Molecular and cellular analysis of the endophyte *Neotyphodium uncinatum* and its association with Festulolium

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science
In
Biotechnology

At Massey University, Palmerston North
New Zealand

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2013

Abstract

Epichloë and *Neotyphodium* endophytes are well known for the fitness benefits they impart on the cool-season grasses they inhabit. The production of secondary metabolites, in particular lolines, which deter insect predation, is one such benefit and is of particular interest in pastoral grass development. The identification, testing and implementation of novel endophyte-grass associations resulting in high production of lolines is highly valued in the development of grass cultivars in New Zealand.

An in depth analysis of the two simple sequence repeats (SSR) used to identify endophyte species showed that the repeat structure is unique for some endophyte species and that ancestral relationships of interspecific hybrids may be inferred from the repeat structure. One family of SSRs was found to be enriched in exonic regions of a number of genes and may be an important factor in gene innovation and adaptation. Levels of loline production by *N. uncinatum* was found to be strain specific with the highest production by the strain, U10. *N. uncinatum* colonising intergenic hybrids of *Festuca pratensis* and *Lolium perenne* (Festulolium) displayed incompatibility in older tissue through cell wall thickening, degeneration of cytoplasm and production of dense inclusions around hyphae and in the plant intercellular space. The production of dense inclusions actively degrading hyphae indicated a plant response to hyphal colonisation.

Results of this study indicate the importance of repeat structure in strain identification, repeat elements in genes, the testing of loline alkaloids *in planta* and the barriers to establishing novel endophyte-grass associations.

Acknowledgments

Firstly I would like to thank my supervisors, Barry Scott and Carla Eaton, for their guidance and support throughout my masters. A special thanks to Barry for introducing me to the world of fungal endophytes, I have enjoyed it thoroughly.

Thanks to the Scottbase members Arvina, Daniel, Gemma, Ruth, Conni, Tetsuya, Yvonne, Philippa, Alex and Yonathan for their help and guidance around the lab and making the lab an enjoyable place to work even when experiments are going wrong.

Thank you to Cropmark Seeds Ltd who made this study possible. Thank you to Nick Cameron for hosting me in Darfield and Tim for his help in the lab and testing of my many plant samples.

Thanks to IMBS and all the staff, especially Pat, Ann and Cynthia, who have helped along the way. Thanks to everyone at the Manawatu Microscopy and Imaging Centre, particularly Jianyu but also Jordan, Lawrie and Doug for their help with TEM and Confocal microscopy.

I would like to thank my partner Erin for supporting me through my masters and bringing me sanity with the many excursions away from Palmerston North to see her. Lastly thanks to my family for supporting me throughout my studies, it has meant a lot.

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Common Abbreviations

Amp	Ampicillin
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide database search using a nucleotide query
d	Days
°C	Degrees Celsius
cDNA	Complementary DNA
cv	Cultivar
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
g	Gravity
gDNA	Genomic DNA
h	Hours
H_2O	Dihydrogen Monoxide
kb	Kilo base pairs
LB	Luria-Bertani medium
M	Molar
min	Minutes
µg	Micro-gram
mg	Milli-gram
µL	Micro-litre
mL	Mill-litre
µM	Micro-molar
mM	Milli-molar
NCM	Nitrocellulose membrane
NRPS	Non-ribosomal peptide synthetase
NAL	N-Acetylloline
NFL	*N*-Formylloline
NML	*N*-Methylloloine
NANL	*N*-Acetylnorololine
PCR	Polymerase chain reaction

PD	Potato dextrose medium
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SSR	Simple Sequence Repeat
TBE	Tris Borate EDTA buffer
v/v	Volume/volume ratio
WGA	Wheat Germ Agglutinin
w/v	Weight/volume ratio

1. Introduction

1.1 Background

Grass endophytes make up a group of filamentous fungi comprised of the sexual *Epichloë* and asexual *Neotyphodium* species (Moon *et al.*, 1999). The mutualistic benefits of both the sexual and asexual endophyte species have been extensively studied for their use in agricultural systems due to the ability to enhance host grass persistence and provide tolerance to biotic and abiotic stresses (Rodriguez *et al.*, 2009). The best documented of these benefits is an increased resistance to insect herbivory due to the production of secondary metabolites (Bush *et al.*, 1997). Use of endophytes in pastoral systems can also result in the production of anti-mammalian metabolites which cause problems in grazing livestock such as fescue toxicosis and ryegrass staggers (Gallagher *et al.*, 1981), (Bacon, 1995), while the anti-insect metabolites may reduce pasture damage from the insect pests they act on (Wilkinson *et al.*, 2000). The discovery of endophyte strains that produce a desired profile of secondary metabolites conferring insect resistance, without the detrimental effects to grazing livestock, has been of great interest in the pastoral farming sector.

Endophyte strains that produce a desirable metabolite profile must also be matched with compatible host grasses for a beneficial association to be established. Methods are available for the inoculation of grasses with endophyte (Latch & Christensen, 1985). This gives the potential to inoculate a desired endophyte into a new host better suited to pastoral farming to maximise the benefit of insect resistance as well

as grass persistence and palatability, so long as the grass-endophyte association is compatible and persistent in ongoing generations. These synthetic associations, however, have been shown to impact on the production of the secondary metabolites with some metabolites expressed at higher or lower levels relative to expression in the natural host (Easton *et al.*, 2009). Therefore, a compromise must be reached between the suitability of a grass host for grazing and the ability of the endophyte to produce specific secondary metabolites in that host.

The endophyte species *Neotyphodium uncinatum,* a natural symbiont of meadow fescue (*Festuca pratensis*), produces high levels of the anti-insecticidal loline metabolites without the production of the anti-mammalian ergot alkaloid and lolitrem compounds (Leuchtmann *et al.*, 2000). This endophyte has received attention as an ideal endophyte for New Zealand's pastoral farming sector. However its natural host, meadow fescue, is not the most suitable grass for New Zealand's intensive grazing due to its lower yield and palatability (Thomas & Humphreys, 1991). Therefore, the potential exists to use inoculation methods to infect this endophyte into a more suitable grass host. However, compatibility barriers exist for inoculation into new host species such as perennial ryegrass, the most widely used forage grass in New Zealand (Williams *et al.*, 2007). By using hybrids between perennial ryegrass and meadow fescue the potential exists to retain the colonisation of *N. uncinatum* while still utilising the pastoral benefits of perennial ryegrass.

1.2 Fungal endophytes of grasses

The major pasture grass endophytes belong to the *Epichloë* and *Neotyphodium* genera (Ascomycota, Clavipitaceae) which form associations with cool-season grasses within the subfamily Pooideae (Tanaka *et al.*, 2012). These endophytes have been of great interest due to their ability to enhance tolerance to both abiotic and biotic stresses in the host species they colonise (Schardl *et al.*, 2009). Use in the pastoral sector sees the production of secondary metabolites by these fungi which have effects on both grazing livestock and insect herbivory (Williams *et al*., 2007). Endophytic hyphae in grasses were first thought to have little or no effect on the host grasses they colonised, but studies linking endophytes with fescue toxicosis and ryegrass staggers opened the field for further research (Fletcher & Harvey, 1981, Bacon *et al.*, 1977). It was soon found that endophyte symbiosis, including their production of secondary metabolites, were responsible for not only major health effects on grazing animals but also increases in grass yields and resistance to insect herbivory (Siegel *et al.*, 1987). These early findings led the way for a greater interest by both the scientific and commercial communities and a further understanding of the grass-endophyte association.

1.2.1 Growth and life cycle

Epichloë and *Neotyphodium* endophytes have similar growth patterns *in planta,* while the life cycle of *Epichloë* differs from *Neotyphodium* endophytes due to the ability of *Epichloë* species to enter the sexual cycle. However, both follow a distinct mode of growth originating from heavily branched mycelium in the shoot apical meristem (SAM) from which hyphae enter the meristematic tissue where new shoots develop. Hyphae are proposed to then grow by polarized tip growth in order to form the branched mycelial network. Once hyphae colonise intercellular spaces and attach to cells within the leaf expansion zone they undergo a change in growth pattern from tip growth to that of intercalary expansion (Christensen *et al.*, 2008). This change is thought to occur due to stretching of hyphal cells exposed to expanding leaf tissues. Intercalary extension allows hyphae to synchronise their growth with plant cell growth and leaf expansion to form hyphal networks within the intercellular spaces, that are rarely branched and aligned parallel to the longitudinal leaf axis (Fig.1.1) (Christensen *et al*., 2008). Hyphae are attached physically to host cell walls throughout their growth in leaf tissues. This attachment may increase the ability for nutrient uptake and signalling between host and symbiont. Endophyte, particularly those of *Neotyphodium* species, may also be present in and around the vascular bundles of the plant. This pattern of colonisation is enhanced when inoculation of seedlings is used, but may still occur rarely in natural associations. The colonisation of vascular bundles themselves may be detrimental to the plant whereby hyphae disrupt nutrient supply to the plant cells. However, some plants with highly colonised vascular bundles are not noticeably affected and maintain a normal growth pattern (Christensen *et al.*, 2001). Hyphal growth in and around vascular bundles

may also represent an important mode for transport of signalling molecules and metabolite compounds throughout the plant.

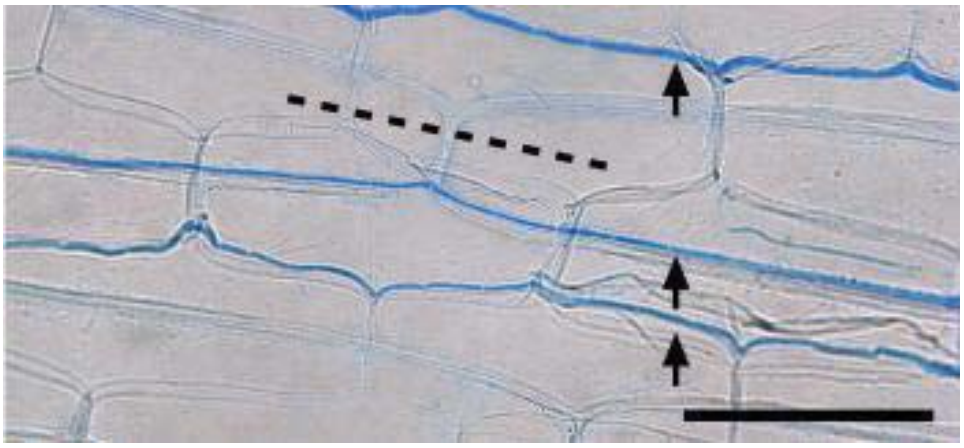


Figure 1.1: Endophyte growth *in planta*. Adapted from Christensen *et al* (2008). *N. lolii* hyphae in a ryegrass leaf sheath. Hyphae, stained with aniline blue, are orientated parallel to the longitudinal leaf axis (dashed). Bar = 100 μm

The *Epichloë* endophytes may transmit either horizontally as part of the sexual cycle or vertically as part of the asexual cycle, while the *Neotyphodium* endophytes are known only to be transmitted vertically (Fig. 1.2) (Schardl *et al.*, 2004). During horizontal transmission and sexual reproduction hyphae undergo proliferative growth on leaves surrounding the host inflorescence. Mycelia form a stroma around the developing inflorescence inhibiting its emergence and stopping seed production on the affected tiller (Schardl *et al*., 2004). These structures (stroma) contain conidia (mitotic spores) which can act as spermatia when transferred from one mating type to that of a compatible opposite mating type. This transfer is often mediated by flies of the genus *Botanophila* which lay their eggs on the fungal stroma and transfer spermatia from one stroma structure to another (Clay & Schardl., 2002). Fruiting

structures develop on fertilized stroma and eventually ascospores are released which are able to infect seeds or neighbouring plants (Chung & Schardl, 1997b, Schardl *et al*., 2004).

However, vertical transmission of endophyte in the plant is the most prevalent method of reproduction. Endophyte hyphae grow into a developing inflorescence and eventually into the developed seed where it is ready to propagate once germination occurs. This occurs initially before anthesis by growth through the rachilla into the lemma and palea and also into the base of the ovary. Endophyte hyphae colonise the ovule but are restricted to nucellar tissue and do not enter the embryo sac. Hyphae in post-fertilisation tissue are found in a dense layer colonising the scutellum before eventually penetrating the embryo sac. Endophyte hyphae are then able to readily colonise the new plant tissue germinating from infected seeds and maintain colonisation in successive generations (Majewska-Sawka & Nakashima, 2004, May *et al.*, 2008).

Vertical transmission also ensures endophyte survival in the next population and may be linked to endophytes that have highly mutualistic relationships with their host grasses (Selosse & Schardl, 2007). Some *Epichloë* endophytes are not restricted to either horizontal or vertical transmission but rather use a mixed strategy whereby some tillers undergo stroma formation arresting inflorescence development, while other tillers on the same plant may develop inflorescence and produce endophyte infected seed. This strategy allows for the maintenance of endophyte infected host plants through seed, while still allowing sexual reproduction required for quick adaptation to changing conditions or niche host populations (Clay & Schardl, 2002).

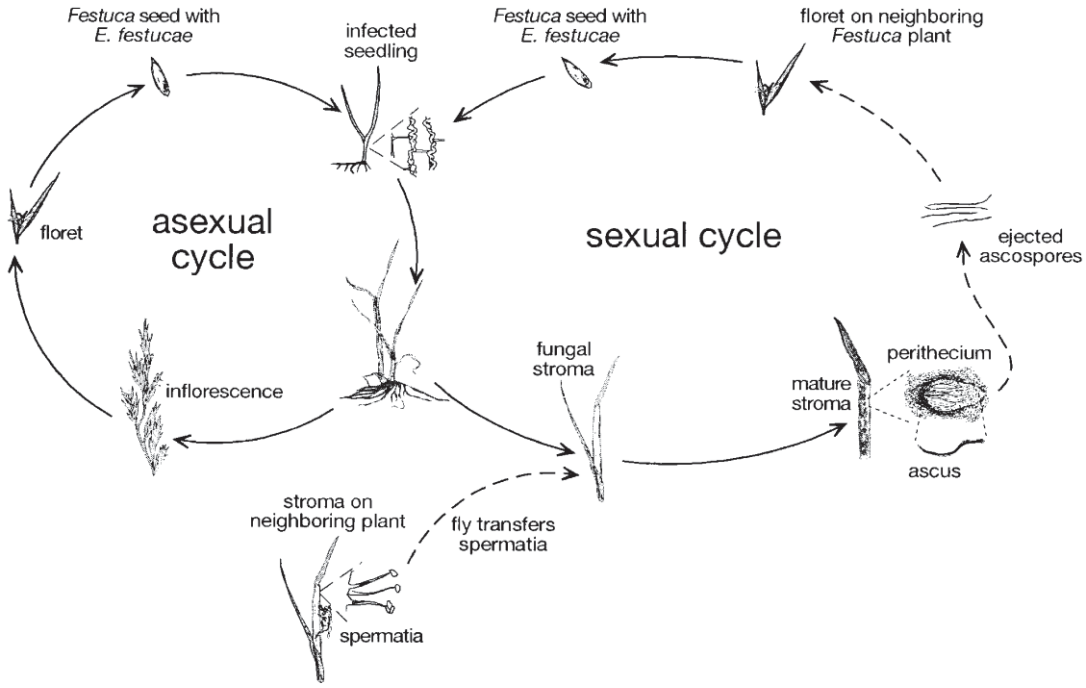


Figure 1.2: Life cycle of Epichloë endophytes. Life cycle of *Epichloë festucae* showing both asexual (vertical) and sexual (horizontal) reproductive methods. Reproduced from Clay & Schardl, (2002).

1.2.2 *Neotyphodium* endophytes and *N. uncinatum*

The *Neotyphodium* endophytes have been of great interest agronomically as they are naturally associated with some of the most important forage grasses, such as perennial ryegrass. These *Neotyphodium* endophytes are asexual derivatives of the sexual *Epichloë* endophytes and are thought to have arisen mainly via interspecific hybridisation between two or more *Epichloë* species (Moon *et al.*, 2004).

Of particular interest in this study is *Neotyphodium uncinatum,* which naturally associates with the grass host *Festuca pratensis* (meadow fescue) and produces high levels of the potent anti-insect compounds, lolines (Craven *et al.*, 2001). *N.*

uncinatum is proposed to be an interspecific hybrid between *E. typhina* and *E. bromicola* (Moon *et al*., 2004). It is a high producer of loline alkaloids yet its proposed extant ancestors do not synthesise these compounds. *E. typhina* and *E. bromicola* may once have had the ability to synthesise lolines but have since lost it. Evidence of this is shown by *Neotyphodium* species, with *E. typhina* as a proposed ancestor, containing nearly identical copies of the loline synthesis genes suggesting these were derived from an *E. typhina* ancestor that contained the ability to synthesise lolines. As *N. uncinatum* contains two loline gene clusters it is thought the second locus was gained from the *E. bromicola* ancestor during hybridisation rather than a recent duplication of the first cluster (Kutil *et al.*, 2007).

1.2.3 Interspecific hybridisation in fungi

Interspecific hybridisation has been shown in plants (Abbott, 1992), but the ability of fungi to undergo these hybridisation events is much less recognised. Fungi may undergo this hybridisation event as a result of a partial or complete sexual process or by a parasexual process (Schardl & Craven, 2003). In sexual hybridisation, gametes of the male and female structures fuse to form a dikaryotic state. This state can then be resolved by the fusing of nuclei and meiosis. The length of time that the dikaryotic state is maintained depends on the fungal species, with some fusing and entering meiosis quickly while others may maintain the state indefinitely. Hybrids that enter meiosis may resolve to form the euploid state, commonly haploid for many fungi, or abnormal meiosis can occur to form a heteroploid hybrid (Schardl & Craven, 2003). In the parasexual process, vegetative cells fuse resulting in

multinucleate cells that can continue in the heterokaryon state or spawn homokaryon cells. The nuclei may also fuse and undergo mitotic crossovers to generate a unique genotype with the potential for chromosomal loss in order to form a more stable state (Clutterbuck, 1996).

The parasexual process of hybridisation is typical of many fungi in the Ascomycota family which includes *Neotyphodium* species and is thought to have occurred to give rise to these asexual hybrids (Moon *et al*., 2004). However in most fungi the parasexual process is severely restricted by genetic recognition systems that determine vegetative incompatibility groups. For the hybridisation and formation of viable heterokaryon the two individuals must share common alleles at several incompatibility regulating loci (Leslie, 1993). These incompatibility systems may act to conserve the genetic identity of the individuals as well as provide a defensive barrier against foreign elements such as viruses (Schardl & Craven, 2003). These systems would reduce the potential for a hybridisation event that would lead to the formation of the interspecific hybrids that exist today. However, importantly in the grass endophyte species *Epichloë,* no evidence for these incompatibility systems has been reported (Chung & Schardl, 1997a), further enhancing the view that the asexual *Neotyphodium* species have arisen due to interspecific hybridisation of their sexual *Epichloë* relatives (Moon *et al.*, 2000).

In parasexual hybridisation the resulting hybrid fungus often has a heteroploid genome resulting in the loss of its sexual ability. Parents may differ in one or many chromosomes, disrupting pairing and normal meiosis that would result in a euploid genome (Schardl & Craven, 2003). The loss of chromosomes may also occur in an

attempt to stabilise the genome but in some cases, as in *N. uncinatum* the dominant state is heteroploid and they possess some or all of the genetic information from both their ancestors resulting in a larger genome (Kuldau *et al.*, 1999, Moon *et al*., 2000, Schardl & Craven, 2003). This heteroploid state may be further stabilised by the added benefit to the endophyte of allowing adaptation to unique hosts or environments that were previously unable to be utilised by their sexual ancestors (Schardl & Craven, 2003).

1.3 Identification of Endophytes

Endophyte species were previously defined on the basis of morphology and host specificity (Christensen *et al.*, 1993). More robust methods for identification were subsequently developed using molecular phylogenetic analysis of intron sequences from the β-tubulin and translation elongation factor 1-α genes which allowed for more distinct taxonomic groupings and identification *(*Moon *et al*., 2004). Ten *Epichloë* species have been identified so far using these methods. The phylogeny of the asexual *Neotyphodium* species has also been studied using these techniques and shows the interspecific hybridisation of the sexual *Epichloë* ancestors that gave rise to the asexual *Neotyphodium* species (Moon *et al*., 2004).

A PCR based method for rapid *in planta* strain identification of endophytes was also developed (Groppe & Boller, 1997). This method uses PCR to assess random amplified polymorphic DNA and Simple Sequence Repeats (SSR) for the identification of distinct endophyte species and strains (Moon *et al*., 1999). SSR

regions are known to be scattered throughout eukaryotic genomes with the high frequency of polymorphism making them ideal to distinguish between individuals (Tautz & Renz, 1984). So far the PCR amplification and analysis of SSR loci has been the most informative way of easily identifying endophyte species. These repetitive sequences consist of repeated units with the number of repeated units varying between individuals, allowing them to be distinguished by the length of common SSR loci (de Jong *et al.,* 2003, Dracatos *et al.,* 2006, Moon *et al*., 1999).

The most informative of these are the B10 and B11 SSR loci (Moon *et al*., 1999, Moon *et al*., 2000). Used in combination, the length of these B10 and B11 loci are able to distinguish between species by nucleotide unit (ntu) length and also differentiate between strains of the same species (Table 1.1). The use of these SSRs allows for the fast identification of different strains and species yet little is known about how the DNA structure of these repeats varies between species and strains and where in the genome these repeats are located.

Table 1.1: **Endophyte allele groupings from SSR PCR assays.** Reproduced from Moon *et al* (1999).

Species	Isolate	Host grass	DNA preparation	Allele size (ntu)[a] B4	B6	B9	B10	B11
LpTG-2	Lp1[c]	*L. perenne*	culture	100.0 119.8	172.4 187.6+	247.4+	169.3+ 178.1+	119.8
N. uncinatum	Fp1[c]	*F. pratensis*	culture	84.8 104.4	178.7	—	160.7+ 195.8+	120.7
Epichloë spp.				83.6				
E. amarillans J.F. White	200743[d]	*Sphenopholis obtusata*	culture		177.9+	—	169.8	141.5+
E. baconii J.F. White	200745[d]	*Calamagrostis villosa*	culture	—	—	—	169.7	150.1+
E. brachyelytri Schardl et Leuchtm.	200752[d]	*Brachyelytrum erectum*	culture	93.8	178.3+	—	169.4	137.0+
E. bromicola Leuchtm. et Schardl	201558[d]	*Bromus benekenii*	culture	84.1	178.2+	—	185.9	112.7+
E. clarkii J.F. White	90168[d]	*Holcus lanatus*	culture	118.0+	—	—	186.8	—
E. elymi Schardl et Leuchtm.	201551[d]	*Elymus canadensis*	culture	82.9	178.3+	—	194.1	128.8+
E. festucae Leuchtm. et al	201550[d]	*F. rubra* subsp. *commutata*	culture	99.0	186.5+	274.5+	172.5	166.0+
E. glyceriae Schardl et Leuchtm.	200747[d]	*Glyceria striata*	culture	81.7	—	—	158.7	120.7
E. sylvatica Leuchtm. et Schardl	200748[d]	*Brachypodium sylvaticum*	culture	111.0+	—	—	172.2	—
E. typhina (Pers.: Fr.) Tul.	E348	*Phleum pratense*	culture	127.8	173.6	—	191.9	—
E. typhina	201667[d]	*Poa nemoralis*	culture	102.3	—	—	180.3+	—
E. typhina	200740[d]	*Dactylis glomerata*	culture	119.1	173.2	—	178.4	—

[a] + = another peak approximately 1 ntu larger was also observed; — an allele was not observed at this locus.
[b] Peak was very large, possibly affecting accuracy of the size determination.
[c] Microsatellite profiles were determined by Moon et al 1999.
[d] American Type Culture Collection accession number.

1.4 Production of alkaloids by endophytes

Secondary metabolites can be classified into four main groups: pyrrolizidines, ergot alkaloids, indole diterpenes and pyrrolopyrazines (Bush *et al*., 1997). The production of these metabolites varies depending on the grass-endophyte association, with a variety of metabolite profiles possible (Table 1.2).

Table 1.2: Alkaloid profiles from grass-endophyte associations. Reproduced from Bush *et al* (1997).

Host Grass	Symbiont	Alkaloids[b]				Association
		E	L	LM	P	
F. arundinacea	*N. coenophialum*	0.5	1100	0	2	Natural
F. arundinacea	*N. lolii*	1.2	0	23	18	Artificial
L. perenne	*N. lolii*	1.3	0	4.7	19	Natural
L. perenne	*N. coenophialum*	2.5	1000	0	29	Artificial
L. perenne	*Epichloë typhina*	0	0	0	53	Natural
L. perenne	*N. lolii* × *E. typhina*[c]	4.8	0	0.4	22	Natural
F. pratensis	*N. uncinatum*	0	5600	0	0	Natural
Festuca gigantea	*E. festucae*	0	300	0	4	Natural
Festuca longifolia	*E. festucae*	0.9	0	4.0	22	Natural
Festuca rubra subsp. *rubra*	*E. festucae*	1.2	0	0	0	Natural

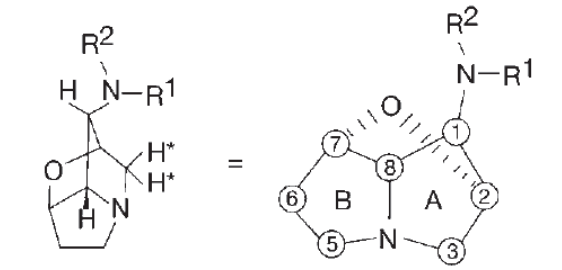
Alkaloid profiles for a variety of grass-endophyte associations[a]

[a] Approximate concentrations given in $\mu g\ g^{-1}$ dry wt. [b] Abbreviations: E, ergovaline; L, lolines; LM, lolitrems; P, peramine.
[c] A naturally occurring interspecific hybrid.

1.4.1 Pyrrolizidines

Pyrrolizidines, better known as lolines, are potent insecticidal compounds produced by both *Neotyphodium* and *Epichloë* species including *N. uncinatum, N. coenophialum, N. segalii* and some *E. festucae* strains. These lolines are responsible for the anti-insecticidal properties that provide protection to the plant from herbivory, yet exhibit no anti-mammalian effects. This insecticidal benefit allows for a significant advantage in areas susceptible to high insect predation (Riedell *et al.*, 1991, Bush *et al*., 1997).

Lolines have a unique structure with various 1-amino substituents of a saturated pyrrolizidine and an oxygen bridge between carbons two and seven (Fig. 1.3). The different 1-amino groups are responsible for the varying activities between the loline compounds (Fig. 1.3).



	R^1	R^2
N-formylloline (**1**)	HC=O	CH_3
N-acetylnorloline (**2**)	CH_3C=O	H
loline (**3**)	CH_3	H
N-methylloline (**4**)	CH_3	CH_3
N-acetylloline (**5**)	CH_3C=O	CH_3
norloline (**6**)	H	H
N-formylnorloline (**7**)	HC=O	H

Figure 1.3: The structure of different loline alkaloids produced by *Neotyphodium uncinatum*. Carbon number and ring assignments are indicated. Reproduced from Blankenship *et al.,* (2005).

Lolines have been shown to be produced *in planta* at levels of up to 2% of total dry mass and may accumulate in both the roots and shoots (Wilkinson et al., 2000, Patchett *et al.*, 2008a). Loline production has also been shown to be wound inducible, such as occurs with insect feeding, resulting in an increase in the production of lolines (Bultman *et al.*, 2004, Craven *et al*., 2001). Plant distribution of lolines can also be altered to enhance protection in response to insect attack. Loline levels previously low in the roots of meadow fescue infected with *N. uncinatum* were shown to increase upon grass grub attack suggesting a mechanism for the redistribution of lolines in response to insect attack (Patchett *et al.*, 2008b). This ability for activated distribution and induction in response to insect predation makes the ability of lolines to protect their host more efficient than constitutive high level expression.

Lolines are produced from the amino acids L-proline and L-homoserine (Blankenship *et al.*, 2005) and are regulated by a *LOL* gene cluster (Spiering *et al.*, 2005). Two *LOL* gene clusters were found in *N. uncinatum*, *LOL1* and *LOL2*, each containing nine protein encoding genes, designated *lolF, lolC, lolD, lolO, lolA, lolU*, *lolP*, *lolT* and *lolE* in reference to their predicted functions (e.g. *lolD* relates to a decarboxylase). However, one cluster is non-functional due to a mutation in the *lolP* gene (Spiering *et al*., 2005). These genes were found in a 25 kb region and were shown to be expressed with loline alkaloid production. Using RNA interference it was shown that knock down of *lolC*, proposed to be involved in the first step of the loline pathway, reduced loline synthesis and demonstrated the importance of these genes in loline production (Spiering *et al*., 2005).

The biochemical pathway for the production of lolines begins with a γ-substitution reaction yielding structure 14 (Fig. 1.4). This is followed by an oxidative decarboxylation preceding a second decarboxylation to yield structure 16 (Fig. 1.4). The pathway then follows by formation of the *exo*-1-aminopyrrolizindine (structure 17, Fig. 1.4) followed by the addition of the oxygen atom between carbons 2 and 7 to yield norloline. This can then undergo methylation to create other loline structures (Schardl *et al.*, 2007).

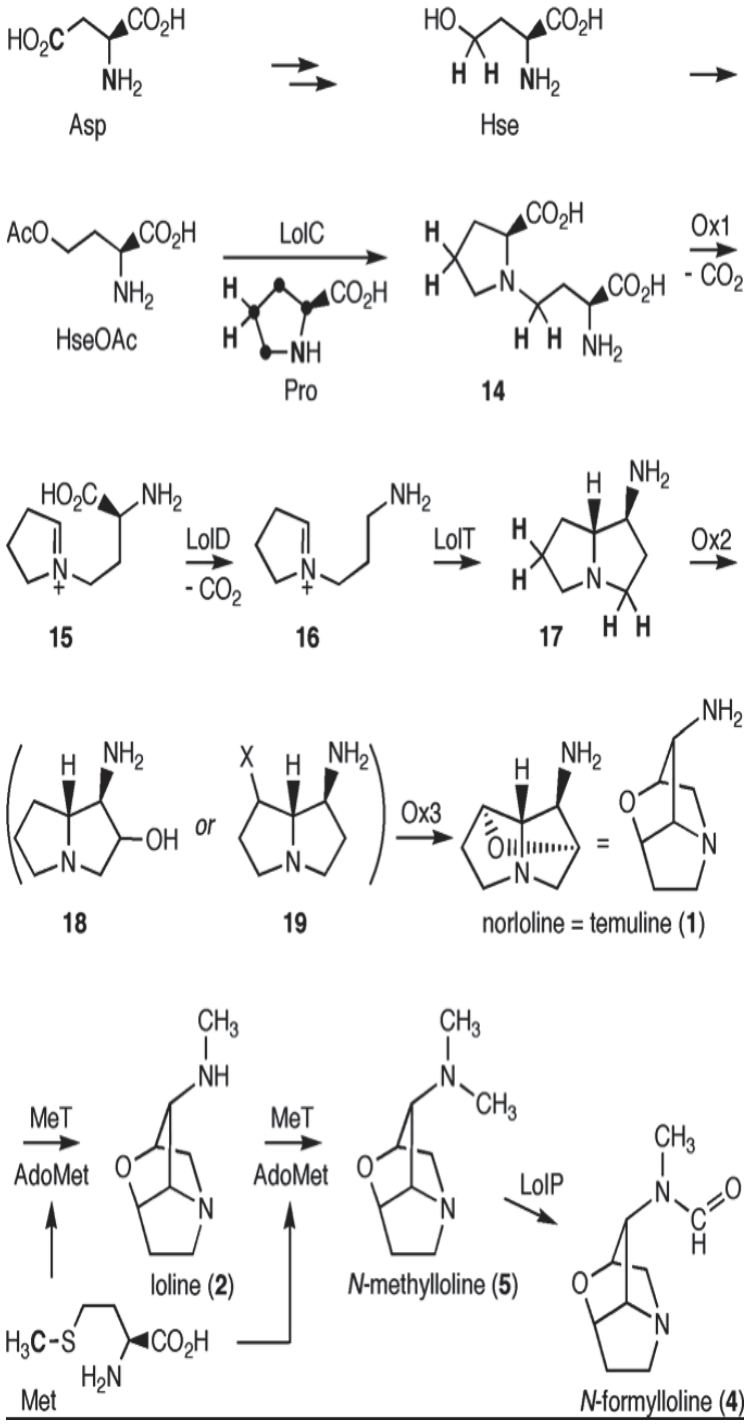


Figure 1.4: Proposed biochemical pathway for the production of lolines. Labelled atoms – indicated by dots or bold letters – were incorporated into lolines as predicted by the pathway shown here. Enzymes likely to catalyse pathway steps are designated above arrows. Genes for LolC, LolD, LolT and LolP are clustered in the *LOL* locus, along with genes for three enzymes (LolE, LolF, and LolO) proposed to carry out the oxidative steps labelled Ox1–Ox3. Reproduced from Schardl *et al,* (2007).

1.4.2 Ergot alkaloids

Ergot alkaloids produced by both *Epichloë* and *Neotyphodium* species have been implicated in the toxicity of feeding livestock, causing significant impacts on the agricultural industry (Thompson & Stuedemann, 1993, Hoveland, 1993). In particular the ergot alkaloid ergovaline has been linked to this negative effect but intermediate compounds are also thought to play a role in toxicosis (Fleetwood *et al.*, 2007). These alkaloids are produced by nonribosomal peptide synthetase (NRPS) enzymes, the first step of which catalyses the addition of a tripeptide to lysergic acid. This product can then interact with additional NRPS enzymes, including LPS1 and LPS2 for the addition of specific amino acids to create the desired ergopeptide compound (Fleetwood *et al*., 2007).

1.4.3 Indole diterpenes

Indole diterpenes are found in both the *Neotyphodium* and *Epichloë* species, mainly *N. lolii* and *E. festucae*, and are responsible for anti-mammalian effects. The main compound produced is that of lolitrem B, the causative agent of ryegrass staggers in grazing animals (Gallagher *et al*., 1981). The synthesis of the indole diterpene lolitrem B requires a complex cluster of 10 genes interspersed with transposon relics (the *LTM* cluster) (Young *et al.*, 2006). These genes encode the enzymes for catalysing the various biochemical steps, although only four of these genes are required to synthesise the intermediate compound paspaline (Young et al., 2006). For active synthesis of lolitrem B the *LTM* cluster must be complete and to date only

two endophytes (*N. lolii* and *E. festucae)* contains the complete *LTM* cluster, although lolitrem synthesis genes have been found in other species (Young *et al.*, 2009).

1.4.4 Pyrrolopyrazines

Peramine is the only known pyrrolopyrazine produced by grass endophytes (Bush *et al*., 1997). This metabolite is an insect feeding deterrent, especially against Argentine stem weevil and is an ideal secondary metabolite for expression in pasture systems because of its activity against insects (Rowan, 1993). This is especially the case in New Zealand where the Argentine stem weevil is an invasive pest causing significant damage to perennial ryegrass (Rowan *et al.*, 1990). The biosynthesis of peramine has been shown to be due to the presence of the *perA* gene. This gene is solely responsible for peramine production and encodes a two module NRPS enzyme that is proposed to catalyse all the steps in peramine synthesis (Tanaka *et al.*, 2005). Studies of the *perA* NRPS gene in *Epichloë* and *Neotyphodium* endophytes have found the *perA* gene is present in different species, while their distribution suggests a common ancestor (Johnson *et al.*, 2007). However, not all species that carry the gene are able to synthesise peramine. *N. uncinatum* is one such species and is shown to carry at least one homologue of the *perA* gene thought to be contributed from its ancestor *E. typhina or E. bromicola* (Johnson *et al.*, 2007). However peramine has not been detected in plants infected with *N. uncinatum,* suggesting that the gene has undergone mutation and is no longer functional.

1.5 Agricultural Application of Grass-Endophyte Associations

Grass endophytes have a wide practical application within pastoral systems due to their ability to enhance their hosts against both biotic and abiotic stresses (Rasmussen *et al*., 2008, Rodriguez *et al*., 2009). A major benefit to pasture growers from endophytes is their production of secondary metabolites conferring resistance to insect herbivory and thus reducing pasture losses *(*Patchett *et al.,* 2008a, Bush *et al*., 1997). However while these secondary metabolites may deter insect herbivory they may also have effects on grazing animals, resulting in significant production losses. Therefore a balance between the use of endophytes to increase pasture growth and reduce insect herbivory while incurring potential negative effects on grazing animals must be established (Williams *et al*., 2007). Not all endophytes produce the same metabolite profiles and some may not produce negative metabolites at all, which may better suit pasture grazing situations. However a large part of the production of metabolites and an endophytes ability to enhance the host depends on the grass-endophyte association itself.

1.5.1 Natural Associations

Endophytes will naturally associate and form symbiotic relationships with particular grass species. When using a single grass for agricultural purposes it is common that one endophyte species will dominate within the pasture population. For instance the major forage crop of New Zealand, perennial ryegrass, naturally associates with

Neotyphodium lolii. The resulting association produces a specific range of metabolites that may protect against insect herbivory yet also have the potential to have negative effects on grazing animals. While perennial ryegrass is highly suited to grazing livestock its association with *N. lolii* results in production of lolitrems and ergovaline which may have major negative effects on grazing livestock (Bush *et al*., 1997, Gallagher *et al*., 1981, Lean, 2001, Rowan & Shaw, 1987). The grass host would benefit strongly in a symbiotic relationship with endophytes that deterred not only insect herbivory but animal herbivory as well. It is only with human intervention and utilisation of grass for animal production that this relationship and protection for the grass is seen as a negative one.

1.5.2 Establishing synthetic associations

While the production of secondary metabolites may be beneficial to the grass, it may not always be beneficial in pasture systems. Metabolites such as lolitrem and ergovaline may have significant effects on animals and where possible pasture grasses that do not produce or produce very low amounts of these negative metabolites are desired (Lean, 2001). Therefore the potential exists, using inoculation techniques, to infect grass hosts with endophytes that only produce anti-insect metabolites for greater pasture crops (Williams *et al*., 2007). While these inoculation techniques allow new endophyte-host interactions they may also significantly add to the breeding process due to increased testing of each new association and its stability (Lee *et al.*, 2011). Perennial ryegrass, being the major

forage crop, is the focus of many of these synthetic associations often with the aim of producing a secondary metabolite range with a higher or broader resistance to insect pests. Often these metabolites are targeted towards a specific pest species that is problematic in certain areas or towards certain cultivars (Lee *et al*., 2011, Williams *et al*., 2007). For instance endophytes from meadow fescue have been shown to produce the secondary metabolites lolines, which give high protection against insect pests such as grass grub, while current ryegrass endophytes give little activity against grass grub (Lee *et al*., 2011). Therefore a major goal of seed companies is to artificially associate a ryegrass cultivar with loline producing endophytes, however this is difficult due to incompatibility barriers between potential hosts and endophytes.

To improve inoculation of new hosts, the use of a hybrid grass cultivar to overcome incompatibility between endophyte and host may be a desirable alternative. Hybrid cultivars between perennial ryegrass and meadow fescue exist that may contain the necessary genetic backgrounds from both parents to potentially overcome incompatibility barriers to colonisation by certain endophyte species, yet retain agronomically important ryegrass characteristics (Hopkins *et al.*, 2009). In the case of *N. uncinatum,* which is unable to efficiently infect perennial ryegrass, a hybrid cultivar between ryegrass and the *N. uncinatum* natural host, meadow fescue, may provide a more stable genetic background to permit colonisation. However, it is yet to be determined how the genetic makeup of such hosts may affect endophyte colonisation potential or the production of specific secondary metabolites.

1.5.3 Festulolium Hybrids

Festulolium refers to intergenic hybrids obtained through natural or synthetic breeding of *Festuca* (fescue) and *Lolium* (ryegrass) species (Ghesquière *et al.*, 2010b). The *Festuca* and *Lolium* grasses contributing to these hybrids are often from commercially important species with the aim of utilising grass characteristics from each of the cultivars (Ghesquière *et al.*, 2010a). These grasses share many characteristics and both are used for pasture production in New Zealand. *Lolium* species, in particular *Lolium perenne,* are preferred due to higher yields and nutritional quality. However, they lack resilience against abiotic stress and for this reason it is ideal to create hybrids with *Festuca* species, which have high abiotic stress tolerance (Ghesquière *et al*., 2010a, Williams *et al*., 2007). The resulting hybrid Festulolium offspring may result in characteristics of both parents to achieve high yield, nutritional quality and abiotic stress tolerance. These hybrid grasses may carry over the endophyte colonisation from one of the host grasses (Chikusansochiken, 2002) or can be further enhanced by association with a particular endophyte through manual inoculation techniques. Manual inoculation could utilize the genetic background of hybrids in order to use endophytes from *Fescue* species that cannot normally colonise into *Lolium* species and vice versa. However, the proportion of compatible parent genome in hybrids that would allow such colonisation to occur is unknown.

Genomic *in situ* hybridisation can be used to determine the genetic make up of Festulolium hybrids and allows the contribution from each parent to be determined (Zwierzykowski *et al.*, 2011). Future experiments could allow the genetic

contribution to be determined and elucidate the minimum amount of a particular host grasses genomic contribution to allow for its associated endophyte to colonise a hybrid grass. The use of chromosome mapping and molecular markers may also be able to determine specific genome regions and genes associated with endophyte colonisation in general.

1.6 Aims and Objectives

SSR loci have previously been used for rapid identification of endophyte strains and species (Moon *et al*., 1999). Using the B10 and B11 repeats *N. uncinatum* strains can be separated into four clonal groups based on PCR product size. However, it is unknown what the SSRs themselves look like within these products and whether they can distinguish individuals within clonal groups. Therefore one objective of this thesis was:

- To analyse the sequence of B10 and B11 SSRs between *N. uncinatum* strains to determine if SSR sequence can distinguish between individuals in clonal groups.

Endophyte strains may produce higher or lower levels of secondary metabolites depending on their host, environmental conditions and exposure to stress conditions. For commercial benefit the strains that produce the highest levels of secondary metabolites, such as lolines, will be of higher value in plant breeding development than low producers. Therefore a key objective of this thesis was to:

- Measure loline levels produced by strains of *N. uncinatum* in a common host plant, under controlled environmental conditions to determine whether production of lolines is strain specific.

Commercialisation of endophyte strains producing high levels of the protective metabolite lolines in a perennial ryegrass host has been a significant objective for plant breeders. However many endophytes used in ryegrass produce lolines as well as undesirable metabolites with negative health effects on animals. The endophyte *N. uncinatum* however, has been found only to produce lolines, and may represent a key species for introducing high loline levels to pasture grasses. Unfortunately compatibility barriers exist between *N. uncinatum* and perennial ryegrass, which do not allow for direct inoculation and colonisation. Therefore hybrid cultivars between perennial ryegrass and meadow fescue have been used which allow for the inoculation and colonisation of endophyte into a ryegrass-like host to generate hybrids with both ryegrass plant characteristics and endophyte loline production. Cropmark Seeds Ltd (Darfield, Canterbury) has developed such hybrid grasses and holds the patent to the *N. uncinatum* strain 'U2', a high producer of lolines. They aim to produce a commercial grass with high agronomic quality and also high loline levels utilising inoculation techniques. Understanding how *N. uncinatum* colonises these hybrid hosts will be important for the future development of these hybrid grasses. Therefore key objectives of this study were:

- To study the colonisation of *N. uncinatum* strains within different host grasses to determine association stability and growth patterns

- To study the effect of seed transmission on *N. uncinatum* endophytes in hybrid plants.

2. Materials and Methods

2.1 Biological materials

Table 2.1: Bacterial strains, fungal strains and plant material

Biological material	Relevant characteristics	Reference
Fungal strains		
E. festucae		
PN2278 (Fl1)	Wild type	Young et al. 2005
N. uncinatum		
PN2807	Wild type U2	This study
PN2809	Wild type U12	This study
PN2810	Wild type U3	This study
PN2811	Wild type U4	This study
PN2812	Wild type U6	This study
PN2813	Wild type U10	This study
PN2815	Wild type U13	This study
PN2816	Wild type U5	This study
PN2817	Wild type U7	This study
Bacterial strains		
E. coli		
DH5α	F^-, ϕ80*lacZ*, ΔM15, Δ(*lacZYA-argF*), U169, *recA1*, *endA1*, *hsdR17* (r_k^-, m_k^-), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*	Invitrogen
Plasmids		
pGEM®-T	Amp^R	Promega
Plant Material		
L. perenne	cv.Samson:Fl1	This study
F. pratensis	cv.FHAB0802:U2,U3,U4,U5,U6,U7,U9,U10, U12,U13	This study
Festulolium	cv.FHBDF0802:U2	This study
Festulolium	cv.FL1466:U2	Cropmark Seeds Ltd
Festulolium	cv.FL1436:U2	Cropmark Seeds Ltd
Festulolium	cv.FL1432:U2	Cropmark Seeds Ltd

2.2 Common stocks, growth media and conditions

2.2.1 Growth media

All media were prepared with milli-Q water and sterilised at approximately 121°C for 15-20 min.

2.2.1.1 Luria Bertani medium (LB)

LB medium contained 85 mM NaCl, 1% (w/v) tryptone and 0.5% (w/v) yeast extract, with 1.5% (w/v) agar added if required.

2.2.1.2 Malt Extract Agar

Malt Extract medium contained 1.275% (w/v) maltose, 0.275% (w/v) dextrin, 0.235% (w/v) glycerol, 0.0078% (w/v) peptone and 1.5% (w/v) agar.

2.2.1.3 Potato Dextrose medium (PD)

PD medium contained 2.4% (w/v) potato dextrose with 1.5% (w/v) agar if required.

2.2.1.4 Regeneration medium (RG)

RG medium contained 2.4% (w/v) potato dextrose, 0.8 M sucrose and 1.5% (w/v) agar.

2.2.1.5 SOC medium

SOC medium contained (per L) 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4.7H_2O$, 20 mM glucose, 2% (w/v) tryptone and 0.5% (w/v) yeast extract.

2.2.2 Growth conditions

2.2.2.1 *Escherichia coli*

E. coli cultures were grown at 37°C overnight on LB plates or in LB broth with shaking at 200 rpm. If required ampicillin was added to 100 µg/mL. Cultures were maintained at 4°C for short periods.

2.2.2.2 *Epichloë festucae*

E. festucae cultures were grown at 22°C on PD plates for 5-7 days or in PD broth with shaking at 150 rpm for 4-6 days.

2.2.2.3 *Neotyphodium uncinatum*

N. uncinatum cultures were grown at 22°C on Malt extract agar plates for 25-50 days, or in PD broth with shaking at 150 rpm for 10-16 days. Cultures were stored at 4°C over periods of several months, or at -80°C in 50% (v/v) glycerol for long term storage.

2.2.2.4 Plant growth conditions

L. perenne, F. pratensis and Festulolium *cv.* were grown under controlled conditions at 22°C with a photoperiod of 16 hrs.

2.3 Standard *E. coli* methods

2.3.1 DNA cloning into an *E. coli* plasmid vector

Cloning of PCR fragments into an *E. coli* plasmid vector was performed using pGEM®-T cloning kit (Promega) following the manufacturer's instructions.

2.3.2 *E. coli* transformation

One tube of DH5α chemically competent cells per transformation was thawed on ice and 500 ng of plasmid was added and incubated on ice for 20 min. Cells were then heat shocked at 42°C for 1 min and placed in ice for a further 2 min. Nine hundred µL of SOC media was added and incubated at 37°C with shaking for 1 hr. After which 30 and 300 µL of transformant mixture was plated onto LB agar plates containing 100 µg/mL ampicillin for selection.

2.3.3 Screening of *E. coli* transformants

E. coli colonies transformed with plasmid were selected for white colonies after streaking on LB agar plates with 100 µg/mL ampicillin, 0.1 mM IPTG and 40 µg/mL X-Gal. Colonies were transferred using a sterile toothpick and grown on LB agar plates with 100 µg/mL ampicillin, and plates were incubated at 37°C overnight. Resulting colonies were picked using a sterile yellow (2-200 µL) pipette tip and

resuspended in screening PCR mixture. A screening PCR was run and resulting PCR fragments analysed by gel electrophoresis for presence of plasmid containing insert.

2.3.4 Plasmid isolation

Plasmids were isolated using the commercially available High Pure Plasmid Isolation Kit (Roche), as per manufacturer's instructions.

2.4 Standard *N. uncinatum* cell methods

2.4.1 Fungal DNA extraction

Fungal colonies were grown on malt extract agar at 22°C for approximately 14-30 d. A 0.5 cm^2 piece of mycelia from colony outer edge was ground in a 1.7 mL microfuge tube using a micropestle, and used to inoculate 50 mL of PD broth that was then incubated at 22°C with 200 rpm shaking. Once the culture had grown sufficiently (~ 14 d) the mycelia was filtered through a nappy liner, washed with H_2O and frozen at -80°C. Frozen mycelium was then freeze dried and stored at -20°C. Freeze dried material of 0.015 g was ground under liquid nitrogen in a mortar and pestle. Ground mycelia was resuspended in 800 µL DNA extraction buffer (150 mM EDTA, 50 mM Tris, 1% (w/v) SDS, pH 8.0) and proteinase K added to 2 mg/mL followed by incubation at 37°C for 20 min. The resulting solution was centrifuged at 13000 rpm and the supernatant transferred to a new tube. Half volumes of phenol and chloroform were added followed by brief vortex and centrifugation for 10 min at 13000 rpm. The aqueous phase was removed to a new tube and the phenol-chloroform extraction step repeated twice more. Following this step 1 volume of chloroform was added to the resulting aqueous phase, mixed and

centrifuged for 10 min at 13000 rpm. The aqueous phase was then removed and added to 1 volume isopropanol, mixed and left for 10 min at room temperature to precipitate the DNA followed by centrifugation for 10 min at 13000 rpm. The supernatant was discarded and the DNA pellet washed with 1 mL of 70% (v/v) ethanol. The pellet was air dried at room temperature until all ethanol was removed and resuspended in sterile Milli-Q H_2O.

2.5 Standard plant methods

2.5.1 Plant inoculation

Seeds were surface sterilised by soaking in 50% bleach solution (sodium hypochlorite 4.2% w/v) for 20 min followed by rinsing in sterile Milli-Q H_2O for 30 seconds. This was repeated 3-5 times depending on the seed line used. Seeds were then soaked in sterile Milli-Q H_2O for 2 min, air dried on filter paper and plated onto 3% (w/v) water agar plates. Seedlings were then germinated in the dark at 22°C for 7 days. After germination, a shallow 2-3 mm longitudinal slit between the mesocotyl and coleoptiles was made and a small piece of fungal mycelia inserted into the cut. Seedlings were then returned to the dark at 22°C for a further 7 days before being transferred to light for a further 7 days at 22°C. Healthy seedlings were then planted into root trainers containing potting mix.

2.5.2 Immunoblotting of plants

Four to six weeks after seedlings were planted (providing sufficient plant growth) a tiller was cut as close to the base as possible from each plant, outer and dead leaf sheaths were removed and the exposed cut pressed onto a nitrocellulose membrane

(NCM). The process was repeated for all plants to be tested. The NCM was immersed into freshly made blocking solution (20 mM Tris, 50 mM NaCl, 0.5% (w/v) non-fat milk powder) for 2 hours at room temperature. The blocking solution was then decanted off and replaced with 5 mL of blocking solution containing 5 µL of primary antibody (1:1000) (polyclonal rabbit antibodies raised against homogenised mycelium of *Neotyphodium lolii*, Christensen *et al*., 1993) and incubated with slow shaking (15 rpm overnight at 4°C. The solution was then decanted off and the NCM washed 3 times in fresh blocking solution. Five millilitres of fresh blocking solution and 2.5 µL of secondary antibody (1:10000) (goat anti-rabbit antibody with an alkaline phosphatise conjugate, Sigma) was then added and incubated with shaking for 2 hrs before decanting the solution and washing the membrane in fresh blocking solution three times. Developing solution was prepared using SIGMAFAST^{TM} Fast Red TR/Naphthol AS-MX tablets, as per the manufacturer's instructions. This was then added to the membrane and incubated with shaking at room temperature for 15 min before decanting off and washing in sterile H_2O. The membrane was then dried on blotting paper and the results visualised.

2.5.3 Preparation of infected grass for confocal microscopy

Pseudostem samples were taken from endophyte infected plants by cutting near the base using a scalpel, removing any dead outer layers and peeling of sections of leaf tissue from the pseudostem. The bottom 1 cm was removed and sections cut in half longitudinally before incubated in 95% (w/v) ethanol at 4°C for at least 24 hours. Samples were then treated in 10% (v/v) potassium hydroxide for 3 h, washed three times in PBS (137 mM NaCl, 2.7 mM KCL, 8.1 mM Na_2HPO_4, 1.76 m M KH_2PO_4,

pH 7.4), and incubated in staining solution (20 µL of 1% (w/v) aniline blue, 10 µL of 2% (v/v) Tween 20, 10 µL of 1 mg/mL Alexa Fluor® 488 WGA (Invitrogen), made up to 1 mL in PBS) for 30 mins, including a 10 min vacuum infiltration.

2.5.4 Confocal microscopy

Imaging of samples was done using a Leica SP5 DM6000B confocal microscope at the Manawatu Microscopy and Imaging Centre. A final magnification of 400× was used, and images were taken of hyphae just below the plant epidermal cells. Optical sections of 2 µm were taken with a final depth of 50 µm. Image modifications were performed using the ImageJ processing package *(*http://rsb.info.nih.gov/ij/).

2.5.5 Transmission Electron Microscopy (TEM)

Small pieces (0.5-1 mm thick) of pseudostem tissue from endophyte infected plants were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2 for 1 h and then transverse sections prepared for light microscopy and TEM (Spiers & Hopcroft, 1993), by the Manawatu Microscopy and Imaging Centre. For light microscopy, the sections were stained with toluidine blue (Christensen *et al.*, 2002). A Philips CM10 transmission electron microscope was used to examine hyphal structure and images were recorded using a SIS Morada digital camera.

2.5.6 PCR test of endophyte localisation in plant

Plant material from endophyte infected plants was harvested from pseudostem, leaf blade and root tissue and frozen at -80°C. Plant material was ground in liquid nitrogen with a mortar and pestle after which DNA was extracted using the DNeasy

Plant Mini Kit (Qiagen) as per manufacturer's instructions. PCR was performed using Phusion® High Fidelity DNA Polymerase (NEB) with primers Tef 1d and Tef 1u to yield an endophyte specific product of 864 bp.

2.6 Standard DNA methods

2.6.1 Measurement of DNA concentration

The concentration PCR products and plasmid DNA was measured on an Implen Nanophotometer™ as per the manufacturer's instructions. gDNA concentration was measured by gel electrophoresis with standards of known concentration. After staining with Ethidium bromide and visualisation on a Geldoc, the intensity of the gDNA sample and standards were compared to predict concentration and visualise any degradation of gDNA.

2.6.2 DNA sequencing

DNA sequencing was performed by the Massey Genome Service using BigDye™ Terminator (version 3.1) Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Samples containing 500 ng of purified plasmid and 6.4 pmol primer were made up to 15 μL using milli-Q H_2O. Sequence analysis was performed using MacVector® version 10.0.2

2.6.3 Polymerase Chain Reaction (PCR)

Primers used in this study are listed in Table 2.2

Table 2.2: Primers used in this study

Nam	Sequence (5'-3')	Used for
B10.1	CGCTCAGGGCTACATACACCATGG	Amplify B10
B10.2	CTCATCGAGTAACGCAGGCGACG	Amplify B10
B11.1	CATGGATGGACAAGAGATTGCACG	Amplify B11
B11.4	TTCACTGCTACAATTCTGTCCAGC	Amplify B11
B12.1	TGTAGGAGGCCAACCTTTTG	Amplify B12
B12.2	CTTGTTCACCACTCGAAGCA	Amplify B12
bZ1.1	ATGAATCGATCACCATCAGC	Expression analysis of B10
bZ1.2	CATCGAGTAACGCAGGCGAC	Expression analysis of B10
bZ1.3	AAACGAAAGGGCACCCGAAG	Expression analysis of B10
M13F	TGTAAAACGACGGCCAGT	Sequencing
M13R	GAGCGGATAACAATTTCACACAG	Sequencing
Tef 1d	GGGTAAGGACGAAAAGACTCA	PCR plant screen
Tef 1u	CGGCAGCGATAATCAGGATAG	PCR plant screen

2.6.3.1 Standard PCR

Standard PCR amplification was performed with Taq DNA polymerase (Roche Diagnostics). PCR reaction mixtures contained 20.4 µL sterile Milli-Q H_2O, 2.5 µL PCR buffer (supplied with polymerase), 0.5 µL primer 1 (10 pM), 0.5 µL primer 2 (10 pM), 1µL dNTP mix (1.25 mM), 0.1 µL Taq DNA polymerase (5 U/µL) and 1 µL of template DNA (10 ng/µL) to a total of 25 µL. The reaction mixture was then gently mixed and run in a thermocycler using the following scheme: Heat lid 95°C; 1. 95°C 2 min; 2. 95°C 30 sec; 3. 60°C* 1 min; 4. 72°C 1 min; Go to 2, repeat x30; 5. 72°C 10 min; Hold at 4°C.

* Actual annealing temperature dependent on T_m of primers used.

2.6.3.2 Phusion high fidelity PCR

PCR products to be used for the determination of nucleotide sequence were amplified using Phusion® High Fidelity DNA Polymerase (NEB). Reaction mixtures contained 16.25 µL sterile Milli-Q H_2O, 5 µL PCR buffer (supplied with polymerase), 1.25 µL primer 1 (10pM), 1.25 µL primer 2 (10 pM), 1 µL dNTP mix (1.25 mM), 0.25 µL polymerase and 5 µL of template DNA (1 ng/ µL) to a total of 25 µL. The reaction mixture was then gently mixed and run in a thermocycler using the following scheme: Heat lid 98°C; 1. 98°C 2 min; 2. 98°C 30 sec; 3. 60°C* 1 min; 4. 72°C 1 min; Go to 2, repeat x30; 5. 72°C 10 min; Hold at 4°C.

* Actual annealing temperature dependent on T_m of primers used.

2.6.4 Gel electrophoresis

Agarose gels were made by heating the desired amount of agarose (0.7% w/v to 1.8% w/v) with 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) in a microwave until fully dissolved. The liquid agarose was cooled and held at 56°C before being poured into a gel tray with desired well moulds inserted and left to set. Once set well moulds were removed and the gel immersed in 1x TBE buffer. Samples were mixed with SDS loading dye and inserted into wells before apparatus was turned on with the power pack set to the desired voltage. Once run the gel was removed and submersed for 10-15 min in ethidium bromide solution (1 mg/mL ethidium bromide in Milli-Q H_2O). The gel was then destained for 15 min in tap water and visualised using a UV Transilluminator Gel Documentation System (Bio-Rad).

2.6.5 A-tailing PCR fragments

To facilitate TA cloning into pGEM-T vector PCR products produced by the proof reading enzyme, Phusion, were A-tailed as follows. One μL of Taq polymerase was mixed with PCR products and 1 μL of dNTPs added. The reaction was mixed well and incubated at 70°C for 30 min in a thermal cycler.

2.7 Standard RNA methods

2.7.1 RNA isolation

Total RNA was extracted from *E. typhina* E8 mycelium using TRIzol® reagent (Invitrogen). For isolation approximately 1 g of mycelium was ground in liquid nitrogen in a mortar and pestle before addition of 1 mL of TRIzol. The mixture was allowed to thaw at room temperature before being transferred to a 15 mL tube and centrifuged at 9,700 rpm at 4°C for 10 min. The supernatant was transferred to a fresh tube and 200 μL of chloroform added. The solution was mixed thoroughly and allowed to sit at room temperature for 3 min before being centrifuged at 9,700 rpm at 4°C for 15 min. The aqueous phase was then transferred to a new tube and 500 μL of isopropanol added before incubation at room temperature for 10 min to allow RNA to precipitate. Samples were then centrifuged at 9,700 rpm at 4°C for 10 min. The supernatant was discarded and 1 mL of 75% EtOH added to the RNA pellet before being centrifuged at 6,700 rpm at 4°C for 5 min. The pellet was air dried and resuspended in 100 μL of DEPC-treated H_2O for further analysis.

2.7.2 RT-PCR

One microgram of *E. typhina* total RNA was heat denatured and reverse transcribed using SuperScript™ II RT (Invitrogen) according to the manufacturer's instructions. cDNA was then used as a template for PCR reactions specific to genes of interest.

2.8 Loline Analysis

2.8.1 Growth and harvest of plant material

Seedlings of the plant variety *Festuca pratensis* FHAB0802 were grown and infected with *N. uncinatum* strains as previously described (section 2.5.1). After potting into root trainers seedlings were grown to mature plants. After 15 weeks, plants were removed and root material washed with tap water to remove excess soil. Tissue from root, leaf blade and pseudostem were then isolated and frozen at -80°C, after which material was freeze dried and stored at -20°C. A minimum of three plants were infected with each strain to be used for analysis.

2.8.2 Loline extraction

Material was analysed at Cropmark Seeds Ltd where 0.25 g plant material was added to 5 ml of solvent (95:5 dichloromethane : ethanol) in an 8 ml glass vial. This solvent also contained phenylmorpholine (6 mg/100 ml) as an internal standard. 250 µl of saturated sodium bicarbonate solution (2 g/10 ml) was added to the vial. The vials were shaken on an orbital shaker for 1 h. The vial was left to settle and the supernatant filtered using a plugged Pasteur pipette into a clean vial. 1 ml of this extract was then transferred to a GC vial for analysis. GC analysis was then performed by Cropmark Seeds Ltd.

2.8.3 Statistical analysis

The statistical difference between loline levels within plants were analysed using the *t*-test and the Tukey post hoc analysis using Minitab® Statistical Software. F-tests were performed to establish equality of variance before *t*-tests were performed. Two-tailed *t*-tests were used with significance level of $P<0.05$.

2.9 Bioinformatic methods

2.9.1 Sequence comparison and domain characteristics

Comparison of sequences was performed at the National Centre for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/) using the BLASTn, BLASTp and BLASTx algorithms. Amino acid and nucleotide sequence similarity was assessed using the MacVector™ ClustalW program. Predicted intron/exon boundaries were analysed by Fgenesh at the Softberry site (http://linux1.softberry.com/berry). Predicted protein domain structures were analysed using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/).

2.9.2 Sequence comparison of SSRs

SSR sequences from the Clavicipitaceae were obtained from the University of Kentucky Genome projects site (http://www.endophyte.uky.edu/). Sequence of *E. bromicola* 799 was obtained through direct sequencing. Sequence comparison was performed using the MacVector™ ClustalW program.

3. The use of SSR loci sequences to distinguish Epichloë endophytes

3.1 Localisation and polymorphism of B10 and B11 SSR

SSR loci consist of repeating sequences of 2-6 basepairs of DNA and have found wide use in identification of strains and species. In particular, the length of the B10 and B11 SSR loci have been used to identify endophyte species yet little is known of the repeat structure contained within them. The repeat structure of B10 and B11 loci was analysed from available Clavicipitaceae genome sequences (http://www.endophyte.uky.edu/) to understand how these repeats affect sequence structure and where they are located in the genome.

The genome sequences of *E. festucae* Fl1 and E2368, *E. brachyletri* E4808, *E. glyceriae* E277, *E. amarillans* E57, *E. typhina* E5819 and E8 were interrogated using BLASTn with the consensus sequence of the B10 and B11 SSR loci as a query (Accession: B10 AF063093, and B11 AF063094). The location within the genome was noted with respect to proximity to any flanking genes for each strain and the repeat sequence was aligned (Fig. 3.1, Fig. 3.2).

3.1.1 The B10 SSR

The B10 SSR was found to be within a gene encoding a putative bZIP transcription factor (EfM2.086180) and was polymorphic across the endophyte strains analysed (Fig. 3.1). The B10 repeat is made up of three separate trinucleotide repeats CAG, CAT and CAA, with each polymorphic between species and strains. The two *E. festucae* strains analysed had repeat sequences which were identical, except for an expansion of two trinucleotide CAG repeats found within the Fl1 strain which were absent from the E2368 strain. A comparison of *E. typhina* E5819 and E8 showed E8 contained two SNPs that generated CCG trinucleotides and an extra CAA repeat within the CAG and CAA regions. The length of trinucleotide repeats was polymorphic across endophyte species, while *E. glyceriae* and *E. typhina* both contained mixtures of trinucleotide units in regions previously consisting of only one repeated trinucleotide (Fig. 3.1).

3.1.2 The B11 SSR

The B11 repeat was found to be located immediately downstream of the 3' end of a predicted pyrazinamidase/nicotinamidase encoding gene (EfM2.098210), with one of the primers used for amplifying the SSR binding within the coding region of the gene and the repeat itself falling outside of the gene (Fig. 3.2). The B11 repeat was polymorphic in repeat length across most endophyte species, although *E. festucae* Fl1 and *E. typhina* E5819 contained the same repeat sequence. Single nucleotide polymorphisms were found to be present within the repeat sequences of *E. festucae*

Fl1, *E. amarillans* E57 and *E. typhina* E8. The B11 repeat of *E. festucae* E2368 was much larger than the Fl1 repeat, while the *E. typhina* E8 repeat was larger than E5819 and also contained an inserted G nucleotide (Fig. 3.2).

3.1.3 The B10 SSR is found within a hypothetical protein

Analysis of the location of the B10 SSR found that the repeat region was contained within the exon of a gene encoding a putative bZIP transcription factor (EfM2.086180). The repeat unit was found to code for a glutamine repeat with a histidine core. An example of how the SSR sequence can change the protein sequence is illustrated by a comparison of the *E. glyceriae* and *E. amarillans* repeats (Fig. 3.3). Alignment of the amino acid sequence between these endophyte strains showed high levels of conservation throughout the protein, except for the region containing the SSR (Fig. 3.4). The polypeptide sequence for each of the species and strains analysed showed that polymorphism and alteration of the SSR results in increased or decreased glutamine and histidine composition between species depending on which of the trinucleotides is altered (Fig. 3.4).

The protein sequence for the bZIP transcription factor from *E. festucae* Fl1 was used as a query with the protein domain prediction software SMART (http://smart.embl-heidelberg.de/smart) resulting in prediction of a coiled coil domain between amino acids 39 and 100. No signal peptide or transmembrane domains were predicted within the protein. Alignment of amino acid sequences from other fungal species showed high conservation of this coiled coil domain (Fig. 3.5). The B10 repeat region was conserved with the *Claviceps paspali* sequence and to a lesser extent

with that of *Periglandular ipomoeae,* but was not conserved in more distantly related species including *Magnaporthe oryzae, Neurospora crassa* and *Fusarium oxysporum*. The repeat region from *C. paspali* was much larger than that contained in *E. festucae* while the repeat region in *P. ipomoeae* was significantly smaller, containing few repeated residues (Fig. 3.5). RT-PCR showed that the gene was expressed within *E. typhina* and cDNA sequencing confirmed the presence of the SSR within the coding sequence. Size difference between gDNA and cDNA corresponded to predicted introns and confirmed splicing of the expressed product (Fig. 3.6).

3.1.4 Additional B10-like repeat within a copper sensing transcription factor

While analysing the B10 SSR, a second B10-like repeat was found within *E. festucae* Fl1. This was named B12 to follow the convention used previously to describe the repeats B1-B11 (Moon *et al*., 1999). This repeat consisted of a CAG-CAT-CAA repeat locus and was found within a gene encoding a putative copper sensing transcription factor (EfM2.078910). The repeat was further studied in related endophyte species and shown to be polymorphic across species (Fig. 3.7). Protein domain prediction using SMART prediction software revealed a putative copper fist domain at the N-terminus between amino acid residues 1 and 41, while no other functional regions were identified. Amino acid alignment across endophyte species showed high conservation across the entire protein, while polymorphism could be seen within the repeat region (Fig. 3.8). Alignment with more distantly related species *C. paspali, P. ipomoeae, M. oryzae, N. crassa* and *F. graminearum*

showed the repeat sequence was not conserved, while the N-terminus region containing the copper-fist domain remained highly conserved (Fig. 3.9).

Primers were designed to conserved regions identified using multiple sequence alignments of endophyte species and used to determine if *N. uncinatum* strains contained this repeat, and whether a single or double allele was present. PCR showed a single product of the same size (~300 bp) present in each ecotype and sequencing of this revealed no difference between repeats in the strains U2, U3, U4 and U5 representative of 4 separate ecotypes. Strain U6 from ecotype one was also sequenced and revealed a loss of a single CAG and CAA repeat and gain of a single CAT repeat. The repeat sequence from all ecotypes was similar to the proposed ancestral species *E. typhina*. Sequence of the other proposed ancestor, *E. bromicola*, was unavailable for comparison but is presumably lost or absent in *N. uncinatum*.

3.1.5 B10-like repeats are enriched within exons of the *Epichloë* genome

Upon finding a second B10-like repeat within the genome and contained within the exon boundaries of a second gene, a search was performed to determine the abundance of these repeats and their location. A BLASTn search for repeat elements consisting of different lengths of CAG, CAT and CAA was performed in both *E. festucae* Fl1and *E. typhina* E8, and the gene in which they were contained was noted (Table 3.1)(Appendix 7.1). Regions containing repeats were found to be within the exon boundaries of a variety of different genes, while only two repeats were found within intronic or non-coding regions. Repeats often coded for a string of glutamine

residues but depending on reading frame and the direction of translation a variety of amino acid repeats are predicted to be produced. In some genes multiple repeat elements were present that did not consist of repeated glutamine or histidine residues and were derived from sequence other than CAG, CAT or CAA repeats. Searches within the related species *Magnaporthe*, *Neurospora*, *Fusarium*, *Claviceps* and *Periglandular* were used to determine if repeats were specific to Epichloë endophytes, the Clavicipitaceae or common across distantly related fungi (Table 3.1). Of the 19 genes examined, five were found to be specific for the Epichloë endophytes while six were found within *Epichloë* and *Claviceps* or *Periglandular* representative of the Clavicipitaceae. The other eight genes were found to have similar repeats in other distantly related fungi including *Fusarium*, *Magnaporthe* or *Neurospora* (Table 3.1)

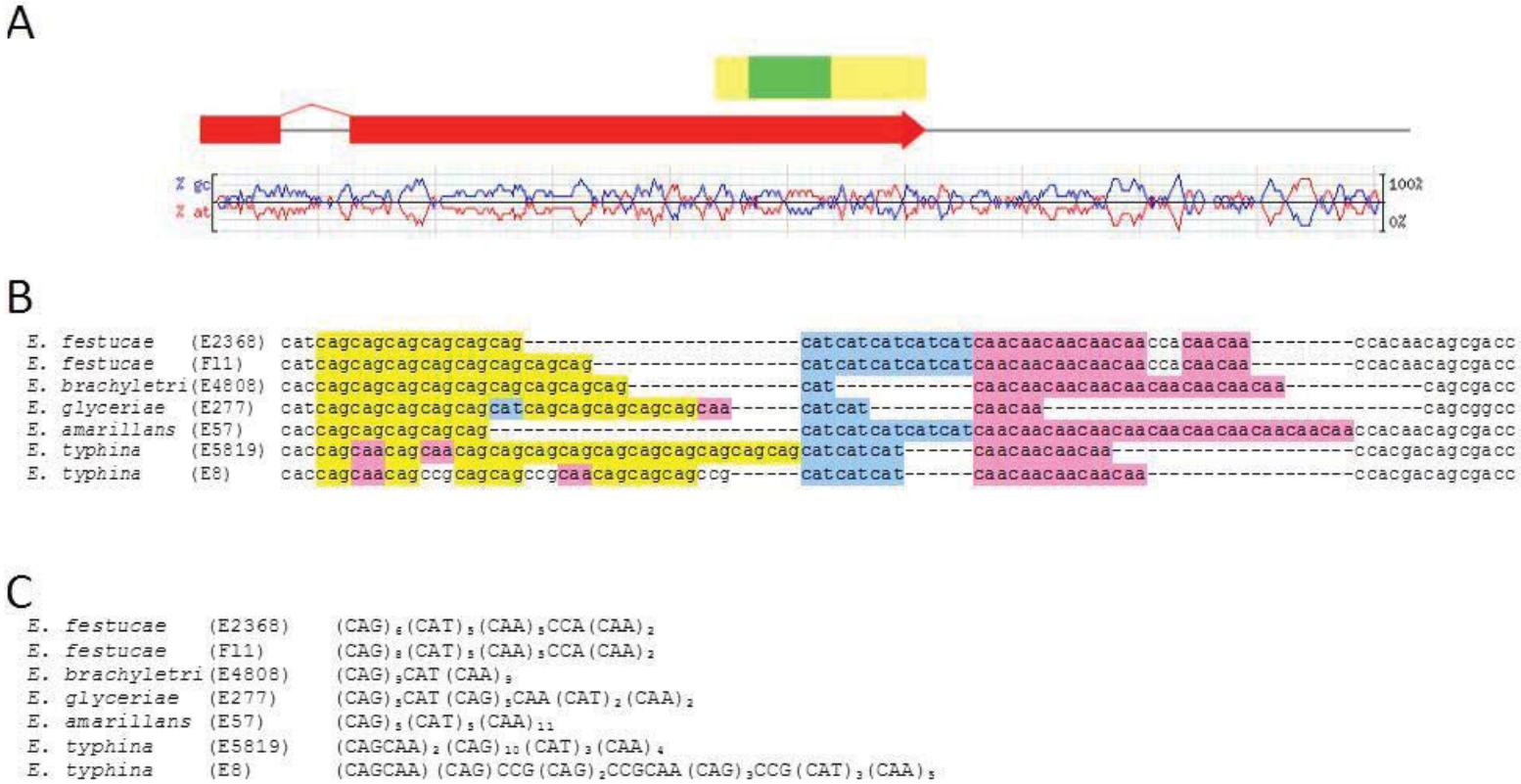


Figure 3.1: Overview of B10 SSR. A) Genomic region containing the B10 SSR. B10 primer amplified region as per primers B10.1 and B10.2 (yellow) and SSR (green). SSR length is representative of the *E. festucae* Fl1 B10 repeat. The SSR is within an exon of a putative bZIP Transcription Factor (EfM2.086180) (red). The region shown corresponds to a 1kb fragment centred on the SSR. B) Repeat sequence of (CAG)n(CAT)n(CAA)n between endophyte strains coloured by trinucleotide repeats, CAG (yellow), CAT (Blue), CAA (pink). Single nucleotide polymorphisms resulting in changes in trinucleotide repeats (CCG,CCA) remain uncoloured. C) Consensus sequences for each strain of endophyte identifying variety in repeat units.

Figure 3.2: Overview of the B11 SSR. A) Region containing the B11 SSR. B11 primer amplified region as per primers B11.1 and B11.4 (yellow) and SSR (green). SSR length is representative of the *E. festucae* Fl1 B11 repeat. The SSR lies 3′ of the end of a putative pyrazinamidase/nicotinamidase encoding gene (EfM2.098210) (red). Region shown corresponds to a 1kb fragment centred on the SSR. B) Repeat sequence of (GACA)n between endophyte strains coloured by green and yellow. One nucleotide difference in trinucleotide repeats are shown in red and coloured black. C) Consensus sequences for each strain of endophyte identifying variety in repeat units.

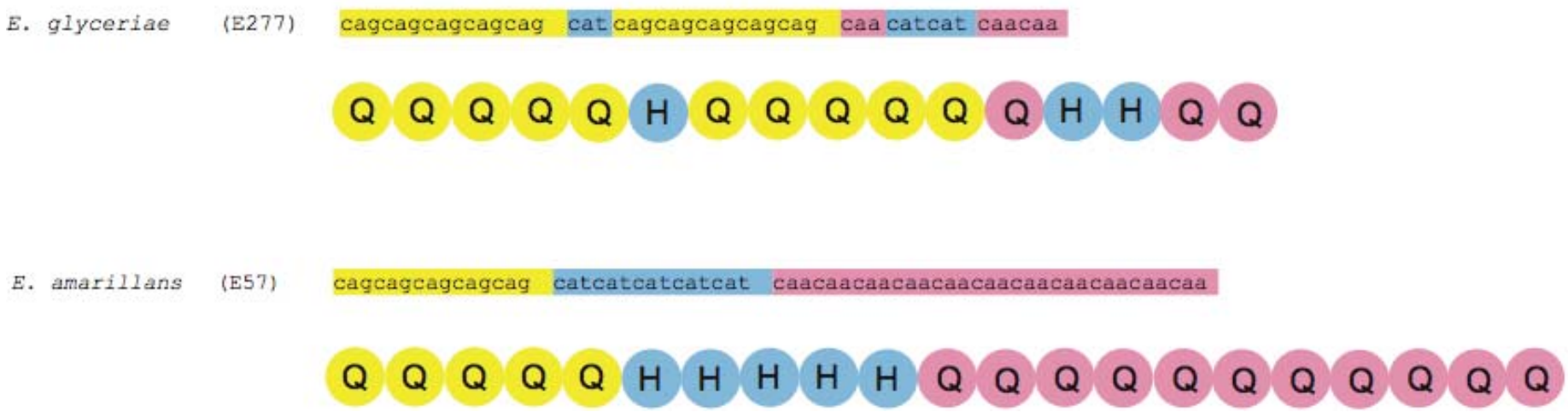


Figure 3.3: Consequences of B10 repeat changes on peptide sequence. Comparison of the B10 repeats of *E. glyceriae* and *E. amarillans* and the amino acid each trinucleotide produces. Repeats are coloured CAG (yellow), CAT (blue) and CAA (pink). Amino acids produced are coloured for the repeat sequence from which they are derived.

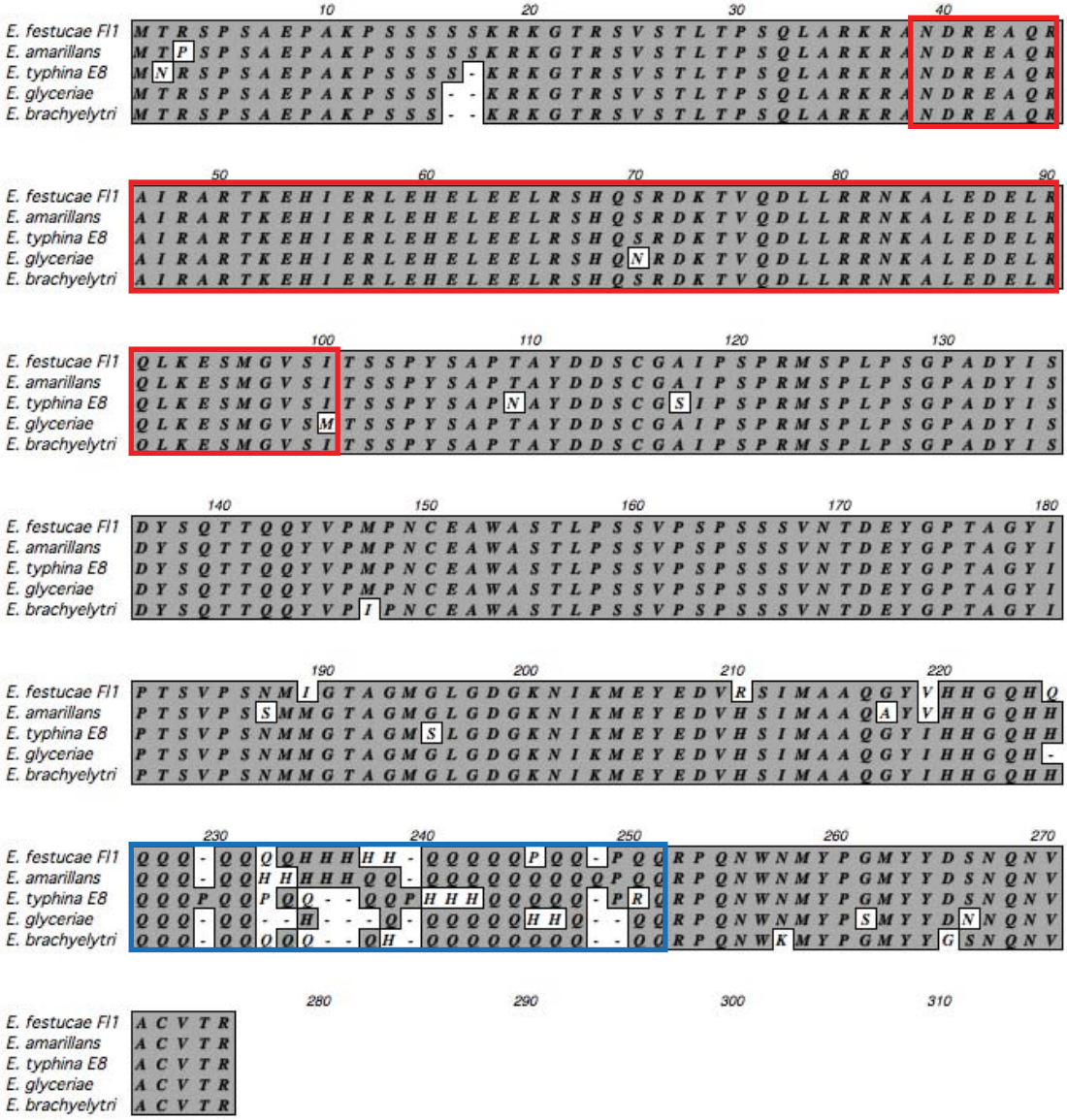


Figure 3.4: Amino acid alignment of bZIP transcription factor within *Epichloë* endophytes. Multiple sequence alignments of *Epichloë* species bZIP transcription factors. Predicted coiled-coil domain boxed in red. B10 SSR region boxed in blue.

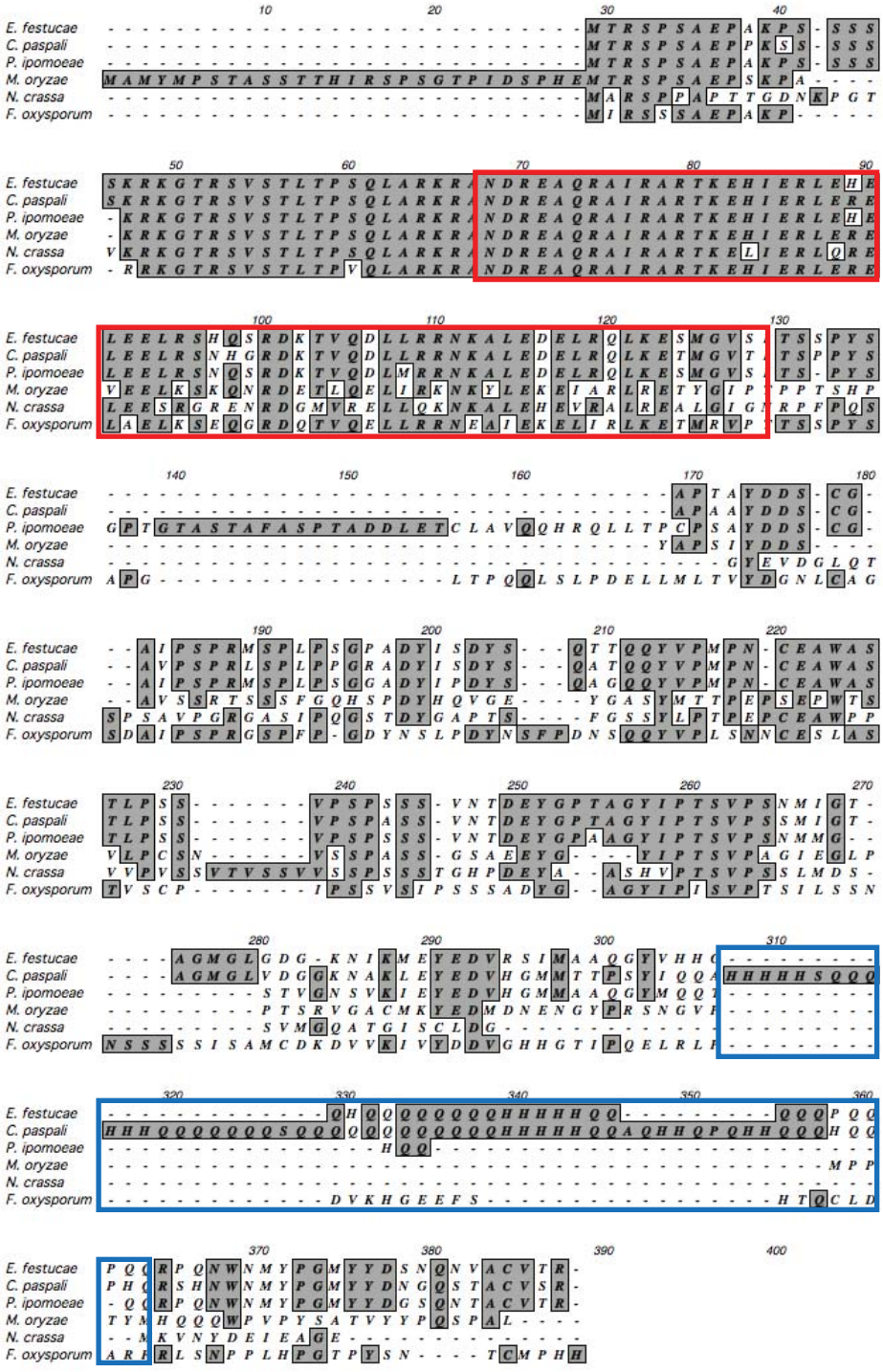


Figure 3.5: Amino acid alignment of bZIP transcription factor between *Epichloë festucae* Fl1 and related fungal species. Multiple sequence alignment showing conservation of domains and the B10 repeat region. Conserved coiled-coil domain boxed in red. Region containing B10 repeat boxed in blue.

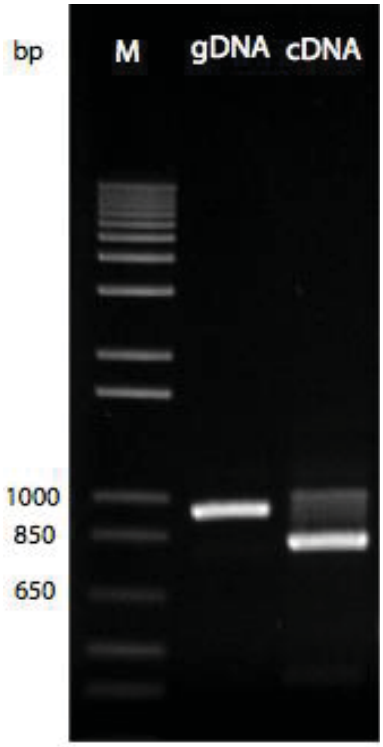


Figure 3.6: Expression of the bZIP transcription factor in *E. typhina*. Lane gDNA indicates genomic *E. typhina* DNA used as template. Lane cDNA indicates complementary DNA from RT-PCR used as template. Lane M shows 1kb+ ladder with corresponding size of relevant bands on the left. Size shift in band indicates splicing out of introns.

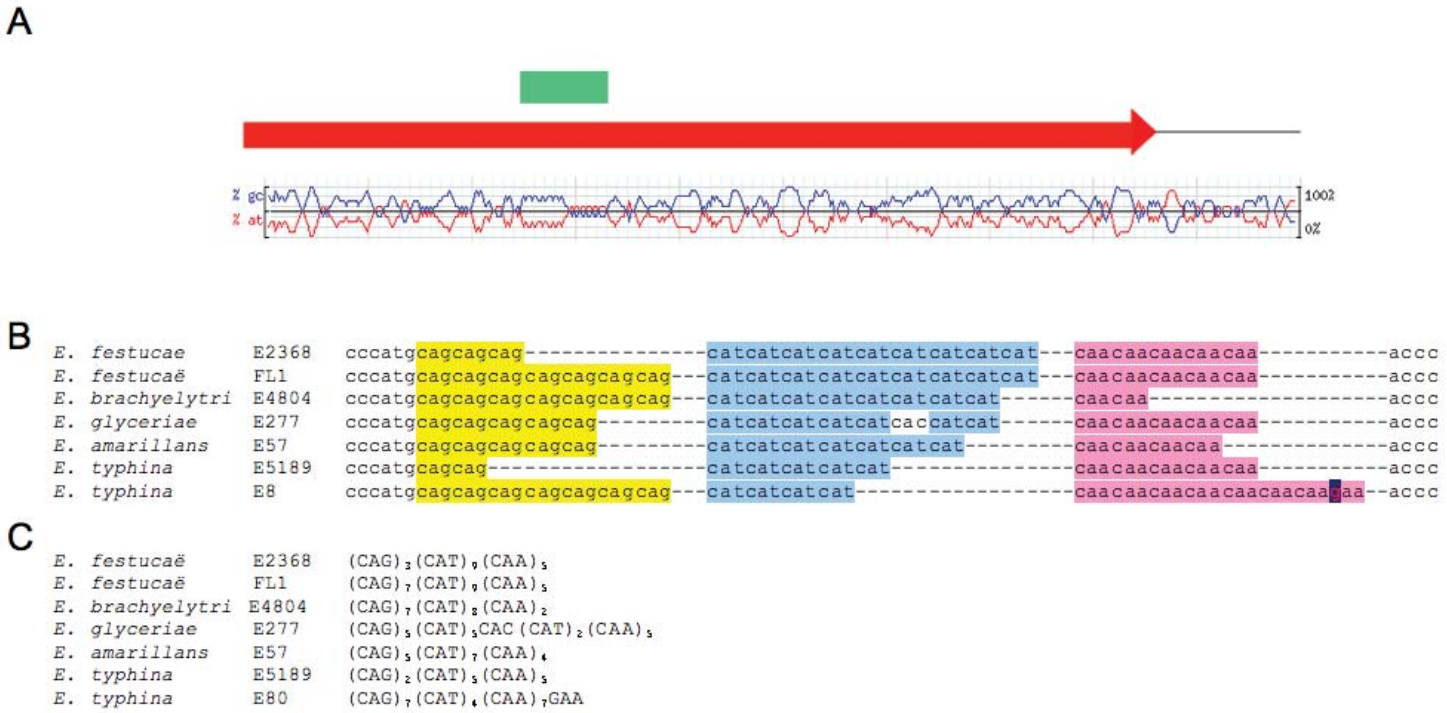


Figure 3.7: Overview of the B12 SSR. A) Region containing B12 SSR (green). SSR length is representative of the *E. festucae* Fl1B12 repeat. The region overlaps with a putative copper sensing transcription factor (EfM2.078910) encoding gene (red). The region shown corresponds to a 1kb fragment around the SSR. B) A consensus sequence of (CAG)n(CAT)n(CAA)n between endophyte strains coloured by trinucleotide repeats, CAG (yellow), CAT (Blue), CAA (pink). Single nucleotide polymorphisms resulting in changes in trinucleotide repeats (CAC) remain uncoloured. C) Repeat sequences for each strain of endophyte highlighting the variable repeat unit structures.

Figure 3.8: Amino acid alignment of the putative copper sensing transcription factor within *Epichloë* endophytes. Multiple sequence alignment of *Epichloë* species copper sensing transcription factors. Predicted copper fist domain boxed in red. B12 SSR region boxed in blue.

Figure 3.9: Amino acid alignment of the putative copper sensing transcription factor across the Sordariomycetes. Multiple sequence alignment showing conservation of domains and the B12 repeat region. Conserved copper fist domain boxed in red. Region containing B12 repeat boxed in blue.

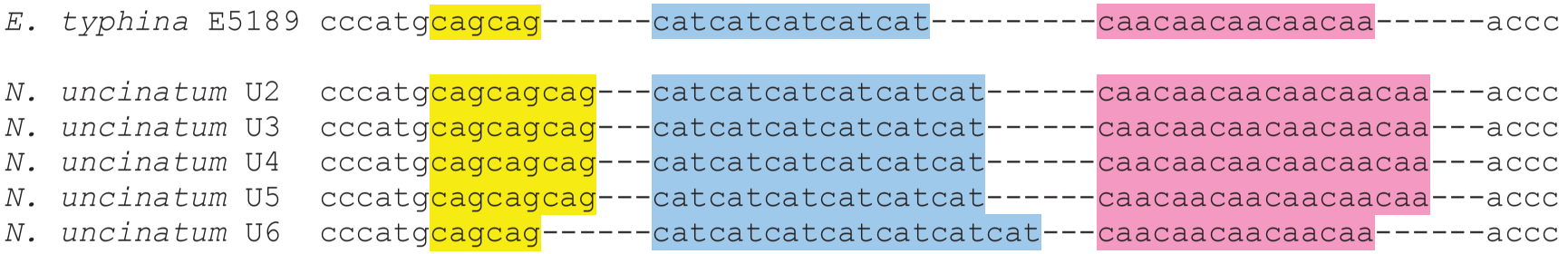


Figure 3.10: B12 repeat in *N. uncinatum*. B12 repeats of *N. uncinatum* including proposed ancestral species *E. typhina*. Trinucleotide repeat sequence CAG (yellow), CAT (blue) and CAA (purple) is highlighted.

Table 3.1: B10-like repeat search in fungal species

Repeat query	Top gene hits	Polypeptide repeats found	Specificity
CAG_5CAT_5			
	G protein coupled receptor	QQQQQQHHHHHHHPH	Epichloë
	Copper sensing transcription factor	QQQQQQQHHHHQQQQQQ	Epichloë
	Hypothetical protein	QQQQQQQHHH EDEDEDEDEDEDEDEDEDEDE	Epichloë
	Putatative GNAT family acetyltransferase	DDDNDDDDNDDNDDDDNDDNDDDDNDDNDDDDDDDD	Epichloë
	polyA nuclease	QQQQQQQQQQQQHHQQPQQ	Common
	Cyclin dependent protein kinase complex component	QQQQHQQHQHHHQQHQHHHQQHQQHQHYQQQHH	Common
	Hypothetical protein	QHQHQHQHQQQQQQHHHHHHQ	Clavicipitaceae
$CAG_5CAT_5CAA_5$			
	Hypothetical protein	YYYYYYYYYYYYYYYYYYYYYYYY HNHHSHHHNQQQQQQQQQQQQQQQQ HHHHHQQQQQ	Clavicipitaceae
	Complex I intermediate associated protein 30	SSSSSSSTPTTT	Clavicipitaceae
	bZIP transcription factor	QQQPQQPQQQQPHHHQQQQQ	Clavicipitaceae
	Ribosomal protein L24E	HQQHHHQAHQQHHQAHQQHQAHHHQQQQQQQQQQQQQQQ QQQQHH	Clavicipitaceae
	Putative sulfide quinine reductase	HHHHHQHQHHH	Epichloë
	Hypothetical protein	QQQQQQQQQQQQQQQQQQQQQQQQQQAAAQA	Common
CAG_5CAA_5			
	Nucleoporin NIC96	QQQQQQQQQQQQQ QQQQQQQQQQ	Common
	Transcriptional corepressor cyc8	QQQQQQQQQQQQQQQQQQQQQQQQQQQQ	Common
	75 gamma secalin	QQQQQQ QQQQQQQQQQQQHQ PEPEPEPEPEPEPEPEPEPE	Common
	Transmembrane anterior posterior transformation 1 / Puatative cytomegalovirus gH-receptor family protein	QQQQQQQQQQQQQ	Clavicipitaceae
	C6 zinc finger domain containing protein	QQQQQQQQQQQQQAAAA	Common
	Topoisomerase II associated protein	QQQQQQQQQQHQQQ QQQQQQQQQQQQQQQHQQQQQQHHQHQQQHHQQQQLQQQ QQQQQGQQQQ QQQQQQQHQQ AAAAAAAA	Common

*Common indicates presence of repeat within Epichloë, Clavicipitaceae and a Sordariomycetes species

3.2 *N. uncinatum* strains vary in morphology, and SSR length groups strains according to ecotypes

N. uncinatum strains were provided by Cropmark Seeds Ltd from four different ecotypes grouped by differences in B10 and B11 SSR length and country of origin (Fig. 3.11) (Table 3.2). The morphology and growth of strains was recorded to provide insight into the distinguishing features of each ecotype (Fig. 3.11). Strain morphology was variable both within and between the different ecotypes although major differences in growth pattern and growth rate were not observed. Cultures grew radially with white mycelia forming circular colonies. Aerial hyphae were generally not present and most colonies grew with a waxy appearance. No difference in growth rates were observed between strains. Calcofluor white staining showed mycelia were highly branched and growing radially from the centre. Hyphal tips stained brightly due to high chitin content, but hyphal cell walls stained less brightly possibly because of lower exposed chitin in fungal cell walls. Hyphae grew to form a dense matrix closer to the centre of the colony (Fig. 3.12).

Table 3.2: B10 and B11 alleles of *N. uncinatum* and proposed ancestors

Species	Strain	B10[+]	B11[+]
E. typhina	E8	178.1	-
E. bromicola	ATCC 200750	189.6	110.7
N. uncinatum	U2, U6, U10, U11, U12 & U13	159, 194	119
N. uncinatum	U3, U7, U9	159, 177	119
N. uncinatum	U4	171, 191	119
N. uncinatum	U5	159, 196	119

*Source: Cropmark Seeds Ltd (Darfield)

[+]Alleles sizes are given in nucleotide units (ntu)

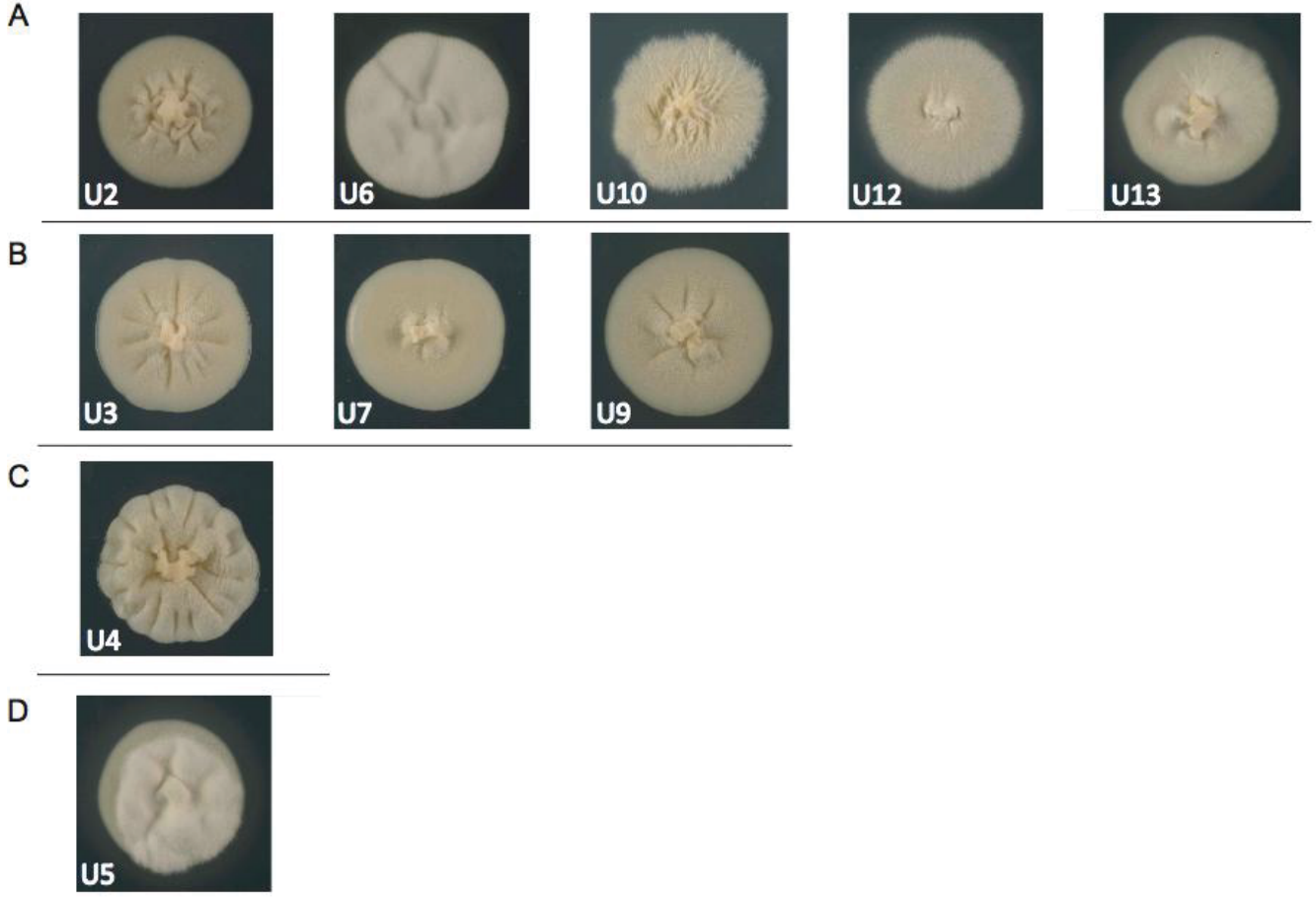


Figure 3.11: Colony morphology of *N. uncinatum* ecotypes. Photographs of colonies after 2 months of growth on malt extract agar. A) Ecotype 1 strains U2, U6, U10, U12 and U13.(Norway). B) Ecotype 2 strains U3, U7 and U9.(Bulgaria). C) Ecotype 3 strains U4.(Germany). D) Ecotype 4 strain U5 (Germany).

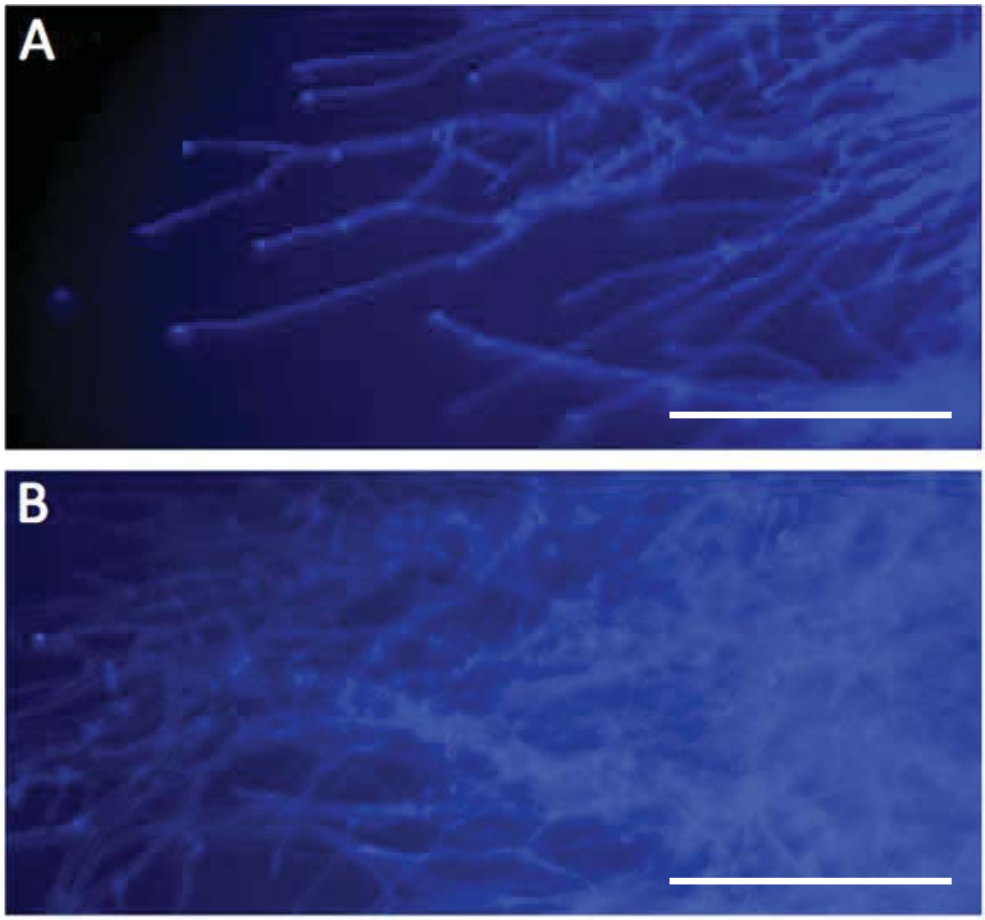


Figure 3.12: Calcofluor white stained fluorescent microscopy of *N. uncinatum* strain U2. A) Growth of the outer edge of *N. uncinatum* colony. Hyphae fluoresced blue while tips fluoresce bright blue. Hyphae grow radially from centre of colony and are highly branched. Bar = 100 μm. B) Hyphal growth within centre of colony. Hyphae form a dense matrix of highly branched strands overlapping each other. Bar = 100 μm.

3.3 *N. uncinatum* B10 and B11 repeats are conserved within ecotypes but sequence structure may indicate ancestral species

While morphology between *N. uncinatum* strains was only slightly variable, the B10 and B11 loci showed variation between the different ecotypes. *N. uncinatum* contains two different length alleles of the B10 SSR within its genome, but the structure of the repeats was not known. The repeat structure of the SSRs found within strains from different ecotypes was analysed to determine if any variation in repeat unit sequences occurred which could differentiate strains within the same ecotype.

3.3.1 Repeat structure of *N. uncinatum* strains

The B10 and B11 SSR loci were amplified by PCR using primers specific for B10 and B11, cloned into pGEM-T vector and sequenced to determine the repeat structure. Two alleles of the B10 repeat were found within all *N. uncinatum* strains, while only one B11 allele was found. The B10 alleles consisted of one large and one smaller allele of around 200 and 160 bp respectively.

The B10 repeat from *N. uncinatum* strains was found to be polymorphic between ecotypes, while conserved within ecotypes for both large and small repeats, with the exception of the U6 large repeat which contained a CAA repeat within its CAG region differing from other strains within that ecotype (Fig. 3.13). The repeated units of CAG, CAT and CAA were found to be polymorphic only in ecotype 3 (strain U4)

for the small repeats, but polymorphic across ecotypes for all large alleles. Variation in repeat structure was high between the large and small alleles. Small alleles contained a CAG repeat interspersed with CAA followed by distinct small CAT and CAA repeats. Large alleles generally had a large CAG repeat followed by a mixed CAT and CAA repeat region (Fig. 3.13). Flanking regions around repeats were highly conserved across all endophyte strains.

Sequencing of the B11 allele in *N. uncinatum* strains revealed conservation of sequence across ecotypes was high. The B11 repeat was identical across five strains representative of each the ecotypes (Fig. 3.14). As polymorphism did not exist across ecotypes, the B11 repeat from each strain within ecotypes was not analysed as it was likely to also be identical. Flanking regions around the B11 repeat were also highly conserved indicating the strains are closely related.

3.3.2 *N. uncinatum* B10 repeat shows similarity to ancestral species.

N. uncinatum is an interspecific hybrid between the proposed ancestors of *E. typhina* and *E. bromicola* (Moon *et al.,* 2004), which may indicate that each of the B10 alleles was inherited from these ancestors. To test this hypothesis, the B10 repeat of *E. bromicola* E799 was sequenced following PCR amplification and cloning into pGEM-T. *E. typhina* E5819 and *E. typhina* E8 sequences were obtained from available genomic data (http://www.endophyte.uky.edu/). These sequences were then used for comparison to the *N. uncinatum* B10 alleles. *E. typhina* contained a single B10 allele in each strain while the structure was similar to that of the *N. uncinatum* small repeat. *E. bromicola* contained a single allele of B10 with

structural similarity to that of the large repeat from *N. uncinatum* (Fig. 3.13). Both *E. typhina* strains and small repeats of *N. uncinatum* contained CAA repeats within the CAG region. The position of these CAA repeats from E5819 matched those of the small repeat in *N. uncinatum*. SNP's resulting in CCG trinucleotides in E8 were not seen in any of the *N. uncinatum* repeats. The *E. bromicola* repeat and the large repeats from *N. uncinatum* both contained distinct regions of mixed CAT and CAA repeats. This region was identical between ecotype 1 of *N. uncinatum* and *E. bromicola* but the CAG region between the two was different with the *N. uncinatum* ecotype containing an extended CAG repeat of three units. This CAT/CAA region was also very similar in the other ecotypes and matched closely to that of *E. bromicola* however the CAG region did not match any of the strains or ecotypes (Fig. 3.13). The repeat structure from *N. uncinatum* of the small or large alleles did not match any of the other previously analysed endophyte species B10 repeat structures.

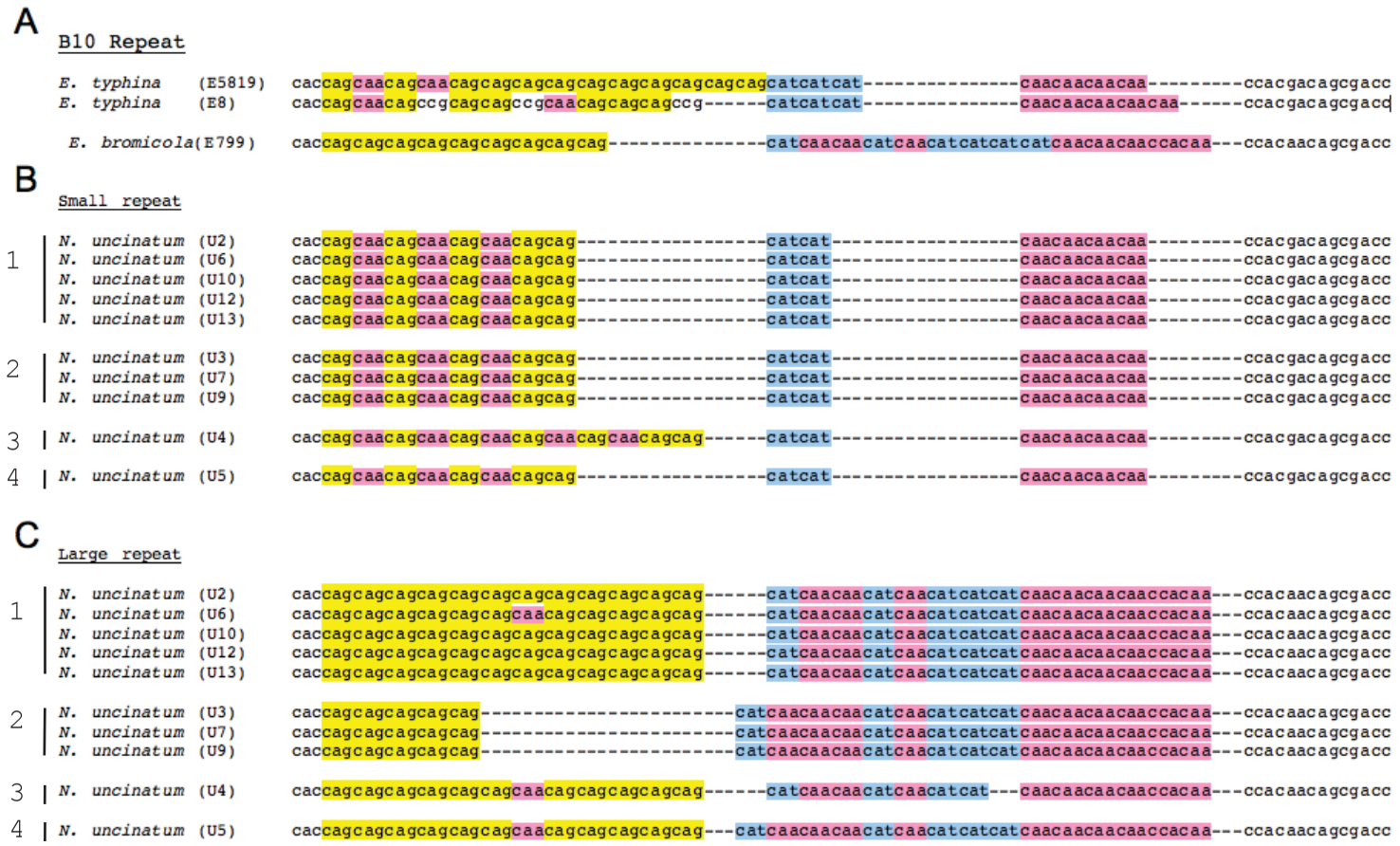


Figure 3.13: B10 SSR from *N. uncinatum* strains. A) B10 repeats of *N. uncinatum* proposed ancestral species *E. typhina* and *E. bromicola*. Trinucleotide repeat sequence CAG (yellow), CAT (blue) and CAA (purple) is highlighted. B) Sequences of the small repeats contained within each *N. uncinatum* strain. Strains are grouped and aligned for the different ecotypes (1-4). C) Sequences of the large repeats within *N. uncinatum* strains.

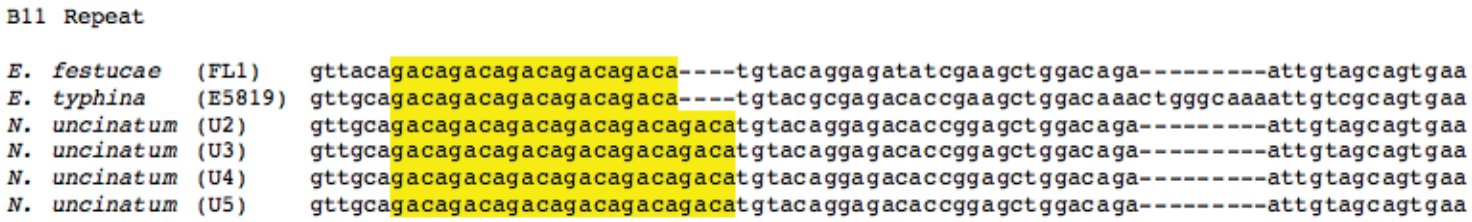


Figure 3.14: B11 repeat found within different *N. uncinatum* ecotypes. GACA repeat is highlighted yellow and found within a conserved B11 region. Repeat is identical within ecotypes 1-4 (U2-U5) and similar to known B11 repeats from *E. festucae* and *E. typhina* species. *E. typhina* contains a small insertion upstream of B11 repeat.

3.4 Discussion

The use of SSR markers for the identification of endophyte strains and species based on loci length has been shown to be both practical and informative (Moon *et al*., 1999). However, little was known about the location of these SSRs within the genome and how the individual repeat structure affects SSR regions and their genomic consequences.

Alignment of available sequence across a range of *Epichloë* species showed polymorphism in repeat length for both the B10 and B11 SSRs. While the B11 repeat contained only one repeated tetranucleotide, the B10 repeat contained three distinct repeated trinucleotide regions. This made the B10 repeat more informative and strains not only showed variation in overall length, but also in the number of each of the trinucleotide repeats. For example the SSR length of *E. amarillans* E57 and *E. typhina* E8 were the same, yet the repeat structure contained within each was completely different. *E. amarillans* contained a much smaller CAG repeat and much larger CAA repeat than that of the *E. typhina* strain. PCR analysis of an unknown endophyte producing a B10 product corresponding to this size could be defined as either *E. typhina* or *E. amarillans* based on length alone, yet further analysis of the repeat structure is able to resolve the endophyte species. Varying strains within a species may not conform to an exact repeat structure of defined CAG, CAT and CAA lengths but it appears that a consensus pattern between endophyte strains within a species may be able to be defined. Of the species used in this study only *E. festucae* and *E. typhina* had multiple genomes available for comparison. Similarity was seen between both the *E. festucae* strains with a difference of only two CAG

repeats, while both had a unique CCA polymorphism within the CAA region. The repeats of *E. typhina* were also similar with a large CAG repeat interspersed with CAA and CCG while the CAT and CAA repeats were the same. These were not similar to other endophyte species analysed and the potential may exist for a preliminary classification of endophytes based on the B10 repeat structure alone, although with the ease and robustness of *tefA* (translation elongation factor) and *tubB* (β-tubulin) sequencing for classification this may not be a practical alternative.

While the B11 SSR was contained outside of the boundaries of adjacent genes, the B10 repeat was found to fall within the exonic region of a putative bZIP transcription factor. RT-PCR showed that the gene was expressed in *E. typhina* and sequencing of the cDNA showed that the spliced transcript included the repeat region. The predicted amino acid sequence showed that the SSR coded for a glutamine rich repeat with a histidine core in the centre. Alignment within *Epichloë* species showed high conservation across the entire amino acid sequence excluding the repeat region. Alignment with more distantly related species showed that the repeat was contained within *C. paspali* and *P. ipomoeae,* while absent from *M. oryzae*, *N. crassa* and *F. oxysporum*. Species that contained the B10 repeat within the gene that encoded a protein change, all belonged to the Clavicipitaceae and may represent a specific change found in this family.

SSRs within genes have the potential to provide fast adaption to changes in environmental conditions (Li *et al.*, 2004), which may be beneficial to endophytes and plant pathogens due to changing host conditions or the establishment of new host niches. The location of this repeat within a transcription factor has the potential

to impact on the many other genes regulated by this gene product. A further search for B10-like repeats found that these SSRs were scattered through a wide variety of gene families (Table 3.1). Analysis of a second B10 like repeat, designated B12, showed that it was located in a putative copper sensing transcription factor and also conserved within the Epichloë endophytes yet lost within closely related Clavicepitaceae species *C. paspali* and *P. ipomoeae*. A further repeat found in a G-protein receptor was also conserved within *Epichloë* but again lost within related species. Searches for further repeats within genes in *Epichloë* species found a variety of repeat elements in a wide range of genes (Table 3.1). While some repeats were specific to *Epichloë* species, others were conserved in *Claviceps* and *Periglandular*, representative of the Clavicipitaceae, and in some cases found in more distantly related species of *Fusarium, Neurospora* and *Magnaporthe*. Genes found with repeats specific for *Epichloë* species did not follow a common gene family or function with transcription factor, receptor, reductase and acetyltransferase genes identified (Table 3.1). Similarly, genes found to have repeats conserved within the *Clavicipitaceae* did not follow a specific gene function or family.

Glutamine-rich repeats may specifically be enriched within some Epichloë endophyte genes in order to provide a selective advantage or adaptation to the unique host or environment they inhabit. Work in a variety of organisms has begun to show the importance of repeats within gene regions, including a study of bovine proteins containing polyQ repeats which showed these repeats were highly polymorphic, enriched in genes with functions associated with transcriptional regulation, and caused phenotypic variation (Whan *et al.*, 2010). A glutamine SSR in *Candida albicans* within a transcription factor gene, *RLM1*, found to be

polymorphic across strains suggested that elongation of the repeat was associated with an increased resistance to stress agents (Sampaio *et al.*, 2009). Glutamine repeats have also been found to be related to onset of neurodegenerative diseases due to expansion of long chains of glutamate residues (Zoghbi *et al.*, 2000, Perutz, 1996).

Grass endophytes exhibit a high level of host specificity and colonise defined host species (Clay & Schardl, 2002, Saikkonen *et al.*, 2004). Potentially, the polymorphism of SSRs in a wide variety of genes could be partially responsible for this high host specificity. Each species could achieve a unique distribution of genes with different polymorphic lengths of SSRs within certain genes important in endophyte compatibility, allowing for a tailored response to the particular host background in which it will inhabit. This would be even more prominent in species that undergo interspecific hybridisation as they would acquire genes from each parent and potentially be able to colonise new hosts, yet the loss of a sexual cycle may inhibit transmission to these new hosts, and correspondingly result in loss of any genetic advantage overtime as the genome stabilises. Taking into account the large variety of genes within *Epichloë* found to contain glutamine repeats, which may represent only a small snapshot of their occurrence, and the evidence for their effect on adaption and phenotype, it is not unreasonable to assume that changes within the repeat regions of these SSRs may allow higher adaptation of endophytes. This may provide enhancement of the endophytes association with the host, ability to adapt to environmental fluctuations or exploitation of new host niches.

While a single allele of B10 was present in the *Epichloë* species, two alleles were present within *N. uncinatum*. Sequencing of the large allele and small allele showed similar repeat structure across all the *N. uncinatum* strains. As *N. uncinatum* is an interspecific hybrid, it was thought that each of the two alleles may have originated from the proposed ancestors of this hybridisation event. The B10 sequences of proposed ancestor species from *E. typhina* and *E. bromicola* were obtained and used to compare to the large and small repeats of *N. uncinatum*. The large repeat matched that of *E. bromicola* while the small repeat matched that of *E. typhina*. Although not exact matches, the repeat structure between ancestors and those of *N. uncinatum* strains was very similar. This strongly suggests that these B10 repeats were obtained from each of the proposed parents rather than a recent duplication event (Fig. 3.15). The use of the B10 repeat from interspecific hybrids may be able to show ancestral relationships in other *Neotyphodium* species if two alleles are retained. The length of each CAG, CAT and CAA repeat as well as unique polymorphisms in each repeat element may give insight into parentage of interspecific hybrids.

The analysis of a second repeat labelled B12, which was shown to be polymorphic in *Epichloë* species, was analysed in *N. uncinatum* to determine if it could also provide information on ancestral linkage or be used to distinguish strains within clonal groups. While no differences in repeat structure were found between representative strains U2, U3, U4 and U5 (ecotypes 1-4) a difference was present in strain U6 (ecotype 1). Sequencing of U6 was performed as it had previously shown a polymorphism in the B10 large repeat, which differed from the other strains in its ecotype. When compared to other *N. uncinatum* strains, the U6 PCR product was

only 3 nucleotides different to the other strains yet it held a 9 nucleotide difference within the repeat region with losses of CAG and CAA repeats and a gain of CAT. This further emphasises the benefits of using the sequence and repeat structure of these elements rather than the length of SSR loci alone. This difference may also indicate that U6 belongs to a separate ecotype rather than ecotype 1. Although grouped in ecotype 1 due to its B10 and B11 allele lengths, the difference in sequence structure of the B10 large repeat and B12 shows its divergence from this group.

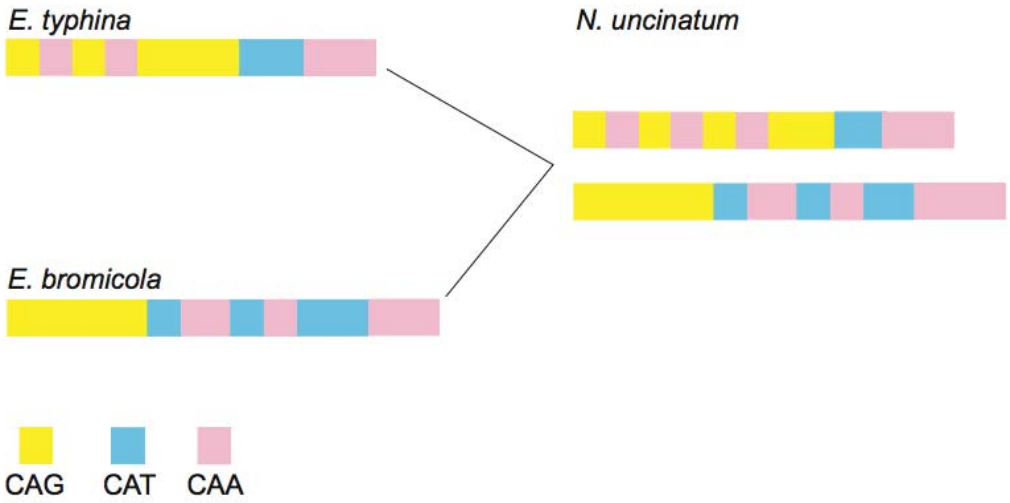


Figure 3.15: SSR structure conservation between proposed ancestor species. Schematic representation of the B10 repeat structures from *N. uncinatum* and its proposed ancestors. Repeat structure is coloured to represent repeated units CAG (yellow), CAT (blue) and CAA (pink). A single B10 allele is found within *E. typhina* and *E. bromicola* while two alleles are contained within *N. uncinatum*. Repeat structure of *E. typhina* and *E. bromicola* are similar to that of *N. uncinatum*.

4. Analysis of loline production by various *N. uncinatum* strains in association with meadow fescue

4.1 *N. uncinatum* grows within leaf and pseudostem but not root tissue

The colonisation pattern of the *N. uncinatum* strain U2 was tested to determine endophyte localisation within the natural host, meadow fescue, and also in the Festulolium hybrid host. PCR was performed using primers specific for the translation elongation factor (TefA) gene using DNA isolated from endophyte infected plants. This method showed that the endophyte *N. uncinatum* U2 was able to colonise the pseudostem and leaf blade of both the natural host and Festulolium hybrids. No endophyte was detected in the root tissue of either host. A control using *E. festucae* Fl1, which is known to grow within both the leaf blade and pseudostem, was used to confirm PCR specificity and showed the expected endophyte localisation within leaf blade and pseudostem while no endophyte was found in the root tissue (Fig. 4.1).

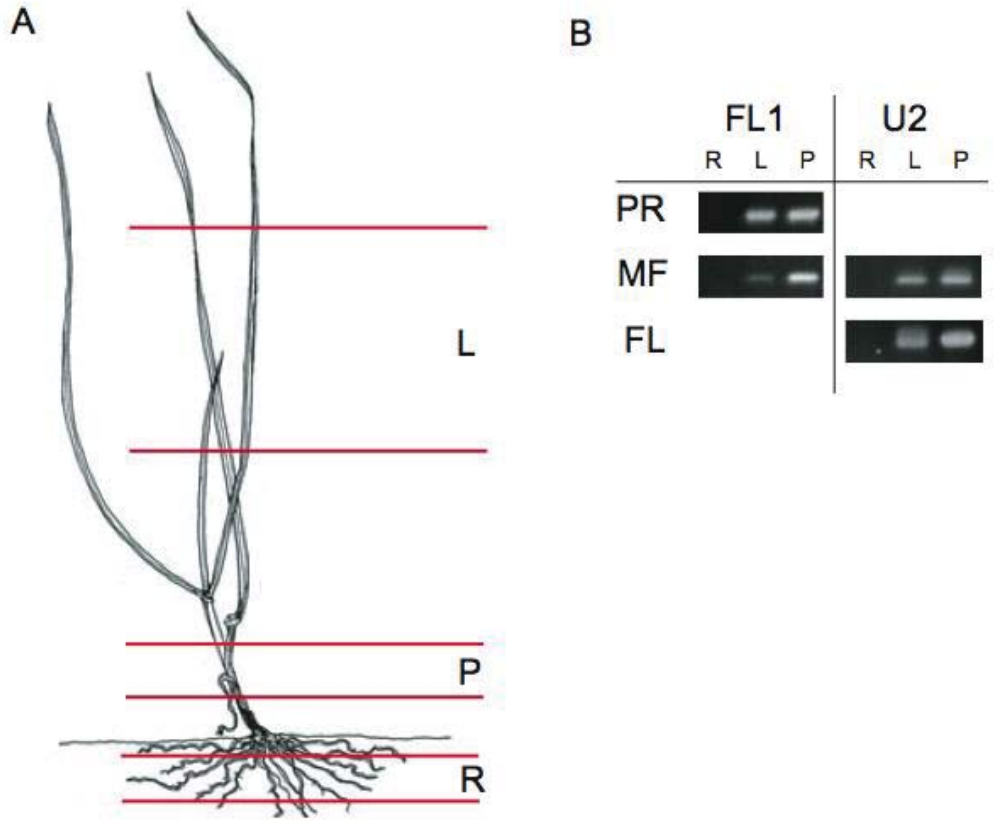


Figure 4.1: Colonisation pattern of endophytes within plant. A) Diagram of grass plant indicating locations of tissue samples taken. B) Amplification of endophyte DNA of *E. festucae* strain Fl1 and *N. uncinatum* strain U2 in root (R), pseudostem (P) and leaf (L) tissue for perennial ryegrass (PR), meadow fescue (MF) and Festulolium hybrids (FL). Total DNA extracted from tissue was used to amplify endophyte specific *tefA* product of 864 bp using primers Tef 1d, Tef 1u. Amplicon product indicates presence of fungal biomass within tissue.

4.2 *N. uncinatum* strains vary in production of total lolines

N. uncinatum is known to produce lolines, yet certain strains may be able to produce more lolines than others. Ten strains were inoculated into multiple plants ($n > 3$) of a single meadow fescue cultivar and grown under controlled environmental conditions to determine if strain variation alone affected loline production. Meadow fescue was used as it is a natural host of *N. uncinatum* and represents a natural association producing lolines. Lolines were produced by all strains (Fig. 4.2) and the presence of lolines was detected in all host tissues including the leaf blade, pseudostem and root tissue (Fig. 4.2). The average level of lolines across all tissues in the common host plant was determined to be strain dependent with statistically significant differences between group means as determined by one-way ANOVA ($F(9,29) = 2.60$, $p = 0.025$). Post hoc comparisons using the Tukey HSD test indicated that the total loline levels of U10, U2 and U9 were significantly different from other *N. uncinatum* strains ($p < 0.05$). Of the strains analysed, U10 was the highest producer while U2 and U9 were lower loline producers (Fig. 4.2). Tissue specific distribution of total lolines showed that all strains showed the highest levels within the pseudostem with the exception of U3 which contained the highest loline levels within the leaf blade. All strains showed the lowest levels of lolines within the root tissue (Fig. 4.3).

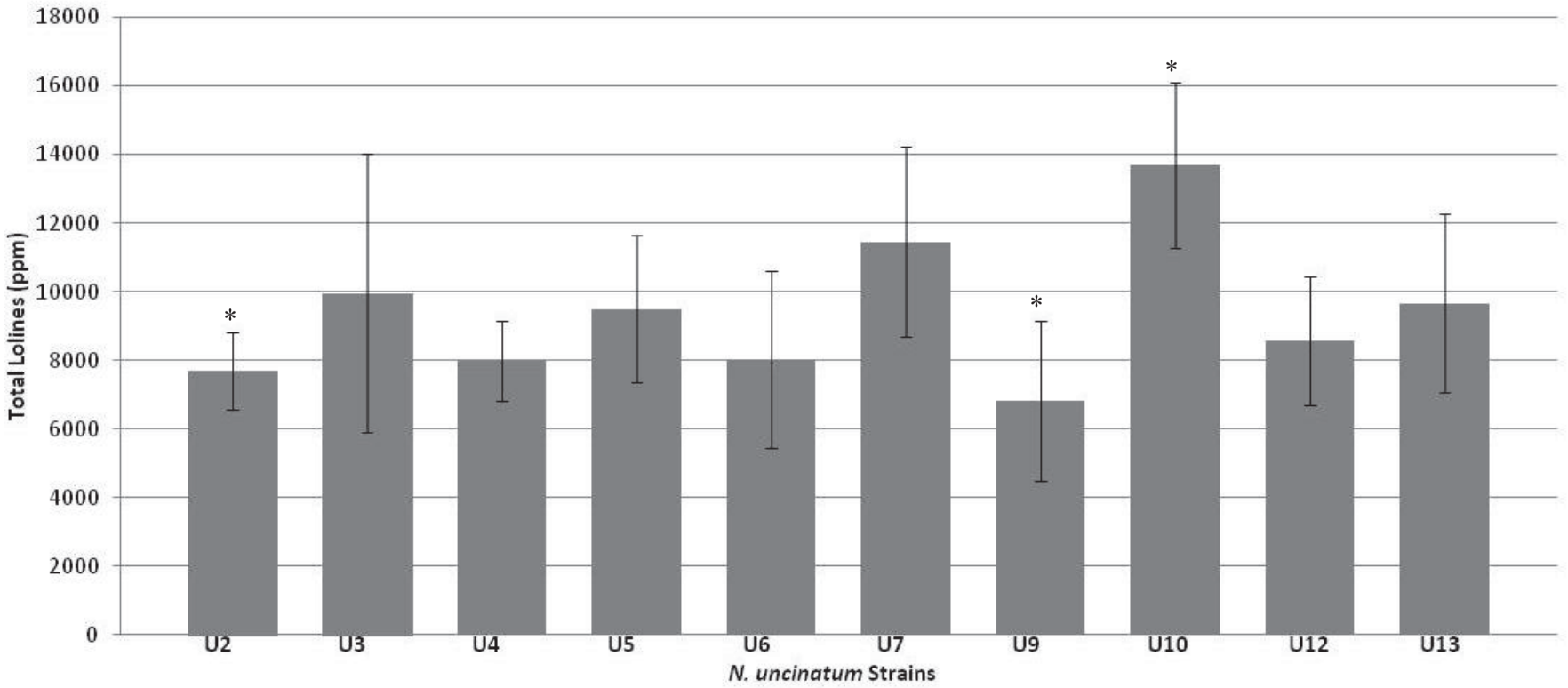


Figure 4.2: Analysis of loline production by *N. uncinatum* strains. Graph showing average level of lolines across root, pseudostem and leaf blade tissue for each of the *N. uncinatum* strains tested (n > 3). Error bars show ± standard deviation. * indicates significant difference from the group mean ($p<0.05$)

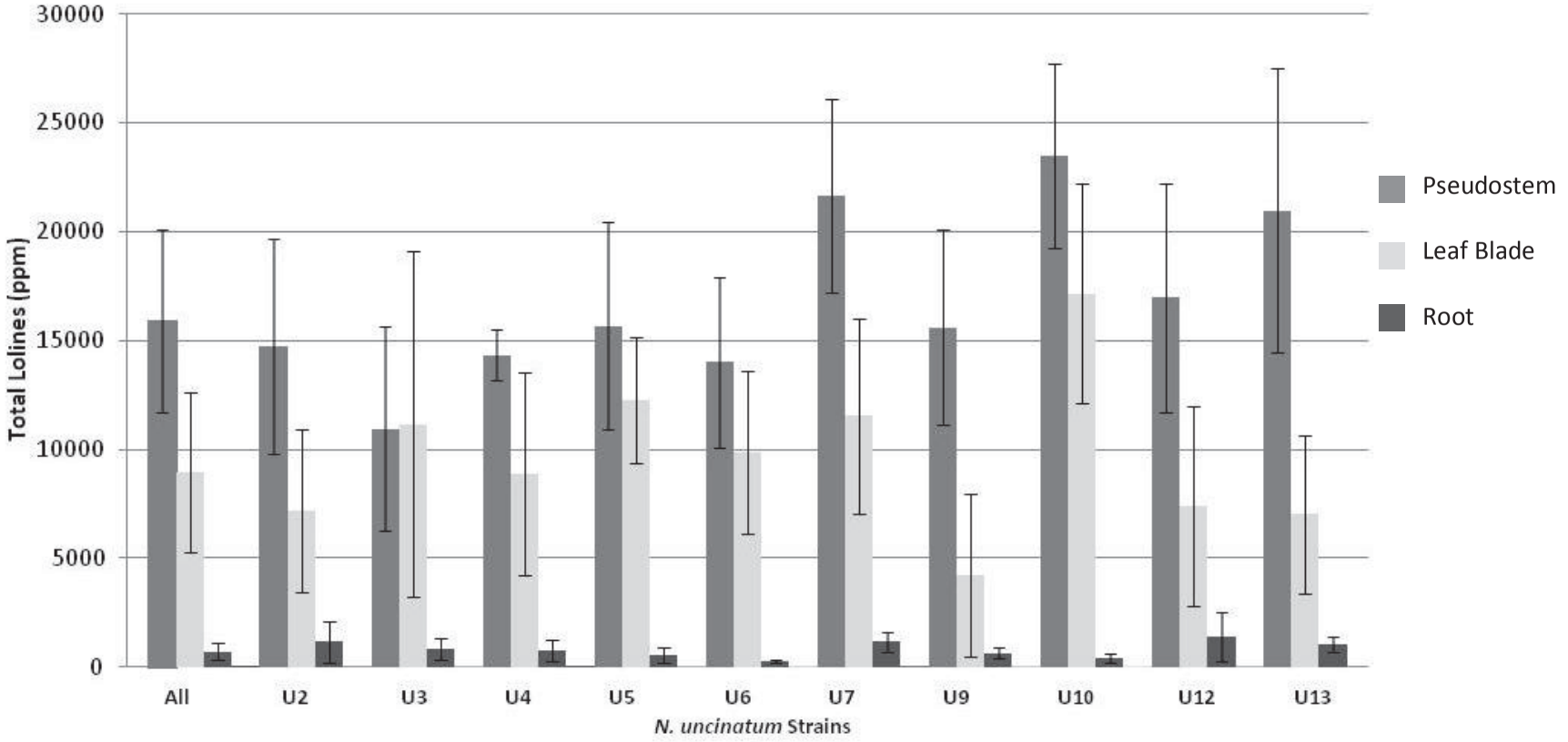


Figure 4.3: Comparison of loline levels in various tissues by *N. uncinatum* strains. Graph showing total loline levels in each tissue type for each endophyte strain (n > 3). (All) shows the average levels across all strains. Error bars show ± standard deviation.

4.3 Level of different lolines in plant tissue

To determine if different lolines were enriched within certain tissues a comparison was performed of each loline detected in the separate tissues. The loline that accumulated the highest in each tissue was *N*-formyl loline (NFL), with the levels of all other lolines significantly lower, independent of tissue type (Fig. 4.4). Levels of *N*-acetylloline (NAL), *N*-acetylnorololine (NANL) and *N*-methylloline (NML) were very low (<20 ppm) in the root tissue but were present in higher concentrations in both pseudostem and leaf blade (Fig. 4.4). NFL comprised 95% of total loline levels within root tissue, and 77 and 72% of total lolines detected in leaf blade and pseudostem tissue respectively.

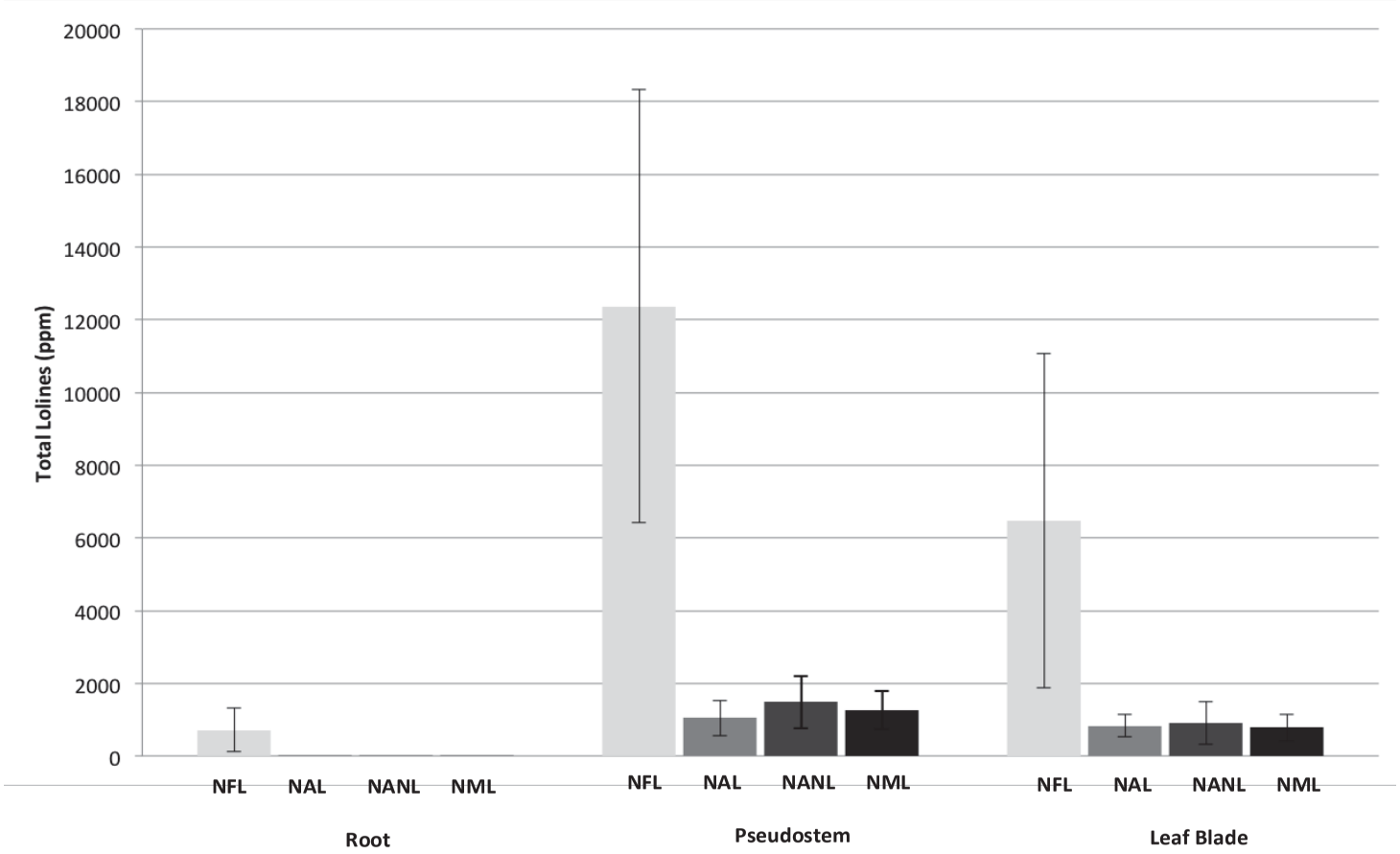


Figure 4.4: Comparison of different loline accumulation in plant tissue. Average amounts of *N*-formylloline (NFL), *N*-acetylloline (NAL), *N*-acetylnororololine (NANL) and *N*-methylloline (NML) in individual tissues across all *N. uncinatum* strains. Error bars show ± standard deviation.

4.4 Endophyte inoculation of plant does not affect loline production

To test whether inoculation of plants affects the production of lolines, a comparison between plants manually inoculated and those grown from seed naturally infected with *N. uncinatum* strain U2 was performed (Fig. 4.5). Standard two-tailed t-test analysis was used to determine if loline levels were significantly different between groups. Average loline level in the plant was not found to be statistically significantly different ($t(5) = 0.362$, $P = 0.732$) and loline levels in the roots ($t(5) = 2.01$, $P = 0.101$), leaf blade ($t(5) = 1.55$, $P = 0.180$) and pseudostem ($t(5) = 1.23$, $P = 0.273$) were also found not to be statistically significantly different ($P < 0.05$). The production of lolines was found to be independent of manual or natural inoculation of plants.

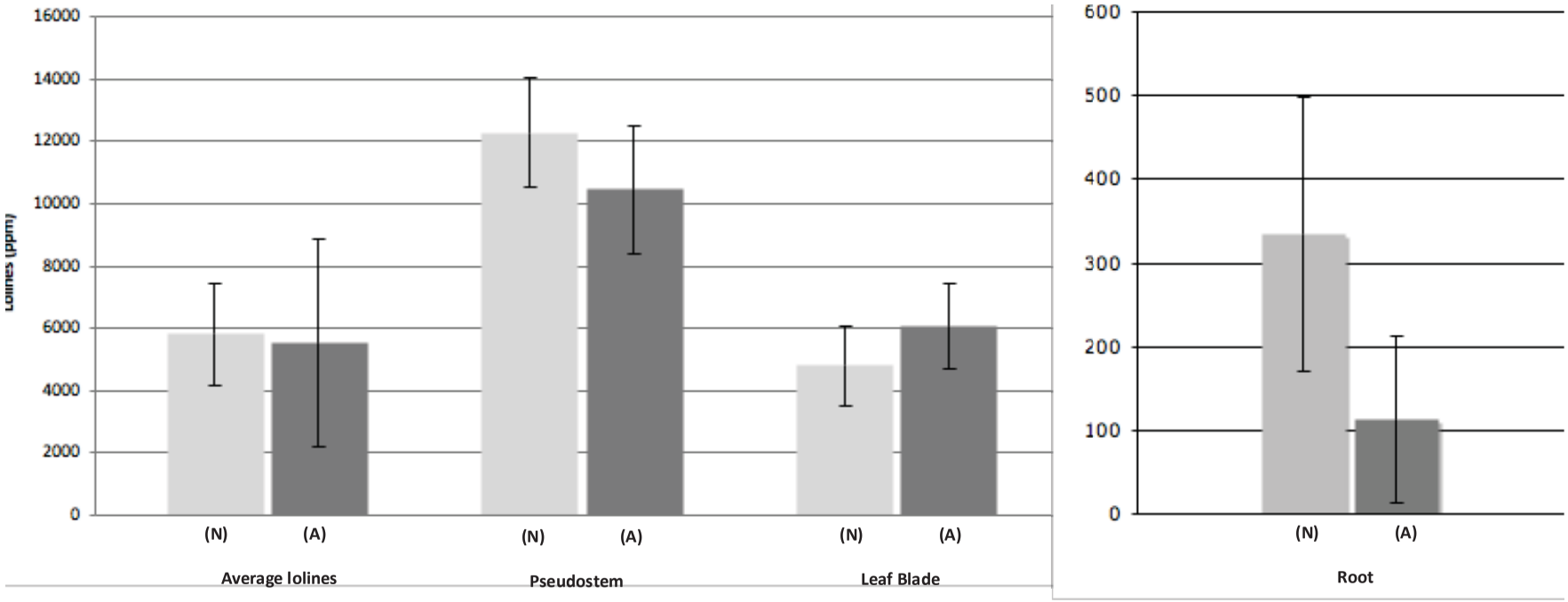


Figure 4.5: Effect of colonisation process on loline production. Meadow fescue naturally infected plants (N) and manually infected plants (A) loline production for the strain *N. uncinatum* U2 (n > 3). Average lolines represents the average level over all plant tissue. Total loline levels were measured in pseudostem, leaf blade and root tissue. Error bars show ± Standard deviation.

4.5 Discussion

While each *N. uncinatum* strain was known to have the capability to produce lolines it was unknown whether potential existed for variation in strains causing higher or lower loline production. Inoculation into multiple plants of a single meadow fescue cultivar to determine each strain's potential for loline production showed that U10, U2 and U9 differed significantly from all other strains in the ability to produce lolines. U10 was the highest producer while U2 and U9 were low level producers of lolines. While these differences were shown to be statistically significant, the average amount of lolines produced in the plants across all strains were higher than the levels thought to be needed for resistance to relevant insect pests (Patchett et al., 2008a, Wilkinson et al., 2000, Popay & Lane, 2000). Growth of the plant is also thought to have a major impact on the production of lolines with high levels in late spring and the lowest levels in autumn (Patchett *et al.*, 2011). The growth conditions used in this study are more comparable to those found in the field in late spring and early summer so it was expected that plant loline levels would be high, which was indeed the case.

The growth of *N. uncinatum* was restricted to the leaf blade and pseudostem and not found within the root tissue, supporting previous studies that showed no endophyte colonisation of roots and the transport of lolines to root tissue by the plant through the vascular tissue (Patchett *et al*., 2008b, Koulman *et al.*, 2007, Schardl *et al*., 2004). This transport is especially useful for redistribution of loline compounds in the presence of insect attack on root tissue (Patchett *et al*., 2008b). While plants in

this study showed root loline content at medium to high levels they may not reflect the true potential of root loline accumulation of the plant as root damage did not occur. The levels measured here may simply represent the basal level accumulation of lolines after transport to root tissue, which upon insect attack or stress response would have the capacity to increase dramatically (Patchett *et al*., 2008b, Bultman *et al*., 2004).

N-formylloline (NFL) was found to be highest in all tissues while *N*-acetylloline (NAL), *N*-acetylnorololine (NANL) and *N*-methylloline (NML) were all found at much lower levels, matching previous results that showed NFL is the major loline produced in plant (Patchett *et al*., 2011, Justus *et al.*, 1997, Yates *et al.*, 1990). The production of lolines are regulated by the *LOL* gene cluster encoding proteins for the production of the loline alkaloids (Spiering *et al*., 2005). The end product of this pathway is NFL while the other lolines NAL, NANL and NML are pathway intermediates. The high accumulation of NFL in plant tissue is therefore expected, while low accumulation of the other lolines may represent a build up of intermediates. The high accumulation of NFL in the roots may also indicate that this compound is more efficiently transported throughout the plant than other lolines.

Manual inoculation of seedlings is used to produce synthetic grass-endophyte associations and is particularly useful for testing the production of alkaloids by particular endophytes within different grass hosts. However the inoculation technique involved is very different from the natural vertical and horizontal transmission used by many of these endophytes to colonise grass hosts. Manual inoculation also involves the wounding of plants which may have unknown effects

on endophyte within the plant during the initial colonisation step. Plants manually infected with endophyte were not seen to have a statistically significant difference in loline content in the whole plant, pseudostem, leaf blade or root and were comparable to lolines produced from naturally infected plants. Overall, the inoculated plants showed lower loline levels than the naturally infected plants with the exception of the leaf blades which had slightly higher levels. The root tissue of inoculated plants contained much lower levels of lolines than naturally infected plants. There was also large variation in levels and the difference in levels between natural and manual infection was not statistically significant ($p<0.05$). The results suggest that loline production from inoculated plants is similar to that of naturally infected plant material.

Despite using a single cultivar of meadow fescue that was grown under controlled conditions of light and temperature there was still a high level of variability in loline production within strains. The high variation observed suggested that the results found should be viewed cautiously. High variation may have been due to plant conditions that could not be fully controlled, such as individual plant characteristics and the watering of each plant between replicates. Although inoculated into a common host plant the plants used were simply from the same cultivar and did not represent a clonal population. Natural variation in growth of plants grown from individual seeds could mean loline production will be affected independent of endophyte strain inoculated. However since it would be very hard to inoculate plants produced by cloning (Latch & Christensen, 1985), the use of seed from a single cultivar was the best option to get some indication of each strain's ability to produce lolines. Variation in watering may have also occurred as a strict watering routine

was not in place and plants were watered simply when needed. Variation in root mass caused by differences in watering may have caused changes in root loline levels while in some cases large root mass meant plants were beginning to become root bound. Root bound or water deficient plants may begin to become stressed and this in turn may activate the natural induction of loline production in response to plant stress (Malinowski & Belesky, 2000, Bultman & Bell, 2003, Spiering, 2000) resulting in artificially induced higher levels of lolines within the plant. While care was taken to maintain healthy plants, these factors may have made a significant contribution to the high variation seen within replicates.

5. The growth, stability and transmission of *N. uncinatum* in both natural and synthetic host associations

5.1 *N. uncinatum* U2 growth in meadow fescue is stable

In order to compare growth within hybrid plants, the growth characteristics of *N. uncinatum* strain U2 was first established in its natural host, *Festuca pratensis* (meadow fescue). Endophyte free grass seed was provided by Cropmark Seeds Ltd and seedlings were inoculated with *N. uncinatum* U2 (Method 2.5.1). Samples were taken from the leaf sheath of infected plants and stained with a mixture of aniline blue and WGA-Alexafluor 488 before examination by confocal microscopy. Images were taken of hyphae below the epidermal cell layer. Hyphae were found to be abundant in the leaf sheath tissue, grew parallel to the leaf axis and were rarely branched. All hyphae appeared to grow within the intercellular spaces between plant cells and were uniform in both thickness and spacing of septa. Septa stained well with WGA-Alexafluor 488 and no deregulation in chitin production, as determined by aberrant green fluorescence, was observed (Fig. 5.1).

Further analysis of infected plants was performed by light and transmission electron microscopy of transverse sections from the pseudostem. Hyphae stained with toluidine blue were deep blue under light microscopy, regular in shape, abundant within the leaf sheath tissue and present in the intercellular spaces of the plant.

Hyphae did not appear to be within or concentrated around vascular bundle tissue and showed an even distribution throughout plant tissue. Hyphae were not discernible within the young/immature tissue at the centre of the pseudostem. (Fig. 5.2)

Transmission electron microscopy (TEM) of pseudostem tissue was used to provide more detailed information on hyphal morphology within the plant. Hyphae were observed within the intercellular spaces between plant cells, with multiple hyphae present in some spaces. Hyphae had electron dense outer cell walls and cytoplasm. Organelles were often visible within the hyphae and the cytoplasm lacked large vacuoles or lipid bodies. Hyphae appeared to adhere strongly to plant cell walls, and in some cases were surrounded by a mucilage layer or fibrous network contained within the intercellular space (Fig. 5.3). TEM analysis of plants infected with *N. uncinatum* strains U3 and U4 revealed a similar pattern of host colonisation to that of U2 and indicated a stable association (Appendix. 7.2-7.4)

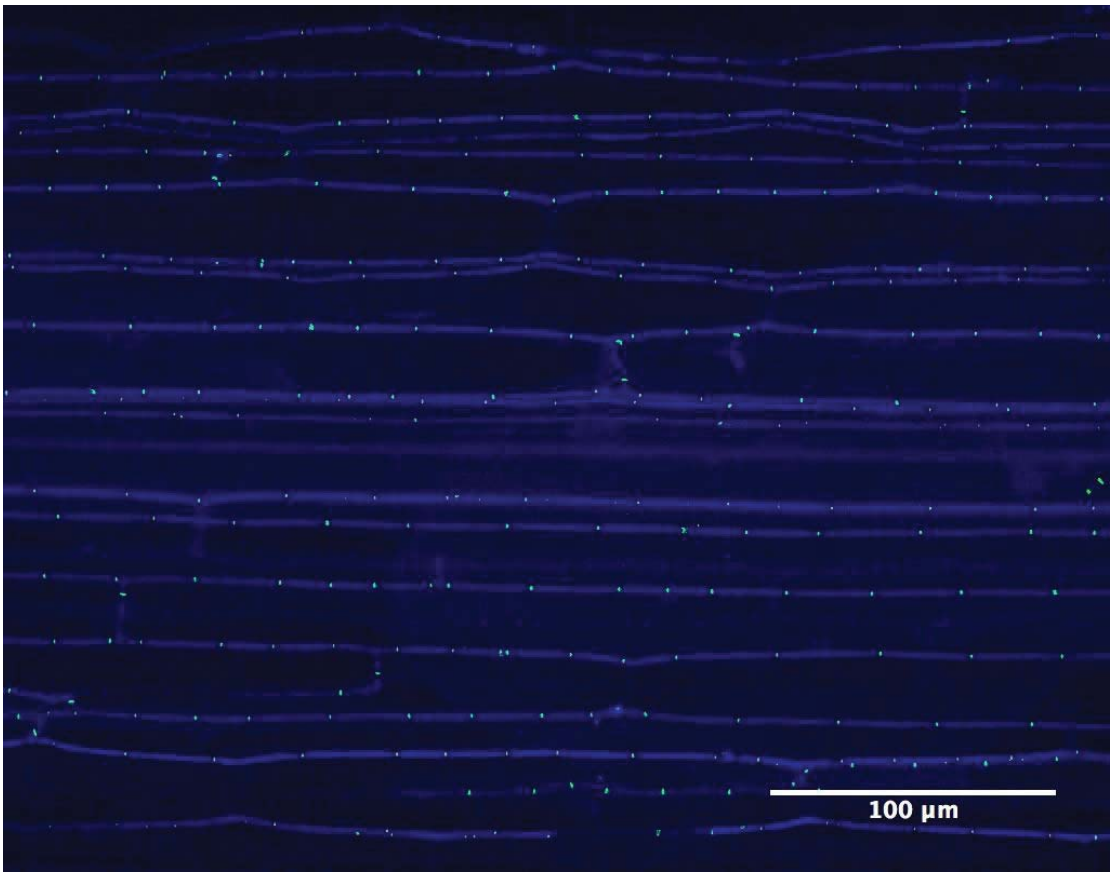


Figure 5.1: Growth of *N. uncinatum* strain within a *Festuca pratensis* host. Confocal depth series image of longitudinal sections through *Festuca pratensis* (meadow fescue) leaves showing U2 hyphae stained with WGA-Alexafluor 488 and aniline blue. The image shows hyphae (fluorescent blue) growing in close association with plant cells. Strongly illuminated green points indicate hyphal septa.

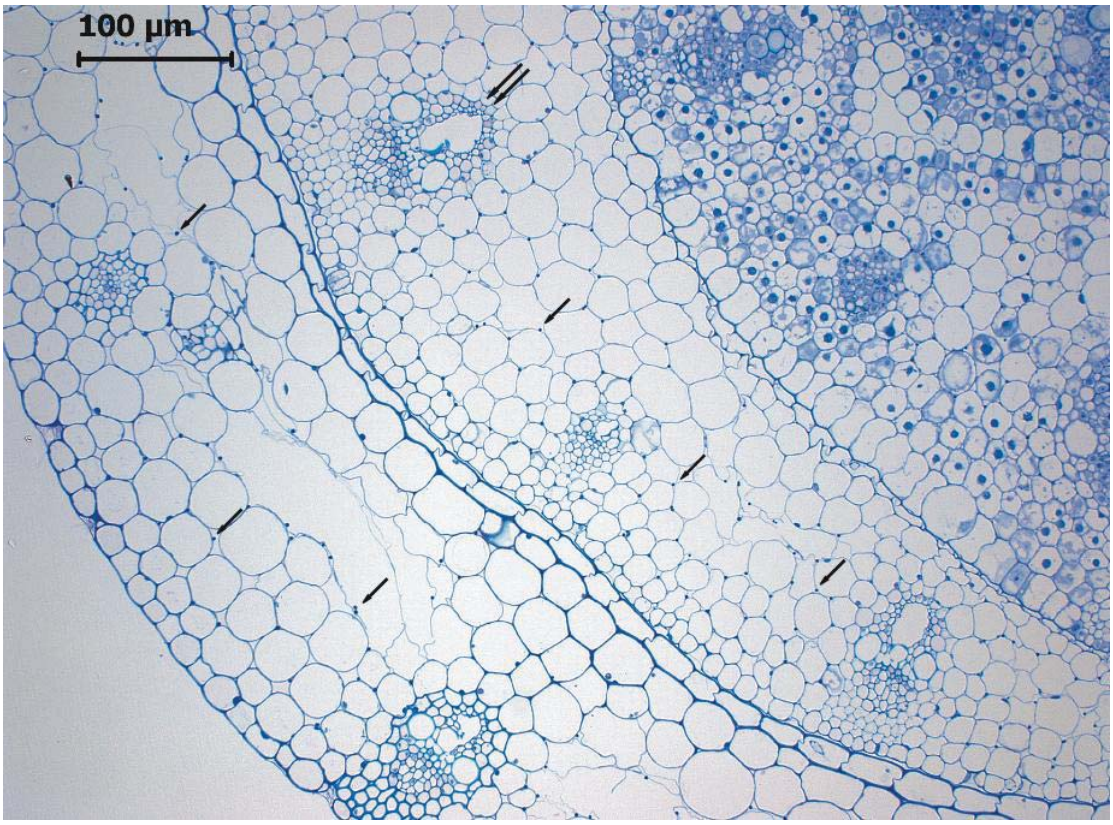


Figure 5.2: Light micrograph of *Festuca pratensis* pseudostem infected with *N. uncinatum*. Pseudostem transverse section show U2 hyphae stains deep blue and appears as dots within the intercellular spaces of plant cells (arrows). Hyphal colonisation can be seen in leaf layers with exception of young/immature tissue (top right). Vascular bundle tissue (double arrow).

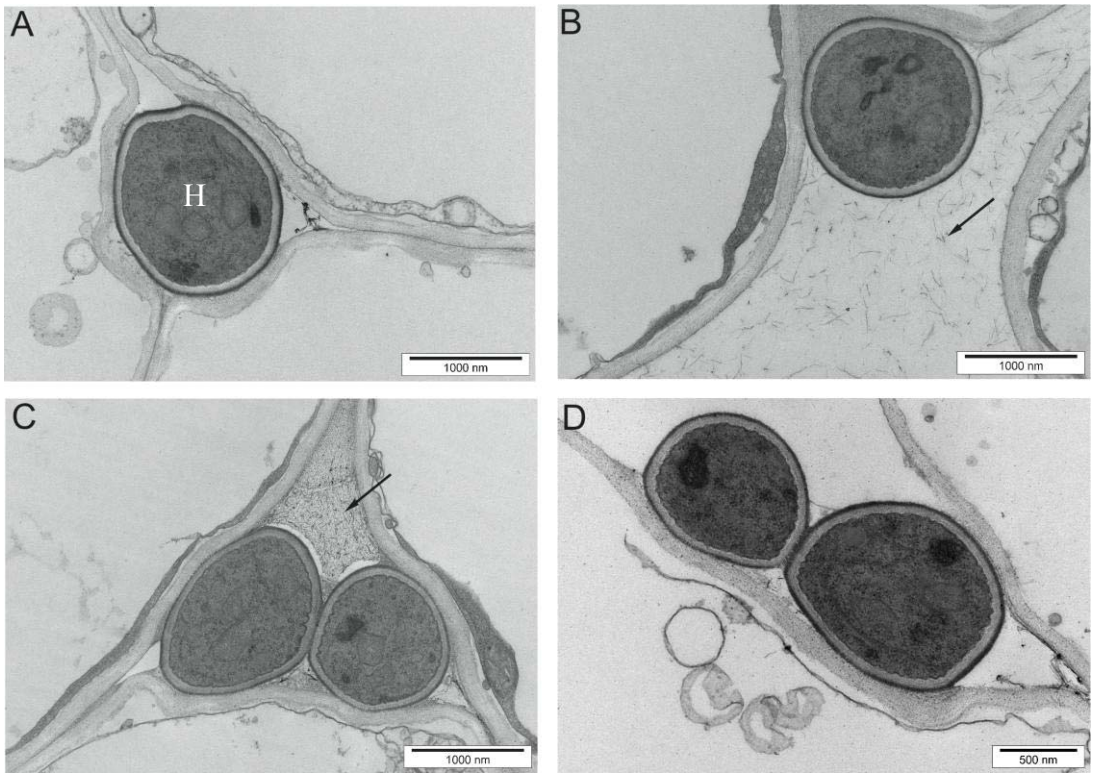


Figure 5.3: Transmission electron micrographs of *Festuca pratensis* containing *N. uncinatum*. A) Transverse sections of pseudostem containing hyphae (H) of strain U2 within a plant intercellular space. Cytoplasm appears electron dense and hyphae are in contact with plant cell walls. B,C) Hyphae may be surrounded by an extracellular substance within intercellular space (arrows). C,D) Multiple hyphae within the same intercellular space and adhered to plant cell walls.

5.2 Low levels of vascular bundle colonisation observed in manually inoculated seedlings

To determine if inoculation of endophyte free seedlings could be responsible for any difference in growth or morphology of *N. uncinatum, F. pratensis* plants were grown from seed already infected with endophyte (naturally infected) and endophyte free seedlings manually inoculated with endophyte (manually infected, method 2.5.1). The model endophyte *E. festucae* Fl1 was also inoculated into *Lolium perenne* (perennial ryegrass) as a control. No major differences in growth or morphology of the endophyte were seen between naturally infected plants compared to that of manually infected plants. However, a difference in vascular bundle colonisation was observed. Vascular bundles of *F. pratensis* manually infected with *N. uncinatum* strains U3 and U4 were shown to have low levels of fungal colonisation, a feature not present in plants naturally infected (Fig. 5.4). Manual inoculation of *L. perenne* with *E. festucae* Fl1 also resulted in low levels of vascular bundle colonisation (Fig. 5.4). All hyphae found to be colonising vascular bundles had electron dense cytoplasm and thin outer cell walls. Hyphae did not appear to contain any large vacuoles or lipid bodies and in general looked to be maintained in a healthy state similar to hyphae within the intercellular spaces of plant mesophyll cells.

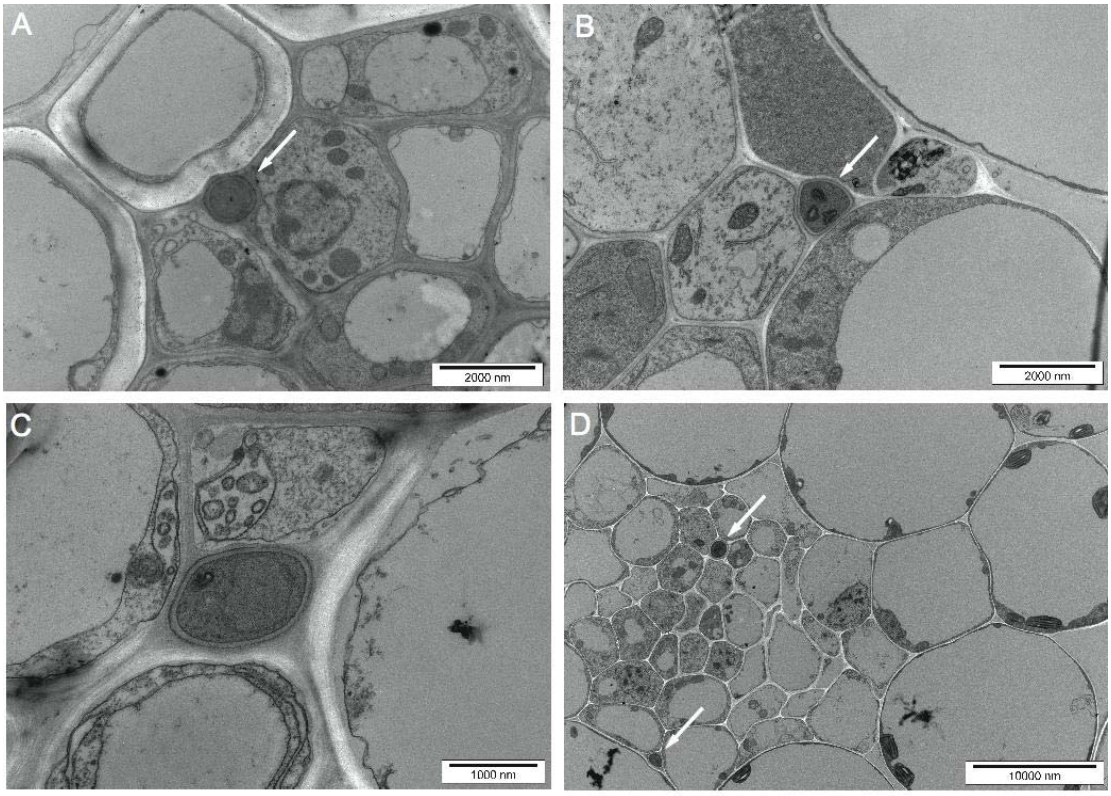


Figure 5.4: Transmission electron micrographs of vascular bundle colonisation in manually infected plants. A) *N. uncinatum* strain U3 within developing vascular bundle tissue (arrow) of a *F. pratensis* host. B,C) Hyphae of *N. uncinatum* strain U4 within vascular bundle tissue of a *F. pratensis* host. D) Hyphae of *E. festucae* strain Fl1 contained in a vascular bundle within a *L. perenne* host (arrows).

5.3 *N. uncinatum* may possess a unique cell wall structure

While the endophyte *E. festucae* has been studied extensively, not much is known about the growth or morphology of *N. uncinatum*. During *in planta* TEM analysis, it was observed that the cell wall structure of *E. festucae* Fl1 was different to that of *N. uncinatum* U2 within a *F. pratensis* host (Fig 5.5). The outer edge of Fl1 was fibrous in nature, less defined and less electron dense than that of U2. Hyphae of Fl1 were surrounded by more mucilage from the intercellular space than that of U2 which was

rarely observed and only showed mucilage where hyphae were in direct contact with the plant cell walls. Apart from these differences, the structure of Fl1 and U2 hyphae in *F. pratensis* were very similar.

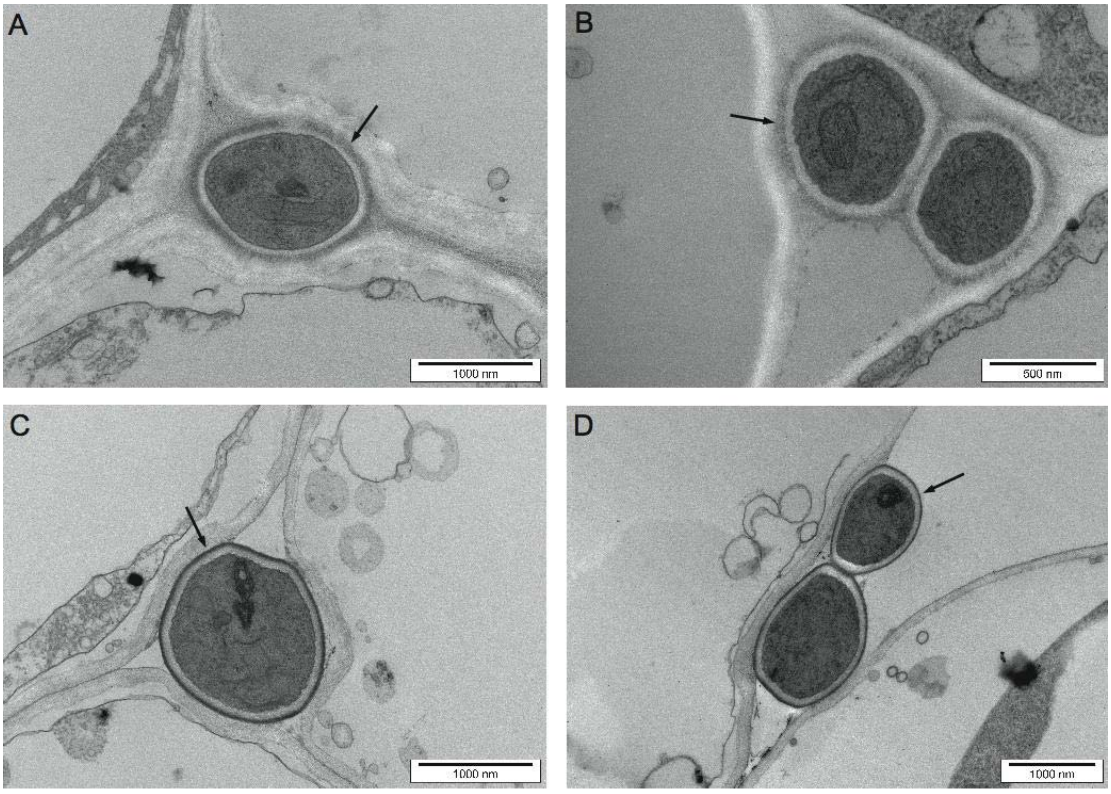


Figure 5.5: Transmission electron micrographs of *E. festucae* and *N. uncinatum* within *Festuca pratensis*. A,B) Hyphae of *E. festucae* within intercellular spaces show an outer cell wall that is fibrous in nature (arrows). This fibrous layer was most prominent at the contact point with plant cell walls. C,D) Hyphae of *N. uncinatum* within intercellular spaces show a thin electron dense outer cell wall (arrows). No fibrous layer can be seen around hyphae.

5.4 *N. uncinatum* shows signs of incompatibility in the outer leaf layers of Festulolium hybrids.

WGA-Alexafluor-488 analine blue staining of *N. uncinatum* U2 hyphae within the leaf sheaths of Festulolium hybrids showed a variation in endophyte growth, with some samples containing well aligned linear hyphae while others contained fewer hyphae, had a deregulated pattern of growth, were not aligned to the leaf axis and were highly branched. Samples taken from the first leaf sheath (oldest tissue) were found to have very few or no hyphae present (not imaged). Samples of younger tissue from the second leaf sheath showed a low number of hyphae present, that appeared to have a deregulated pattern of growth compared to the natural association. Hyphae in this layer were generally found to grow in an unstable fashion compared to the straight hyphae seen in the natural host (Fig. 5.6). WGA-Alexafluor 488 staining showed that chitin production in the septa of some hyphae was abnormal, with large areas of chitin exposed. Hyphae within the newest leaf sheath (youngest tissue) grew with characteristics similar to U2 within its natural host (Fig. 5.6). Hyphae were aligned parallel to the leaf axis, straight and rarely branched. A much greater fungal biomass was present within this younger tissue when compared to older second and first leaf sheaths.

To better understand the colonisation of Festulolium hybrids by U2, pseudostem tissue of plants infected with endophyte were analysed by light microscopy and TEM. Light microscopy showed that hyphae were present within younger tissue but were generally absent from older tissue (Fig. 5.7). Hyphae that were present in inner layers stained densely and appeared healthy within the intercellular spaces of the

plant tissue. Older tissue that did not contain any hyphae had signs of senescence and in some areas the breakdown of leaf tissue was observed.

Further independent samples were analysed to confirm this initial observation was unique to the *N. uncinatum*-Festulolium association. Light microscopy from these samples showed that colonisation of the outer layer of plant tillers was quite variable, from abundant colonisation to very low colonisation (Fig. 5.8, 5.9). The morphology of those hyphae present in outer layers were different to those found in the inner layers. The morphology of the outer layer hyphae ranged from hyphae that were distinct and well stained to irregular shaped hyphae that lacked cytoplasmic staining (Fig. 5.8, 5.9). Many of the irregular shaped hyphae found within the outer layers were also much larger than the inner layer hyphae or hyphae seen in the natural association of U2 in *F. pratensis*. No irregular shaped or cytoplasmically distorted hyphae were found within inner sheath layers.

To better understand the changes in hyphal structure seen in outer leaf layers, samples were analysed by TEM. Hyphae in the inner layers were found to be similar to those seen in the natural association between U2 and *F. pratensis* (Fig. 5.10). These hyphae had a distinct outer cell wall that was electron dense and retained a densely stained cytoplasm. Hyphae were found within the intercellular spaces and appeared to be attached to plant cell walls through a mucilage layer. In some intercellular spaces electron dense inclusions could be seen within the mucilage layer that surrounded the hyphae, although these inclusions were not present in all intercellular spaces.

Hyphae within the outer leaf layers had an irregular cell shape and degenerated cytoplasm (Fig. 5.11). Single and multiple hyphae were found within intercellular spaces. In addition to the degenerated cytoplasm, hyphae in the outermost layers often had a thickening of the cell wall and presence of a dense covering around the cells walls. Some hyphae formed large vacuoles and lipid body structures within the cytoplasm (Fig. 5.11). These features are indicative of incompatibility between the host and endophyte in the outer leaf layers.

Hyphae displaying incompatibility as evidenced by a degenerated cytoplasm, production of vacuoles or irregular cell shape were often associated with an electron dense covering around the outside of the fungal cell wall (Fig. 5.12). This layer was not always associated with distorted hyphae but was prevalent in many cases where hyphae showed incompatibility with the host. Electron dense inclusions were observed within the mucoid layer of the intercellular spaces where hyphae were present. These dense inclusions also covered the outside of hyphal cell walls, often accumulating between hyphae. This dense covering was not observed where hyphae were attached to cell walls by way of a mucoid layer but was formed where hyphal walls were exposed to empty intercellular space (Fig. 5.12).

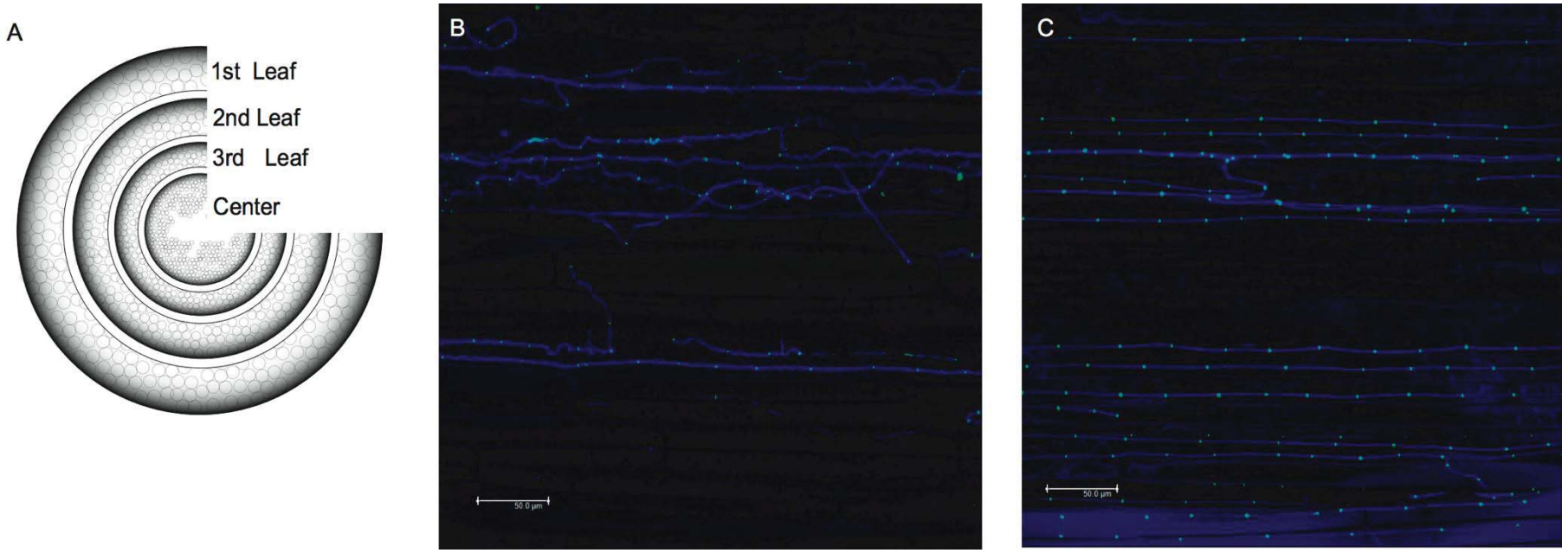


Figure 5.6: Confocal microscopy analysis of single leaf layers of Festulolium plants infected with *N. uncinatum* U2. A) Schematic of plant tiller showing leaf layers where tissue samples were taken. B) Confocal depth series image of longitudinal section from leaf sheath of the second leaf. Hyphae stained with Alexafluor (WGA-AF488) and aniline blue. The image shows hyphae (blue) growing in an unrestricted pattern within intercellular spaces of plant cells. Strongly illuminated green points indicate hyphal septa. C) Confocal depth series image of longitudinal section from leaf sheath of the third leaf. Hyphae stained with Alexafluor (WGA-AF488) and aniline blue. Hyphae (blue) grow in a restricted pattern aligned parallel to the leaf axis and within intercellular spaces. Strongly illuminated green points indicate hyphal septa.

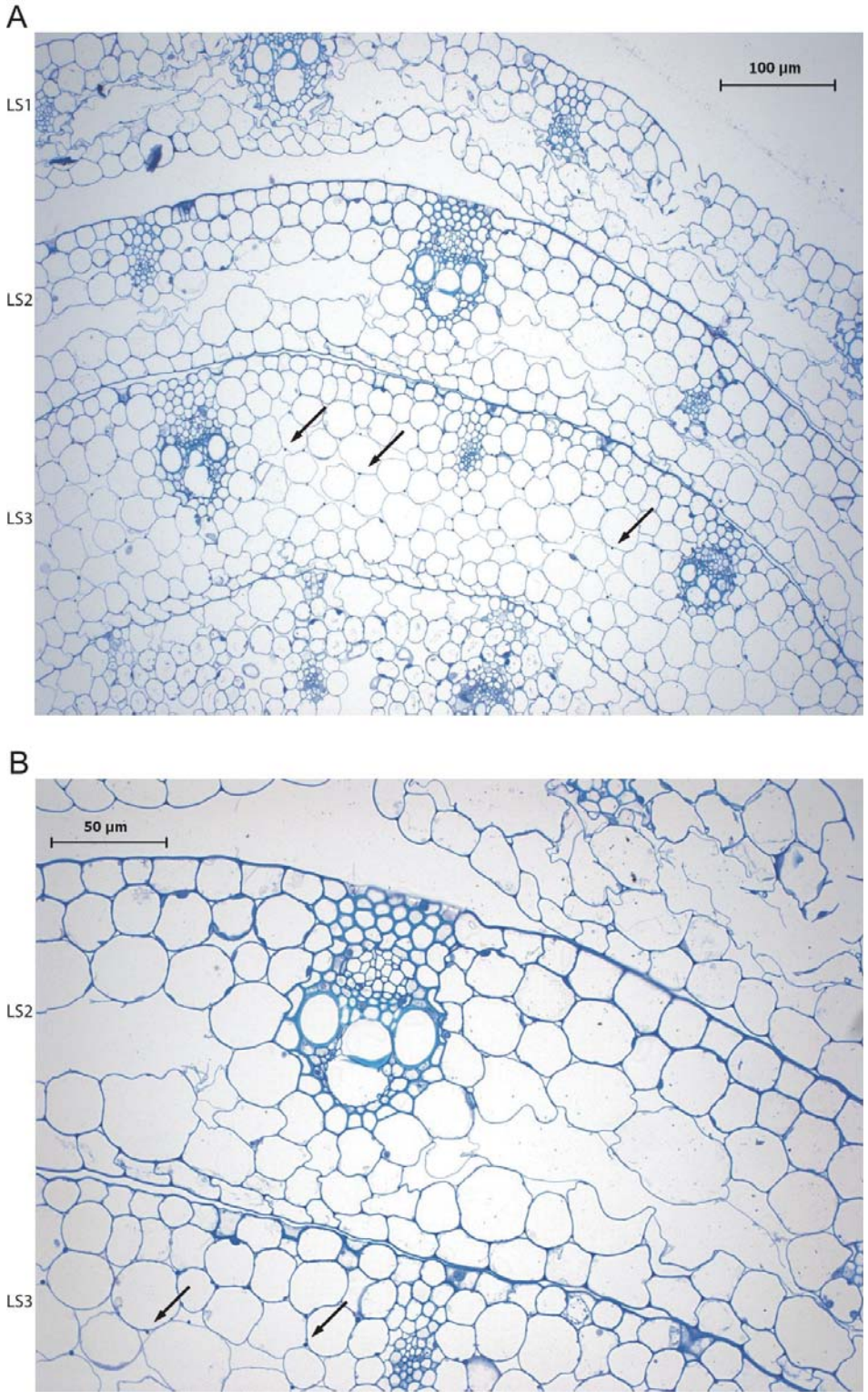


Figure 5.7: Light micrograph of Festulolium hybrid pseudostem infected with *N. uncinatum*. A) Pseudostem transverse section shows layers of plant leaf sheaths (LS1-3) growing from a single tiller. Hyphae of *N. uncinatum* strain U2 can be seen in only the innermost sheath, LS3 (arrows). B) Expanded view of leaf layer showing hyphae present in inner layer LS3 (arrows) but an absence of hyphae in the adjacent layer LS2.

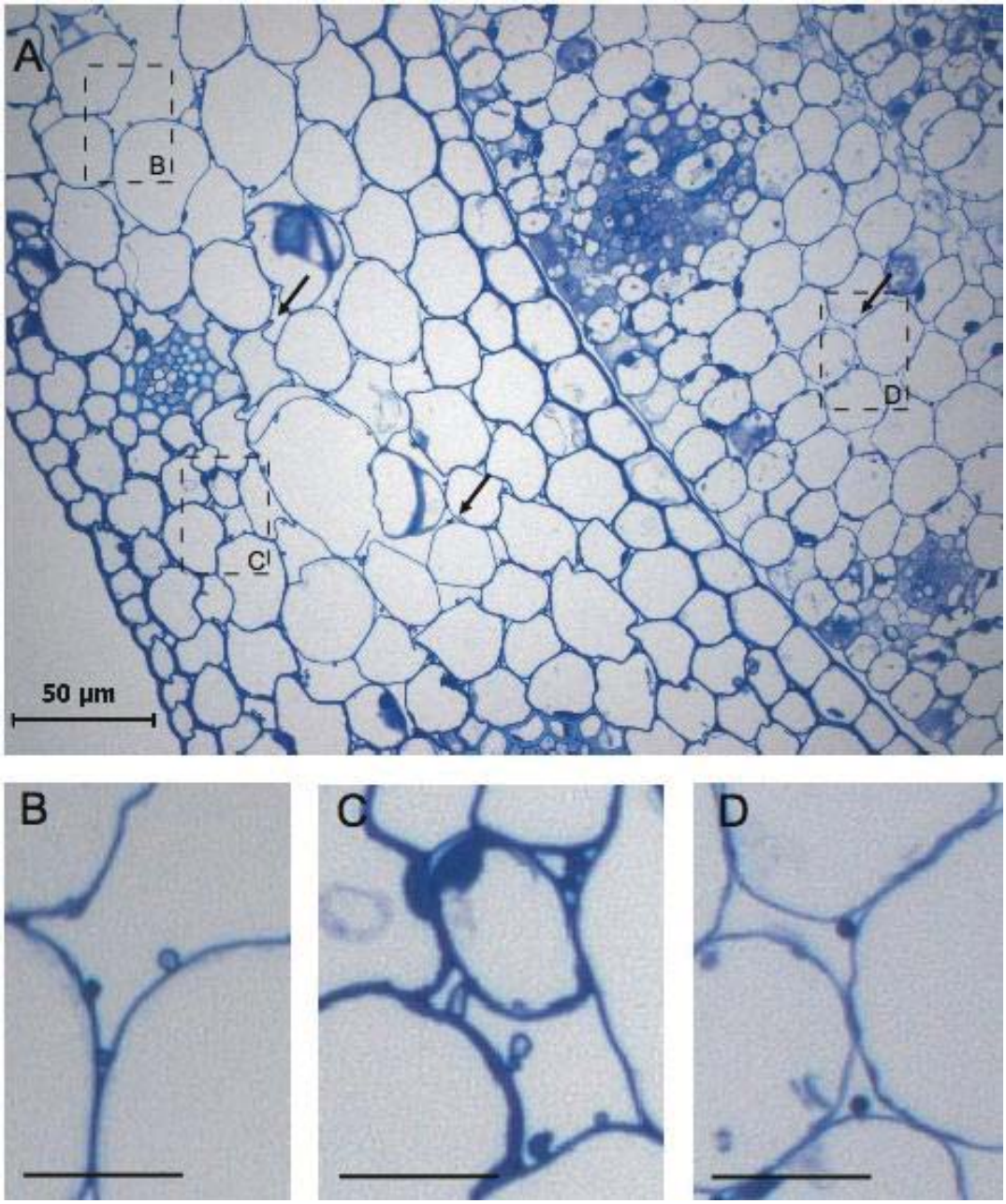


Figure 5.8: Light micrograph of Festulolium pseudostem infected with *N. uncinatum* showing incompatibility in outer leaf layers. A) Transverse section of pseudostem with inner and outer layers colonised by endophyte hyphae (arrows). B,C) The outer leaf layers contain multiple hyphae within intercellular spaces. Hyphae appear misshapen in some cases and the cytoplasm stained poorly. Bar = 12.5 µm. D) Hyphae within inner layers are well stained and form uniform circular hyphae within the intercellular spaces. Bar = 12.5 µm

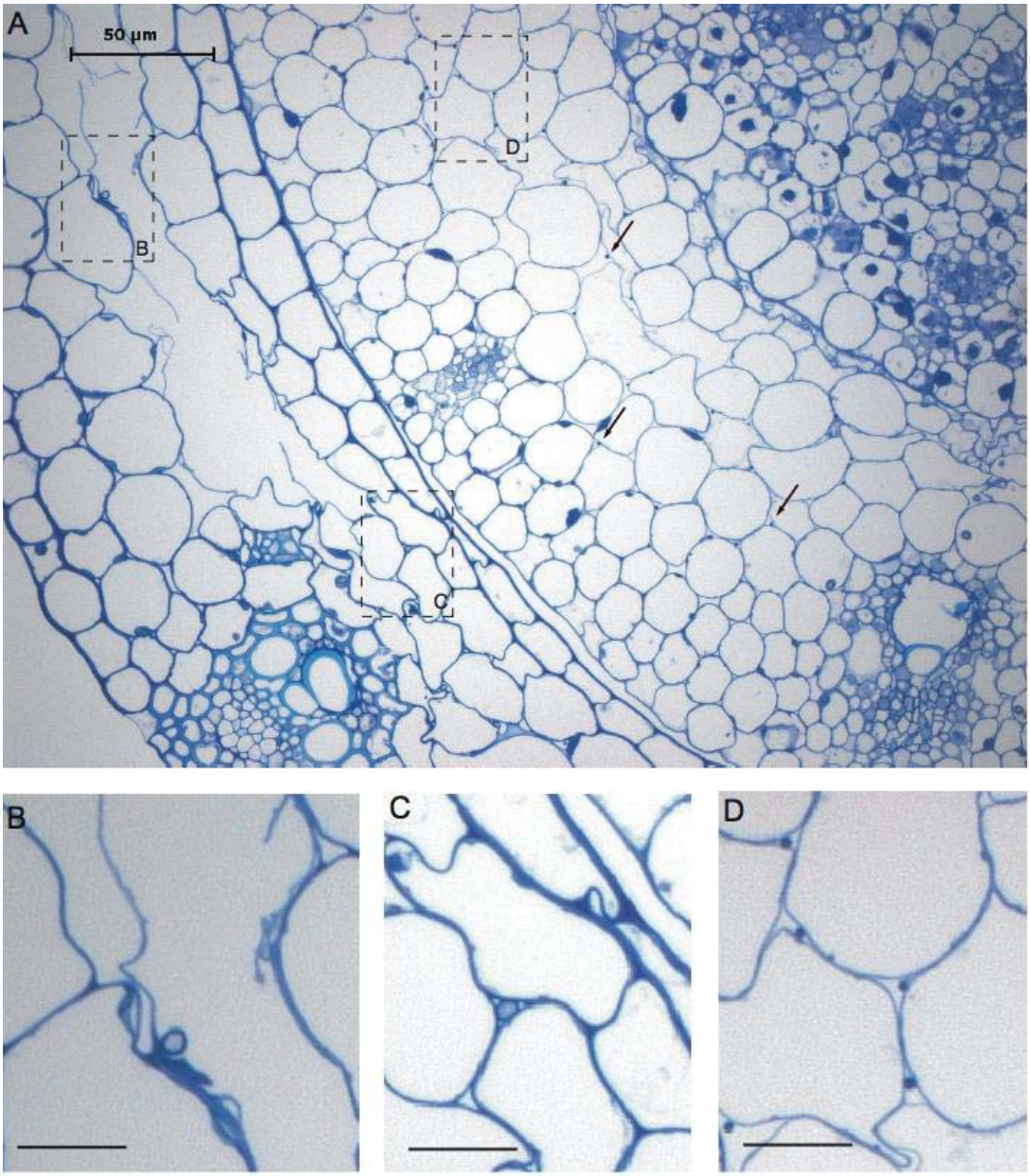


Figure 5.9: Light micrograph of Festulolium pseudostem infected with *N. uncinatum* showing decreased colonisation and incompatibility in outer leaf layers. A) Transverse section of pseudostem with inner and outer layers of plant pseudostem colonised by endophyte hyphae (arrows). The outer layers have little to no colonisation. B,C) Hyphae within intercellular spaces of outer layers. Hyphae are much larger and show distorted cytoplasm. Bar = 12.5 µm. D) Hyphae within inner layers are well stained and form uniform circular hyphae within the intercellular spaces. Bar = 12.5 µm

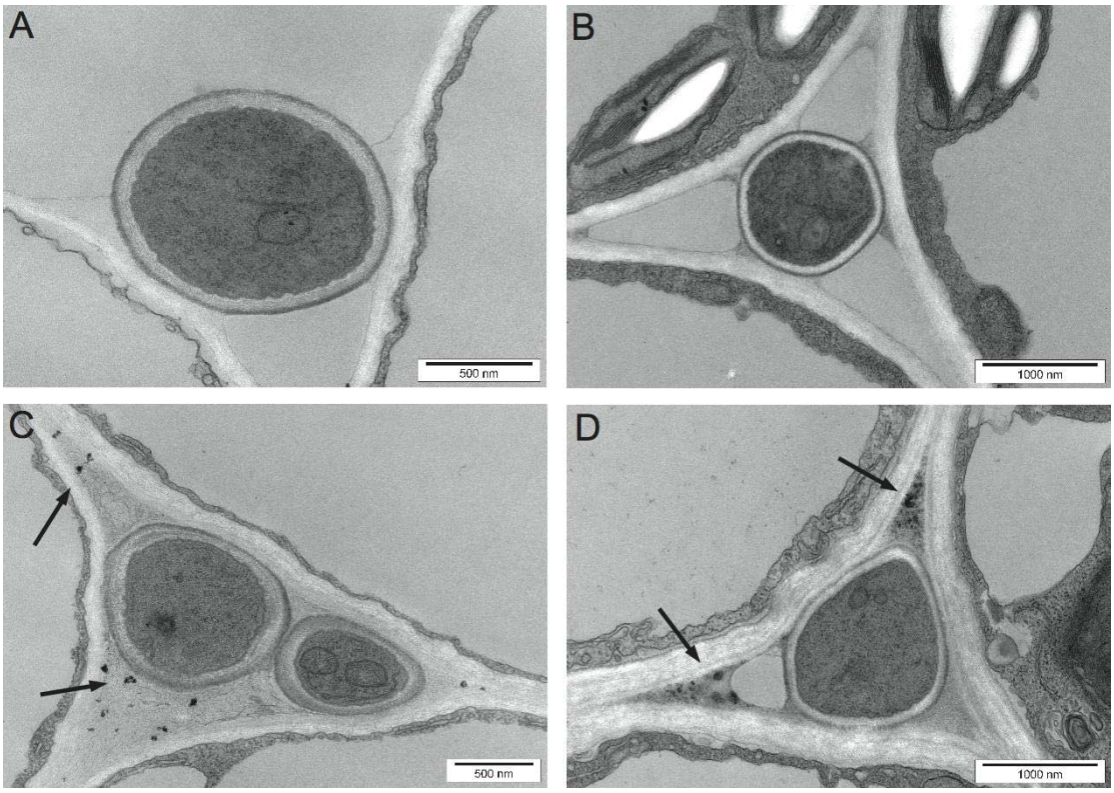


Figure 5.10: Transmission electron micrographs of *N. uncinatum* colonising inner leaf layers of a Festulolium hybrid. A,B) Hyphae within the intercellular space are cytoplasmically electron dense and are firmly attached to cell walls. A mucilage layer surrounds the hyphae and is prominent at the point of attachment to plant cells. C,D) Hyphae within the intercellular space show the presence of densely stained inclusions within the mucilage layer around the hyphae (arrows) Hyphae remain firmly attached to cell walls within intercellular space and are cytoplasmically dense.

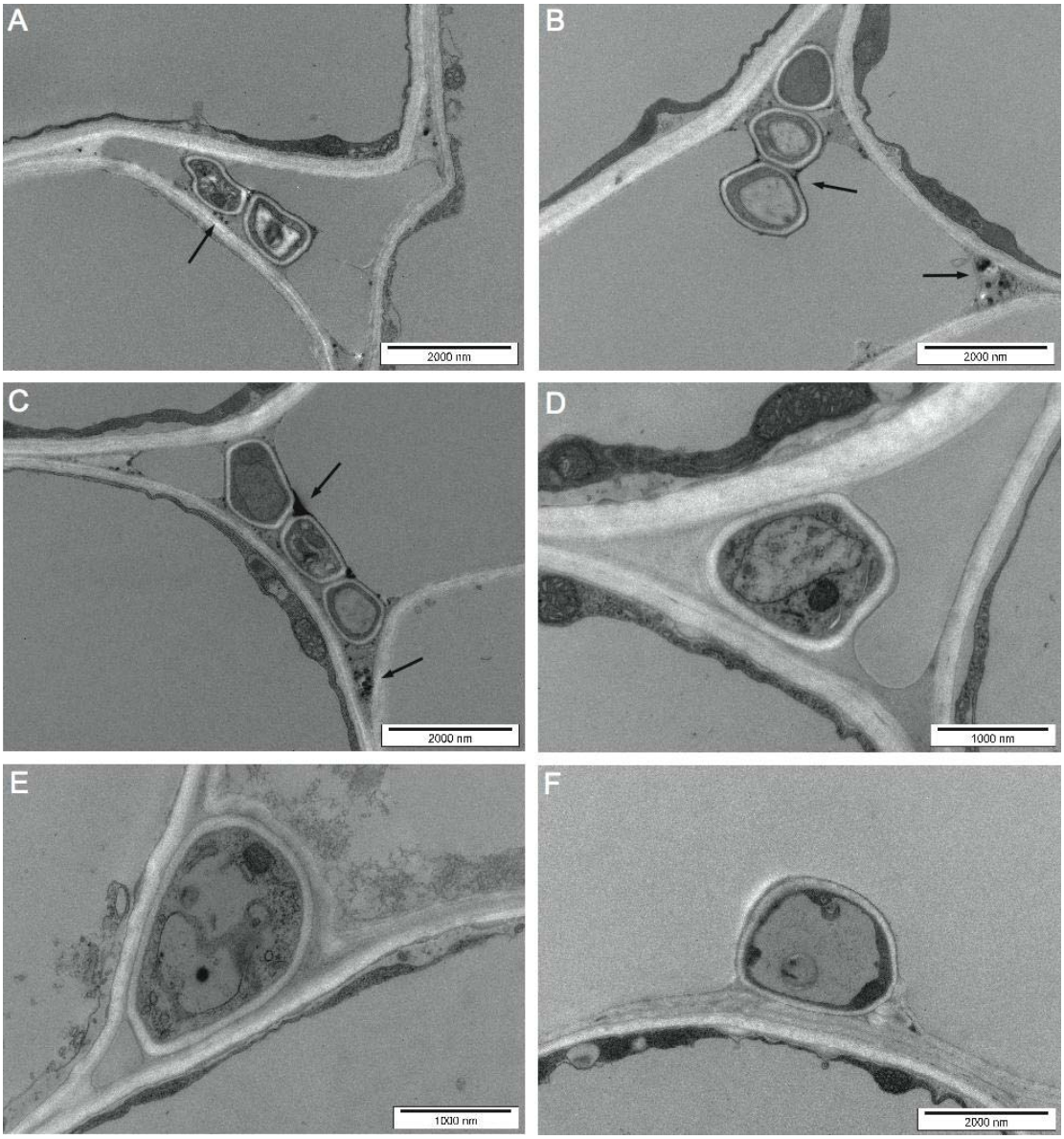


Figure 5.11: Transmission electron micrographs of *N. uncinatum* colonising Festulolium in outer leaf layers. A-C) Multiple hyphae within intercellular spaces attached to plant cell walls. Hyphae are attached to plant cells by way of a mucoid layer directly or through contact with other hyphae (B). Densely stained inclusions are present within the mucoid layer, which appears to form a coating around some hyphae (arrows). Hyphae appear misshapen (A) and may contain large vacuoles (B,C). D-F) Single hyphae within the intercellular spaces of outer layers. Hyphae lack densely stained cytoplasm and cell organelles are visible. Hyphae are surrounded by a mucoid layer and firmly attached to plant cells, although no dense inclusions are present.

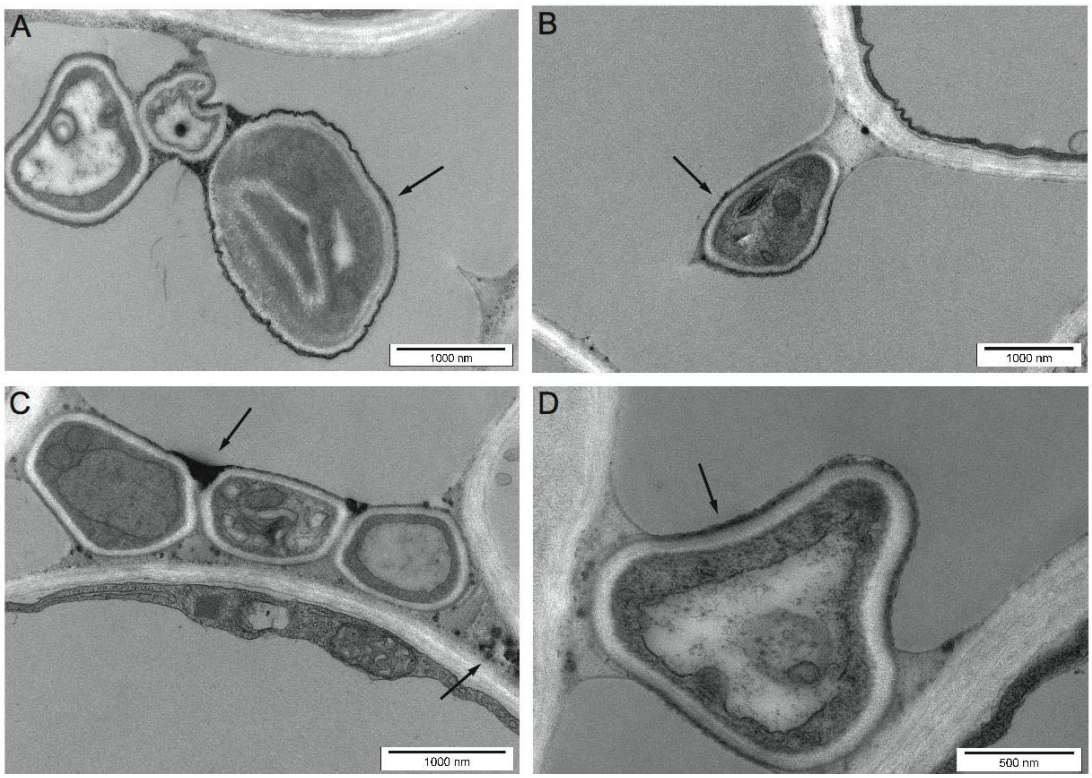


Figure 5.12: Transmission electron micrographs of *N. uncinatum* within the outer layers of a Festulolium hybrid show dense outer wall coverage. A) A cluster of hyphae attached by a thin strand of mucoid layer to the plant cell wall. Hyphae are misshapen and contain distorted cytoplasmic contents. The largest hypha is coated in an electron dense layer that completely surrounds the outer cell wall (arrow). B) A single hyphae attached to plant cell wall via mucilage layer showing thin outer coating of electron dense material (arrow). C) Multiple hyphae firmly attached within the plant intercellular space. A large electron dense layer is seen to coat the outer surface of hyphae not in contact with plant cell wall (arrow). Dense inclusions are also present within mucoid layer (arrow). D) Close up of misshapen hyphae lacking cytoplasmic contents with electron dense coating forming around hyphae (arrow). Note the coating does not form where hyphae are attached to cell walls by way of a mucoid layer.

5.5 Naturally infected Festulolium hybrids also display incompatibility.

To determine whether the incompatibility observed in artificially inoculated hybrids was retained upon seed transmission or a more stable association formed, seed was provided by Cropmark Seeds Ltd from three separate Festulolium plant lines which exhibited different rates of endophyte transmission. The transmission of endophyte to seed was 29% successful for line FL1432, 52% for FL1436 and 77% for FL1466 (T. Gillanders 2012, pers. comm.). Pseudostem tissue of these plants were transversely sectioned, stained with toluidine blue and analysed by light microscopy. These samples also showed evidence of incompatibility. Hyphae in the outer layers were distorted with misshapen cell walls and lacked a densely stained cytoplasm. In contrast to the artificially inoculated plants, few hyphae with densely stained cytoplasm and regular cell shape were present within the younger tissue. The plant line FL1466 which had the best endophyte transmission had the greatest number of regular growing hyphae within younger tissue than the other two seed lines. Hyphae in the outer layers of FL1466 still showed incompatibility as indicated by decreased cytoplasmic staining, although, regular densely stained hyphae were also present (Fig. 5.13). Plants from FL1436 also showed a similar pattern of colonisation with few normal hyphae in the younger tissue and hyphae in older tissue showing signs of incompatibility (Fig. 5.14). Plant A from FL1432 showed incompatibility with distorted hyphae in the older tissue but regular hyphae were also seen in this tissue. Hyphae biomass however was very low within the younger tissue of this plant (Fig. 5.15). Plant B of FL1432 showed many hyphae present within leaf layers colonising young and older tissue yet these hyphae were highly distorted and lacked cytoplasm

staining (Fig. 5.16). Overall each plant from the different lines exhibited some form of incompatibility as previously seen in artificially inoculated Festulolium hybrids.

TEM images of these plants showed similar incompatibility characteristics to Festulolium plants artificially infected with *N. uncinatum*. Only a small number of hyphae had growth and morphology similar to those of the association in the natural host with dense cytoplasm, regular cell shape and distinct cell wall structure (Fig. 5.17). Hyphae in some cases had thicker cell walls that were less electron dense, while the distinct thin outer layer present in the natural host association appeared to be lost (Fig. 5.18). The intercellular spaces of infected plants also contained an accumulation of electron dense product. This product was found in nearly all intercellular spaces and accumulated whether hyphae were present or not. As plant cell walls aged and intercellular spaces became large, the dense accumulations where seen to cover the outside of plant cells and appeared to be contained within a plant mucilage layer or middle lamella (Fig. 5.19). This electron dense product was seen to accumulate and form a dense layer around the outside of hyphae but was not present where hyphae were in direct contact with plant cells. Hyphal cell walls covered in this product showed small areas of degradation in the cell wall (Fig. 5.20). Hyphae also showed degeneration of cytoplasm with the presence of large vacuoles and loss of electron dense staining of the cytoplasm (Fig. 5.21).

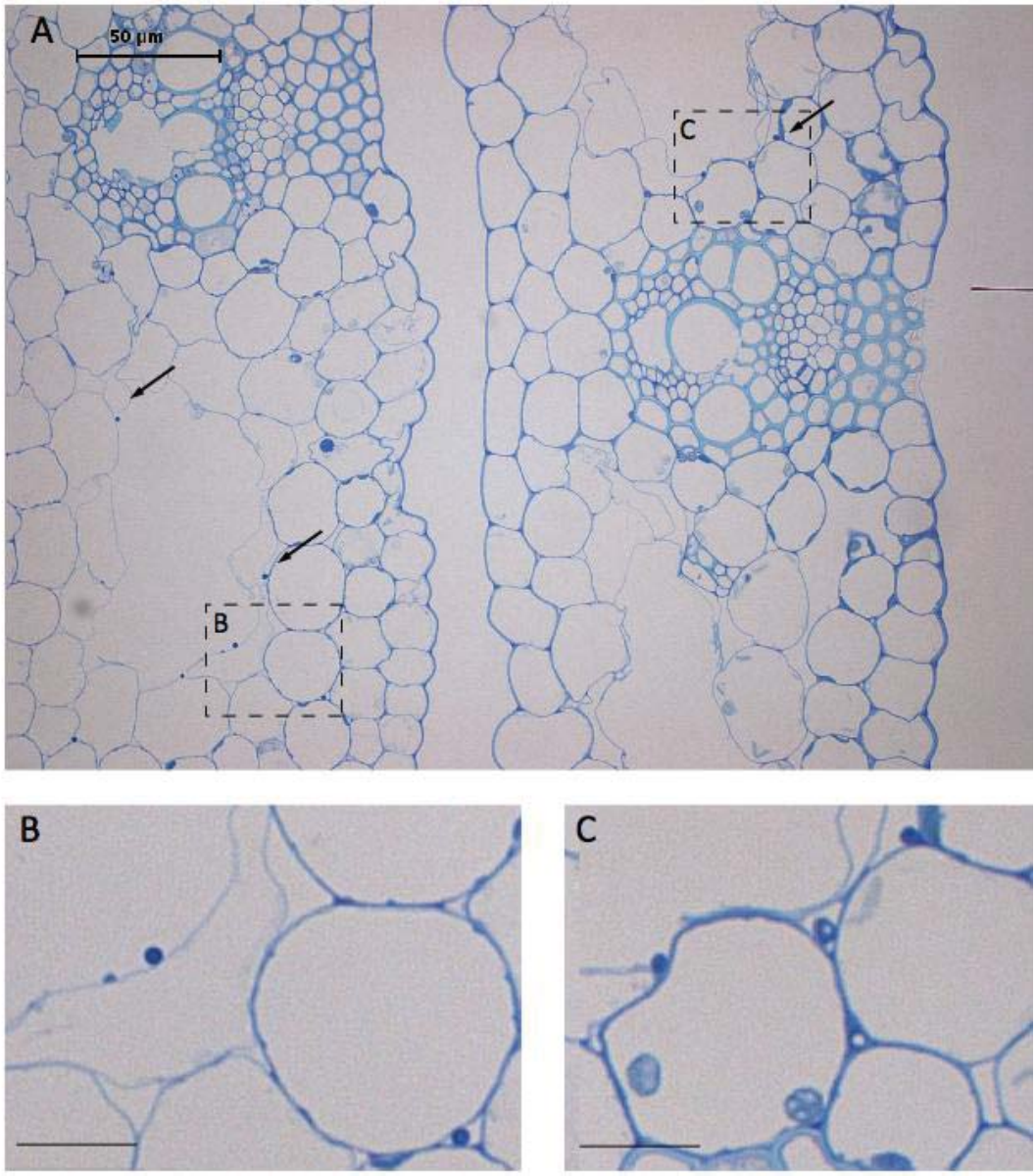


Figure 5.13: Light micrograph of Festulolium (FL1466) naturally infected with *N. uncinatum*. A) The outer layers of plant leaf tissue showing hyphal colonisation within the intercellular spaces of the plant (arrows). B) Hyphae within the inner layer show dense cytoplasmic staining and regular cell shape. C) Hyphae within the outer layers lacking complete cytoplasm staining. Bar = 12.5 µm.

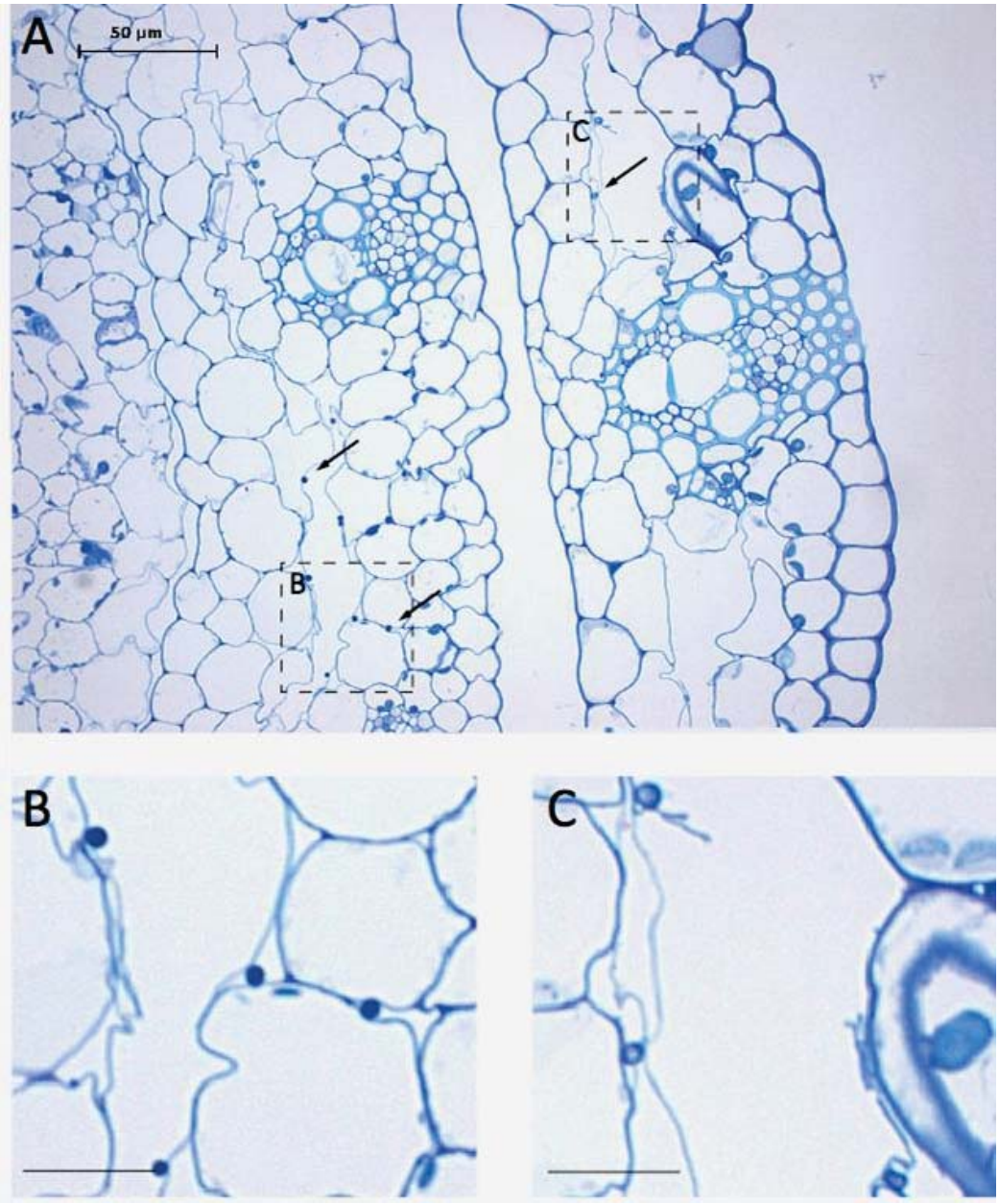


Figure 5.14: Light micrograph of Festulolium (FL1436) naturally infected with *N. uncinatum*. A) The outer layers of plant leaf tissue showing hyphal colonisation (arrows). B) Hyphae within the inner layer with dense cytoplasmic staining and regular cell shape. Bar = 12.5 µm. C) Hyphae within outer layers lack complete cytoplasmic staining. Bar = 12.5 µm.

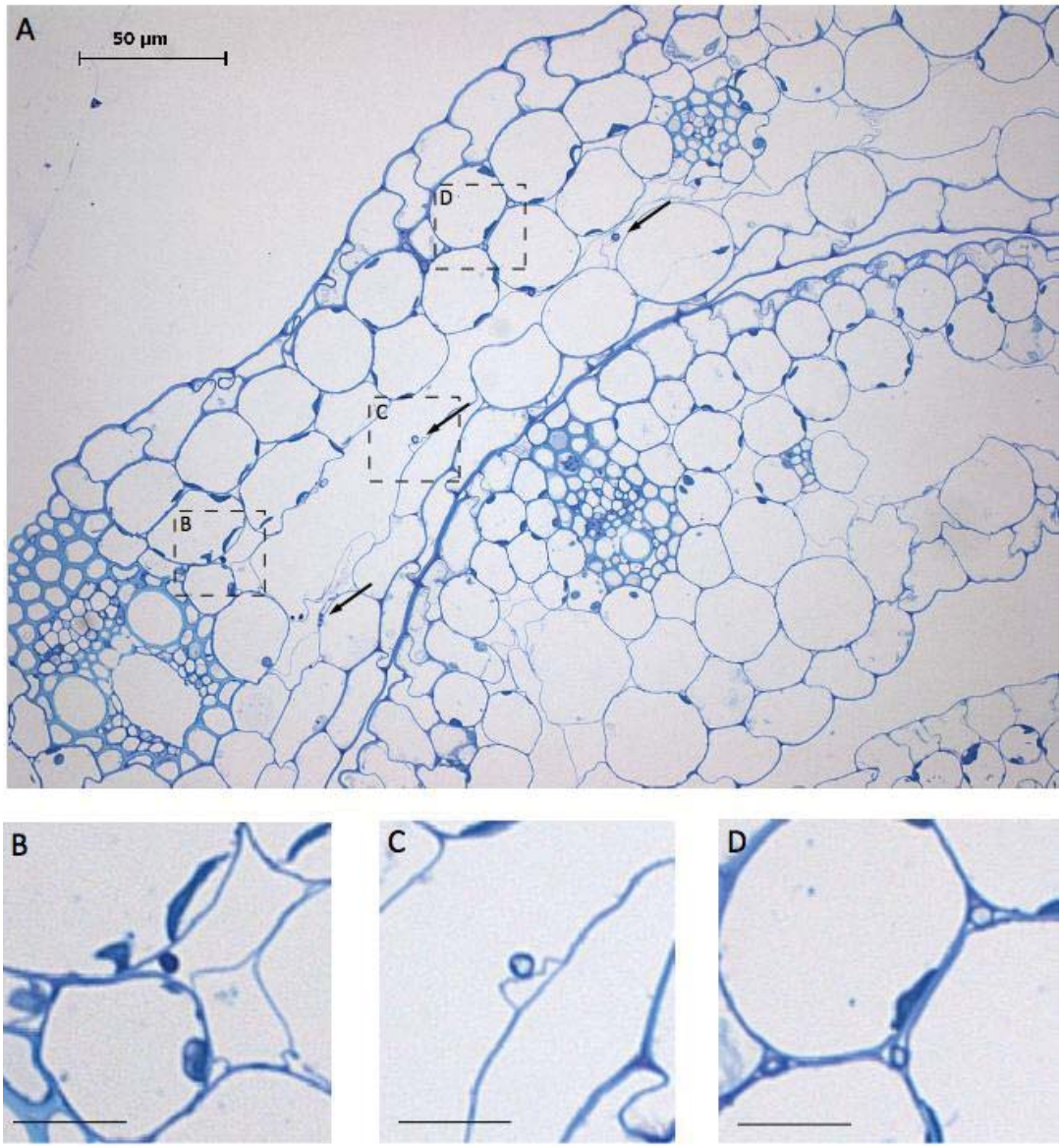


Figure 5.15: Light micrograph of Festulolium (FL1432 A) naturally infected with *N. uncinatum*. A) The outer layers of plant leaf tissue showing hyphae colonisation. Hyphae are within the outermost layer (arrows) colonising the intercellular spaces but appear to be absent from inner layers. B) Hypha within the outer layer with dense staining of cytoplasm and regular cell shape. Bar = 12.5 µm. C,D) Hyphae within the outer layers showing lack of cytoplasmic staining. Bar = 12.5 µm.

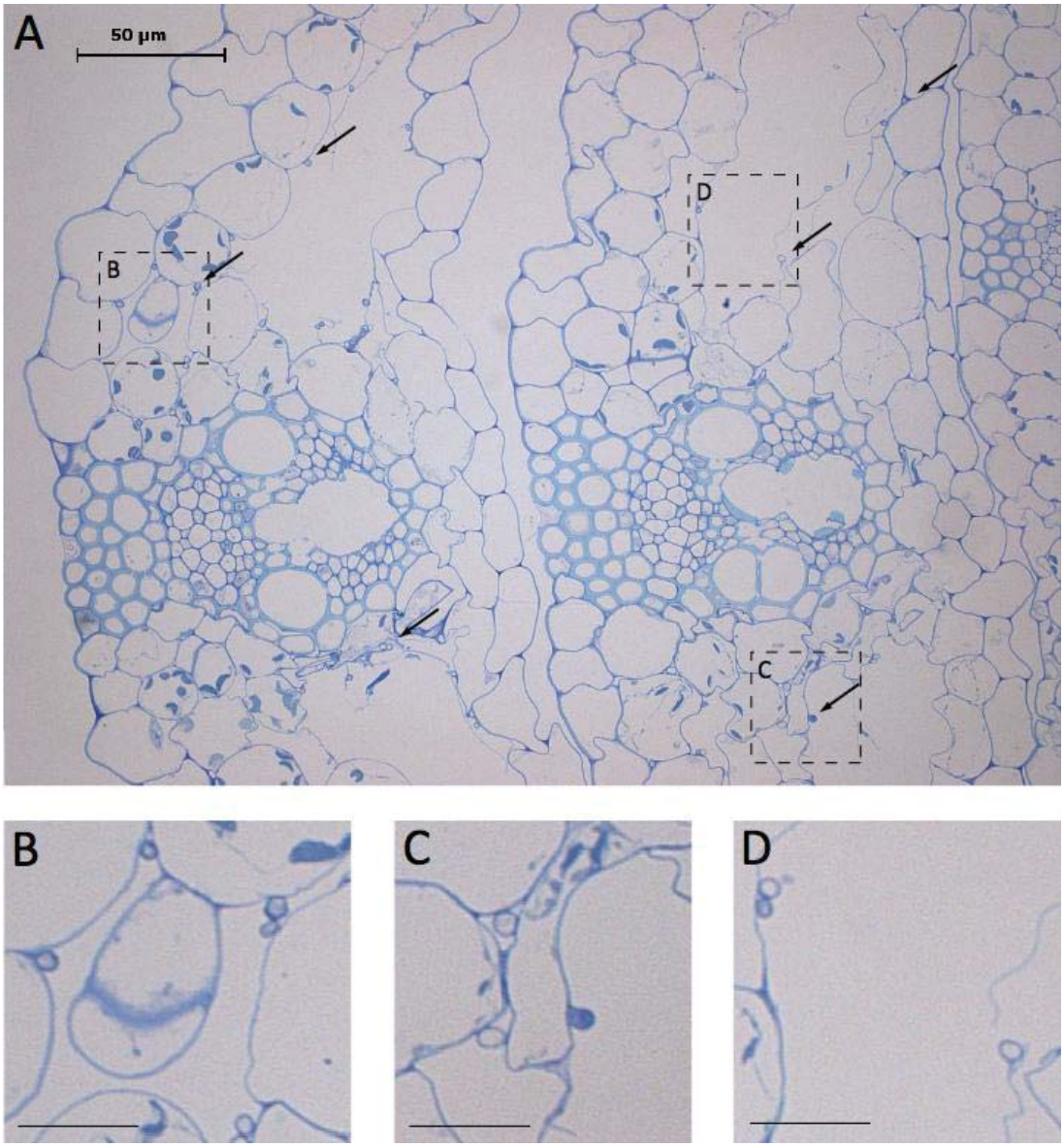


Figure 5.16: Light micrograph of Festulolium (FL1432 B) naturally infected with *N. uncinatum*. A) The outer layers of plant leaf tissue showing hyphae colonisation within all leaf layers (arrows). Hyphae colonise the intercellular spaces of the plant and lack cytoplasmic staining. B) Hyphae within the outer layer show a lack of cytoplasmic staining and irregular cell shape. Bar = 12.5 µm. C,D) Hyphae within the inner layers with irregular cell shape and a lack of dense cytoplasmic staining. Bar = 12.5 µm.

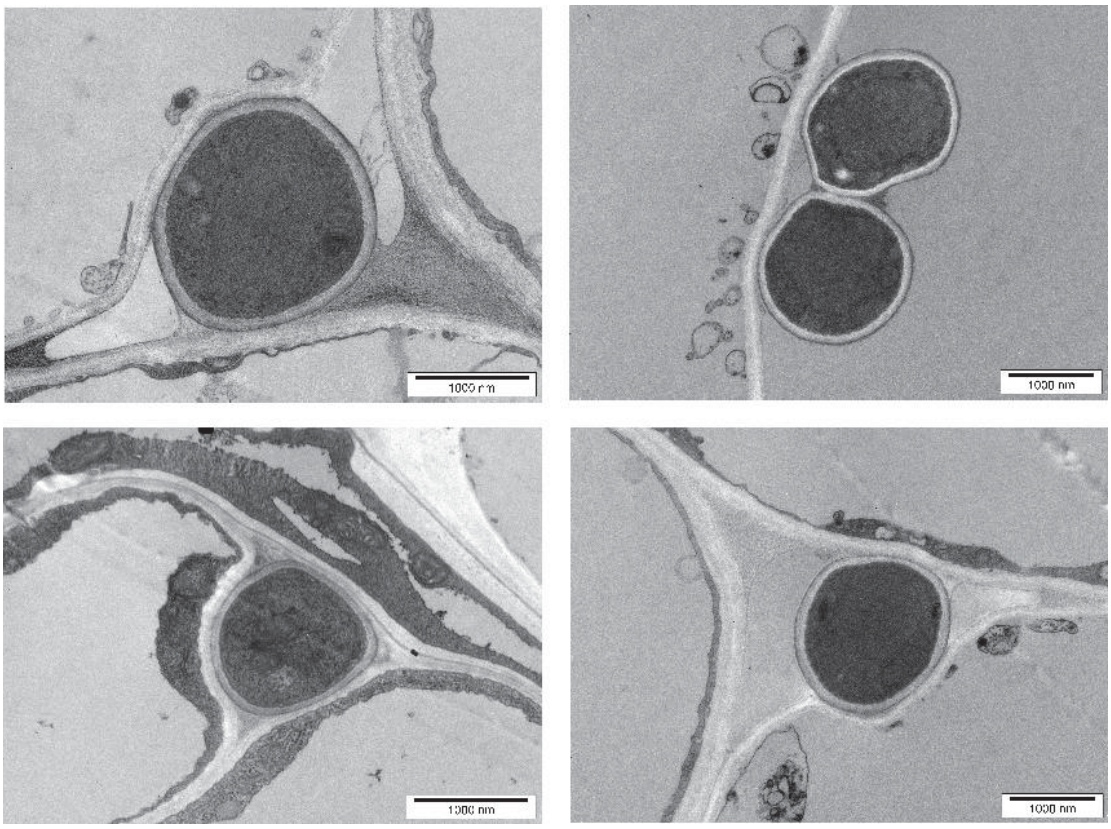


Figure 5.17: Transmission electron micrographs of *N. uncinatum* showing regular hyphae within seed infected Festulolium plants. Hyphae are within the intercellular spaces of plant tissue and have regular cell shape and electron dense cytoplasm. Hyphae show thin cell walls with a distinct outer layer. Hyphae appear to be strongly attached to plant cell walls by way of a mucilage layer and no electron dense inclusions are seen within the intercellular space.

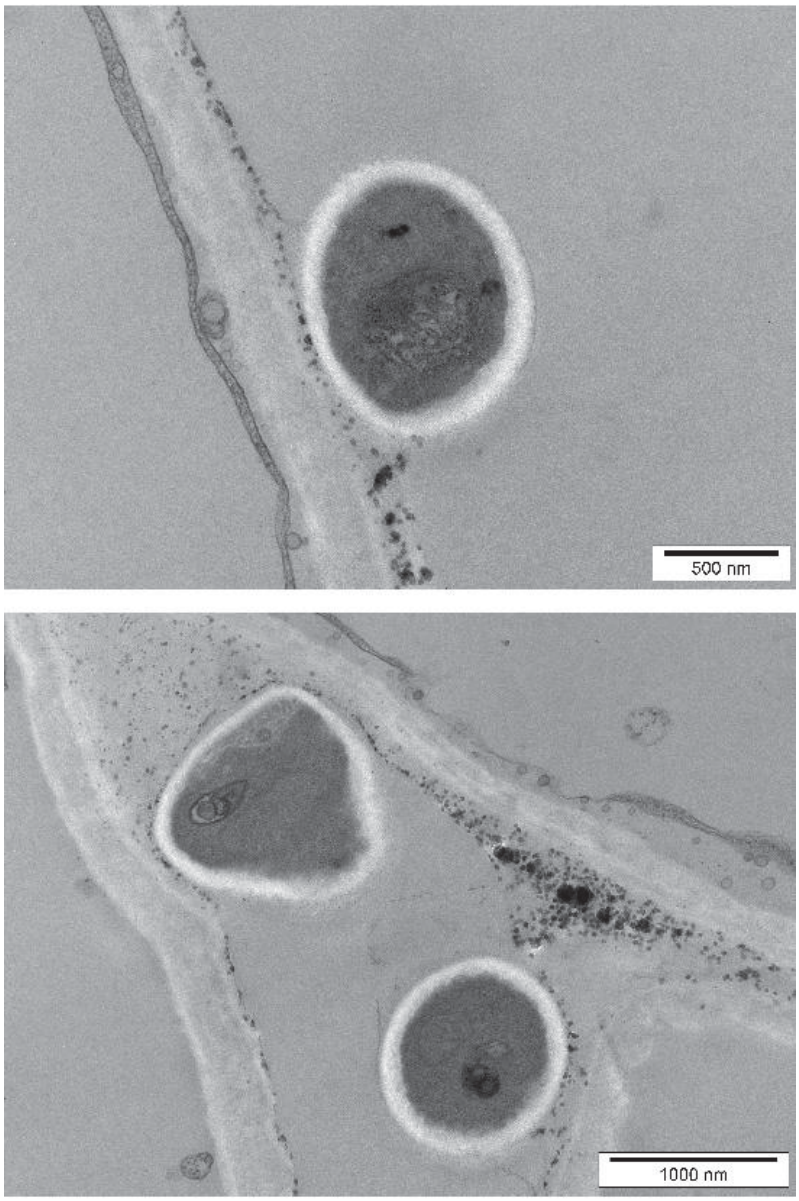


Figure 5.18: Transmission electron micrographs of *N. uncinatum* showing cell wall thickening of hyphae. U2 hyphae within seed infected Festulolium. Cell walls exhibit loss of the distinct dense outer layer, appear thicker and more electron neutral. Dense inclusions are present around hyphae in the mucoid layers of the intercellular space. Hyphae retain cytoplasmic staining and do not show degeneration of cytoplasmic contents.

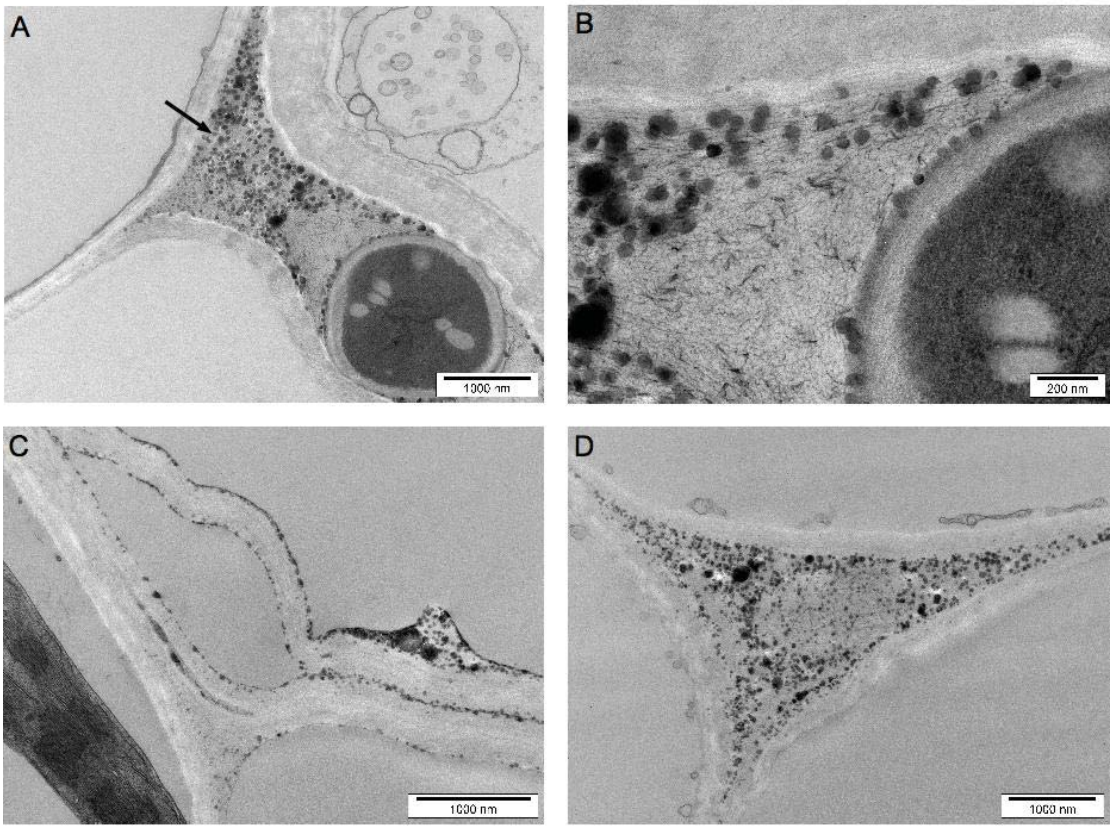


Figure 5.19: Transmission electron micrographs of *N. uncinatum* from seed infected Festulolium showing presence of dense inclusions within intercellular spaces. A) Hypha within the intercellular space surrounded by small dense inclusions (arrow). Hypha has regular cell shape and cytoplasm staining and is attached to plant cell walls. B) An enlarged view of the intercellular space showing presence of dense inclusions and fibrous product surrounding hypha. C) Dense product is present on the surface of plant cell walls. D) Dense inclusions are produced in the intercellular spaces where hyphae are not present.

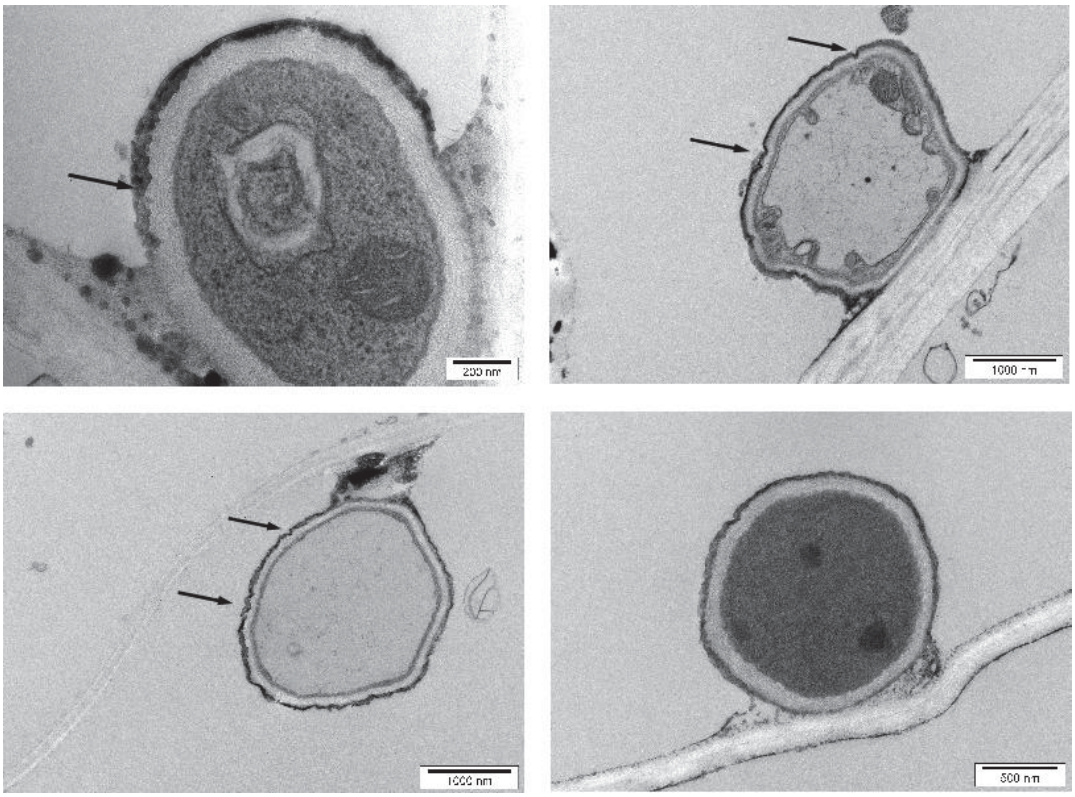


Figure 5.20: Transmission electron micrographs of *N. uncinatum* from seed infected Festulolium showing dense accumulations around hyphal cell walls. Electron dense product forms over the outer surface of hyphal cell walls not in contact with plant cells or mucoid layer. Hyphal cell walls in contact with dense layer appear to be degraded in some areas (arrows). Hyphal cytoplasm ranges from electron dense to highly vacuolated and degenerated.

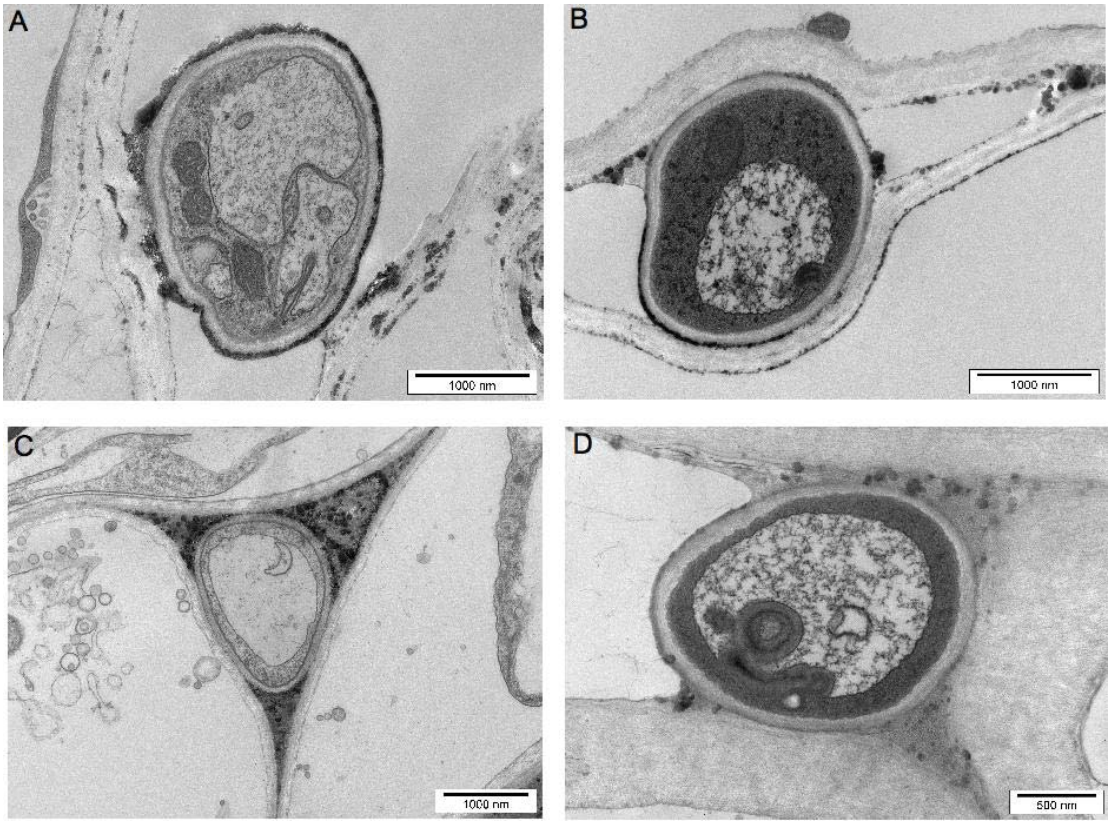


Figure 5.21: Transmission electron micrographs of *N. uncinatum* from seed infected Festulolium showing degeneration of cytoplasm. A) Hypha surrounded by dense inclusions with considerable degeneration of cytoplasm. Large vacuoles are present within the hypha and cytoplasm is no longer electron dense. B) Hypha within the intercellular space containing a large vacuole in the cytoplasm. Hypha is attached to plant cell walls by way of a mucilage layer which contains dense inclusions. C) Highly degenerated hypha within the intercellular space. Hyphae is completely surrounded by mucoid layer which contains numerous electron dense inclusions. D) A single hypha showing large vacuole within the intercellular space, firmly attached to plant walls by way of the mucilage layer which contains few electron dense inclusions.

5.6 Colonisation of a hybrid cultivar causes lower loline production

Festulolium plants manually infected with U2 endophyte were tested for loline production after 15 weeks of growth under controlled environmental conditions. Plants were found to have much lower loline production compared to that of the natural association (Fig. 5.22). Within meadow fescue, the average loline production was around 7800 ppm while the Festulolium host produced an average of around 1500 ppm. Festulolium plants lacked lolines within the root and for two of the three replicates lolines were only found within leaf tissue. *N*-formylloline (NFL) was produced at the highest level while *N*-methylloline (NML) was the second highest. Only trace amounts of the other lolines were detected in the Festulolium host.

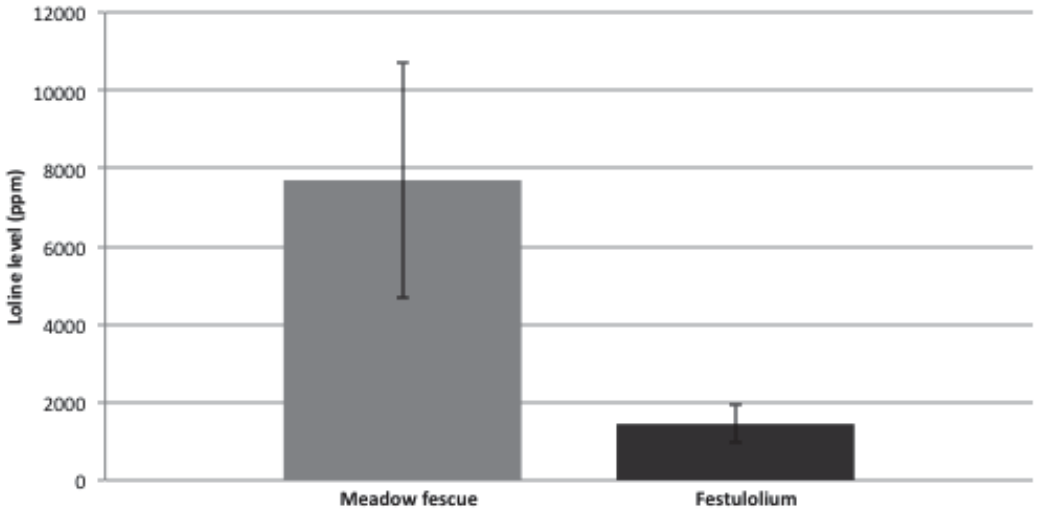


Figure 5.22: Loline production by *N. uncinatum* in meadow fescue and Festulolium. Average loline levels (ppm) across all tissue types compared between meadow fescue and Festulolium hosts infected with *N. uncinatum* U2 (n > 3). Error bars ± standard deviation.

5.7 Discussion

Before the growth and colonisation of *N. uncinatum* in Festulolium hybrids could be compared, a thorough understanding of its growth within the natural host meadow fescue was needed. Infection and colonisation in this host was analysed using a variety of microscopy techniques and showed that hyphae grew within the intercellular spaces, were highly regulated, stained electron dense and were abundant within all leaf tissue; observations indicative of a stable endophyte-grass association. While the natural association was stable, hyphae were found to colonise the vascular tissue at low levels when manual inoculation was used to infect plants with endophyte. Similar vascular bundle colonisation has been reported previously (Christensen *et al*., 2001), although the tissue analysed in the current study had lower colonisation. Plants grown from seed infected endophyte did not show any vascular bundle colonisation and may indicate that the manual inoculation technique results in very low levels of vascular bundle colonisation. However this colonisation was not seen to have any impact on the growth of host plants.

Hyphae of *N. uncinatum* were seen to have a cell wall that appears to be substantially different to that of the endophyte *E. festucae* Fl1. The outside of the hyphal cell wall is much denser and thinner in *N. uncinatum* than in *E. festucae,* which has a thick fibrous coating indicative of a different cell wall composition. Little is known of the composition of the cell walls of these endophytes, however analysis in other fungi has shown the presence of two distinct layers of the cell wall (Pettolino *et al.*, 2009, Osumi, 1998). Analysis of the two distinct layers found in the plant pathogen *Rhynchosporium secalis* showed that the outer layer was

rhamnomannan, protein and glycoprotein rich while the inner layer was dominated by (1,3/1,6)-β-D-glucans, galactomannans, rhamnomannans, (1,3;1,4)-β-D-glucans and chitin (Pettolino *et al*., 2009). If this is similar in *E. festucae* and *N. uncinatum* then the difference seen in this study would indicate a difference in the protein rich composition of the outer layer in the hyphal walls. The cell wall of fungi is particularly important for plant associations as this is the component involved in innate cell immunity (Chisholm *et al.*, 2006). In the case of *E. festucae* and *N. uncinatum* the difference in this outer cell layer may be important for suppression or evasion of the plant immune response in the different hosts they colonise. Further analysis of the cell wall composition of endophytes may help determine their ability to colonise plant hosts while eliciting no immune response by the plant. Particular focus on the differences in cell wall composition and the host range of endophytes may help identify specific factors needed to evade host immunity and allow colonisation in particular host backgrounds. The difference in cell wall structure was also apparent in attempts to obtain protoplasts from *N. uncinatum* and *E. festucae* (data not shown). While *E. festucae* yields high numbers of protoplasts using standard lysing enzymes, very low yields are obtained from *N. uncinatum* under the same protocol. The difference in cell wall composition may cause enzymes previously effective against *E. festucae* to be ineffective on *N. uncinatum*.

While *N. uncinatum* was known to colonise Festulolium hybrids, the colonisation characteristics and morphology of *N. uncinatum* within Festulolium hybrids was largely unknown. Confocal microscopy analysis of leaf blade found a variation in colonisation pattern dependent on the age of tissue. The pattern of colonisation in young tissue was similar to that of the natural association while older tissue in the

outer leaf layers showed distorted hyphal growth. Older leaf tissue was largely devoid of any hyphae and overall it appeared that hyphal colonisation within the plant was lost as the tissue aged. Light microscopy analysis of toluidine blue stained transverse sections of pseudostem showed lower hyphal biomass within older tissue as well as hyphae lacking cytoplasmic staining with irregular cell shape. TEM analysis of pseudostem showed hyphae exhibited incompatibility with the host, characterised by cell wall thickening, degeneration of cytoplasm and an electron dense covering around hyphae. These characteristics were not seen in hyphae colonising the natural host plant and appear to be unique to hyphae within the Festulolium host. A similar incompatible phenotype has been observed in other endophyte associations with loss of hyphae in older tissue and distorted cytoplasm (Koga *et al.*, 1993). The incompatibility in the current study does not result in the loss of hyphae completely from the plant and fresh tillers remained colonised, indicating a healthy endophyte population within the meristematic tissue. Hyphal loss within outer leaves was not thought to be a due to a delay in colonisation by the endophyte of new tillers, but more due to incompatibility and degradation of hyphae in older tissues.

The incompatibility seen between *N. uncinatum* and Festulolium may be the result of a variety of mechanisms. While it is difficult to determine from electron micrographs the nature of the mechanism, the appearance of hyphae with degenerated cytoplasm and large vacuoles may indicate a restriction in the nutrient uptake by the hyphae. Further support for this hypothesis is the thickening of cell walls and the production of a dense covering around cells. It is possible that hyphae slowly lose the ability to obtain nutrients from the plant as the tissue ages. The

presence of large vacuoles within hyphae may also indicate restricted nutrient uptake resulting in autophagy and degradation of cytoplasmic content in order to survive (Kourtis & Tavernarakis, 2009).

The accumulation of a dense product within the intercellular matrix around hyphae may also indicate a plant host element to this incompatibility. The accumulation of this dense material over time may be involved in the degeneration of hyphae in the intercellular spaces. This dense material is most likely plant produced as production was seen in areas not in direct contact with hyphae, and the dense accumulation only in older tissue suggests a build up over time rather than a direct rapid response. This may be a plant produced product formed in the intercellular matrix of the host naturally in response to endophyte. Importantly, as Festulolium is a cross between perennial ryegrass and meadow fescue, this product may occur in both or only one of these hosts. In the natural association with meadow fescue, the endophyte may be able to metabolise or breakdown this product so no accumulation is seen. However, in the Festulolium hybrid this product may be unable to be metabolised or broken down by *N. uncinatum* resulting in its dense accumulation in the intercellular space and around hyphae. This accumulation may act to decrease nutrient uptake or actively assist in degradation of fungi, which may explain some of the incompatibility seen. As this dense product was not seen in the natural association between *N. uncinatum* and meadow fescue it is most likely a characteristic of the perennial ryegrass parent that has been carried over into the hybrid host. This accumulation was not seen in all cases where hyphae were distorted and may be only one factor contributing to the incompatibility seen in this study. The possibility that the plant produces a product that is utilized or evaded by some endophyte

species, while able to cause incompatibility to others may represent plant host selection of endophyte species. For instance endophyte species with only the ability to evade or utilise the product produced may be selected for, while others unable to evade or utilise the product are selected against. This may represent a novel action whereby plants have evolved to preferentially allow endophyte associations most beneficial or suited to their own requirements and conditions. For example, selection for endophytes with increased drought tolerance in plant species susceptible to drought would be highly beneficial, or selection for endophytes producing specific secondary metabolites able to resist insect pests highly prevalent in the hosts natural areas of distribution. As a wide range of endophytes may fit the requirements of the host the evolved mechanism selecting for or against endophyte is proposed to be broad without high specificity occurring. Also as there may be a cost to the host in supporting endophyte colonisation (Faeth, 2002, Bazely *et al.*, 2007, Rodriguez *et al*., 2009) it would be important that endophyte species which provide the most benefit for the host plant will preferentially form an association.

While the hyphae in Festulolium became distorted in older tissues, the hyphae in young tissue appeared healthy and lacked incompatibility characteristics. New tillers remained colonised and suggested a healthy endophyte population within the meristematic tissue. Seed transmission was seen to be variable with rates of 30-70% transmission in Festulolium hybrids (T. Gillanders 2012, pers. comm). Seeds produced from three separate lines were grown and analysed by light and TEM to determine whether the incompatibility was still present or whether the endophyte stabilised in its association within the host. Plants from the different seed lines were

first viewed by light microscopy and showed that incompatibility was present in all lines. The seed line with the highest rates of transmission, FL1466, had the best hyphae colonisation with more regular shaped and densely stained hyphae, although distorted hyphae were still present. The plant line with the least transmission had fewer hyphae with densely stained cytoplasm and the majority of hyphae in the plants were distorted. The results indicate higher transmission to seed results in better potential for a stable association.

Although the light microscopy showed that hyphae similar to those in the natural association were seen from plants grown with higher seed transmission rates, TEM analysis showed that incompatibility was highly prevalent in all plant lines. The TEM analysis showed that similar incompatibility of endophyte occurred as seen in the artificially inoculated Festulolium plants displayed by the degeneration of cytoplasm, presence of vacuoles and dense covering over outer cell walls. There was also a much larger accumulation of the electron dense product within the intercellular spaces of the plant. This high accumulation was not seen to the same level with artificially inoculated plants and appears much more prevalent in the naturally infected plants. These dense accumulations and inclusions formed within the mucoid layer or intercellular matrix and were present even in spaces not occupied by hyphae. As plant cells aged, these dense inclusions were seen to form a coating over plant cells, most likely due to the extracellular matrix spreading over the outer plant cell layer as the intercellular spaces enlarge due to aging. The accumulation over the surface of plant cell walls was not seen within the artificially infected plants and may represent an enhanced response to the hyphae in these naturally infected plants. This dense accumulation on both the outer surface of

hyphae and also on plants cell walls may act to restrict nutrient uptake by hyphae much more strongly, resulting in the higher levels of hyphal degeneration seen in these plants. In some cases the outer wall of hyphae looked to be actively degraded by the covering of this dense product and may indicate that this product directly acts against the hyphae themselves. Hyphal cell walls showed degradation while plant cell walls did not, even though both had this thick covering, suggesting a fungal specific activity. This most likely indicates a chitinase activity as chitin is enriched within fungal cell walls but not plant cell walls and would allow for a fungal specific response. Chitinases have also been shown to be important in many plant defence responses (Collinge *et al.*, 1993, Ferreira *et al.*, 2007). It could be that these dense inclusions form to not only restrict nutrient uptake but also act to actively degrade the fungal cell walls of hyphae in the intercellular spaces.

The transmission of endophyte in Festulolium hybrids did not stabilise the association in the resulting progeny. In fact, the results seem to show that the association between endophyte and host retained the same or slightly higher level of incompatibility previously seen in the first generation of artificially inoculated plants. As the Festulolium hybrid is comprised of two plant parents, *Festuca pratensis* and *Lolium perenne*, the genomic contribution from each may be a key factor in how the endophyte associates with the host. Successive breeding of Festulolium plants has been shown to alter the genomic balance in favour of *Lolium* (Zwierzykowski *et al*., 2011, Barth *et al.*, 2013). Plants tested in the current study represent only those from a single cross, so the amount of genomic rearrangement may have been low. *Lolium* does not allow colonisation by *N. uncinatum* so it may be that more of the *Lolium* genome is present within these plants, which could help

account for any increased instability of endophyte within these hosts. Further breeding may result in a larger *Lolium* contribution to the hybrid and the resulting incompatibility may become greater or result in complete loss of endophyte colonisation. This highlights the importance of breeding Festulolium that maintains enough *Festuca* genome or important genome elements, to allow suitable genetic backgrounds for *N. uncinatum* colonisation. Methods such as genomic *in situ* hybridisation (GISH) has been shown to be a useful tool in identifying genomic contribution of Festulolium hybrids (Kopecky *et al.*, 2005, Barth *et al.*, 2013, Zwierzykowski *et al*., 2011, Kopecky *et al.*, 2006) and may be important for the further development of hybrids able to retain strong endophyte colonisation.

The major aim of using Festulolium with *N. uncinatum* was to produce lolines within a perennial ryegrass-like host. However, comparison showed that lower loline levels were detected within the Festulolium host when compared to the natural meadow fescue host. Taking into account the incompatibility and variation in hyphal growth seen in these Festulolium plants this was not unexpected. As secondary metabolites are produced during the late growth phase the decreased production of lolines within the Festulolium host may be due to hyphal degradation in older tissue. Hyphae were also seen to be largely missing from older tissue and so may simply not be metabolically active long enough or able to maintain sufficient biomass in the host to be able to produce lolines at high levels. *N*-formylloline (NFL) was produced at the highest level while *N*-methylloline (NML) was produced at the second highest. Only trace amounts of the other lolines were detected in the Festulolium tissue. Of the plants analysed two were found to accumulate lolines only within the leaf blade. This is in contrast to the natural host were lolines

accumulate at the highest levels within the pseudostem tissue. Why lolines would only accumulate in leaf blade tissue in some Festulolium plants is unknown and unexpected as leaf blade tissue is older than pseudostem tissue and therefore would likely hold poorer endophyte colonisation. However detection of lolines in leaf blades does not necessarily mean that endophyte within this tissue has produced the lolines detected. Lolines are detected in root tissue in most associations yet endophyte does not colonise the roots of plants. The detection of lolines within leaf blades may be due to increased transport to the leaf in favour over all other tissues. Why lolines would be favourably transported to leaf blades in Festulolium is unknown. Lolines have been shown to be redistributed in response to plant attack (Patchett *et al*., 2008b) yet no plant wounding was present within leaf blades of these plants. Lolines are especially valuable when transported to the root as they provide high resistance to grass grub and porina attack. However no loline was detected in the roots of the Festulolium plants tested, although plants accumulated lolines in other tissues. This indicates transport to root tissue did not occur under the conditions used in this study, but this does not rule out their transport and accumulation in other circumstances, such as that of insect attack or root tissue wounding. It must also be noted that only one Festulolium line was used in regards to loline testing and represents the potential for production in a specific *Lolium:Festuca* genomic ratio. Other combinations with different ratios of *Lolium:Festuca* genome may exhibit much higher loline production.

6. Conclusions

The development of new endophyte-host associations will be particularly useful in the production of enhanced grass cultivars. This study looked at several aspects of endophyte development, with particular focus on *N. uncinatum* strain U2, from the identification and ancestral links of SSR structure, to the production of secondary metabolites and colonisation of natural and unnatural host plants. The results highlight the difficulties in establishing new endophyte associations and provide insight into the incompatibility that may arise from these new associations.

The use of the B10 and B11 SSR loci was unable to distinguish between clonal groups of *N. uncinatum,* yet analysis of sequence and repeat structure was able to indicate ancestral species. The finding of multiple repeats within the exonic regions of endophyte specific genes also highlighted the potential of these SSRs in gene innovation and adaptation through altered function.

The production of lolines by *N. uncinatum* was found to be strain dependent and the identification of high and low producing strains will be of particular importance in establishing high loline producing associations. The high variation in loline production found in this study may indicate that very controlled conditions or high numbers of replicates are necessary for distinct comparison of secondary metabolite production within plants in future.

Analysis of the colonisation of *N. uncinatum* within Festulolium showed that the colonisation of young tissue was stable, yet as the plant tissue aged the endophyte displayed signs of incompatibility. Loline production in Festulolium was markedly decreased as compared to the natural host, likely due to the endophyte incompatibility within Festulolium. Endophyte transmission through seed did not stabilise the association and incompatibility was present within plants grown from seed. Production of electron dense compounds in the intercellular space by the plant in response to hyphae may represent a novel response by the host against endophyte colonisation. The genomic contribution may also play an important role on endophyte stability and the contribution of *Fescue* and *Lolium* to Festulolium hybrids will be of particular interest in establishing any future endophyte associations.

7. Appendices

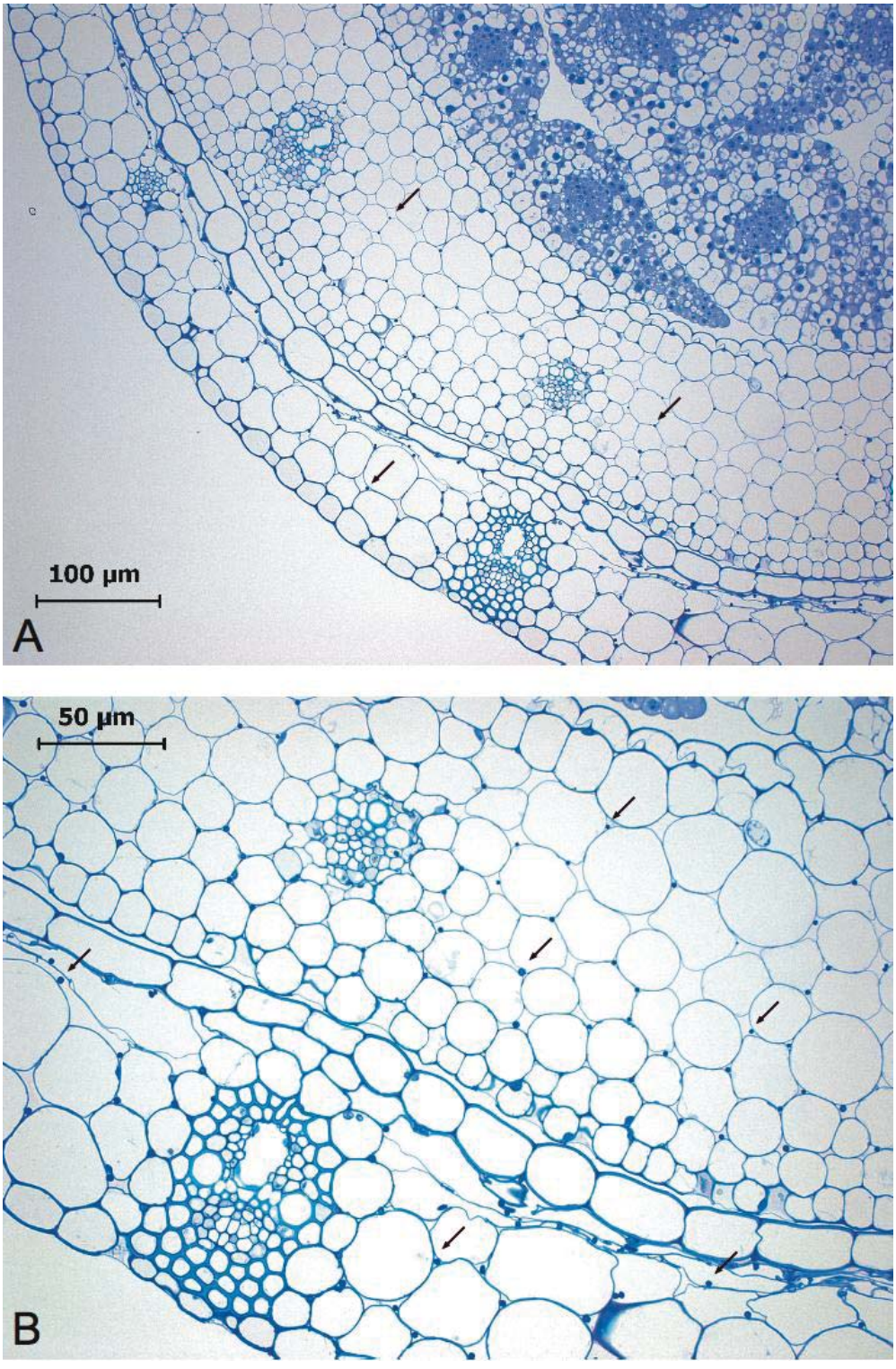
7.1 B10-like gene identifiers

Table 7.1: Identifiers for genes containing repeats within *E. festucae* and *E. typhina*

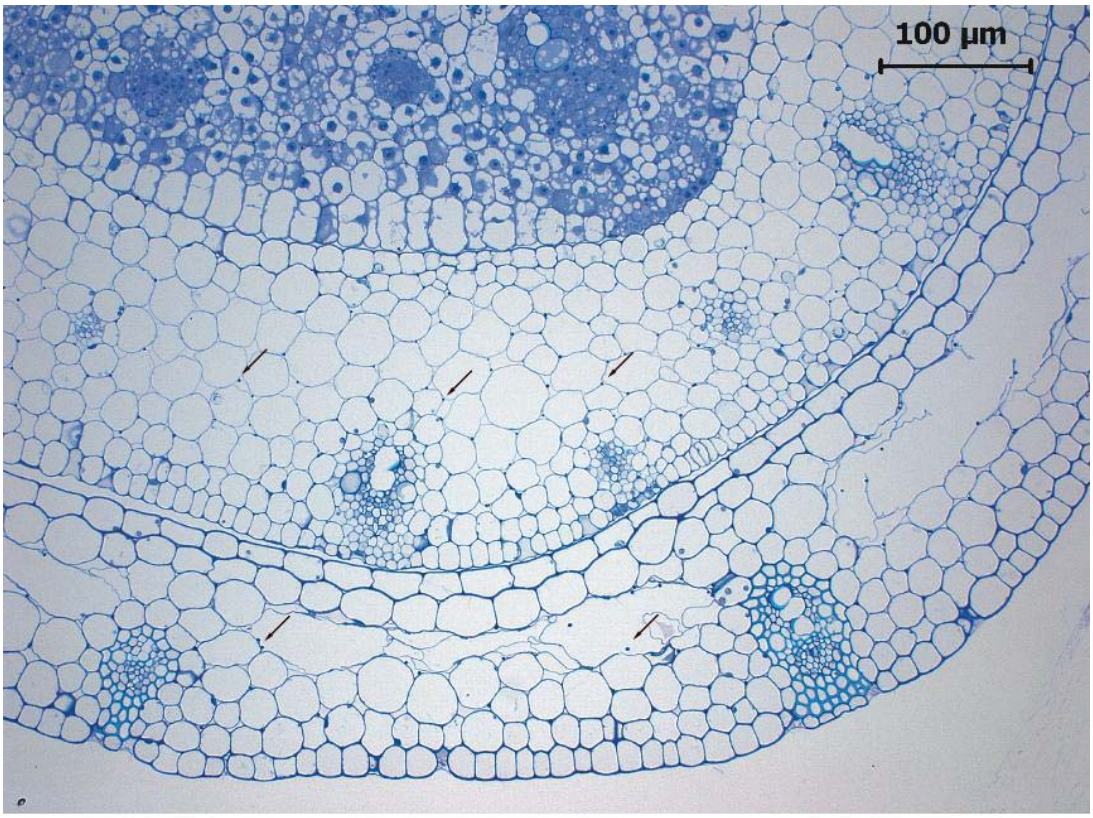
Top gene hits	Identifier
G protein coupled receptor	EfM2.062910
Copper sensing transcription factor	EfM2.078910
Hypothetical protein	EfM2.069880
Putatative GNAT family acetyltransferase	EFY98236
polyA nuclease	EfM2.024400
Cyclin dependent protein kinase complex component	EfM2.071750
Hypothetical protein	EfM2.066230
Hypothetical protein	EfM2.083030
Complex I intermediate associated protein 30	EfM2.001460
bZIP transcription factor	EfM2.027780
Ribosomal protein L24E	EfM2.006610
Putative sulfide quinine reductase	EfM2.089400
Hypothetical protein	EfM2.086110
Nucleoporin NIC96	EfM2.050430
Transcriptional corepressor cyc8	EFY87433
75 gamma secalin	EfM2.060710
Transmembrane anterior posterior transformation 1 / cytomegalovirus gH-receptor family protein	EfM2.086800

C6 zinc finger domain containing protein	EfM2.085550
Topoisomerase II associated protein	EfM2.015830

7.2 Light micrographs of U3 and U4 in meadow fescue

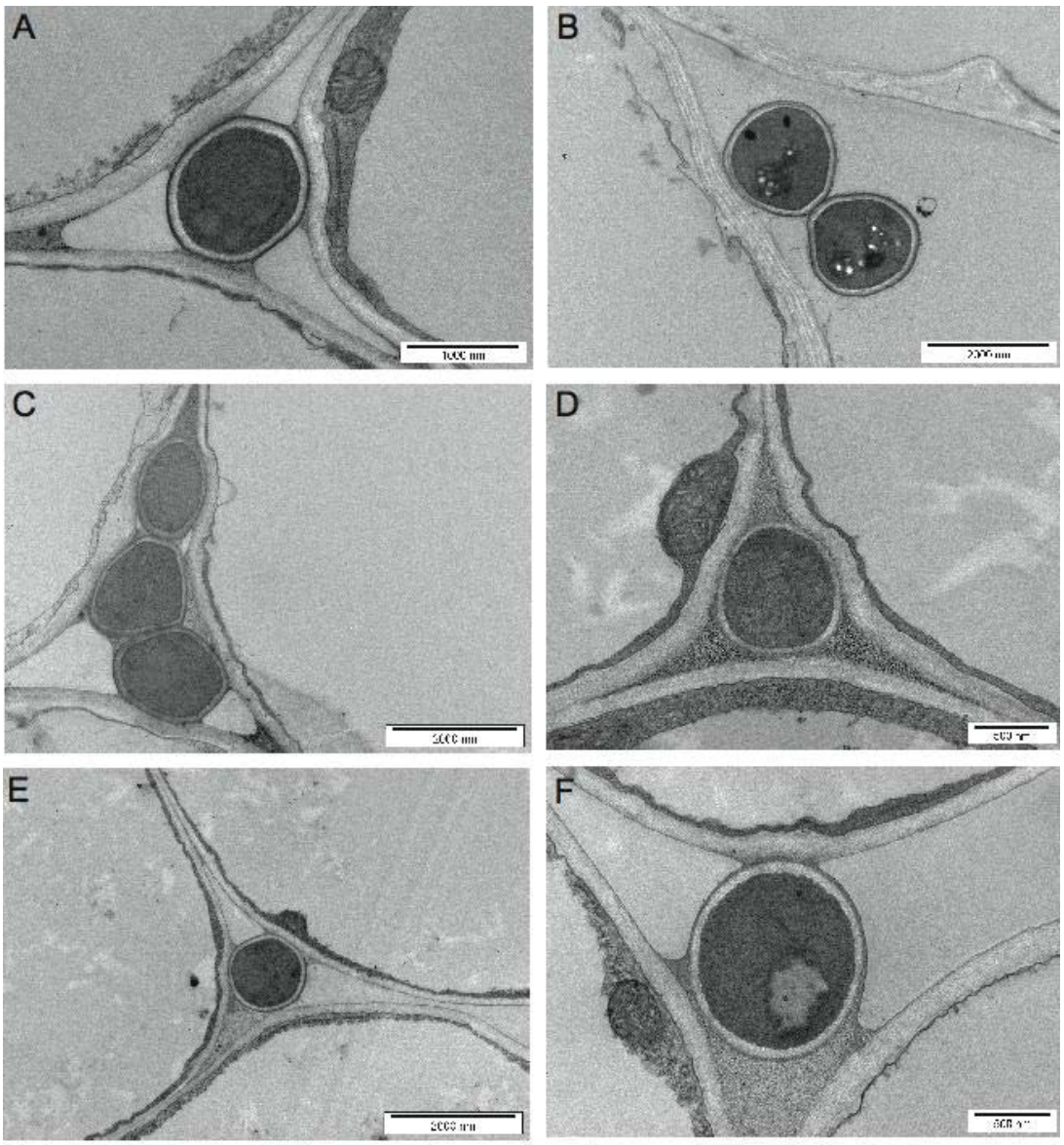


Appendix 7.2: Light micrograph of meadow fescue infected with *N. uncinatum* strain U3. A) Toluidine blue stained transverse section of pseudostem showing colonisation of layers by hyphae (arrows). B) Expanded view of hyphae within layers showing regular appearance with dense cytoplasm staining and regular cell shape contained within the intercellular spaces.



Appendix 7.3: Light micrograph of meadow fescue infected with *N. uncinatum* strain U4. Toluidine blue stained transverse section of pseudostem showing colonisation of layers by hyphae (arrows). Hyphae colonise all leaf layers appear densely stained and are contained within the intercellular spaces of the plant.

7.3 TEM analysis of U3 and U4



Appendix 7.4: Transmission electron micrographs of *N. uncinatum* strain U3 and U4 colonising meadow fescue. A-C) Hyphae of *N. uncinatum* U3 colonising meadow fescue. Hyphae show dense cytoplasm staining, regular cell shape and appear to be firmly attached to plant cell walls. Multiple hyphae are present within some intercellular spaces D-F) Hyphae of *N. uncinatum* U4 colonising meadow fescue. Single hyphae colonising the intercellular spaces showing electron dense cytoplasm, regular cell shape and firm attachment to plant cell walls through mucilage layers.

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