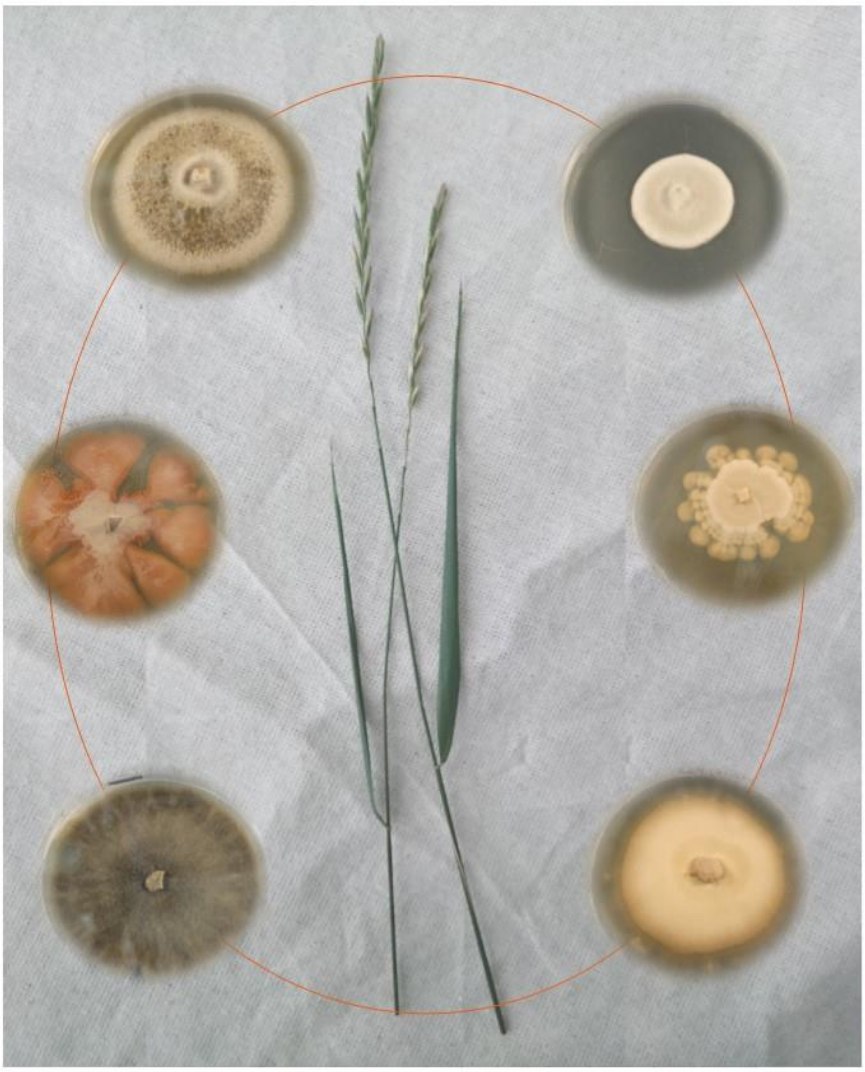
A thesis submitted for the degree of Doctor of Philosophy

Isolation and characterisation of novel fungal root endophytes from *Elymus repens* (Poaceae) for resistance to *Fusarium*, *Gaeumannomyces* and *Pyrenophora*



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Declaration

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Summary

Barley is an important crop worldwide with production largely used for animal feed and alcoholic beverages. Diseases are a major limiting factor to its production, which have, up until recently, been controlled by agrochemicals. However, legislation on the use of agrochemicals, especially within the European Union, is being tightened and there is growing interest in integrated pest management. This means that there is an increasing focus on controlling diseases using biological control. Living microorganisms that are applied as biological control agents (BCAs) to either soil, seed or leaves can have difficulty in persisting once applied. Therefore, the focus of this study is on endophytes, which can be defined as microorganisms that live inside plants without causing symptoms of disease. In this thesis, we discuss the different approaches for finding and testing beneficial endophytes as well as the endophyte host range. Furthermore, we undertook a comprehensive literature search to summarise previous studies that have investigated the use of endophytes as well as other BCAs against barley diseases.

The fungal root endophytic community of *Elymus repens*, a crop wild relative of barley, was investigated using culture dependent and direct amplicon sequencing approaches from the same root systems. Plants were sampled from five agricultural sites in Ireland that had high disease pressure from *Fusarium* spp. and *Gaeumannomyces graminis*. It was hypothesised that these sites would harbour endophytes that could be used as BCAs. Endophytes were cultured on three different types of media (PDA, MEA and 2 % MEA) and their isolation efficacy was estimated. Furthermore, three DNA barcoding regions (ITS, LSU and TEF1α) were used for the identification of the cultured endophytes and their effectiveness compared. Lastly, the cultured endophyte community was compared to the community identified by direct amplicon sequencing from root samples. The number of cultured fungal isolates from the different sites did not correspond to the OTU richness determined by direct amplicon sequencing. The ITS barcoding region identified the largest number of cultured OTUs (27) compared to LSU (23) and TEF1α (13), and the ITS region was

primarily used for identification of the cultured endophytes. The OTU richness of cultured endophytes was influenced by the types of media used. The majority of OTUs were cultured on PDA (18) however, without the inclusion of MEA and 2 % MEA approximately half of the total number of unique OTUs would not have been discovered. A total of 715 different fungal OTUs were discovered across all five sites by direct amplicon sequencing and they belonged to 31 different taxonomic classes from 8 different divisions. From site III, 349 OTUs were identified by direct amplicon sequencing but only 66 OTUs were cultured. The two communities shared ten OTUs and only four of them were among the 48 OTUs that were found in all plants from site III determined by direct amplicon sequencing. The direct sequencing techniques have therefore revealed the full diversity of fungal endophytes and demonstrated that there is a high degree of heterogeneity among communities across different sites and individual plants. The cultured community from one site represented only a small subset of the full diversity but was still diverse and hosted several previously tested control agents including *Epicoccum nigrum* and *Periconia* sp. as well as many previously untested species.

Twenty four of the fungal endophytes isolated from *Elymus repens* were screened in barley against foot and root rot caused by *Fusarium culmorum* and net blotch caused by *Pyrenophora teres*, under controlled conditions. In all experiments the endophytes were applied as seed dressings. Treatment with *Periconia macrospinosa* E1 significantly ($P \leq 0.05$) reduced disease symptoms in two out of four experiments. A subset of endophytes consisting of eight isolates was also tested against the leaf pathogen *Pyrenophora teres.* Seed treatment with Lasiosphaeriaceae sp. E10 reduced net blotch symptoms significantly, but was only effective in one out of three experiments. Often a change in fungal lifestyle is reported in relation to changes in host genotype as well as biotic and abiotic conditions. However, here endophytic strains from *E. repens* appeared to remain neutral and in a few cases were antagonistic to fungal pathogens in a controlled environment when tested in barley.

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Chapter 1

1. General introduction

This thesis reviews the current literature on biological control of barley diseases and experimentally explores the communities of root endophytes in *Elymus repens* and their role in disease resistance. Chapter 2 uses the literature to explore several questions including, among others, how do studies find their biocontrol agents? And how are they tested? Chapter 3 explores the fungal root endophyte community of *Elymus repens* with the aim of discovering new biocontrol agents. It investigates and compares the fungal root endophyte community using two approaches, one method that depends on direct sequencing and another method that depends on culturing. The chapter also describes endophyte communities of individual plants which has not been done before. The final experimental chapter (Chapter 4) tests 24 of the cultured endophytes as biocontrol agents of three important diseases of barley and Chapter 5 discusses the overall findings of the thesis and their significance in a wider context.

1.1 Important diseases of barley in northern Europe

Barley is the fourth most cultivated cereal in the world, with a global production of more than 141 million tonnes (FAOSTAT 2018) and it is the most important cereal in Ireland (Tyndall National Institute 2017). Barley is primarily used as animal feed (55-60 % of total production), and for malting in the production of alcoholic beverages (30-40 %). Additionally, barley crops are used for seed production (5 %) and a small proportion is used for human consumption (2-3 %, Ullrich 2011). It is estimated that pre-harvest losses in cereals can be as high as approximately 35 % of the yield (Akar *et al.* 2004). Diseases in particular can cause severe damage and there are many important barley diseases worldwide. Some of the most important diseases in northern Europe are: powdery mildew (*Blumeria graminis* f. sp. *hordei*), leaf blotch or scald (*Rhynchosporium graminicola*), rusts such as brown rust (*Puccinia hordei*) and yellow rust (*P. striiformis* f. sp. *hordei*), net blotch (*Pyrenophora teres*), Ramularia leaf spot (*Ramularia collo-cygni*) and barley yellow dwarf (*BYDV*) (Walters *et al.* 2012; Oldach 2018), which all cause

symptoms on leaves (Mathre 1982; Videira *et al.* 2016). In addition, Fusarium head blight (*Fusarium* spp.) affects the malting quality and yield of malt which is important when producing beer and whiskey (Oliveira *et al.* 2012; Nielsen *et al.* 2014; Potterton and McCabe 2018). Furthermore, *Fusarium* spp. can decrease the quality of the grain by producing mycotoxins that are harmful to animals including humans (Wegulo *et al.* 2015). *Fusarium* spp. cause early symptoms in roots and later symptoms occur in the heads (Mathre 1982; Scherm *et al.* 2013). In addition, take-all (*Gaeumannomyces tritici*), a root disease, has become a problem in countries relying on monotonous crop rotation practices (Mathre 1982; Minnock 2017).

This thesis focuses on two soil-borne diseases caused by *Fusarium culmorum* and *Gaeumannomyces tritici*, respectively, as well as a leaf disease caused by *Pyrenophora teres. Fusarium culmorum* can cause foot and root rot (also known as Fusarium crown rot) as well as Fusarium head blight in barley and wheat (Scherm *et al.* 2013). *Fusarium* spp. survive between cropping seasons in crop residues or in the seeds as mycelium, chlamydospores or ascocarps (perithecia) (Mathre 1982; Wegulo *et al.* 2015). *Fusarium* spp. have been shown to be able to survive in soil for 5-8 years as chlamydospores (Mathre 1982). Some of the symptoms of foot and root rot include seedling death before or after emergence and brown discolouration of roots and coleoptiles (Figure 1.1A, Scherm *et al.* 2013). The main symptom of Fusarium head blight is the bleaching of one or more spikelets in the spike. The disease can cause up to 74 % yield loss which is mainly the result of spikelet sterility and reduced kernel size (Wegulo *et al.* 2015). The infection can also reduce quality through kernel discolouration (Fusarium-damaged kernels) and the presence of the mycotoxins including deoxynivalenol (DON), nivalenol and sterol zearalenone (Wegulo *et al.* 2015). As an example, DON is toxic to animals and studies have shown a linear relationship between the degree of Fusarium head blight disease severity and the concentration of DON (Wegulo *et al.* 2015). Different factors have been shown to increase disease severity including soil temperatures of 20-30 °C and high water content of the soil. No

resistant barley cultivars are available and the disease is primarily managed by fungicidal seed dressings (Mathre 1982).

Gaeumannomyces tritici is a major problem in wheat, but can also cause symptoms in barley and rye (Kwak and Weller 2013). Furthermore, the fungus has been shown to infect wild grasses within the genera *Agropyron*, *Agrostis* and *Bromus* (Mathre 1982). Some of the symptoms of take-all are yellowing of the shoots, stunted growth, root rot and nutrient deficiency. Take-all produces characteristic lesions on the roots that are dark brown in colour (Kwak and Weller 2013). *Gaeumannomyces tritici* survives as mycelium in host debris, in roots of other grasses or occasionally as perithecia on host debris (Mathre 1982) from where ascospores are spread (Kwak and Weller 2013). Hyphal infections in the soil are believed to be the most important means of pathogen spread and when hyphae reach the root, they will colonise the root surface and penetrate (Mathre 1982).

Figure 1.1. Disease symptoms caused by two diseases of barley. A) Increasing severity, from left to right, of symptoms of foot and root rot (*Fusarium culmorum*) with brown discolouration of roots and coleoptiles as well as seedling death. B) Symptoms of net blotch (*Pyrenophora teres*) with dark-brown lesions surrounded by chlorosis on leaves.

The symptoms of take-all are often seen in patches in the field and thus it is considered that the pathogen spreads between plants via runner-hyphae which are darkly pigmented hypha that grow on the surface of the root (Mathre 1982). Moderate moisture levels will often promote patches of disease in the field while high moisture levels can often cause more comprehensive spread and also lead to the pathogen infecting the lower part of the shoot, in addition to the root. Take-all infection blocks the water and nutrient flow of the plant and this will finally lead to water stress and nutrient deficiency (Kwak and Weller 2013). Additionally, take-all is favoured by high pH as well as nitrogen- and phosphorous deficient soils. Occasionally, in soils with low pH, ammonium based fertilizer has worked to reduce incidence of the disease (Mathre 1982). However, liming has been shown to cancel the positive effect of the ammonium based nitrogen fertilizer and N fertilizer, in the form of nitrate, makes disease symptoms worse (Mathre 1982). Take-all resistant *Hordeum* cultivars are not available (Mathre 1982) but the active compounds silthiofam available in Europe and fluquinconazole available in Australia have been made into respective seed dressings, which are available against take-all, but are expensive for farmers to use (Bayer 2018; CERTIS 2018; Fitzgerald 2018). Apart from chemical control, a crop rotation with break crops is the most efficient control measure, but it is not always feasible in today's management regimes (Asher and Shipton 1981). Take-all decline is the development of a suppressive soil after repeated growth of plants that are susceptible to take-all and some farmers will wait for take-all decline so that they can continue with a mono-cropping regime (Kwak and Weller 2013). Take-all decline occurs because of a change in the microbial composition in the soil or the rhizosphere that results in a suppression of the pathogen (Schlatter *et al.* 2017). 2,4-diacetylphloroglucinol producing fluorescent *Pseudomonas* spp. has been shown to be responsible for take-all decline in Washington State and in the Netherlands (Raaijmakers and Weller 1998; De Souza *et al.* 2003). One strain of these bacteria have been shown to have almost 6 % of its genome dedicated to the production of antibiotic compounds (Loper and Gross 2007).

Net blotch is a fungal disease caused by *Pyrenophora teres*. The fungus primarily infects barley, but it has been shown to infect, among others, wild *Hordeum* species as well as *Avena, Bromus, Elymus* and *Triticum* species (Liu *et al.* 2011). Net blotch reduces grain yield as well as quality and is estimated to cause yield losses of around 10-40 % (Mathre 1982). The disease symptoms are dark-brown, narrow, longitudinal and transverse striations on leaves (Figure 1.1B, Liu *et al.* 2011). Chlorotic tissue will form around the brown lesions and if the infection is severe the whole leaf will become necrotic. *Pyrenophora teres* has also been shown to infect caryopses, but the symptoms look very similar to other fungal infections (Mathre 1982). *Pyrenophora teres* survives as seed-borne mycelium or as pseudothecia in host residue and it can stay viable for more than two growing seasons (Mathre 1982). The origin of the primary inoculum is not completely understood. Perhaps it is from plants that survived the winter, from plant debris or from seed-borne mycelium (Liu *et al.* 2011). Conidia formed from the primary lesion makes up the secondary inoculum and the spores are spread with the wind at least as far as seven meters (Mathre 1982). High nitrogen status of the plant is correlated with high disease severity. Furthermore, the pathogen thrives in humid and warm conditions (15-25 °C, Mathre 1982). *Pyrenophora teres* resistant barley cultivars exist (Liu *et al.* 2011), but the disease is primarily controlled by fungicide applications (Rehfus *et al.* 2016). However, *P. teres* isolates have been found which are resistant to the most commonly used fungicides (Rehfus *et al.* 2016).

1.2 Biological control of plant diseases

The control of these highlighted diseases (Section 1.1) mainly relies on the use of fungicides. However, regulations within the European Union aims at securing sustainable use of pesticides and promoting integrated pest management (Department of Agriculture 2013) as part of the Directive 2009/128/EC (European Parliament 2009). Integrated pest management (IPM) has been defined by ENDURE (2008) as “a sustainable approach to managing pests by combining biological, cultural and chemical

tools in a way that minimises economic, environmental and health risks". IPM is the combination of different practices that together can overcome the limitations of the individual practices (Chandler *et al.* 2011). IPM is not a new invention, perhaps just a principle that has not been put into practice for a long time (Kendrick 1977). One of the many tools in IPM is the use of biological control (Lewis and Papavizas 1991; Chandler *et al.* 2011). Biological control is a subgroup known under the term "biopesticides" which is frequently used within Europe. The term biopesticides, is often used to describe a broad range of products that can be used to protect crops against diseases, pests and weeds. Biopesticides can be divided into two groups that either rely on the use of 1) living organisms directly, or 2) naturally occurring compounds, including extracts from organisms as well as volatiles (Balog *et al.* 2017). Only a limited number of registered biopesticide products are available in Europe and they are based on bacteria, fungi or viruses (Kabaluk *et al.* 2010; Balog *et al.* 2017). The products that control plant diseases are most often based on a species that is different from the plant pathogen. However, sometimes weak strains or non-pathogenic isolates of the same pathogen species are used (Punja 1997; Kabaluk *et al.* 2010). Biological control appears especially desirable for diseases where no good plant genetic resistance exists within the gene pool of the crop and where pesticides are inefficient or expensive to apply such as for the control of take-all in wheat and barley and clubroot in cruciferous crops (Butt *et al.* 2001; Fitzgerald 2018).

Within the scientific literature, the term "biological control" is commonly used (Brodeur *et al.* 2018), but there is no common agreement about the definition. Biological control of plant diseases has most often been defined as the use of living organisms for crop protection (Pal and Gardener 2006). However, from now onwards, in this thesis, the term biological control is used more narrowly to describe the control of plant diseases by microorganisms. A variation of different biological control agents including endophytes, which are microorganisms living inside plants, have been used successfully to protect barley against diseases (Chapter 2).

1.2.1 Mechanisms of biological control

Plants have several ways of defending themselves against pathogens. Plants can have morphological obstructions including waxy and hairy surfaces as well as chemical barriers including secondary metabolites of which glucosides and nicotine are examples (Hammond-Kosack and Jones 1996). Plants also have induced defence responses which can be triggered either by microbe-associated molecular patterns (MAMPS) or by the recognition of effectors so called effector triggered immunity (Bernoux *et al.* 2011).

When microorganisms protect plants against pathogens they can use one or a combination of four general mechanisms, namely 1) antibiosis, 2) competition, 3) induction of host plant resistance and 4) parasitism (Guetsky *et al.* 2002; Alabouvette *et al.* 2006). To study mechanisms, it is essential that the appropriate disease symptoms are evaluated and it is vital to choose relevant control treatments. It can be difficult to design an experiment so that all possible mechanisms can be tested and, therefore, usually only one or a few mechanisms are examined per study, even though all four may contribute to the control *in planta*.

The first mechanism that control agents can use to supress disease is antibiosis, which results in the production of a compound that is harmful to the pathogen (Fravel 1988). For example, *Trichoderma* spp. have been shown to produce a range of different antibiotic compounds including gliotoxin, gliovirin, alamethicin and trichovirin II (Mukherjee *et al.* 2012). Another example is the fungus *Verticillium biguttatum*, which has been shown to produce two antimicrobial compounds bigutol and methylbigutol (Morris *et al.* 1995).

The second mechanism, when antagonists control disease through competition, works because the antagonist can be a better competitor for nutrients and/or for space (Ghorbanpour *et al.* 2018). Card *et al.* (2009) showed that *Trichoderma atroviride* was a better competitor for glucose under glucose-limited conditions and could reduce the germ tube length of *Botrytis cinerea* by 25 % and thereby the number of infection sites.

Kirk and Deacon (1987) showed that *Microdochium bolleyi* reduced disease symptoms by competing for space with *Gaeumannomyces tritici* on wheat roots.

The third mechanism, involves the induction of host plant resistance. Traditionally, two types of induced resistance are distinguished, namely systemic acquired resistance (SAR) depending on pathways controlled by salicylic acid and induced systemic resistance (ISR) which is dependent on jasmonate and ethylene controlled pathways (Vallad and Goodman 2004). In practice it can be tricky to determine which type of induced resistance is at work because often the defence responses are studied and not the signalling pathways that initiated the response. The biological control agent *Fusarium oxysporum* (Fuchs *et al.* 1997; Veloso and Díaz 2012) and *Rhizoctonia* spp. (Xue *et al.* 1998) are examples of fungi that have been shown to exhibit biological control through induced resistance. In order to show that induced resistance is activated, it is a prerequisite that the defence response(s) of the plant is significantly increased in response to the inducer (Kloepper *et al.* 1992). Examples of some of the defence responses that have been measured in order to show induced resistance include 1) fungal cell wall degrading enzymes such as β-1,3-glucanases and chitinases (Xue *et al.* 1998; Bowman and Free 2006), 2) reinforcement of the cell and restriction of pathogen growth by increased papillae formation, catalase activity, peroxidase, polyphenol oxidase and phenols (Jørgensen *et al.* 1998; Xue *et al.* 1998; Li and Steffens 2002; Anand *et al.* 2007; Mhamdi *et al.* 2010; Daayf *et al.* 2012; Rios *et al.* 2014), and 3) general defence reactions through phenylalanine ammonia lyase and upregulation of pathogenesis related (PR)-protein genes (Bevan *et al.* 1989; Khan *et al.* 2006; Anand *et al.* 2007). It is logical that if studies conclude that their biological control agent works through induced resistance then the investigation will have to be done *in planta*. Also, it is important that the elevated plant defence responses are relevant to stop the specific pathogen. Furthermore, studies that quantify defence responses in plants have to include treatments with pathogen and antagonist present together in order to compare with treatments with pathogen alone.

The fourth mechanism, occurs when antagonists actively parasitize the pathogen. *Chaetomium globosum, Clonostachys rosea* and *Trichoderma* spp. are examples of fungi that have been shown to be able to degrade cell walls through mycoparasitism *in vitro* (Zeilinger *et al.* 1999; Inglis and Kawchuk 2002). To show that the control organism is working through parasitism it is important to show that nutrients are taken up from the pathogen by the control agent which can be done by for example showing penetration of the target organism. Studies *in planta* are less frequent, but Flores *et al.* (1997) tested a mutant of *Trichoderma harzianum* overproducing a cell wall degrading enzyme and found that disease symptoms of *Rhizoctonia solani* was reduced in cotton plants.

In general, firm conclusions of the mechanisms in play cannot be based on *in vitro* studies lacking the plant. Mechanisms like antibiosis, competition and parasitism can be difficult to show *in planta*, thus, investigations often rely on dual culture *in vitro* studies without plant to give indications of the mechanism.

1.2.2 Commercialisation of biological control products

In 1991, the European Union (EU) began a review of the existing active compounds found in plant protection products (European Council 1991). Due to a number of factors including withdrawal from industry, failing the review and missing or incomplete documentation, 74 % of the active compounds were banned from the European market on the grounds that they were deemed unsafe for human health and the environment (European Commission 2009). In 2009, new approval criteria on active substances were implemented and it is at EU-level that the active compounds are approved, while member states authorize the use of the plant protective products that contain the active substance(s) (European Commission 2009).

Within the European Union, 24 microorganisms have been approved for use against fungal pathogens of which eight are bacterial, fifteen are fungal and one is an oomycete (Table 1.1). The majority of the bacterial species belong to the genus

Bacillus and the majority of fungal species belong to the genus *Trichoderma.* In Ireland, only two bacterial and six fungal strains have been approved and they can be used against fruit damage caused by grey mould (*Botrytis cinerea*), against soil borne fungi of potatoes, and against *Fusarium* spp., powdery mildew, *Pythium* spp., *Rhizoctonia* spp. and *Sclerotinia* spp. of horticultural crops (European Commission 2016). Furthermore, an all-round product is also available which can be used against seed, soil and foliar diseases of horticultural crops (European Commission 2016). So far, no biological control product has been approved in Ireland to treat fungal diseases of agricultural crops, however products are approved in other European countries.

Biological control organisms and chemical pesticides are regulated in the same way by the European Union and on average it takes seven years to get biopesticides registered (Balog *et al.* 2017). Some groups advocate that biopesticides relying on living organisms should not go through the same registration process as regular pesticides because they claim that the use of microorganisms is sustainable and environmentally safe (Ehlers 2006). However, it could be wrong to make such an assumption because some of our most toxic compounds are found in nature and produced by living organisms (Schmidt *et al.* 2009). Therefore, Brimner and Boland (2003) argue that more research into the non-target effects of biological control agents are needed. However, before commercialisation becomes a reality, many additional tests will have to be performed (reviewed by Köhl *et al.* 2011) and they include, among other steps, formulation, mass production, risk assessments and incorporation into a crop protection programme.

Table 1.1. Overview of the microorganisms approved as biological control agents against fungal pathogens within the European Union and specifically in Ireland. Names are given according to the EU pesticides database (European Commission 2016).

Organism	Species	Strain	Approved in Ireland
Bacteria	*Bacillus amyloliquefaciens*	MBI600	No
		FZB24	No
	Bacillus amyloliquefaciens ssp. *plantarum*	D747	No
	Bacillus pumilus	QST 2808	In progress
	Bacillus subtilis	QST 713	Yes
	Pseudomonas chlororaphis	MA342	No
	Pseudomonas sp.	DSMZ 13134	Yes
	Streptomyces sp.	K61	No
	Streptomyces lydicus	WYEC 108	No
Fungi	*Ampelomyces quisqualis*	AQ10	Yes
	Aureobasidium pullulans	DSM 14940	No
		DSM 14941	No
	Candida oleophila	O	No
	Coniothyrium minitans	CON/M/91-08	Yes
	Gliocladium catenulatum	J1446	Yes
	Phlebiopsis gigantea	Several strains	No
	Saccharomyces cerevisiae	LAS02	No
	Trichoderma asperellum	ICC012	No
		T25	No
		TV1	No
	Trichoderma asperellum	T34	Yes
	Trichoderma atroviride	IMI 206040	No
		T11	No
	Trichoderma atroviride	I-1237	No
		SC1	No
	Trichoderma gamsii	ICC080	No
	Trichoderma harzianum	T-22	Yes
		ITEM 908˚	Yes
	Trichoderma polysporum	IMI 206039	No
	Verticillium albo-atrum	WCS850	No
Oomycetes	*Pythium oligandrum*	M1	No

1.3 Endophytes

The term “endophyte” was first used by Anton de Bary in 1884 and his original definition was “an endophyte is a parasite living inside its hosts organ” (de Bary 1884). The definition has since then been broadened to include all organisms living inside plants (Wilson 1995; Schulz and Boyle 2005) and in mycology, endophytes are commonly defined as microorganisms living inside plants without causing symptoms of disease (Wilson 1995). The symbiosis between plants and endophytes is long-established, as endophytes have been found in 400 million year-old fossil plants

(Krings *et al.* 2007, 2009). All present-day plants from tropical to arctic regions are believed to harbour endophytes (Fröhlich and Hyde 1999; Higgins *et al.* 2007; Arnold and Lutzoni 2007) and they can be found within all tissue types including roots, stems, leaves, flowers, fruits and seeds (Oliveira *et al.* 2013; Bezerra *et al.* 2015; Comby *et al.* 2016). Some endophytes are systemic while others will occupy a more restricted niche within the plant (Stone *et al.* 2004). Certain species of endophytes are host specific but, in contrast, some species can be found in multiple hosts (Petrini 1986). The life cycles of most endophytes are not completely understood and some endophytes do not remain exclusively within the plant throughout their whole life cycle, which means that they can potentially be latent pathogens (Comby *et al.* 2016), or latent saprotrophs, or can represent early colonisation by rhizobia or mycorrhizal fungi (Porras-Alfaro and Bayman 2011).

Endophytes have gained a lot of attention from ecologists, agronomists and pharmacists. Endophytes have been shown to be able to shape the plant community (Clay and Holah 1999) and their associated food webs (Omacini *et al.* 2001). Furthermore, some species of endophytes have been shown to provide plants with benefits such as drought tolerance (Naveed *et al.* 2014), heat tolerance (Hubbard *et al.* 2014), salt stress tolerance (Rodriguez *et al.* 2008), improved mineral nutrition (Taghinasab *et al.* 2018), as well as protection against diseases (Deshmukh and Kogel 2007; Wicaksono *et al.* 2017) and pests (di Menna *et al.* 2012). In addition, useful secondary metabolites have been isolated from endophytes such as sphaeropsidin A, sphaeropsidin D and acetylsphaeropsidin A which have shown anti-cancer properties and they were isolated from a fungal endophyte of a moss, *Ceratodon purpureus* (Wang *et al.* 2011) In addition, subglutinol A isolated from a fungal endophyte of a vine, *Tripterygium wilfordii,* has shown immunosuppressive properties (Lin *et al.* 2014).

There is often a clear distinction between bacterial and fungal endophyte investigations, both when it comes to community studies and when it comes to evaluating their positive impact on plants (Maciá-Vicente *et al.* 2008; Ghimire *et al.*

2011; Ferrando *et al.* 2012; Wicaksono *et al.* 2017). Thus, fewer studies have examined a combination of bacterial and fungal endophytes. However, community studies including both fungi and bacteria as well as investigations on beneficial consortia containing both groups have emerged recently (Senthilraja *et al.* 2010; De Souza *et al.* 2016). Perhaps the distinction between bacterial and fungal endophyte studies can be explained by the fact that research groups will specialize in one or the other group because the microbes belong in different domains of life (Doolittle 2000).

1.4 Factors shaping the endophyte community

It is commonly recognized that the composition of the endophyte community is dependent on plant species (Wearn *et al.* 2012; Nissinen *et al.* 2012). However, studies also frequently find generalist endophytes, which are able to colonize taxonomically unrelated plant groups (Mandyam *et al.* 2012; Nissinen *et al.* 2012; Unterseher *et al.* 2013). Many other factors have been proposed to influence the composition of the endophytic community within a particular plant species and examples include: latitude (Arnold and Lutzoni 2007; Herrera *et al.* 2010), season (Rodrigues 1994; Lingfei *et al.* 2005; Unterseher *et al.* 2007), plant growth stage (Rodrigues 1994; Van Overbeek and Van Elsas 2008; Douanla-Meli *et al.* 2013; Jin *et al.* 2013), host organ (Sieber *et al.* 1988; Comby *et al.* 2016) as well as soil properties and management (Bala *et al.* 2003; Seghers *et al.* 2004; Long *et al.* 2010; Nissinen *et al.* 2012).

It is debated whether differences in plant genotype within a species can significantly shape the endophyte community composition. Hardoim *et al.* (2011) and Bulgarelli *et al.* (2015) found that the bacterial communities of roots of rice and barley, respectively, was dependent on the plant genotype. Also, Bálint *et al.* (2013) and Müller *et al.* (2015) found that the poplar and olive genotype shaped the fungal and bacterial community of leaves, respectively. Van Overbeek and Van Elsas (2008) and Saikkonen *et al.* (2003) showed that some genotype differences influenced the bacterial community of potato and some genotype differences influenced the fungal

community of *Betula* spp. leaves, respectively. However, Oliveira *et al.* (2013) found that the bacterial community within coffee drupes was not dependent on genotype and also Comby *et al.* (2016) found no correlation between fungal and bacterial endophytes and wheat genotype. It is speculated that results that show a positive dependency of endophyte composition on genotype will be published with higher frequency than those that report a corresponding negative result.

Furthermore, it is debated whether plant health status can determine the endophyte composition. Bulgari *et al.* (2012) investigated the difference in the bacterial root community of healthy apple trees and trees diagnosed with apple proliferation, which is a phytoplasma-disease transmitted by phloem feeding insects. They found that the bacterial endophyte community was dependent on whether the apple tree roots were infected. In contrast, Araujo *et al.* (2002) investigated a bacterial disease of *Citrus*, citrus variegated chlorosis, and found that there was no correlation between the isolation frequency of bacterial endophytes found in branches and the health status of the trees defined as uninfected, asymptomatic and symptomatic. This area is in need of more research for more firm generalisations to be drawn.

1.5 Clavicipitaceous and non-clavicipitaceous endophytes of grasses

Fungal endophytes of grasses are typically divided into two major groups 1) the clavicipitaceous endophytes and 2) the non-clavicipitaceous endophytes (Rodriguez *et al.* 2009; Sánchez Márquez *et al.* 2012). In the review by Rodriguez *et al.* (2009) they further divide endophytes into four functional groups based on many criteria including colonized tissue, host range and range of colonisation where the clavicipitaceous endophytes form class 1 and the non-clavicipitaceous endophytes form classes 2 to 4.

The Clavicipitaceae is a species-rich family with approximately 800 species and includes pathogens of arthropods, parasites of fungi as well as pathogens, endophytes and epiphytes of grasses and sedges (Clay and Schardl 2002; Sung *et al.* 2007). The clavicipitaceous endophytes are characterized by growing systemically within the grass

throughout their life (Clay and Schardl 2002) and dependent on whether they are asexual, sexual or pleiotropic, they will be transmitted vertically directly via the seed, horizontally from one plant to the next via sexual reproduction or as a combination of the two (Bush *et al.* 1997). *Epichloë* spp. are examples of species that are vertically transmitted and have co-evolved with their cool season (temperate) grass hosts (Saikkonen *et al.* 2016). Clavicipitaceous endophytes received much attention in the 1970's when it was recognised that an endophyte, *Neotyphodium lolii* treated as part of *Epichloë* in recent classifications, present in perennial ryegrass, *Lolium perenne* (di Menna *et al.* 2012) was causing the neurological disease, ryegrass staggers, in grazing animals (Keogh 1973; Bacon *et al.* 1977). The endophyte was protecting the grass by producing several secondary metabolites of which the alkaloid lolitrem B is toxic to grazing animals (Gallagher *et al.* 1981) and the alkaloid peramine is toxic to feeding insects (di Menna *et al.* 2012). Research has subsequently gone into the development of forage grasses containing endophytes that keep insects away, but do not harm grazing animals (Young *et al.* 2013). These have been commercialised by the company called Grasslanz based in New Zealand (Grasslanz 2010).

Non-clavicipitaceous endophytes are a taxonomically artificial group found across the plant kingdom. They can be systemic or non-systemic and often their mode of transmission is not known (Rodriguez *et al.* 2009; Sánchez Márquez *et al.* 2012). Also, this group includes another artificial assemblage known as the dark septate endophytes, which are grouped together on the basis of their dark melanised septa (Jumpponen and Trappe 1998; Rodriguez *et al.* 2009). The non-clavicipitaceous endophytes of grasses have been reviewed by Sánchez Márquez *et al.* (2012), who found that the dominant endophyte genera in temperate grasses are *Alternaria, Acremonium, Aureobasidium, Cladosporium, Epicoccum* and *Penicillium* (Sánchez Márquez *et al.* 2012).

1.6 Isolation and identification of non-clavicipitaceous fungal endophytes of grasses

Because many factors have been demonstrated to shape the endophyte community (Section 1.4), these factors are worth considering before sampling and isolating endophytes in order to get the 'snap shot' of interest from the huge diversity of endophytes that exist (Hardoim *et al.* 2015). Endophyte communities can be studied using culture dependent methods or direct molecular methods. Surface sterilization of the plant tissue is crucial no matter what approach is taken and best practice is the verification of the technique. Hallmann et al. (2007) lists three ways to ensure this: 1) imprinting the surface plant tissue onto nutrient medium, 2) spreading aliquots of water from the last rinsing step onto nutrient agar and 3) dipping the tissue into nutrient broth. When studies use the culture dependent method, the surface sterilized tissue is placed on an agar based medium and, once the endophyte grows out, they can be maintained in pure culture followed by identification on the basis of morphology as well as using DNA sequencing. Another possibility is to identify endophytes directly from the plant material using DNA sequencing. In this case, DNA is extracted from the surface sterilized tissue and a set of primers are used to obtain sequences of interest. The obtained sequences are then compared to sequences with 'known identity', often but not always, using a public database (Hallmann *et al.* 2007). The choice of primers and databases are discussed later in this section.

It is generally accepted that not all fungi will grow on all artificial media (Guo *et al.* 2000) and studies have shown that different media influenced the number of isolated endophytes as well as the species richness (Bills and Polishook 1992; Elvira-Recuenco and van Vuurde 2000; Verma *et al.* 2011). Verma et al. (2011) used malt yeast extract agar (MYA), mycological agar (MCA), potato dextrose agar (PDA) and nutrient agar (NA) to isolate fungal endophytes from fruit and root of the Neem tree. They found that NA gave the lowest species richness (number of different species) together with MYA. Furthermore, the maximum species richness was recovered from

MCA while the maximum number of endophytes was recovered from PDA. Elvira-Recuenco and van Vuurde (2000) used 5 % tryptic soy agar (TSA), Reasoner´s 2A agar (R2A) and synthetic complete medium (SC) to isolate bacterial endophytes from the stem of different pea cultivars. They found that 5 % TSA recovered bacterial endophytes significantly better than the other two media. Despite this fact, it is common practice to use one type of medium only when isolating non-clavicipitaceous fungal endophytes of grasses (Table 1.2) – only four out of 32 studies used more than one type of medium (Pelaez *et al.* 1998; Herrera *et al.* 2010; Tejesvi *et al.* 2010; Potshangbam *et al.* 2017). Multiple studies suggest that the most commonly used media for isolating fungal grass endophytes are PDA, followed by MEA and 2 % MEA (Table 1.2).

It is also recognised that a proportion of endophytes will be left undiscovered by using the culture method. Herrera *et al*. (2010) compared the communities discovered on MEA and PDA with the community discovered by direct sequencing from roots of the blue grama grass, *Bouteloua gracilis,* and found that the cultured community was a small subset of the community discovered by direct sequencing. In contrast, Tejesvi *et al.* (2010), found no overlap between the cultured community on MEA and PDA, and the directly sequenced community of fungal endophytes of wavy hair grass, *Deschampsia flexuosa.*

Relatively few studies rely solely on morphology for their identification and these investigations are generally from a time when sequencing was not as cheap as it is today (Sieber *et al.* 1988; Fisher and Petrini 1992; Fisher *et al.* 1992; Pelaez *et al.* 1998; Schulthess and Faeth 1998). It can be challenging to identify fungi solely on morphology. Some of the features that are used to identify fungi are the texture, growth rate, smell and colour of the mycelium (Barnett and Hunter 1972; Domsch 1980). However, some fungal species may have several morphotypes (Burgess *et al.* 2001) and the characteristics of the mycelium can be dependent on the media used, the age of the culture and whether the culture has been grown in light or dark (Domsch 1980).

In addition, important traits include the morphology and colour of specialized structures like the conidiophores which are not always available or produced (Barnett and Hunter 1972). Furthermore, isolates can be identified by the number, size, colour and morphology of the different types of spores (Barnett and Hunter 1972; Domsch 1980). Thus, it can be challenging to identify fungi to species level (Arnold *et al.* 2000) not to mention getting further than phylum if the fungus is not sporulating in culture (Santamaría and Bayman 2005; Arnold and Lutzoni 2007).

The majority of recent studies used DNA sequencing to aid the identification of fungal cultures and most of them used primers targeting the ITS (internal transcribed spacer) region of nuclear ribosomal DNA (Table 1.2). A few studies used additional barcoding regions for identification and they included the SSU (small subunit) and LSU (large subunit) of nuclear ribosomal DNA and TEF (translation elongation factor, Comby *et al.* 2016; Lindsay Higgins *et al.* 2011; Morakotkarn *et al.* 2007; Saunders & Kohn 2009). The rate of evolution in these DNA regions determines how useful they are at placing fungi at different taxonomic resolution. The SSU has evolved relatively slowly and is commonly used to determine taxonomic level down to family, the LSU contains slightly more variation and can be used down to genera in most fungi and down to species in some, whereas the ITS region has the highest amount of variation and is often used down to species level (Schoch *et al.* 2012; Raja *et al.* 2017). The ITS region is the 'official' barcoding region for fungi (Schoch *et al.* 2012). However, Lindahl *et al.* (2013) recommend using a combination of primers if the aim is to obtain the highest possible diversity and most accurate identification from PCR based methods especially in metabarcoding approaches from environmental samples. Brown *et al.* (2014) compared the fungal species richness, diversity and evenness in stored Sorghum biomass using both the ITS and the LSU barcoding regions and found similar patterns with each region. Also, Demirel (2016) found that the ITS and LSU gave similar phylogenetic topology for 43 species belonging to the family Trichocomaceae.

The TEF barcoding region is promoted by Stielow *et al.* (2015) and is used in the study by Saunders and Kohn (2009) to aid the discrimination between *Fusarium* isolates.

Relying on identification using only DNA is also problematic as the identification is only as accurate as the previous submissions to the public databases (Kang *et al.* 2010; Nilsson *et al.* 2016) and relies on accurate morphological identification of the species in the first place. Multiple studies indicate that the NCBI database is the most commonly used and that investigations rely on only one database for identification (Table 1.2). Comparisons of many studies suggest that very few investigations will use several databases for fungal identification (Tejesvi *et al.* 2010; Luo *et al.* 2017). Some mycologists promote the UNITE database because the species identification relies on two ITS sequences, there are well-defined herbarium voucher specimens and the database also includes ecological data as well as quality control by for instance third party annotation (Kõljalg *et al.* 2005, 2013; Lindahl *et al.* 2013).

Community diversity is in its simplest definition described by species richness. By increasing the number of sampled individuals, the number of recorded endophyte species will increase until an asymptote is reached. To begin with, the increase in newly sampled species will be very steep but, as sampling progresses, a point is reached where sampling another individual only adds very rare taxa (Gotelli and Colwell 2001). When isolating endophytes it is interesting to know how many plants need to be sampled in order to obtain the full species richness of endophytes. A growing body of literature has not reached a consensus or a general rule on how many plants should be sampled (Table 1.2) and everything from 5 to 640 plants have been sampled. A scatterplot was created here (Figure 1.2), which is based on the studies found in Table 1.2 and which plots the number of sampled plants and the corresponding fungal species richness. It is perhaps not justifiable to fit a rarefaction curve to the data points because the data are limited. However, with a rarefaction curve, it would be possible to predict how many plants it is necessary to sample in order to isolate the full endophytic species richness. Studies sometimes use the

Michaelis-Menten equation to fit an asymptotic function to a rarefaction curve although many other asymptotic functions have been used (Keating *et al.* 1998).

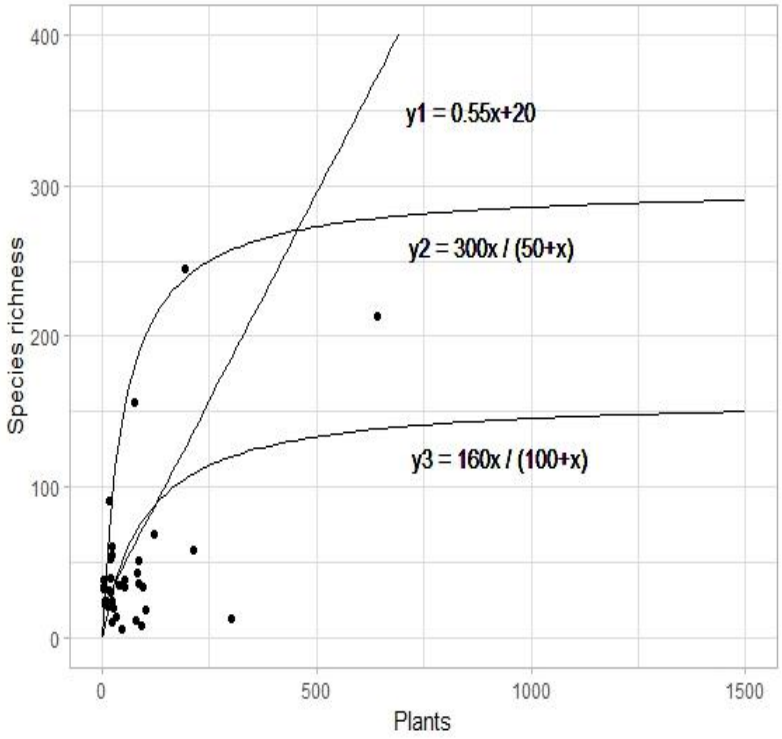

Figure 1.2. Scatter plot of the number of individual grasses sampled in the literature and the reported richness of non-clavicipitaceous fungal endophytes. Data are taken from Table 1.2. Three functions are plotted onto the data, a linear regression and two Michaelis-Menten equations. The functions are examples of functions that could be fitted to the data but does not represent the best fit.

In Figure 1.2, two different Michaelis-Menten based rarefaction curves have been drawn and from their individual equations, it is possible to determine that the maximum number of isolated fungal species would be 160 and 300, respectively. As the function is an asymptote, it will never be possible to obtain the full species richness, but to collect 90 % of the species, 450 plants (function 2) and 900 plants (function 3) would have to be sampled. This infers that if studies have sampled around 100 plants, then they would have collected 10-20 % of the species richness dependent on the function. Perhaps a linear regression is a better match for the data points, but with a linear equation, it would also be impossible to isolate the full diversity of endophytes. It is debateable whether the species richness from these individual studies is comparable

as they might have used different definitions of a species/OTU (operational taxonomic unit). In addition, the species richness has been measured across different tissue types and the fungi were isolated on different media. With the accumulating number of factors described to shape the endophyte community, a linear regression might describe the pattern recorded in these studies of endophytes in grasses best. It is perhaps merely the first part of an asymptote taking shape and indicates that many more plants need to be sampled before the line levels off. However, more studies are clearly needed to explore this topic further.

Table 1.2. Overview of studies isolating non-clavicipitaceous fungal endophytes of grasses using culturing dependent methods or direct DNA sequencing. Studies are ordered in alphabetic order according to plant species.

Grass species	Plants sampled	Tissue	C or D[A]	Medium[B]	Primers	Database	Richness	Author
Ammophila arenaria *Elymus farctus*	84 54 84 54	Shoot Rhizome Shoot Rhizome	C	PDA	ITS5 ITS4	NCBI	51 38 36 34	Sánchez Márquez *et al.* 2008
Arundo donax *Phragmites australis* *Stipa tenacissima*	5 5 5	Shoot Shoot Shoot	C	4 media[C]	-	-	34 32 38	Pelaez *et al.* 1998
Bouteloua gracilis	32	Shoot Root Seed	C D	MEA PDA	ITS1-F ITS4	NCBI	>14[D] 80[E]	Herrera *et al.* 2010
Bouteloua gracilis *Sporobolus cryptandrus*	8 8	Root	C	MEA	ITS1-F ITS4	NCBI	24 22	Khidir *et al.* 2010
Bouteloua gracilis	18	Root	C	PDA	ITS1-F ITS4	NCBI	91	Porras-Alfaro *et al.* 2008
Chusquea simpliciflora *Ichnanthus pallens* *Lithachne pauciflora* *Olyra latifolia* *Oplismenus hirtellus* *Orthoclada laxa* *Panicum pilosum* *Pharus latifolius* *Rhipidocladum racemiflorum* *Streptogyna americana* *Streptchaeta spicata* Total	18 18 18 18 18 17 18 18 15 18 16 192	Shoot	C	2 % MEA	ITS5 ITS1-F ITS4 LR3 nssu131 nssu97a NS17 NS22 nssu1088 LR0R LR7[F]	NCBI	245	Lindsay Higgins *et al.* 2011
Dactylis glomerata	120 82	Shoot Root	C	PDA	ITS5 ITS4	NCBI	69 43	Sánchez Márquez *et al.* 2007
Deschampsia antarctica	91	Shoot	C	PDA	ITS1 ITS4	NCBI	8	Rosa *et al.* 2009
Deschampsia flexuosa	24	Shoot Root Seed	C	MEA	ITS1 ITS4	NCBI	24 (7) (10) (7)	Poosakkannu *et al.* 2015
Deschampsia flexuosa	24	Root	C	50 % MMN[G]	ITS1 ITS4	NCBI	10	Tejesvi *et al.* 2013
Deschampsia flexuosa	46	Root	C D	MEA PDA	ITS1 ITS1-F ITS4 ITS4-B	NCBI UNITE	6	Tejesvi *et al.* 2010
Deschampsia flexuosa	30	Root	C	MEA	ITS1-F ITS4	NCBI	?	Zijlstra *et al.* 2005
Dichanthelium acuminatum *Panicum virgatum*	20 20	Root Root	C	2 % MEA	ITS1 ITS4	AFTOL NCBI UNITE	39 52	Luo *et al.* 2017
Festuca arizonica	>300	Shoot	C	1 % MEA[H]	-	-	13	Schulthess & Faeth 1998
Holcus lanatus	75	Shoot Root	C	PDA	ITS5 ITS4	NCBI	77 79	Sánchez Márquez *et al.* 2010
Hordeum maritimum	27	Shoot Root	C	PDA	ITS1 ITS4	NCBI	20	Hammami *et al.* 2016
Hordeum murinum	?	Root	C	50 % MEA	ITS1 ITS4	NCBI	33[I]	Murphy *et al.* 2015
Oryza granulata	40	Shoot	C	2% MEA	ITS1 ITS4 ITS1-F ITS4-A	NCBI	35	Yuan *et al.* 2011

[A] Cultured (C) or direct DNA sequencing (D).
[B] MEA – malt extract agar, PDA – potato dextrose agar.
[C] Four different media – the substances that the media have in common are mentioned, but are otherwise not described.
[D] Culturable core of the roots.
[E] OTUs from direct sequencing.
[F] The primers amplify three barcoding regions including ITS, SSU and LSU.
[G] Modified 1/2 MMN medium with malt extract.
[H] Growth medium containing 2 % agar, 1 % malt, 1 % glucose and 0.2 % yeast.
[I] Unknown counted as one.

Oryza granulata	15	Root	C D	MEA	ITS1 ITS1-F ITS4	NCBI	31	Yuan *et al.* 2010
Oryza sativa *Zea mays*	? ?	Shoot Root Shoot Root	C	CMA[J] CZA[K] MEA OMA[L] PDA SDA[M] YEMA[N]	ITS1 ITS4	NCBI	57 66	Potshangbam *et al.* 2017
Oryza sativa	80	Shoot Root Seed	C	1.5 % MEA	-	-	11	Fisher & Petrini 1992
Panicum virgatum	214	Shoot Root	C	PDA	ITS1-F ITS4	NCBI	58	Ghimire *et al.* 2011
Phragmites australis	12	Shoot Root	C	2 % MA[O]	ITSI-F ITSl ITS4	NCBI Distance matrix	21	Wirsel *et al.* 2001
Phyllostachys edulis	100[P]	Seed	C	2 % PDA	ITS1-F ITS4	NCBI	19	Shen *et al.* 2014
Phyllostachys edulis	20[Q]	Branch	C	PDA	ITS1-F ITS4	NCBI	30[R]	Shen *et al.* 2012
Phyllostachys spp. *Sasa* spp.	23 23	Shoot Shoot	C	PDA	NS1-1 SR6-1 ITS1-1 ITS4	?	60 24	Morakotkarn *et al.* 2007
Saccharum officinarum	12	Shoot Root	D	-	ITS9 ITS4	UNITE	434[S]	De Souza *et al.* 2016
Stipa grandis	96	Shoot Root	C	2 % MEA	ITS5 ITS4	NCBI	34	Su *et al.* 2010
Triticum aestivum	24	Shoot Root Seed	C	MA[T]	ITS5 ITS4 LROR LR6	NCBI	55	Comby *et al.* 2016
Triticum aestivum	640	Shoot Root Seed	C	2 % MEA	-	-	213	Sieber *et al.* 1988
Zea mays	?	Shoot Root	C	PDA	ITS1-F NLB-3 TEF-1 TEF-2	NCBI	43	Saunders & Kohn 2009
Zea mays	58	Shoot	D	-	ITS1-F ITS4	NCBI	19	Pan *et al.* 2008
Zea mays	8	Root Seed	C	R2A[U]	EF4f NS3r	-	V	Seghers *et al.* 2004
Zea mays	10	Shoot Seed	C	1.5 % MEA	-	-	23	Fisher *et al.* 1992

[J] Corn meal agar.
[K] Czapek-Dox agar.
[L] Oat meal agar.
[M] Sabouraud dextrose agar with chloramphenicol.
[N] Yeast extract mannitol agar.
[O] Malt agar.
[P] More than 100 seeds.
[Q] Branches (not necessarily different plants – description not clear).
[R] 30 representatives were identified.
[S] 434 represent the core fungal OTUs which are present in 90 % of the plant samples.
[T] Malt agar.
[U] Reasoner's 2A agar.
[V] Fungal community was described using DGGE (Denaturing Gradient Gel Electrophoresis) patterns.

1.7 Aims

The aim of this project was to investigate the fungal endophyte community of the roots of a wild relative of barley, *Elymus repens* (L.) Gould. *Elymus repens* was chosen as host because it is a perennial grass that is native to Europe, which is found in agricultural fields and which is also a close relative of barley within the same taxonomic tribe Triticeae (Mason-Gamer 2008). For these reasons high diversity and endophyte compatibility with barley as a host were expected. Another aim was to examine whether a subset of isolated endophytes could be used as biological control agents of *Fusarium*, *Gaeumannomyces* and *Pyrenophora* in barley. Based on experimental work and review of relevant literature, the following broad questions were addressed:

- Do biological control agents, including endophytes, have the ability to control important barley diseases?
- What are the characteristic features of the endophyte community of *Elymus repens*? Are there community differences between sampling sites and individual plants? And how do species richness and community composition estimates vary between culture dependent and culture independent methods?
- Can endophytes isolated from *Elymus repens* reduce disease caused by *Fusarium*, *Gaeumannomyces* and *Pyrenophora* in barley? And if so, can a transformed fluorescent isolate elucidate endophyte colonisation?

1.8 Limitations

The project focused on barley, 24 endophytes - a subset of the total culturable endophyte community of *Elymus repens* and three pathogens *Fusarium*, *Gaeumannomyces* and *Pyrenophora*. Thus, conclusions made are only founded on these combinations. In addition, for experimental treatments, plants were grown in a growth chamber to reduce the number of variables in the experiments. The description of and the results from the *Gaeumannomyces* experiments can be found in Chapter 4, Supplement 4.4.

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Chapter 2

Emerging methods for biological control of barley diseases including the role of endophytes

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Abstract

Barley is an important crop worldwide with production largely used for animal feed and alcoholic beverages. Diseases are a major limiting factor to its production, which have, up until recently, been controlled by agrochemicals. However, legislation on the use of agrochemicals, especially within the European Union, is being tightened and there is growing interest in integrated pest management. This means that there is an increasing focus on controlling diseases using biological control. Living microorganisms that are applied as biological control agents (BCAs) to either soil, seed or leaves can have difficulty in persisting. Therefore, the focus of this review is on endophytes, which are microorganisms that live inside the plant without causing symptoms of disease and have the potential of staying protected as well as being beneficial to the plant and effective against multiple diseases. In this review, we discuss the different approaches for finding and testing beneficial endophytes and for determining the endophyte host range. Furthermore, we undertook a literature search to summarise previous studies that have investigated the use of endophytes as well as BCAs against barley diseases.

Keywords Barley disease, biological control, endophyte*, Bipolaris sorokiniana, Blumeria graminis* f. sp. *hordei, Fusarium* spp.*, Gaeumannomyces tritici, Hordeum vulgare, Pyrenophora teres*

2.1 Important diseases of barley in Northern Europe

Barley is an important crop worldwide and its uses, production and most important diseases in northern Europe has been described in Chapter 1. It is expected that the use of some agrochemicals will be banned or restricted in the future (Oldach 2018), which means that the reliance on chemical inputs will need to be reduced. Furthermore, organic agriculture has increased by almost 20 % a year globally (Nandwani and Nwosisi 2016) and farmers would also benefit from an alternative non synthetic solution.

A European Union regulation (Directive 2009/128/EC; European Parliament 2009) has already been implemented, which is encouraging the sustainable use of pesticides and promoting integrated pest management (Department of Agriculture 2013). Integrated pest management is defined by ENDURE (2008) as “a sustainable approach to managing pests by combining biological, cultural and chemical tools in a way that minimises economic, environmental and health risks". The use of biopesticides is promoted as an environmentally friendly alternative to synthetic pesticides. Within Europe, the term “biopesticides” is often used to cover a range of products that can be used to protect crops from diseases, pests and weeds. The products can be divided into two subgroups that either rely on the use of 1) living organisms or 2) naturally occurring compounds, including extracts from plants and microorganisms as well as volatiles (Balog *et al.* 2017). There are a limited number of registered biopesticide products in Europe and they are based on bacteria, fungi or viruses (Kabaluk *et al.* 2010; Balog *et al.* 2017). In most cases, the control agent is a

different species to the plant pathogen. However, in some cases weak strains or non-pathogenic isolates of the same pathogen species are used (Punja 1997; Kabaluk *et al.* 2010). Within the scientific community, biological control has most often been defined as the use of living organisms for crop protection (Pal and Gardener 2006) and here the term biological control is more precisely defined as the control of plant diseases by microorganisms. Biological control of plant diseases can work through one or a combination of four general mechanisms which are 1) parasitism, 2) antibiosis, 3) competition and 4) induction of host resistance (Guetsky *et al.* 2002; Alabouvette *et al.* 2006). Integrated control of foliar barley diseases is reviewed by Walters *et al.* (2012). However, they do not include the use of biological control.

2.1.1 Endophytes used for biological control of plant diseases

The interest in using microorganisms as biological control agents of plant diseases is increasing (Broadfoot 2016), especially for diseases that are otherwise difficult to control (Walters 2009). The plant microbiome consists of epiphytes and endophytes (Lindow and Brandl 2003; Müller *et al.* 2016). Epiphytes are the microorganisms that live on plant surfaces and they can be further divided into organisms that inhabit the rhizosphere, the phyllosphere (Müller *et al.* 2016) and the spermosphere (Lindsey *et al.* 2017). The term “endophyte” was first used by Anton de Bary in 1884. He described an endophyte as a parasite living inside its host’s organ (de Bary 1884). The definition has since then been broadened and endophytes are generally defined as microorganisms living inside plants without causing symptoms of disease (Wilson 1995). Some endophytes have been shown to provide plants with benefits such as drought tolerance (Naveed *et al.* 2014), heat tolerance (Hubbard *et al.* 2014), improved mineral nutrition (Murphy *et al.* 2015), salt stress tolerance (Rodriguez *et al.* 2008) and protection against disease (Maciá-Vicente *et al.* 2009). While endophytes are not the only biocontrol approach to have received attention in recent years, they compare favourably to other microorganisms which may have difficulty persisting and/or

remaining competent when they are applied to the leaves, the seeds or the soil (Walker *et al.* 2002; Ting *et al.* 2009; Buddrus-Schiemann *et al.* 2010). Thus, the use of endophytes may keep the BCAs protected within the plant (Eevers *et al.* 2015) and provides the possibility of control of several stresses without losing efficacy over the growing season (Wilkinson *et al.* 2000). In our research, focus is put on generalist endophytes, which can be transferred from crop wild relatives (CWRs) and promising results have been obtained in barley (A. K. Høyer, unpublished results). Thus, this review will emphasise the targeted search for plant protecting endophytes as well as previous studies of biocontrol in barley.

2.1.2 Endophyte host range and the targeted search for beneficial endophytes

All plants in natural habitats are believed to harbour endophytes (Aly *et al.* 2011) and they can be tissue-type specific or systemic (Zabalgogeazcoa 2008). The life cycles of the majority of endophytes are not completely understood, but it is clear that some endophytes do not remain exclusively within the plant throughout their whole life cycle, which means that they can potentially be latent pathogens (Comby *et al.* 2016), or latent saprotrophs, or can represent early colonisation by rhizobia or mycorrhizal fungi (Porras-Alfaro and Bayman 2011). The diversity of different taxonomic groups of endophytes that has been elucidated recently has been summarised in the meta-analysis by Hardoim et al. (2015). The most frequently reported sequences of prokaryotic endophytes were from Proteobacteria (54 %), Actinobacteria (almost 20 %) and Bacilli (15 %) whereas eukaryotic sequences were mostly from Glomeromycotina (40 %, arbuscular mycorrhizal fungi), Ascomycota (almost 31 %, with the subordinate class Dothideomycetes accounting for 15 %), Basidiomycota (20 %, with Agaricomycetes accounting for 18 %).

Many factors have been shown to influence the endophyte community composition and one of the important factors is host plant species (Nissinen *et al.* 2012; Wearn *et al.* 2012). Nissinen *et al.* (2012) showed that several bacterial genera

were tightly associated with particular arcto-alpine plant species (*Oxyria digyna*, *Diapensia lapponica* and *Juncus trifidus*). In total, they identified 58 different bacterial genera. Of the major bacterial genera, six were exclusively associated with *J. trifidus* (*Acido Gp1*, *Arthrobacter*, *Knoellia*, *Paenibacillus*, *Paracoccus* and *Rhodanobacter*), four were specific to *O. digyna* (*Agreia, Ancylobacter*, *Rhizobium* and *Rhodococcus*), and one was exclusively associated to *D. lapponica* (*Pedobacter*). However, some groups of endophytes are generalist and are able to colonise plants of unrelated taxonomic identity. Interestingly, all three plant species were colonised by *Burkholderia*, *Mucilaginibacter*, *Nocardioides* and *Sphingomonas*. Wearn *et al.* (2012) explained that part of the fungal communities of grassland forbs (*Cirsium arvense, Plantago lanceolata* and *Rumex acetosa*) were host plant specific. Thus, 48 % of the fungal community belonging to *C. arvense* was generalist endophytes, with 58 % and 72 % generalists for *P. lanceolata* and *R. acetosa*, respectively (Wearn *et al.* 2012). In grasses, generalist endophytes are, for instance, found in the groups of clavicipitaceous endophytes and dark septate endophytes (DSE) (Clay 1990; Jumpponen and Trappe 1998; Mandyam *et al.* 2010). Known generalists of clavicipitaceous endophytes in temperate grasses are *Epichloë coenophiala* and other *Epichloë* ssp., which infect grasses in the subfamily Pooideae and *Atkinsonella* spp. which infects *Danthonia* spp. and *Stipa* spp. (Clay 1990). For generalists within the DSE, Mandyam *et al.* (2010) showed that the roots of four C_4 grasses (*Andropogon gerardii*, *Sorghastrum nutans*, *Schizachyrium scoparium* and *Panicum virgatum*) normally had two DSE in common, i.e. *Periconia macrospinosa* and *Microdochium* sp.

Several approaches have been explored to isolate potential endophytes that confer protection against diseases. Most studies have cultured endophytes from healthy looking plants that live in an environment that has a particular disease stress. In this case, it is hypothesised that the endophytes contribute to plant health and that they are able to relieve the stress (Araujo *et al.* 2002). An alternative strategy has been suggested by Ellis (2017), who proposed looking for biocontrol agents in diseased

tissue because organisms can persist in a pathogen infected tissue and hence potentially act as control agents. Although this may appear as counter-intuitive, Ellis (2017) gives an example of control of crown gall in stone fruit and furthermore, Köhl *et al.* (2009) found antagonists supressing apple scab using this approach.

There are different approaches when it comes to both selecting target plant species as sources of beneficial endophytes and selecting a plant species to test the biological control properties. Most studies have isolated endophytes from a crop species and then tested the biological control effects in the original crop (Kirk and Deacon 1987; Coombs *et al.* 2004; Silva *et al.* 2012). Some studies isolated endophytes from related taxonomic groups of a crop species and tested the effect in the crop (Maciá-Vicente *et al.* 2008). CWRs are valuable resources in crop breeding programmes and have been used to transfer disease resistance (Zeng *et al.* 2013; Brar and Hucl 2017; Fedak *et al.* 2017). Likewise, CWRs can be a unique source of potential biocontrol agents (Maciá-Vicente *et al.* 2008; Brian R. Murphy *et al.* 2015). CWRs will have their own microbiome and, although not adequately tested, could be expected to host some endophytes not ordinarily present in the crop species. Due to the close taxonomic affinity to the crop plants, they could also be expected to be more compatible to the target species than endophytes isolated from an unrelated species (Murphy *et al.* 2018). In addition, some endophytes are isolated from an unrelated plant and then tested on crops. For example, *Serendipita indica* (formerly *Piriformospora indica*; Weiß *et al.* 2016) is a basidiomycete endophyte that has been tested on many different crops, which are not closely related to the original host (Kumar *et al.* 2009; Harrach *et al.* 2013; Rabiey *et al.* 2015; Wang *et al.* 2015). *Serendipita indica* was isolated from the rhizosphere of two woody shrubs, *Prosopis juliflora* and *Zizyphus nummularia*, in desert soils of Rajasthan in India (Varma *et al.* 2012). This fungus has been tested as a BCA in several crop species including wheat (*Triticum aestivum;* Serfling *et al.* 2007; Rabiey *et al.* 2015), barley (*Hordeum vulgare;* Harrach *et al.* 2013),

maize (*Zea mays;* Kumar *et al.* 2009) and tomato (*Solanum lycopersicum;* Roylawar *et al.* 2015; Wang *et al.* 2015).

2.2 Previous studies of endophytes and other BCAs controlling barley diseases

An extensive literature survey, conducted here, revealed a total of eight studies reporting the control of barley diseases by endophytes and 21 studies reporting control by other BCAs (Table 2.1). Only studies using living microorganisms were included in the review. Eight different fungal endophyte species were tested in the endophyte studies and *Serendipita indica* was tested in four of the investigations. In the studies reporting control by BCAs, several different organisms were used, with the majority using fungi. *Pseudomonas* spp. strains were widely used followed by *Trichoderma* spp. and *Clonostachys rosea.* Although not tested as endophytes in the investigations reviewed here, these commonly used organisms have often been widely isolated as endophytes (Evans *et al.* 2003; Høyer *et al.* 2016; Mercado-Blanco *et al.* 2016).

Table 2.1. Overview of the investigations on biological control of barley diseases.

The first part of the table lists studies using endophytes and the second part of the table lists studies using other types of biological control agents. The investigations are organised firstly according to pathogen, secondly according to efficiency of disease control and thirdly according to year of publication. The investigations are evaluated on a scale where ’0’ is given to reports where there was no disease control, ‘+’ is given to reports were disease control efficiency of 0.01-33 % are described, ‘+ +’ are reports of 33-66 % efficiency and ‘+ + +’ are reports of 66-100 % efficiency. The minus symbol indicates that the investigation did not look into disease control or the mechanism of control. Evaluations are made on the basis of the test system. Names of organisms are given according to Species Fungorum and Catalogue of Life (http://www.indexfungorum.org/).

Pathogen	**Endophyte**	**Endophyte origin**	**Test system**	**Disease control efficiency**	**Suggested mechanism of control**	**Author**
Bipolaris sorokiniana *Pyrenophora teres*	*Chaetomium globosum*	Barley leaves	Dual culture	**+**[1]**/++**[2,3]	Antibiosis and competition[4] Competition and mycoparasitism[5]	Moya *et al.* 2016
Bipolaris sorokiniana *Blumeria graminis* f. sp. *hordei*	*Serendipita indica*	Roots of woody shrubs	Detached-leaf-segment assay Pot trials	**++**[6] **0**[7]	Systemic induction of resistance associated with elevated	Waller *et al.* 2005

[1] For *Bipolaris sorokiniana.*
[2] For *Pyrenophora teres.*
[3] Some treatments gave reductions of 0-33% / others gave reductions of 33-66% / while others gave reductions of 66-100%.
[4] For *Bipolaris sorokiniana.*
[5] For *Pyrenophora teres.*
[6] For *Blumeria graminis* f. sp. *hordei*.
[7] Methods of evaluation was insufficient.

Fusarium culmorum					antioxidative capacity	
Blumeria graminis f. sp. *hordei*	*Dichotomopilus funicola*	Tomato roots	*In vitro* spore germination test	**++**	Antibiosis	Vilich *et al.* 1998
	Chaetomium globosum	Laboratory strain[8]	Pot trials	**+/++**[9]	-	
Fusarium culmorum	*Serendipita indica*	Roots of woody shrubs	Pot trials with soil	**+**	Elevated antioxidative capacity	Harrach *et al.* 2013
Fusarium graminearum	*Serendipita indica*	Roots of woody shrubs	Dual culture	**0**[10]	Not antibiosis	Deshmukh and Kogel 2007
			Pot trials with soil	**+++**	Not induced resistance	
Fusarium graminearum	*Serendipita indica*	Roots of woody shrubs	Pot trial with soil	**0**[11]	Growth acceleration	Achatz *et al.* 2010
Gaeumannomyces tritici	*Acremonium furcatum*	24 plant species	Dual plate bioassay	**+**	-	Maciá-Vicente *et al.* 2008
	Dactylaria sp.		Tube with vermiculite	**+++**	-	
	Fusarium equiseti					
	Phoma herbarum					

[8] Perhaps it originated from twigs of *Quercus* sp., however it is unclear.
[9] For *Chaetomium globosum.*
[10] No inhibition zone was formed.
[11] Methods of evaluation were insufficient.

Gaeumannomyces tritici	*Fusarium equiseti* *Metacordyceps chlamydosporia*	*Corynephorus canescens* and *Lygeum spartum.* *Heterodera avenae* infected eggs and *Meloidogyne* sp.	Tube with vermiculite	**++**	**-**	Maciá-Vicente *et al.* 2009
Pathogen	**BCA**	**BCA origin**	**Test system**	**Disease control efficiency**	**Suggested mechanism of control**	**Author**
Bipolaris sorokiniana	*Clonostachys rosea*	Barley roots	Pot trial with sand	**++/+++**[12]	**-**	Jensen *et al.* 2002
Bipolaris sorokiniana *Blumeria graminis f. sp. hordei* *Pyrenophora teres* *Rhynchosporium graminicola*	*Bipolaris maydis* *Parastagonospora nodorum*	Maize Wheat	Pot trial with soil	**+**[13]**/++**[14]**/+++**[15]	-	Jørgensen *et al.*1996
Bipolaris sorokiniana	*Clonostachys rosea*	Barley roots	Pot trial with soil	**0**[16]**/++**[17]**/+++**[18]	Direct inhibition. Reduced conidial	Jensen *et al.* 2016

[12] Dependent on storage conditions.
[13] For *Bipolaris sorokiniana* and *Blumeria graminis* f. sp. *hordei.* Results are cultivar dependent.
[14] For *Bipolaris sorokiniana, Pyrenophora teres* and *Rhynchosporium graminicola.* Results are cultivar dependent.
[15] For *Rhynchosporium graminicola.* Results are cultivar dependent.
[16] For *Blumeria graminis* f. sp. *hordei.*
[17] For *Bipolaris sorokiniana* and *Pyrenophora teres.*
[18] For *Rhynchosporium graminicola.*

Blumeria graminis f. sp. *hordei* *Pyrenophora teres* *Rhynchosporium graminicola*					germination and appressorium formation[19]	
Bipolaris sorokiniana *Microdochium nivale* *Pyrenophora graminea* *Pyrenophora teres* *Ustilago hordei* *Ustilago nuda*	*Pseudomonas chlororaphis*	Soil	Field experiments	**+**[20]**/++**[21]**/+++**[22]	**-**	Johnsson *et al.* 1998
Bipolaris sorokiniana	*Acremonium sp.* *Chaetomium sp.* *Clonostachys rosea* *Fusarium roseum* *Humicola* sp. *Microdochium bolleyi* *Trichoderma* sp.	Barley seed Barley seed Organic soil Conventional soil Conventional hay Organic hay Roots	Pot trial with sand Pot trial with soil Field experiments	**+/++** **+/++** **+/++**	- - -	Knudsen *et al.* 1995
Bipolaris sorokiniana	*Microdochium bolleyi*	Grassland	Cellulose filter paper rolls	**+**	Induced resistance	Liljeroth and Bryngelsson 2002

[19] For *Bipolaris sorokiniana.*
[20] Against *Ustilago nuda*
[21] Against *Bipolaris sorokiniana* and *Microdochium nivale.*
[22] Against *Pyrenophora graminea, Pyrenophora teres, Ustilago hordei* and *Ustilago nuda.*

			Pot trials with soil Field experiment	**++** -[23]		
Bipolaris sorokiniana	*Microdochium bolleyi*	Wheat coleoptile	Field experiments	**+**	-	Duczek 1997
Blumeria graminis f. sp. *hordei*	*Bacillus thuringiensis* (14 isolates)	Faecal samples from mammalian species	Pot trial	**+/+++**	**-**	Choi *et al.* 2007
Fusarium culmorum	*Clonostachys rosea*	Barley roots	Pot trial with sand Field experiments	**++/+++** **++/+++**	-	Jensen *et al.* 2000
Fusarium culmorum	*Actinomycetes* (133 strains)	Saharan soil	Streak method Petri dishes with filter paper	-[24] **+/++/+++**	- -	Yekkour *et al.* 2012
Fusarium culmorum *Fusarium graminearum* *Fusarium poae*	*Acinetobacter* sp. *Chryseobacterium* sp. *Pseudomonas fluorescens* *Pseudomonas frederiksbergensis* *Pseudomonas* sp. unidentified isolate	Cereal plant and soil	Dual culture Pot trial with soil	**+/++/+++** **+/++/+++**	Not antibiosis Induced resistance[25]	Khan *et al.* 2006
Fusarium culmorum	*Acinetobacter* sp.	Cereal plant and soil	Pot trial with soil	**+/++**[26]	-	Khan and Doohan 2008a

[23] No disease symptoms occurred before ear emergence.
[24] No control treatment – difficult to estimate the disease reduction. Antagonists were tested against several *Fusarium* ssp., *Aspergillus* ssp. and *Penicillium* ssp. at this step.
[25] Results are from wheat not barley.
[26] Dependent on timing of BCA application

Fusarium graminearum *Fusarium poae*	*Chryseobacterium* sp. *Pseudomonas fluorescens* *Pseudomonas frederiksbergensis* *Pseudomonas* sp.		Field experiment	**++**	-	
Fusarium culmorum	*Pseudomonas fluorescens*	Barley rhizosphere soil	Pot trial with soil Field experiment	**++** **++**	- -	Khan and Doohan 2008b
Gaeumannomyces tritici	*Metacordyceps chlamydosporia* *Metapochonia rubescens* *Lecanicillium lecanii*	Nematophagous fungi	Petri dish experiment Tube with vermiculite	**++/+++** **+**	- -	Monfort *et al.* 2005
Pyrenophora graminea *Pyrenophora teres*	*Streptomyces* ssp. (5 strains) *Trichoderma* spp. (5 strains)	- -	Pot trial with soil	**+/++/+++**	**-**	Koch *et al.* 2006
Pyrenophora teres	Actinomycete *Albifimbria verrucaria* *Trichoderma viride* *Trichoderma pseudokoningii* *Trichoderma* sp.	Soil and straw	*In vitro* straw assay Tube with cotton Pot trial[27]	**+++** **++/+++** **+++**	**-** **-** **-**	Mostafa 1993
Pyrenophora teres	400 bacterial isolates *Pseudomonas* isolates	Roots of wild and cultivated plants	Pot trial with soil Field experiment	**+/++/+++** **+++**	- -	Hökeberg *et al.* 1997

[27] Substrate not mentioned.

Pyrenophora teres	*Micromonospora* *Trichoderma koningii* *Trichoderma pseudokoningii* *Trichoderma viride* Five unidentified fungi	Barley straw	*In vitro* straw assay	**+/++/+++**	**-**	Ali-Hoimoud *et al.* 1993
Pyrenophora teres	Nine fungal antagonists[28]	Soil and roots of barley sprouts	Pot trial with sand	**+/++/+++**	**-**	Abrahamsen 1992
Pyrenophora teres	*Bipolaris maydis* *Parastagonospora nodorum*	Maize Wheat	Pot trial with soil	**+/+++**	Induced resistance	Jørgensen *et al.* 1998
Pseudomonas syringae	*Pantoea agglomerans*[29]	Barley seeds	Pot trial[30] Field experiments	**++/+++** **+/++/+++**	-[31]	Braun-Kiewnick *et al.* 2000

[28] Identity not mentioned.
[29] Several strains.
[30] Substrate not mentioned.
[31] Investigated but mentioned in a different paper.

All the studies collectively focus on a limited number of pathogens and they include the leaf pathogens *Bipolaris sorokiniana, Blumeria graminis* f. sp. *hordei, Pseudomonas syringae, Pyrenophora graminea, Pyrenophora teres, Rhynchosporium graminicola, Ustilago hordei, U. nuda* as well as the soil pathogens *Bipolaris sorokiniana, Fusarium culmorum, F. graminearum, F. poae, Gaeumannomyces tritici* and *Microdochium nivale.*

The rationale behind the selection of host plant species as a source of biological control agents has often not been described sufficiently well in the studies. The endophytes were sourced from barley (Moya *et al.* 2016), grasses including *Ammophila arenaria* ssp. *australis*, *Corynephorus canescens* and *Lygeum spartum* (Maciá-Vicente *et al.* 2008, 2009) and unrelated plant species (Vilich *et al.* 1998; Waller *et al.* 2005; Maciá-Vicente *et al.* 2008; Deshmukh and Kogel 2007; Achatz *et al.* 2010; Harrach *et al.* 2013). Among the studies using other BCAs, one study did not describe where the BCA originated from (Koch *et al.* 2006). Seven out of 21 studies used antagonists that originated from barley (Abrahamsen 1992; Ali-Hoimoud *et al.* 1993; Knudsen *et al.* 1995; Braun-Kiewnick *et al.* 2000; Jensen *et al.* 2000, 2002, 2016), five studies used BCAs from other cereals or what was termed “straw” (Mostafa 1993; Knudsen *et al.* 1995; Duczek 1997; Jørgensen *et al.* 1996, 1998) and two studies used antagonists originating from unspecified grasses (Hökeberg *et al.* 1997; Liljeroth and Bryngelsson 2002). One study used BCAs from unrelated plant species (Hökeberg *et al.* 1997) and seven used samples from soil (Mostafa 1993; Knudsen *et al.* 1995; Johnsson *et al.* 1998; Khan *et al.* 2006; Khan and Doohan 2008a, 2008b; Yekkour *et al.* 2012). Two studies used nematophagous fungi (Monfort *et al.* 2005; Maciá-Vicente *et al.* 2009), one used fungi isolated from insects (Maciá-Vicente *et al.* 2009) and one study found their control agents in mammalian faeces (Choi *et al.* 2007).

Four different reasons for working with a specific endophyte species were given (Table 2.1) including getting good results from a preliminary *in vitro* study (Maciá-Vicente *et al.* 2008), the fact that the endophyte belonged to a genus which is known

for the production of secondary metabolites (Vilich *et al.* 1998), previous success with the organism within the research group (Maciá-Vicente *et al.* 2009; Achatz *et al.* 2010) or a literature review (Deshmukh and Kogel 2007; Achatz *et al.* 2010; Harrach *et al.* 2013; Moya *et al.* 2016). The choice of BCA species in the other studies was often explained by the fact that the organisms had been used successfully in previous studies by the same authors or research group (Knudsen *et al.* 1995; Jensen *et al.* 2000, 2002, Koch *et al.* 2006; Khan and Doohan 2008b; Jensen *et al.* 2016). However, a short literature review of the abilities of the BCAs in different crops or against specific diseases was more common (Mostafa 1993; Duczek 1997; Braun-Kiewnick *et al.* 2000; Liljeroth and Bryngelsson 2002; Monfort *et al.* 2005; Choi *et al.* 2007; Khan *et al.* 2006; Khan and Doohan 2008a; Yekkour *et al.* 2012). Much rarer reasoning was that the organisms were screened *in planta* in the actual study (Hökeberg *et al.* 1997; Johnsson *et al.* 1998), or were non-pathogens of barley (Jørgensen *et al.* 1996, 1998). Sometimes no reason was given (Abrahamsen 1992; Ali-Hoimoud *et al.* 1993).

2.2.1 Experimental test systems

There is increasing financial expense in progressing from *in vitro* studies, to pot trials, to field experiments. There is, however, also an increase in the value of the knowledge produced, at least if the aim is to reduce disease pressure in the field. *In vitro* studies are, in general, controversial because there is often not a good correlation between *in vitro* results and results obtained from more complex growth systems (Renwick *et al.* 1991; Fravel 1988; Khan *et al.* 2006; Deshmukh and Kogel 2007).

In two of the biocontrol investigations (Table 2.1), long term field trials were used (Duczek 1997; Johnsson *et al.* 1998). However, the most common experimental test system is pot trials (Abrahamsen 1992; Jørgensen *et al.* 1996, 1998; Jensen *et al.* 2002; Koch *et al.* 2006; Choi *et al.* 2007; Achatz *et al.* 2010; Harrach *et al.* 2013; Jensen *et al.* 2016), combined with *in vitro* testing (Mostafa 1993; Vilich *et al.* 1998; Khan *et al.* 2006; Waller *et al.* 2005; Deshmukh and Kogel 2007) or followed by field

experiments (Knudsen *et al.* 1995; Hökeberg *et al.* 1997; Jensen *et al.* 2000; Braun-Kiewnick *et al.* 2000; Liljeroth and Bryngelsson 2002; Khan and Doohan 2008a 2008b). Different substrates have been used in pot trials including vermiculite (Ali-Hoimoud *et al.* 1993), sand (Jensen *et al.* 2002) and soil (Jørgensen *et al.* 1996). The more complex the pot trial system, the better it represents field conditions; thus it is preferable to use soil instead of vermiculite. However, when disease symptoms are evaluated on roots, it can ease the work flow not to use soil. In the less complex end of the spectrum, Yekkour *et al.* (2012) used Petri dishes with filter paper and four studies used tube assays with either vermiculite (Monfort *et al.* 2005; Maciá-Vicente *et al.* 2008, 2009) or cotton (Mostafa 1993). Two studies used *in vitro* experiments of BCA and pathogen only (Ali-Hoimoud *et al.* 1993; Moya *et al.* 2016). Ali-Hoimoud *et al.* (1993) used a cut straw assay because they were interested in biocontrol of the survival structures of *Pyrenophora teres* on crop residues. The study by Moya *et al.* (2016) performed a “classical” dual culture test using only one type of medium. This could be considered controversial because various studies have shown that type of media and water potential within the medium will influence growth rates, production of secondary metabolites and hyphal interactions between antagonist and pathogen (Whipps 1987; Whipps and Magan 1987).

Six of the eight endophyte studies checked whether their control agent could colonise barley as an endophyte (Vilich *et al.* 1998; Waller *et al.* 2005; Deshmukh and Kogel 2007; Maciá-Vicente *et al.* 2008, 2009; Achatz *et al.* 2010). If the reduction in disease symptoms is linked to the lifestyle of the microorganism as an endophyte then it is relevant to show that the endophyte colonises the plant in question as an endophyte, especially, but not exclusively, if the endophyte has been sourced from a different species than the crop.

2.2.2 Biological control efficiency

Biological control efficiency varies among experiments with the best results for the most complex test systems summarised in Table 2.2. It is clear that barley diseases can be controlled using BCAs as well as endophytes in pot and in field trials. The beneficial microorganisms have been discovered from many and varying places and the best ones have originated from barley itself, marram grass (*Ammophila arenaria* ssp. *australis*), wild and cultivated plants and mammalian faeces (Table 2.2).

Table 2.2. Summary of the best biological control results from the most complex systems obtained against five of the most commonly studied pathogens in barley. The origin of the biological control organism is also listed. Names of organisms are given according to Species Fungorum (www.speciesfungorum.org).

Pathogen	Disease control (%)	Test system	Origin of BCA or endophyte	Author
Bipolaris sorokiniana	43	Field experiments	Soil	Knudsen *et al.* 1995
Blumeria graminis f.sp. *hordei*	70	Pot experiment	Mammalian faeces	Choi *et al*. 2007
Fusarium culmorum	73	Field experiment	Barley roots	Jensen *et al.* 2000
Gaeumannomyces tritici	88	Tube with vermiculite	Endophyte of *Ammophila arenaria* ssp. *australis* (Poaceae)	Maciá-Vicente *et al*. 2008
Pyrenophora teres	98	Field experiments	Roots of wild and cultivated plants	Hökeberg *et al.* 1997

The experimental test system will influence the reported outcomes. The fewer the variables in the experiments, the easier it will be to obtain efficient biocontrol results. In the investigations, which tested biological control agents first in pot trials and later in the field, there was a tendency for the efficiency of the control agents to be 4-35 % lower in the field (Knudsen *et al.* 1995; Hökeberg *et al.* 1997; Braun-Kiewnick *et al.* 2000; Jensen *et al.* 2000; Khan and Doohan 2008a, 2008b). As an exception, Hökeberg *et al.* (1997) reported a specific *Pseudomonas* strain (MA 342), which controlled disease slightly better in the field (98 %) compared to the pot trial (75 %). If

the treatment works in a pot experiment it will have a higher likelihood of success in the field than if the BCA was identified *in vitro.*

2.2.3 Biocontrol mechanisms used against barley diseases

Ten studies (Table 2.1) have investigated the mechanisms behind the biological control, but rigorous evaluations are rare. In many cases, the potential involvement of all the possible mechanisms in biological control (antibiosis, competition, parasitism and induced resistance) have not been studied or even been possible to study simply because an appropriate experimental setup has not been applied. For example, to show that induced resistance is involved in biological control, requires plant experiments to be performed and defence responses to be studied.

Two of the studies used *Chaetomium* spp. endophytes as BCAs and only mechanisms inferred from *in vitro* assays were reported (Vilich *et al.* 1998; Moya *et al.* 2016). Both studies showed that *Chaetomium* spp. worked through antibiosis against leaf pathogens of barley *in vitro*. Vilich *et al.* (1998) concluded that their fungal isolate reduced spore germination of barley powdery mildew by antibiosis. They spread conidia of *Blumeria graminis* f. sp. *hordei* on malt extract agar plates that contained a filtrate of the BCA. However, they did not outline their control treatment, which makes it difficult to evaluate their findings and furthermore, since the pathogen is an obligate biotroph, their *in vitro* setup may yield results different from a more realistic situation using barley leaves. In subsequent pot experiments, a BCA spore suspension was coated onto the seeds and the pathogen was inoculated onto the leaves. It is, however, difficult to make firm conclusions on the mechanism *in planta* from the *in vitro* study. Thus, it is not known whether compounds of the endophyte reached the leaves, which would be a prerequisite for concluding that metabolites produced by the BCA was responsible for any disease reducing effect. Moya *et al.* (2016) performed a dual culture test where they placed a plug of the *Chaetomium* antagonist on a PDA plate and three days later placed a plug of either *Bipolaris sorokiniana* or *Pyrenophora teres*

at a distance of 4 cm away from the first plug. The control treatment was the pathogen alone, which is perhaps not the optimal control as it may be argued that a proper control would have been a pure agar plug placed on a plate and a pathogen plug added three days later to exclude any effect of the agar. The conclusion was that the *Chaetomium globosum* isolates worked through antibiosis and competition against *Bipolaris sorokiniana* and through competition and mycoparasitism against *Pyrenophora teres.* These conclusions are all based on evaluations using a microscope and unfortunately, these observations stand alone. Thus, it is unknown whether the endophytes had a similar behaviour *in planta* or whether they would be able to induce resistance against the pathogen.

Four studies investigated the mechanisms of control exerted by the endophyte *Serendipita indica* (Waller *et al.* 2005; Deshmukh and Kogel 2007; Achatz *et al.* 2010; Harrach *et al.* 2013). All studies used pathogens from the genus *Fusarium* and Waller *et al.* (2005) also included *Blumeria graminis* f. sp. *hordei* and *Bipolaris sorokiniana*, all in separate experiments. Harrach *et al.* (2013) and Waller *et al.* (2005) both concluded that elevated antioxidative capacity was the mechanism for disease control whereas Achatz *et al.* (2010) suggested that the endophyte used plant growth promotion to avoid disease and Deshmukh and Kogel (2007) concluded that pathogenesis-related (PR) proteins were not involved in protection. The main aim of the investigation by Achatz *et al.* (2010) was to show that *S. indica* relieved plants from nutrient stress and *Fusarium* sp. was used as an additional biotic stress. They showed that plants with and without *Fusarium* infection had equivalent grain yields. As grain yield is not a reliable measure of biological control and disease symptoms were not evaluated, it is difficult to discern if the pathogen was established and one must therefore be cautious in interpreting the results. Harrach *et al.* (2013) used *S. indica* against *F. culmorum* in a pot trial. No direct symptom scoring was made, but they used the shoot/root biomass as a proxy for disease scoring and they did quantify pathogen biomass as an indication of disease pressure. Antioxidant status of the roots was examined through ascorbate

and glutathione levels as well as antioxidant enzyme activity. It was concluded that *S. indica* altered the antioxidant status of the cells so that they could detoxify excess reactive oxygen species (ROS) produced by the pathogen. However, in the literature used to indicate how the pathogen is affected by reactive oxygen species, the authors only show “plausible” correlations between *F. culmorum* and ROS production in *Arabidopsis* floral tissue. So it is not entirely clear whether these responses can explain reductions in disease in barley.

The study of Waller *et al.* (2005) also used shoot/root biomass as an indicator of biological control for *F. culmorum* and the data for *B. sorokiniana* are not shown. It is suggested that the mechanism cannot be antibiosis because this was ruled out in a study in axenic culture, but data are not shown. Furthermore, it is not clear how plant inoculation with *S. indica* took place and therefore, it is difficult to evaluate the relevance of the *in vitro* study. Antioxidant capacity was also studied when inoculating roots with and without *S. indica.* Since the pathogen was not present in these experiments, it is difficult to make conclusions about the mechanisms of control. Waller *et al.* (2005) also examined the control of *Blumeria graminis* f. sp. *hordei* and used a disease index to show reduction in disease symptoms in a detached leaf assay, but again the antagonist delivery system is not clear. This time, systemic resistance was suggested.

Deshmukh and Kogel (2007) also ruled out antibiosis based on dual culture tests, although the nature of these experiments was not fully described. Perhaps it is too early to rule out antibiosis when there have been no additional tests of whether the endophyte can produce antagonistic compounds within the plant. The authors found that PR-protein genes were expressed at lower levels when *S. indica* was present with *F. culmorum* compared to plants inoculated with *F. culmorum* alone. They therefore concluded that PR-proteins were not involved in the protection induced by the endophyte.

Khan *et al.* (2006) also examined the biocontrol of *Fusarium* spp., but they used bacteria as their control agents. Antibiosis was excluded as a potential mechanism using *in vitro* inhibition zone studies on one type of medium. Again, it is perhaps premature to completely rule out antibiosis because of the absence of an inhibition zone when it is not clear what the BCA produces *in planta*. Their subsequent *in planta* study was only conducted on wheat and the results indicate that induced resistance is the mechanism involved. They examined the expression of a PR-gene (class III peroxidase), which is known to be upregulated in wheat in response to *Fusarium* infection. In this experiment, they worked with *Pseudomonas fluorescens* (MKB 156) and *Pseudomonas* sp. (MKB 158) and they were only able to show induced resistance for one of the strains (MKB 158).

The three last studies (Table 2.1) concern control of *Bipolaris sorokiniana* (Liljeroth and Bryngelsson 2002; Jensen *et al.* 2016) and/or *Pyrenophora teres* (Jørgensen *et al.* 1998; Jensen *et al.* 2016). Furthermore, Jensen *et al.* (2016) also included *Blumeria graminis* f. sp. *hordei* and *Rhynchosporium graminicola.* All three investigations used different BCAs. Thus, Jørgensen *et al.* (1998) used two non-barley pathogens to control diseases, whereas Liljeroth and Bryngelsson (2002) used *Microdochium bolleyi* as a BCA and Jensen *et al.* (2016) used *Clonostachys rosea*. Jørgensen *et al.* (1998) found that induced resistance was probably the main mechanism involved in the local protection exerted by the two non-barley pathogens. They showed that appressoria-formation was reduced and that papillae formation was increased. In the study by Jensen *et al.* (2016), *C. rosea* was able to control *Bipolaris sorokiniana, P. teres* and *R. graminicola*, but mechanisms of control were only evaluated for *B. sorokiniana*. It was concluded that the inhibition was direct and therefore probably involved mycoparasitism, competition and/or antibiosis. This was based on the fact that germination of pathogen conidia and inhibition of appressorial formation was observed. Induced resistance was ruled out because expression of three PR-protein genes was not increased in plants treated with antagonist and pathogen

compared to the control, and furthermore, there was no increase in defence responses when evaluated under the microscope. In the study by Liljeroth and Bryngelsson (2002), *B. sorokiniana* was suggested to be controlled by induced resistance and this was shown by the elevated expression of PR-protein genes. However, results for pathogen and antagonist treated plants were performed separately and there was no treatment with both pathogen and antagonist together so it is not clear whether the antagonist can upregulate PR-protein genes in the presence of the pathogen.

2.3 Conclusion

There is a need to address the heavy reliance on agrochemicals in barley production and improve the environmental sustainability of the industry. Integrated pest management is encouraged within the European Union and biological control can be incorporated into this approach. It is evident from the literature review conducted here that living microorganisms can control barley diseases in controlled laboratory experiments and, more importantly, under field conditions. It is furthermore clear that some of the most problematic diseases of barley in Northern Europe, including rusts, Ramularia leaf spot and barley yellow dwarf have not been challenged using biological control in agricultural systems. There is a trend in legislation for restricting the use of certain agrochemicals and organic agriculture is increasing globally each year which means that there will be a huge demand for non-chemical control methods for these diseases in the future.

Another finding is that there is no particular, specific niche from where to isolate biocontrol antagonists. It appears that it is possible to find antagonists in many types of environments. However, the majority of studies sourced their control agents from barley plants, other cereals or wild grasses and some of the best results were also obtained with BCAs obtained from such hosts. Endophytes also showed good results and they were sourced from barley leaves, wild grasses and from unrelated plant

species. There is very little known about the host range of endophytes and it seems theoretically more likely to obtain reliable results when looking for endophytes from the crop of interest or its wild relatives because the chance of successful establishment within the plant is increased. Also, work with endophytes is recommended because endophytes can be protected within the plant and also have a biocontrol potential for multiple diseases.

Very few investigations have examined the mechanisms behind the biological control reported in barley, and within these, rigorous investigations were found to be infrequent. There is a need for the biocontrol research community to agree on standards in order to conclusively demonstrate biological control and determine the mechanisms involved. Appropriate disease symptoms must be evaluated and it is essential to choose relevant control treatments. Furthermore, gene expression studies or other studies to quantify defence responses in plants need to include treatments with pathogen and antagonist present together to compare with treatments with pathogen alone and quantify defence responses with a documented effect against the pathogen in question. When using endophytes to control diseases, it is also important to show that the endophyte can establish within the plant.

The trend in biological control research is to isolate control agents that can reduce symptoms from more than one disease or combine control agents in synergistic consortia. Such BCAs should have different modes of action. Ideally, a control agent should also be found which controls the pathogens in such a way that the pathogen does not evolve quickly to overcome the mechanism. Induced resistance is one such example because it generally elicits multiple defence reactions in the plant and thereby becomes difficult to overcome. Conversely, antibiosis might not be the best approach for biocontrol in barley and other crops because the pathogen population might develop tolerance to the active compound, as they are known to do with agrochemicals.

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Chapter 3

Fungal root endophyte discovery from *Elymus repens* (Poaceae) using DNA barcoding of cultured endophytes and direct metagenomic amplicon sequencing

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Abstract

The fungal root endophytic community of *Elymus repens* was investigated using a culture dependent and a direct amplicon sequencing method from the same root systems. Plants were sampled from five agricultural sites in Ireland that had high disease pressure from *Fusarium* spp. and take-all. It was hypothesised that these sites would harbour endophytes that could be used as biological control agents. Endophytes were cultured on three different types of media (PDA, MEA and 2 % MEA) and their isolation efficacy was estimated. Furthermore, three DNA barcoding regions (ITS, LSU and TEF1α) were used for the identification of the cultured endophytes and the effectiveness was compared. Lastly, the cultured endophyte community was compared to the community identified by direct amplicon sequencing from root samples. The number of cultured fungal isolates from the different sites did not correspond to the operational taxonomic unit (OTU) richness determined by direct amplicon sequencing. The ITS barcoding region identified the largest number of cultured OTUs (27) compared to LSU (23) and TEF1α (13), and the ITS region was primarily used for identification of the cultured endophytes. The OTU richness of cultured endophytes was influenced by the types of media used. The majority of OTUs were cultured on PDA (18) however, without the inclusion of 2% MEA and MEA approximately half of the unique OTUs would not have been discovered. Combinations of media are therefore

highly recommended for endophyte isolation purposes. Across all five sites, 715 different fungal OTUs were discovered by direct amplicon sequencing and they belonged to 31 different classes from 8 different divisions. From site III, 349 OTUs were identified by direct amplicon sequencing but only 66 OTUs were cultured. The two communities shared ten OTUs and only four of them were among the 48 OTUs that were found in all plants from site III determined by direct amplicon sequencing. In the search for biocontrol agents direct sequencing techniques revealed the full diversity and the heterogeneity across different sites and individual plants. The cultured community from one site was a small subset of the full diversity and hosted previously tested control agents including *Epicoccum nigrum* and *Periconia* sp. as well as many untested species.

Keywords DNA barcoding, *Elymus repens*, fungal root endophytes, high throughput amplicon sequencing, ITS, LSU, MEA, PDA, TEF1α

3.1 Introduction

There is an increasing interest in discovering plant beneficial microorganisms by companies (Broadfoot, 2016) as well as by the scientific community where more than 47,000 papers were published on biopesticides in a period from 2000-2015 (Balog *et al.* 2017). Plants are surrounded by microorganisms living on seeds, roots, leaves and flowers (Vorholt 2012; Bulgarelli *et al.* 2015; Torres-Cortés *et al.* 2018). Microorganisms found within plants, which are classified as endophytes (de Bary 1884), are of special interest, because some endophytes have been shown to promote plant growth (Taghavi *et al.* 2009; Maciá-Vicente *et al.* 2009) as well as providing increased tolerance to biotic (Omacini *et al.* 2001; Deshmukh and Kogel 2007) and abiotic stresses (Naveed *et al.* 2014; Taghinasab *et al.* 2018). Endophytes of wild grasses have been shown to be able to colonise crop plants and give protection

against disease (Maciá-Vicente *et al.* 2008) as well as improve growth under nutrient deficient conditions (Murphy *et al.* 2015).

Elymus repens was targeted as a potential host of beneficial root endophytes in this study because it is a wild relative of many important cereals and its endophytes might be more compatible with its closely related crop species than unrelated sourced plants. *Elymus repens* is placed in the tribe Triticeae that also includes barley, rye, and wheat, but the relationships of taxa within this tribe are debated (Kavanagh *et al.* 2010). *Elymus repens* is an allohexaploid (2n = 6x = 42) and three donors have contributed to its genome which include *Hordeum*, *Pseudoroegneria* and an unknown donor (Mason-Gamer 2008). The perennial *Elymus repens* is native to Europe and Asia (Mason-Gamer 2008) and is capable of extensive vegetative spread via rhizomes. Perenniality theoretically offers a longer period for endophyte colonization than that offered in annual plants (although we are unaware of any studies that have empirically tested this assumption). Individual plants were sampled from an environment with high disease pressure from *Fusarium* spp. and take-all caused by *Gaeumannomyces graminis* to target potential disease suppressing endophytes.

A culture system is needed when studying disease protection using biological control. It is commonly accepted that not all fungi will grow on all artificial media (Guo *et al.* 2000) and studies have shown that different isolation media can influence the number of recovered endophytes as well as the species richness (Bills and Polishook 1992; Elvira-Recuenco and van Vuurde 2000; Verma *et al.* 2011). Still, the majority of studies investigating the non-clavicipitaceous fungal endophytes of grasses only use one type of medium (see Chapter 1; Su *et al.* 2010; Ghimire *et al.* 2011; Hammami *et al.* 2016). Here the three most commonly used media in studies of non-clavicipitaceous fungal endophytes of grasses were used (PDA, MEA and 2 % MEA) to compare their efficacy and to maximize the diversity of cultured endophytes recovered. The cultured endophyte community identified by Sanger sequencing was compared to the fungal endophyte community revealed by direct high throughput amplicon sequencing

(hereafter direct amplicon sequencing) of the same individual root systems. Direct amplicon sequencing using an Illumina platform relies on the principles of Sanger sequencing where fluorescent nucleotides are added to a growing DNA template strand and each nucleotide is recorded (Shendure and Ji 2008). However, a large difference between the approaches is that direct amplicon sequencing can sequence many DNA fragments at the same time whereas Sanger sequencing only works on one fragment at a time. The Illumina platform uses bridge PCR where amplicons arising from the same template are clustered to one physical location on an array (Shendure and Ji 2008).

To obtain a higher and more precise taxonomic identification from the cultured endophytes, three DNA barcoding regions were applied and compared including the ITS, LSU and TEF1α. ITS has been proposed as the universal barcoding region for fungi because the probability of successful identification is high for the widest range of fungal taxa (Schoch *et al.* 2012). However, the ITS locus has limitations and some of the commonly isolated fungi including *Cladosporium, Fusarium* and *Penicillium* have been shown to share ITS sequences for the congeneric species examined (Schoch *et al.* 2012; Demirel 2016). Furthermore, within *Aspergillus* there is no variation within the ITS region for the species included by Schoch *et al.* (2012). Stielow *et al.* (2015) promote the use of the TEF1α locus for species identification because they found it to have superior resolution compared to the ITS locus.

We evaluate the effect of site differences, individual plant (genet or ramet) and culture medium in respect to the full diversity (as determined by direct amplicon sequencing from roots) and the cultured diversity (as determined by endophyte isolation and Sanger sequencing). We also examine the efficacy of three DNA barcoding regions ITS, LSU and TEF1α, and their primers, for fungal culture identification. The results help to understand and optimise the discovery efficiency of endophytes and better understand the factors influencing their diversity.

3.2 Materials and methods

3.2.1 *Elymus repens* sampling

Plant collections were initiated in August 2016 from a total of five fields in Ireland. Site I was situated in Johnstown in Kildare (53.22884N; -6.61186W) where the present crop was barley, site II and III were at Kildalton Agricultural College in Kilkenny (52.34397N; -7.30638W and 52.35636N; -7.31603W) with barley and wheat. The last two sites IV and V were situated in Cork (51.81678N; -8.49056W and 51.8526N; -8.04323W) where the crop was winter wheat and barley. From each field, ten plants were sampled except from site V where only eight plants were sampled. Plants were sampled from field margins where barley or wheat had been grown and there was a record of high disease pressure from *Fusarium* spp. and take-all caused by *Gaeumannomyces graminis*. Individual plants were kept at 4 ˚C in their clump of soil until they could be processed.

3.2.2 Root surface sterilisation method

Endophytes were isolated from roots of *Elymus repens.* Roots were washed in plenty of tap water. For each plant the cleanest roots were cut from the root system and surface sterilised. The surface sterilisation was done in six steps. Between each step the roots were transferred to a new sterile 50 mL tube with ethanol wiped forceps. The sterilisation was done as follows: I. 25 mL autoclaved ultrapure water (Purite Select Fusion; max. 18.2 MΩ.cm) shaken at 350 rpm for 1 minute (min); II. 25 mL 70 % ethanol shaken at 350 rpm for 3 min; III. 25 mL 5 % sodium hypochlorite (NaOCl) shaken at 350 rpm for 10 min; IV-VI. 25 mL autoclaved ultrapure water shaken by hand for 1 min at each round. After the third washing step the roots were transferred to an empty Petri dish and cut into 2 mm long pieces. Five root pieces were placed on three types of media, PDA (potato dextrose agar), MEA (malt extract agar) and 2 % MEA, and 1 min imprints of five root pieces were also made on PDA to test for possible epiphytic contamination. The surface sterilisation technique was found to be efficient in eliminating epiphytes. Subcultures were made on the original medium.

3.2.3 High throughput amplicon sequencing

Surface sterilised root material from individual plants was taken aside, stored at -80 ˚C and was used to run direct amplicon sequencing of root DNA on a high throughput Illumina paired end sequencing platform. Roots were freeze dried and each sample was disrupted using a mixer mill (Retsch MM 300) with three surface sterilized 4 mm glass beads at 30 freq 1/s for 5-30 min dependent on the toughness of the sample. Novogene Co. Ltd. performed the DNA extraction and sequenced the nrDNA of ITS2 of individual samples at 100,000 raw tags/sample using fITS7 (Ihrmark *et al.* 2012) and ITS4 (White *et al.* 1990).

3.2.4 Fungal DNA extraction, amplification and Sanger sequencing

DNA for Sanger sequencing was extracted using predominately the DNeasy Plant mini Kit from Qiagen. For samples where this procedure did not work the NucleoSpin plant kit from Macherey-Nagel was utilized. Independent of the kit the subsequent steps were done. Under sterile conditions 1/8th of fungal culture growing on a Petri plate was scraped with a sterile scalpel and put into a 1.5 mL microcentrifuge tube. A sterile metal bead was added to the tube and the sample was disrupted using mixer mill (Retsch MM 300) for 30 s at 20 1/s frequency. The final volume was 50 µL for both kits.

PCR was prepared for a total volume of 12.5 µL using BioMix from Bioline. For the first 96 well plate, 0.5 µL DNA template was used and for the subsequent plates 1 µL DNA template (approximately 100 ngµl^{-1}) was used as it had a higher success rate. DNA was extracted from each fungal culture and ITS (internal transcribed spacer 1 and 2 of nuclear ribosomal DNA, ITS1 and ITS4, White *et al.* 1990), LSU (large subunit of nuclear ribosomal DNA, LROR and LR5, Stielow et al. 2015) and TEF1α (transcription elongation factor 1, TEF1-983F and TEF1-1567R, Rehner and Buckley 2005) was amplified (see Supplement 3.1 and Supplement 3.2). PCR products were purified using ExoSAP-IT^{TM} (Thermo Fisher Scientific) and sequenced using automated Big Dye terminator Sanger sequencing (by Macrogen Inc.).

3.2.5 Endophyte identification

Neighbour-joining trees based on p-distance were made for each barcoding region using the software MEGA7: molecular evolutionary genetics analysis across computing platforms (Kumar *et al.* 2018). Sequences were edited and trimmed in MEGA7. Then individual trees were built for each taxonomic class of fungi separately to examine if the same number of operational taxonomic units (OTUs) would be determined. OTUs define individual sequences which are closely related (Blaxter *et al.* 2005). Clusters were defined as OTUs if their members had at least 99 % sequence similarity.

To assign a name to the OTU clusters the ITS sequences were compared to the UNITE database (https://unite.ut.ee/, Nilsson *et al.* 2018) and assigned a rank if the percentage identity was 99-100 %. When there were discrepancies for the identification within an OTU cluster, the following steps were taken to allocate the taxonomic name and manage incongruence: 1) evaluate the quality of the sequence and 2) compare levels of percentage identity (only 99-100 % was accepted). When identity was lower than 99 % the cluster was assigned to the consensus taxonomic class.

3.2.6 Bioinformatics

Bioinformatics was undertaken with demultiplexed paired-end reads using the microbiome analysis package Qiime 2, version qiime2-2018.6. Sequences were denoised, trimmed, joined, chimera were removed and sequences were quality filtered using Dada2 (Callahan *et al.* 2016) following essentially the “Moving Pictures” tutorial https://docs.qiime2.org/2019.1/tutorials/moving-pictures/ (Supplement 3.4). Furthermore, all sequences that were 95 % identical to, and had 95 % overlap with, a selection of plant ITS sequences were removed from the dataset according to a BLAST search. Classification was done using the UNITE developers classifier, UNITE Community (2017): UNITE QIIME release. Version 01.12.2017. UNITE Community. The data were not rarefied (McMurdie and Holmes 2014; Bálint *et al.* 2016) and low frequency clusters were not removed (Kauserud *et al.* 2012).

3.2.7 Statistical analyses

The data was analysed with the software package R i386 3.4.3 (https://cran.r-project.org/bin/windows/base/old/3.4.3/). Linear models with the appropriate random effects were fitted and tested against each other using ANOVA. The data followed a normal distribution and the residuals were homogenous and independent. Multiple comparisons were made using Bonferroni adjusted *P*-values with significance level ($P \leq 0.05$).

Non-metric multidimensional scaling (NMDS) was performed using the R package vegan (Oksanen 2016). Bray-Curtis distances for binary data was used with 100 iterations and the stress was <0.05 and thus, provided an excellent representation in reduced dimensions.

Beta diversity was calculated using the following equations first for all sites and then for site III specifically. The gamma diversity is the total number of recorded species in the area of interest and alpha diversity is the average number of recorded species across all plants or each plant in site III.

$$Beta\ diversity_{all\ sites} = \frac{gamma\ diversity_{all\ sites}}{average(alpha\ diversity_{all\ 48\ plants})}$$

$$Beta\ diversity_{site\ III} = \frac{gamma\ diversity_{site\ III}}{average(alpha\ diversity_{each\ plant\ in\ site\ III})}$$

3.3 Results

3.3.1 Endophyte isolation

Overall, 394 endophyte isolates were obtained from 48 plants of *Elymus repens* on three different media from five different sites. A total of 165 isolates were cultured on PDA, 147 on MEA and 82 on 2 % MEA (Figure 3.1A). Significantly more endophytes were isolated from site III compared to sites I, IV and V. Also, significantly more endophytes were isolated from site III on MEA compared to the other four sites (Figure

3.1B). The number of isolated endophytes from individual plants on different media showed high variation (Figure 3.1C) and on average 8 isolates were cultured per plant.

3.3.2 Cultured OTU richness from site III

Cultures from site III were chosen for DNA barcoding identification because the site showed the largest number of isolated endophytes and it was hypothesised that it could contain the highest total species richness. For 12 cultures out of 151 it was not possible to amplify any DNA using the three sets of primers. The amplification was most successful using LSU, which was followed by ITS and TEF1α (Table 3.1). Only Ascomycota were cultured and all three loci identified three classes of fungi including the Dothideomycetes, Leotiomycetes and Sordariomycetes. An additional class was identified by the ITS locus: the Pezizomycetes (Table 3.1). The ITS identified the largest OTU richness, followed by LSU and TEF1α. In addition, the ITS locus discriminated better between OTUs within the Dothideomycetes and Sordariomycetes compared to LSU and TEF. However, the LSU discriminated better between the OTUs within the Leotiomycetes compared to the other two loci. As TEF1α was amplified less successfully there were fewer sequences to form OTUs with.

The OTU richness was highest for the ITS locus and therefore ITS sequences were subjected to BLAST analysis within the UNITE database to assign a taxonomic name to the different OTUs. Four classes of fungi were discovered including 15 OTUs within the Dothideomycetes, two OTUs within the Leotiomycetes, one OTU within the Pezizomycetes and nine OTUs within the Sordariomycetes (Table 3.1 and Table 3.2). Unfortunately, 12 isolates could not be identified because DNA was not successfully extracted, and for an additional 27 isolates no ITS sequence was amplified.

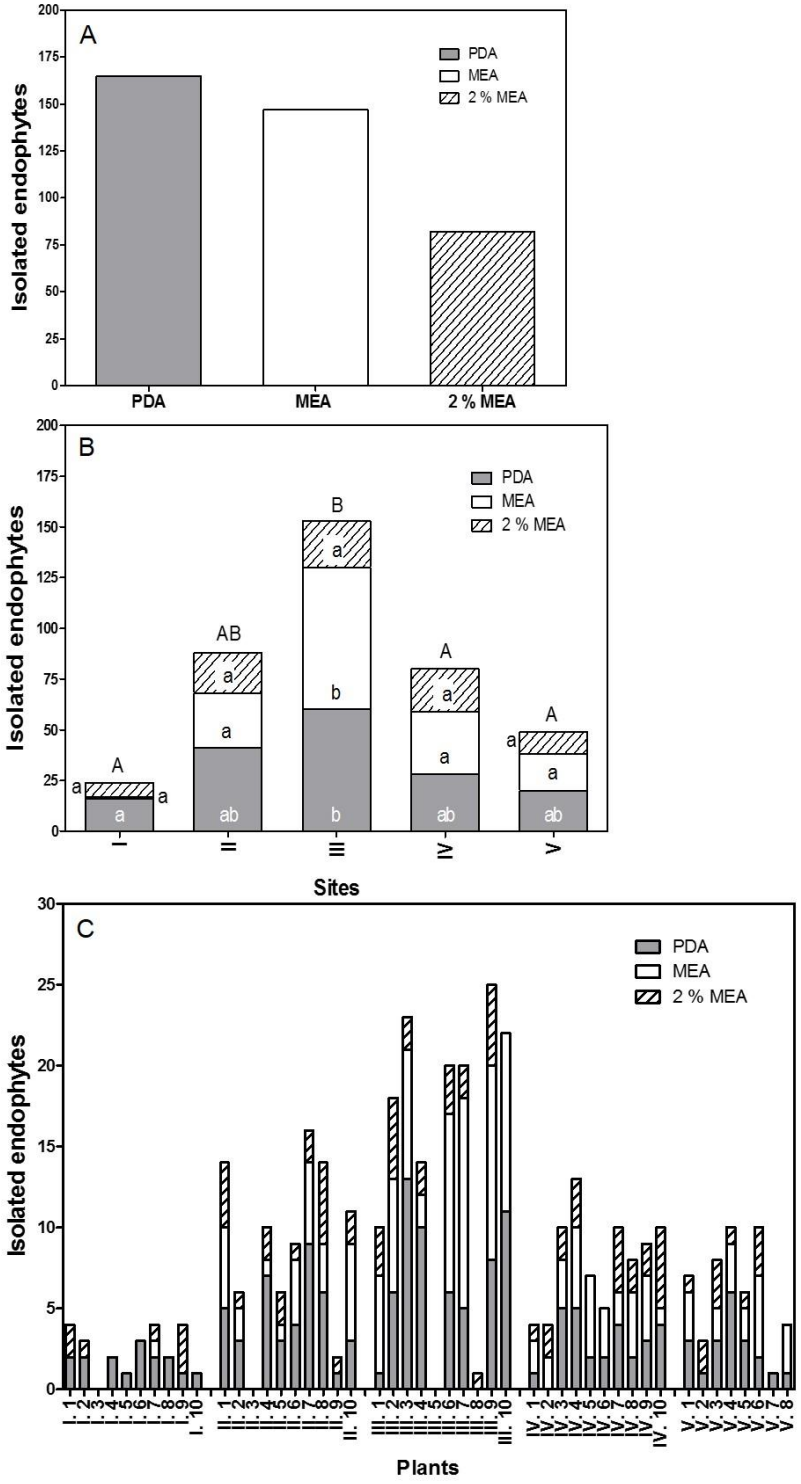

Figure 3.1. Number of endophyte isolates recovered on three different media from five different sites. A) Number of endophytes isolated on three different media. B) Number of endophytes isolated from each of five sites (I to V) on three different media. Sites sharing the same letter are not significantly different ($P \leq 0.05$). Capital letters show differences in mean number of endophytes isolated per site. Small letters show differences between numbers of endophytes isolated on a specific medium compared between sites. C) Number of endophytes isolated from each plant on three different media grouped by site (I to V).

Table 3.1. Overview of the number of OTUs, categorised by fungal class, identified using three different barcoding loci. The number of OTUs is based on individual neighbour-joining trees constructed, using p-distance, on the listed number of sequences with 1 % dissimilarity as the cut off value.

	ITS		LSU		TEF1α	
Fungal class	**OTUs**	**Sequences**	**OTUs**	**Sequences**	**OTUs**	**Sequences**
Dothideomycetes	15	48	11	61	7	15
Leotiomycetes	2	36	4	37	2	18
Pezizomycetes	1	2	0	0	0	0
Sordariomycetes	9	18	8	17	4	6
Total	27	104	23	115	13	39

Media influence on OTU richness

The three types of media influenced the species richness of retrieved endophytes. The highest OTU richness was discovered on PDA with 18 OTUs, followed by MEA with 10 OTUs and 2 % MEA with 9 OTUs (Figure 3.2). Only three OTUs could be discovered by all media and included Dothideomycetes sp. 2 (OTU3), Dothideomycetes sp. 7 (OTU8) and *Leptodontidium* sp. (OTU17, Figure 3.2 and Table 3.2). The media PDA and MEA additionally shared four OTUs which included Chaetosphaeriaceae sp. (OTU19), *Clohesyomyces* sp. (OTU1), Lasiosphaeriaceae sp. (OTU23) and *Periconia* sp. 1 (OTU13). The remaining 20 OTUs were only found on one specific medium and therefore using PDA only would have excluded approximately 50 % of the unique OTUs.

Inter-plant variation in OTU richness

On average six OTUs were isolated from each plant root system from site III and the combination of OTUs isolated from each of the individual plants was unique (Figure 3.3A). All plant roots had one OTU in common identified as *Leptodontidium* sp.

Table 3.2. Taxonomic identification of OTUs from the cultured endophytes from site III using ITS sequences. The sequences were compared using BLAST through the UNITE database. For a more detailed examination of the identification see Supplement 3.3. 12 cultures could not be identified because no DNA was extracted and for 27 cultures no ITS sequence was amplified so these cultures were categorised as individual OTUs. Names of organisms are given according to Species Fungorum (http://www.indexfungorum.org/).

OTU	Sequence(s)	Class	Identification
1	4	Dothideomycetes	*Clohesyomyces* sp.
2	1		Dothideomycetes sp. 1
3	8		Dothideomycetes sp. 2
4	1		Dothideomycetes sp. 3
5	1		Dothideomycetes sp. 4
6	1		Dothideomycetes sp. 5
7	1		Dothideomycetes sp. 6
8	15		Dothideomycetes sp. 7
9	6		*Epicoccum nigrum*
10	1		*Epicoccum* sp.
11	2		*Ophiosphaerella korrea*
12	1		*Ophiosphaerella* sp. 1
13	4		*Periconia* sp. 1
14	1		*Periconia* sp. 2
15	1		Pleosporaceae sp.
16	2	Leotiomycetes	*Glarea* sp.
17	34		*Leptodontidium* sp.
18	2	Pezizomycetes	*Pyronema domesticum*
19	7	Sordariomycetes	Chaetosphaeriaceae sp.
20	1		*Diaporthe sp.*
21	2		*Falciphora* sp.
22	1		*Gaeumannomyces graminis*
23	2		Lasiosphaeriaceae sp.
24	1		Sordariomycetes sp. 1
25	1		Sordariomycetes sp. 2
26	2		Sordariomycetes sp. 3
27	1		Xylariaceae sp.
28-54	27		Fungus sp. 28-54
55-66	12		Fungus sp. 55-66

(OTU17). The second most dominating OTU was Dothideomycetes sp. 7 (OTU8 – possibly *Ophiosphaerella* sp., Supplement 3.3) isolated from four plant roots, followed by Chaetosphaeriaceae sp. (OTU19), Dothideomycetes sp. 2 (OTU3), *Epicoccum nigrum* (OTU9) and *Periconia* sp. 1 (OTU13) isolated from three plants. Plant 2 and plant 3 had four OTUs in common which was the highest number of shared OTUs. Remarkably, plant 2 and plant 3 were not among the plants situated closest together (Figure 3.3B). Unique OTUs were isolated from all plant root systems, except plant 7. The number of unique OTUs in each root system ranged from two (plant 1, 2, 6, 10) to five (plant 9).

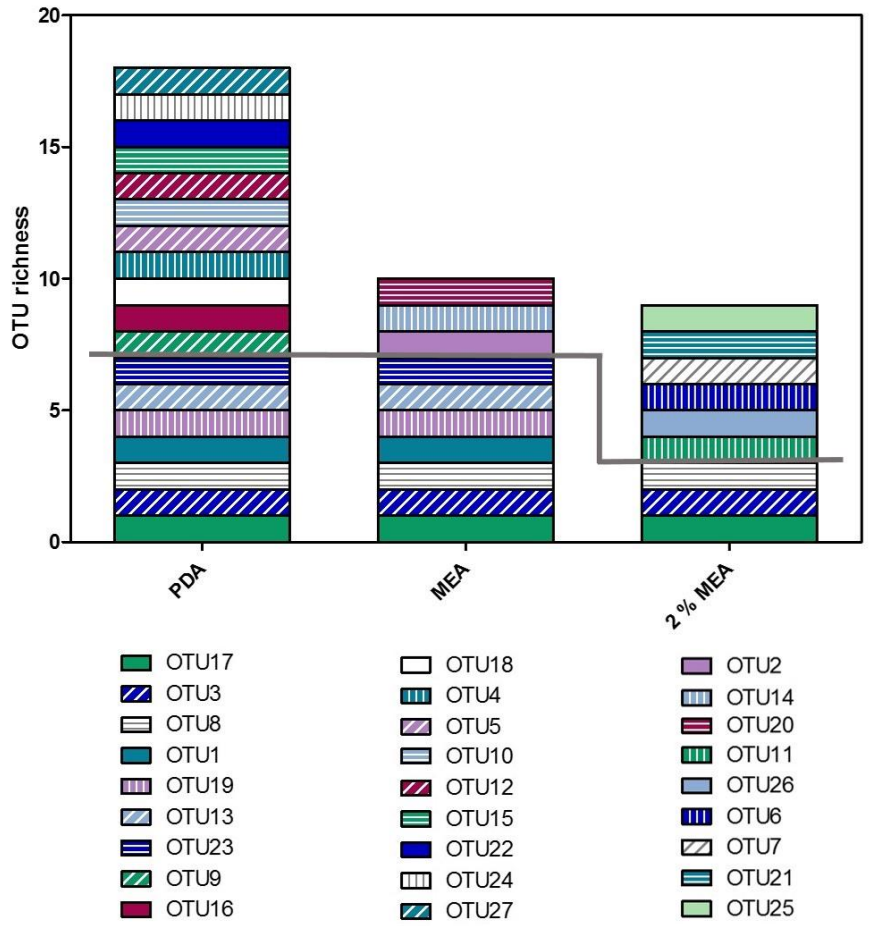

Figure 3.2. OTU richness isolated on three types of media. Each colour pattern represents a different OTU and their taxonomic identification can be found in Table 3.2. The OTUs below the grey line can all be isolated on PDA whereas the OTUs above the grey line were only isolated on a specific medium.

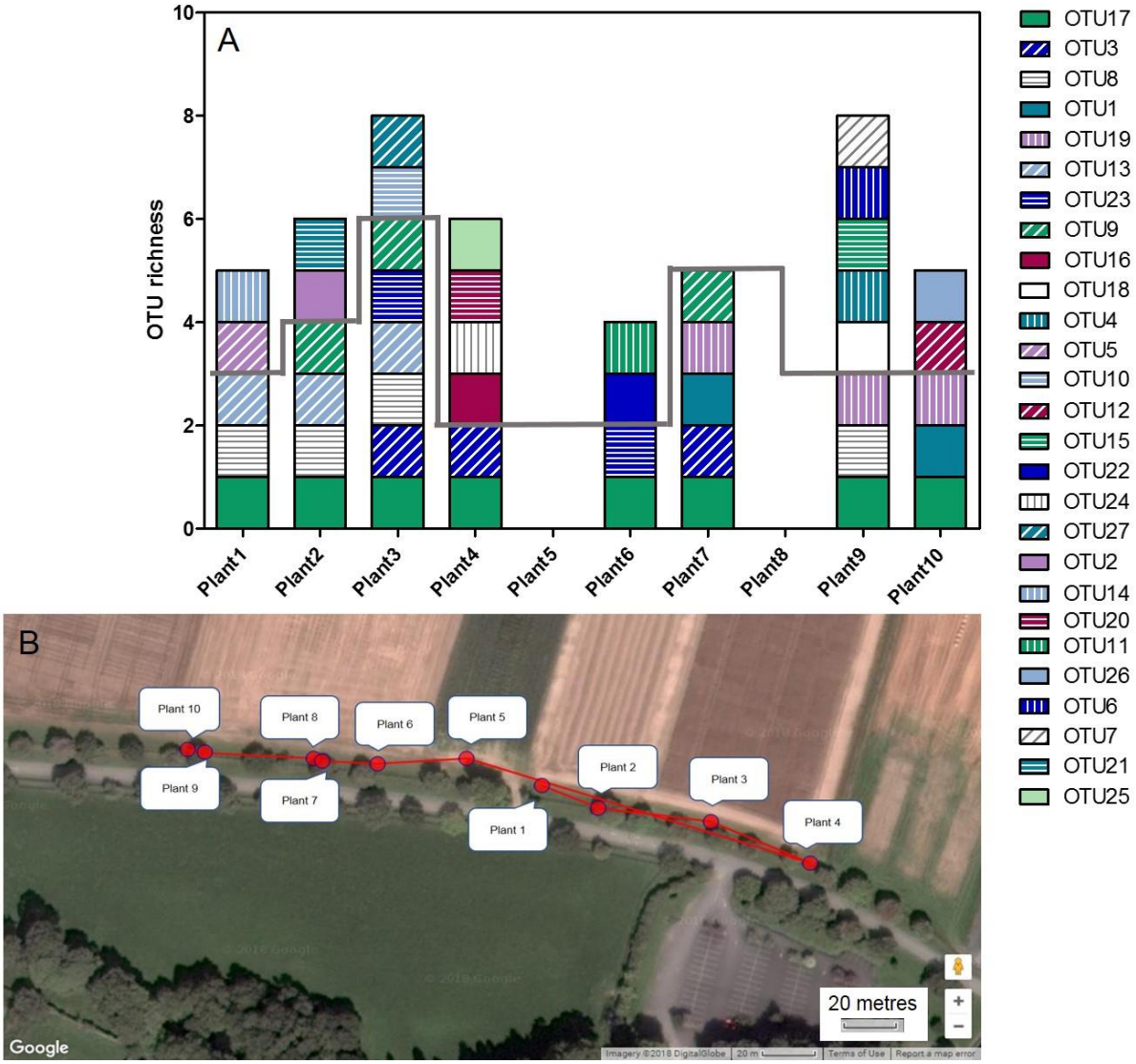

Figure 3.3. OTU richness discovered from individual plants. A) Each colour pattern represents a different OTU and their taxonomic identification can be found in Table 3.2. The OTUs below the grey line can all be found in more than one plant and the OTUs above the grey line are unique to the specific plant. No endophytes were cultured from plant 5 and no ITS sequence was amplified from the cultures isolated from plant 8. B) The individual plant's location in relation to each other from site III.

3.3.3 OTU richness described by direct amplicon sequencing

The OTU richness and community structure of all sites

From all five sites, three different kingdoms of organisms were discovered living as endophytes within *Elymus repens* roots, using direct amplicon sequencing, including the Chromista, Fungi and Rhizaria (Table 3.3). In total 715 different fungal OTUs were discovered from the five sites and they belonged to 8 different divisions and 31 classes (Table 3.3). The widespread classes that were found within all plants included Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes, Agaricomycetes, Glomeromycetes and Mortierellomycetes.

To our knowledge, this is the first time the communities of individual plants have been studied in grasses because other studies pool their samples. There was a large variation in the number of OTUs identified from individual plants ranging from 96 OTUs (site I, plant 4) to 239 OTUs (site II, plant 4), with a mean of 151 OTUs identified across all 48 plants (Figure 3.4B). Significantly more OTUs were identified from site II compared to site IV and V (Figure 3.4A). Interestingly, site II harboured a kingdom and three classes of fungi that were not present in the other sites including the Chromista, Taphrinomycetes, Xylonomycetes and Pucciniomycetes. However, site II was not significantly different from site I and site III.

Across all 48 plants, the beta diversity quantified 4.7 communities which corresponded well with the non-metric multidimensional scaling which suggested that all five sites had unique communities, with the communities in site III and site IV being the most similar (Figure 3.5).

Table 3.3. Overview of the different classes of endophytes identified from *Elymus repens* roots from all sites. It lists the number of plants that contained each fungal class and in how many sites the class was present.

Kingdom	Division	Class	Number of plants	Site				
				I	II	III	IV	V
Chromista	-	-	2		x			
Fungi	Ascomycota	Archaeorhizomycetes	12	x	x	x	x	x
		Dothideomycetes	48	x	x	x	x	x
		Eurotiomycetes	48	x	x	x	x	x
		Lecanoromycetes	13	x	x	x	x	x
		Leotiomycetes	48	x	x	x	x	x
		Orbiliomycetes	16	x	x	x	x	x
		Pezizomycetes	34	x	x	x	x	x
		Saccharomycetes	43	x	x	x	x	x
		Sordariomycetes	48	x	x	x	x	x
		Taphrinomycetes	1		x			
		Xylonomycetes	2		x			
	Basidiomycota	Agaricomycetes	48	x	x	x	x	x
		Agaricostilbomycetes	1				x	
		Exobasidiomycetes	1				x	
		Malasseziomycetes	32	x	x	x	x	x
		Microbotryomycetes	26	x	x	x	x	x
		Pucciniomycetes	4		x			
		Tremellomycetes	47	x	x	x	x	x
		Tritirachiomycetes	1	x				
		Ustilaginomycetes	17	x	x	x	x	x
		Wallemiomycetes	7	x		x	x	x
	Chytridiomycota	Rhizophydiomycetes	1	x				
		Spizellomycetes	4	x	x		x	x
	Glomeromycota	Archaeosporomycetes	6	x	x			
		Glomeromycetes	48	x	x	x	x	x
		Paraglomeromycetes	12	x	x	x	x	x
	Mortierellomycota	Mortierellomycetes	48	x	x	x	x	x
	Mucoromycota	Endogonomycetes	13	x	x	x	x	x
		Mucoromycetes	32	x	x	x	x	x
	Olpidiomycota	Olpidiomycetes	3		x	x		
	Rozellomycota	-	5	x	x			
Rhizaria	Cercozoa	-	2	x			x	

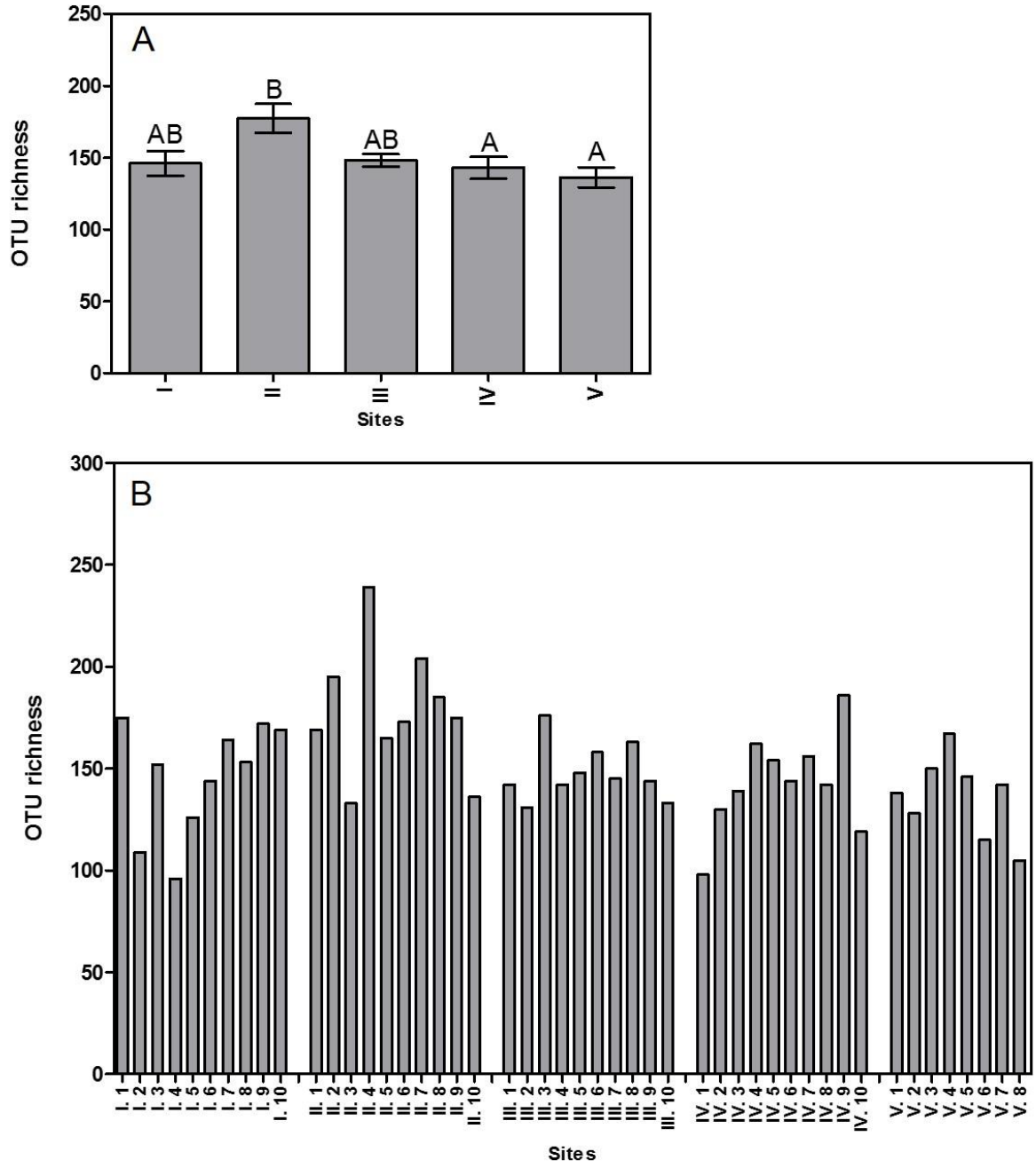

Figure 3.4. OTU richness determined by direct amplicon sequencing of roots from five sites. A) Mean number of OTUs is given +/- SEM present in five sties (I to V). Capital letters show differences in mean number of OTUs identified per site. Sites sharing the same letter are not significantly different ($P \leq 0.05$). B) The OTU richness determined for individual plants from five sites.

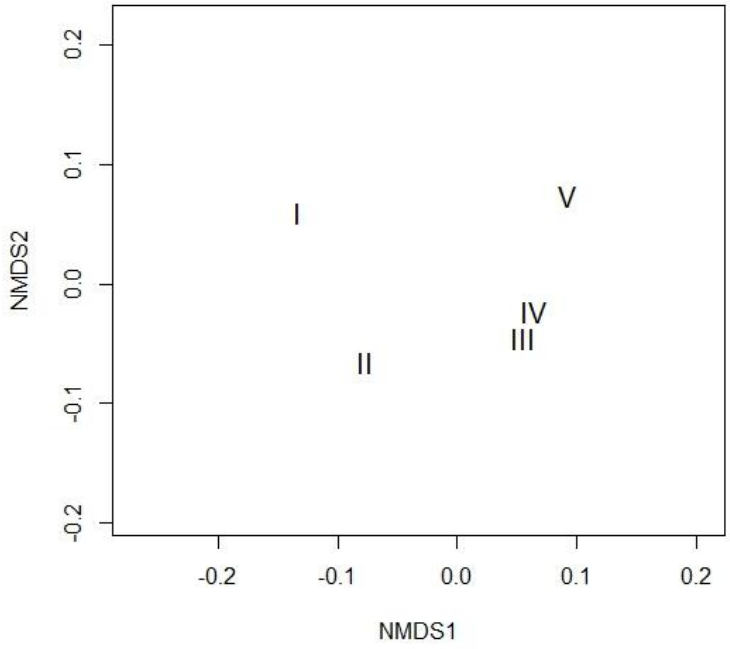

Figure 3.5. Non-metric multidimensional scaling of the communities found within roots from the five sites, stress was <0.05.

The OTU richness and community structure of site III

Only fungi were identified from site III which belonged to 21 different classes including nine classes within the Ascomycota, six classes within the Basidiomycota, two classes within the Glomeromycota, one class within the Mortierellomycota, two classes within the Mucoromycota and one class within the Olpidimycota (Table 3.3).

A total of 349 different OTUs were identified from site III (Figure 3.4) and the average OTU richness per root system was 148. A subset of 48 OTUs could be found widespread in all root systems and they belonged to seven different classes including the Dothideomycetes (16 OTUs), Eurotiomycetes (two OTUs), Leotiomycetes (11 OTUs), Sordariomycetes (13 OTUs), Agaricomycetes (one OTU), Glomeromycetes (one OTU) and Mortierellomycetes (one OTU, Table 3.4).

Across all 10 plants in site III, the beta diversity quantified 2.3 communities with separation for these communities apparent in the non-metric multidimensional scaling (Figure 3.6).

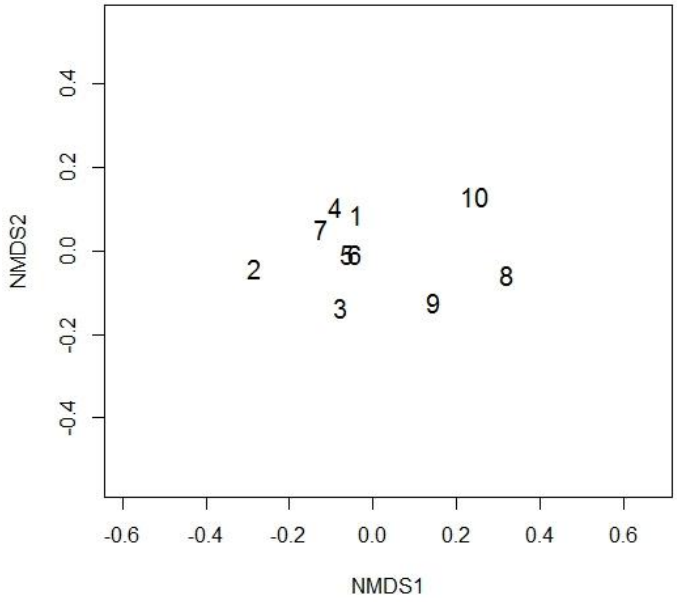

Figure 3.6. Non-metric multidimensional scaling of the communities found within roots from the ten plants sampled within site III, stress was <0.05.

3.4 Discussion

3.4.1 Cultured endophytes of *Elymus repens*

This is the first report describing the cultured root endophyte community of *Elymus repens* and to our knowledge the first comparison of endophytes cultured and identified by direct amplicon sequencing at individual plant level.

Endophyte isolation per site and per root system

There was large variation in the number of endophytes isolated from individual sites and it ranged from on average 2 cultures per plant (site I) to 15 cultures per plant (site III). Tejesvi et al. (2010) also reported large variation between sites in Finland.

Table 3.4. The 48 OTUs that were present in all plants from site III and their identification. Names of organisms are given according to Species Fungorum (http://www.indexfungorum.org/).

Class	Species	Class	Species
-	Fungi sp. 1	Leotiomycetes	*Articulospora* sp.
-	Fungi sp. 2		*Glarea* sp.
-	Ascomycota sp.		*Hymenoscyphus* sp.
Dothideomycetes	Dothideomycetes sp.		*Tetracladium* sp.
	Capnodiales sp.		*Tetracladium marchalianum*
	Pleosporales sp.		*Tricladium splendens*
	Xenopyrenochaetopsis pratorum		*Rhexocercosporidium panacis*
	Didymellaceae sp.		*Microscypha* sp.
	Neoascochyta graminicola	Sordariomycetes	Sordariomycetes sp.
	Didymosphaeriaceae sp.		*Codinaea acaciae*
	Stagonospora pseudovitensis		*Pseudolachnella* sp.
	Melanommataceae sp.		*Gibellulopsis nigrescens*
	Periconia sp.		*Dactylonectria macrodidyma*
	Ophiosphaerella sp.		*Gaeumannomyces graminis*
	Phaeosphaeria triglochinicola		*Slopeiomyces cylindrosporus*
	Phaeosphaeriaceae sp.		*Myrmecridium* sp.
	Alternaria sp.		*Pleotrichocladium opacum*
	Alternaria hordeicola		*Schizothecium glutinans*
	Drechslera sp.		*Microdochium* sp.
Eurotiomycetes	*Exophiala* sp.		*Microdochium bolleyi*
	Aspergillus sydowii		*Microdochium phragmitis*
Leotiomycetes	Helotiales sp.	Agaricomycetes	Agaricomycetes sp.
	Helotiaceae sp. 1	Glomeromycetes	Glomeraceae sp.
	Helotiaceae sp. 2	Mortierellomycetes	*Mortierella exigua*

They isolated endophytes from roots of three plant species including the perennial grass *Deschampsia flexuosa,* the dwarf shrubs *Empetrum nigrum* ssp. *hermaphroditum* and *Vaccinium vitis-idaea* from five sites. Tejesvi *et al.* (2010) were able to isolate an average of 0.5 to 1.5 fungal endophytes per root system on PDA and MEA. The isolation success from our study was much higher than Tejesvi et al. (2010) even if only isolation from PDA and MEA is included. An average of 6.5 endophytes were

isolated from *E. repens* root systems from five sites. However, comparisons between these two studies can be difficult, as the difference in number of isolated endophytes could be explained by the difference in plant species, the difference in sites and perhaps also by latitude. Tejesvi *et al.* (2010) examined endophyte communities in Northern Finland and some studies have found that high latitude endophyte communities include fewer species but from a larger number of fungal classes compared to tropical regions where the community will be represented by a few classes with many different species (Arnold and Lutzoni 2007). It is not known whether the latitude difference between Finland and Ireland is enough to explain the difference in number of cultured root endophytes alone.

Sánchez Márquez *et al.* (2008) investigated the endophyte community of *Elymus farctus* rhizomes and cultured a total of 34 species from 48 rhizomes. *Elymus repens* and *E. farctus* are in the same genus and could thus potentially have a similar endophyte composition. However, *E. farctus* is predominantly a coastal sand dune species and *E. repens* a more widespread species that is also a troublesome weed in some environments. Sánchez Márquez *et al.* (2008), identified species belonging to four fungal classes, the Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes. Commonly, it is accepted that the specific plant species will shape the endophyte community (Wearn *et al.* 2012) but it is still a little surprising that these two *Elymus* species do not share any species in their below ground tissue. Many additional factors have been proposed to influence the community composition including host organ (Wearn *et al.* 2012; Hammami *et al.* 2016) and it is possible that the slightly increased species richness could be explained by the fact that the rhizome is a more permanent structure compared to the root and could possibly host a higher diversity of fungal species. Sánchez Márquez *et al.* (2008) may have recorded a greater species richness due to more sites being sampled, with 12 being surveyed in total. Number of sampled sites has been demonstrated to be a factor influencing the recorded endophyte community composition and total taxon number, with endophyte

richness increasing with increasing number of sampled sites (Tejesvi *et al.* 2013). However, the two plant species were sampled from completely different types of habitats the beach versus a margin of a field which could also explain the differences.

Amplification of barcoding regions and OTU clusters

The estimation of species richness of endophytes will be heavily influenced by the methods used to delimit a species. The ITS barcoding region defined the largest number of OTUs (27) compared to LSU (23) and TEF1α (13). This was not expected as a higher amplification success was seen for LSU (115/151, 76 %) compared to ITS (108/151, 72 %). Similarly, Stielow *et al.* (2015) compared 14 primer pairs and found that the ITS and LSU loci were amplified with similar success. On the other hand, TEF1α showed very low amplification success (44/106, 42 %) which was not the finding of Stielow *et al.* (2015) using the same primer pairs. They found that the TEF1α barcoding region was amplified to their satisfaction and they state that it had "relatively consistent amplification". The amplification and identification was so successful that they promoted the TEF1α locus as a secondary barcoding region for fungi.

Many additional sequences were amplified within the Dothideomycetes by the LSU compared to the two other barcoding regions. Perhaps, the additional sequences belonged to some of the same species that were already amplified for the ITS region or the LSU has less resolution within the Dothideomycetes compared to ITS. Demirel (2016) found that the ITS gave better resolution compared to the LSU but that the branching pattern for the two trees was fully congruent. They compared the identification success of the ITS and LSU for 43 isolates belonging to three genera within the Eurotiomycetes, *Aspergillus*, *Penicillium*, and *Talaromyces*. However, Schoch *et al.* (2012) compared the ITS and LSU barcoding regions for 742 strains across the kingdom of fungi and found no significant difference between the probability of correct identification using ITS (0.66) and LSU (0.63) within the Pezizomycotina a

subdivision that includes among others the Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes and Sordariomycetes.

Three barcoding regions were used in our study of cultured endophytes and DNA sequences were clustered into OTUs using 99 % sequence similarity. In studies identifying the non-clavicipitaceous fungal endophytes of grasses it is very common for researchers to use only ITS and group sequences into OTU clusters based on 97 % similarity (Sánchez Márquez *et al.* 2007, 2010; Porras-Alfaro *et al.* 2008; Herrera *et al.* 2010; Khidir *et al.* 2010). There is no one universally applicable percentage cut off value, however, 3 % has become widely used (Hughes *et al.* 2009). Gazis *et al.* (2011) and Luo *et al.* (2017) compared diversity determined by 1 % and 3 % clustering criteria. Luo *et al.* (2017) examined the root endophyte community from rosette grass, *Dichanthelium acuminatum;* switchgrass, *Panicum virgatum*; and pitch pine, *Pinus rigida* and found that the two cut off values resulted in similar community structure estimations. In contrast, Gazis *et al.* (2011) studied three species complexes within the Sordariomycetes and found that increasing the percent similarity cut off value increased the number of OTUs. The intraspecific variation within the ITS region from fungi within the INSD database was examined by Nilsson *et al.* (2008) and they found species with very low intraspecific variation 0.2 % (*Aspergillus fumigatus* and *Candida albicans*) and species with very high variation 24.2 % (*Xylaria hypoxylon*). The big difference in intraspecific variation between species might explain why Gazis *et al.* (2011) and Luo *et al.* (2017) had conflicting results. Across all the examined species, Nilsson *et al.* (2008), found that the majority of species had intraspecific variability of 0-1 % and thus a 1 % cut off value was adopted in this study.

OTU richness based on identification using ITS through the UNITE database

Only members of the Ascomycota were cultured which is a division of fungi that usually dominates other reports of non-clavicipitaceous fungal endophytes of grasses (Sánchez Márquez *et al.* 2012). Using the ITS barcoding region, four classes of fungi

and 27 OTUs were identified with the majority of OTUs identified from the Dothideomycetes (15) followed by Sordariomycetes (8), Leotiomycetes (2) and Pezizomycetes (1). Notably, this order of richness matches the order reported in the extensive review by Hardoim *et al.* (2015) of the number of sequences identified to individual classes within the Ascomycota.

Two ITS sequences contributed to the identification of the class Pezizomycetes, which was not identified by the other loci. The two ITS sequences aligned poorly to the sequences within the other three classes and through UNITE they were identified as *Pyronema domesticum* (Pezizomycetes) at 100 % identity. The two LSU sequences which were of good quality aligned well both to the Dothideomycetes and the Leotiomycetes and the UNITE database grouped one sequence to each of the classes. Only one TEF sequence, also good quality, was obtained from the two cultures and it did not align very well to the other Dothideomycetes sequences which was the class it should belong to according to UNITE. Based on this, the Pezizomycetes were identified by the ITS locus but not by the other two loci. Perhaps there was insufficient resolution within the LSU barcoding region to distinguish between species within the Pezizomycetes. In any case it would have been better to have more sequences within the Pezizomycetes to confidently identify the class.

The widespread OTUs and their potential as biocontrol agents

Species that were only isolated once were in majority and widespread species found in most samples were much rarer. This is a common pattern for fungal endophyte communities (Arnold and Lutzoni 2007; Comby *et al.* 2016). *Leptodontidium* sp. (OTU17) was a widespread species isolated from all plant roots from site III. *Leptodontidium* sp. has not previously been isolated as an endophyte from *Elymus farctus* (Sánchez Márquez *et al.* 2008) nor from wheat, as an example of a crop relative of *Elymus* (Comby *et al.* 2016). However, according to Sánchez Márquez *et al.* (2012), *Leptodontidium* sp. has been isolated from both the grasses *Dactylis glomerata* and

Holcus lanatus but is not considered a common non-clavicipitaceous endophyte of temperate grasses. Jumpponen and Trappe (1998) report that *Leptodontidium orchidicola* is a common dark septate endophyte of 23 trees and forbs. Whether or not *Leptodontidum* sp. could be a good biocontrol candidate is not explored extensively, but, one species, *Leptodontidium trabinellum*, has been associated with sooty blotch disease of apples (Belding *et al.* 2000).

The other relatively widespread species that were isolated from three to four plant root systems included among others Chaetosphaeriaceae sp. (OTU19), Dothideomycetes sp. 2 (OTU3, top hit Pleosporales sp. potentially *Camposporium cambrense*, see Supplement 3.3), Dothideomycetes sp. 7 (OTU8, top hit fungi potentially *Ophiosphaerella* sp., see Supplement 3.3), *Epicoccum nigrum* (OTU9) and *Periconia* sp. 1 (OTU13). The genera *Epicoccum* and *Periconia* are frequently isolated as endophytes of grasses (Sánchez Márquez *et al.* 2007, 2012; Mandyam *et al.* 2012; Comby *et al.* 2016). As biocontrol agents, *Epicoccum* ssp. are interesting, because *Epicoccum* spp. have shown promising biocontrol results in previous studies, both against soil-borne (Hamza *et al.* 2013; El-Gremi *et al.* 2017), leaf-infecting (Li *et al.* 2013) and post-harvest diseases (Larena *et al.* 2005). *Periconia macrospinosa* has been tested as a biocontrol agent once in a study by Kirk and Deacon (1987) against the root disease of wheat; take-all, *Gaeumannomyces tritici*. However, the fungus was not able to control the root disease. Other *Periconia* isolates have received a lot of attention for their production of secondary compounds that have been proposed to have anti-cancer and anti-viral properties (Zhang *et al.* 2016; Liu *et al.* 2016, 2017).

According to the review by Sánchez Márquez *et al.* (2012) of the non-clavicipitaceous endophytes of grasses neither Chaetosphaeriaceae nor *Ophiospharella* are among the taxa that dominate temperate grasses. However, there are reports of their isolation in temperate grasses. Sánchez Márquez *et al.* (2007) isolated Chaetosphaeriaceae sp. from cat grass, *Dactylis glomerata* and interestingly De Souza *et al.* (2016) found Chaetosphaeriaceae sp. as a core OTU in roots of

sugarcane (Panicoideae) which is of course not a temperate grass (Pooideae). *Ophiospharella* ssp. are associated with disease of many grasses including barley, *Hordeum vulgare;* bermudagrass, *Cynodon dactylon*; creeping bentgrass, *Agrostis stolonifera;* and red fescue, *Festuca rubra* (Nus and Shashikumar 1993; Câmara *et al.* 2000; Corwin *et al.* 2007; Hong *et al.* 2019). Chaetosphaeriaceae sp., *Epicoccum nigrum, Leptodontidium* sp. and *Periconia sp.*, were all selected as potential biocontrol agents in this thesis (Chapter 4).

The Influence of media on OTU richness

This is the first evaluation of how the most commonly used media for isolation of endophytes of grasses can influence the isolation success. The majority of OTUs were discovered on PDA (18) followed by MEA (10) and 2 % MEA (9). The overall difference in these three media is the sugar source and the strength with PDA composed of dextrose a monosaccharide and potato extract while the ingredients in MEA is dextrin, maltose a disaccharide and vegetable peptone (Merc 2019). A few studies of endophytes of grasses have isolated endophytes on several media but they do not discuss the influence on endophyte diversity (Pelaez *et al.* 1998; Herrera *et al.* 2010; Tejesvi *et al.* 2010; Potshangbam *et al.* 2017). Verma *et al.* (2011) isolated endophytes from the neem tree on four different media. They also found that the maximum number of endophytes was recovered from PDA.

Three OTUs (OTU3, OTU8, OTU17) were versatile and could grow on all three types of media and four OTUs (OUT1, OTU13, OTU19, OTU23) could grow on both PDA and MEA. Thus, the majority of OTUs could only be isolated on one type of media.

The only known previously successful biocontrol agent, *Epicoccum* ssp. (OTU9 and OTU10) was only isolated on PDA. *Gaeumannomyces graminis* (OTU22), a known pathogen of barley and wheat (Kwak and Weller 2013), was only isolated on PDA. In contrast, *Ophiosphaerella* spp. (OTU11, OTU12 and OTU3) was isolated on all three

media and has also been reported as a pathogen of a range of grasses. Known pathogens are often found as endophytes within non symptomatic plants (Fisher and Petrini 1992; Sánchez Márquez *et al.* 2007) which highlights the knowledge gap of the functional roles of endophytes and the abiotic as well as biotic cues that might change those roles.

3.4.2 Endophyte community described by direct amplicon sequencing

Endophyte identification from all five sites

Three kingdoms including Chromista, Fungi and Rhizaria were identified as root endophytes of *Elymus repens* by direct amplicon sequencing of roots. Plant associated organisms are found within the Chromista including plant pathogens belonging to the Oomycetes such as *Phytophthora* sp. causing as examples potato late blight (Birch and Whisson 2001) and collar rot of Kauri, *Agathis australis* (Than *et al.* 2013). The kingdom Rhizaria belong to the paraphyletic protists (Cavalier-Smith and Chao 2003) and it was an OTU within the phylum Cercozoa that was identified from two individual plants of *E. repens*. There are several root endophytic and plant pathogenic Cercozoa (Flues *et al.* 2018) including as examples *Plasmodiophora brassicae* causing clubroot in crucifers and *Spongospora subterranea* causing potato powdery scab disease (Niwa *et al.* 2011).

There was a large variation in the OTU richness identified from each root system from the five sites. Interestingly, there was no correlation between the OTU richness determined by direct amplicon sequencing of roots (Figure 3.4) and the number of cultured endophytes isolated from the individual sites (Figure 3.1B and C). Across all sites each plant had an average of 151 OTUs determined by direct amplicon sequencing and an average of 8 isolates were cultured from each plant from a total pool of 715 different OTUs determined by direct amplicon sequencing. Depending on classification, the UNITE database identifies approximately 56 fungal classes of which

the fungal OTUs identified by direct amplicon sequencing from *E. repens* belonged to 31 classes (Table 3.3, https://unite.ut.ee/, Nilsson *et al.* 2018).

A limited number of studies have explored the fungal endophyte community of grass roots using next generation sequencing and de Souza *et al.* (2016) found 8,750 OTUs in the roots of sugarcane with 434 OTUs being part of the core which they defined as being present in 90 % of all plant samples. The study by de Souza *et al.* (2016) is primarily interested in plant development and the changes in the endophyte community. Their plants were grown in the greenhouse but perhaps being a tropical grass is the determining factor in species richness as Arnold and Lutzoni (2007) found that endophyte incidence and diversity increased from arctic to tropical regions. Yu *et al.* (2018) found distinct communities of fungi associated with axial and lateral roots of maize. It is possible that the endophyte communities also differ according to root type and perhaps differences in OTU richness cannot be compared at such a coarse scale as the root system. De Souza *et al.* (2016) does not describe exactly what type of roots they used in their study and it is possible that the OTU richness cannot be compared because different types of roots were used.

It is noteworthy that the number of represented classes within the core community, defined as present in 90 % of all plant samples, equals nine for both the study by de Souza *et al.* (2016) and the present study of endophytes in *Elymus repens.* Seven of these nine classes are shared and include the Dothideomycetes, Eurotiomycetes, Saccharomycetes, Sordariomycetes, Agaricomycetes, Tremellomycetes and the Glomeromycetes. These classes might represent the core fungal classes found within grasses.

Known biocontrol organisms were present as endophytes in *E. repens including Clonostachys rosea* present in site I, II, IV and V, *Trichoderma* spp. present in site I, II, III and IV and *Verticillium* spp. present in all sites. Unfortunately, these fungi were not among the cultured endophytes of site III. Another example of plant beneficial fungi that were not cultured belong to the Glomeromycota. This division include mycorrhizal fungi

of high value to nutrient acquisition and stress tolerance in plants (Chen *et al.* 2018) and arbuscular mycorrhizal fungi have been described to colonise barley, *Elymus repens and* wheat (Wang and Qiu 2006). Arbuscular mycorrhizal fungi are biotrophic and thus, usually identified using spores or direct sequencing (Krüger *et al.* 2009).

Endophyte community from site III and a comparison to the cultured community

The endophyte community identified by direct amplicon sequencing was much richer than the endophytes recorded in the cultured community. A total of 349 OTUs belonging to 21 classes and six divisions was identified from site III using amplicon sequencing (Table 3.3 and Figure 3.4). In comparison, only 27 OTUs from four classes belonging to one division was identified using cultures (Table 3.2). Using direct amplicon sequencing it also became clear that all plants hosted endophytes which were not evident or detectable from the culturing technique alone.

It was hypothesised that the most widespread fungal species would also be the ones that were predominantly cultured. 48 OTUs were identified across all plants of site III using amplicon sequencing and interestingly, only four of these OTUs/species names were shared with the cultured community. The overlapping species included *Ophiosphaerella* sp. and *Periconia sp.* from the Dothideomycetes, *Glarea sp*. belonging to the Leotiomycetes and *Gaeummanomyces graminis* from the Sordariomycetes. *Ophiosphaerella* sp. and *Periconia sp.* were among some of the species cultured relatively frequently however, *Glarea sp*. and *Gaeummanomyces graminis* were only isolated once. An additional six species identifications were shared between the two types of methods and included Dothideomycetes sp. 2 and 3 (OTU3 and OTU4 – possibly Pleosporales sp., Supplement 3.3), Chaetosphaeriaceae sp. (OTU19), *Diaporthe* sp. (OTU20), Lasiosphaeriaceae sp. (OTU23), Sordariomycetes sp. 1 (OTU24 – possibly *Falciphora* sp.) and Xylariaceae sp. (OTU27).

The endophyte that was cultured from all roots *Leptodontidium* sp. (OTU17) is surprisingly not on the list of endophytes found in all plants from site III identified by

direct amplicon sequencing. The identification of OTU17 was not straight forward (Supplement 3.3) and if this OTU had been identified as Helotiales sp. then there would have been a match to the 48 OTUs that were present in all ten plants of site III. Several culturable fungi were found in the amplicon sequencing dataset with examples such as *Alternaria* spp., *Aspergillus* spp., *Trichoderma* spp. and *Verticillium* spp. which were not cultured. This suggests, that the cultured endophyte community is a fraction of what could potentially be cultured. In addition, most of the widespread fungi from direct amplicon sequencing were not recovered.

A limited number of studies have compared the community obtained by direct sequencing with the community obtained by culturing methods in grasses. Herrera *et al.* (2010), found that the cultured community on MEA and PDA was a small subset of the community discovered by direct sequencing from the blue grama grass, *Bouteloua gracilis*. However, Tejesvi *et al.* (2010) did not find any similarities between the cultured community on MEA and PDA, and the directly sequenced community of fungal root endophytes of the wavy hair grass, *Deschampsia flexuosa.* The study of root endophytes of *E. repens* show that the cultured endophytes are both a subset of the total community explored with direct amplicon sequencing and that the majority of cultured endophytes do not overlap with the amplicon dataset. The non-existing overlap for the majority of OTUs could reflect errors in the identification process, however high percent identity scores were used and the same database (UNITE) as well as barcoding region (ITS) were employed. Perhaps the lack of overlap in the two communities is due to the use of different forward primers. For direct amplicon sequencing fITS7 was used whereas ITS1 was used for the cultured communities. It is also possible that the time lapse between querying UNITE about individual sequences made a difference to identification. However, only approximately three months passed between identifying the cultures and the sequenced community. It is therefore most probable that the pattern is real. Jayawardena *et al.* (2018) suggest that the fast growing fraction is cultured and that these fungi might not represent the most

widespread in the community. Some endophytes could be antagonistic to others on isolation media and some could be more sensitive to the surface sterilisation procedure than others.

This study illustrates many of the issues at the core of endophyte discovery. PDA medium recorded the highest species richness but also excluded many rare species while the ITS barcoding region identified most species but also left a lot of cultures unidentified. Only a fraction of those endophytes that could potentially be isolated were cultured and did not represent the most widespread species. Furthermore, large variation in the fungal species richness highlights the high heterogeneity at both plant and site level. Despite the attention received, the field is still some way off in developing a satisfactory methodology with the desired outcomes.

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Supplement 3.1

Table s3.1. Primers used to amplify DNA from fungal endophytes, the primer sequence and the reference.

Locus	Primer	Primer sequence	Reference
ITS	ITS1	5’ TCCGTAGGTGAACCTGCGG 3’	White *et al.* 1990
	ITS4	5’ TCCTCCGCTTATTGATATGC 3’	White *et al.* 1990
LSU	LROR	5’ ACCCGCTGAACTTAAGC 3’	Stielow *et al.* 2015
			Johnson and Vilgalys 1998
	LR5	5’ TCCTGAGGGAAACTTCG 3’	Stielow *et al.* 2015
TEF1α	EF1-983F	5’ ACHGTRCCRATACCACCSATCTT 3’	Rehner and Buckley 2005
	EF1-1567R	5’ GCYCCYGGHCAYCGTGAYTTYAT 3’	Rehner and Buckley 2005

Supplement 3.2

Table s3.2. PCR cycles used for the individual primers. Temperature [˚C] and time [minutes].

Primer	TEF1α		ITS		LSU	
	Temperature	Time	Temperature	Time	Temperature	Time
Premelt	94	1.30	94	1.30	94	1.30
Denature	94	0.45	94	1.30	94	0.30
Anneal	61	1.00	58	1.00	53	1.00
Extension	72	1.00	72	1.00	72	1.00
Final extension	72	7.00	72	7.00	72	7.00
No. cycles	30	-	30	-	30	-

Supplement 3.3

Table s3.3. Taxonomic identification of OTUs from the cultured endophytes from site III using ITS sequences. The sequences were blasted through the UNITE database. In the first section of the table the number of sequences used to form the OTU, the class and the final identification are listed. In the next two sections the top hit, the next best hit as well as the percentage identity and the accession numbers are listed. The next best hit is listed when the top hit was below 99 % identity. The next best hit is defined as the hit that gave more resolution than the top hit. 12 cultures could not be identified because no DNA was extracted and for 27 cultures no ITS sequence was amplified so these cultures were grouped into individual OTUs. Names of organisms are given according to Species Fungorum (http://www.indexfungorum.org/).

OTU	Sequence(s)	Class	Final identification	Top hit	Identity %	Accession number	Next best hit[A]	Identity %	Accession number
1	4	Dothideomycetes	*Clohesyomyces* sp.	*Clohesyomyces* sp.	99	KT269257	-	-	-
2	1		Dothideomycetes sp. 1	Fungi	96	KT203006	*Ophiosphaerella* sp.	96	KT269297
3	8		Dothideomycetes sp. 2	Pleosporales sp.	98	GQ996144	*Camposporium cambrense*	96	KY853428
4	1		Dothideomycetes sp. 3	Pleosporales sp.	79	EU490184	*Phaeosphaeria* sp.	79	EF590323
5	1		Dothideomycetes sp. 4	*Clohesyomyces* sp.	90	JQ435795	*Clohesyomyces* sp.	94	KT269257
6	1		Dothideomycetes sp. 5	Fungi	87	UDB028789	*Abrothallus suecicus*	88	UDB018278
7	1		Dothideomycetes sp. 6	*Clohesyomyces* sp	94	JQ435795	*Clohesyomyces aquaticus*	91	JX276948
8	15		Dothideomycetes sp. 7	Fungi	99	KT203006 KC007324 KT269297	*Ophiosphaerella* sp.	99	KT269297
9	6		*Epicoccum nigrum*	*Epicoccum nigrum*	100	KX426949 MF509753	-	-	-
10	1		*Epicoccum* sp.	*Epicoccum* sp.	99	MF788189	-	-	-
11	2		*Ophiosphaerella korrea*	*Ophiosphaerella korrea*	99	AF486626	-	-	-

[A] This column represents the next best identification that gives a better resolution compared to the top hit which had a lower identity than 99 %. Sometimes the identity percentage is the same or higher than in the top hit column because the sequences are scored on multiple criteria, not listed here, which also influences their ranking. As an example, if the top hit is an order then a lower rank is listed.

12	1		*Ophiosphaerella* sp. 1	*Ophiosphaerella* sp	99	KT692575	-	-	-
13	4		*Periconia* sp. 1	*Periconia* sp.	99	HG936263	-	-	-
14	1		*Periconia* sp. 2	*Periconia* sp.	99	KT269450	-	-	-
15	1		Pleosporaceae sp.	Pleosporaceae sp.	100	EU754971	-	-	-
16	2	Leotiomycetes	*Glarea* sp.	*Glarea* sp.	99	KT268823	-	-	-
17	34		*Leptodontidium* sp.	*Leptodontidium* sp. (17)[B]	100	KU886584	*Cadophora* sp.	100	KT269226
				Cadophora orchidicola (1)	99	KX440156	*Leptodontidium* sp.	99	KF428333
				Lasiosphaeriaceae sp. (7)	100	KY430565	*Cadophora* sp.	100	KT269262
				Helotiales sp. (9)	100	HG936155	Lasiosphaeriaceae sp.	99	KY430518
18	2	Pezizomycetes	*Pyronema domesticum*	*Pyronema domesticum*	100	HQ115722	-	-	-
19	7	Sordariomycetes	Chaetosphaeriaceae sp.	Chaetosphaeriaceae sp.	99	GU327452	-	-	-
20	1		*Diaporthe sp.*	*Diaporthe sp.*	100	KP984752	-	-	-
21	2		*Falciphora* sp.	*Falciphora* sp.	99	HG937137	-	-	-
22	1		*Gaeumannomyces graminis*	*Gaeumannomyces graminis*	99	KT819302	-	-	-
23	2		Lasiosphaeriaceae sp.	Lasiosphaeriaceae sp.	100	KY430501 KY430565	-	-	-
24	1		Sordariomycetes sp. 1	*Falciphora* sp.	98	KX306546	*Falciphora oryzae*	94	EU63699
25	1		Sordariomycetes sp. 2	*Dictyochaetopsis gonytrichoides*	97	AF178556	*Dictyochaeta simplex*	95	AF178559
26	2		Sordariomycetes sp. 3	_[C]	-	-	-	-	-
27	1		Xylariaceae sp.	Xylariaceae sp.	100	KX067819	-	-	-

[B] In brackets are the number of sequences that contributed to the identification. The majority of sequences (27 out of 34) put this OTU in the Leotiomycetes and the majority was followed for the final naming of this OTU.
[C] The ITS sequences forming this OTU were contaminated with *Penicillium sp.* therefor the LSU sequences were trusted and they grouped this OTU to the Sordariomycetes.

28-54	27[D]	Fungus sp.		
55-66	12[E]	Fungus sp.		

[D] For 27 cultures DNA was extracted but not amplified using the ITS primers.
[E] For 12 cultures DNA was not successfully extracted.

Supplement 3.4

Code for bioinformatics analysis of demultiplexed paired-end reads in Qiime2.

```
# Directory overview, folders are in bold
      # 2NGS
            #data
                  # 00.RawData
                  # manifest_raw
                  # Elymus_features
                  # UNITE_classifier


# Activate qiime
source activate qiime2-2018.6

# Import data into Qiime2
qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path manifest_raw \
  --output-path paired-end-demux.qza \
  --source-format PairedEndFastqManifestPhred33

# Generate summary of demultiplexed data, determine how many
# sequences were obtained per sample, and also get a summary
# of the distribution of sequence qualities at each position in
# your sequence data
qiime demux summarize \
  --i-data paired-end-demux.qza \
  --o-visualization paired-end-demux.qzv

# Remove the primer sequence from the raw sequences
qiime cutadapt trim-paired \
  --i-demultiplexed-sequences paired-end-demux.qza \
  --p-front-f GTGARTCATCGAATCTTTG \
  --p-front-r TCCTCCGCTTATTGATATGC \
  --p-error-rate 0.1 \
  --p-cores 6 \
  --o-trimmed-sequences trimmed-seqs.qza

# Dada2: denoising, trimming, chimera removal
qiime dada2 denoise-paired \
  --i-demultiplexed-seqs trimmed-seqs.qza \
  --p-trim-left-f 0 \
  --p-trim-left-r 0 \
  --p-trunc-len-f 222 \
  --p-trunc-len-r 220 \
  --o-table table_trimdada.qza \
  --o-representative-sequences rep-seqs_trimdada.qza \
  --o-denoising-stats denoising-stats_trimdada.qza

# Create FeatureTable and FeatureData summaries
qiime feature-table summarize \
  --i-table table_trimdada.qza \
  --o-visualization table_trimdada.qzv \
```

```
# Created a folder, Elymus_features, with the following plant
ITS
# fasta files:

# Elymus_5.8S.fasta FJ793076.1
# Elymus_ITS1.fasta FJ793076.1
# Elymus_ITS2.fasta FJ793076.1
# Elymus_antiquus.fasta AY740818.1
# Elymus_caninus.fasta AY740897.1
# Elymus_fullsequence.fasta FJ793076.1
# Elymus_lanceolatus.fasta EF396961.1
# Elymus_sibiricus.fasta EF396962.1
# Elymus_tauri.fasta EF014244.1
# Elymus_tauri_short.fasta EU617238.1
# Agrostis_gigantea KY872905.1
# Agrostis capillaris KX872899.1
# Agrostis stolonifera KX872911.1
# Agrostis hallii  KX872907.1
# Elymus tangutorum KF905148.1
# Elymus repens FJ793087.1
# Hordeum brachyantherum MG215969.1
# Elymus atratus KJ526331.1
# Kengyilia gobicola JF976721.1
# Agropyron krylovianum KJ561240.1
# Hordeum roshevitzii KU513502.1
# Elymus_virginicus.fasta MG215649.1
# Elymus_glaberrimus.fasta AY740844.1
# Elymus_stipifolia.fasta EU617049.1
# Prunus_domestica.fasta KX166465.1
# Prunus_spinosa.fasta KX167489.1
# Prunus_bokhariensis.fasta GQ179665.1
# Prunus_armeniaca.fasta JF978104.1
# Prunus_sibirica.fasta AF318739.1
# Elymus_nutansxkengyilia.fasta JQ670990.1
# Trebouxia_impressa.fasta KX181276.1

# Combined the plant ITS sequences using cat
cat Elymus_5.8S.fasta Elymus_ITS1.fasta Elymus_ITS2.fasta
Elymus_antiquus.fasta Elymus_caninus.fasta
Elymus_fullsequence.fasta Elymus_lanceolatus.fasta
Elymus_sibiricus.fasta Elymus_tauri.fasta
Elymus_tauri_short.fasta Agrostis_gigantea.fasta
Agrostis_capillairs.fasta Agrostis_stolonifera.fasta
Agrostis_hallii.fasta Elymus_tangutorum.fasta
Elymus_repens.fasta Hordeum_brachyantherum.fasta
Elymus_atratus.fasta Kengyilia_gobicola.fasta
Agropyron_krylovianum.fasta Hordeum_roshevitzii.fasta
Elymus_virginicus.fasta Elymus_glaberrimus.fasta
Elymus_stipifolia.fasta Prunus_domestica.fasta
Prunus_spinosa.fasta Prunus_bokhariensis.fasta
Prunus_armeniaca.fasta Prunus_sibirica.fasta
Elymus_nutansxkengyilia.fasta Trebouxia_impressa.fasta >
Plant_features.fasta
```

```
# Imported the combined Plant_features into Qiime2 as .qza
qiime tools import \
  --input-path Plant_features.fasta \
  --output-path Plant_features.qza \
  --type 'FeatureData[Sequence]'

# Excluded different plant sequences with 95 % identity to
sequences # within Plant_features.qza
# Removed anything that has a match with at least 95 % identity
over at least 95 % of the sequence length

qiime quality-control exclude-seqs \
  --i-query-sequences rep-seqs_trimdada.qza \
  --i-reference-sequences Elymus_sequences/Plant_features.qza \
  --p-method blast \
  --p-perc-identity 0.95 \
  --p-perc-query-aligned 0.95 \
  --o-sequence-hits hits95_trimdada.qza \
  --o-sequence-misses misses95_trimdada.qza

qiime feature-table filter-features \
  --i-table table_trimdada.qza \
  --m-metadata-file hits95_trimdada.qza \
  --o-filtered-table no-Plant95_trimdada-table.qza \
  --p-exclude-ids

#Visualise no-Plant table
qiime feature-table summarize \
  --i-table no-Plant95_trimdada-table.qza \
  --o-visualization no-Plant95_trimdada-table.qzv \
  --m-sample-metadata-file metadata2019-2.txt

# List of blastable miss sequences
qiime feature-table tabulate-seqs \
  --i-data misses95_trimdada.qza \
  --o-visualization misses95_trimdada.qzv

# Classifier training (tutorial
https://github.com/gregcaporaso/2017.06.23-q2-fungal-tutorial)
# Obtaining and importing reference data sets (from UNITE) to
the
# folder UNITE_classifier
https://files.plutof.ut.ee/doi/0A/0B/0A0B25526F599E87A1E8D7C612D
23AF7205F0239978CBD9C491767A0C1D237CC.zip
# importet files from the developer folder
      # sh_refs_qiime_ver7_97_01.12.2017_dev.fasta
      # sh_refs_qiime_ver7_99_01.12.2017_dev.fasta
      # sh_taxonomy_qiime_ver7_97_01.12.2017_dev.txt
      # sh_ taxonomy _qiime_ver7_99_01.12.2017_dev.txt
      # sh_refs_qiime_ver7_dynamic_01.12.2017_dev.fasta
      # sh_ taxonomy _qiime_ver7_dynamic_01.12.2017_dev.txt

# qiime tools import \
 --type FeatureData[Sequence] \
 --input-path sh_refs_qiime_ver7_99_01.12.2017_dev.fasta \
 --output-path unite-ver7-99-seqs-01.12.2017_dev.qza
```

```
# Train classifier
qiime feature-classifier fit-classifier-naive-bayes \
 --i-reference-reads unite-dynamic-seqs.qza \
 --i-reference-taxonomy unite-dynamic-reftax.qza \
 --o-classifier unite-dynamic-classifier.qza

# Taxonomy at 95 %
# Renaming two files
mv misses95_trimdada.qza rep-seqs95_trimdada.qza
mv no-Plant95_trimdada-table.qza table95_trimdada.qza

# Using classifier for taxonomic analysis
qiime feature-classifier classify-sklearn \
  --i-classifier UNITE_classifier/unite-dynamic-classifier.qza \
  --i-reads rep-seqs95_trimdada.qza \
  --o-classification taxonomy95_trimdada.qza

qiime metadata tabulate \
  --m-input-file taxonomy95_trimdada.qza \
  --o-visualization taxonomy95_trimdada.qzv

qiime taxa barplot \
  --i-table table95_trimdada.qza \
  --i-taxonomy taxonomy95_trimdada.qza \
  --m-metadata-file metadata2019-2.txt \
  --o-visualization taxa-bar-plots95_trimdada.qzv
```

Chapter 4

Biological control of *Fusarium culmorum* and *Pyrenophora teres* in barley using fungal endophytes isolated from *Elymus repens,* a wild relative of barley

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Abstract

Twenty four fungal endophytes isolated from a wild relative of barley, *Elymus repens,* were screened in barley against foot and root rot caused by *Fusarium culmorum* and net blotch caused by *Pyrenophora teres*, under controlled conditions. In all experiments the endophytes were applied individually as seed dressings. Treatment with *Periconia macrospinosa* E1 significantly ($P \leq 0.05$) reduced disease symptoms in two out of four experiments. Furthermore, a *gfp* transformed *Periconia macrospinosa* E1 isolate was present on roots 14 days after sowing. A subset of endophytes consisting of eight isolates was also tested against the leaf pathogen *Pyrenophora teres.* Seed treatment with Lasiosphaeriaceae sp. E10 reduced net blotch symptoms significantly, but only in one out of three experiments. Often a change in fungal lifestyle is reported in relation to changes in host genotype as well as biotic and abiotic conditions. However, here endophytic strains from *E. repens* appeared to remain neutral and in a few cases antagonistic when changing the setting to a barley host attacked by fungal pathogens in a controlled environment.

Keywords: barley, biological control, *Elymus repens,* endophyte, *Fusarium culmorum, Periconia*, *Pyrenophora teres*

4.1 Introduction

Barley is the fourth most produced cereal crop in the world (FAOSTAT 2018) and barley diseases result in great yield losses. Some of the most important diseases in Northern Europe include net blotch (*Pyrenophora teres*, Walters *et al.* 2012; Oldach 2018), which causes symptoms on leaves and kernels (Mathre 1982) and Fusarium head blight (*Fusarium* spp.), which reduces yield and quality of malt when producing beer and whiskey (Oliveira *et al.* 2012; Nielsen *et al.* 2014; Potterton and McCabe 2018) with early symptoms occurring in roots and late symptoms occurring in the heads (Mathre 1982; Scherm *et al.* 2013).

Conventional agriculture relies on chemical inputs to control diseases, but organisms infecting leaves are becoming tolerant to the fungicide treatments used (Walters *et al.* 2012) and soil-borne diseases are particularly challenging to target with chemical control (Haas & Défago 2005). In addition, it is anticipated that the use of some agrochemicals will be banned or restricted in the future (Oldach 2018) and furthermore, the European Union is promoting the sustainable use of pesticides and integrated pest management as part of their “Directive 2009/128/EC” (European Parliament 2009; Department of Agriculture 2013). Farmers need alternatives to chemical control and therefore there is growing interest in using microorganisms as biological control agents of plant diseases (Broadfoot 2016).

A constraint in the application of microorganisms for biological control is that they may have difficulties in persisting and/or remaining active when they are applied to the leaves, the seeds or the soil (Walker *et al.* 2002; Ting *et al.* 2009; Buddrus-Schiemann *et al.* 2010). In contrast, endophytes, which are microorganisms living inside plants without causing symptoms of disease (Wilson 1995), are potentially favourable because the plant can protect the microorganism (Eevers *et al.* 2015).

When protected within the plant, endophytes have the potential to provide control of several stresses without losing efficacy over the growing season. As an example, *Epichloë* spp. of forage grasses have been shown to provide both abiotic and biotic stress relief and the vertical transmission affirms the mutual beneficial symbiosis (Bush *et al.* 1997; di Menna *et al.* 2012). Some endophytes have been shown to protect plants against disease (Maciá-Vicente *et al.* 2009; Wicaksono *et al.* 2017; Vinayarani and Prakash 2018) and the use of endophytes against barley diseases was reviewed by Høyer *et al.* (2019).

The objective of this study was to screen fungal root endophytes isolated from *Elymus repens* for their potential to control the foot and root rot pathogen *Fusarium culmorum* and the net blotch pathogen *Pyrenophora teres.* It was hypothesised that *E. repens* as a perennial wild relative of barley, would host endophytes that would be compatible for use in barley.

4.2 Materials and methods

4.2.1 Plant and fungal material

The spring barley (*Hordeum vulgare*) cultivar Chapeau was grown in a growth chamber under the following conditions: cycles of 16 h of light (Philips Master IL-D 36 w/865, France, 200 µmol m^{-2} s^{-1}) and 8 h of darkness. Day and night temperatures were maintained at 20 °C (60 % relative humidity) and 15 °C (80 % relative humidity), respectively.

The pathogens used were *Fusarium culmorum* strain 5 and *Pyrenophora teres* strain CP2189. Twenty four endophytes were previously isolated from roots of *Elymus repens* (Table 4.1, for details see Chapter 3). The endophytes came from plants sampled from a field margin sown with wild flowers at Kildalton Agricultural College, Kilkenny, Ireland (52.35636N; -7.31603W). The field had a history of high disease pressure from Fusarium infection (Hyland 2016). The biocontrol candidates were picked from a large culture collection based on their ability to sporulate and their

difference in morphology. The endophytes were grown on potato dextrose agar (PDA) or malt extract agar (MEA) according to which medium they were originally isolated on (Table 4.1). Single spore cultures were prepared and stored as follows. Under sterile conditions, 10 µL autoclaved ultrapure (MiliQ) water was added to an empty Petri dish. With a sterile scalpel, 4 mm^2 of a growing fungal culture was scraped and comminuted in the 10 µL of water. With a loop, the fungal material was smeared out on two water agar plates. The plates were checked under the microscope for germinating spores the following day. Germinating spores were picked out using a sterile needle prepared from a small capillary tube (Pyrex 1.3-1.5 mm x 100 mm). The single spore plug was placed on the type of medium that the original culture grew from. When the single spore culture had grown in size, plugs were frozen in 10 % glycerol at -80 °C for storage.

Endophyte identification was based on morphology and DNA sequences using three different barcoding loci. Cultures were examined for identifiable characters such as conidiophores and spores. If suitable spores were present, the length, width and additional features were measured for 100 individual spores using an Olympus BX60 light microscope. DNA was extracted from each fungal culture and ITS (internal transcribed spacer 1 and 2 of nuclear ribosomal DNA), LSU (large subunit of nuclear ribosomal DNA) and TEF (transcription elongation factor 1) was amplified (see Supplement 4.1 and Supplement 4.2), purified using ExoSAP-IT™ (Thermo Fisher Scientific) and sequenced using automated Big Dye terminator Sanger sequencing (by Macrogen Inc.). Neighbour-joining trees based on p-distance were made for each barcoding region using the software MEGA7: molecular evolutionary genetics analysis across computing platforms (Kumar *et al.* 2018). First trees were built with all sequences and afterwards individual trees were built for each class of fungi separately to examine if the same number of OTUs would be determined. The sequences were clustered into OTUs using 99 % sequence similarity. Consensus clusters were prepared by comparing the groups assigned by the different phylogenetic trees. If there was incongruence between OTUs among the gene regions used, the OTU determined

by the most discriminating gene was chosen (best coverage and highest variability). To assign a name to the OTU clusters all sequences were put through NCBI standard nucleotide blast (https://www.ncbi.nlm.nih.gov/) and the UNITE database (https://unite.ut.ee/, Nilsson *et al.* 2018). When there were discrepancies between the best hit of the different barcoding regions the following steps were taken to allocate the taxonomic name and manage incongruence: 1) evaluate the quality of the sequence, 2) compare levels of percentage identity (only 99-100 % was accepted), 3) compare spore morphology where possible and 4) give priority to the barcode hit determined by the most discriminating gene.

4.2.2 Endophyte and pathogen inoculation

Inoculation of seeds with endophyte

A total of nine experiments were conducted. Sporulating cultures of endophytes were harvested by adding 5 mL deionised water to each Petri dish and the suspension was filtered through a layer of cheese cloth. The spore concentration was adjusted to 10^7 spores/mL. In all the experiments, seeds were soaked in the endophyte spore suspension (1:2 w/v) except in one experiment (exp. 5) where the spore suspension was (1:1 w/v). The seeds were placed for 10 min on a shaker at 130 rpm, except in one experiment (exp. 9) where the seeds were shaken for 24 h in the spore suspension. The seeds were dried for 30 min on filter paper in a laminar flow cabinet for all experiments except two where seeds where dried for 2 hours (exp. 9) and where seeds were dried overnight (exp. 6). Soaking in deionised water was used as a control treatment.

Inoculation of seeds with Fusarium culmorum

Fusarium culmorum was grown on PDA for 14-21 days. Spores were harvested as described for endophyte inoculation and the spore concentration adjusted to 1.5×10^6 spores/mL. Seeds were inoculated by soaking in the spore suspension (1:1 w/v) for 30

min on a shaker at 130 rpm. In all experiments seeds were dried for 24 h on filter paper in a laminar flow bench except for one experiment were seeds were dried for 1 h (exp. 6).

Inoculation of leaves with Pyrenophora teres

Pyrenophora teres was grown on grass agar (filtrate of 32.5 g/L of boiled clover-rich grass fodder pills for cattle and 20 g/L agar, Jørgensen *et al.* 1996) for 14 days. Spores were harvested by adding 5 mL deionised water to each Petri dish and the suspension was poured into a 50 mL tube and shaken vigorously by hand. Subsequently, the suspension was filtered through a layer of cheese cloth. The spore concentration was adjusted to 10^3 spores/mL. The suspension was sprayed onto the adaxial side of the leaves until run-off using a glass hand sprayer.

4.2.3 Sand assay to evaluate the efficacy of endophyte seed coating against seed-borne *Fusarium culmorum*

The protocol of Jensen *et al.* (2000) was followed. All treatments consisted of two seed dressing steps as shown in Table 4.2. The pathogen treatment was added before the endophyte treatment to mimic the seed borne transmission of *Fusarium culmorum.* A total of six experiments were conducted and each experiment had a water treatment, a *F. culmorum* treatment and 3-8 treatments with *F. culmorum* + endophyte, dependent on the experiment (Figure 4.2, Figure 4.3).

Sowing and experimental design

Sand (grain size 0.4-0.8 mm) and tap water was mixed (3:1 v/v) and filled into strips of four plastic pots (Figure 4.1B). A template was used to make 1 cm deep and 2 cm in diameter wide holes in the sand. Three seeds were sown per hole and then covered with moist sand. There were four replications per treatment and each replication

consisted of 12 seeds. The experiment was conducted using a fully randomized block design. Each strip of four plastic pots was placed in a saucer. Strips and saucers were placed in trays that were covered with a plastic bag in order to keep the humidity high. After 8 days, the seedlings were watered in the saucer with 50 mL of fertilizer solution (Pioner Brun, NPK 14-2-23, adjusted to pH = 6.3 with nitric acid, Azelis, Denmark) and otherwise watered every 3rd day with tap water.

Disease index and disease incidence

Disease symptoms of foot and root rot were scored at 14 days after sowing. The roots were washed free of sand and the disease severity was scored using a scale from 0 to 4 described by Knudsen *et al.* (1995) where: 0: healthy plant; 1: slightly brown root and/or coleoptile; 2: moderately brown root and coleoptile; 3: severe browning of coleoptile; 4: dead plant (Figure 4.1A). A disease index was calculated using the following equation where non-germinated seeds that were red and very soft were also scored as a category 4.

$$\text{Disease index} = \frac{(0 \text{ scored plants} \times 0 + 1 \text{ scored plants} \times 1 + 2 \text{ scored plants} \times 2 + 3 \text{ scored plants} \times 3 + 4 \text{ scored plants and non-germinated seeds} \times 4)}{\text{Total number of plants}}$$

Disease incidence was evaluated on the same plants used for the disease index. Plants were scored as either healthy (corresponding to disease index 0) or sick (corresponding to disease index 1-4).

Table 4.1. List of endophytes (E1-E24) tested as biological control agents in nine different experiments against the two pathogens Fusarium culmorum and Pyrenophora teres. “Plant” indicates which host plants the endophytes were originally isolated from (1-10). The medium from which endophytes were originally isolated from and the taxonomic identification, according to morphology and DNA barcoding, of the species are also given. Names of fungi follow Species Fungorum (www.indexfungorum.org).

Endophytes				Experiments								
				Fusarium culmorum						*Pyrenophora teres*		
Label	**Identification**	**Plant**[a]	**Medium**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
E1	*Periconia macrospinosa*	1	MEA	X			X	X	X		X	X
E2	*Periconia macrospinosa*	1	MEA	X								
E3	*Slopeiomyces cylindrosporus*	2	MEA	X								
E4	*Epicoccum nigrum*	2	PDA	X			X	X			X	X
E5	*Leptodontidium* sp.	3	PDA	X								
E6	*Slopeiomyces cylindrosporus*	6	PDA	X								
E7	*Leptodontidium* sp.	6	PDA	X			X					
E8	*Epicoccum* sp.	7	PDA	X								
E9	*Periconia* sp.	2	PDA		X					X		
E10	Lasiosphaeriaceae sp.	3	PDA		X			X		X	X	X
E11	*Leptodontidium* sp.	3	PDA		X					X		
E12	*Leptodontidium* sp.	6	MEA		X							
E13	Lindgomycetaceae sp.	7	MEA		X		X			X		
E14	Chaetosphaeriaceae sp.	10	PDA		X							
E15	*Dictyochaeta siamensis*	10	MEA		X					X		
E16	*Leptodontidium* sp.	10	MEA		X					X		
E17	*Diaporthe sp.*	4	MEA			X						
E18	*Slopeiomyces cylindrosporus*	4	PDA			X						
E19	*Mycochaetophora* sp.	4	PDA			X						
E20	*Leptodontidium* sp.	6	MEA			X						
E21	Unidentified	9	MEA			X						
E22	*Clohesyomyces aquaticus*	9	MEA			X						
E23	*Ophiosphaerella* sp.	9	MEA			X						
E24	*Dictyochaeta siamensis*	7	MEA			X						
E1gfp10	*Periconia macrospinosa* gfp10	-	-						X			

[3] Endophytes were isolated from ten different plants and the plants were given a number from 1-10. As an example the endophytes E3, E4 and E9 were all isolated from *Elymus repens* plant number 2 (Chapter 3).

Table 4.2. Overview of the two seed dressing steps for the treatments used in the experiments with *Fusarium culmorum*.

Name of treatment	First dressing	Second dressing
Water	Deionised water	Deionised water
F. culmorum	*F. culmorum*	Deionised water
F. c + E(number)	*F. culmorum*	Endophyte (number)

4.2.4 Root colonisation by *Periconia macrospinosa* E1 – transformed with GFP

Agrobacterium tumefaciens mediated transformation with GFP

The protocol of Mullins *et al.* (2001) was essentially followed for the *Agrobacterium* mediated transformation. The strain E1 was transformed using the *A. tumefaciens* strain AGL1 containing the GFP transformation plasmid pPZP201-GG-BH. The promoter region of the *Blumeria graminis gpd* (glyceraldehyde-3-phosphate dehydrogenase) gene regulates the *gfp* gene and the *B. graminis* β-tubulin promoter region regulates the *hph* gene encoding hygromycin B resistance.

Fungal tolerance to hygromycin B was tested on PDA supplemented with hygromycin B in the following concentrations 0, 50, 100, 150, 200, 250 and 300 µg/mL. Bacteria containing the GFP plasmid were grown on LB plates with half salt concentration and 0.1 mg/mL ampicillin, 0.05 mg/mL kanamycin and 0.05 mg/mL rifampicin for 2 days at room temperature. *Agrobacterium* cultures were transferred into an autoclaved glass tube with screw cap containing 10 mL half salt LB media and 0.1 mg/mL ampicillin, 0.05 mg/mL kanamycin and 0.05 mg/mL rifampicin, and incubated tilted for 24 h at 28 °C at 220 rpm. A culture solution OD660 of 0.45 was measured in a 1:10 (v/v) dilution with MiliQ water and the standard blank reference used was diluted LB medium with half salt concentration and 0.1 mg/mL ampicillin, 0.05 mg/mL kanamycin and 0.05 mg/mL rifampicin. 5 mL *Agrobacterium* culture was spun down at 1449 x *g* for 3 min. The pellet was resuspended in 10 mL induction medium (IM:

composition see Supplement 4.3) with 200 µM acetosyringone and incubated for 3 h at 28 °C.

Spores from endophytes were harvested in IM media and spore suspensions were filtered through cheese cloth. The spore concentration was adjusted to 2 x 10^6 spores/mL. Four sterile filter papers (Black round 7.5 cm diameter filters, Frisinette Aps) were cut into 16 pieces. Four pieces were placed on each plate of CM agar (same as IM, but instead of 10 mM glucose, 5 mM glucose and 15 g Bacto agar was used) supplemented with 200 µM acetosyringone. Equal volumes of *Agrobacterium* and fungal spore solutions were mixed. 25 µL of the mix was pipetted out on to the individual filters and spread over them with a spatula. Plates were incubated at 25 °C for two days with the lid side up. Individual filters were transferred to Czapek Dox agar supplemented with 100 µg/mL hygromycin B and 95 µg/mL cefoxitin. Plates were incubated at 25 °C for 4-8 days. Fungal cultures that grew successfully from the filters into the medium were selected. A volume of 10-15 µL MiliQ water was pipetted up and down on the part of the fungal culture growing on the medium. The spore suspension was spread with a Drigalski spatula on PDA plates containing 100 µg/mL hygromycin B and 95 µg/mL cefoxitin. Plates were left to incubate at 25 °C for 2-3 days. Individual germinating spores were transferred to PDA with 100 µg/mL hygromycin B.

Confirmation of the transformation

The transformation was checked under a fluorescence microscope (Olympus BX60) using excitation filters for GFP (excitation 455-495 nm, dicroitic mirror DM 505, barrier filter 510-555 nm). Seven individual transformants were prepared and plugs stored in 10 % glycerol at -80 °C. Three replications were made of the wildtype and the seven transformants and their growth was measured at three time points, at 5, 7 and 9 days. Furthermore, the intensity of their fluorescence emission was evaluated on a scale from 1-5.

Root colonisation experiment

Experiment 6 was set up in order to examine whether the transformed isolate of E1 would behave as the wildtype and in order to follow the root colonisation. The transformed *Periconia* macrospinosa isolate E1gfp10 was tested in a *Fusarium* assay with a total of six treatments including "water", "*F. culmorum*", "E1", "E1gfp10", "*F. culmorum* + E1" and "*F. culmorum* + E1gfp10". Colonisation was checked under the fluorescence microscope before germination and on the third day after sowing. Furthermore, 14 days after sowing, four root pieces from plants treated with *F. culmorum,* E1 gfp10 and *F. culmorum* + E1 gfp10 were washed in tap water and placed on 1/5 PDA to check the colonisation.

4.2.5 Net blotch assay to evaluate the efficacy of endophyte seed coating against *Pyrenophora teres*

For the net blotch experiments, the protocol prepared by Jørgensen *et al.* (1996) was followed with a few modifications. Thus, the plants were grown in the growth chamber and the endophyte antagonist was coated on the seeds. First, the seeds were dressed in endophyte spore suspension or water and sown. After 14 days, leaves were sprayed with the pathogen inoculum. A total of three experiments were conducted (Figure 4.6).

Sowing and experimental design

Rectangular pots (11.5 x 10 cm) were filled with Pindstup potting mix (Pindstrup Substrate no. 2, Pindstrup Mosebrug, Ryomgård, Denmark) and 10 seeds were sown in a row at a distance of 1/3 of the shortest side of the pot. There were four replications per treatment and each replication consisted of 10 seeds. 24 h before pathogen inoculation, the 14 day old plants had their second leaf mounted horizontally on a bent plastic plate with the adaxial side up, using two unbleached cotton strings (Figure 4.1C). The fixed leaves were inoculated with *Pyrenophora teres* and pots were placed

in trays covered with plastic bags. Trays were kept in the dark overnight and plastic bags were opened the following day. The experiments were conducted with a fully randomized design.

Disease scoring

Seven days after pathogen inoculation, images of the fixed leaves were recorded and percent lesion area was scored using the software Assess 2.0 Image Analysis Software for Plant Disease Quantification (https://my.apsnet.org/ItemDetail?i ProductCode=43696m5, American Phytopathological Society).

4.2.6 Statistical analyses

The data was analysed with the software package R i386 3.4.3 (https://cran.r-project.org/bin/windows/base/old/3.4.3/). Linear and logistical models with the appropriate random effects of “pot” and “plant” were fitted and tested against each other using ANOVA. The models were validated by checking the assumption that the observed data followed a normal distribution and that the residuals were homogenous and independent. Pairwise comparisons were made for all experiments and in addition in experiment 6 Tukey’s range test was used. All comparisons were adjusted using Bonferroni adjusted *P*-values at the significance level $P \leq 0.05$. All histograms represent means of raw data from individual experiments and error bars show the standard error of the mean (SEM). Treatments that were significantly different to the “*F. culmorum*” treated plants in the *Fusarium* assays or the “water” treatment in the net blotch assays were given an asterisk. In experiment 6, all treatments were compared to each other and given letter codes to indicate dissimilarities.

Figure 4.1. Disease symptoms in barley from *Fusarium culmorum* and *Pyrenophora teres*. A) Disease symptoms on a scale from 0-4 caused by *F. culmorum* in barley. B) Experimental set up of the sand assay screening candidates against *F. culmorum* showing seven day old plants. C) Fixed leaves of three week old plants showing symptoms of *P. teres*.

4.3 Results

4.3.1 Sand assay to evaluate the efficacy of endophyte seed coating against seed-borne *Fusarium culmorum*

The ability of 24 endophytes to control *F. culmorum* was tested in a sand assay (exp. 1-3) and three endophyte treatments were found to significantly reduce disease index, namely *Periconia macrospinosa* E1, *Epicoccum nigrum* E4 and *Slopeiomyces cylindrosporus* E18 (Figure 4.2). When a subset of treatments were repeated (exp. 4-6), *P. macrospinosa* E1 was able to reduce disease symptoms in experiment 4 but not in experiments 5 and 6. However, in experiment 6, it was evident, that treatment with *P. macrospinosa* E1 or E1gfp10 alone was not making disease symptoms worse

compared to the *F. culmorum* treated plants. Treatment with *Epicoccum nigrum* E4 had no effect in the experiments 4 and 5 (Figure 4.2).

The initial screening (Figure 4.3, exp. 1-3) resulted in the recording of five endophyte treatments that could significantly reduce incidence of disease, namely *Periconia macrospinosa* E1 and E2, *Epicoccum nigrum* E4*, Leptodontidium* sp. E7 and *Slopeiomyces cylindrosporus* E18. Interestingly, E1 and E2 were both isolates of *P. macrospinosa* cultured from the same plant (Table 4.1). However, when a subset of treatments was repeated there were no significant differences between endophyte treatments and the *F. culmorum* only control (exp. 4-6).

4.3.2 Root colonisation by *Periconia macrospinosa* E1 – transformed with GFP

Periconia macrospinosa E1 was successfully transformed with GFP. The growth rates of the transformed strains were not different to the wildtype (data not shown). The intensity of the emission was evaluated on a subjective scale from 0-5 for seven strains of E1gfp and, based on the intensity, E1gfp10 was chosen for the root colonisation experiment (Figure 4.4 and Figure 4.5A-F).

Periconia macrospinosa E1gfp10 was present on roots 14 days after sowing (Figure 4.5H) and this was confirmed in culture under fluorescent light. E1gfp10 was present on 23 out of 24 root pieces. However, colonies of *P. macrospinosa* E1gfp10 were not present in Petri dishes when competing against *F. culmorum* (Figure 4.5I).

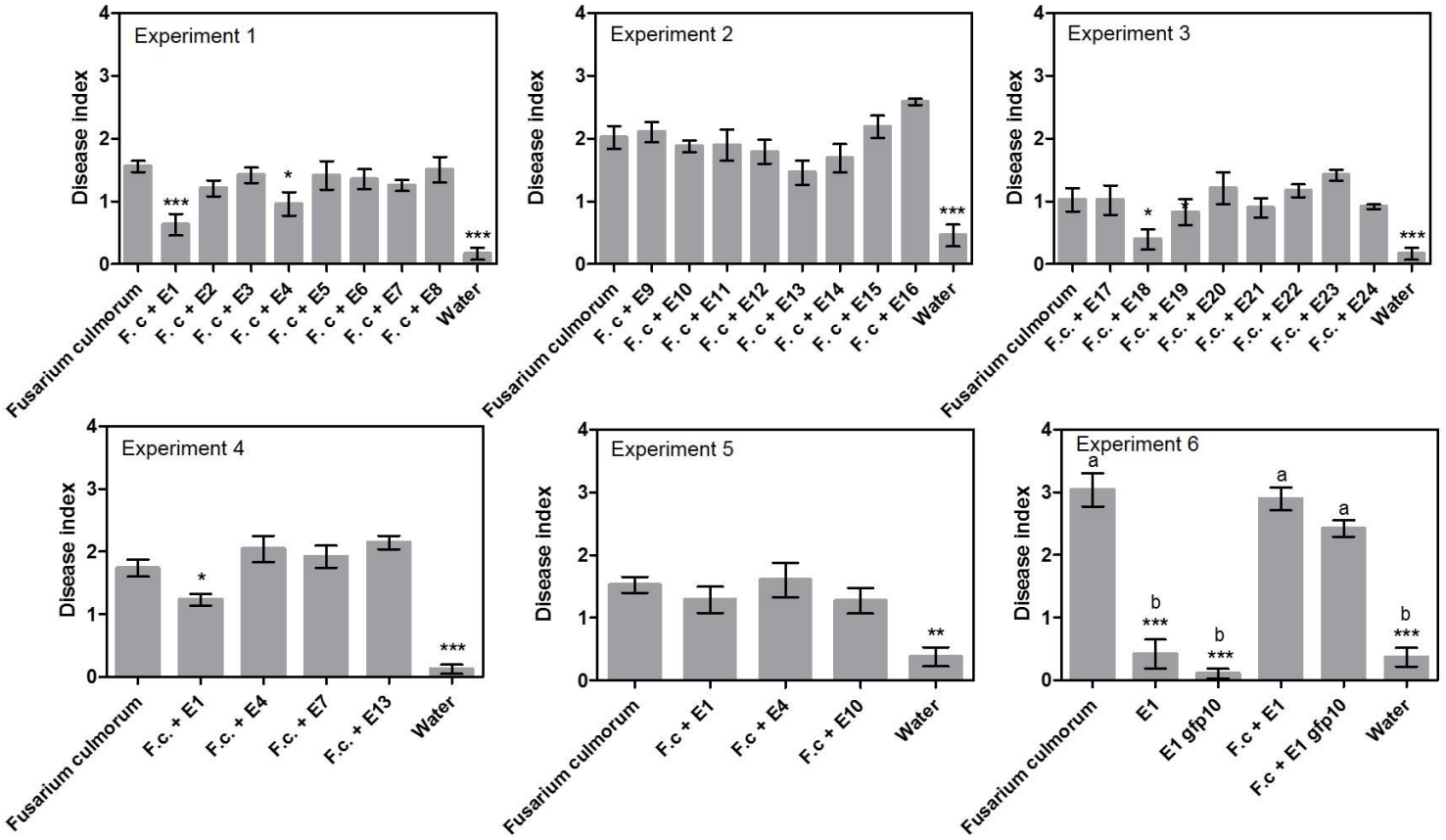

Figure 4.2. Disease index results from the sand assays testing 24 different endophytes against seed-borne *Fusarium culmorum*. Disease index is given +/- SEM. Each column represents the mean of 48 plants. Significant differences are compared to the "*Fusarium culmorum*" treated plants and are shown with asterisks ('*' $0.01 < P \leq 0.05$, '**' $0.001 < P \leq 0.01$, '***' $P \leq 0.001$). In experiment 6, all treatments were compared to each other and given letters to indicate dissimilarities ($P \leq 0.001$).

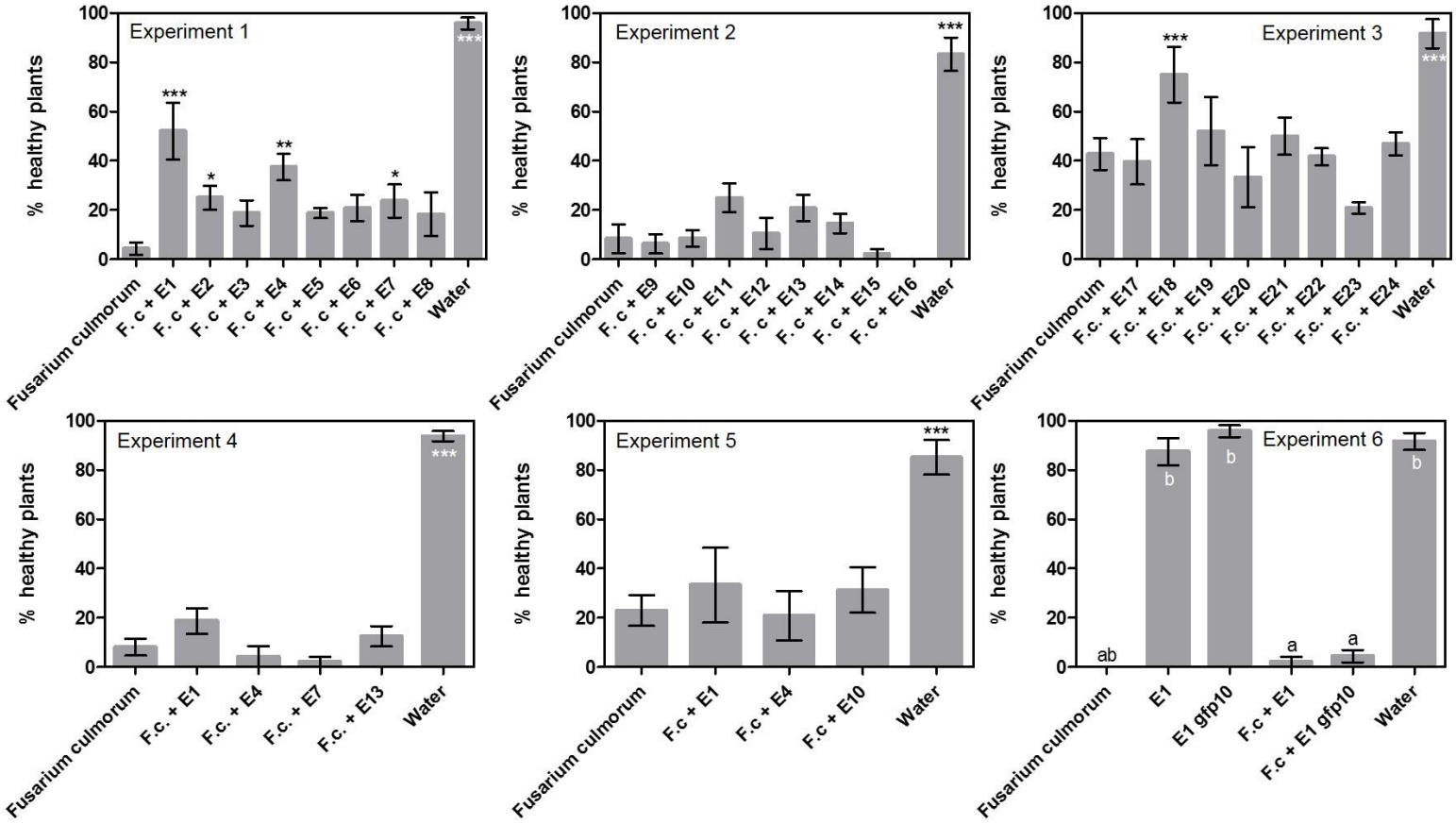

Figure 4.3. Disease incidence results from the sand assays testing 24 different endophytes against seed-borne *Fusarium culmorum*. Percent healthy plants is given +/- SEM. Each column represents the mean of 48 plants. Significant differences are compared to the "*Fusarium culmorum*" treated plants and are shown with asterisks ('*' $0.01 < P \leq 0.05$, '**' $0.001 < P \leq 0.01$, '***' $P \leq 0.001$). In experiment 6, all treatments were compared to each other and given letters to indicate dissimilarities ($P \leq 0.001$).

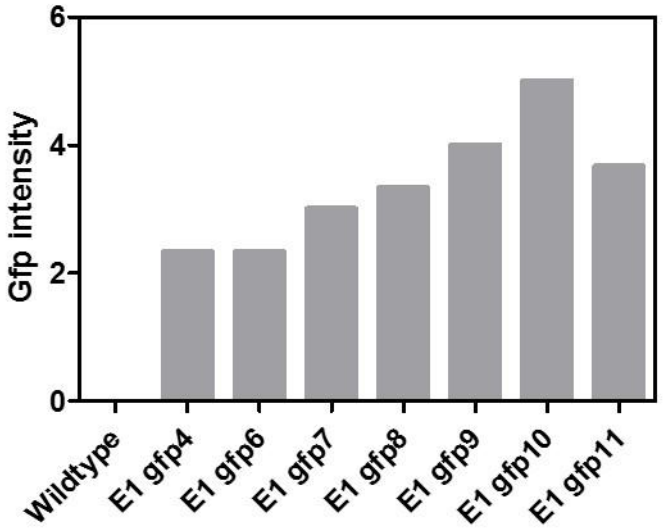

Figure 4.4. The GFP intensity of seven gfp strains and the wildtype evaluated on a subjective scale from 0-5. Each column represents the mean of three Petri dishes. No statistical evaluation was made because of the subjective nature of the assessment.

4.3.3 Net blotch assay to evaluate the efficacy of endophyte seed coating against *Pyrenophora teres*

The ability of a subset of endophytes to control leaf borne *Pyrenophora teres,* when the endophyte was dressed on barley seeds, was examined in experiments 7 to 9. In experiment 7, three endophyte isolates were able to significantly reduce percent lesion area of net blotch, namely Lasiosphaeriaceae sp. E10, Lindgomycetaceae sp. E13 and *Leptodontidium* sp. E16 (Figure 4.6). The three isolates were all cultured from different plants (Table 4.1). However, no significant differences in percent lesion area were found with Lasiosphaeriaceae sp. E10 in the replicate experiments 8 and 9. Treatment with *Periconia macrospinosa* E1 and *Epicoccum nigrum* E4 in experiments 8 and 9 also failed to significantly reduce percent lesion area despite having been successful against *F. culmorum* in previous experiments.

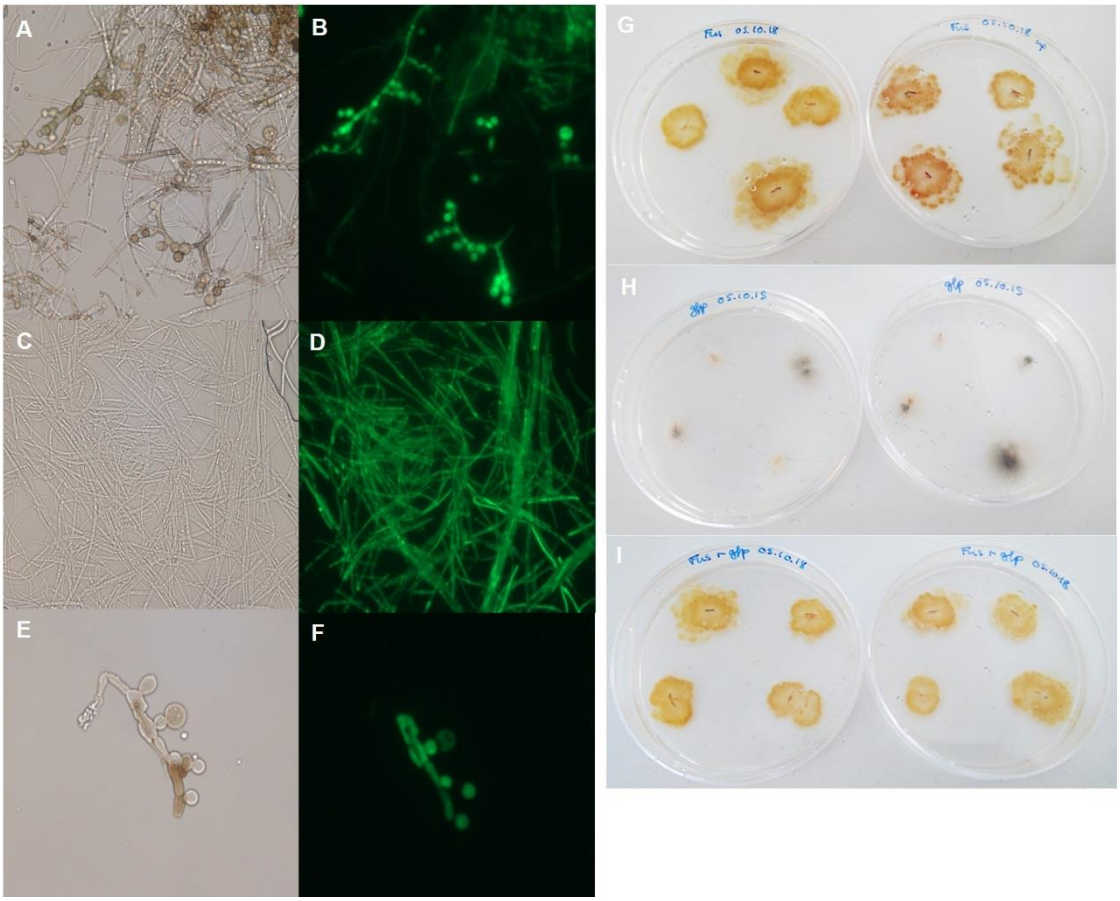

Figure 4.5. Intensity of E1 transformants and growth from washed root pieces 14 days after sowing from experiment 6. A) Hyphae, conidiophores and conidia of E1gfp9 under normal light, B) Hyphae, conidiophores and conidia of E1gfp9 under fluorescent light, C) Hyphae of E1gfp10 under normal light, D) Hyphae of E1gfp10 under fluorescent light, E) Conidiophores and conidia of E1gfp11 under normal light, F) Conidiophores and conidia of E1gfp11 under fluorescent light, G-I) Growth from four root pieces per Petri dish. Root pieces were taken from 14 day old plants from experiment 6. G) *Fusarium culmorum* treated plants. H) E1gfp10 treated plants and I) *Fusarium culmorum* and E1gfp10 treated plants.

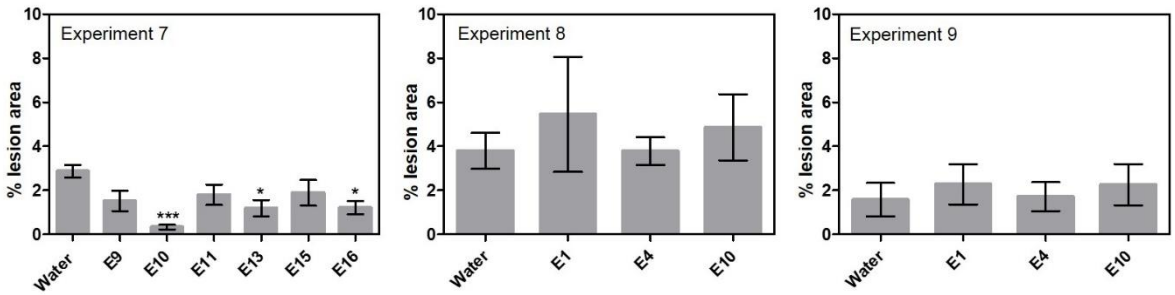

Figure 4.6. Results from the net blotch assay are given +/- SEM. Each column represents 40 plants. Significant differences are compared to the “water” treated plants and are shown with asterisk (‘*’ $0.01 < P \leq 0.05$, ‘**’ $0.001 < P \leq 0.01$, ‘***’ $P \leq 0.001$).

4.4 Discussion

In this study, endophytes isolated from a wild relative of barley, *Elymus repens*, were tested against both a soil pathogen and a leaf pathogen of barley. The 24 tested endophytes were originally isolated from eight different *E. repens* plants and interestingly, seven out of eight plants contributed an isolate that significantly reduced disease of either *Fusarium culmorum* or *Pyrenophora teres* in at least one experiment.

To our knowledge this is the first report of *Periconia macrospinosa* being tested against barley diseases. Two isolates of *P. macrospinosa,* E1 and E2, showed promising results against *F. culmorum* where they were able to reduce disease incidence in the initial experiment. Interestingly, the two isolates came from the same plant. Furthermore, isolate E1 also reduced disease severity in two out of four experiments.

Periconia macrospinosa is a species commonly associated with grasses as an endophyte (Sánchez Márquez *et al.* 2007; Mandyam *et al.* 2012; Comby *et al.* 2016) and was previously tested as a biocontrol agent by Kirk and Deacon (1987) against the take-all (*Gaeumannomyces tritici*) root disease of wheat. However, they found that *P. macrospinosa* could not control take-all even when tested at very high concentrations. Instead of using seed coating they inoculated the plants by mixing the inoculum of pathogen and control agent into the soil. Kirk and Deacon (1987) did evaluate the establishment of *P. macrospinosa* on the roots, but the results are not mentioned. In our experiments *P. macrospinosa* conidia applied to the seed resulted in root colonisation 14 days after sowing. However, in the presence of *F. culmorum*, *P. macrospinosa* could not be re-isolated. Caution should be taken when interpreting *in vitro* growth experiments like these since *F. culmorum* might be a better competitor on 1/5 PDA compared to *P. macrospinosa.*

The *Epicoccum nigrum* isolate E4 reduced *Fusarium culmorum* disease severity and incidence in one out of three experiments. *Epicoccum* spp. have shown good biocontrol results in previous studies, both applied to seeds against soil-borne diseases

(Hamza *et al.* 2013; El-Gremi *et al.* 2017) as well as sprayed for leaf-infecting (Li *et al.* 2013) and post-harvest diseases (Larena *et al.* 2005). El-Gremi *et al.* (2017) showed that an *Epicoccum* isolate could significantly decrease disease symptoms under field conditions of kernel black point caused by *Bipolaris sorokiniana*, *Alternaria alternata* and *Fusarium graminearum* in wheat. Likewise, Hamza *et al.* (2013) showed that *E. nigrum* could significantly reduce disease symptoms of late wilt of maize caused by *Magnaporthiopsis maydis.* In the study by El-Gremi *et al.* (2017) the antagonist suspension was applied to individual seeds at a concentration of 5 x 10^7 spores/mL, whereas, Hamza *et al.* (2013) prepared the *E. nigrum* inoculum at a concentration of 3 x 10^5 spores/mL. It would be interesting to test *E. nigrum* isolate E4 at a higher concentration. Not all *Epicoccum* isolates are reported to be able to reduce disease. Kortekamp (1997) found that *E. nigrum* applied to leaves was not antagonistic against *Plasmopara viticola*, the downy mildew pathogen of grape, and Busby *et al.* (2016) found that when an isolate of *E. nigrum* was applied to leaves of black cottonwood tree, *Populus trichocarpa,* the disease severity of the rust hybrid, *Melampsora* x *columbiana* was increased. Other screening systems, more similar to field conditions could have been used in our study to test the biocontrol agents against *F. culmorum.* However, Jensen *et al.* (2000) found that the performance of their biocontrol agent in the same type of sand assay showed high correlation to their results in subsequent field trials.

Eight of the 24 endophytes were tested against *Pyrenophora teres*. In two experiments, the promising candidates *Periconia macrospinosa* E1 and *Epicoccum nigrum* E4 could not reduce disease caused by *P. teres.* In contrast, seed treatment with Lasiosphaeriaceae sp. E10 reduced percent lesion area significantly in one out of three experiments, but Lasiosphaeriaceae sp. E10 had no effect against *Fusarium culmorum*. Also, Lindgomycetaceae sp. E13 and *Leptodontidium* sp. E16 reduced net blotch symptoms significantly. But when challenged with *Fusarium*, Lindgomycetaceae sp. E13 and *Leptodontidium* sp. E16 had no positive effect. Better results might have

been obtained if the endophytes had been sprayed onto the leaves before inoculation with the pathogen.

Studies have shown that cultivars can respond differently to the same biological control treatment. Osborne *et al.* (2018) found that different wheat cultivars showed a difference in their ability to support the colonisation of a beneficial root fungus, *Gaeumannomyces hyphopodioides*. Jørgensen *et al.* (1996) found that their biocontrol agent could reduce disease in seven different barley cultivars, however, the magnitude of the disease reduction depended on the cultivar. Thus, potentially more cultivars should be tested in order to explore the full potential of a biocontrol agent.

Only a few studies have investigated the control of barley diseases using endophytes. Previous studies of endophytic control of *Fusarium culmorum* have been done with *Serendipita indica*, an Indian isolate which is most likely not going to be released commercially in Europe (Harrach *et al.* 2013). In addition, Moya *et al.* (2016) controlled *Pyrenophora teres* using *Chaetomium globosum,* originally isolated as an endophyte, in dual culture experiments. However, several studies have shown that there is a poor correlation between results obtained in such dual culture *in vitro* experiments and results obtained from more complex growth systems including plant hosts (Renwick *et al.*, 1991; Fravel, 1988; Khan *et al.*, 2006; Deshmukh and Kogel 2007).

Successful endophytic control agents of barley diseases have been sourced from varying plants species including wild grasses such as *Ammophila arenaria* ssp. *australis* and *Corynephorus canescens* (Maciá-Vicente *et al.* 2008, 2009) as well as from two woody shrubs, *Prosopis juliflora* and *Zizyphus nummularia* (Deshmukh and Kogel 2007; Achatz *et al.* 2010; Harrach *et al.* 2013). It would appear logical that there is a potential for isolating effective control agents from *E. repens,* a grass found within the same tribe (Triticeae) as barley (Kavanagh *et al.* 2010), because *E. repens* is more closely related to barley than *A. arenaria* ssp. *australis* and *C. canescens* (both tribe Aveneae) which hosted beneficial control agents in the studies by Maciá-Vicente *et al.*

(2008). However, none of the 24 tested endophytes showed consistently significant results in the biocontrol experiments presented here. Originally, around 150 fungal root endophytes were obtained from *Elymus repens* (Chapter 3) and it is possible that there are successful control agents among the non-tested isolates. The subset of endophyte cultures that were tested against barley diseases were picked based on their ability to sporulate. However, some sporulating cultures were not tested. It is sometimes suggested that hundreds to thousands of microorganisms have to be screened in order to find a few beneficial ones (Glare *et al.* 2012; Broadfoot 2016), however studies also get away with less (Maciá-Vicente *et al.* 2008). Perhaps it is still necessary to screen many strains because we have little understanding of the ecological functions of endophytes within plants, including beneficial species. In the present investigation, two isolates of *Periconia macrospinosa,* E1 and E2, originating from the same plant showed very different results in the *Fusarium* assays. This emphasises the importance of the "isolate" and illustrates why only specific isolates can be patented and made into commercial products (European Commission 2016).

Endophytes are often defined as microorganisms living inside plants without causing symptoms of disease (Wilson 1995). However, this definition does not correlate well with the fact that known pathogens are often isolated as endophytes in the plants in which they can cause disease (Photita *et al.* 2004; Comby *et al.* 2016). It has been proposed that the role of the endophyte can range from latent pathogen to latent saprotroph and from neutral to antagonistic (Schulz and Boyle 2005). The endophyte community can protect the plant against pathogens (Arnold *et al.* 2003; Lee *et al.* 2009) but, studies have also shown that sometimes the endophytes can make the pathogen infection worse (Saunders and Kohn 2008; Kurose *et al.* 2012; Busby *et al.* 2016). Saunders and Kohn (2008) speculate that the endophyte community can in some cases detoxify the secondary metabolites produced by the plant and make it easier for the pathogen to colonise the plant; that is how some endophytes will seem like pathogen facilitators. Kurose *et al.* (2012) speculate that either pathogen and

pathogen facilitator work in synergy or that a pathogen can create a stressed environment that an endophyte can use to its advantage as it changes niche.

Biological control agents have frequently been found to produce inconsistent disease control (Glare *et al.* 2012). Such inconsistency is often explained by changes in the environment including, among other factors, the plant genotype, soil properties or growth conditions (Danielsen and Jensen 1999; Martín *et al.* 2015). In addition, Adame-Alvarez *et al.* (2014) found that the order of endophyte colonization could change the lifestyle of the endophyte from antagonistic to disease facilitator. However, our results show that endophytes can change functional role in an apparently constant experimental environment. In general, more consistent results would have to be shown in growth chamber experiments before trials could be recommended under field conditions.

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Supplement 4.1

Table s4.1. Primers used to amplify DNA from fungal endophytes, the primer sequence and the reference.

Locus	Primer	Primer sequence	Reference
ITS	ITS1	5’ TCCGTAGGTGAACCTGCGG 3’	White *et al.* 1990
	ITS4	5’ TCCTCCGCTTATTGATATGC 3’	White *et al.* 1990
LSU	LROR	5’ ACCCGCTGAACTTAAGC 3’	Stielow *et al.* 2015
			Johnson and Vilgalys 1998
	LR5	5’ TCCTGAGGGAAACTTCG 3’	Stielow *et al.* 2015
TEF1α	EF1-983F	5’ ACHGTRCCRATACCACCSATCTT 3’	Rehner and Buckley 2005
	EF1-1567R	5’ GCYCCYGGHCAYCGTGAYTTYAT 3’	Rehner and Buckley 2005

Supplement 4.2

Table s4.2. PCR cycles used for the individual primers. Temperature [˚C] and time [minutes].

Primer	**TEF1**		**ITS1-4**		**LSU**	
	Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
Premelt	94	1.30	94	1.30	94	1.30
Denature	94	0.45	94	1.30	94	0.30
Anneal	61	1.00	58	1.00	53	1.00
Extension	72	1.00	72	1.00	72	1.00
Final extension	72	7.00	72	7.00	72	7.00
No. cycles	30	-	30	-	30	-

Supplement 4.3

Table s4.3. Composition of 1000 mL induction medium (IM) + acetosyringone

Volume	Concentration
10 mL	1M K_2HPO_4
10 mL	1M KH_2PO_4
2.5 mL	1M NaCl
4 mL	1M $(NH_4)_2SO_4$
10 mL	50% (w/v) Glycerol
910 mL	MiliQ water
Autoclave for 20 min at 120 ˚C	
1 mL	9mM $FeSO_4$ (filter sterile)
10 mL	1M Glucose (autoclaved)
40 mL	1M MES (pH=5.3; filter sterile*)
	* make freeze aliquots of 40 mL
1.4 mL	0.5M $CaCl_2$ (autoclaved)
2 mL	1M $MgSO_4$
200µM acetosyringone = 20 mL 10 mM AS	

Supplement 4.4

Take-all assay to evaluate the efficacy of endophyte seed coating against soil borne *Gaeumannomyces tritici* and *Agrobacterium tumefaciens* mediated transformation with red fluorescent protein DsRed

Two types of experiments were conducted with take-all. The first was a soil assay which was used to determine the effect of the endophytic biocontrol agents against soil borne *Gaeumannomyces tritici* (Ggt) and for the second type agrobacterium mediated transformation was used to insert the red fluorescent protein DsRed in Ggt in order to follow the root colonisation and interaction with the biocontrol agents.

Materials and methods

Plant and fungal material

The plant material was the same and grown under the same conditions as described in section 4.2.1. The pathogen used were two isolates of *G. tritici* GgtNZ1610b and GgtNZ1611a (kindly provided by Rothamsted Research, isolated from post-harvest soil cores on the Rothamsted Farm, Harpenden, Hertfordshire, United Kingdom in 2016). Four single spore cultures of, *Periconia macrospinosa* E1, Lindgomycetaceae sp. E13, *Slopeiomyces cylindrosporus* E18 and Unidentified E21, were used (Table 4.1). For details about endophyte isolation see section 4.2.1.

Endophyte and pathogen inoculation

Inoculation of seeds with endophyte

Two experiments were conducted. Spores from endophyte cultures were harvested according to section 4.2.2. The spore concentration was adjusted to 10^7 spores/mL. Seeds were inoculated by soaking in the endophyte spore suspension (1:2 w/v) for 24 hours on a shaker at 130 rpm. The seeds were then dried for 2.5 hours on filter paper

in a sterile laminar flow chamber. For control treatments, deionised water was used in the same volumes as the endophyte treatment.

Preparation of Gaeumannomyces tritici (Ggt) inoculum

The protocol from McMillan *et al.* (2014) was followed, however, barley was used instead of wheat. Briefly, 500 mL conical flasks were filled with 3 g maize flour (full grain, organic), 100 g sand grain size 0.4-0.8 mm was mixed and 10 mL ionised water was added. The conical flasks were autoclaved twice with 48 hours between autoclaving. 6 plugs (cork borer 6 mm diameter) from Ggt growing on PDA was added to the flasks and grew for 6 weeks with weekly shaking of the inoculum. Two isolates GgtNZ1610b and GgtNZ1611a were inoculated separately. Four days before the experiment was sown, the sand inoculum was blended for 30 seconds individually. The inoculum concentration was estimated using a dilution series of 0.5 g inoculum diluted in 1 mL water (Table s4.4.1). To use as a control treatment, 7 g of Ggt inoculum from each isolate was mixed together before it was autoclaved twice with 48 hours between autoclaving.

Table s4.4.1. Inoculum estimations for experiment 1 and 2 for the two Ggt isolates.

	GgtNZ1610b	**GgtNZ1611a**
Experiment 1	1400 cfu/g	300 cfu/g
Experiment 2	800 cfu/g	1420 cfu/g

Sowing and experimental design

In the biocontrol experiments, seeds were dressed in either endophyte solution or deionised water and sown in soil with Ggt inoculum or double autoclaved Ggt inoculum (Table s4.4.2). Pots with a 9 cm diameter were used and 16 pots were used for each treatment. The experiment was set up as a 6 block experimental design. All pots were given a bottom layer of coarse damp sand, grain size 1.0-2.0 mm.

Table s4.4.2. Overview of the types of treatments prepared for the biocontrol of Ggt experiments.

Name of treatment	Seed treatment	Soil inoculum
Autoclaved Ggt	Ionised water	Autoclaved Ggt inoculum
Ggt	Ionised water	Ggt inoculum
Ggt + E(number)	Endophyte (number)	Ggt inoculum

For 16 pots the bottom layer was prepared as follows: 1.5 kg sand was mixed with 200 mL water and 98 g was distributed into each pot. Afterwards the 16 pots were filled with a mix of 1596 g coarse damp sand grain size 1.0-2.0 mm, 1500 g Pindstup potting mix (Pindstrup Substrate no. 2, Pindstrup Mosebrug, Ryomgård, Denmark), and 480 g sand grain size 0.4-0.8 mm with 1:50 w/w dilution of the blended Ggt inoculum. One seed was sown in each pot and covered with 20 mL vermiculite. The pots were watered with tap water in their tray three times a week.

Disease scoring

After five weeks, roots were washed free of soil and evaluated using the six point scale provided by McMillan *et al.* (2014).

***Agrobacterium tumefaciens* mediated transformation with DsRed**

Two isolates of *Gaeumannomyces tritici*, GgtNZ1610b and GgtNZ1611a, were transformed individually using the *A. tumefaciens* strain AGL1 containing DsRed in the plasmid PCA1 dsRed. The protocol by Mullins *et al.* (2001) was essentially followed see section 4.2.4, however mycelium from the two Ggt isolates growing on ¼ PDA with yeast extract were harvested in IM media separately (Supplement 4.3) and the hyphal concentration was adjusted to approximately 5 x 10^5 hyphae/mL.

Confirmation of the transformation

The transformation was checked under different fluorescent microscopes using DsRed excitation filters (Leica M205FA, excitation 546/10 and Leica DM 5000B, excitation 515-560; dicrotic mirror 580).

Results and discussion

Take-all assay to evaluate the efficacy of endophyte seed coating against soil borne *Gaeumannomyces tritici*

Symptoms of Ggt did not establish, thus, it was impossible to evaluate the experiments. It would have been interesting, if time had allowed, to test whether higher inoculum concentrations would have established the disease.

Agrobacterium tumefaciens mediated transformation with DsRed

Hyphal cultures were made from cultures that grew successfully from the filters and showed tolerance to hygromycin. Unfortunately, these pure cultures did not emit red light under a fluorescent microscope. There are many ways to optimise the protocol including adjusting the hyphal concentration, the age of the culture that is used as starting material, the *A. tumefaciens* strain, the ratio between bacterial and fungal material, the acetosyringone concentration which can influence the number of transformants, the filters, pH and temperature during co-cultivation (Michielse *et al.* 2005). Unfortunately, time did not allow for these adjustments to be made.

Chapter 5

5. General discussion

This thesis investigated the fungal root endophyte community of *Elymus repens* and aimed at describing the characteristic features and the differences between individual sites and plants. In addition, it investigated how different methods including direct sequencing and culturing influenced the estimates of species richness. Furthermore, an aim was to investigate whether a subset of endophytes isolated from *Elymus repens* could reduce disease symptoms of three important diseases of barley. Lastly, with the use of a literature study a review of biological control studies in barley was done to investigate whether biological control including endophytes can be used to reduce disease symptoms in barley.

5.1 The endophyte community and the variation among sites and individual plants

The root endophyte communities of the sampled *E. repens* plants formed 4.7 communities based on beta diversity which indicates that the five sites harboured substantially different communities. However, the communities from sites III and IV showed the most similarities as determined by NMDS (Chapter 3). The general root endophyte community of *E. repens* consisted of Chromista, Fungi and Rhizaria. From the five sites, 715 different fungal OTUs were discovered and they belonged to 8 different divisions and 31 classes of which the widespread classes, identified from all plants, included Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes, Agaricomycetes, Glomeromycetes and Mortierellomycetes (Chapter 3). Site II had a significantly higher OTU richness than sites IV and V, however site I and III were not significantly different form site II nor sites IV and V in terms of OTU richness. The mean richness from each site was 151 OTUs (Chapter 3).

Usually in studies, the endophyte communities of individual plants within populations are not described separately and to our knowledge, this is the first time the communities of individual plants have not been pooled. There was a large degree of

variation in the OTU richness identified from the individual root systems ranging from 96-239 OTUs per root system (Chapter 3). This difference could perhaps be explained by the size of the individual root systems. Bigger root systems could potentially hold a higher diversity. Yet, many other factors including soil properties and management regimes (Bala *et al.* 2003; Seghers *et al.* 2004; Long *et al.* 2010; Estendorfer *et al.* 2017) as well as genotype of the plant (Bulgarelli *et al.* 2015), have been proposed to influence the communities of the different plants. Bulk soil samples were taken from each plant and, if time allowed, it would have been interesting to analyse the fungal community of the bulk soil from each plant and the chemical composition of the soil. This would have given a more thorough understanding of how different the five sites and the individual plants were as well as the factors contributing to these differences. It would also have been interesting to determine the genotypes of the individual *E. repens* plants as this grass can reproduce vegetatively (Cope and Gray 2009). But, whether host genotype influences the community composition is a debated topic. Bulgarelli *et al.* (2015) showed that the bacterial endophyte community of barley was dependent on host genotype and Van Overbeek and Van Elsas (2008) found that some genotypes of potato had an effect on the community of bacterial endophytes. In contrast, Comby *et al.* (2016) found no correlation between two wheat genotypes and their fungal and bacterial communities.

From site III, 21 different taxonomic classes including nine from within the Ascomycota, six within the Basidiomycota, two within the Glomeromycota, one within the Mortierellomycota, two within the Mucoromycota and one within the Olpidimycota were identified with direct amplicon sequencing (Chapter 3). In contrast, the cultured community only consisted of four classes within the Ascomycota which corresponds to what most studies find when culturing endophytes from grasses (Sánchez Márquez *et al.* 2012). There was a small overlap between the cultured community and the community determined by direct amplicon sequencing with ten species shared by the two datasets. Surprisingly, 17 OTUs within the cultured community were not present in

the amplicon dataset for site III. Studies in grasses, trees and vines either show no overlap (Duong *et al.* 2006; Tejesvi *et al.* 2010) or only little overlap (Herrera *et al.* 2010; Prihatini *et al.* 2016; Jayawardena *et al.* 2018) in the communities of fungi identified using culture or direct sequencing approaches. It is possible that differences in OTU clustering could explain why there are differences in the communities identified with direct sequencing and culturing approaches. To add to the complexity, several fungi that are known to be cultured on standard media were identified by the direct amplicon sequencing but were not isolated from site III. Thus, the cultured community is not only a fraction of the total community but also a fraction of what could potentially be cultured.

If further direct amplicon sequencing are done in the future it is recommended that the guidelines of Nilsson *et al.* (2018) should be followed including at least three replicates per sample. They also recommend using a negative control which consist of no sample as well as two positive controls 1) with a known synthetic community and 2) with known species which are not expected to be present in the samples of interest to limit the number of sequences identified due to technical error.

5.2 Cultured OTU richness and the dependency on media and barcoding region

It is obvious that what we find depends on how we look. The diversity of endophytes isolated on three of the most commonly used media for endophytes of grasses were examined in Chapter 3. The largest number of OTUs were discovered on PDA but, it was also apparent that many OTUs would not have been discovered without the use of the other two media, MEA and 2 % MEA. In fact, the majority of OTUs were only isolated on one type of medium and were predominately only isolated once. It is possible that the media preference is an artefact of the isolation method. Perhaps it is merely by chance that a less abundant species ended up being cultured on medium “x” and had it been placed on one of the two other media it would have grown just as well.

Another possibility, is that some isolates will be out competed on medium “x” but thrive on media “y” and thus, they can grow on all media but not in the presence of certain other species.

Also, the threshold used to cluster OTUs can influence the species richness statistics that studies report. Higgins *et al.* (2007) showed that the higher the cut off value the larger number of species was found. There is no universally correct threshold, at least not if the barcoding region is ITS because this region has a high amount of intraspecific variation. Nilsson *et al.* (2008) showed that the intraspecific ITS variability is dependent on species so there is no common yardstick for the variation expected in a fungal genus, family or any higher taxon. To obtain a more precise identification, three barcoding regions (ITS, LSU and TEF1α) were used (Chapter 3 and Chapter 4) and all sequences were compared to two DNA sequence databases (NCBI and UNITE). The databases would most often agree on the identification but unfortunately, having three barcoding regions complicated identification much more than anticipated. Barcoding regions, would very often not agree on the identification and would sometimes group a single culture to completely different taxonomic classes all with very high percent similarity. Studies will often explain that they assigned a name to their OTU based on the percent similarity obtained from the top hit. This means that if the top hit is more than 97 % similar to their OTU they will trust the species or genus listed, if the top hit is 95-97 % identical then they will assign it to the genus and with identity of less than 95 % they will say that the OTU is unidentified. Caution is necessary when interpreting results as two barcoding regions with 100 % similarity have, in this project, been shown to group a culture into different fungal classes using the public sequence databases. Well curated databases are a must when using a molecular technique for identification and so is the use of morphology. Morphology was a very useful tool when trying to resolve the incongruences in database identification, however for many of the cultures no characteristic spore or other identifiable feature was obtained. Perhaps in the future, with lower prices, the

whole genome could be sequenced. With a full genome the identification would be more precise but, the discussion about similarity threshold would possibly still exist. It will also take many years for a large database of entire sequences covering a high proportion of fungal species to be amassed.

5.3 The cultured endophyte community of *Elymus* repens and their biocontrol potential

Endophytes were cultured from roots of *Elymus repens* (Chapter 3). The fungal community cultured from *E. repens* did not show a high degree of similarity to previously described communities of temperate grasses. The community was represented by four taxonomic classes including 15 OTUs within the Dothideomycetes, two OTUs within the Leotiomycetes, one OTU within the Pezizomycetes and 9 OTUs within the Sordariomycetes (Chapter 3).

Leptodontidium sp. (OTU17) was isolated from all root systems from site III. *Leptodontidium* sp. is not considered common in grasses but has been isolated extensively from forbs and trees. One species, *Leptodontidium trabinellum,* has been reported to cause sooty blotch disease of apples (Belding *et al.* 2000). In Chapter 4, six *Leptodontidium* isolates (E5, E7, E11, E12, E16 and E20) were tested as biocontrol agents of *Fusarium culmorum* in barley. Interestingly, one isolate (E7) could significantly reduce incidence of disease in the first experiment but not when repeated (in experiment 4). Also, *Leptodontidium* sp. E16 was tested against *Pyrenophora teres* and was able to reduce percent lesion area significantly (Chapter 4). If time had allowed, it would have been exciting to repeat this experiment to test whether the result was reproducible. These results, again, highlight the importance of the “isolate”.

Among the other relatively widespread OTUs were *Periconia* sp. (OTU13, Chapter 3) and two isolates, E1 and E9, were tested against *Fusarium culmorum* (Chapter 4). *Periconia macrospinosa* E1 was able to significantly reduce disease

symptoms in two out of four experiments and significantly reduce disease incidence in one out of four experiments. *Periconia macrospinosa* E1 did not harm the plants when it was introduced in solo, and in the presence of *F. culmorum* it was either antagonistic or neutral.

Lasiosphaeriaceae sp. (OTU 23) was isolated from two plants and one of the isolates (E10, plant 3) could significantly reduce percent lesion area against *Pyrenophera teres* in one experiment and in the following two experiments there was no effect of the seed treatment (Chapter 4). Exploring the biocontrol abilities of the second isolate, from plant 6, would be a potential next step and to try adjusting the inoculum concentration to test whether that would influence the outcome. Getting a seed treatment to reduce symptoms of a leaf infecting pathogen could indicate that the treatment is working through induced resistance and this prospect could also be explored further.

Gaeumannomyces graminis was recorded in all plants according to the direct amplicon sequencing. Therefore, the cultured endophytes persisted in a pathogen infected environment and could according to Köhl *et al.* (2009) and Ellis (2017) potentially work as biocontrol agents because of the pathogens presence. Thus, it would have been useful to establish a successful take-all assay. Attempts were made to develop such an assay but more work is needed to develop an efficient system (Chapter 4, Supplement 4.4).

To summarise, a subset of the cultured endophytes were tested against a seed borne (*Fusarium culmorum*) and leaf borne disease (*Pyrenophora* teres) of barley and the candidates showed inconsistent results. It would be interesting to examine why some isolates made shifts in their lifestyle from antagonistic to neutral. Perhaps RNA sequencing could be used to study the events associated with an antagonistic versus neutral lifestyle (Sjokvist *et al.* 2019). Afterwards the information about the genes of interest could be studied in more detail. It is also possible that the change in lifestyle is due to a change in the nutritional status of the plant. The hemibiotrophic fungus,

Zymoseptoria tritici, lives as an endophyte just after penetration of wheat leaves and later becomes a pathogen (Rohel *et al.* 2001). Rohel *et al.* (2001) found that the endophyte begins to produce spores, and adopt a pathogen lifestyle, once the fungus starts to starve carbon.

In addition, there are many untested isolates in the collection from *Elymus*, obtained in this PhD study (in total 151 isolates from site III), which could be examined as biological control agents. Also, experiments using consortia could be tested as they sometimes show better results compared to the use of single strains (Jain *et al.* 2015; Palmieri *et al.* 2017). Some important diseases of barley in Northern Europe including Ramularia leaf spot (*Ramularia collo-cygni*), rusts such as brown rust (*Puccinia hordei*) and yellow rust (*P. striiformis* f. sp. *hordei*), as well as barley yellow dwarf (*BYDV*) have never been challenged with biological control and are obvious diseases to target for future research. As an example, Ramularia leaf spot is primarily controlled using foliar fungicide application (Oxley *et al.* 2006), but the pathogen is also seed-borne (Oxley *et al.* 2006) and can grow endophytically without visible symptoms of disease (Salamati and Reitan 2006). Future research could aim to develop a seed dressing that could control the seed borne load as there are no available fungicidal seed treatments on the market (Bayer Crop Science). If the mechanism of biological control is induced resistance, then this seed treatment could also possibly work against infections later in the season. As symptoms of Ramularia leaf spot have been seen on *E. repens* (Salamati and Reitan 2006) it is possible that our collection harbour fungi that can control Ramularia leaf spot because the sampled plants were disease free. The research in biological control of Ramularia leaf spot has perhaps not been carried out yet because it is challenging to work with the pathogen and the symptoms of disease are evident late in the growing season (Salamati and Reitan 2006).

5.4 Biological control of barley diseases and selecting suitable biocontrol endophytes

It is evident from the review that biological control agents and endophytes can be used to control diseases of barley in controlled growth experiments and also under field conditions (Chapter 2). For example, the biocontrol agents that controlled *Fusarium culmorum* and *Pyrenophora teres* best in individual field trials were *Clonostachys rosea* (IK726) isolated from barley roots (Jensen *et al.* 2000) and *Pseudomonas* isolates isolated from wild and cultivated plants (Hökeberg *et al.* 1997), respectively. Other successful biocontrol agents of barley diseases have been isolated from a variety of sources including cultivated barley, wild grasses, unrelated plants, soil, insects and mammalian faeces (Høyer *et al.* 2019).

Most studies that isolate biocontrol agents from a plant, will isolate the agents from plants that are not showing symptoms of disease and thus, hypothesise that the microorganism is contributing to the health status of the plant. However, a few studies have targeted diseased tissue as a potential source of endophytes because they are either seeking an organism that can persist in the diseased tissue and thus act as a control agent, or they are hypothesising that the biocontrol agent might be recruited in response to the disease infection (Chapter 1).

Several different reasons have been proposed to justify the choice of specific biocontrol organisms applied to plants and they include 1) prior knowledge in the literature of for instance the production of secondary metabolites or 2) prior knowledge of control of specific diseases, having already tested the organism *in vitro* or *in planta;* 3) having already worked with the organism within the research group or 4) the fact that the organism was not a pathogen of barley (Chapter 2).

It is reported that as many as a hundred to a thousand biocontrol agents may need to be tested to find a few suitable candidates (Glare *et al.* 2012). It is difficult to design a high throughput screening system that can evaluate the control of disease. Köhl *et al.* (2011) describes a stepwise screening system for biocontrol organisms.

Some of the first steps include evaluating the market size, knowledge of the pathogen and isolation of the antagonist. Afterwards in the high throughput screening system candidates are excluded if they are a health risk to humans and their tolerance to pH, temperature, UV and commonly used fungicides are tested as well as the ease of spore production. All these steps are relatively cheap compared to the biocontrol assays. It should be noted that the biological control assays are not defined as high throughput. To screen for biocontrol properties it is necessary to have host or host tissue, pathogen and candidate antagonist interacting under controlled conditions that are representative of the end production system (Köhl *et al.* 2011; Høyer *et al.* 2019). It is essential that results are reproducible. It is also recommendable to test the candidates under different environmental conditions in order to test their suitability for field trials and testing against more pathogens. Biological control evaluations are always going to be labour intensive if their results are to be of high importance (Chapter 2).

Another approach to finding biocontrol agents could be the application of functional genomics. Here it is necessary to sequence the full/partial genome or transcriptome of the candidate, assemble the genome, annotate the genes and search for genes that produce beneficial proteins in a biocontrol context. For example, if the candidates should work through mycoparasitism, then the genes coding for chitinases, β-1,3-glucanases, β-1,6-glucanases and proteases could be of interest. If the candidate should have tolerance to harmful chemicals produced by the pathogen, polyketide synthases (PKSs) and ATP-binding cassette (ABC) transporters could be of interest (Karlsson *et al.* 2015). To date, 37 strains of ascomycetes and 8 strains of basidiomycete genomes have been completely assembled and submitted to the NCBI database (NCBI genomes 2019). The already sequenced strains are predominantly fungi that infect human tissue, are plant pathogens or are yeasts. It is possible that genome annotation could work as a guide for picking the candidates that continue to the biocontrol assays. However, the presence of genes does not guarantee expression

and thus, the biocontrol experiments must be executed anyway. With a limited number of genome references in the databases and with genome annotation not being very cheap this step is perhaps still premature for some applications.

5.5 Mechanisms of biological control

The extensive review in Chapter 2 revealed that only a small number of investigations have examined the mechanism behind the biocontrol effect in barley and rigorous evaluations are in fact rare. In general, biological control agents are of highest value if they can target more than one disease (Köhl *et al.* 2011). With more than one target they might also work through different modes of action. Strong conclusions about the mechanism of disease control cannot be based on studies that do not include a plant or plant tissue. *In vitro* dual culture studies are cheap, but most often, controversial because there is commonly not a good correlation between *in vitro* results and results obtained from more complex growth systems that include the plant (Fravel 1988; Renwick. *et al.* 1991; Khan *et al.* 2006; Deshmukh and Kogel 2007; Adame-Alvarez *et al.* 2014).

Biological control agents that work through antibiosis are perhaps not the most desirable as they will possibly work in the same way as fungicides and have the same limitations. One problem could be that the pathogen population evolves to overcome this metabolite and then the search for a new biocontrol agent has to begin again. The other three mechanisms; competition, induced resistance and mycoparasitism, seem more promising. The mechanisms behind the positive effect of a biocontrol agent can be difficult to show for microorganisms and especially for endophytes. It is crucial to evaluate appropriate disease symptoms and to include all the relevant treatments in the experiment. In studies that quantify defence responses or gene expression it is important to include a treatment with pathogen and antagonist present together and compare that to a treatment with pathogen alone. Also, it is necessary to document a link between the defence response and the biology of the pathogen. For example, with

mycoparasitism it is necessary to show an uptake of nutrients which can be done in several ways such as showing penetration of the target organism by the endophyte.

While steps forward have undeniably been made, especially in recent years, better dialog between academia and industry as well as more targeted funding towards core research would be greatly beneficial in advancing the field and ultimately producing a more widely applicable technology.

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