because soils high in nitrogen such as those on agricultural land can trigger increased alkaloid production (Cheplick & Faeth, 2009). For example, Lyons *et al.* (1986) found that treatment with nitrogen fertilizer significantly increased the ergot alkaloid concentrations in greenhouse-grown tall fescue.

5.3 Lawns in public areas

Many birds naturally rest on open grasslands, either to graze or to eat the worms and insects that are found there. Unfortunately it is undesirable for birds to inhabit golf courses and recreation areas in towns and cities, as they may cause huge amounts of damage. Moreover, this behaviour can be life-threatening at airports due to the risk of collisions between birds and aeroplanes that are landing or taking off (‘bird strike’). Thousands of bird strikes are reported every year; in Sweden, they are most common between April and June, when migrating birds return to the country and need to rest after a long flight. In other parts of the world, endophyte-infected tall fescue have been planted around airports and have significantly reduced the frequency of bird strikes by repelling birds from the area (Pennell *et al.*, 2010). However, existing tall fescues are not sufficiently winter-hardy to be used in this way in northern Sweden, and it is not realistic to resow grasslands at airfields every year.

5.4 New climate

The usage of perennial ryegrass, tall fescue, and rye fescues (*Festulolium* spp.) has been increasing in Sweden. Rye fescues are created by crossing meadow fescue or tall fescue with either perennial or Italian ryegrass. New grass species are welcome in Sweden, but it is important to be aware of the endophyte infections that may come with them. It would not take long for seed distributors to test for endophytes when importing a new grass species or cultivar, and the process need not be expensive relative to the potential cost of using grasses with endophytes that produce alkaloids that are harmful to livestock.

One of the original purposes of the study presented in paper III was to determine whether meadow fescue plants infected by *E. uncinata* exhibited any enhanced degree of winter-hardiness, compared with uninfected meadow fescue. This turned out not to be possible, because essentially all of the infected and uninfected plants survived the winter in this study. However, it would be interesting to do a similar study with other forage grasses. Until recently, perennial ryegrass was exclusively regarded as an annual crop in Sweden. However, breeding programs focused on winter survival have yielded plants that can be grown in southern Sweden for at least two years and with expected changes in climate that period may increase. It is currently not clear how the country’s climate will change in the coming years. Many different scenarios are possible according to the Swedish Meteorological and Hydrological Institute (SMHI) but all of them involve rising temperatures and increased precipitation, particularly in northern regions. Previous studies on *Epichloë* endophytes have shown that they can help the meadow fescue host grass withstand snow mold (*Typhula ishikariensis* Imai) infection (Wäli *et al.*, 2006). Snow mold need a moist environment and a snow cover to grow. With increased precipitation it is possible that mold and similar diseases that cause winter damage thrive. Several studies have demonstrated that endophyte infection can increase the drought-tolerance of perennial ryegrass and tall fescue. Even if levels of precipitation are about to increase in Sweden due to a changing climate, there may still be periods of soil moisture deficit during spring when the ground is no longer covered with snow but temperatures are low and the wind is strong. Therefore, it would be interesting to do a new study focusing on winter survival, using endophyte-infected and uninfected perennial ryegrass and tall fescue in different parts of Sweden.

6 Conclusions and future perspectives

Given the extensive cultivation of meadow fescue around the world, there is a surprising lack of knowledge about the positive and negative effects that *Epichloë* spp. may have on the grass in colder climates. Most studies that have been reported to date have focused on the effects of nutrient availability and alkaloid toxicity (Lehtonen *et al.*, 2006; Wäli *et al.*, 2006). For example, little is known about loline effects on insects common to northern Europe. It would therefore be desirable to investigate the effects lolines and peramine have on insects' common to northern Europe, especially as temperatures are expected to increase and insects may migrate further north.

During recent years, perennial ryegrass and tall fescue have become increasingly popular in forage grass mixtures in the south of Sweden. Under appropriate conditions, perennial ryegrass can produce more biomass than meadow fescue. However, there are no regulations that require the determination of endophyte infection frequencies in grass seeds before they are imported into Sweden; instead, it is left to the commercial seed companies to decide how to test new seeds before selling them on the Swedish market. This would be simplified if there were facilities to easily and accurately screen a seed lot for endophyte infections. Also, it would be desirable to have easy access to analyses of toxic alkaloids in both fresh and conserved grasses.

In this study *Epichloë uncinata* did not show any major effects on meadow fescue, it is however interesting that the superior cultivars of meadow fescue on the Swedish market during many years were endophyte-infected. This may indicate that there are aspects of this symbiosis that are still to discover.

7 Populärvetenskaplig sammanfattning

I Sverige är vi vana att se gräs i trädgårdar och parker, på ängar, odlade vallar och i skogarna. Gräsen utgör rekreationsplatser för människor och föda för många olika djur. Vad vi inte alltid kan se är alla de mikroskopiska organismer som lever på och i gräsen. En av dessa organismer är svampendofyten *Epichloë*. Ordet endofyt innebär att det är en organism, vanligen svamp eller bakterie, som lever inuti en växt. Endofyter i gräs blev kända på 1970-talet då forskare i USA och Nya Zeeland upptäckte sambandet mellan sjuk boskap och endofyters produktion av kvävebaserade ämnen, så kallade alkaloider. Under de årtionden som följt har man främst studerat *Epichloë coenophialum,* som kan infektera rörsvingel (*Festuca arundinacea* L.) och *Epichloë typhina* var. *lolii* som kan infektera engelskt rajgräs (*Lolium perenne* L.). Anledningen till att just dessa endofyter och gräsarter har varit i fokus är främst deras produktion av alkaloider som är giftiga för kor, hästar och får.

Ett av Sveriges vanligaste vallgräs är ängssvingel (*Festuca pratensis* Huds.). Det är ett härdigt gräs i större delen av landet och ger bra skördar. Den endofytiska svampen *Epichloë uncinata* (tidigare *Neotyphodium uncinatum*) förekommer i ängssvingel. *E. uncinata* producerar alkaloider ur gruppen loliner. Loliner är inte giftiga för större gräsätare såsom boskap, men verkar avskräckande på somliga insekter.

Arter av *Epichloë,* lever i ovanjordiska växtdelar. De kan reproducera sig antingen horisontellt, via sporer som sprids till plantor i närheten eller vertikalt, då endofyten växer i skotten på en infekterad planta, in i blomställningarna och vidare in i de frön som bildas. *Epichloë uncinata* i ängssvingel sprids bara via frön. Normalt blir alla frön på en infekterad planta infekterade, men ibland kan några frön förbli oinfekterade. Dessa frön ger då plantor som saknar endofyten.

Denna avhandling beskriver hur ängssvingel påverkas av att vara infekterad, med fokus på frögroning, respons på kvävegödsling och vinteröverlevnad. Alkaloidproduktion och hur man förvarar fröna på bästa sätt när man vill behålla vitalitet hos både fröet och endofyten har också studerats.

Eftersom endofyten hos ängssvingel lever hela sitt liv inuti växten bildas inga symptom som vi kan se med blotta ögat. För att lokalisera *Epichloë* i gräsplantorna har vi främst använt mikroskop och immunoblotting. Immunoblotting är en metod där man låter proteiner i endofyten utsöndras och fästa vid ett membran. Genom att sedan behandla membranet med olika antikroppar och färgämne framträder märken på membranet där proteinerna har fäst. I den mån det är möjligt rekommenderas att skottbasen av färskt gräs används för denna analys, men man kan även använda frysta skott, vilket kan underlätta när ett stort antal plantor ska skördas och analyseras samtidigt.

Att förvara frön så att man får en hög och synkroniserad frögroning eftersträvas av både praktiska och ekonomiska skäl. En frögroningsstudie visade att det fanns en högre andel endofytinfekterade frön bland frön som grodde tidigt jämfört med frön som grodde sent. Ju högre tusenkornsvikt fröna hade desto snabbare grodde de och tyngre frön hade även en högre grobarhet. För att bibehålla en hög vitalitet hos både frö och endofyter bör man helst förvara endofytinfekterade ängssvingelfrön i papperspåsar i frys.

Ett av målen med denna avhandling var att studera endofytens eventuella inverkan på vinteröverlevnad hos ängssvingel i norra Sverige. Närmare 1000 plantor i fält markerades och följdes under två vegetationsperioder. Det visade sig att endofytinfekterade plantor producerade fler skott än oinfekterade, men det var ingen skillnad på den totala biomassaproduktionen. Vinteröverlevnaden var mycket god både hos endofytinfekterade och oinfekterade plantor under den gynnsamma vintern med tidigt och långvarigt snötäcke. De endofytinfekterade plantorna producerade loliner i olika koncentrationer, men inga andra alkaloider påvisades.

I Sverige har vi ännu inte haft några bekräftade fall av alkaloidrelaterade skador på boskap. Det kan delvis bero på att fodergräsen i Sverige oftast odlas i en mix tillsammans med andra gräs och klöver. Odlar man enbart ett gräs i monokultur, till exempel rörsvingel, och det är endofytinfekterat kan boskapen få i sig höga halter alkaloider. Delvis kan det också bero på att användningen av gräsarter med alkaloider som är giftiga för boskap hittills har varit låg. Detta är dock på väg att förändras. Rörsvingel och engelskt rajgräs introduceras i allt större del av landet. Om någon sort av dessa gräs har endofyter kan de påverka djurens hälsa negativt, varför det kan vara lämpligt att fröfirmor börjar kontrollera endofytförekomsten i fröpartierna.

Den spontana reaktionen angående endofythaltiga gräs kan vara att döda de endofyter som producerar alkaloider som är giftiga för boskap, men i vissa fall gör de nytta. Många insekter och andra djur undviker endofytinfekterat gräs. På platser där djur kan göra skada eller skadas genom sin närvaro, som till exempel i parker, på golfbanor eller flygplatser kan endofytinfekterat gräs göra nytta. Varje år inträffar incidenter där flygplan, främst under start och landning, krockar med fåglar. Fåglarna dras till flygplatsernas öppna fält för rekreation och födointag. På flera håll i världen görs försök där endofytinfekterat gräs används runt start- och landningsbanorna, vilket gör att fåglarna söker sig till andra områden som man kan anlägga på ett betryggande avstånd från flygplatsen. Endofytinfekterat rörsvingel är då ett lämpligt gräs att använda, men då detta gräs fortfarande inte är vinterhärdigt i hela Sverige behövs mer forskning.

Ängssvingel är känd för att vara ett vinterhärdigt gräs, särskilt när det gäller resistens mot utvintringssvampar. Då nästan alla plantor överlevde vintern i fältförsöket kunde det inte avgöras om endofyten på något sätt påverkade plantorna. Det skulle vara intressant att göra en liknande studie med gräsarter och sorter som hittills inte klarar svensk vinter lika bra, t ex rörsvingel och engelskt rajgräs, för att se om deras endofyter har någon inverkan på vinteröverlevnaden.

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