Choke disease in timothy

– seed borne disease and mycotoxins

Kolvsjuka i timotej – fröburen smitta och mykotoxiner

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Abstract

Epichloë spp. and their asexual state, formerly named *Neotyphodium*, are endophytes of the phylum Ascomycota that are symbionts of cool-season grasses. There has been an estimation that one third of all cool-season grass species all over the world are associated with these endophytes. The endophytes inhabit the above ground part of the plant and can transmit vertically through intercalary growth depending on the host-species interaction. Eventually, infections of the seeds occur through the intercalary growth, or horizontally through dispersion of conidia and ascospores. Sexually reproducing *Epichloë* spp. are able to produce a light-colored stroma on the leaf sheath of the host. The stroma prevents the inflorescence from emerging and this condition is referred to as choke disease. *Epichloë* spp. are known for producing alkaloids toxic to vertebrates and invertebrates but can also help the host tolerate various stress factors.

Choke disease is of importance in timothy (*Phleum pratense*) since it leads to severe yield losses for seed producers due to the choked inflorescences. There is also a concern for mycotoxins toxic to livestock. *Epichloë typhina* is the only species known to associate with timothy and has never been confirmed to transmit vertically in this host. Given the importance of timothy and the concern from the industry due to the severe outbreaks of choke disease in Sweden 2014 and 2019, the objective of this thesis was to (i) examine possible presence of *Epichloë* spp. in timothy seeds, (ii) to identify the species of *Epichloë* present in Swedish timothy, (iii) examine the presence of the mycotoxins ergovaline and lolitrem B in Swedish cool-season grasses and (iv) to better understand the life cycle of *Epichloë* spp. in timothy.

No sign of *Epichloë* were to be found in any of the timothy seeds through examination with PCR or microscopy. It was not possible to determine the species of *Epichloë* through isolation of the fungi or directly from stromata, possibly due to old material. The concentration of ergovaline was below the detection limits in all the samples sent for analysis and lolitrem B could only be detected in four samples, however at low concentrations. The samples sent for analysis were various samples of hay, silage and green mass. Of the 17 samples analyzed, three contain solely timothy and in two of these lolitrem B was found.

The results indicate that *Epichloë* spp. are not seed borne in timothy, or at least occurs only rarely in seeds. Even though it was not possible to determine the species present in Swedish timothy it can be assumed that the species present is *E. typhina* since it is the only species reported in timothy. The results from the mycotoxin analysis suggest that there was no obvious risk for livestock to graze on ley infected with *Epichloë* spp. or to be fed with fodder containing *Epichloë* spp. in Sweden 2019.

Keywords: timothy, *Phleum pratense*, *Epichloë*, stroma, stromata, seeds, microscopy, mycotoxins, alkaloids, ergovaline, lolitrem B

Populärvetenskaplig sammanfattning

Många gräsarter i världen lever i symbios med endofyter. Endofyter är vanligtvis svampar eller bakterier som lever inuti sin värd. *Epichloë* är ett svampsläkte av endofyter, som bland annat infekterar timotej och lever i värdens ovanjordiska delar. Endofyter av detta släkte får näring av växten i utbyte mot skydd från växtätare och torka med mera. *Epichloë* spp. kan antingen spridas genom att den inifrån värdväxten, växer in i värdväxtens frön och på sätt sprider sig till nästa generation. *Epichloë* spp. kan också sprida sig med hjälp av sporer till andra värdväxter. För att föröka sig sexuellt måste *Epichloë* spp. producera en fruktkropp på värdens översta del, varifrån så kallade ascosporer sprids och infekterar nya värdväxter. Fruktkropparna ser ut som kolvar och tillståndet på värdväxten har därför fått namnet kolvsjuka. *Epichloë* spp. anses generellt vara nyttiga för gräsen som infekteras, men ibland kan problem uppstå.

Timotej är en ekonomiskt viktig gröda i Sverige och odlas ofta på betesmarker och ingår i grovfoder till boskap. När *Epichloë* spp. producerar kolvar förhindras utvecklingen av blomställningen, vilket resulterar i att produktionen av frön förhindras. Detta är mycket problematiskt i fröodlingar för timotej, då det är fröna som är ekonomiskt viktiga för odlarna. En annan problematik för oss människor är det skydd som *Epichloë* spp. erbjuder värdväxten. I särskilda kombinationer mellan svampart och värdväxt, producerar svampen gifter av en kemisk grupp som kallas alkaloider. Vissa av dessa alkaloider är giftiga för boskap, till exempel ergovalin och lolitrem B. Djurägare är därför väldigt måna om att inte låta sina djur beta mark eller utfodra dessa med foder som kan innehålla giftiga alkaloider. Stora utbrott av kolvsjuka obseverades i timotej åren 2014 och 2019, vilket gjorde fröproducenter samt djurägare oroliga.

Det här examensarbetet har gått ut på att studera (i) förekomsten av *Epichloë* spp. i frön av timotej, (ii) försöka ta reda på vilken art av *Epichloë* vi har i timotej i Sverige, (iii) att analysera förekomsten av ergovalin och lolitrem B i svenskt vallgräs och (iv) att generellt bättre förstå biologin för *Epichloë* spp. i timotej. Arbetet genomfördes genom insamling av timotejfrön, kolvar och växtmaterial från olika fält, samt analyser av dessa. Det gick inte att hitta några spår av *Epichloë* spp. i timotejfröna som undersöktes och det gick inte heller att fastställa vilken art av *Epichloë* som fanns i kolvproverna. Ergovalin kunde inte upptäckas i proverna och bara ett fåtal prover innehöll låga koncentrationer lolitrem B.

Att inga spår av *Epichloë* spp. hittades i timotejfröna kan troligtvis förklaras av att den enda arten som bekräftats i timotej är *E. typhina* och just den arten har aldrig bekräftats sprida sig systemiskt till fröna i timotej. Det skulle kunna vara så att att sporer kan spridas från infketerade naturliga populationer eller timotejfält, till friska timotejfält och skulle på så sätt kunna infektera frisk vävnad, exempelvis blommorna vilket kan resultera i infekterade frön. En annan infektionsväg är stubb efter det att vallen skördats. Två olika tillvägagångssätt användes för att försöka ta reda på vilken svampart kolvproverna innehöll. Detta lyckades dock inte, troligtvis på grund av att materialet var för gammalt för att det skulle fungera. Skulle man göra om försöken så skulle man testa färskt material istället. De låga koncentrationerna svampgift som upptäcktes i några prover låg alla under tröskeln för att avara giftig för boskap. Resultaten och litteraturen indikerar att det inte finns någon uppenbar fara för boskap att beta timotej eller utfodras med timotej i Sverige.

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1. Introduction

Epichloë spp. are endophytes of the phylum Ascomycota that are natural symbionts of cool-season grasses (Schardl et al. 2004). They are almost only found on grass species belonging to the sub-family Pooideae and many of them are host specific (Kauppinen et al. 2016). There has been an estimation that one third of all cool-season grass species all over the world are associated with *Epichloë* spp. (Leuchtmann 1992). *Epichloë* spp. inhabit above-ground plant tissue without necessarily causing any visible disease symptoms (Saikkonen et al. 1998). They are, however, capable of synthesizing alkaloids that function as insect deterrents, and are toxic to livestock (Vikuk 2019). Sexually reproducing *Epichloë* spp. are able to produce a light-colored stroma composed of a dense mass of hyphae that proliferates specifically on the leaf sheath that surrounds the non-emerged inflorescence (Figure 1), a symptom that is mostly referred to as choke disease (Western and Cavett, 1959; Schardl et al. 2004).

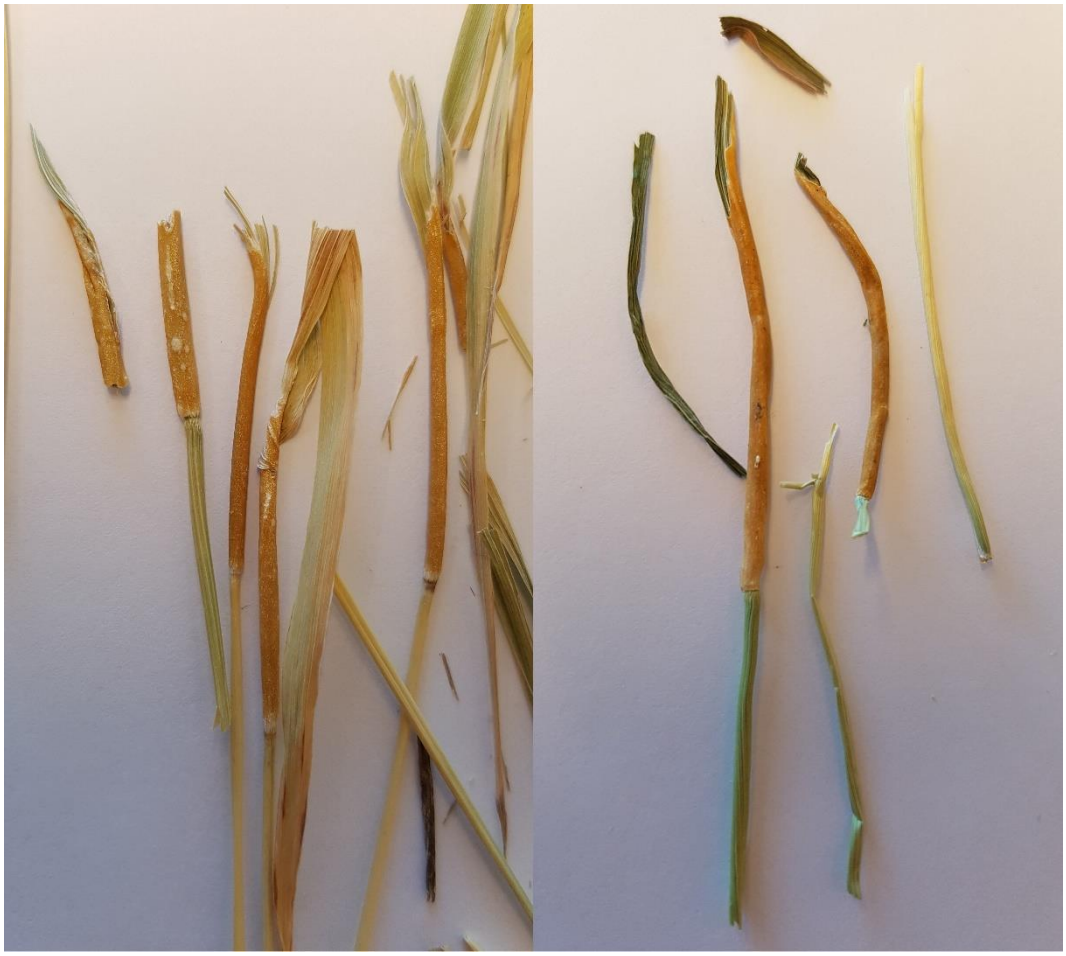

Figure 1. Timothy with choke disease (cultivar Lischka to the left and unknown cultivar to the right*).*

Choke disease has become economically important in grass species grown in seed lots like timothy (*Phelum pratense*) and orchard grass (*Dactylis glomerata*) since the stroma that develop on the emerging inflorescence partly or completely prevent the inflorescence from emerging (Cagaš and Macháč 2012), resulting in severe yield losses. Choke disease usually first appears as a light infection scattered over the field in the first cropping year. Infected plants seem to produce stromata every year that increase in proportion over the field (Western and Cavett 1959). From the second to third and later years, disease incidence in the field could in extreme cases be as high as 80% of infected tillers in orchard grass (Western and Cavett 1959). In older literature timothy seed yield loss due to choke disease has been reported to be up to two thirds in Sweden (Eriksson 1904).

In the growing season 2019, there was a severe outbreak of choke disease in timothy in Sweden which initiated this thesis. Many questions arose: “what factors leads to outbreaks of choke disease in timothy?”, “Is the fungus seed borne?”, “Is it safe for livestock to be fed timothy from fields with choke disease?”, “How can seed pro-

ducers tackle the problem?" and so on. Similar outbreaks of choke disease in timothy has been seen before in Sweden, for example in 1901, 1902 (Eriksson 1904) and in 2014 (J Kroon 2020, personal communication). According to Cagaš and Macháč (2012), also long-term observations shows an increasing frequency of choke disease in grass seed lots in the Czech Republic.

Besides causing problems with seed production and toxins, there are several benefits for the host of microbial symbionts like *Epichloë* spp., offering increased tolerance to various stress factors (Saikkonen et al. 1998). Beneficial effects of *Epichloë* spp. interactions with plants include enhanced growth of the host (Kauppinen et al. 2016), increased pest and pathogen protection (Bacetty et al. 2009; Hume et al. 2016; Kauppinen et al. 2016), drought avoidance and tolerance (Malinowski and Belesky 2000; Wäli et al. 2011). Drought avoidance and tolerance is a result of endophyte-related mechanisms expanding the root system, control of transpiration, water storage in plant tissues and osmotic adjustment etcetera (Malinowski and Belesky 2000).

2. Objective

The objective of this thesis was to:

- Examine presence of *Epichloë* spp. in timothy seeds, using PCR and microscopy.
- Examine presence of mycotoxins ergovaline and lolitrem B caused by *Epichloë* spp. in Swedish hay, silage and green mass.
- Identify species of *Epichloë* present in Sweden from samples of stromata.
- Better understand the life cycle of *Epichloë* spp. in timothy, through literature studies.

3. Background

3.1. Taxonomy

The species of the genus *Epichloë* and their anamorphic stages, formerly *Neotyphodium* (Leuchtmann et al. 2014; Hume et al. 2020) are ascomycetes belonging to the family Clavicipitaceae. Due to the principle "one fungus = one name" (Hawksworth 2004; Hawksworth et al. 2011a, 2011b; Norvell 2011), Leuchtmann et al. (2014) grouped together all species of *Epichloë* and *Neotyphodium* with the exception of *N. starrii* and *N. chilense*, into one genus.

3.2. Biology of *Epichloë* spp.

3.2.1. Vertical transmission

Epichloë spp. have the ability to colonize most part of the plant and for some species, the vertical transmission is very important for the asexual life cycle (Clay and Schardl 2002). In general, the endophyte will spread in the leaf sheaths of seedlings, meristems of flowering tillers and the inflorescence of the host plant through intercalary growth and persist as the cells of the host plant elongates (Christensen et al. 2008). As the host plant develops, the endophytes will grow into and within the ovaries of the florets (Freeman, 1904; Leuchtmann and Schardl 1998) and eventually the developing seeds will be infected through colonization of the embryonic cells (Zhang et al. 2017).

After germination of the seed, the endophyte will grow into the shoot of the embryo and further into the above ground parts of the host and eventually close the vertical transmission cycle (Leuchtmann et al. 2014). The proportion of infected seeds produced by infected host plants varies depending on the combination of host species and symbiont species. Often, the vertical transmission causes infection in a majority of the seeds (Afkhami and Rudgers 2008), sometimes up to 100% (Leuchtmann et al. 2014).

3.2.2. Horizontal transmission

The external transmission of *Epichloë* spp. is referred to as the horizontal transmission, it is however important to note that the horizontal transmission also can be asexual through direct infection by conidia. The sexual life cycle starts in the same way as the vertical asexual life cycle, with intercellular growth in the plant. Later, stromata are formed on the leaf sheath surrounding the undeveloped inflorescences, where conidia are produced (Tadych et al. 2014). *Epichloë* is a bipolar heterothallic genus and therefore needs conidia of two mating types, spermatia, for fertilization and production of perithecia and ascospores. The transmission of the conidia produced on the stroma is most likely vectored through flies of the genus *Botanophila* (Kohlmeyer and Kohlmeyer 1974; Bultman et al. 1995). After fertilization, the stromata change color to bright yellow or orange (Tadych et al. 2014), perithecia develop with asci that each contain eight meiotic ascospores. The ascospores are then ejected with force (Chung and Schardl 1997). The ascospores will germinate directly when reaching a new substrate and produce conidiophores or hyphae (Moy et al. 2000; Alderman 2013). In this way, ascospores can for example cause infection of the florets and finally the seeds of the host (Chung and Schardl 1997). In general, *Epichloë* spp. only sporulate on some of the tillers in the host population and leave other to develop normally, however producing *Epichloë* infected seeds in some species (White 1988). For the general life cycle of *Epichloë* endophytes (not including horizontal asexual transmission) see Figure 2.

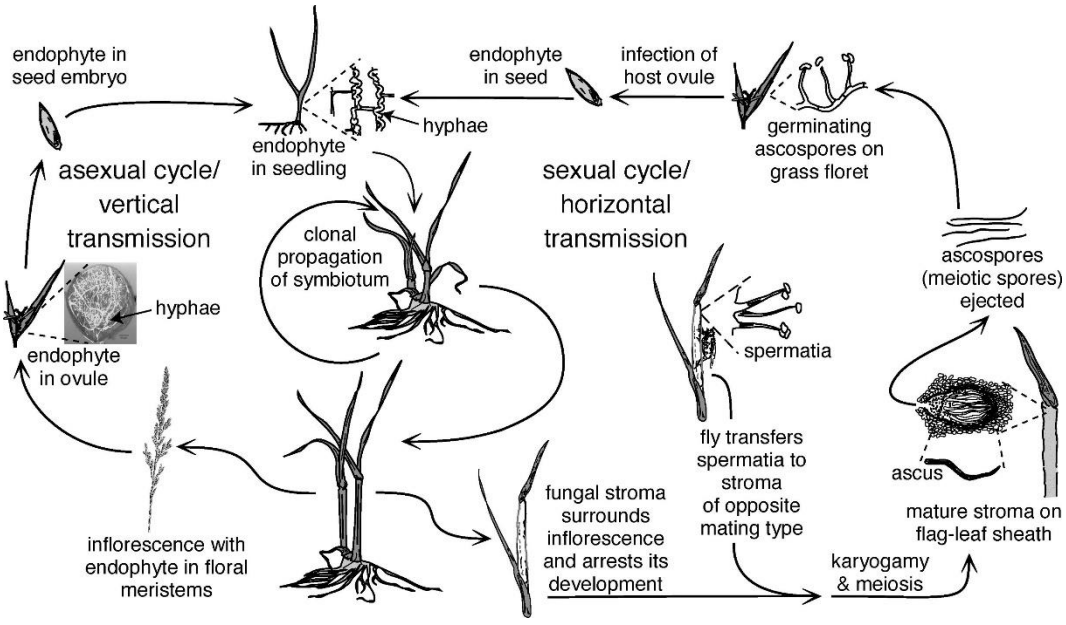

Figure 2. Life cycles of Epichloë spp. in their host grasses. Some Epichloë spp. are capable of undergoing both sexual and asexual life cycles. Obligately sexual Epichloë spp. can only transmit horizontally. Obligately asexual Epichloë spp. do not produce stromata. The micrograph at left shows an ovule infected with E. festucae expressing a gene for cyan fluorescent protein. From Schardl (2010). The Epichloae, Symbionts of the Grass Subfamily Poöideae. Vol. 97(4), p. 650. Reproduced with permission from Missouri Botanical Garden Press, St. Louis.

Transmission of conidia and ascospores

Many *Epichloë* species produce conidia not only on the stroma, but also on leaves of the host (Moy et al. 2000). Stromata and mycelial networks with conidiogenous cells are the two external structures that allow horizontal transmission of the fungi (White et al. 1996). In addition to vectoring flies (Kohlmeyer and Kohlmeyer 1974; Bultman et al. 1995), conida produced by *E. typhina* subsp. *poae* have been reported to spread contagiously through water (Tadych et al. 2012). Ascospores are wind-dispersed and germinate to form conidia, hyphae or both (White 1997; Tadych et al. 2012¸ Alderman 2013). Reported infection sites are the meristematic zones of tillers and seedlings (Tadych et al. 2012), infection by hyphae formed by conidia or ascospores, entering stubble or wounds (Western and Cavett 1959; Alderman 2013), conidiogenous (infection by conidia) infection of leaves (Alderman 2013) and as mentioned above, infection of the florets (Chung and Schardl 1997). Alderman (2013) has reported that ascospore germination on leaves mainly are hyphal at wound sites and iterative (conidiogenous) on non-wound sites.

In a study by Western and Cavett (1959) it was reported that more ascospores were found to be released during the latter part of the day compared to the morning for the host-species interaction between *E. typhina* and orchard grass. Also, more conidia are released when exposed to humid air streams compared to dry ones. In the same study, spore release was tested at seven different locations in a field near York (UK) and conidia dispersion was highest during anthesis. High ascospore production did not occur until well after anthesis.

3.2.3. Uncertainty of the sexual reproduction

Stroma formation is required for the sexual reproduction of *Epichloë* spp. However, completion of the sexual stage for *E. stromatolongum* (Ji et al. 2009) and *E. poae* strains (Tadych et al. 2012) have never been reported, even though stromata have been observed. Several asexual *Epichloë* species that are interspecific hybrids, lack the ability of sexual reproduction (Moon et al. 2007). At the same time, the *E. liyangensis*, a hybrid related to *E. yangzii* and members of the *E. typhina* complex clade, is able to produce perithecia with ascospores on its host, although in sparse numbers (Yan et al. 2011).

3.2.4. Uncertainty of variation in transmission between species

The majority of sexually propagating *Epichloë* species can transmit both horizontally and vertically (Leuchtmann et al. 2014). There are however 24 species that only propagate clonally and those include *E. uncinata*, *E. coenophiala* (Leuchtmann et al. 2014) along with a number of interspecific hybrids (Tsai et al. 1994; Moon et al. 2004). There are also species that are only known to propagate hori-

zontally and those include *E. baconii* and *E. glyceriae*. Another example of variation in transmission is *E. bromicola* and *E. typhina* which are able to transmit contagiously in some hosts and only vertically in other hosts (Leuchtmann and Schardl 1998).

Epichloë typhina is the only species of *Epichloë* that has been found in timothy and orchard grass, and it has been reported to only transmit horizontally on these hosts (Tadych et al. 2014). In an experiment by Chung and Schardl (1997), *E. typhina* was not reported to transmit vertically in perennial ryegrass or orchard grass. In a survey by Saikkonen et al. (2000) the seeds of five wild populations of timothy (47 individuals) were examined. Only the seeds of one population contained endophytes (33% incidence of endophyte infected plants), but it is unlikely that this population was infected by *Epichloë* spp. (M Helander 2020, personal communication). In a Swedish survey, no *Epichloë* infections were found in any of 39 timothy samples collected from agricultural sites, using immunoassay or microscopy to examine seeds and seedlings (Huss-Danell 2008).

3.3. Environmental effects on *Epichloë* spp.

There is evidence that environmental factors have a role in the incidence of *Epichloë* spp. (Victoria Novas et al. 2007; Iannone et al. 2013) and for stroma production. In one study, stroma production was significantly different between years and populations, thus indicating an environmental effect (Tintjer et al. 2008). For example, fragmentation of habitat has been reported to induce stroma production (Groppe et al. 2001). Several studies have reported the effect of nutrient abundance on stroma production. Wennström (1996) reports a higher occurrence of stroma in wet and nutrient rich soils and Meijer and Leuchtmann (2000) reported a slight stimulation of stroma production when applying fertilizer. Exposing *Epichloë* infected plants to higher concentrations of CO_2 has been reported to enhance stroma production (Groppe et al. 1999; Meijer and Leuchtmann 2000). Other growth enhancing factors has been reported to either reduce stroma formation (Kirby 1961) or enhance it (White and Chambless 1991). Shading has also been reported to increase stroma production (Meijer and Leuchtmann 2000). Alderman (2013) showed that production of stromata by *E. typhina* could be induced by vernalization of orchard grass in a growth chamber.

The distribution of stromata formation usually does not follow the geographical distribution of their most common hosts, but is probably a complex interaction between fungal-plant genetics and various environmental factors (Tadych et al. 2014), with emphasis on genotypic variation of the fungus (Meijer and Leuchtman 2000).

3.4. Symbiotic relationship

The exact mechanisms of the symbiosis between *Epichloë* spp. and their hosts are not known since there is a high variation between these relationships. It has been suggested that the symbiosis is of an antagonistic-mutualistic nature depending on the genotype of the host and the endophyte-haplotype combination as well as environmental factors (Saikkonen et al. 2002; Brosi et al. 2011). The benefits of the symbiosis for endophytes are the potential sources of nutrients that they obtain in the apoplast of the plant. These are macromolecules in the intercellular matrix, amino acids, simple sugars and other metabolites that are either transported or leaked into the apoplast (Schardl 1996).

3.4.1. Alkaloid production

The production of alkaloids with neurotoxic activities on invertebrates and vertebrates are more common in plants with asexual endophytes compared to those produced by sexual *Epichloë* species which produce stromata (Schardl et al. 2012). The alkaloids protect the plant from herbivores such as livestock (Figure 3) and insects, but also pathogens (Bacetty et al. 2009; Hume et al. 2016; Kauppinen et al. 2016) and nematodes (Bacetty et al. 2009). The loline alkaloids N-formylloline, N-acetylloline and N-acetylnorloline as well as the pyrrolopyrazine alkaloid peramine are potent deterrents of insects (Scott 2001; Fuchs et al. 2020) and are regarded as useful for agronomical and biotechnological purposes (Kauppinen et al. 2016). The alkaloids ergovaline (ergot alkaloid) and lolitrem B (indole diterpene alkaloid) are both toxic to livestock, with the former being associated with tall fescue toxicosis (Belesky et al. 1988) and the latter with ryegrass staggers (Gallagher et al. 1981).

Figure 3. Some alkaloids produced by Epichloë spp. have neutrotoxic activities on for example cattle and can cause diseases such as tall fescue toxicosis and ryegrass staggers.

The concentration of alkaloids in the plant are influenced by endophyte species, plant genotype, tissue type, season and environmental factors. In a study by Lyons et al. (1986), ergot alkaloid concentrations were elevated when greenhouse-grown tall fescue were fertilized with nitrogen, with a higher concentration found in leaf sheaths in comparison to leaf blades. Results from Arechavaleta et al. (1992) indicates that drought stress is promoting ergot alkaloid production in plants. Malinowski et al. (1998) reported that ergot alkaloid accumulation in the roots is positively correlated with phosphorus availability in the soil. In another study ergovaline concentrations peaked in spring and autumn, but declined during midsummer to one third of the autumn peak (Belesky et al. (1988).

3.4.2. Use of *Epichloë* endophytes in agriculture

The ability of *Epichloë* spp. to synthesize alkaloids as well as providing other benefits of host plants are regarded as useful for biological, agronomical and biotechnological exploitation (Aiken et al. 2012). Since microbial endophytes are increasingly regarded as important for sustainable agriculture in the form of biological plant protection, several patents have been issued for clavicipitaceous grass endophytes, their metabolites and genes (Leuchtmann et al. 2014). There are compounds produced by *E. typhina* in timothy with antifungal activities, for example sesquiterpenes- chokols A-G that has found to be fungicidal to *Cladosporium phlei* which cause the leaf spot disease and aliphatic compounds (Kumar and Kaushik 2013). It is however important to be cautious since the alkaloid production is highly variable

and seem to depend on the specific endophyte-grass interaction (Leuchtmann et al. 2000; Vikuk et al. 2019).

3.5. Management and inoculation of *Epichloë* spp.

Management and manipulation of *Epichloë* spp. infection in forage grasses is of importance due to a variety of reasons and the type of manipulation will depend on whether the effect of the endophyte is regarded as beneficial or detrimental. First, these fungal infections can be manipulated to prevent disease on domestic animals, due to the possible production of toxins by *Epichloë* spp. in infected grass. It is also important to prevent yield loss for the seed production industry due to the inhibition of inflorescence in some grass species. In other cases, inoculation of *Epichloë* spp. can be used to improve for example, drought resistance or pest control. Manipulation of *Epichloë* spp. is also important to study the evolution and ecology of grass-endophyte interactions (Wäli et al. 2011).

3.5.1. Storage

Seed transmission of *Epichloë* spp. depends on the specific species of *Epichloë* and grass host (Tadych et al. 2014). In case of seed transmitted *Epichloë* species, a treatment method such as long-term storage can be used (Latch 1983). Long-term storage combined with high temperature and high moisture have been reported to decrease the viability of endophytes in seeds (Figure 4). The importance of temperature and relative humidity on endophyte viability is unclear. The effect relative humidity has on the endophyte viability is due to changes in seed moisture, since they are correlated to each other. Other variables that might affect the viability of the endophyte are variation in grass species, cultivar, field and endophyte strains. One downside of the long-term storage treatment is the loss of viability in seed germination (Welty et al. 1987; Hume et al. 2013).

Figure 4. Timothy seeds of cultivar Lischka.

3.5.2. Hot-water treatment

Another faster method to treat seeds are through hot-water treatment (Latch 1983; Wäli et al. 2011). In this treatment, a combination of water temperature and incubation time is determining the results. Higher water temperature and longer incubation time will decrease the viability of the endophyte more efficiently, but at the same time it will lead to the reduction of germination rates in the seeds. For example, the best temperature-incubation time for red fescue was +55-56°C with an incubation time of 20 minutes, but this would decrease the germination rates of the seeds by 30% (Wäli et al. 2011).

3.5.3. Fungicide treatment of seeds

The most effective way to treat endophyte infected seeds is by systemic fungicide treatment (Latch 1983). In an experiment by Harvey et al. (1982), treatment of ryegrass seeds with prochloraz was the most efficient fungicide seed treatment, whereas seed treatment with propiconazole only resulted in partial control. Prochloraz is regarded as an efficient fungicide due to its penetrating action. Latch and Christensen (1982) reports that prochloraz (authorized in the European Union, but is not approved for use in Sweden or Denmark; European Commission 2020), propiconazole (since the 19th of March 2020, propiconazole is prohibited within the European Union; European Commission 2020) and etaconazole (not authorized in the European Union; European Commission 2020) works for fungicide treatment of ryegrass seeds, although etaconazole is less efficient. Treatments with all three

fungicides were phytotoxic, seedling emergence rate were lower, emerged seedlings had distorted, curled and very dark green first leaves. Seedlings from seeds treated with prochloraz were less affected compared with the other two. According to Latch (1983), the seedlings recover quickly from the phytotoxic effects of this type of fungicide treatment.

3.5.4. Soil drenching

Soil drenching with fungicides is a treatment that can be used to produce endophyte free seedlings in laboratory experiments or for production of commercial endophyte free cultivars. When growing infected plants in pots, any traces of the endophytes would disappear within two months (Latch 1983) after drenching the soil with a suspension of benomyl at 0,1 g/L. This is probably the simplest way to produce endophyte free parent plants and afterwards bulk up on endophyte free seeds (Latch and Christensen 1982). It is also possible to treat infected seedlings in water containing fungicides (Kauppinen et al. 2016).

3.5.5. Spraying with fungicides on foliage

In cases where endophyte infections are not a result of seed transmission, other treatments could be applied. For example, spraying with systemic fungicides in the growing crop to manage endophyte infected fields has been reported to be successful (Harvey et al. 1982; Latch 1983; Hill and Brown 2000). According to Latch (1983), prochloraz can be used to kill the fungi in the foliage.

In a study by Harvey et al. (1982), seven fungicides reported to have either systemic or translaminar movement were sprayed on ryegrass infected with *Lolium* endophytes. The results were a bit contradictory compared to Latch's (1983) results. Prochloraz did not have any effect when sprayed on the plants in pots, but seemed to stimulate regrowth of the plants. The *Lolium* endophyte was, however, very sensitive to prochloraz in culture (PDA) at concentrations of 10 ppm or more. At 100 ppm fungicides imazalil (authorized in the European Union, but is not approved for use in Latvia, Malta and Slovenia; European Commission 2020) and propiconazole showed good activity. Benomyl, diclobutrazol and triadimefon (all three not authorized in the European Union; European Commission 2020) showed some activity at 1000 ppm and the only fungicide that did not show any activity in culture at this concentration was procymidone (not authorized in the European union; European Commission 2020). At 5000 ppm all fungicides showed effect. In the *in vivo* studies, propiconazole (25 % 1,25 g or mL/L) was the only fungicide tested that resulted in a significant ($P<0,01$) reduction of the endophyte in the plants, but none of the seven fungicides tested eliminated the endophyte completely at the application rates used. The reason why prochloraz and imazalil have a strong effect in culture but, poor performance when sprayed on the plants, may be that prochloraz

only has translaminar movement and is not actually a true systemic fungicide (moving from old and new tissues). Imazalil is not normally used on monocotyledonous plants and may therefore not be systematically active in ryegrass (Harvey et al. 1982).

In a study by Hill and Brown (2000), two contact fungicides, chloroneb (not authorized in the European Union; European Commission 2020) and etridiazole (authorized in the European Union, but only approved for use in Greece, Spain and the Netherlands; European Commission 2020) and one systemic fungicide propiconazole were tested on tall fescue infected with *E. coenophiala*. Weekly fungicide applications with propiconazole decreased endophyte infection of potted plants but infection rates increased when applying chloroneb or etridiazole, compared to the control. In a second experiment, the effect of variations of application dates on endophyte infection were examined for propiconazole. Infection rates were lower in the treatments where propiconazole was applied early post germination compared to later. Repeated applications seemed to suppress endophyte infection as well. In all treatments where the plants were not treated seven days post-germination (earliest application in the experiment), endophyte infections were high.

3.5.6. Preventing infection by *Epichloë* spp. in the field

Additional measures that in theory could reduce the risk of horizontal transmission of *Epichloë* spp. could be (i) an intense weed management program (ii) to get rid of hosts in the edges of the fields (see Figure 5) (iii) using barrier crops around fields for seed production to reduce the inflow of wind carried ascospores to the field and (iv) avoid cutting the foliage when it is very humid and the conidia and ascospore production are at their peaks. The effect of these measures is for the moment unclear. To completely stop the spread of *Epichloë* spp. in a field, ploughing and incorporating the crop into the soil is a possible control measure (Cagaš and Macháč 2012).

Figure 5. Timothy plant with choke disease in edge of spring barley field.

3.6. Swedish monitoring of choke disease in timothy

In 2014 and 2019, monitoring of choke disease in Swedish timothy fields were carried out by the Seed Unit of the Swedish Board of Agriculture. The difference between counties, cropping years and cultivars were noted.

Table 1. Observations of choke disease in timothy in the year of 2014. Difference between counties from field inspections carried out by the Seed Unit of the Swedish Board of Agriculture (J Kroon 2020, personal communication). Presented in number of inspected fields and fields with choke disease in number as well as percentage.

County	**Nr. of fields**	**Nr. of fields with choke disease**	**% fields with choke disease**
Skåne	38	14	37
Södermanland	19	5	26
Uppsala county	7	3	43
Värmland	7	0	0
Västmanland	28	10	36
Västergötland	85	27	32
Örebro county	30	16	53
Östergötland	35	7	20
Total	249	82	33

In 2014, an average of 33% of inspected timothy fields in Sweden showed symptoms of choke disease. The two counties that had most fields with choke disease were Uppsala county and Örebro county, with 43% and 53% respectively (Table 1).

Table 2. Observations of choke disease in timothy in the year of 2019. Difference between counties from field inspections carried out by the Seed Unit of the Swedish Board of Agriculture (J Kroon 2020, personal communication). Presented in number of inspected fields and fields with choke disease in number as well as percentage.

County	**Nr. of fields**	**Nr. of fields with choke disease**	**% fields with choke disease**
Skåne	22	0	0
Södermanland	12	6	50
Uppsala county	7	0	0
Värmland	3	2	67
Västmanland	17	9	53
Västergötland	63	24	38
Örebro county	24	12	50
Östergötland	22	9	41
Others	5	0	0
Total	175	62	35

In 2019, an average of 35% of inspected timothy fields in Sweden had plants showing symptoms of choke disease. The two counties that had most fields with choke disease were Värmland and Västmanland with 67% and 53% respectively (Table 2). The percentage of fields with choke disease were slightly higher in 2019 compared to 2014. However, the total number of fields with choke disease were lower in 2019 compared to 2014. It is difficult to speculate whether there actually was a lower disease pressure in Sweden in 2014 compared to 2019 (Table 1 and 2).

Table 3. Incidence of choke disease in timothy seed lots sown between 2015-2019 (and in some cases before 2015), in the year of 2019. From field inspections carried out by the Seed Unit of the Swedish Board of Agriculture (J Kroon 2020, personal communication). Presented in number of inspected fields and fields with choke disease in number as well as percentage. In total, 40% of the fields with choke disease had more than a couple plants with stromata and 2014 were similar in this regard as well.

Cropping year	**Nr. of fields**	**Nr. of fields with choke disease**	**% fields with choke disease**
I.	21	1	5
II.	62	19	31
III.	56	24	43
IV. or older	36	17	47
Total	175	61	35

The incidence of choke disease in timothy fields seems to increase with crop age (Table 3) which has been observed before by Western and Cavett (1959) and Cagaš and Macháč (2012).

Table 4. Incidence of choke disease in timothy for different cultivars in the year of 2014. From field inspections carried out by the Seed Unit of the Swedish Board of Agriculture (J Kroon 2020, personal communication). Presented in number of inspected fields and fields with choke disease in number as well as percentage.

Cultivar	**Nr. of fields**	**Nr. of fields with choke disease**	**% fields with choke disease**
Comer	1	1	100
Grindstad	41	12	29
Jonatan	2	1	50
Lidar	2	0	0
Lischka	45	16	36
Ragnar	21	9	43
Rakel	1	0	0
Rhonia	1	0	0
Rubinia	1	0	0
Snorri	3	0	0
Switch	104	38	37
Tryggve	18	2	11

Cultivar	Nr. of fields	Nr. of fields with choke disease	% fields with choke disease
Vega	11	3	27
Winnetou	2	0	0
Total	253	82	32

The cultivars with the highest incidence (>20%) of choke disease and a considerable number of fields inspected (>10) in 2014 were Grindstad, Lischka, Ragnar, Switch, and Vega (Table 4).

Table 5. Incidence of choke disease in timothy for different cultivars in the year of 2019. From field inspections carried out by the Seed Unit of the Swedish Board of Agriculture (J Kroon 2020, personal communication). Presented in number of inspected fields and fields with choke disease in number as well as percentage.

Cultivar	Nr. of fields	Nr. of fields with choke disease	% fields with choke disease
Baronaise	2	2	100
Comer	5	4	80
Dorothy	5	2	40
Grindstad	9	5	56
Gunnar	1	0	0
Haukila	1	0	0
Lischka	16	5	31
Natsupirika	1	0	0
Polarking	8	1	13
Rakel	19	13	68
Rhonia	26	5	19
Snorri	4	0	0
Switch	56	20	36
Tryggve	17	5	29
Vega	3	0	0
Winnetou	2	0	0
Total	175	62	35

The cultivars with the highest incidence (>20%) of choke disease and a considerable number of fields inspected (>10) in 2019 were Lischka, Rakel, Switch and Tryggve (Table 5). Incidence of choke disease was high for Lischka and Switch in both years. The incidence of disease in Tryggve was quite low in 2014 (11%) but high in 2019 (29%). It is hard to speculate about differences between the cultivars since the number of fields for each cultivar is very different for each year and that the number of inspected fields are lacking for some cultivars to be able to draw any conclusions. The monitoring was also only done two times (2014 and 2019). In addition, location of the fields and the environmental factors affecting those fields

might affect disease incidence as well (Tadych et al. 2014). The information from the field inspections could however be used as an indicator for which cultivars that are more affected by the disease.

Table 6. Incidence of choke disease in timothy (Phleum pratense) in the Czech Republic for different cultivars (monitored 2008-2010). Presented in number of monitored fields and fields with choke disease in number as well as percentage (Cagaš and Macháč 2012).

Cultivar	**Nr. of fields**	**Nr. of fields with choke disease**	**% fields with choke disease**
Comer	10	5	50
Ragnar	17	1	6
Winnetou	48	11	23
Total	75	17	23

When comparing the Swedish monitoring of choke disease in 2014 and 2019 to the monitoring in the Czech Republic (Table 6) by Cagaš and Macháč (2012), there were not many similarities. The incidence of choke disease in Ragnar was much higher in Sweden (2014; Table 4) compared to the Czech Republic. The number of fields inspected for Comer and Winnetou in Sweden were too few to compare the Swedish results with the results from the Czech Republic.

4. Material and methods

4.1. Overview

To fulfil the objective of this thesis, samples were collected and examined. Stroma samples from Swedish grass including timothy and orchard grass, were collected to be used for attempting to isolate *Epichloë* spp., to identify specific *Epichloë* spp. through Sanger sequencing and as positive controls for PCR. The seed samples were collected to examine presence of *Epichloë* spp. through PCR and microscopy and possibly identification of specific *Epichloë* spp. through Sanger sequencing. Samples of hay, silage and green mass were sent for toxin analysis to give an overview of the situation regarding mycotoxins in Swedish ley.

4.2. Collection of samples

Samples of timothy seeds, stroma samples from infected grass and various samples for mycotoxin analysis were collected. This was done by contacting companies affiliated with seed producers to gather the seed samples (Figure 6). The Seed Unit of the Swedish Board of Agriculture was also contacted for this purpose. The stroma samples used in this study came from the Plant Protection Centre in Uppsala (Swedish Board of Agriculture) and the National Veterinary Institute, SVA. The samples sent for mycotoxin analysis were collected by the National Veterinary Institute. In total 35 seed samples were collected (Table 8, see Appendix I), where 23 of the samples were Swedish and some of these originated from the same seed lot. Six stroma samples were collected, where two samples were confirmed of containing only timothy (Table 9, see Appendix II) and 17 samples were collected for mycotoxin analysis (Table 7 and 10, see Appendix III).

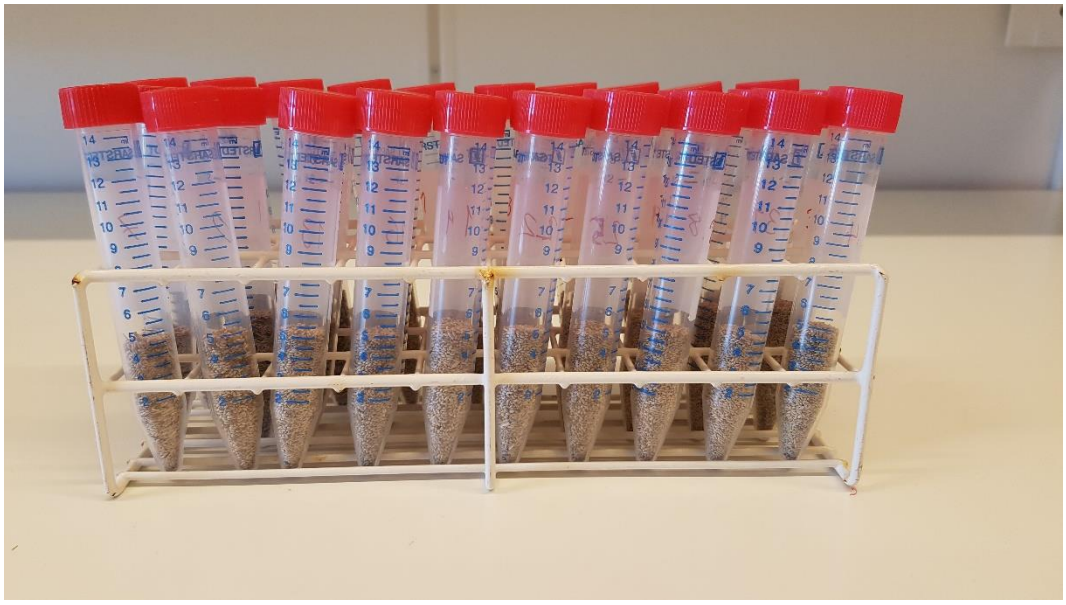

Figure 6. Timothy seed samples.

4.1. Isolating *Epichloë* spp. from stromata and infected stems

Half a liter of Potato Dextrose Agar (VWR Chemicals), PDA, was prepared and autoclaved. The PDA was then poured into petri dishes in sterile conditions and left to cool off. A 6% sodium hypochlorite solution was prepared. Stromata and stems were cut in approximately 0,5x0,5 cm cuttings. The cuttings were sterilized in sodium hypochlorite for 3 minutes. After sterilizing the cuttings, they were transferred to three water baths with ultrapure water for washing. The cuttings were washed in each bath for 20 seconds and then dried on Munktell filter papers in slightly open petri dishes for 15 minutes. When dried, the cuttings were placed on individual PDA plates and labelled. The plates were left in a window at ambient room temperature. When necessary, 0,5x0,5 cm cuttings from the PDA plates overgrown with mycelia, were transferred to a new PDA plate.

4.2. DNA extraction

DNA from all samples were extracted using the NucleoSpin Plant II Genomic DNA Purification kit with lysis buffer systems based on the CTAB method. The protocol “Genomic DNA from plant” was used but in step 2a the suspension was incubated

for 30 minutes instead of ten. To prepare the samples for DNA extraction, a mortar and pestle was used to grind the samples into a fine homogenized powder. About 90-100 mg of the seed samples and 10-20 mg of the stroma samples were used for extraction. Stroma samples were used as positive controls in the PCR.

4.3. Nanodrop

The concentrations and purity of DNA extracts were checked using the Nucleic Acid program in Nanodrop. Ultrapure water was used as blank and 1,5 µL of each sample was used for the measurement. The products from the column purification (see chapter 4.8) were measured using the elution buffer as blank.

4.4. Diluting primers and DNA

To prepare for PCR, all DNA samples were diluted to 3 ng/µL with an end volume of 100 µL. Genus specific primers for *Epichloë spp*. tef1-exon1d-1 (GGG-TAAGGACGAAAAGACTCA) and tef1-exon6u-1 (CGGCAGCGATAATCAG-GATAG; Vikuk et al. 2019) were diluted to 10 ng/µL.

4.5. PCR for *Epichloë* spp. detection

PCR detection of *Epichloë* spp. was performed in a total volume of 25 µL containing 12,5 ng DNA, 1 U DreamTaq DNA Polymerase (5 U/µL), 5 µL 10X DreamTaq Green Buffer (including 20 mM $MgCl_2$), 0,2 mM dNTP, 2 mM $MgCl_2$, and 1 µM target specific primers. The PCR conditions were 94 °C for 1 minute, then 30 cycles of 94 °C for 15 seconds, 56 °C for 30 seconds, and 72 °C for 45 seconds, finishing with 72 °C for 10 minutes.

After PCR, all products were analyzed by gel electrophoresis. For each sample 5 µL was loaded into wells of an 1,5 % agarose gel in SB buffer at 250 V, 400 mA for 20 minutes. The DNA fragments were visualized with Nancy-520 by UV trans-illumination. The expected product size was 860 bp (Vikuk et al. 2019).

4.6. Microscopy method

To prepare the seeds for microscope, a NaOH solution was prepared mixing 95 mL water, 5 mL ethanol and 2,5 g NaOH. The NaOH was added to 1,5 mL Eppendorf tubes together with about 100 seeds per sample and soaked for 12-16 hours. Any

remaining NaOH after the soaking was discarded, and the seeds were rinsed two times in water. Seeds were then examined by crushing the soaked seeds between a microscope slide and a cover glass, observing the embryonic cells of the seed (Zhang et al. 2017), 50 seeds per sample were examined. For reference see Figure 7 and 8.

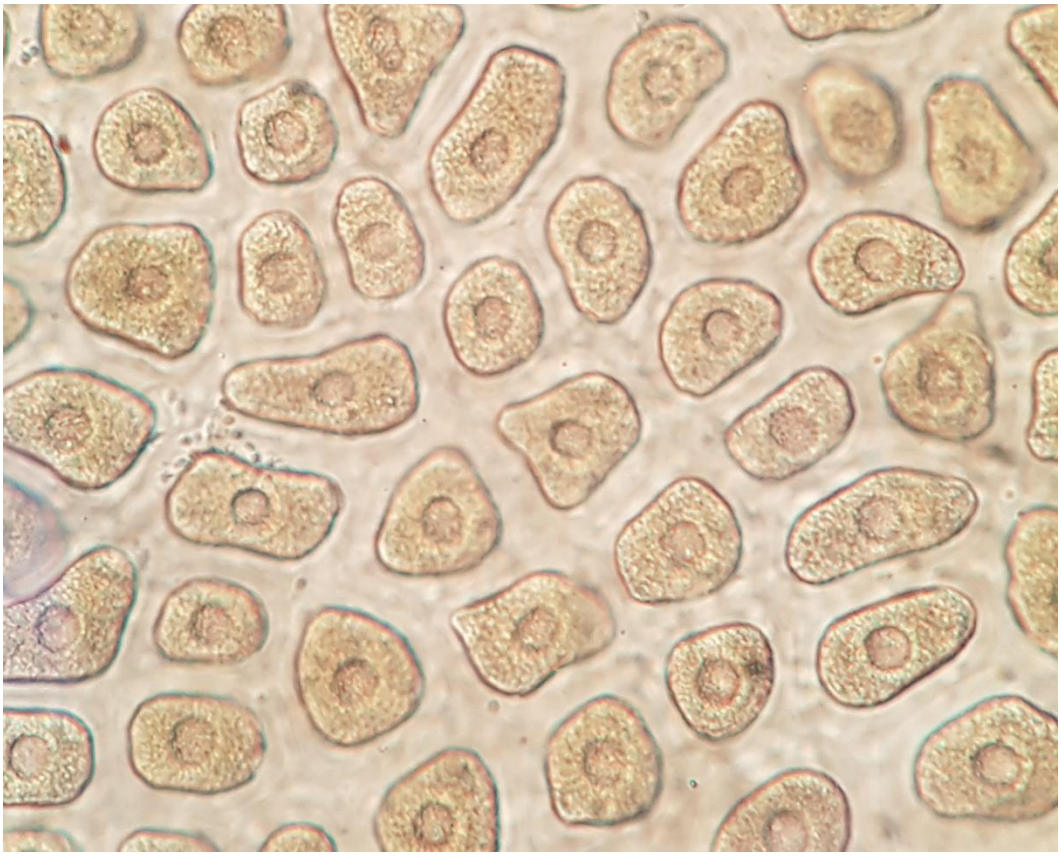

Figure 7. Embryonic cells of uninfected timothy seed.

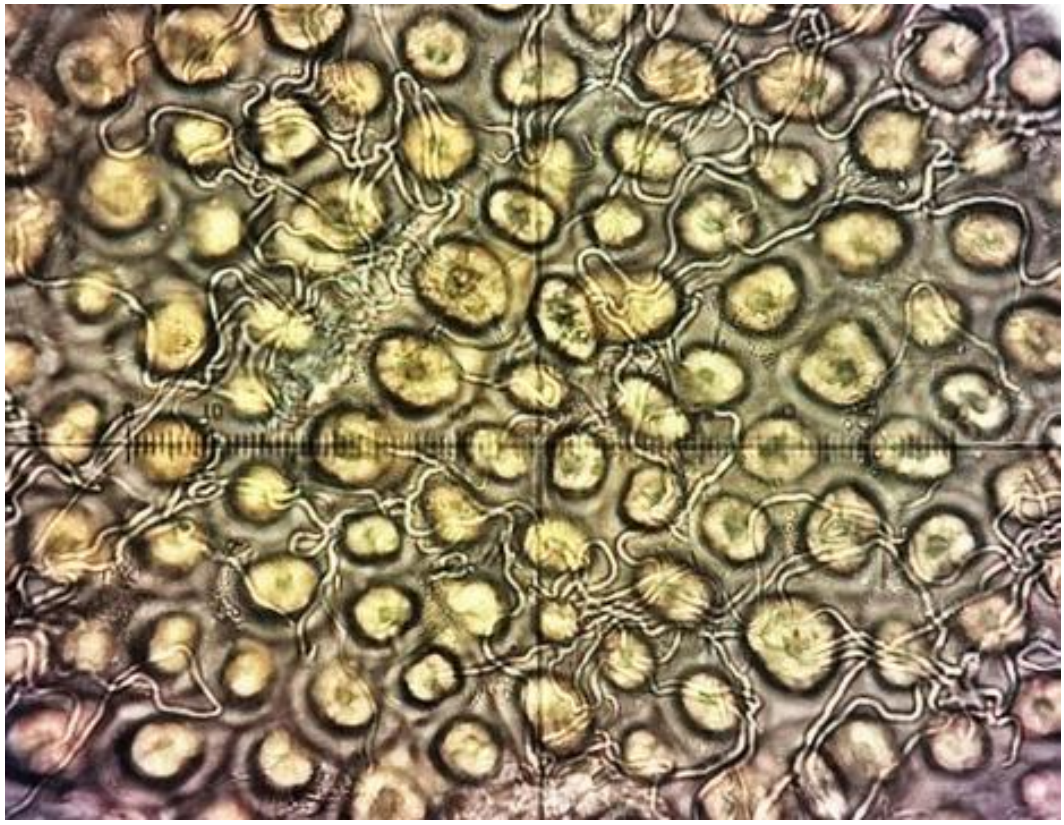

Figure 8. Embryonic cells of tall fescue seed infected by E. coenophiala. Photo: Miika Laihonen

4.7. PCR for Sanger sequencing

PCR for sequencing of the positive controls derived from the original DNA extraction was performed in a total volume of 50 µL using proportionate volumes of the same reagents as for detection of *Epichloë* spp., but instead using fungal specific primers ITS1F (Gardes and Bruns 1993), which targets a site in the ribosomal small subunit (SSU) encoding region, and ITS4 (White et al. 1990). The cycling parameters were 95 °C for 5 minutes, then 35 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds, finishing with 72 °C for 7 minutes.

After PCR, the products were analyzed by gel electrophoresis. For each sample 5 µL was loaded into wells of an 1% agarose gel in SB buffer at 250 V, 400 mA for 20 minutes. The DNA fragments were visualized with Nancy-520 by UV transillumination.

4.8. Column purification and Sanger sequencing

Before sending *Epichloë* spp. samples for Sanger sequencing to Macrogen Europe B.V., the E.Z.N.A Cycle Pure Kit was used to purify the PCR products from five

of the positive controls from the 50 µL cycling, only one of the timothy samples was purified. Isopropanol was used in the protocol and the product was eluted with 50 µL elution buffer. The concentrations of the samples were measured with Nanodrop. Finally, 250 ng of each sample were pipetted into 1,5 mL Eppendorf tubes and dried over night at 37 °C. The samples were posted the following day together with 4 µL of ITS1F primer per sample and instructions to dilute the samples with 20 µL.

4.9. Analyzing Sanger sequences

To determine which *Epichloë* spp. that were present in the five stroma samples sent for sequencing, SeqMan Pro (software by DNASTAR) were used to analyze and trim the sequences. After preparation of the sequences, BLASTn was used to search for matching sequences.

4.10. High-performance liquid chromatography

A collection of Swedish samples of hay, silage and green mass with or without stromata were sent to Oregon State University, Corvallis Oregon, USA for analysis of ergovaline and lolitrem B through high-performance liquid chromatography (HPLC), which with high precision separates molecules from complex mixtures found in various chemical and biological systems. In total 17 of the samples were analyzed. To guarantee that the desired components are detected, the detection approach is critical. One of the more common detectors in HPLC would be UV detection. In this case fluorescence detection was used, which is about 10-1000 times more sensitive than the UV detector and is one of the most sensitive liquid chromatography detectors (Siddiqui et al. 2017). Fluorometric detection is also more specific than UV which also strengthens the identification of the substances (E Nordkvist 2020, personal communication).

5. Results

5.1. Isolation of *Epichloë* spp. from stromata and infected stems of timothy

To carry out this part of the experiment, the method of Bacon et al. (1977) was used, however in that paper, fresh grass culms infected with *E. typhina* were used. The attempt to isolate *Epichloë* spp. on PDA failed. Nothing grew on the plates with stem cuttings from infected plants. On most of the plates with stroma cuttings nothing grew and on a few there seemed to grow bacteria instead of fungi. Fungi were only growing on one of the plates.

5.2. Incidence of *Epichloë* spp. in timothy seeds

Epichloë was not detected in any seed samples. Neither from seeds originating from fields where symptoms were observed or from symptomless fields, when using PCR and microscopy.

5.3. Sanger sequences

The Sanger sequences from the stroma samples did not match any *Epichloë* spp. when using BLASTn. Only a few of the samples had a high percentage identity with other species and those were different kinds of yeast fungi.

5.4. HPLC results

Table 7. Samples of hay, silage and green mass for analysis of mycotoxins Ergovaline and Lolitrem B [ppb].

Nr.	Type	Ergovaline [ppb]	Lolitrem B [ppb]
1	Hay	<100	<100
2	Hay	<100	<100
3	Silage	<100	<100
4	Green mass	<100	213
5	Green mass	<100	<100
6	Green mass	<100	<100
7	Hay	<100	118
8	Hay	<100	<100
9	Green mass	<100	<100
10	Hay	<100	<100
11	Hay	<100	<100
12	Hay	<100	<100
13	Green mass	<100	<100
14	Hay	<100	<100
15	Hay	<100	<100
16	Hay	<100	170
17	-	<100	102

In all samples analysed for mycotoxins, ergovaline concentrations were under the detection limit (100 ppb) and lolitrem B was only detected in four of the samples tested (sample 4, 7, 16 and 17). The concentrations of lolitrem B were low in the four samples where it was detected. Sample 4 had the highest concentration of lolitrem B and was a sample which only contained grass stems with stromata. It is also noticeable that the lolitrem B concentration was higher in sample 16 which did not contain stromata, compared to sample 7 and 17 which contained stromata (Table 10). It has been reported before that production of alkaloids with neurotoxic activities are more common in symptomless symbiosis (Leuchtmann et al. 2000; Schardl et al. 2012). In the three samples confirmed of containing only timothy, ergovaline could not be detected and the concentration of lolitrem B that were detected in sample 16 was quite low and the concentration detected in sample 17 was barely over the detection limit (Table 7 and 10).

6. Discussion

6.1. Isolation of *Epichloë* spp. from stromata and infected stems of timothy

If fungi with the same morphology would have grown on all or most of the plates with stroma cuttings it could have been assumed to be *Epichloë* spp. (M Helander 2020, personal communication) and the mycelia could have been used to send for sequencing. This was unfortunately not the case. Without extracting DNA from the fungus and running PCR as well as analyzing the product through gel electrophoresis, it cannot be determined if the fungus was of the genus *Epichloë* or not. Isolation from fresh stroma and fresh cuttings of infected plants should be used if this is to be tried again.

The results could have been expected since the material used was quite old by the time the experiment began. The plant tissue and stromata were collected in the growing season of 2019, the attempt to grow the fungi on PDA started early February. In observations by Western and Cavett (1959) examining *E. typhina* in orchard grass, the longevity of ascospores were tested from stromata collected over three seasons. The stromata were stored under partial cover or in open air. During all three years the stromata had a very high germination rate in July followed by a steady decline until November, were only 14% of the stromata yielded any viable ascospores at all and those were few. At the end of January, no viable ascospores were possible to obtain at all. Hyphal growth from the stromata were not tested, but there is a possibility that this type of growth could have been possible even though no viable ascospores could be harvested. The results from Western and Cavetts' experiment likely explains the result for the attempts to isolate the fungus reported here. In an experiment by Alderman (2013), between 20-60% of ascospores from *E. typhina* were viable after 16 days of desiccation.

6.2. Incidence of *Epichloë* spp. in timothy seeds

Epichloë spp. was not detected in any seed samples from symptomatic or symptomless fields by either of the two methods used, PCR and microscopy. This suggests there is a lack of vertical transmission and horizontal transmission resulting in seeds infected with *Epichloë* spp. in timothy, or that the horizontal transmission resulting in infected seeds at least is uncommon. These results are supported by the literature. The only observed *Epichloë* species on timothy is *E. typhina* and vertical transmission has never been observed in that particular host-species interaction (Scharld 2010; Tadych et al. 2014).

The results of this study are supported by the survey of Huss-Danell (2008) as well, where *Epichloë* infection in timothy seeds and seedlings collected from agricultural sites in Sweden were absent. In another survey by Saikkonen et al. (2000) endophyte infections were only found in the seeds of one of five natural populations collected in Finland. It was never verified if the endophytes in the seeds of the natural population were *Epichloë* spp.

In an experiment by Chung and Schardl (1997), the possibilities of both vertical and horizontal transmission by *E. typhina* were tested under growth chamber conditions. The progeny (germinated seeds) of individuals of perennial ryegrass and orchard grass where *E. typhina*'s stromata did not completely sterilize the inflorescence were all tested negative for *E. typhina*. The progeny was tested through tissue-print immunoblot and for some individuals, microscopy as well. Thus, further supporting the lack of vertical transmission amongst *E. typhina*, for at least perennial ryegrass and orchard grass. When placing ascospores around uninfected perennial ryegrass plants with emerging inflorescence, 1,3% of the progeny tested positive by tissue-print immunoblot, thus confirming horizontal transmission (Chung and Schardl 1997).

Similar results were observed by Western and Cavett (1959) when harvesting seeds from infected second year plants of orchard grass which had developed partial heads. The microscopy study done reported here showed that many of the seeds were infected but that they had stopped developing at an early stage. All stages of the harvested seeds were found, ranging from completely aborted to viable seeds. Of the viable seeds, 116 of them were possible to grow and to be examined for infection over the following three cropping years, but with negative results. This further suggests that *E. typhina* is not seed borne through vertical transmission, since infected seeds can be harvested but they seem to be non-viable.

A possible issue with the method used in the study presented here could have been the seed collection. Even though the seeds were selected as random as possible, there could always be a risk that the selection was not random enough, implying

that more material could have been examined in the two different approaches. Using microscopy as method is a common way to examine endophyte infection of seeds and has been used in studies before (Western and Cavett 1959; Saikkonen et al. 2000; Zhang et al. 2017), 50 seeds were chosen to be examined (see Saha et al. 1988) due to time constraint, but 100 seeds per sample as in Saikkonen et al. (2000), would have been preferred. The detection limit of PCR could always be an issue but at the same time less material was used for the DNA extraction of the positive controls compared to the seed samples and were still tested positive for *Epichloë*.

6.3. Transmission of *Epichloë* spp. in timothy

The most likely scenario for infection of timothy in the first-year crop would be through in-blown conidia or ascospores (Western and Cavett 1959) that germinate to form conidia on healthy plant tissue or hyphae on wounded plant tissue (White 1997; Alderman 2013). In-blown spores may origin from infected weeds or other infected fields. The mechanism of infection by ascospores has previously been reported to be through infection of the inflorescence (Chung and Schardl 1997) and through hyphae from conidia or ascospores entering stubble or wounds (Western and Cavett 1959, Alderman 2013). The exact mechanisms for infection by ascospores and conidia are still somewhat unclear (Tadych et al. 2014). Conidia are transferred between hosts through *Botanophila* flies (Kohlmeyer and Kohlmeyer 1974; Bultman et al. 1995) or water (Tadych et al. 2012). Ascospores are carried with wind (White 1997; Tadych et al. 2012) and the most probable place for infection for them would be the meristematic zones of tillers and seedlings according to Tadych et al. (2012). After the initial infection of a field, a few stromata can cause the disease to spread rapidly under conducive conditions in the following years (Western and Cavett 1959).

Humidity seems to be of importance for the spread of choke disease. During dry conditions spores will shrivel or die (Western and Cavett 1959; Alderman 2013), and the host plant material dries up and becomes harder to penetrate for the endophyte. The combination of environmental factors and harvest time will probably partly determine the amount of infection in the following years of a newly sown crop, for example, high humidity during harvest might increase the risk for infection. An average of 5,4% infection was obtained when orchard grass was cut and artificially brushed with stromata (Western and Cavett 1959). Infections from old stromata or plant debris may be unlikely since the longevity of stromata seems to be poor (Western and Cavett 1959; Alderman 2013). In addition, stromata seem to be appealing to soil fauna and insects and is therefore most likely destroyed before the following growing season and may therefore not act as an infection source. Stromata have also been observed to be heavily overgrown with molds such as

Cladosporium and *Penicillium*. Animals that feed on the stromata may spread them to various locations through movement, thus possibly mediating infection of healthy plants (Western and Cavett 1959).

6.4. Sanger sequences

Unfortunately, the species of *Epichloë* present in the stroma samples could not be determined. Instead DNA from other fungi got amplified when using fungal specific primers ITS4 and ITS1F. Because the PCR primers used to detect *Epichloë* spp. are not species specific, they were not used for sequencing. The sequences matched mostly with different yeast species and this result might be due to old sample material. It is possible for other fungi to colonize the stromata during the cropping year and this is what might have happened. The samples were collected during the cropping year of 2019 and were not analyzed until early 2020. The observations by Western and Cavett (1959) indicates that colonization of stromata by other fungi are not unreasonable. If this method is to be used again to identify which *Epichloë* spp. that are present in Swedish timothy, fresh stromata and tissues of infected plants should preferably be used.

6.5. Ergovaline and lolitrem B

As mentioned before, ergovaline is responsible for tall fescue toxicosis (Belesky et al. 1988) and lolitrem B for ryegrass staggers (Gallagher et al. 1981), thus making these two alkaloids very important to control for the ruminant husbandry branch of agriculture and the horse industry. The threshold level for ergovaline is 400-750 ppb in cattle, but in one study 475 ppb ergovaline did not cause any clinical symptoms. The threshold levels are 500-800 ppb in sheep and 300-500 ppb in horses. For lolitrem B, the threshold levels are 1800-2000 ppb for cattle and sheep (Hovermale and Craig 2001) and for horses it is 800 ppb (Jensen 2005), which is considerably lower than for cattle and sheep. It is however reported in Easton et al. (1996), that livestock grazing on tall fescue containing lower than 200 ppb ergovaline showed clinical symptoms of fescue toxicosis and in another experiment, physical effects were reported when livestock were fed a diet containing as low as 50 ppb ergovaline. Cattle are more susceptible to clinical disease due to ergovaline under heat stress (Hovermale and Craig 2001).

All samples in the study reported here contained less than the threshold limit for both ergovaline and lolitrem B for cattle, sheep and horses. These results indicate that there might not be an urgent risk for livestock to graze on Swedish pasture lands or be fed forage, at least for the cropping year 2019. The risk of physical

effects from as low as 50 ppb ergovaline could always be present in Swedish agriculture, but it is not possible to evaluate this since the method used to detect ergovaline does not detect levels lower than 100 ppb. Early literature describing animals allegedly poisoned or injured by *E. typhina*, were not confirmed by any critical tests (Sampson, 1933), and Eriksson (1904) reports that there were no reports of poisoned or injured animals in Sweden after the severe outbreak of choke disease in timothy 1901 and 1902. The sample which had the highest concentration of lolitrem B was a sample which only contained stems with stromata, thus meaning a very high incidence of *Epichloë* spp. in the sample and it is therefore not very surprising that the sample in question had the highest concentration of lolitrem B.

Earlier studies report that neither ergot alkaloids (including ergovaline) or indole diterpenes (including lolitrem B) are synthetized by *E. typhina*. In Vikuk et al. (2019), *E. typhina* in orchard grass is only reported to synthetize peramine, an insect deterrent (Hume et al. 2016). Leuchtmann et al. (2000) reported that there is no synthetization of alkaloids by *E. typhina* in host-species interactions with sweet vernal grass, heath false brome, orchard grass, timothy, except for rough bluegrass where peramine was synthetized. This suggests that there should be a low risk for livestock to be fed with timothy, orchard grass and other species only known to be infected by *E. typhina*. It is however interesting that in two of three of the samples confirmed to contain only timothy lolitrem B was found. This suggests that lolitrem B could possibly be synthesized by *E. typhina* since it is the only confirmed species in timothy. However, contamination of the samples by other grass species might have affected this result.

7. Conclusions

Since the sequencing of the positive controls and isolation of *Epichloë* spp. from stromata in this study failed, it is impossible to tell which species of *Epichloë* that is present in timothy in Sweden. The efforts to isolate *Epichloë* spp. also failed probably due to the age of the stromata (Western and Cavett 1959; Alderman 2013), which further adds to the difficulties to draw any conclusions. The reason why the sequencing of the positive controls did not give any satisfying results, was probably because of colonization of the material by other fungi due to the age of it. However, since *E. typhina* is the only species that has been confirmed in timothy, it can be assumed that this is the species present in Swedish timothy and that it is responsible for the severe outbreaks of choke disease in the cropping year of 2019.

No *Epichloë* spp. could be verified in the seeds by any of the two methods used in this study. This suggests that *Epichloë* spp. seed infection in timothy is absent or at least very rare. Similar results have also been found in timothy, orchard grass and perennial ryegrass in other studies (Western and Cavett 1959; Chung and Schardl 1997; Saikkonen et al. 2000; Huss-Danell 2008). It is possible that infection of timothy seeds occurs through infection of florets by iterative germination of ascospores (Chung and Schardl 1997; Schardl 2010).

The nature of *Epichloë* spp. initial infection in timothy could be a result of horizontal transmission from wild populations or other agricultural fields of grass hosting the endophyte. Infection is probably a result of in-blown ascospores that germinate to infect florets (Chung and Schardl 1997; Schardl 2010) or wounds (Western and Cavett 1959; Alderman 2013) in the plants. Whilst in the field, the endophyte might spread contagiously through ascospores, water-dispersed conidia (Tadych et al. 2012), through vectoring flies (Kohlmeyer and Kohlmeyer 1974; Bultman et al. 1995) or possibly through external mycelial networks (White et al. 1996). The best way to manage infection in seed lots would probably be through spraying with systemic fungicides (Harvey et al. 1982; Latch 1983; Hill and Brown 2000) in the growing crop early on and with frequent intervals (Hill and Brown 2000) to get rid of or suppress the endophyte, securing the yield for timothy seed producers. Avoiding cutting the crop when it is very humid might also prevent infection. Ploughing is another measure to stop the spread of the disease to surrounding timothy fields (Cagaš and Macháč 2012).

The fact that the only *Epichloë* spp. reported on timothy is *E. typhina* (Tadych et al. 2014) and that the literature report that the only alkaloid produced by *E. typhina* is peramine (Leuchtmann et al. 2000; Vikuk et al. 2019) suggests that there is no apparent danger for livestock to graze on timothy or to be fed with timothy fodder. The results from the present study are a bit conflicting with the literature, since lolitrem B was detected in two samples that were confirmed to contain only timothy. This would indicate that *E. typhina* actually could synthesize lolitrem B, but it can also be explained by the presence of other infected grass species in the analyzed samples. Although very little information of each sample was available, concentrations of egovaline and lolitrem B were below the detection limit in most samples and in the four cases where lolitrem B could be detected, the concentrations were at low levels, and under the risk thresholds for cattle, sheep and horses. The results indicate that there was no obvious risk regarding toxic alkaloids for livestock in Sweden in 2019.

It is not possible to draw any conclusions from the Swedish field monitoring of choke disease in 2014 and 2019, regarding the cultivars or counties. There were only two cultivars that had a high incidence of choke disease (present in >20% of the fields) and a considerable number of fields inspected (>10) during the two occasions, Lischka and Switch. This information could be interpreted that these two cultivars are more susceptible to choke disease than the other cultivars, but there are no other studies to support this hypothesis. The results in the survey by Cagaš and Macháč (2012), did not share any similarities with the Swedish monitoring. The disease incidence is likely a combination of geography, fungal-plant genetics and environmental factors (Tadych et al. 2014). The number of fields with choke disease in Sweden were similar both years and it was more commonly observed in older fields than younger fields, this has been reported in other studies (Western and Cavett 1959).

The objective of this study has been accomplished in general through the laboratory work and the literature review. There are however some unclarities that would need to be resolved, such as the exact life cycle of *Epichloë* spp. in timothy, what factors that triggers the outbreaks and if there is any true variation between cultivars. It would also be favorable to identify which species of *Epichloë* that are present in timothy in Sweden since this study failed to identify which species that were present in the positive controls that were used. Also, more samples of for example timothy and orchard grass should be analyzed for mycotoxins to clarify whether lolitrem B actually could be synthesized in these hosts. Choke disease is an important disease for grass seed producers and should be further investigated.

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Appendix I

Table 8. List of seed samples used in this study, presented in cultivar, country of origin and if choke disease was present in the field or not.

Sample	Cultivar	Country of origin	Choke disease in field
1	Lischka	Sweden	No
2	Lischka	Sweden	No
3	Lischka	Sweden	No
4	Rhonia	Sweden	No
5	Rhonia	Sweden	No
6	Polarking	Sweden	No
7	Switch	Sweden	Yes
8	Switch	Sweden	Yes
9	-	Sweden	No
10	-	Sweden	No
11*	-	Sweden	Some of the fields
12	-	Sweden	No
13**	-	Sweden	Yes
14****	-	Sweden	Yes
15	-	Sweden	No
16*	-	Sweden	Some of the fields
17***	-	Sweden	No
18**	-	Sweden	Yes
19****	-	Sweden	Yes
20***	-	Sweden	No
21*	-	Sweden	Some of the fields
22*	-	Sweden	Some of the fields
23*	-	Sweden	Some of the fields
24	Classic	Denmark	-
25	Comer	Denmark	-
26	Comtal	Netherlands	-
27	DOLINA	Belgium	-
28	Motim	Netherlands	-

Sample	Cultivar	Country of origin	Choke disease in field
29	Moverdi	Netherlands	-
30	Promesse	Denmark	-
31	Richmond	Canada	-
32	Sleipnir	Finland	-
33	Summergraze	Denmark	-
34	Tiller	Denmark	-
35	Winnetou	Denmark	-

* The samples are from the same two seed lots

** The samples are from the same seed lot

*** The samples are from the same seed lot

**** The samples are from the same seed lot

Appendix II

Table 9. List of stroma samples used in this study, presented in species and cultivar.

Sample	Species	Cultivar
100	Timothy	Lischka
101	-	-
102	-	-
103	Mixed ley	-
104	Timothy	-
105	Orchard grass	-

Appendix III

Table 10. Detailed list for samples of hay, silage and green mass for analysis of mycotoxins Ergovaline and Lolitrem B [ppb].

Nr.	Sample ID	Type	Comment	Ergovaline [ppb]	Lolitrem B [ppb]
1	19-FOD001796	Hay	-	<100	<100
2	19-FOD001797	Hay	-	<100	<100
3	19-FOD001938	Silage	Timothy and meadow fescue from one bale, no visible stromata, 5-year-old ley	<100	<100
4	19-FOD001940	Green mass	Ley, only grass stems with stromata from bales before wrapping	<100	213
5	19-FOD001941	Green mass	Ley with root and soil, visible stromata	<100	<100
6	19-FOD001942	Green mass	Samples from approximately	<100	<100

Nr.	Sample ID	Type	Comment	Ergovaline [ppb]	Lolitrem B [ppb]
			20 bales before wrapping, no visible stromata		
7	19-FOD002336	Hay	Stromata visible	<100	118
8	19-FOD002337	Hay	No stromata, 3-year-old ley	<100	<100
9	19-FOD002340	Green mass	Collected before baling	<100	<100
10	19-FOD002341	Hay	Harvested 2018	<100	<100
11	19-FOD002342	Hay	Harvested 2018	<100	<100
12	19-FOD002343	Hay	Harvested 2018	<100	<100
13	19-FOD002344	Green mass	Timothy	<100	<100
14	19-FOD002345	Hay	-	<100	<100
15	19-FOD002346	Hay	-	<100	<100
16	19-FOD002364	Hay	Timothy, no stromata, 4-year-old ley	<100	170
17	20-FOD000020	-	Timothy, cultivar Lischka, 2-year-old seed lot, 5% choke disease, 2019	<100	102