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**A high frequency change, which is both
inducible and reversible, results in altered
colony morphology of a fungal symbiont
(*Neotyphodium lolii*) and dwarfing of its
grass host (*Lolium perenne*)**

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Abstract

Fungal endophytes of the genus *Neotyphodium* form stable symbiotic associations, with grasses, that are symptomless and generally considered to be mutualistic. The benefits that these fungi confer to their grass hosts are exploited in pastoral agriculture systems. The production of a range of secondary metabolites, specifically alkaloids including peramine and ergovaline can give their host plants an ecological advantage in certain environments. *Neotyphodium* endophytes are asexual and have lost the ability to transfer horizontally between hosts making seed transmission a vital feature of the association.

This thesis reports the occurrence of phenotypically different perennial ryegrass plants (*Lolium perenne*) in a population infected with *Neotyphodium lolii*. Here we show that the change in the plants is directly attributable to a variant endophyte that they host. Isolation of the variant endophyte reveals a change in colony growth compared to the wild-type resident endophyte in the population, which has a white and cottony phenotype. Colonies of the variant endophyte are smaller than wild-type colonies and mucoid, with hyphal filaments forming aggregates. Evidence shows that the switch between colony morphologies occurs at a very high frequency, is reversible, and appears to be environmentally induced. This suggests that the switching phenomenon involves gene regulation rather than mutation. When endophyte-free plants are infected, with either white and cottony (wild-type) or mucoid (variant) fungal colonies, they assume a morphology consistent with the state of the fungus at the time of inoculation, that is normal or dwarfed, respectively. In addition, re-isolation of endophyte from either normal or dwarfed plants always yields white and cottony or mucoid colonies, respectively, suggesting that the host environment stabilizes the state of the fungus. Proteomic profiling revealed differences in protein expression between plants infected with either the wild-type or mucoid fungus. Furthermore, host plants containing the mucoid fungus have never flowered or produced seed. Thus, if this change in the fungal symbiont occurs in a competitive natural environment the mucoid fungus and its host plant may not persist beyond the first generation. This thesis provides insights into the plastic nature of fungal endophyte/grass symbiote and discusses possible mechanisms for the observed morphological switching in culture and host dwarfing.

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Chapter 1 Introduction

1.1 *Epichloë*/*Neotyphodium* endophytes (epichloae)

Neotyphodium endophytes are asexual derivatives of *Epichloë* species and as such an understanding of the entire *Epichloë* complex gives insight into the nature of *Neotyphodium* endophytes.

1.1.1 *Epichloë* endophytes

Epichloë endophytes are fungal symbionts of C3 grasses that form long term systemic infections that vary within the symbiotic continuum from antagonism to mutualism and can be transmitted vertically (via seed) and horizontally (from plant to plant) via ascospores (Schardl et al. 1997). *Epichloë* are ascomycetes (family Clavicipitaceae) with a bipolar heterothallic mating system, forming fungal stroma, an external mycelial structure that gives rise to spermatia. A fly of the genus *Phorbia* is responsible for the transfer of these spermatia (Schardl 1996).

Epichloë endophytes have three distinct dispersal mechanism types; type I where stroma are obligatory on infected plants, type II where stroma are optional and type III where no stroma are formed (Leuchtman and Clay 1997). *Epichloë* endophytes are intercellular colonisers and do not give rise to visible symptoms of infection for most of the lifecycle of the symbiosis, the only outward sign of infection is seen when flowering commences. During flowering, stroma can form giving rise to ‘choking’ of the inflorescence where it is subsumed by the stromal mycelium of the fungus (Craven et al. 2001). With the exception of the period when stroma are formed in type I and type II infections the colonisation of grasses with *Epichloë* endophytes is asymptomatic and as such, infected plants are not distinguishable from uninfected plants. In type III infections endophyte-infected and endophyte-free plants are indistinguishable for the duration of the lifecycle of the symbiosis.

Colonisation by *Epichloë spp* has been shown to enhance host plant protection against both vertebrate and invertebrate herbivores via four classes of alkaloidal secondary metabolites; ergovaline, lolitrem B, peramine and lolines (Bush et al. 1997; Clay et al. 1993; Schardl 2001; Siegel et al. 1990).

Ergovaline is an ergot alkaloid and its biosynthesis involves a complex gene cluster. (Fleetwood et al. 2007). Lolitrem B forms part of a structurally diverse group of indole-diterpene mycotoxins and also requires a complex gene cluster for biosynthesis (Young et al. 2006). Peramine is a pyrrolopyrazine, the putative product of a two-module non-ribosomal peptide synthetase (Tanaka et al. 2005). Lolines are comprised of a saturated 1-aminopyrrolizidine-ring system and their biosynthesis in *N. uncinatum* involves two homologous gene clusters LOL-1 and LOL-2 (Spiering et al. 2005b). These compounds are produced in various combinations throughout the *Epichloë* species as well as in their asexual derivatives the *Neotyphodium* spp.

1.1.2 *Neotyphodium* endophytes

Neotyphodium endophytes are asexual derivatives of the above mentioned *Epichloë* and similarly infect a number of cool season grasses of the subfamily Pooideae (Christensen et al. 2002; Clay 1993; Schardl 1996). The asexual *Neotyphodium* form asymptomatic and for the most part mutualistic symbioses with their hosts and transmit vertically via host seed colonisation (Schardl et al. 1997). Although *Neotyphodium* endophytes have been widely described as mutualistic, this claim has been challenged and it has been suggested that although the interaction with agronomic grasses such as perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*) tends toward a tight mutualism, within the wider context grass host/endophyte interactions range between antagonism and mutualism (Easton 2007; Faeth 2002; Saikkonen et al. 1998).

The genus *Neotyphodium* was previously known as *Acremonium*. A review of the taxonomy was made following an examination of the molecular phylogeny of *Acremonium*. Glenn et al. used parsimony analysis of 18S rDNA sequences of a number of fungal orders including *Clavicipitales* to reclassify the anamorphs of *Epichloë* and related mutualists, forming the genus *Neotyphodium* (Glenn et al. 1996). This nomenclature arose from the fact that Diehl as a convention of convenience, applied *Typhodium* as a form genus and used the term typhoidal when referring to the anamorph (asexual stage) of *Epichloë* (Diehl 1950).

Many *Neotyphodium* are hybrids with ancestors among two or more biological species of *Epichloë*. There are eleven distinct biological species (mating populations) of

Epichloë, most of which exhibit host specificity for groups of related grass genera (Kuldau and Bacon 2008; Moon et al. 2004; Moon et al. 2002).

Interactions of *Epichloë* endophytes with their host grasses span the symbiotic continuum from antagonism to mutualism. Many *Epichloë* species and all of their asexual relatives are transmitted vertically via the seeds of infected plants (Schardl 1996). In the vertical transmission route of asexual and pleiotropic *Epichloë* endophytes the fungus invades the developing ovule and ultimately the embryo and endosperm of the mature seeds (Philipson and Christey 1986). In this clonal and highly efficient means of propagation of the fungus, nearly 100% of seeds from infected mother plants transmit the endophyte (Siegel et al. 1984).

1.2 Features of *Neotyphodium* endophytes

1.2.1 Symbiosis

Neotyphodium endophytes are obligate symbionts. They have no known capacity to exist independent of their host grasses in nature. However, it is possible to isolate them from surface-sterilised tissue in the laboratory and culture them saprotrophically on agar preparations such as potato dextrose agar (PDA) (Latch and Christensen 1985).

The symbioses that *Neotyphodium* form are mutualistic in that both the fungus and the host grass benefit from the association. The fungus benefits from a biological niche with few if any competing organisms and a ready source of nutrients in the host apoplastic fluid along with a mechanism for vicarious dispersal via the host seed. The host benefits from the range of secondary metabolites the fungus produces in the form of alkaloids, many of which have individual and/or multiple activities against different classes of organisms.

1.2.2 Alkaloids

As with the *Epichloë* four classes of symbiota-specific alkaloids have received intensive study in grasses hosting *Neotyphodium* endophytes. These are the pyrrolizidines (lolines), ergot alkaloids (clavines, lysergic acids and derivative alkaloids), indole diterpenoids (lolitremes) and pyrrollopyrazine (peramine) alkaloids (Siegel and

Bush 1997). As mentioned for the *Epichloë*, lolines, clavines and peramine alkaloids individually or in combination can offer endophyte-infected grasses considerable advantage over endophyte-free grasses in that they can confer insect pest resistance and protection to the host. In *Neotyphodium lolii*/*Lolium perenne* associations in New Zealand insect pest resistance is the primary advantage effected via the production of peramine and ergovaline. Peramine is associated with resistance to the pasture pest Argentine Stem Weevil (*Listronotus bonariensis*) (Prestidge et al. 1991) while ergovaline is associated with resistance to African Black Beetle (*Heteronychus arator*) (Ball et al. 1997). The tall fescue endophyte *N. coenophialum* confers primary advantage via drought resistance and is capable of extending the southern range limit of tall fescue grasses in agricultural areas of the southern part of North America. The research into this phenomenon has examined both direct physiological effects of the endophyte symbiont on the physiology of the host plant affecting stomatal conductance and osmotic adjustment (Elmi and West 1995) and indirect effects via differences in nematode populations affecting the host plant (West et al. 1987).

1.2.3 Host specificity

The associations that the various *Neotyphodium* species form are host specific. *Neotyphodium lolii* specifically colonises perennial ryegrass (*Lolium perenne*), *Neotyphodium coenophialum* colonises tall fescue (*Lolium arundinaceum* syn. *Schedonorus phoenix* syn. *Festuca arundinacea*), *Neotyphodium uncinatum* colonises meadow fescue (*Festuca pratensis*) and *Neotyphodium occultans* colonises annual grasses such as *Lolium multiflorum*.

It is thought that speciation of the host has progressed alongside that of the symbiont fungus, contributing to a co-speciation that manifests in this host specificity (Schardl et al. 1997). It is suggested that multiple infections from sexual *Epichloë* spp. have given rise to hybrid asexual endophyte species that essentially become trapped in their host species (Schardl et al. 1991). Schardl *et al.* have shown using molecular techniques that multiple copies of tub2 genes are present in many *Neotyphodium* suggesting that the different species have developed by super-infection and hybridisation while within their host grasses (Schardl et al. 1997).

1.2.4 Observing *Neotyphodium* endophytes

There are various methods employed to either detect or directly observe *Neotyphodium* endophytes in plant tissues. A long employed method for observing endophyte hyphae in fresh tissue involves removing the single cell layer of tissue that forms the epidermis on the adaxial surface of host leaf sheaths and mounting this on a microscope slide with aniline blue stain. Aniline blue stains the cytoplasmic contents of intact hyphae. Using this method the endophyte hyphae appear as long, septate, even width filaments that run parallel to the leaf axis.

Endophytes can also be detected without direct observation using immunological and PCR based techniques (Groppe and Boller 1997; Musgrave 1984; Panaccione et al. 2001; Rasmussen et al. 2007). Two immunological methods commonly used are a microtitre plate based enzyme linked immunosorbance assay (ELISA) and an antigen binding matrix dot blot or immuno-blot. Both of these systems require the production of antibodies raised against the endophyte fungus; this is done by eliciting an immune response in small mammals using a preparation of cultured endophyte.

Immunoglobulins are purified from serum removed from inoculated animals (Musgrave 1984). The use of ELISA facilitates not only endophyte detection but also a degree of quantification. The immuno-blot approach is a quick and simple method of detecting endophyte but it does not allow any form of quantification (Gwinn et al. 1991; Hill et al. 2002).

These two techniques will detect viable endophyte in fresh tissue. It is also possible to detect endophyte in seed but the assays give no indication of fungus viability. Mycelium can be observed directly by staining seed tissues with aniline blue and observing them under a compound light microscope. Endophyte mycelium can be readily detected by removing tissue that includes the aleurone cells of the seed. Hyphae are present in large numbers at the interface of the endosperm and scutellum of infected seed (Philipson and Christey 1986).

Seed can also be assayed using an immuno-technique. Antigen is obtained from seed by soaking in dilute sodium hydroxide and extracting onto a membrane placed on a sponge saturated with an extraction buffer (Hill et al. 2002).

In addition to staining and immunological detection techniques, endophytes can be detected by isolation from either fresh tissue or seed.

1.2.5 Isolation and culture of *Neotyphodium* endophytes

Neotyphodium endophytes are biotrophic in nature and can be thought of as fungi trapped within the grass host plant (Schardl and Clay 1997). In the laboratory however it is possible to isolate and culture them (Latch and Christensen 1985). Isolations involve the surface sterilisation of fresh pseudostem tissue excised from an infected plant that is plated onto a suitable solid media such as Potato Dextrose Agar (PDA) and incubated at 20-25°C. After 3-7 days (sometimes longer depending on the endophyte strain) hyphal filaments will emerge from the tissue (Fig. 1.1) and form colonies on the agar that are visible to the naked eye after 2-3 weeks. The colonies that develop can be used to establish endophyte infections in new uninfected hosts



Fig.1.1 Hyphal filaments growing from surface sterilised host tissue on PDA

1.2.6 Artificial infection of grasses

In nature the asexual *Neotyphodium* endophytes rely on the seeding cycle of the host to disseminate. This reliance on vertical transmission contrasts with the sexual *Epichloë* that can colonise uninfected hosts horizontally via ascospores. It is possible however to infect endophyte-free host grasses artificially in the laboratory and these infections result in plants that are morphologically indistinguishable from uninfected plants. In this way the asexual *Neotyphodium* can be established in novel hosts (Latch and Christensen 1985).

The ability to perform such artificial infections is central to attempts to solve the problem of animal toxicity due to alkaloids while retaining the host protection properties of the endophyte symbiosis. Collections of fungal endophytes have been screened according to their alkaloid profile using HPLC and isolations made from those

strains that do not produce any of the known mammalian toxins. These fungi are then used to infect elite pasture grass germplasm to produce toxin-free pastures that retain resistance to invertebrate pests (Easton 2007).

1.3 *Neotyphodium lolii*

Neotyphodium lolii are non-hybrid asexual endophytes derived directly from *Epichloë festucae*. They occur naturally in perennial ryegrass which, along with white clover (*Trifolium repens*), has historically formed the basis of New Zealand's intensively managed pastures (Woodfield and Easton 2004).

1.3.1 Importance of *N. lolii* in New Zealand

Neotyphodium spp. endophytes have specifically been a subject of study due to their effects in pastoral agriculture.

In New Zealand this focus came about due to the association of the *Neotyphodium* endophyte species *N. lolii* (then known as *Acremonium loliae*) with the animal health condition ryegrass staggers. *Acremonium loliae* had been described in the perennial ryegrass (*Lolium perenne*) of New Zealand pastures by Neill early last century (Neill 1940).

The connection between this fungus and ryegrass staggers was suggested by Fletcher and Harvey (Fletcher and Harvey 1981) when they correlated the level of endophyte infection with scores for severity of the livestock condition in grazing hoggets.

Although ryegrass staggers had been a problem to New Zealand agriculture for some time prior to this, the idea that the systemic fungus described by Neill was responsible had been dismissed by Neill himself and then by Cunningham (Cunningham 1958). The connection was largely dismissed on two grounds; firstly the lack of toxicity of cultures and infected seed feed to birds, rats, mice and sheep and secondly because it was considered by Neill that a widely distributed fungus such as *Lolium* endophyte could not be responsible for localised outbreaks of ryegrass staggers. We now know that regional and sporadic outbreaks can be explained by the fact that alkaloids are produced by the fungus differentially *in planta* and in culture (Blankenship et al. 2001; Tanaka et al.

2005; Young et al. 2006) and that toxin production is affected by the genotype of the host plant and the environment (Easton et al. 2002).

Neotyphodium endophytes are important in agricultural grazing systems that involve cool season grasses such as the above mentioned perennial ryegrass in New Zealand and tall fescue in the United States (Woodfield and Easton 2004).

In the New Zealand context, *Neotyphodium* endophytes have been shown to confer advantages to their host plants via the production of secondary metabolites that have activity against invertebrates (Prestidge and Gallagher 1988) and, contrary to the aims of agriculture, grazing mammals (Smith and Towers 2002). In addition there is evidence that endophyte infection of host grasses can confer drought resistance (Elmi and West 1995). Ryegrass staggers is caused by the neurotoxin lolitrem B, one of several alkaloid metabolites produced by endophyte-infected perennial ryegrass of the New Zealand ecotype (Fletcher and Harvey 1981). Ergovaline is also produced in endophyte-infected New Zealand ecotype ryegrass and can result in low live weight gains and general ill-thrift of grazing animals. A solution to this problem has been to source *Neotyphodium* endophytes with a range of alkaloid phenotypes (chemotypes) including those with anti-insect alkaloids but no anti-mammalian alkaloids. Plant hosts containing such endophytes have been obtained from Europe and collections in the USA. These strains can be isolated in the laboratory and inoculated into novel hosts (Easton 2007; Latch and Christensen 1985).

1.3.2 Variation in *Neotyphodium* endophytes

There are a large number of strains of *Neotyphodium* endophyte. These strains manifest differences not only between broad taxonomic groupings and species (Christensen et al. 1993) but within species also. This is demonstrated in a range of alkaloid and isozyme phenotypes and differences in colony morphologies of the endophytes in culture. The non-hybrid *N. lolii* demonstrate pronounced colony morphology variation with mycelium ranging from sparse to abundant, felted, cottony or aggregated into erect tufts; colour from white to brown; shape from flat, raised, domed, smooth, convoluted, crusted or brain-like and texture from waxy through to yeast-like, dry or cottony (Christensen et al. 1991).

1.4 Phenotypic variation

This thesis describes the identification and characterisation of an *N. lolii* isolate that is able to undergo *en masse* reversible morphological switching and which also has a significant impact on host plant architecture, leading to dwarfism. Results will be discussed in the context of possible mechanisms that are known to lead to these phenomena generally.

1.4.1 Spontaneous mutation

The mutation process ultimately impacts all populations and is relevant to every aspect of genetics and evolution (Lynch et al. 2008). The rate of mutation, defined as the number of mutations per cell division, per generation or per unit of time varies between species and can be categorised as deleterious, neutral or beneficial (Baer et al. 2007). Despite its importance in biology, our understanding of the mutational process and its bearing on organismal fitness remains limited. Most estimates of mutation rate are derived from surveys of visible mutation at reporter loci or from nucleotide-sequence comparisons of silent sites in distantly related species (Lynch et al. 2008). The four main points where mutation rate may be modulated are DNA replication fidelity, mutagen exposure, DNA repair efficiencies and the buffering of mutational effects (Baer et al. 2007).

Within fungi such as the yeast *Saccharomyces cerevisiae* there is debate over whether the vast majority of mutations are deleterious or if the frequency of deleterious versus beneficial are similar with the deleterious mutations having larger average effects (Keightley and Lynch 2003; Shaw et al. 2003). Regardless, the rate of mutation in fungi is low, estimated as 6.3×10^{-5} per haploid genome per generation in *S. cerevisiae* (Joseph and Hall 2004), when compared to phenomena such as phenotypic switching in *Cryptococcus neoformans* for example, where changes occur at rates in the order of $1 \times 10^{-2} - 10^{-5}$ mutations per generation (Fries 2002).

1.4.2 Phenotypic switching

Phenotypic switching is defined as a reversible change of an observable colony phenotype that occurs at a frequency above the expected frequency for somatic mutations (Fries et al. 2002). Phenotypic switching has been described in a range of organisms including bacteria (Hammerschmidt et al. 1996; Silverman et al. 1979), mycoplasma (Lysnyansky et al. 1996) and fungi (Slutsky et al. 1985). We are interested in mechanisms of change such as mutation and phenotypic switching in the context of the ascomycetous fungus *N. lolii*. An existing literature around another ascomycetous fungus, the yeast *Candida albicans*, may offer insight into possible mechanisms of change.

1.4.3 Phenotypic switching in *Candida albicans*

C. albicans is an infectious yeast that can switch between alternative phenotypes that can be distinguished by their colony morphology. Most strains of *C. albicans* are capable of growing in either a budding yeast form, which is similar in morphology and budding pattern to diploid strains of *Saccharomyces cerevisiae*, or an elongated hyphal form. The switching and bud-hypha transition can be distinguished from each other in that switching occurs spontaneously at far lower frequencies. Of all the switch phenotypes described, the most studied is the white-opaque system in strain WO-1 in which smooth white colonies switch to flat grey opaque colonies (Slutsky et al. 1987). Switching is a far more heritable state than the bud-hypha transition and in many cases involves more than two distinct morphologies but most importantly cells of a single strain that are in different switch phases can be induced to undergo the bud-hypha transition (Soll et al. 1993).

Although the bud to hypha transition has been shown to be one of several virulence attributes that enable *C. albicans* to invade human tissues (Brown and Gow 1999), the question of the role of phenotypic switching in the virulence of *C. albicans* remains open to interpretation (Calderone and Fonzi 2001).

1.4.4 Switching and the *C. albicans* mating-type locus

C. albicans is diploid and has been shown to have genes that correspond to the mating type (MAT) genes MATa1, MAT α 1 and MAT α 2 of *Saccharomyces cerevisiae* (Hull and Johnson 1999). More recent studies have shown that although mating-type locus (MTL) heterozygous strains of *C. albicans* do not undergo the white-opaque transition, homozygous MTL derivatives do (Miller and Johnson 2002).

These results indicate that the MTL controls white-opaque switching and suggests that the majority of *C. albicans* strains, which are heterozygous for mating type and do not undergo the white-opaque transition, are capable of switching if they become homozygous at the mating type locus. This proposition was tested and shown to be the case (Lockhart et al. 2002). It is possible for heterozygous strains to become homozygous by the loss of one homologue of the chromosome carrying the MTL (chromosome 5) and subsequent duplication of the remaining homologue or by mitotic recombination (Wu et al. 2007).

Phenotypic switching between the documented white and opaque phases occurs spontaneously at relatively low frequency with around 0.1% of cells of white or opaque populations being of the opposite phase (Soll 1992). The work on MTLs was done with white-opaque switchers but earlier work by Slutsky *et al.* showed a range of different colony phenotypes that arose spontaneously at different frequencies. These distinct phenotypes were shown to switch back and forth with various frequencies with one direction having a higher frequency. With the exception of one, all observed phenotypes could switch to all others (Slutsky et al. 1985).

The molecular mechanism behind the white-opaque switching phenomenon has been shown to involve heterozygous MTL a1- α 2 repression of the WOR1 gene which encodes a transcription factor that induces the white to opaque switch while also up-regulating its own expression (Huang et al. 2006; Zordan et al. 2006). This molecular explanation is consistent with the stochastic nature of white-opaque switching.

However, as will be discussed below, white-opaque switching does not only occur spontaneously at low frequency but can also be induced *en masse* by environmental signals (Ramírez-Zavala et al. 2008).

1.4.5 Mass conversion

Phenotypic switching occurs at frequencies of $10^{-2} - 10^{-5}$ mutations per generation (too high to be mutation which in yeast is 6.3×10^{-5} per haploid genome per generation (Joseph and Hall 2004)) and is reversible (Soll 1992) while the transition from mycelia to yeast-phase fungus involves a mass conversion that can be triggered by environmental cues such as temperature and pH (Brown and Gow 1999).

Recent work by Ramirez-Zavala et al. demonstrates that *C. albicans* can undergo an *en masse* switch from the white to the opaque phenotype. Cultures of an MTL homozygous strain were incubated under anaerobic conditions and subsequently grown under aerobic conditions. Cells switched from the white phase to the opaque phase *en masse* and expressed opaque-phase-specific genes OP4 and SAP1 and had down-regulated expression of the white-phase-specific WH11 gene (Ramírez-Zavala et al. 2008).

This thesis examines an asexual endophyte *Neotyphodium lolii* that has undergone a change that results in alteration of the morphology of its host plant resulting in a dwarf host phenotype. An examination will now be made of plant architecture and factors that impact it, with particular focus on dwarfism.

1.5 Plant architecture

Research examining plant architecture has primarily focused on the activity of shoot apical meristems (SAM), axillary meristems (AM) and root apical meristems (RAM) (Wang and Li 2008). The architecture of a plant is determined by the nature and relative arrangement of its parts and the equilibrium between endogenous processes and exogenous environmental constraints (Barthélémy and Caraglio 2007). Plants showing mutations in a range of vegetative and reproductive organs and tissues have been used to functionally analyse genes that contribute to plant architecture. Dwarfism is one of the phenotypes that lends itself to this type of examination and one that is well researched in both monocotyledonous and dicotyledonous plants. Plant height in rice and other monocots is an agronomically important trait for breeding high yielding cultivars and many dwarf and semi-dwarf mutants have been collected. Most of these mutations involve genes of the gibberellin or brassinosteroid metabolic or signalling

pathways. Gibberellins and brassinosteroids are regarded as major factors that determine plant height (Kurata et al. 2005; Li and Chory 1997; Wang and Li 2008). Gibberellins (GAs) are natural tetracyclic diterpenoid carboxylic acids (Xu et al. 2002), that act as phytohormones involved in many developmental processes in plants. It has been postulated that plants have both membrane bound and soluble GA receptors (Ueguchi-Tanaka et al. 2005). GA promotes plant growth by stimulating degradation of negative regulators of growth called DELLA proteins (Bari and Jones 2009). The presence of DELLA repressors or their GA-dependent turn-over correlates with the repression or derepression respectively of GA-dependent growth responses (Schwechheimer and Willige 2009). As GA promotes cell expansion, exogenous application might be expected to compensate for endogenous GA deficiency; this was investigated in section 3.2.5.

Knowledge of the effect of GAs on plant height has been exploited in agricultural grasses. Wheat and rice varieties have been improved by producing semi-dwarf plants that efficiently use applied nitrogen, utilising it in grain development rather than in vegetative elongation with the advantage that grain lodging associated with wind and rain are reduced (Sakamoto et al. 2003; Wang and Li 2008). Wheat *Rht* and rice *sd1* are two genes utilised to improve crop plants that are involved in gibberellin signalling and biosynthesis respectively (Sakamoto et al. 2003).

In wheat the GA-insensitive alleles *Rht1* and *Rht2* reduce the final sizes of vegetative organs and tissues associated with reproductive structures but the modes of action in different organs differ. With the exception of the flag leaf the dwarfing genes *Rht1* and *Rht2* produced a significant reduction in cell length in wheat plants (Miralles et al. 1998). These genes are exploited in classical breeding programmes and agricultural grasses are genetically modified to study the effects of the manipulation of genes related to GA function. The rice GA2-oxidase encoding gene *OsGA2ox1* when expressed ectopically in transgenic plants inhibits stem elongation and the development of reproductive organs and the plants are deficient in endogenous GA1 (Sakamoto et al. 2001). Gibberellins, with the exception of GA3 are metabolized (hydroxylated) to inactive compounds during active plant growth, GA3 is degraded much more slowly. Conjugates that are inactive can be stored or transported through the phloem and xylem prior to activation in specific tissues and/or at specific times (Sponsel 1995). Compared to gibberellins, brassinosteroids (BRs) are a less well studied class of plant growth substances (Azpiroz et al. 1998). They are plant natural products with structural

similarity to animal steroid hormones and like animal steroids they regulate the expression of specific genes (Clouse 1996). BR-deficient or BR-insensitive mutants display dwarfism, BRs promote growth through genomic pathways and BR-responsive genes have been identified in several species (Müssig 2005). All known mutants of *Arabidopsis* with mutations related to the actions of BR exhibit dwarfism (Tsukaya 2002). Treatment of wild-type *Arabidopsis* seedlings with brassinazole, an inhibitor of BR biosynthesis results in dwarfism due to a reduction in longitudinal growth of cells with reports of no difference in the number of cells along the length of specific organs (Asami et al. 2000), as well as contrasting reports noting that BR is involved in the proliferation of cells (Hu et al. 2000; Nakaya et al. 2002).

Besides hormonal plant growth regulators, various genes that affect cell cycle regulation and meristematic competency are involved in plant dwarfism. An illustration of how these factors may come together to influence final plant architecture is illustrated by the model of *AINTEGUMENTATA* (*ANT*) function in plant organ size control proposed by Mizukama and Fischer. Loss of ANT function reduces the size of all lateral shoot organs by decreasing cell number (Mizukami and Fischer 2000). A simplified model of this is shown below (Fig.1.2) whereby plant growth regulators impact transcriptional regulators that in turn act on meristematic competence, ultimately affecting cell growth and proliferation.

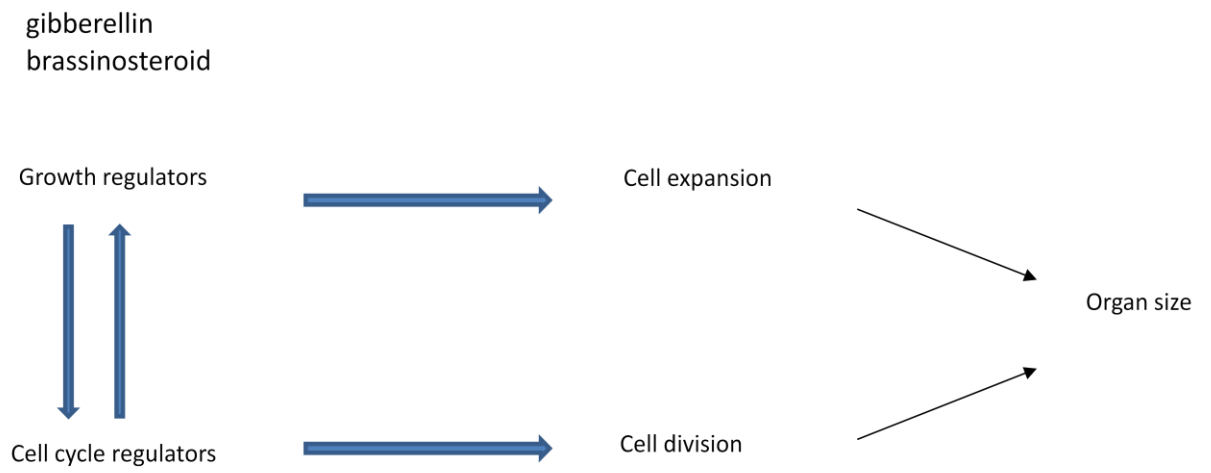


Fig.1.2 Simplified model of plant organ size control. Growth regulators modify cell expansion and/or cell proliferation via mechanisms such as transcriptional regulation.

1.6 Aims

This project is based on an observation of a number of dwarfed perennial ryegrass plants in a population. Isolation of endophytic fungus from these plants has shown that they host a fungus that is culturally distinct from that isolated from non-dwarfed plants.

The aims of this project are to:

- confirm the relationship between the change in the fungus and the dwarfed host phenotype
- establish the identity of the fungus hosted by dwarfed plants
- characterise the changes in the symbiosis using HPLC, SSRs, cell and organ measurement and proteomics

Additionally, when it was demonstrated during the course of this study that the fungus could be induced to change in culture, further aims became to:

- examine the culture conditions that lead to change in the fungus
- document the effect on artificially infected plants

Chapter 2 Materials and Methods

2.1 Table of biological materials

Table 2.1: List of biological materials used in this study

Designation	Name	Description	Reference
FLp119#4	<i>Lolium perenne</i>	Diploid perennial ryegrass	Wrightson's Seeds
Nui D	<i>Lolium perenne</i>	Diploid perennial ryegrass	Spiering et al 2005a
A11104	<i>Lolium perenne</i>	Diploid perennial ryegrass	Margot Forde Forage Germplasm Centre
AR5	<i>Neotyphodium lolii</i>	No lolitrem B <i>in planta</i>	AgResearch Ltd
P41 ¹	<i>Neotyphodium lolii</i>	Isolated from plants of normal appearance. Produces lolitrem B <i>in planta</i> .	This study
P1 ¹	<i>Neotyphodium lolii</i>	Isolated from dwarfed plants. No lolitrem B measured <i>in planta</i> .	This study

¹ Although given different numbers because of their isolation from different plants, P41 and P1 may be the same strain. While P41 was originally isolated from plants of normal appearance, mucoid mycelium of this strain can induce the same dwarfed plant phenotype as P1 mycelium.

2.2 Fungal isolation and culture

2.2.1 Media

2.2.1.1 ABPDA

Solid media was made using proprietary Potato Dextrose Agar (DifcoTM Becton, Dickinson and Co. USA) according to manufacturer's instructions. 19.5g of powder was suspended in 500ml reverse osmosis (RO) water in a 1L Schott bottle and autoclaved at 121°C for 15 min. Melted agar was cooled and poured into sterile plastic Petri plates in a laminar flow cabinet. Just prior to pouring, a filter sterilised tetracycline suspension was added to give a final concentration of 5µg/ml.

2.2.1.2 4% water agar

Water agar (WA) was made by combining 24g of standard agar (Coast Biologicals Ltd, Auckland, NZ) with 600mL RO water in a 1L Schott bottle and autoclaving for 15 min at 121°C. Melted agar was cooled and poured into sterile plastic Petri plates in a laminar flow cabinet.

2.2.1.3 Murashige and Skoog media

Murashige and Skoog media with minimal organics (MSMO) was made using a proprietary preparation (Sigma M6899). MSMO preparation (4.4g) was dissolved in 1L of RO water with 6g of standard agar and decanted into 2 1L Schott bottles (500mL each). The media was autoclaved for 15 min at 121°C, cooled and poured into sterile 98mm tissue culture pots (Vertex Pacific Ltd, NZ) in a laminar flow cabinet.

2.2.1.4 Broth

Liquid media was made using proprietary Potato Dextrose Broth (Difco) according to manufacturer's instructions. 24g of powder was suspended in 1L of RO water and heated with stirring to dissolve the powder. Broth was decanted into Erlenmeyer flasks that were then plugged with non-absorbent cotton wool and covered with aluminium foil. Flasks containing the broth were autoclaved at 121°C for 15 min.

2.2.2 Isolation of fungus on solid media

Fungus was isolated from endophyte-infected plants following surface sterilisation of plant tissue as described by Christensen et al. (Christensen et al. 2002). Tillers were removed from plants by cutting at the base and trimming to ca. 5cm before surface sterilising. Sectioned tillers were surface sterilised by quick rinse with 96% ethanol and a 1min soak in a 10% domestic bleach (Janola) solution followed by rinsing twice in sterile water. Tillers were sectioned transversely, sheath rings were separated and plated to antibiotic potato dextrose agar (ABPDA). Plates were incubated in the dark at 22-25°C for 3-5 weeks.

2.2.3 Liquid culture of *Neotyphodium* endophyte

A 6mm diameter plug was cut from colonies on solid media (ABPDA) using a sterile plug borer. The plug was macerated in 1.5ml potato dextrose broth (PDB) in a sterile eppendorf tube using a sterile plastic pestle. The resulting mycelial suspension was pipetted from the eppendorf tube to an Erlenmeyer flask containing 50ml sterile PDB using a sterile transfer pipette. Flasks were shaken at 150 rpm at 22-25°C for 2 weeks.

2.2.4 Macerate culture of *Neotyphodium* endophyte

A 6mm plug was cut from colonies on solid media (ABPDA) and placed in a sterilised eppendorf tube. 1ml of Potato Dextrose Broth (PDB) was added to the eppendorf using a transfer pipette. The mixture was ground by hand using a sterilised plastic pestle and the resulting macerate transferred to an ABPDA plate. The macerate was spread evenly over the solid media using a sterilised glass spatula.

2.3 Seedling inoculation

Seed was surface sterilised and inoculated as described by Latch and Christensen (Latch and Christensen 1985). Seed was surface sterilised by immersion in a 50% sulphuric acid solution for 15 min followed by a five times rinse with tap water and immersion in a 10% domestic bleach (Janola) solution for 15 min followed by a two times rinse in sterile water. Seed was dried in a laminar flow cabinet on sterile Whatmann filter paper before arranging on 4% water agar Petri plates. Plated seed was germinated in the dark at 22-25°C for 5-7 days and resulting etiolated seedlings were inoculated (Fig.2.1) before being returned to the dark incubator for 7 days. Following this incubation plates were placed under white fluorescent lights for at least 7 days before removing seedlings and planting them in commercial potting mix and growing them in a glasshouse. Plants were grown for ca. 6 weeks before indentifying infected individuals.

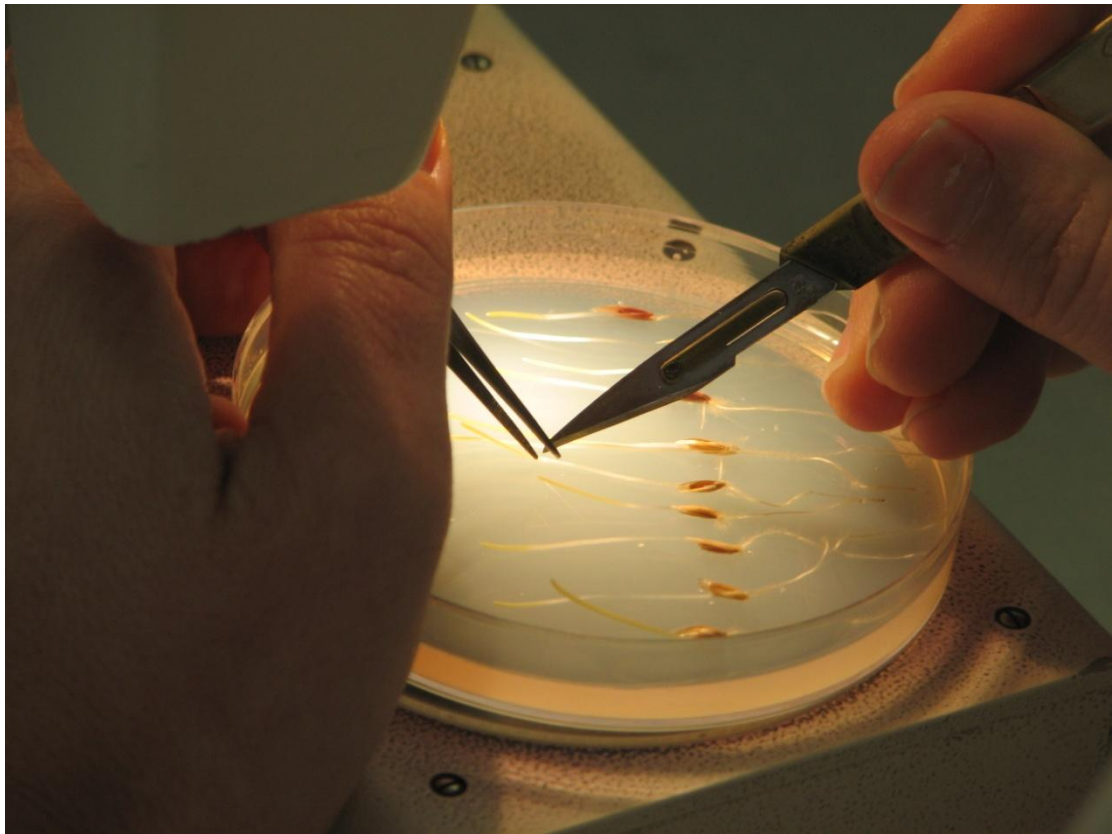


Fig.2.1 Inoculation of surface sterilised and etiolated 7-day old perennial ryegrass seedlings on 4% water agar

2.4 Endophyte detection

2.4.1 Immuno-detection

2.4.1.1 Antigen binding

Plants were grown to at least the 3-4 tiller stage before detection of endophyte was undertaken. Tillers were cut basally ca. 5mm from soil level using a scalpel and #11 blade. Where necrotic sheath tissue was present it was carefully peeled off the tiller before making a transverse cut on a Perspex cutting board. The freshly cut end of the tiller was gently placed onto a nitrocellulose membrane (NCM) (0.45µm) leaving a circular outline of the moist cut end. Tiller blots were arranged on the NCM in a pattern allowing correct identification of the plant source of each blot. A positive and a negative control tiller were blotted to the membrane using plants of known endophyte status. Blotted membranes were ready for processing immediately but could be retained until a

later time. Blotted membranes can be retained for at least three weeks at ambient conditions prior to processing (Wheatley and Simpson 2000) but are ideally kept in cool dark conditions and so were stored in a refrigerator at 4°C until processed.

2.4.1.2 Processing

Surfaces on blotted sheets with no bound protein were blocked by immersion in a milk protein blocking solution (BS) (Tris (hydroxymethyl) methylamine 2.42g, NaCl 2.92g, Non-fat milk powder 5g, 1 M HCl 10ml made up to 1 L with RO water adjusted to pH 7.5) in a 140x140mm (600ml) plastic container. Membranes were shaken on a Bellco mini-orbital shaker (Bellco Biotechnology, Vineland, New Jersey, USA) for at least 2 hours at room temperature. BS was decanted off the membrane and it was rinsed twice with fresh BS before adding 25µl primary antibody (rabbit anti-endophyte produced at AgResearch in conjunction with Massey University's Small Animal Production unit) in 25ml BS (1:1000 dilution). Following 15 min shaking at room temperature the membrane was incubated overnight at 4°C. Excess primary antibody was removed by decanting and rinsing twice in fresh BS. The secondary antibody (goat anti-rabbit IgG-AP, sc-2034, Santa Cruz Biotechnology, USA) was added, 6.25µl in 25ml BS (1:4,000 dilution) and shaken for 15 min at room temperature before incubating at 4°C for 5 hours. Excess secondary antibody was removed by decanting and rinsing twice in BS. Chromogens were prepared by dissolving separately 20mg Fast Red TR (Sigma F-2768) in 12.5ml Tris buffer (Tris (hydroxymethyl) methylamine 24.2g in 1 L RO water adjusted to pH 8.2) and 12.5mg of naphthol AS-MX phosphate (Sigma N4875) in 12.5ml Tris buffer per 10cm² of NCM. Chromogen solutions were combined and the NCM immersed, shaken at room temperature for ca.15 min until red colour develops on control positive blot (Fig.2.2). Development was stopped by rinsing three times in RO water.

2.4.2 Microscopic examination

Tillers were selected from mature plants for endophyte detection. Any necrotic sheath tissue was peeled back off the pseudostem exposing clean, live sheath tissue.

The outermost of the remaining sheaths was removed and manipulated under a Zeiss Stemi DRC dissecting microscope at 16 x magnification. The sheath was laid on a

cutting surface adaxial epidermis facing up, a shallow transverse cut was made with a scalpel and #11 blade and the epidermis gently lifted, separated and pulled off the sheath. The epidermal tissue was mounted on a drop of aniline blue stain (glycerol 50 %, lactic acid 25 %, water 24.95 %, aniline blue 0.05 %) on a 25 x 75 x 1mm microscope slide and covered with a 22 x 22mm coverslip, heated over a naked flame, allowed to cool and examined at 100 x and 400 x using a Zeiss compound microscope (Fig.2.3).

2.4.3 Cell measurement

Sheath tissue was embedded with adaxial epidermis facing down in clear nail polish on a microscope slide. The tissue was removed after setting for 10 min leaving an imprint in the lacquer. With a droplet of water and a #1 cover-glass the imprint was examined using an Olympus BX50 compound light microscope. Images were captured at 400x magnification with an Olympus Colorview Soft Imaging System and cell dimensions measured using analySIS B software. Statistical analysis of data was performed using Excel software.

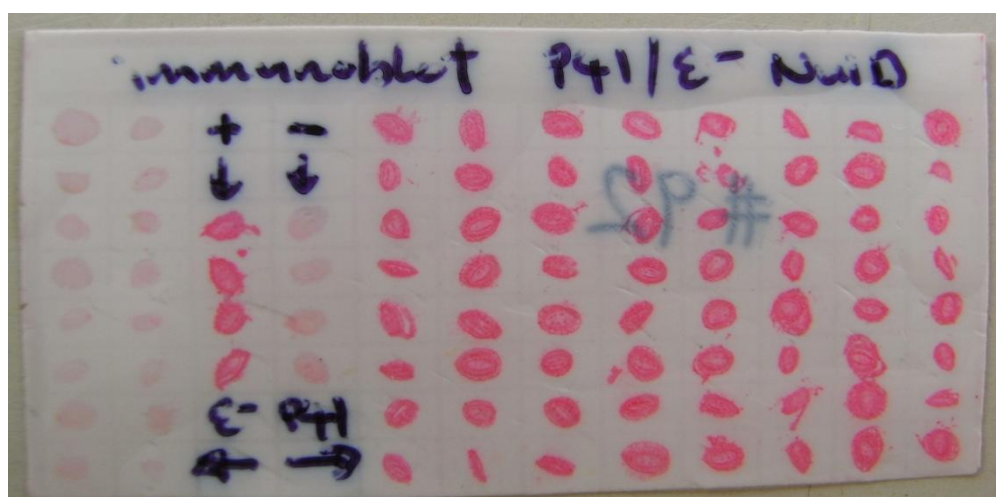


Fig.2.2 Immunoblot: Red chromogen-bound tiller imprints from endophyte-infected plants (right) and unbound imprints from endophyte-free plants (left)

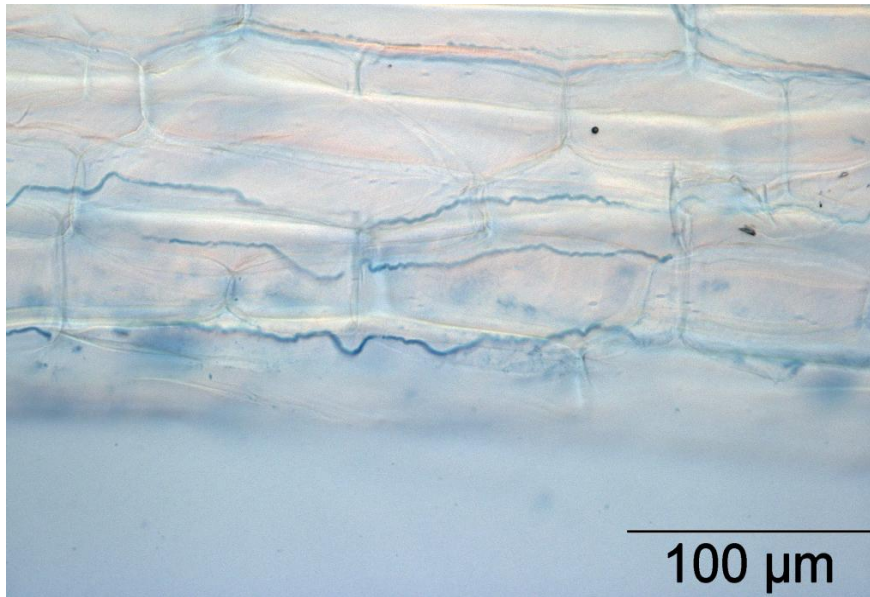


Fig.2.3 Aniline blue stained hyphae of *Neotyphodium* endophyte growing between epidermal cells of ryegrass leaf sheath (400X mag).

2.5 Endophyte elimination

Endophyte was eliminated from infected plants using a procedure similar to that of Latch and Christensen (Latch and Christensen 1982). Intact tillers, including roots, were removed from infected plants and washed thoroughly to remove potting soil then immersed in a 2g/L Benlate (500g/kg benomyl) solution for 3+ hours. Tillers were planted in river sand in pots without drainage and watered to saturation with Benlate solution; watered pots were weighed and regularly watered to weight with fresh water over a period of approximately 6 weeks. Plants were checked for negative tillers and single endophyte-free tillers were potted, confirmed endophyte-free (Methods 2.3) daughter tillers developing from these were then potted individually.

2.6 RAPD analysis

2.6.1 DNA extraction

Genomic DNA of fungal strains AR29, 34, 66, 525 and 542 was kindly donated by Dr Andrew Griffiths, AgResearch (Grasslands) Ltd.

Genomic *N. lolii* DNA was isolated from fungus grown in liquid culture (Methods 2.2.3) was extracted using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research) with

100ug fresh weight of fungal mycelium. DNA concentration was determined using a Nanodrop Spectrophotometer (Thermo Fisher Scientific).

2.6.2 PCR reactions

25µl reactions containing 0.2mM dNTPs, 1.5 mM MgCl₂, 0.2µM RAPD primer (GTAGACCCGT), 1 unit Taq DNA polymerase (Invitrogen), 2.5µl 10X PCR buffer (Invitrogen) and 50ng of template DNA were amplified in a Biorad iCycler as follows: 1 cycle at 95°C for 5 min followed by 45 cycles at 95°C for 1 min, 36°C for 1 min and 72°C for 2 min, with a final extension of 10 min at 72°C.

2.6.3 Gel electrophoresis

The PCR products were separated electrophoretically on 1.5% agarose gels in TBE buffer (45.0 mM Tris-borate, 1.0 mM EDTA). DNA bands were visualized by staining with ethidium bromide (0.5 µg/mL) and observed under UV light.

2.7 Simple Sequence Repeats (SSRs)

For each analysis two tillers cut at soil level were trimmed to ca. 3cm length and transferred to DNA extraction vials. Samples were genotyped by Dr Marty Faville (AgResearch Limited) as follows. Total plant and endophyte DNA was extracted using Q-BIOgene FastDNA kits following manufacturer's instructions for plant samples. Extracted DNA was amplified by PCR as described by Moon et al. (1999) using PCR primers flanking two simple sequence repeat (SSR) markers, B10 and B11, that are known to be polymorphic across strains of *Neotyphodium* endophyte (Moon et al. 1999). During PCR a fluorescent tag was added to the PCR products. The PCR products were run on a capillary electrophoresis system (ABI3100) separating the DNA fragments based on their mobility and thus size.

2.8 Alkaloid determination

Herbage samples were cut to 5mm above soil level, dead material removed, and divided into leaf blade and pseudostem portions prior to freeze drying. Samples were milled in a modified volume reduced domestic coffee grinder.

Ergovaline, combined with its isomer ergovalinine, was estimated by reverse phase HPLC with fluorescence detection (Spiering et al., 2002) using 50mg samples extracted at ambient temperature for 1hr with continuous mixing in 1ml of aqueous 2-propanol (50% v/v) containing 1% lactic acid (w/v) and ergotamine and homoperamine as internal standards. Solid residue was removed from the extract by centrifugation.

Peramine was estimated on the same extract using a different HPLC system based upon Spiering et al., (Spiering et al. 2002) and differing principally in that a cartridge (50 x 2 mm) packed with weak cation exchanger Bond Elute CBA (Varian, Harbor City, CA) was substituted for the initial online ion-exchange separation of interfering compounds.

Lolitrems B was estimated by normal phase HPLC in a simplification of the method of Gallagher et al. (Gallagher et al. 1985). Samples (50mg) were extracted with 1ml of dichloroethane – methanol (9:1 by volume) for 1hr with mixing at ambient temperature and then centrifuged to remove solid residues. The lolitrems were directly separated on a Alltima Silica 5 μ column, 150 x 4.6mm, (Alltech, Deerfield, IL) using solvent of dichloromethane-acetonitrile-water (860:140:1 by volume) and detected by fluorescence (ex.265nm, em.440nm). Peak areas were compared to an external standard of lolitrems B.

2.9 Generation, infection and growth of clonal plant material

2.9.1 Source of clonal plant material

Plant material for the culture of axenic clonal lines was obtained from a single host plant genotype isolated from an out-crossing diploid perennial ryegrass population. The individual plant, Nui D, was sourced from the cultivar Grasslands Nui (Spiering et al. 2006). The selected individual was planted in a 10cm pot using standard potting compost and subsequently confirmed to be endophyte-free by immunoblotting and

examination of aniline blue stained leaf sheath epidermis using a compound light microscope (see section 2.4.2). Plants were grown in the greenhouse for 8 weeks.

2.9.2 Axenic culture of clonal plantlets

Tillers were removed from the potted plant using a Swann-Morton scalpel and #20 blade and washed free of potting soil using tap water. Roots were trimmed from the tillers and the pseudostem (Fig. 2.4) was cut to ca. 6cm. Each tiller was then individually washed under running tap water and surface sterilised by immersion in a 6% solution of sodium hypochlorite for a minimum of two hours. Tillers were then rinsed using sterile water under aseptic conditions and allowed to dry on Whatmann filter paper to remove excess water. Surface sterilised tillers were transferred to sterile 98mm (250g) container (Vertex Pacific Limited) with Murashige and Skoog agar culture media (Sigma M6899 Basal salts MSMO with the macro- and micronutrients and vitamins as described by Linsmaier and Skoog, 1965). Tillers were grown axenically in sealed containers under artificial light (2 X Osram L 36W/72 fluorescent tubes) and developing daughter tillers were removed to fresh media.



Fig.2.4 A tiller prepared for surface sterilisation for the axenic production of clonal plantlets. Roots have been trimmed and the pseudostem cut to ca. 6cm length.

2.9.3 Inoculation of clonal plantlets

When the axenically grown clonal plants developed to the 3-5 tiller stage, individual tillers were removed for inoculation with fungus. Inoculation was performed by making an incision at the base of the tillers using a scalpel and #11 blade and carefully pressing cultured mycelium from a 4 week old colony into the cut. Inoculated tillers were placed in fresh MS media and sealed in sterile containers. After 4 weeks inoculated plantlets were removed from containers and planted in potting soil then grown in the greenhouse for 6 weeks. The infection status of inoculated plantlets was examined using immunoblot and then confirmed using light microscopy.

2.9.4 Growth of clonal plantlets

Plants were grown under controlled conditions prior to harvesting tissue for protein extraction in a growth cabinet set to a constant 15°C and a relative humidity of 45% with a 12hr light cycle at 650-800 $\mu\text{mol}/\text{min}/\text{m}^2$. Plants were watered according to their utilisation which varied as plants developed.

2.10 Proteomics

2.10.1 Harvest and preparation of plant material

To ensure plant tissue was harvested when growth resources were not limiting, clonal plants were grown to a point where tiller numbers were increasing at an exponential rate before transferring to the laboratory for dissection and tissue preparation. Tillers were removed from the plant by making a transverse cut at the base using a Swann-Morton BS 2982 #4 scalpel with a #20 surgical blade. The outer leaf sheath was separated from tillers and discarded. The second leaf sheath was removed and detached from the blade at the ligule (Fig.2.5b). Harvested and sectioned tissue was immediately placed in liquid nitrogen prior to protein extraction.

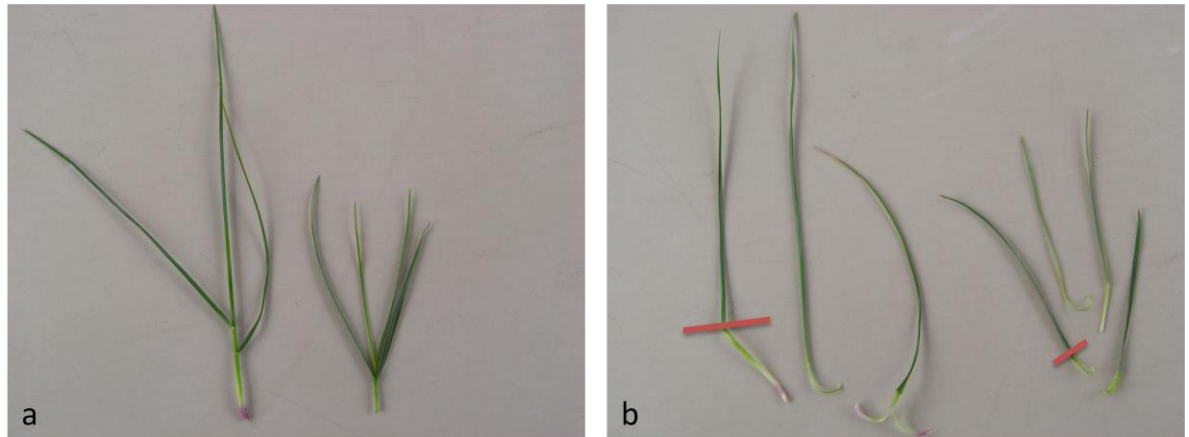


Fig.2.5 Harvesting tissue for protein extraction: Tillers excised from the plant (a); leaf sheaths removed from pseudostem and second leaf sheath cut at intersection of the leaf blade and leaf sheath (b). Red lines indicate excision points. Tiller on left of both (a) and (b) is P41-infected, tiller on right is P1-infected.

2.10.2 Protein extraction

Tissue (ca. 600mg fresh weight) was ground in liquid nitrogen to a powder with a mortar and pestle and transferred immediately to a centrifuge tube, 4ml of extraction buffer (30mM Tris-HCL buffer pH 8.7, 1mM ascorbic acid, 1mM DTT, 1mM EDTA- Na_2 , 5mM MgCl_2 with 1.5mg dithiothreitol (DTT) and 1 tablet of Complete Mini Protease Inhibitor (Roche Applied Science Cat. No. 11 836 153001) per 10ml) was added together with polyvinylpyrrolidone (PVP) at the rate of 2 X fresh weight of the extraction sample and tubes were centrifuged at 17,000 rpm (35,300 x g) using a Sorval SM24 rotor for 15 min at 4°C. The resulting supernatant was transferred to a clean centrifuge tube and an equal volume of sodium dodecyl sulphate (SDS) solution (4% SDS, 5% 2-mercaptoethanol, 5% sucrose) was added. Samples were boiled for 3 min and 8ml of ice-cold acetone containing 5.75 μl mercaptoethanol was added per 2.5ml of supernatant. Proteins were precipitated for 1hr at -18°C then centrifuged at 17,000 rpm (35,300 x g) on a Sorval SM24 rotor for 10 min at 4°C. The resulting pellet was air dried then resuspended in 400 μl of O'Farrel lysis buffer (9.5M urea, 5% 2-mercaptoethanol, 2% Nonidet P40, 2% IPG pH 3-10 ampholytes) and stored at -80°C until required.

2.10.3 Determination of protein concentration

A standard curve was produced using 0, 2, 4, 6 and 8µg of bovine serum albumin (BSA) in 1ml of Coomassie Blue G stain (Sigma). Absorbance readings at 595nm, zeroed with un-amended Coomassie Blue stain, were made on a spectrophotometer (Nova Tech). Unknown samples were diluted (3µl in 1ml of Coomassie Blue stain) and absorbance readings at 595 nm were plotted against the standard curve to quantify sample protein levels.

2.10.4 Protein gel separation/2-D analysis of protein

2.10.4.1 Rehydration

Gels for silver staining were loaded using 100µg of sample protein. 18cm Immobiline™ Drystrip pH 3-10 isoelectric focusing strips (GE Healthcare Bio-Sciences) were laid gel side down in Protean IEF (BIO-RAD) cell apparatus isoelectric focusing tray. Sample protein was prepared by adding 2µl carrier ampholytes (IPG buffer pH3-10) to the sample volume and making up to 350µl in lysis buffer adding a soupçon of Bromophenol Blue. The entire sample was loaded to the well along with two dimensional electrophoresis markers which comprised ovalbumin, (PI 5.1, MW 45,000), carbonic anhydrase (PI 7.0, MW 29,000) and myoglobin (PI 7.6, MW 17,000), (Sigma M 3411) and overlaid with 1ml of mineral oil (DryStrip Cover Fluid, Code No. 17-1335-01, Pharmacia Biotech). Rehydration was performed on a BIO-RAD Protean IEF cell overnight at 30 volts.

2.10.4.2 Isoelectric focusing

IEF electrode covering strips were cut to 5mm length, hydrated in milliQ water and placed over the electrodes at each end of the ceramic wells. The strips were ramped up to 8,000V and run to a total of 40,000Vhrs. Prior to running on the second dimension gel strips were equilibrated using a two buffer system. Strips were agitated in equilibration buffer (50 mM Tris/HCL pH8, 2% SDS, 30% glycerol, 6M urea) with 65mM DTT for 15mins rinsed with milliQ water and then agitated in equilibration buffer with 135 mM iodoacetamide for 15 min.

2.10.5 Second dimension

Reagents for an SDS acrylamide gel (16.44ml milliQ water, 17.5ml 1.5M Tris/HCL pH 8.8, 700µl 10% SDS, 34.98ml 30% acrylamide/Bis 37.5/1) were mixed and degassed with agitation under vacuum for 15 min. 350µl of a freshly prepared 10% solution of ammonium persulphate and 35µl of N,N,N',N' Tetramethylethylenediamine (TEMED) were added immediately prior to pouring into a BIO-RAD ProteanRII xi cell gel casting apparatus using a 5ml pipette. Plates used to construct the casting apparatus were carefully cleaned using detergent and water, dried then wiped using lint-free Kimwipe tissues and ethanol. The poured gel was overlain with saturated butanoyl to level the gel meniscus and to allow polymerisation to take place. The gel was left to polymerise for between 40 min and 1hr then the butanoyl was decanted off, the gel rinsed with distilled water and excess water removed from the edge of the gel using Whatmann filter paper. The cast running gel was rinsed off with glycine buffer and installed on the electrophoresis apparatus. The equilibrated IEF strip was cut to fit the running gel and sealed with molten 1% agarose. The gel was run on a BIO-RAD ProteanRII xi cell using electrophoresis buffer (15g/L Tris, 72g/L glycine, 5g/L SDS in milliQ water) at 60mA until the bromophenol blue front reached the bottom edge of the gel.

2.10.6 Staining and fixing

The gel was fixed in ethanol and acetic acid (50% ethanol, 10% acetic acid, 40% milliQ water), washed three times 5 min in milliQ water then placed in sodium thiosulphate solution (0.2g/L $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) for 2 min then rinsed in milliQ water for 5 min prior to agitating in a 2g/L solution of silver nitrate (AgNO_3). The silver stain was removed using liberal quantities of milliQ water and the gel developed in a sodium thiosulphate/sodium carbonate solution with 37% formalin (5.88% Na_2CO_3 , 0.0185% HCOH , 0.0004% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) for 3-5 min. Gel staining was stopped by adding 5% cold acetic acid for 10 min then washing in milliQ water. The gel was stored in a methanol/glycerol (30% methanol, 3% glycerol) solution and sealed in plastic.

2.10.7 Protein analysis

A differentially expressed spot was identified in the 2D-E gels that showed high expression in the P1 endophyte symbiotum. This protein spot was excised using a clean scalpel blade and placed in an eppendorf tube. The excised spot was sent to Stefan Clerans at the Protein and Structure Group, Growth and Development Section of AgResearch NZ Ltd for LC-MS/MS analysis.

Chapter 3 Results

3.1 A dwarfism-inducing fungus

3.1.1 Dwarf plants observed in a perennial ryegrass population

An observation was made of a number of dwarf seedling plants amongst an otherwise normal phenotype population. Of seventy individual seedlings in the population seventeen were dwarfed (Fig.3.1a) and the remaining plants were of normal phenotype (Fig.3.1b). The dwarf phenotype is marked by short, fine-leaved tillers and dark green pigmentation.

This observation is remarkable given that in mixed populations of wild-type *N. lolii* endophyte-infected and endophyte-free perennial ryegrass seedlings the infection status cannot usually be determined by simply observing the host grass phenotype, in addition individual infected plants cannot be distinguished from other infected individuals. The appearance of these dwarfed plants could be a result of changes in the host plant, the symbiont fungus or both.

The population of plants was derived following germination of seed and artificial inoculation of the seedlings with a characterised *N. lolii* strain, AR5. The seed population prior to inoculation was thought to be endophyte-free but subsequent investigation demonstrated that it was hosting a common-toxic endophyte (section 3.3.2).

3.1.2 *Neotyphodium* endophyte fungus isolated from dwarfed plants differs from that isolated from normal phenotype plants

To test whether a change had occurred to the symbiont *N. lolii* endophyte the fungus was isolated from both normal and dwarfed plants to see if the culture morphology of the fungus differed. Tissue from both normal phenotype plants and dwarf plants from the perennial ryegrass population was surface sterilised and plated on ABPDA to isolate the fungus (Methods 2.2.2). Fungus emerged from tissue of both dwarfed and normal-phenotype plants. The fungus that emerged from normal phenotype plants was typical



Fig.3.1 Phenotype of dwarf plants: (a) dwarfed phenotype plant observed in a population of diploid perennial ryegrass, (b) normal phenotype plant. Potted plants are 6 weeks old and grown under glasshouse conditions.

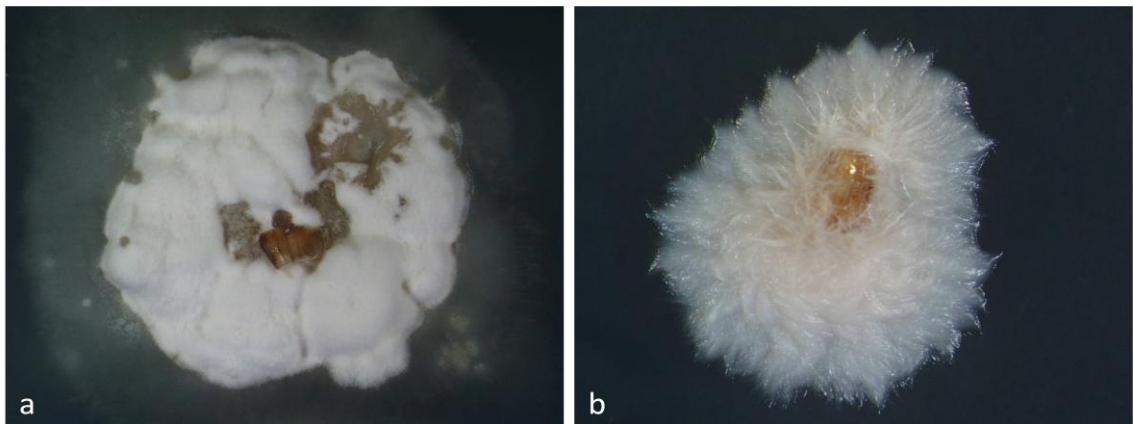


Fig.3.2 Colonies of *Neotyphodium lolii* isolated from perennial ryegrass on antibiotic potato dextrose agar (ABPDA) using surface sterilised tissue from infected plants. Mycelium emerges ca. 5 days from plating and can be observed using sub-stage lighting with a binocular microscope. Colonies shown have been growing for ca. 6 weeks. (a) Colony growing from tissue sourced from a normal-phenotype plant, colony is raised white and cottony, designated P41. (b) Colony growing from a dwarf-phenotype plant, colony grows as aggregated ropes of mycelium rather than discrete filaments contributing to a mucoid appearance, designated P1.

of New Zealand wild-type *N. lolii* being filamentous with aerial hyphae and was designated as P41 (Fig.3.2a) while the fungus isolated from the dwarf plants was atypically mucoid and was designated P1 (Fig.3.2b).

3.1.3 Removal of the mucoid P1 fungus restores the plant phenotype from dwarfed to normal

Dwarfed plants were found to host an endophyte that had a culture morphology that differed from the endophyte isolated from normal phenotype host plants (Results 3.1.2) and wild-type infected plants cannot be distinguished from un-infected plants. To test what happens to dwarf-phenotype plants when the fungus is removed, an experiment was performed whereby dwarf plants were separated into single tillers and treated with fungicide to remove the systemic infection of mucoid *N. lolii* P1 (Methods 2.5). The fungus-free tillers resulting from the fungicide treatment developed into plants that displayed a normal phenotype that when tested using an immunoassay (Methods 2.4.1) were confirmed to be endophyte-free (Fig.3.3).

3.1.4 Infection of endophyte-free seedlings with the mucoid P1 fungus results in dwarfed plants

Having shown that the removal of fungus from a dwarfed plant results in a return to normal phenotype an experiment was performed to see what happens when fungus isolated from a dwarf plant is used to infect an endophyte-free seedling. Mucoid (P1) fungus (Fig.3.2b) was isolated from a dwarf -phenotype plant (Fig.3.1a) and cultured on ABPDA. Mycelium from this culture was used to infect endophyte-free, etiolated, 6 day-old seedlings (Methods 2.3) of a diploid perennial ryegrass (accession A5322). Of 341 seedlings inoculated 81 died, of the 260 living plants 20 became infected (7.7% infection) and developed into dwarf plants while the remaining endophyte-free seedlings developed into normal-phenotype plants (Fig.3.4).



Fig.3.3 A fungicide treated clone (right) derived from a dwarf phenotype P1-infected plant (left). Tillers from the dwarf plant were separated and fungicide treated for 6 weeks. Treated tillers were potted in soil and allowed to grow into multi-tillered plants before testing for endophyte infection.



Fig.3.4 Phenotypic comparison between uninfected and P1-infected plants. Uninfected plants showing normal phenotype (left) and seedlings inoculated with P1 (right) showing a dwarfed phenotype. Seedlings were inoculated at the same age, ca. 6 days old.

3.1.5 Plantlet clones infected with P1 are dwarfed

Experiments to this point elucidate the connection between infection with P1 and the dwarfing effect that it has on its host plant. As perennial ryegrass is an out-crossing species these experiments have utilised populations of individuals with unique genotypes. The fungicide treatment experiment did show that an uninfected clone of a dwarf plant displayed a normal phenotype but for direct comparison to be made between the effect of P1 versus P41 fungus it is necessary to have them in the same host background. For this reason an experiment was performed to see if clonal plantlets could be infected with both fungi. A single perennial ryegrass genotype was grown axenically and divided into a number of cloned plantlets, some were retained as endophyte-free clones others were infected with P1 and others with P41 (Methods 2.9.3). The single P1-infected clone obtained displayed a dwarf phenotype whereas the endophyte-free clones displayed a normal phenotype (Fig.3.5) as did the P41-infected clones.



Fig.3.5 A single genotype clone of diploid perennial ryegrass Nui D; uninfected (left) and infected with P1(right).

3.2 Tillering, flowering and cell size

3.2.1 P1-infected plants produced more tillers than endophyte-free clones although initially at a similar rate

When plants infected with P1 were grown alongside endophyte-free clones under controlled conditions (methods 2.9.4) they produced more tillers. To quantify this, single tillers were potted and the formation of new tillers followed over time. P1-infected plants produced an average of 33 tillers while endophyte-free plants produced an average of 18 tillers.

Tiller growth of variant endophyte-infected and endophyte-free perennial ryegrass plants

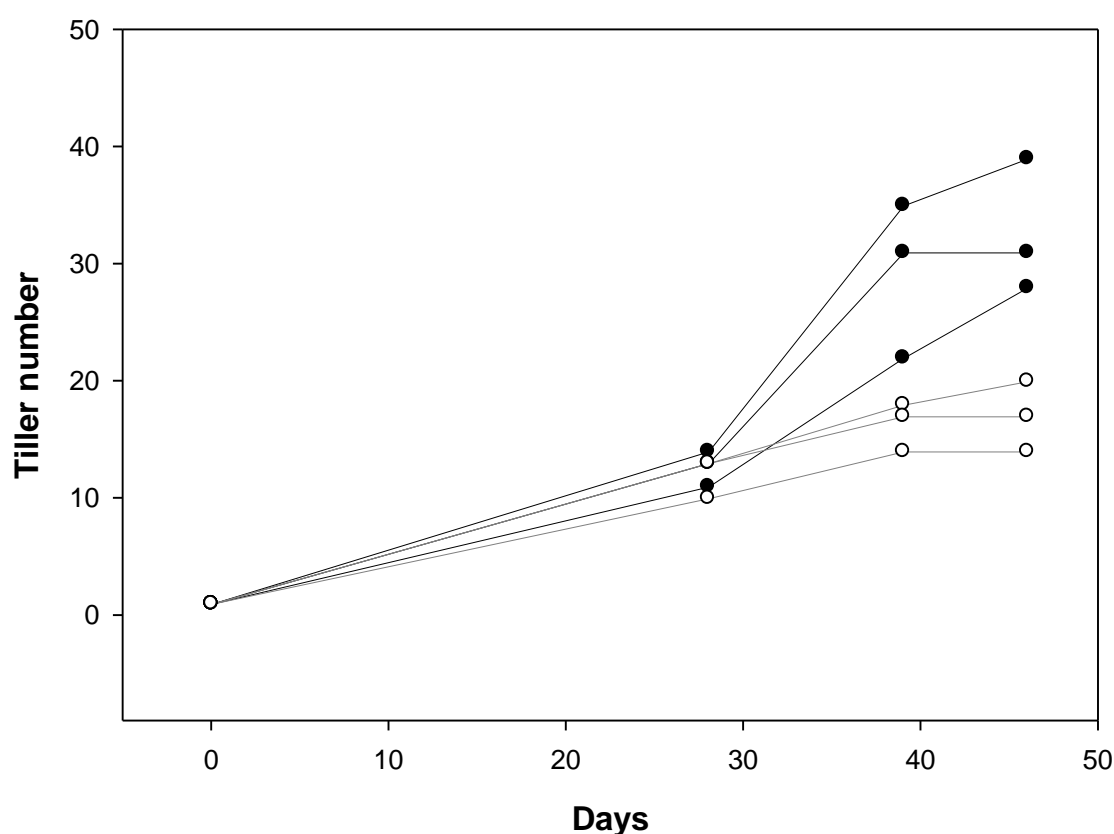


Fig.3.6 Tiller numbers of 3 clones of P1-infected (filled circle) and 3 clones of endophyte-free (open circle) perennial ryegrass grown under controlled conditions sampled over 46 days.

The growth rate and total tiller number of both P1-infected and endophyte-free replicate clones differed. Three clones of P1-infected plants potted as single tillers sampled over 46 days produced 28, 31 and 39 tillers, while 3 clones of endophyte-free plants sampled over the same period produced 14, 19 and 20 tillers (Fig.3.6). From day zero when plants were potted as single tillers to day 28 when the first sampling of daughter tillers was made the P1-infected plants and the endophyte-free plants produced tillers at a similar rate. At the first sampling the initial tillers of the P1-infected clones had produced an average of 12 tillers (10, 12 and 13), at the same sampling time the initial tillers of the endophyte-free clones had produced an average of 11 tillers (9, 12 and 12) (Fig.3.6). Over the next 11 days the rates of tiller production by the P1-infected clones and the endophyte-free clones diverged. At day 39 the P1-infected clones averaged 29 tillers (22, 31 and 35) while the endophyte-free clones averaged 16 tillers (14, 17 and 18) (Fig. 3.6). Infection with *Neotyphodium spp* endophytes has previously been shown to increase tiller production of perennial ryegrass (Latch et al. 1985).

3.2.2 P1 endophyte-infected plants produced a similar number of tillers to P41 endophyte-infected plants

When plants infected with the P1 fungus were grown alongside plants infected with the P41 endophyte under controlled conditions (methods 2.9.4) they produced tillers at a similar rate on average and therefore a similar number of tillers on average (Fig.3.7). P1 endophyte-infected plants produced an average total of 24 tillers over 34 days while P41 endophyte-infected plants produced an average of 21.5 tillers over the same period. The growth rate and total tiller number of both P41 endophyte-infected and P1-infected replicate clones differed (Fig.3.7). At 20 days both P1 and P41- infected plants had produced an average of 9.1 tillers and both exhibited a two-fold range or more of tiller numbers. Eight clones of P1 endophyte-infected plants potted as single tillers and sampled after 20 days produced between 5 and 13 tillers (5, 7, 8, 9, 9, 11, 11 and 13), while 8 clones of P41 endophyte-infected plants grown under the same conditions produced between 6 and 12 tillers (6, 7, 8, 8, 8, 12, 12 and 12) (Fig.3.7).

Tiller development of variant endophyte and wild-type endophyte infected perennial ryegrass clone Nui D

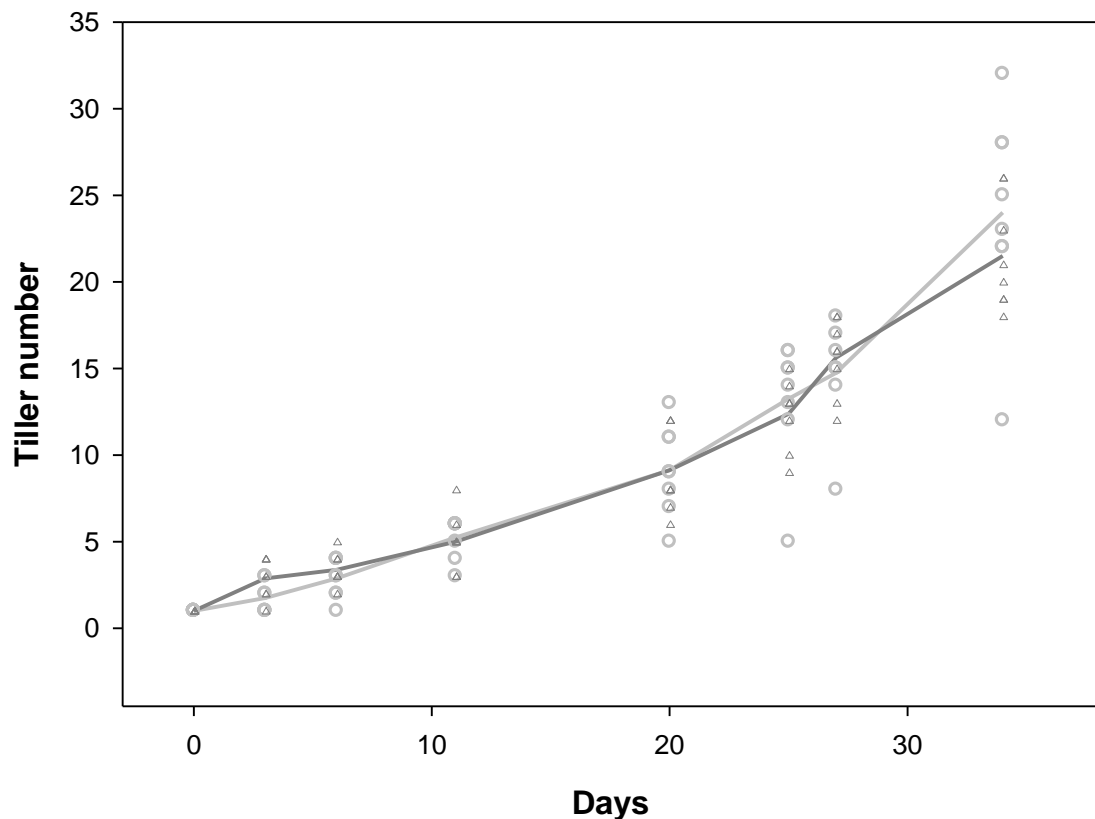


Fig.3.7 Tiller numbers of perennial ryegrass clone Nui D infected with P1 and P41 grown in controlled conditions measured over 34 days. P1-infected plants (circles), grey line mean trend; P41-infected plants (triangles), black line mean trend. Single tillers were potted for each clone at day zero.

3.2.3 Dwarfed plants infected with P1 do not flower under long day conditions following vernalisation

To determine if the dwarf phenotype plants were affected at the vegetative level only or if effects were evident in floral structures also, plants were treated to induce flowering. Dwarfed plants grown in an unheated greenhouse and experiencing short days (vernalising conditions) and subsequently grown under long days did not flower, while normal phenotype P41 endophyte-infected plants growing under the same conditions did (Fig.3.8). The plants were clones, so different flowering response cannot be explained by host genotype differences.



Fig.3.8 Fully vernalised plants of a dwarfed P1-infected plant (left) and normal phenotype P41 endophyte-infected plant (right). Plants were grown outdoors over winter months and brought into the greenhouse to flower under long days. The P41-infected plant flowered while the P1-infected plant did not. Plants were potted as single tillers at the same time.

In an attempt to ensure full vernalisation plants were wintered outdoors and as a consequence exposed to ground frosts. Concerns were held that such treatment would have a marked deleterious effect on the dwarf plants which had been observed to have weak growth in the greenhouse. The dwarf plants did not suffer deleterious effects as a consequence of exposure to frost and surprisingly were observed to thrive under outdoor winter conditions, more so than the normal phenotype P41-infected plants.

3.2.4 Failure of flowers to emerge from P1-infected plants is due to arrest in development of the inflorescence meristems

After full vernalisation and exposure to long days dwarfed plants show no flower emergence, leaving the possibility that they do not make the transition to flowering or that there is a problem in the development of the inflorescence. Tillers of both normal phenotype and dwarfed plants were dissected to directly examine floral meristems over time. Nui D clones infected with P41 and P1 were vernalised, then exposed to long days.

Representative tillers were examined following 0, 5 and 14 days exposure to long days. The development of floral meristems of dwarfed plants was suppressed compared to normal phenotype P41-infected clones. Although some rudimentary development of the floral meristems occurred in the P1-infected plant by day 14, it was insufficient for completion of floral emergence (Fig.3.9).

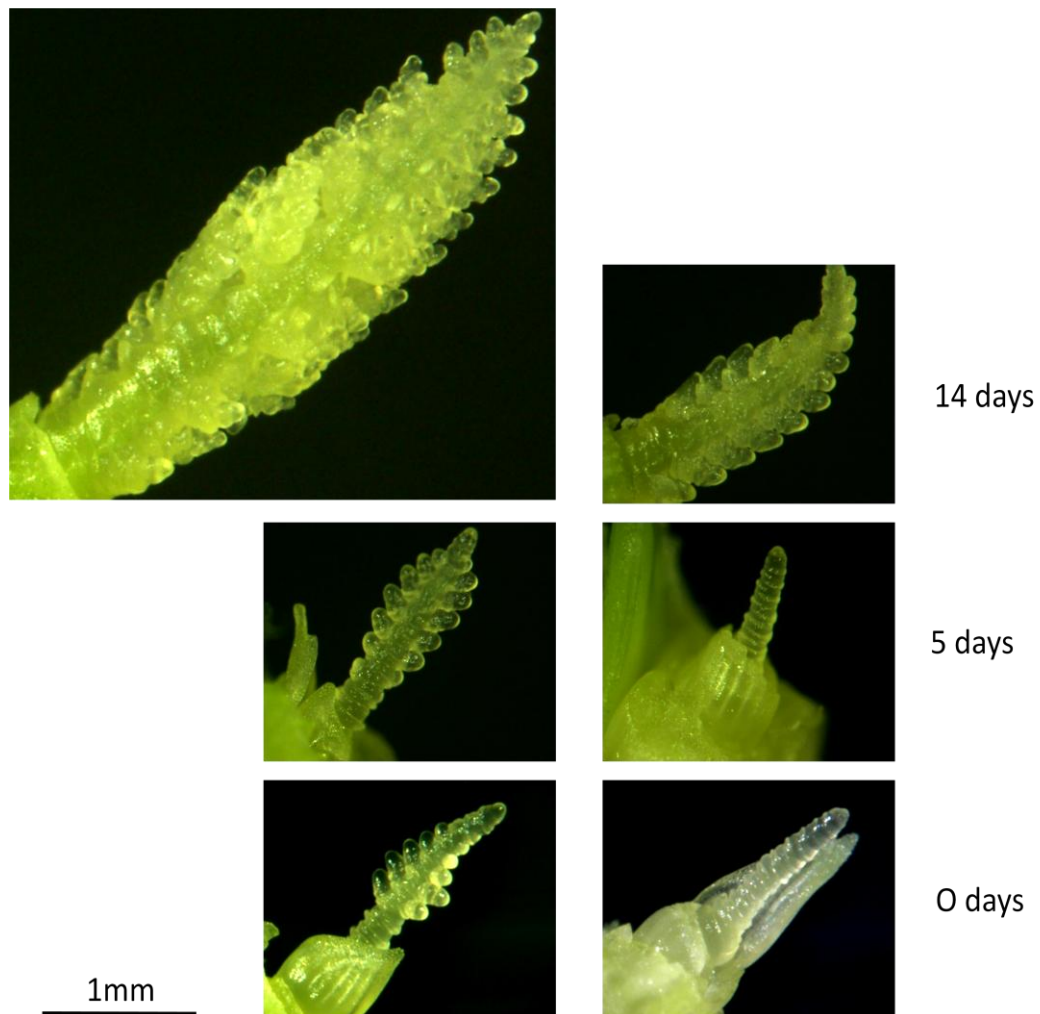


Fig.3.9 Excised tiller meristems of P41-infected perennial ryegrass (left) and P1-infected (right) tillers following vernalisation and 0, 5 and 14 days exposure to long days.

3.2.5 Gibberellin does not restore the dwarf phenotype to normal phenotype

A study by Cooper (1957) examined the application of gibberellin to a genetic dwarf of perennial ryegrass. Gibberellin was applied at a rate of 1ml of a 50mg/L solution (50µg) per week resulting in increased cell elongation in the leaf blade and sheath bringing the genetic dwarf within the normal phenotype for both leaf and inflorescence development (Cooper 1957).

In the current study plants infected with P1 were treated with GA₃ in an attempt to restore the plants to a normal phenotype, endophyte-free plants were also treated as a control. Both the P1-infected plants and the endophyte-free plants increased in size when gibberellin was applied, by 65% and 43% respectively.

The length difference between P1-infected and non-infected plants is on average 208mm, which means the non-infected plants are on average 182% taller than P1-infected plants. When plants were treated with GA₃ this difference in mm was increased to 274 but this is only 146% on average that non-infected plants are taller than P1-infected ones.

Full restoration of tiller length was not achieved by treatment with gibberellin as the P1-infected plants on average were 188mm and untreated non-infected plants were 322mm on average. Even the tallest of the GA₃ treated P1-infected tillers did not reach the size of the smallest untreated non-infected tiller (220 and 275mm respectively) (Fig.3.10). Whilst increasing the concentration or the frequency of application of gibberellins might restore the dwarf plants to a size comparable to that of normal plants, similar to that seen by Cooper (1957), further investigation (Section 3.2.6) shows that this would be unlikely given that the dwarf phenotype is due to less cells rather than smaller cells, implying a different mechanism.

3.2.6 The epidermal cells of dwarfed plants are similar in size to those of normal phenotype plants

Given that the application of GA did not restore dwarf-phenotype plants to a normal phenotype, a result that might be expected if dwarfed plants simply had smaller cells than normal-phenotype plants (Cooper 1957), cell measurements were made. Epidermal cells of dwarfed plants were measured and compared to those of P41-infected normal

phenotype plants to determine if this dwarfism was attributable to simply having smaller cells.

Eighty five cells from the base of a second leaf of a dwarf plant (Fig.3.12a) and a hundred and eight cells from the base of a second leaf of a normal phenotype plant (Fig.3.12b) were measured (Methods 2.4.3). The cells of the leaf from the dwarf plant ranged in length from 94.68 to 413.29 μ m with a mean of 215.71 μ m (SD 72.83) while the cells of the normal phenotype plant ranged from 59.48 to 406.32 μ m with a mean of 214.90 μ m (SD 74.99)

Effect of exogenous gibberellin (GA_3) on tiller length of variant endophyte infected (E+) and endophyte-free (E-) Nui D perennial ryegrass clones

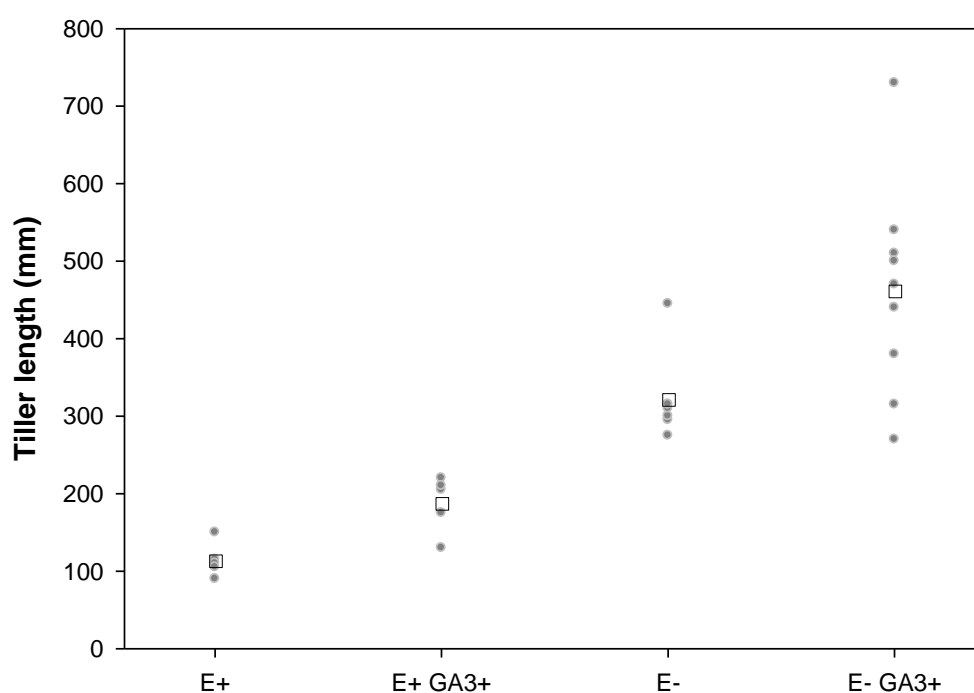


Fig.3.10 Tiller lengths of endophyte-infected and endophyte-free clones treated with gibberellin (100ppm GA_3 Berelex). Gibberellin was applied once as a foliar spray and plants grown for 2 weeks before measuring. Total tiller length from the base of the sheath to the tip of the blade was measured. Tillers measured endophyte infected, E+ (n=5), endophyte-infected + GA_3 , E+ GA_3 + (n=5); endophyte-free, E- (n=7), endophyte-free + GA_3 , E- GA_3 + (n=9). Open squares are means for each treatment.

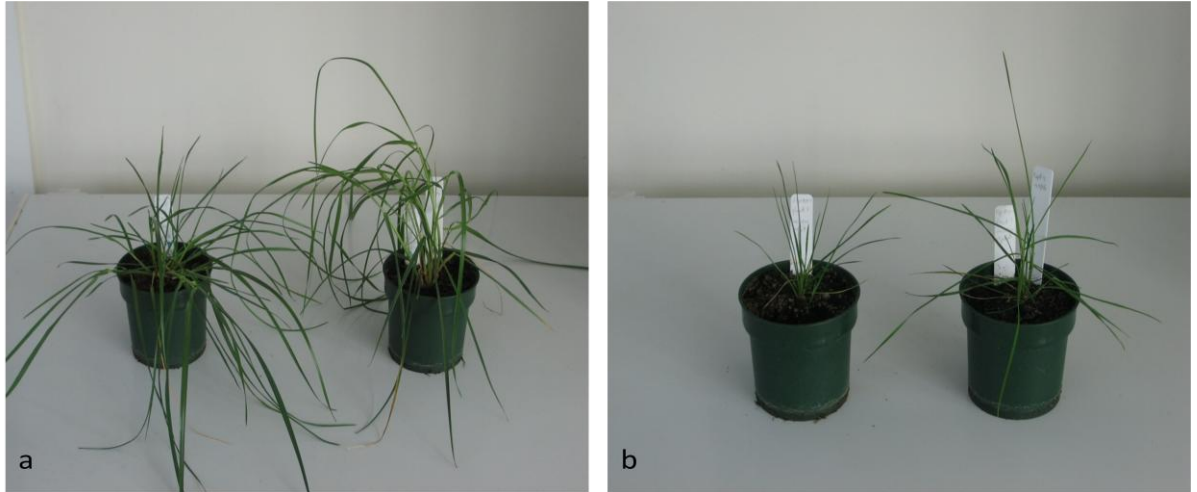


Fig.3.11 Gibberellin treated plants: (a) endophyte-free clone GA_3 minus (control) left and GA_3+ (100ppm GA_3) right, (b) P1-infected clone GA_3 minus left and GA_3+ right. Gibberellin increased the tiller length of both endophyte-free and P1-infected clones but did not restore the dwarf phenotype to normal.

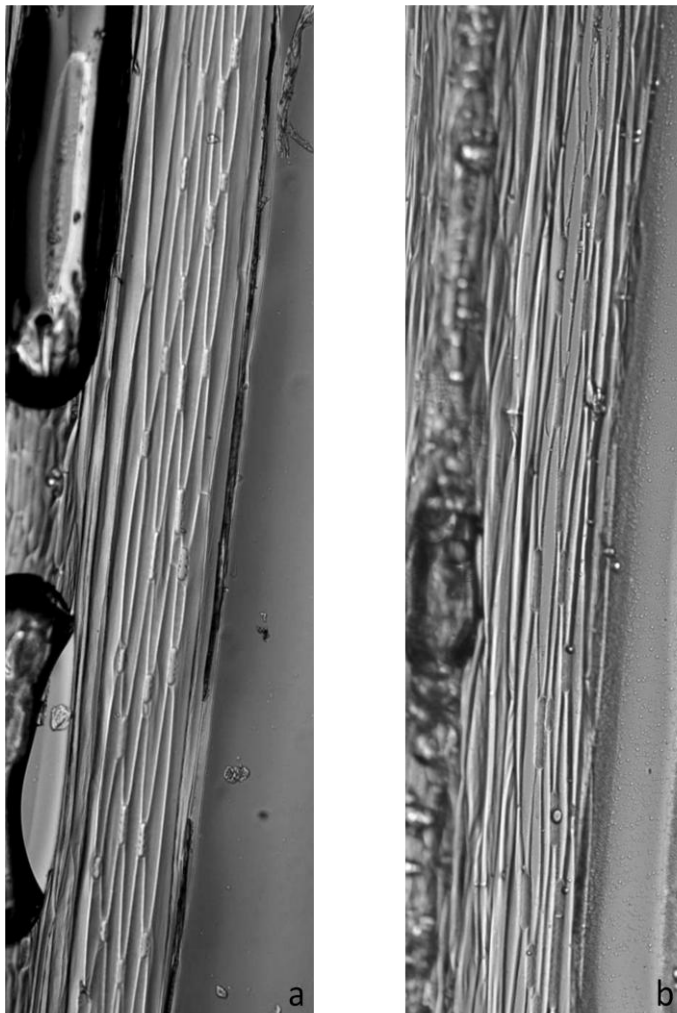


Fig.3.12 Compound light microscope images of leaf tissue imprints examined at 400X magnification. (a) dwarf plant leaf sheath imprint, mean cell length 215.71 μ m, (b) normal phenotype plant leaf sheath imprint, mean cell length 214.90 μ m.

3.3 DNA, protein and metabolic profiling

3.3.1 RAPD analysis shows that P1 has *N. lolii* profile

The P1 fungus isolated from dwarfed plants had an in culture phenotype unlike typical *N. lolii* fungi. A genomic test was made to examine the relationship of P1 to known cultures using randomly amplified polymorphic DNA (RAPD) analysis (Fig.3.13). DNA from a range of known endophytes was used in the analysis. Two *N. lolii* (AR29 and AR34), two *N. coenophialum* (AR525 and AR542) and an *Epichloë festucae* (AR66) were examined alongside the P1 fungus and P41 isolated from a normal phenotype plant from the same population. The analysis showed a difference between P1 and the two *N. coenophialum* but not with the *N. lolii*/*E. festucae* strains. *N. lolii* is a non-hybrid descendant of *E. festucae* whereas *N. coenophialum* is a triple hybrid between three *Epichloë* species (*E. festucae*, *E. baconii* and *E. typhina*). This result shows that the P1 fungus is not some uncharacterised, unrelated fungus that is colonising the dwarfed plants.

3.3.2 Simple Sequence Repeat data indicate that P1 is closely related to wild-type *N. lolii*

The original population from which the dwarf plants were recovered was infected with both a wild-type *N. lolii* (exemplified by strain P41 isolated from one of the normal looking plants) and a non-ergovaline producing *N. lolii* strain, AR5 (section 3.1). To determine which of these strains P1 originated from a further genomic test was undertaken. Tissue from P41-infected normal phenotype plants and dwarf plants was examined to determine the simple sequence repeat (SSR) genotype of the fungi (methods 2.7). DNA was extracted from P41 and AR5-infected normal phenotype plants and P1-infected dwarf plants along with endophyte-free plants as a control. In addition DNA extracted from cultured fungus of AR5 and P1 fungus were examined. SSR loci (microsatellite loci) B10 and B11 (Moon 1999) were used to screen the samples for polymorphism (Table 3.1 and appendix I). All endophyte-infected samples and the cultured fungus showed a peak of 177bp at the B10 locus, the endophyte-free

P1 P41 542 34 525 29 66

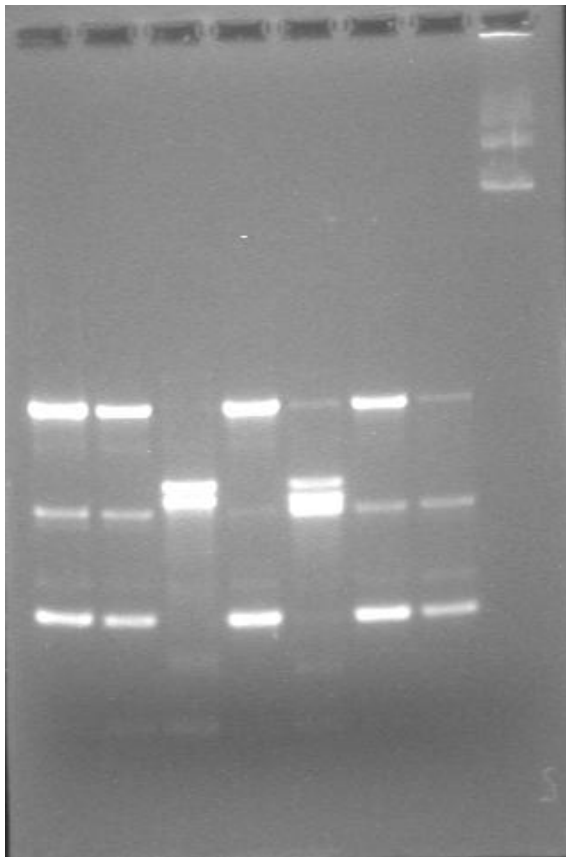


Fig.3.13 RAPD PCR products of endophyte DNA using random primer GTAGACCCGT. Left to right: P1; P41; 542, *N. coenophialum*,; 34, *N. lolii*; 525 *N. coenophialum* ; 29, *N. lolii* and 66, *E. festucae*.

Table 3.1 The presence of peaks at two loci (B10 and B11) using primers specific to *Epichloë* endophyte SSRs.

SSR LOCUS ¹	B10	B11	
Peak size (base pairs)	177	177	238
Dwarf plant, P1-infected	√	√	x
Cultured fungus dwarf	√	√	x
Normal plant, AR5 infected	√	x	√
Cultured fungus AR5	√	x	√
Normal plant, P41-infected	√	√	x
Normal plant, endophyte-free	x	x	x

¹ Dwarf and normal phenotype plants and cultured P1 and AR5 fungus was assayed along with an endophyte-free control plant. √ indicates where a peak was observed, x indicates where no peak of that size was observed at the locus.

plant had no product using PCR primers for this locus. The cultured fungus of AR5 and P1 endophyte also showed a 177bp peak at the B10 locus. At the B11 locus all four of the dwarf plants examined, the cultured P1 fungus and the P41-infected plant showed a 177bp peak while the two AR5 infected plants examined and the AR5 cultured fungus did not have a 177bp peak at this locus nor did the endophyte-free control plant. The AR5 infected plant and cultured fungus did have a peak at the B11 locus of 238bp that was not seen in any of the other material examined (Table 3.1 and appendix I). In summary, P1 showed the same SSR genotype as P41 and other New Zealand wild-type *N. lolii* and was shown to be different from the commercial *N. lolii* strain AR5.

3.3.3 The level of key alkaloids is reduced in symbiosis between P1 and its host compared to P41

A qualitative lolitrem B HPLC analysis of tillers from dwarf plants from the original population (FLp119#4) was made to determine whether they were infected with a non-lolitrem B producing *N. lolii* AR5 or the lolitrem B producing New Zealand wild-type *N. lolii*. When no lolitrem B was detected a quantitative analysis of lolitrem B along with ergovaline and peramine was made for both dwarfed and normal phenotype plants.

The second analysis confirmed that lolitrem B was not being produced at detectable levels ($> 0.1\text{ppm}$) in the dwarfed plant; 13.39ppm lolitrem B was measured in the normal phenotype plant (Fig.3.14b) no lolitrem B was detected in the dwarfed plant (Fig.3.14e). The ergovaline analysis showed depressed levels in the dwarfed plant; 1.2ppm ergovaline was measured in the normal phenotype plant (Fig.3.14a) and 0.3ppm in the dwarfed plant (Fig.3.14d). Peramine was also expressed at depressed levels in the dwarfed plant; 55ppm peramine was measured in the normal phenotype plant (Fig.3.14c) and 2.0ppm in the dwarfed plant (Fig.3.14f).

Given the reduction of both peramine and ergovaline levels it may be that the inability to detect lolitrem B reflects a reduction in production to levels below the level of detection. This might be attributable to reduced levels of fungal biomass or the result of a perturbation of the biosynthetic pathway.

The P1-infected dwarf plants appeared to be non-lolitrem B producers, examination of the chemo-type of the symbioses therefore would give a misleading indication of which

fungal strain the dwarf plants were hosting. SSRs gave a genome based result that was not influenced by the biology of the symbiosis.

3.3.4 Ergovaline HPLC shows extreme difference in peak size at 36.0 -36.2 min

When normal and dwarfed plants from the original population were analysed for ergovaline a large peak was observed in the dwarfed plant sample at ca. 36.0min that was not evident in the normal phenotype plant sample. The original ergovaline analysis was performed on two individuals from an out-crossing population and so represented two distinct genotypes. The analysis was repeated using clonal (Nui D) hosts.

Tissue from P41-infected and P1-infected clones was analysed for ergovaline using HPLC (methods 2.8). A minor accessory peak at 36.0-36.2 min was seen in P41 infected plants (Fig.3.15a). This same peak was seen in P1-infected clones but was over five times higher (Fig.3.15b). This peak is routinely seen in ergovaline analyses of ryegrasses infected with a range of *Neotyphodium* endophytes and endophyte-free plants at the levels seen in the wild-type endophyte-infected material here (Dr W.Mace pers com). The fact that the peak is seen in endophyte-free plant material indicates that it is of plant origin rather than fungal.

The HPLC analysis employed is insufficient to determine what the peak is and substantial further analysis is required to do so (Dr Brian Tapper pers com).

3.3.5 Dwarf P1-infected plants differ from normal P41- infected plants in their protein expression

Nui D clones infected with P41 and P1 were grown under controlled conditions (methods 2.9.4) to standardise the environment under which the symbiota expressed proteins.

Tissue from these plants was harvested and total protein extracted. Extracted proteins were separated according to charge (1st dimension- IEF strip, pH 3-10) and mass (2nd dimension- acrylamide gel) along with 2-D gel marker proteins that separate out at predetermined pH and Pi and silver stained for visualisation (methods 2.10). The protein expression profiles differed between P41 and P1-infected clones (Fig.3.16).

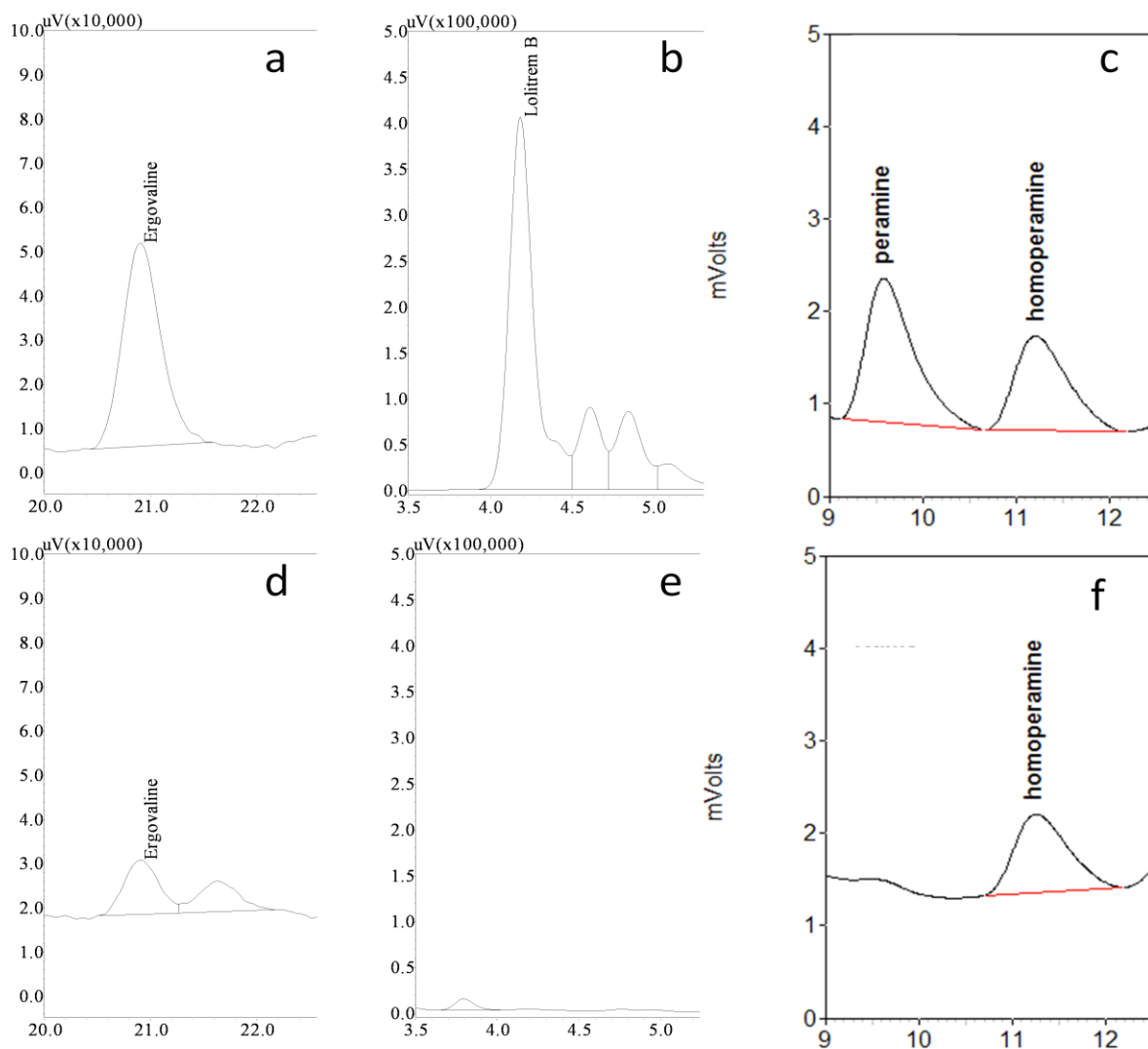


Fig.3.14 HPLC trace of endophyte-infected diploid perennial ryegrass: P41-infected plant; (a) ergovaline; (b) lolitrem B; (c) peramine and P1-infected plant; (d) ergovaline (e) lolitrem B (f) peramine. Limit of detection ergovaline (0.1 ppm), lolitrem B (0.1 ppm), peramine (1.0 ppm)

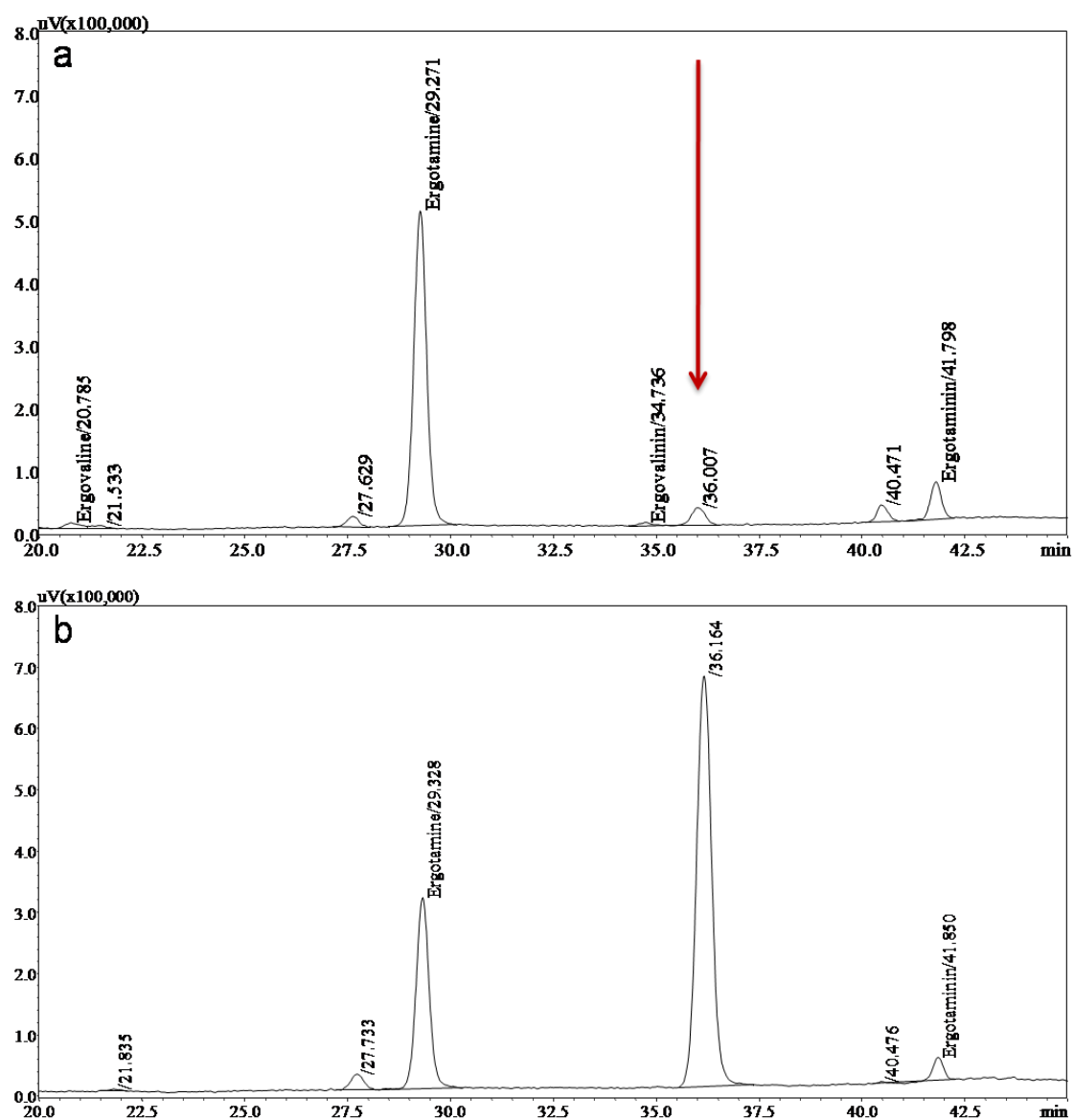


Fig.3.15 HPLC chromatograms of P41-infected perennial ryegrass clone Nui D (a) and P1-infected perennial ryegrass clone (b). The P41-infected clone has a small peak at 36.007 min (red arrow) while the P1-infected has a very large peak around that point at 36.164 min.

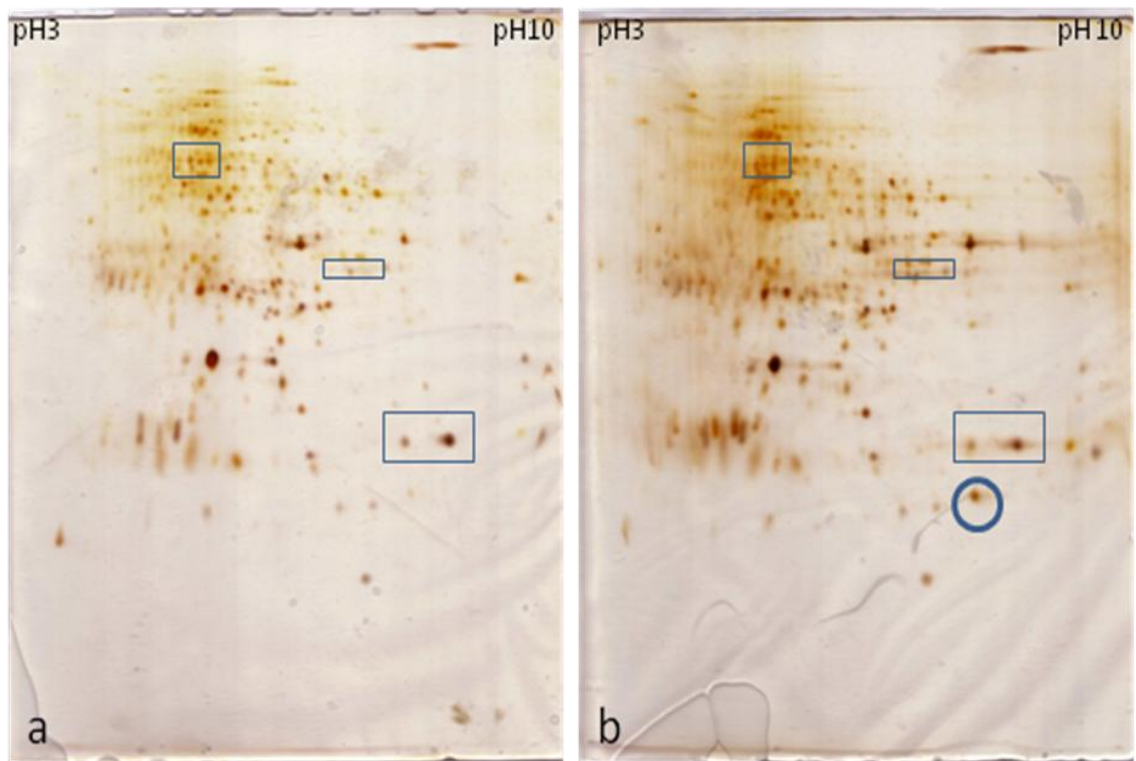


Fig.3.16 2-Dimensional electrophoresis of total protein extracted from P41-infected (a) and P1-infected (b) perennial ryegrass. Three biological replicates were run, all showed differential expression of the protein spot (circled) sent for analysis. Total protein was extracted from clonal plant material, resolved according to charge (PI) on an isoelectric focusing strip (IEF) pH 3-10 and then resolved according to mass on a large format polyacrylamide gel (2nd dimension). Boxes highlight 2D gel marker proteins, top to bottom: ovalbumin, (PI 5.1, MW 45,000), carbonic anhydrase (PI 7.0, MW 29,000) and myoglobin (PI 7.6, MW 17,000). Circle shows protein that was excised and analysed by LC-MS/MS.

3.3.6 LC-MS/MS

The protein spot indicated in figure 3.16 was sent for LC-MS/MS analysis (Methods 2.10.7) and examined in the 25-55 min range. The chromatogram showed heavy polyethylene glycol (PEG) contamination. Despite this there was weak evidence of protein in the form of one, possibly two peptides (LNLSHNL, LNLSHNLL) that matched to a putative phytosulfokine receptor precursor, also known as a putative leucine-rich repeat receptor-like kinase, from rice. Mascot search results of the peptide fragments are shown in appendix II.

3.4 In culture studies

3.4.1 Fungal morphology is uniform when sub-cultured from newly emerged mycelium

Fungus was isolated from surface sterilised tissue (methods 2.2.2) sampled from dwarf plants and normal phenotype plants (Fig.3.1). The fungal colony that formed from the dwarf plant was the mucoid P1 (Fig.3.17a) while the colony that developed from the normal phenotype plant was the cottony P41 (Fig.3.17c). Mycelium was cut from the emerging colonies, macerated and plated onto ABPDA (methods 2.2.4). The fungus that grew from the macerated mucoid mycelium isolated from the dwarf plant formed mucoid colonies (Fig.3.17b) while the fungus that grew from the macerated cottony mycelium isolated from the normal phenotype plant formed cottony colonies (Fig.3.17d).

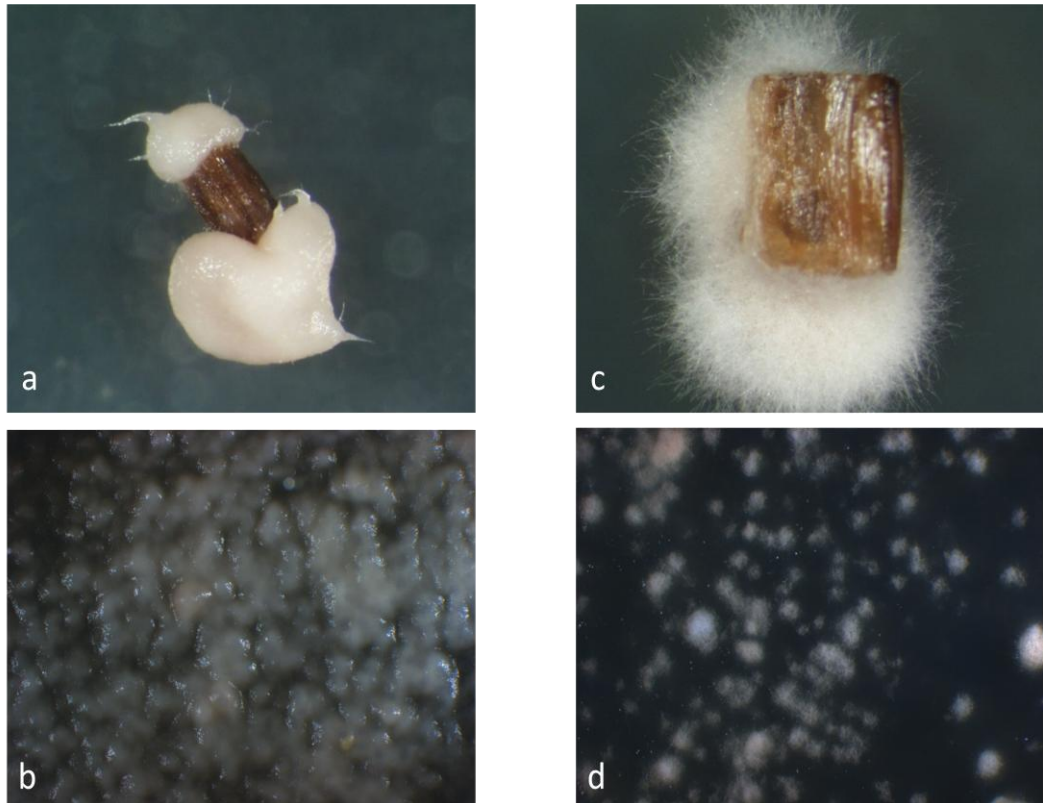


Fig.3.17 Isolation of endophyte fungus on ABPDA. (a) P1 endophyte emerging from surface sterilised tissue, (b) macerate culture of P1, (c) P41 emerging from surface sterilised tissue, (d) macerate culture of P41. Hyphae emerge from surface sterilised tissue incubated at 22°C after ca. 5d, visible colonies evident after 2-3 weeks. Macerate culture inoculum produced by grinding mycelium in PDB and pipetting onto solid media.

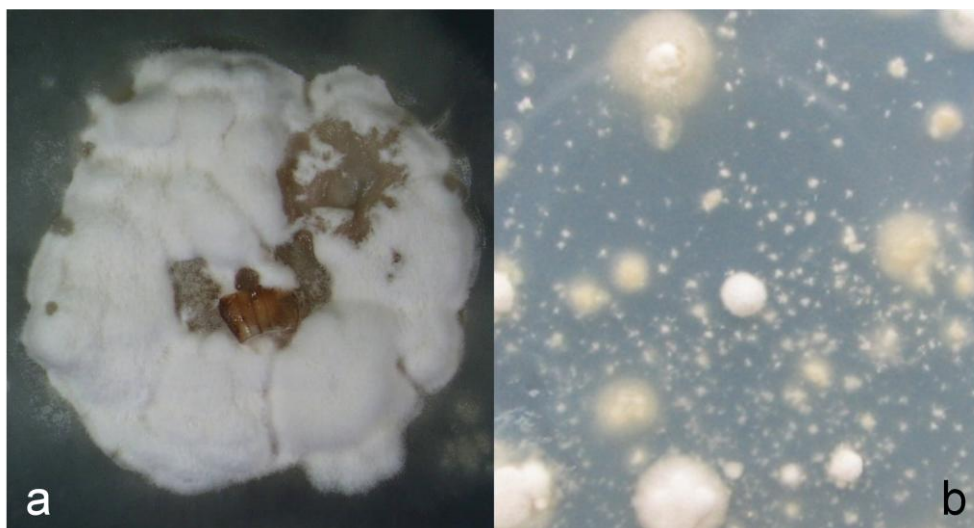


Fig.3.18 (a) Mature colony of P41. (b) Macerate colonies derived from a mature P41 colony.

3.4.2 Fungal morphology is not uniform when sub-cultured from mature P41 colonies

P41 fungus isolated from normal phenotype plants emerges as white cottony filaments that form a uniform white cottony mycelium (Fig.3.17c). As the colony ages it becomes more dense and heterogeneous in colour and texture (Fig.3.18a). Mycelium was cut from a heterogeneous colony, macerated and plated onto ABPDA (methods 2.2.4). The colonies that grew were not uniform, both cottony and mucoid colonies developed from the macerate (Fig.3.18b).

3.4.3 Mucoid phenotype colonies can arise spontaneously in cultures of P41 and induce dwarfing of host plants

A macerate culture (methods 2.2.4) of P41 gave rise to both cottony and mucoid colonies (Fig.3.19a). Endophyte-free seedlings were germinated (methods 2.3) and inoculated with mycelium from each of the two colony types. Seedlings that became infected with cottony fungus developed a normal phenotype (Fig.3.19b top arrow) while seedlings that became infected with mucoid P41 fungus developed a fine-leaved dwarf phenotype (Fig.3.19b bottom arrow).

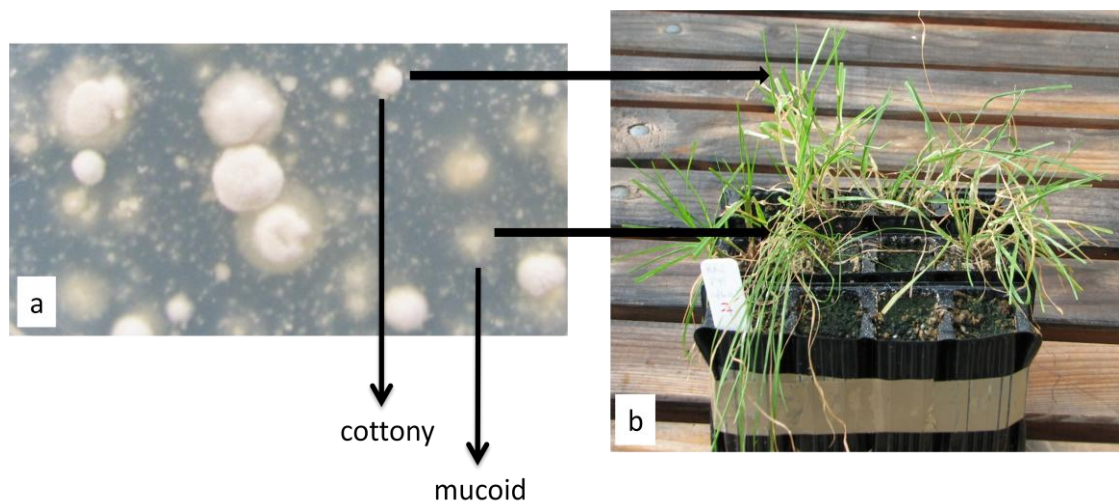


Fig.3.19 Fungus from P41 macerate cultures that developed both cottony and mucoid colony types (a) was used as inoculum to infect endophyte-free perennial ryegrass. Cottony fungus gave rise to normal phenotype plants and mucoid fungus gave rise to dwarf fine-tillered plants (b).

3.4.4 Mass conversions of cottony to mucoid phenotype can be induced in P41 cultures by density

A macerate culture (methods 2.2.4) of P41 was plated at two densities to examine the effect on colony morphology. Fungus plated at low density formed cottony colonies (Fig.3.20 lower images) while fungus plated at high density formed mucoid colonies (Fig.3.20 upper images) when examined following 32d growth. Prior to plating macerate inoculum, samples were examined using a haemocytometer. 3.08×10^5 colony forming units (cfu) were measured in the high density macerate and 4.8×10^4 cfu in the low density macerate.

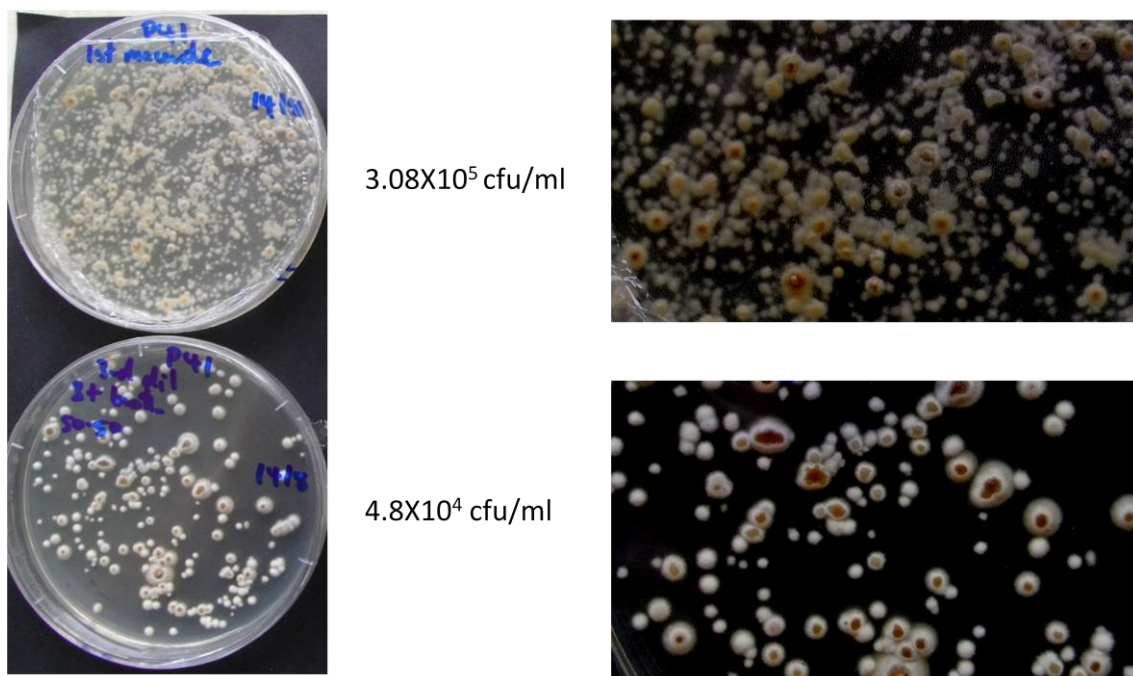


Fig.3.20 Macerated P41 fungus plated at high density (top) produced mucoid colonies while fungus plated at low density produced cottony colonies (bottom). Macerate culture inoculum was produced by grinding mycelium in PDB and pipetting to solid media. Cultures were examined at 32d after plating.

3.4.5 Mass conversions of cottony to mucoid phenotype can be induced in P41 cultures by age

A low density macerate culture of P41 (methods 2.2.4) was plated on ABPDA and examined over 64 days to determine the effect of age on colony morphology. 15 days after sub-culturing cottony colonies visible to the naked eye formed (Fig.3.21a). More

colonies visible to the naked eye developed and at 32 days the colonies maintained a cottony morphology (Fig.3.21b). After 64 days incubation the colonies were displaying a mucoid morphology (Fig.3.21c).

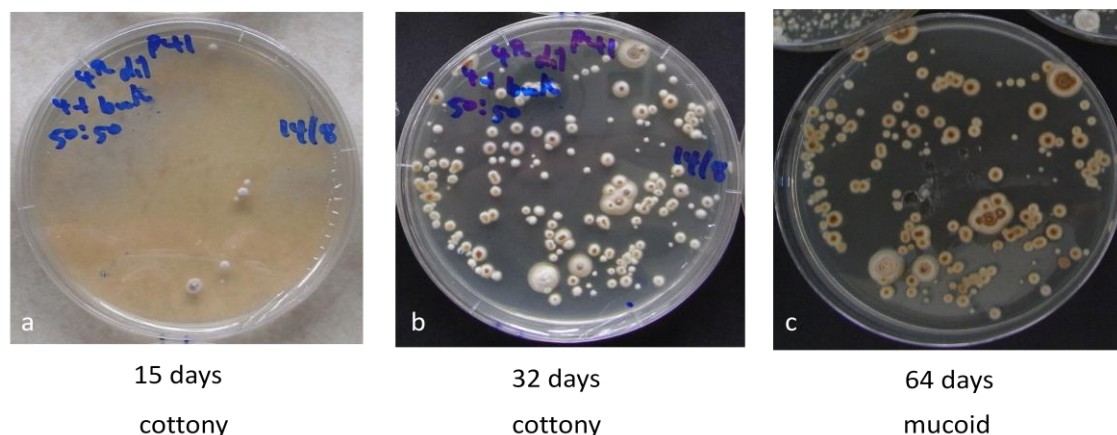


Fig.3.21 Macerated P41 fungus plated on solid media formed cottony colonies (a and b) that became mucoid with age (c). Macerate culture inoculum was produced by grinding mycelium in PDB and pipetting to ABPDA at 2.25×10^4 cfu. Plates were incubated at 22°C for 64 days, observations were made at 15, 32 and 64 days.

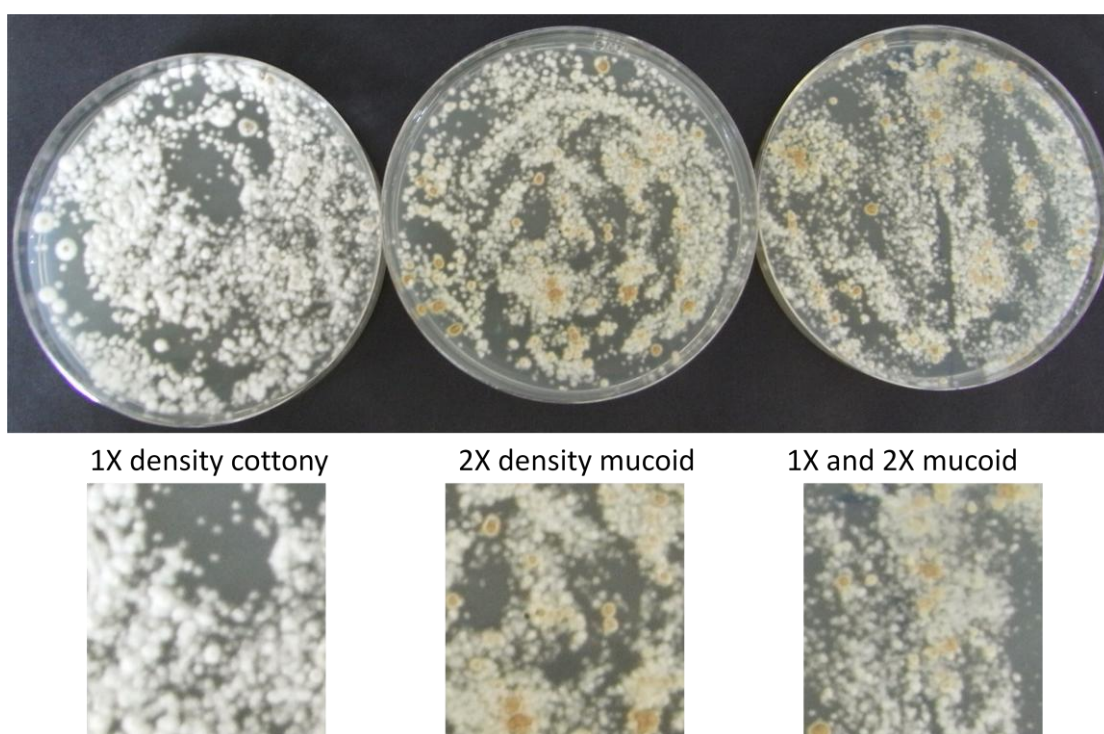


Fig.3.22 Macerated P41 fungus plated to ABPDA at low density (left) produces cottony colonies, at high density (middle) mucoid colonies and when both densities are plated side by side on one plate (right), mucoid colonies.

3.4.6 Evidence that the in culture cottony to mucoid transition is induced by a transmissible substance

A macerate culture (methods 2.2.4) of P41 was plated at high and low density to produce mucoid and cottony colonies respectively. Using the same macerate preparations an ABPDA plate was inoculated with high density macerate on one half of the plate and low density on the other to determine whether the colonies would maintain their respective morphologies or develop as either all cottony or all mucoid colonies. The low density macerate developed into cottony colonies (Fig.3.22, left), the high density macerate developed into mucoid colonies (Fig.3.22, centre). The plate with both high and low density macerate developed mucoid colonies on both halves (Fig.3.22, right).

3.4.7 Conversion from cottony to mucoid phenotype and vice versa can be induced by sub-culturing

En masse conversion of cultures from cottony to mucoid can be induced either by plating macerate cultures or sub-culturing individual colonies, both give rise to opposite colony types. This will be examined in the next two sections.

3.4.7.1 Macerate culture subbing

P41 mycelium that was plated at high density formed mucoid colonies with distinct brown pigmentation. These colonies were used along with cottony white mycelium to produce macerate for spot culturing of the fungus. Macerate cultures (methods 2.2.4) were prepared using cottony and mucoid inoculum obtained from low and high density macerate cultures respectively (results 3.4.4). Inoculum was applied to ABPDA plates in 10µl volumes using a pipette. The white and cottony culture derived from low density macerate cultures were plated as 10µl inoculum spots as were the mucoid culture derived from high density macerate cultures. The cottony culture spots developed mucoid colonies, the mucoid culture spots developed cottony colonies. The cottony colonies that became mucoid maintained cottony borders at the perimeter of the culture and both cottony and mucoid colonies developed occasional spots of the opposite colony type (Fig.3.23).

10µl aliquots of brown
mucoid macerate
inoculum



10µl aliquots of white
cottony macerate
inoculum

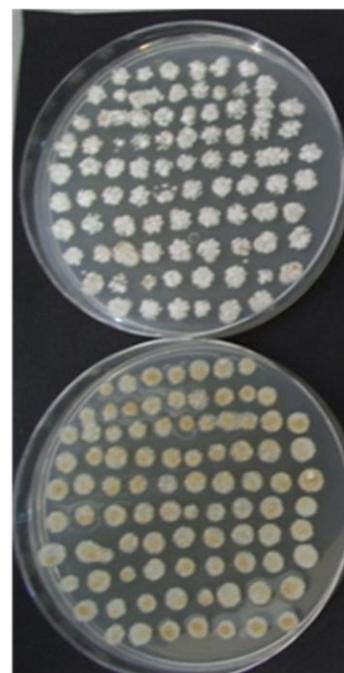


Fig.3.23 Colonies on ABPDA developing from subculture inoculum consisting of 10µl macerated P41 mycelium derived from mucoid and cottony cultures. Top: Cottony colonies developing from mucoid inoculum plated as 10µl spots. Bottom: Mucoid colonies developing from cottony inoculum plated as 10µl spots.

3.4.7.2 Non-macerate subbing

A macerate culture (methods 2.2.4) of P41 gave rise to a heterogeneous population of colonies consisting of both cottony and mucoid types. Non-macerate sub cultures were made by excising colonies with a sterile scalpel and plating to fresh ABPDA media. The 5 cottony colonies sub-cultured in this way all gave rise to mucoid colonies (Fig.3.24 1-5), while the 3 mucoid colonies gave rise to cottony colonies when sub-cultured (Fig.3.24 a-c).

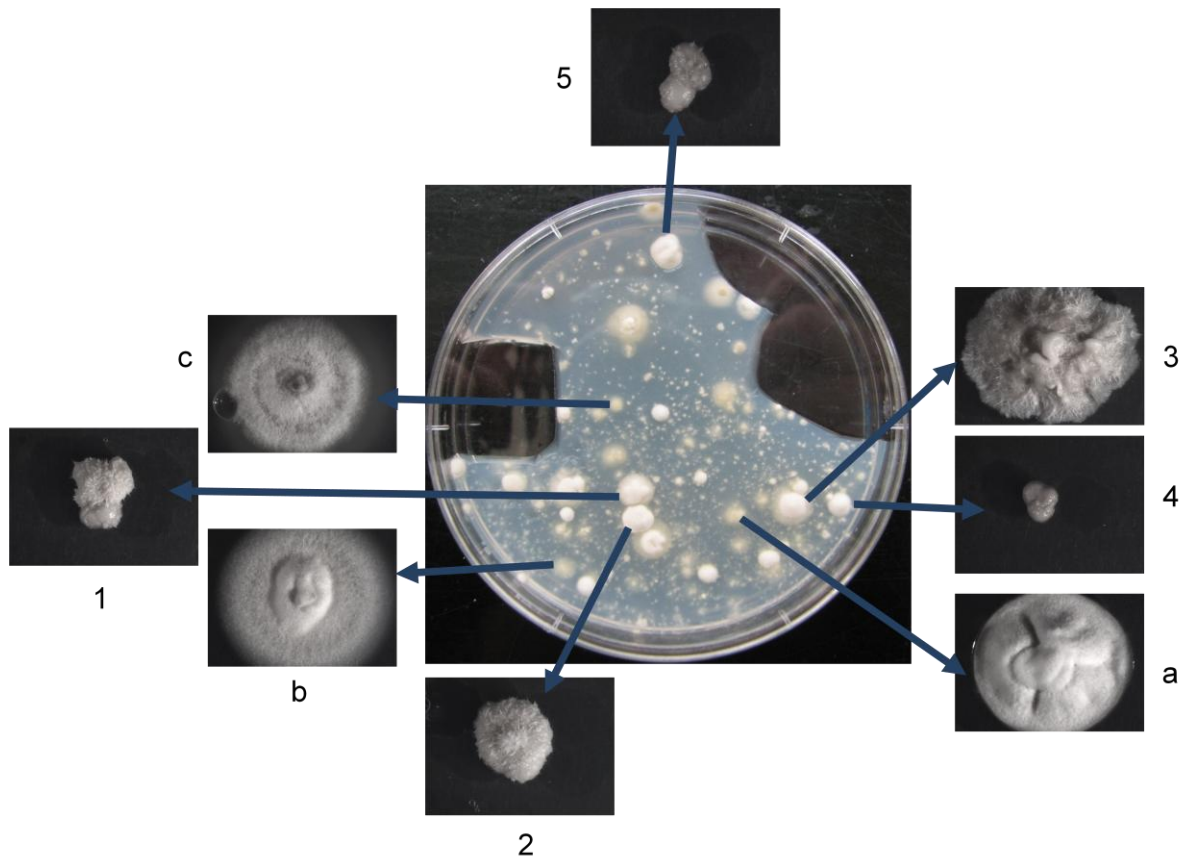


Fig.3.24 Sub-cultured fungus (images a-c and 1-5) from a macerate culture of P41 (centre). The P41 macerate culture (centre) consists of a mixture of cottony and mucoid colonies. The 3 cottony sub-cultures a-c were derived from mucoid colonies from the central plate, the 5 mucoid sub-cultures were derived from cottony colonies from the central plate.

3.4.8 Cottony and mucoid fungus grow differently in liquid culture

Inoculum of the original cottony P41 and mucoid P1 was used to inoculate flasks containing liquid media and incubated with agitation on a shaker (methods 2.2.3). P41 formed ball-like aggregated colonies that settled to the bottom of the liquid media when flasks were allowed to sit without agitation (Fig.3.25 left), P1 did not form aggregated colonies instead the hyphae broke into fragments that remained in suspension giving the media an opaque, soupy appearance (Fig.3.25 right).

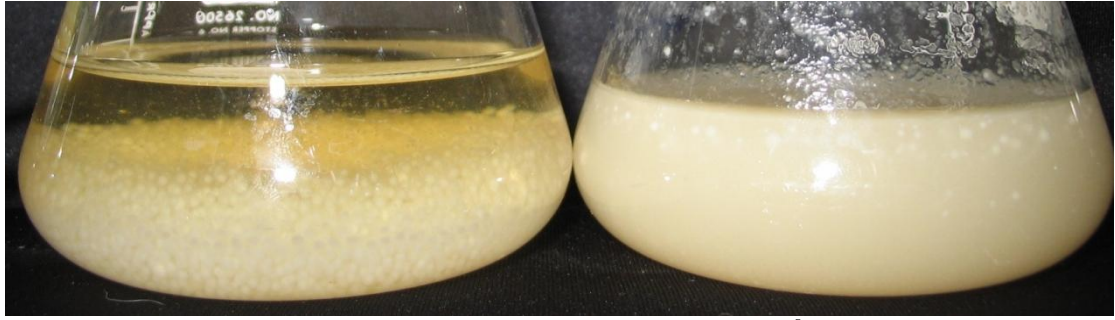


Fig.3.25 Flask cultures of P41 (left) and P1 (right) in PDB incubated at 22°C shaken at 100rpm for 15 days. P41 has formed ball-like aggregations that settle to the bottom of the flask on cessation of shaking while P1 mycelium does not aggregate, instead remaining in solution as hyphal fragments.

3.4.9 Infection of perennial ryegrass seedlings using cottony P41 fungus and mucoid P41 mycelium produced in culture

3.4.9.1 Ryegrass plants artificially infected with cottony P41 fungus display a normal phenotype

Cottony P41 fungus was isolated (methods 2.2.2) from a normal phenotype plant, FLp119#4 plant 41 (Fig.3.17c) and cultured on ABPDA. Mycelium from this culture was used to infect endophyte-free, etiolated, 6 day-old seedlings (methods 2.3) of a diploid perennial ryegrass (accession A5322). Of 96 seedlings inoculated 21 died and 2 were too small to assay for endophyte presence. Of the remaining 73 plants 20 were found to be endophyte-infected using an immuno-assay (methods 2.4.1) giving a live-plant infection rate of 27.4% (Fig 3.26). These plants infected with a cottony P41 fungus displayed a normal phenotype.

3.4.9.2 Ryegrass plants artificially infected with mucoid P41 mycelium that develops in culture have a low infection rate and display a dwarf phenotype

P41 isolated from a normal phenotype plant was macerated and sub-cultured onto ABPDA, after growing for 64 days the colony, originally cottony, became mucoid. The mucoid fungus was used to artificially infect endophyte-free perennial ryegrass seedlings (accession A5322). Of an estimated 98 seedlings inoculated 22 died. Of the remaining 76 seedlings examined by immuno-blot 6 were endophyte infected (7.9% infection; Fig 3.26), all 6 developed into dwarf plants while the remaining endophyte-free seedlings developed into normal phenotype plants.

3.4.9.3 Ryegrass plants artificially infected with cottony P41 fungus that develops from a mucoid colony display a normal phenotype and infection rate

Mucoid fungus derived from cottony colonies was cultured on ABPDA until a number of topical cottony colonies developed. Mycelium from these colonies was used to inoculate endophyte-free perennial ryegrass seedlings. Of 130 seedlings inoculated, 28 died. Of the remaining 102 seedlings examined by immuno-blot 30 were endophyte-infected (29.4%; Fig 3.26) the remaining 72 were endophyte-free. The plants that became infected with the cottony fungus that developed from a mucoid colony displayed a normal phenotype.

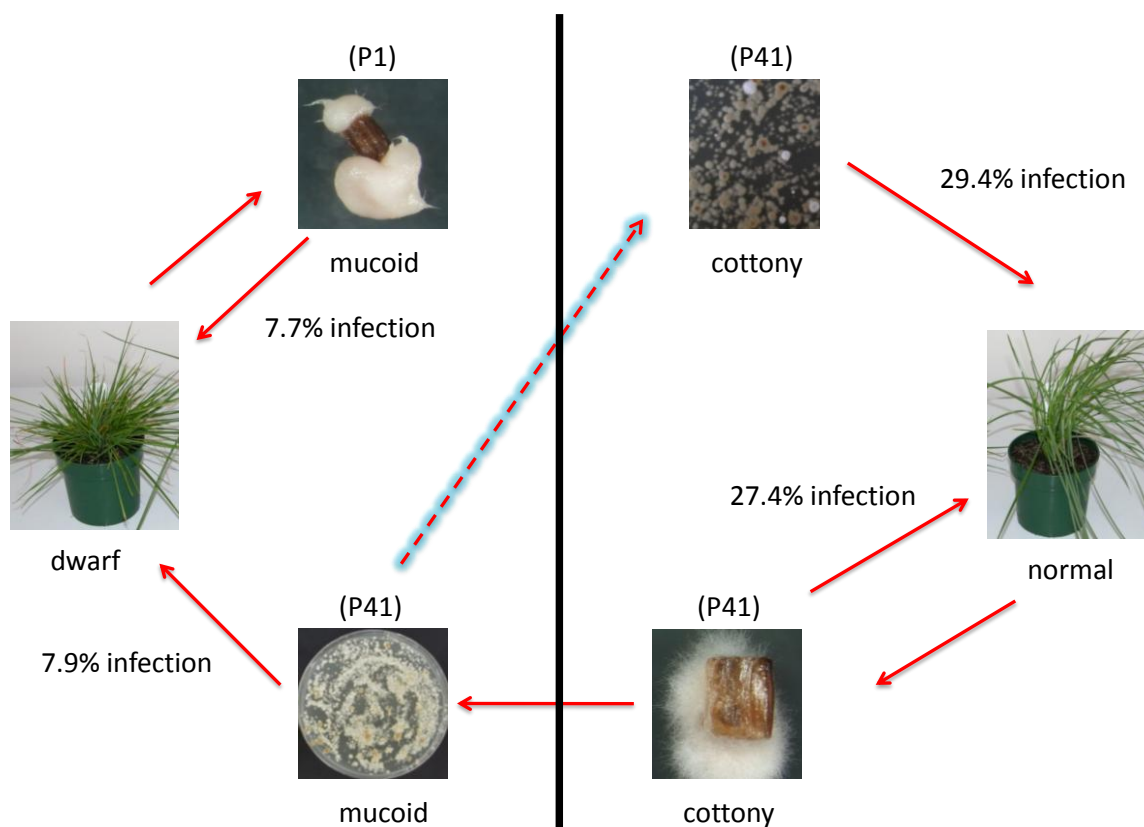


Fig.3.26 Schematic overview of fungal and plant phenotypes following isolation, infection and sub-culturing using both mucoid and cottony *N. lolii*.

Chapter 4 Discussion

Unlike their teleomorphic (sexual) progenitors *Epichloë*, the anamorphic (asexual) *Neotyphodium* endophytes are typically asymptomatic causing no disease or outward signs of infection in their host grasses (Clay 1990). *Neotyphodium* endophytes do not produce the external sexual structure (stromata) that is a clear indicator of infection in the *Epichloë* (Clay and Schardl 2002; Leuchtmann et al. 1994; Schardl 2001; White 1992) and as such unless host grass plants are subject to severe biotic or abiotic stress it is not possible to determine infection status by a macroscopic examination of plants. In this study an observation was made of a number of dwarf seedling plants amongst an otherwise normal phenotype population of seedlings that had been inoculated with the endophyte strain AR5 (Results 3.1.1). AR5 is a *Neotyphodium lolii* endophyte that does not produce the alkaloid lolitrem B *in planta*. Of 70 plants assayed for the presence of lolitrem B 19 tested positive and showed a ‘normal’ phenotype, indicating a resident endemic endophyte was present in the original seedling population. However, within the population there were also 17 individuals displaying a fine-leaved phenotype which were negative for lolitrem B. Endophyte was isolated from both fine-leaved plants and ‘normal’ phenotype lolitrem B negative plants. The endophyte from normal phenotype plants was white and cottony and typical of AR5 colonies whereas the endophyte from fine leaved plants was waxy and wet, forming only small colonies (Results 3.1.2). Studies showed that not only did the waxy endophyte induce a fine-leaved type in all plants it infected, but that curing plants of such infections restored the ‘normal’ plant phenotype (Results 3.1.3). When mycelium from the mutant colonies was used to inoculate endophyte-free plants all infected plants were fine leaved. My original hypothesis was that the waxy endophyte was derived from a mutation of the AR5 endophyte used to inoculate the seedlings because chemistry indicated a non-lolitrem B producing strain (Results 3.3.3). However, a genomic assay (Results 3.3.2), to confirm the identity of the endophyte in the fine-leaved plants, showed in fact that this was not the case. DNA was extracted from both cultured and *in planta* endophytes representing the endemic endophytes (lolitrem B producers), AR5 (non-lolitrem B producer) and P1 (non-lolitrem B producer). Primers for previously identified simple sequence repeats (SSRs) (Moon et al. 1999) were used to amplify the DNA using PCR. Two alleles were examined, B10 which resulted in one peak for all samples and so was not discriminatory and B11 which gave two peaks (Results 3.3.2). The mutant had the same SSR profile as

the endemic endophyte and the AR5 profile differed. The SSR evidence therefore clearly demonstrated that of the two possible sources of the waxy endophyte in the population, it is the endemic wild type not the inoculated *N. lolii* AR5 that is the progenitor fungus. RAPD analysis, performed on a range of different endophyte species (Results 3.3.1), provided further evidence that both the wild type endemic and waxy endophyte belong to *N. lolii*. The failure to detect lolitrem B production by P1 might be explained by either a perturbation of the biosynthetic pathway or possibly by a reduction in hyphal biomass in the symbiosis resulting in a reduction in metabolite production to below the limit of detection (0.1 ppm, Wade Mace pers. com). This could be tested by measuring the expression of genes (if present) involved in lolitrem biosynthesis as well as measuring biomass through RT-PCR or ELISA methodologies (Rasmussen et al. 2007; Spiering et al. 2005a; Young et al. 2006).

Based on these results, my initial hypothesis was that the endemic endophyte had undergone a mutation leading to a change in cultural morphology and that this mutation also had a profound effect on the host plant, something that had only been previously observed in genetically modified endophytes containing gene knockouts in pathways shown to be required for maintenance of symbiosis (Johnson 2008; Tanaka et al. 2006; Tanaka et al. 2008). The mucoid nature and the observation of extreme fragmentation in liquid culture (Results 3.4.8) suggested a possible mutation. The mutant hypothesis was however disproved when in subsequent experiments to study the phenomenon further it was discovered that the wild type endemic cottony endophyte, represented by isolate P41, could be induced to switch to a mucoid phenotype in culture at a high frequency and that this was reversible (Results 3.4.2-3.4.7). A key finding was that P41 mucoid or cottony colonies obtained in this way behaved in a manner similar to the original P1 mucoid and P41 cottony colonies, causing stunting or normal plants, respectively, when inoculated into the host (Results 3.4.3). This type of high frequency in-culture morphological switching has been described for some other fungal species (Griffiths 1992; Hogan 2006; Maheshwari and Navaraj 2008; Oh et al. 2001; Osiewacz 2002; Ramírez-Zavala et al. 2008; Soll 1992; Turker et al. 1987) but has never been observed before in the genus *Neotyphodium*.

4.1 Likely mechanisms leading to the observed morphological switching in culture

One potent inducer of the high-frequency change between phenotypes of the fungus in culture was age (Results 3.4.5). Aging-induced changes in the appearance of colonies are common in many microorganisms but in most cases are readily reversible. When biomass from an old colony is transferred to fresh medium it forms a new colony identical in appearance to the original colony when it was young. This was not observed in this study whereby mycelium from older mucoid colonies also formed mucoid colonies when transferred onto fresh medium. Even on fresh medium, these mucoid types had reduced growth and reduced infectivity as occurs in aging cultures. Macerates of older cottony colonies gave rise to a mixture of cottony and mucoid colonies, and upon prolonged incubation all cottony colonies turned mucoid. On this basis the *N. lolii* system resembles the phenomenon of fungal senescence, a progressive loss of growth potential that cannot be overcome by transfer of mycelium to fresh medium (Griffiths 1992; Maheshwari and Navaraj 2008). The phenomenon of fungal senescence has been most thoroughly examined in the fungus *Podospora anserina* although much work has been done on *Aspergillus amstelodami* along with two strains of *Neurospora*, Kalilo and Maranhar. The common factor in the senescence of all of these fungi is a change in the mitochondrial DNA (mtDNA). In *P. anserina* mtDNA instability leads to the degeneration of mitochondrial function. With *A. amstelodami* amplification of a specific region of the mtDNA is observed but unlike *P. anserina* intact mtDNA is present at easily detectable levels. *Neurospora* grows much faster than *Podospora* yet has a similar manifestation of senescence (Griffiths 1992). Circular DNA species termed α -senDNA have been shown to accumulate in mitochondria of senescent cultures. There is essentially no α -senDNA in juvenile hyphae but during ageing the α -sen-intron becomes systematically liberated and amplified and large parts of the mtDNA are deleted, the vast majority of mtDNA molecules in senescent cultures are extensively rearranged (Osiewacz 2002).

The behaviour of *N. lolii* also resembles senescence in that the mucoid phenotype was not induced in liquid culture (Results 3.4.8). In other fungal systems senescence is triggered by sub-culturing on solid but not on liquid media (Turker and Cummings 1987). Importantly, just as the *N. lolii* mucoid phenotype can be reversed, so can senescence. In *P. anserina* cultures, following a senescence crisis (growth stoppage) growth can resume in part of the mycelium. The new growth contains a novel family of

plasmids with very short monomeric unit sequences referred to as small mitochondrial DNAs (sMt-DNA) (Scheckhuber and Osiewacz 2008). These mtDNAs are derived from a highly ordered 368-base pair region of the mitochondrial genome (Turker et al. 1987). Thus several features of the *N. lolii* system suggest senescence as a potential mechanism underlying the change between the two phenotypes. It may be that deterioration of the mitochondrial DNA is taking place in *Neotyphodium* cultures on solid media resulting in the mucoid phenotype and further that both liquid culture and *in planta* growth select for WT mtDNA and against α -senDNA. For this model to work a wild-type infected plant would have to maintain its WT mtDNA status, while a dwarfed plant would represent one infected with a mixed WT mt DNA/ α -senDNA fungal population either due to a rare event *in planta* or by being artificially infected with a mixed WT mt DNA/ α -senDNA population generated in culture.

Another mechanism to consider either separately or in conjunction with senescence phenomena is quorum-sensing. A quorum-sensing mechanism determines that once a certain colony density is reached an accumulation of a fungal molecule directs a change in the state of the mycelium. Extracellular autoinducing compounds produced by micro organisms that operate in a cell density dependant manner have been described in both bacteria and fungi, in both systems small diffusible molecules accumulate and once these reach a sufficiently high concentration a response regulator is activated in the population leading to coordinated gene expression (Hogan 2006). Examples of this include the regulation of a morphological transition in the ascomycetous fungus, *Candida albicans*. This fungus can excrete an autoregulatory substance, farnesoic acid, into culture media that regulates the yeast to hypha transition favouring a yeast morphology (Oh et al. 2001). The occurrence of a mucoid phenotype in *Neotyphodium* induced by both age and density is consistent with this mechanism. Dimorphism or the ability to switch between two morphological forms is not uncommon in fungi, it is typified by the transition between single celled yeast and multi-celled filamentous forms (Madhani and Fink 1998). This mechanism does not explain the differences observed in this study, however it could provide clues as to possible signalling mechanisms that can bring about mass conversion in response to environmental stimuli. Pathways governing dimorphism in both pathogenic and non-pathogenic fungi have been shown to include both cAMP-dependant protein kinase A (PKA) and the mitogen-activated protein kinase (MAPK) signalling cascades (Nadal et al 2008).

Another known mechanism of rapid and reversible phenotypic change is phenotypic switching, the spontaneous emergence of colonies with altered colony morphology at rates higher than somatic mutation rates (Soll 1992) as described in the introduction (Introduction 1.4.5). The phenomenon observed in *Neotyphodium* involves a mass conversion which although not completely inconsistent with a phenotypic switch scenario would require invoking a selection pressure mechanism to explain the homogeneity of the changed culture. As previously covered (Introduction 1.4.5) however yeasts that display phenotypic switching can also undergo a mass conversion of colony morphology (Ramírez-Zavala et al. 2008).

In summary either a mass conversion as described by Ramirez et al. or senescence due to changes in the mitochondrial DNA seem the most likely mechanisms underlying the change in fungal phenotype. To verify the mechanism mucoid colonies could be examined for the presence of α -senDNA and in the case of topical white and cottony colonies sMt-DNA to assess what correlation, if any, exists between the presence and/or ratios of these DNA species and the respective *Neotyphodium* phenotypes. To examine whether an *en masse* change analogous to that observed in *Candida* is occurring a search could be made for homologues of genes involved in this phenomenon.

4.2 The dwarf plant phenotype

The dwarf phenotype is marked by short, fine-leaved tillers and dark green pigmentation (Results 3.1.1). Although *Neotyphodium* endophytes have impacts on their grass hosts ranging from effects on seed germination (Gundel et al. 2006a; Gundel et al. 2006b), total biomass (Cheplick et al. 1988), herbage dry weight (Lewis 2004), root dry weight and root shoot ratio (Hesse et al. 2005; Hesse et al. 2003), leaf area (Hill et al. 1990), tiller production (Latch et al. 1985), regrowth (Arachevaleta et al. 1989) and nutrient content (Zabalgogazcoa et al. 2006), none of these studies report gross across the board effects on host phenotype as seen in this study. However, some studies have shown that, when the symbiosis is upset by infecting grass hosts with endophytes isolated from other species (Christensen 1995) or with endophytes that have been genetically modified (Takemoto et al. 2006; Tanaka et al. 2006; Tanaka et al. 2008; Zhang et al. 2006), the host phenotype can be impacted. This study describes an endophyte in its natural host that has not been genetically modified.

An obvious question is how the mucoid P1 and the mucoid derivatives of P41 cause their most striking effect, namely how they alter the size of the infected plants. Plants infected with mucoid derivatives have been shown to be small and fine-leaved compared to un-infected individuals, cottony P41-infected individuals and individuals infected with other *N. lolii* wild-types (Results 3.1.1). The fungus that colonises dwarfed plants differs from the fungus that colonises normal phenotype plants (Results 3.1.2) and endophyte-free plants infected with the mucoid fungus isolated from dwarf plants themselves become dwarfed (Results 3.1.4). This sequence of events i.e. identifying a condition (dwarfism), isolating an organism suspected of being the casual agent (the mucoid *N. lolii* fungus), infecting a host free of that organism and then observing in the new host the condition observed in the original host, links the condition with the organism. In addition to this the return to normal phenotype of a dwarfed plant by elimination of the fungus with a fungicide (Results 3.1.3) confirms the link between the mucoid fungus and the dwarfism of its host.

As described in the introduction cell number, not cell size is primarily responsible for the variation in the size of plant organs observed between plant species. I confirmed that this was the case, in the one cell type investigated (epidermal cells of the leaf sheath), in P1- infected plants. Their length in normal phenotype (cottony P41-infected) plants and dwarfed (P1-infected) plants was similar (Results 3.2.6). Additionally I applied gibberellins exogenously (Results 3.2.5) to see if this plant growth regulator would restore the dwarf plants to a normal phenotype. Gibberellin was applied at one concentration only; this treatment resulted in leaf expansion. Both dwarfed and normal phenotype plants increased in size but the gibberellin treated dwarf plants were not restored to normal phenotype (Section 3.2.5). This study did not explore the outcome of increasing the concentration of applied gibberellin, but given our dwarf phenotype is due to less cells rather than smaller cells (Section 3.2.6) full complementation would seem unlikely. Gibberellins are associated with an increase in plant cell size (Cooper 1957) so this result is consistent with the dwarf plant cells being responsive to gibberellin while there being less of them.

Brassinosteoroids however have been reported to be involved in cell proliferation (Introduction 1.5) and taken together with the model for plant organ growth (Introduction Fig. 1.2), results of cell measurement and gibberellin application, a perturbation in this pathway may be responsible for the observed dwarf phenotype (Haubrick and Assmann 2006; Hong et al. 2003; Mori et al. 2002; Müssig 2005; Zhou

et al. 2004). However, there was no opportunity to examine the effect of exogenous application of brassinosteroids in this study but it constitutes a worthwhile experiment for future work.

Looking beyond plant growth regulators or phytohormones, a range of genes have been shown to be involved in plants where cell numbers decrease; a Cdc2 kinase (Hemerly et al. 1995), a cytoplasmic ribosomal protein (Ito et al. 2000), a transcription factor of the AP2-domain family AINTEGUMENTA (Mizukami and Fischer 2000), plant cyclin-dependant kinase inhibitors ICK1 (Wang et al. 2000), KRP2 (De Veylder et al. 2001), a heterotrimeric G-protein GPA1 (Ullah et al. 2001), a phytochrome P450 and gibberellin genes (Tsukaya et al. 2002). There is evidence for a compensation system in leaf morphogenesis whereby a decrease in cell proliferation results in an increase of cell volume (Tsukaya et al. 2002). There are no reports of an increase in cell number resulting in a decrease in cell volume (Tsukaya 2003). Despite the number of studies examining how genetic factors affect cell proliferation, the mechanism by which intrinsic organ size is genetically controlled, or the nature of the developmental regulators involved in plant organ size control, is not well understood (Mizukami and Fischer 2000).

In an attempt to characterise the differences between dwarfed and normal phenotype plants a proteomics approach was undertaken (Methods 2.10). Two dimensional gel electrophoresis (2-DE) provides a system for high level resolution separation of complex protein mixtures. The classical 2-DE protocol separates denatured protein according to two independent properties; isoelectric point (pI) and molecular weight (Carpentier et al. 2008). Proteomic analysis can be used to examine plant/fungus interactions despite the complexity of the biological material involved when two interacting genomes are examined, 2-DE provides a powerful tool to gain a global picture of plant-microbe interactions (Cánovas et al. 2004). In this study total protein was extracted from clonal plant material infected with both the cottony P41 fungus and the mucoid P1 for 2-DE analysis. The use of clonal plants (Methods 2.9) and controlled environmental conditions (Methods 2.9.4) ensured standardised protein expression in the symbiota. The protein spot profiles of P41 and P1 endophyte-infected clones were overall similar but a small number of differences were detected (results 3.3.5). One distinctly differentially expressed protein was identified as up-regulated in the P1-infected symbiosis. Characterisation of this protein spot by LC-MS/MS (Results 3.3.6) identified two overlapping peptides that were most similar to a putative leucine-rich

repeat receptor-like kinase (LRR-RLK) previously identified in rice (Song et al. 1995). Receptor-like kinases (RLKs) are a large family of proteins involved in signalling. They are composed of an extracellular domain connected via a transmembrane domain to a cytoplasmic kinase. The architecture of RLKs indicates that they perceive external signals and transduce them into the cell. More than 600 genes encode RLKs in *Arabidopsis thaliana* and over 1100 in rice (Shiu et al. 2004). They have been associated with functions as diverse as ice recrystallisation and plant defence and importantly the regulation of plant development (Afzal et al. 2008; Dievart and Clark 2004; Middleton et al. 2009). For example, CLAVATA 1, has been shown to impact meristem differentiation in Arabidopsis (Clark et al. 1997) and mutation of the RLK ERECTA has been shown to result in dwarfed plants (Shpak et al. 2004). Likewise mutations in the gene BRI1 that codes for a LRR-RLK exhibit a dwarfed phenotype and are unable to respond to exogenously applied brassinosteroid (Li and Chory 1997; Nam and Li 2002). Interestingly the dwarfed plants in this study show an apparent increase in an RLK like protein which is at odds with a possible explanation of dwarfism affected via interference in production of RLKs such as ERECTA or BRI1. Over-expression of an RLK may not necessarily be associated with a direct impact of the fungus on the host plant morphology. More likely explanations for the increased expression of a RLK in the dwarfed plants in this study is a response to biotic stress. In Arabidopsis seven out of eight biotic stress conditions, including the elicitor flagellin and the oomycete pathogen *Phytophthora infestans*, were shown to be over-represented in the total number of genes up-regulated in a global expression study (Shiu 2009 Plant Physiology Preview). Given that RLKs are involved in stress response and specifically response to pathogens, and that *N. lolii* usually only elicits at best a very weak pathogen response (Zhang et al 2007) it may be that the *N. lolii* mucoid mycelium in this study has undergone an alteration that results in host recognition and up-regulation of an LRR-RLK associated with pathogen response.

Whilst a pathogen response seems the most likely mechanism for up-regulation of a RLK like protein in dwarfed plants an alternative explanation could be the over-expression of a leucine-rich repeat phytosulfokine receptor kinase (LRR-PSR) gene producing large quantities of ice re-crystallisation inhibition (IRI)-like protein. A class of IRI-like proteins that are specific to the Pooideae subfamily lineage have been characterised as conferring frost tolerance (Sandve et al. 2008) and during the course of my study, it was observed that the growth of dwarfed plants compared to normal

phenotype plants was superior under outdoor frost conditions over winter (Results 3.2.3). To determine which scenario, if any, is most likely further characterization of the over-expressed protein and its function is warranted.

4.3 Implications for the symbiosis

In natural associations, *Neotyphodium* endophytes are perpetuated and dispersed via colonisation of the host seed (Philipson and Christey 1986). It is possible for the host plant to lose fungus from vegetative tissues through a failure of the fungus to infect daughter tillers at the axillary bud stage and from successive generations of plants from a failure to infect seed. Transmission of the fungus to successive iterations of vegetative tillers and to host seed is crucial for the survival of the fungus but not necessarily for the grass host. Where there is a fitness advantage to hosting an endophyte any tillers or seed that are not infected will not be competitive and so will be eliminated in the long term. Growth chamber studies have shown a link between endophyte infection and increased tillering (Latch et al. 1985) where a 32 % increase in tillering was reported. In this study, comparisons between clonal material infected with P1 and endophyte-free showed the infected clones grew on average 83% more tillers under controlled conditions (Results 3.2.1). The differences in the increased tillering observed in these studies is likely to reflect genotype differences in both the host grass and the endophyte. Interestingly, the rate of tillering was similar between endophyte-infected and endophyte-free clones for the first 28 days at which point the tillering rate started to diverge (Results 3.2.1). The rate of tillering of cottony P41 and P1-infected plants was similar. Tiller numbers for clones infected with each of the strains increased from 1 at day 0 through to an average of 9.1 at 18 days (Results 3.2.2).

The P1-infected clones produced a similar number of vegetative tillers compared to cottony P41-infected clones but unlike the P41- infected clones the P1-infected clones did not produce any floral tillers (Results 3.2.3). Diploid perennial ryegrass exhibits self incompatibility controlled by two genetic loci designated S and Z (Fearon et al. 1994). They require exposure to cold to induce flowering (vernalisation) and secondary induction in the form of subsequent exposure to long days (Aamlid et al. 2000). The minimum requirement for flower induction is a vernalisation period of 10-12 weeks at temperatures below 5°C followed by a secondary induction long day photoperiod (16hrs light) and temperatures greater than 20°C. The requirement for primary induction varies

greatly between perennial ryegrass genotypes (Milan Gagic PhD thesis 2007). Endophyte-free, cottony P41 and P1-infected plants were kept in an unheated greenhouse over winter, exposing them to vernalising conditions, the following summer the P41- infected and endophyte-free plants flowered while the P1-infected plants did not. To test whether the failure to flower was due to the intensity and duration of the vernalising conditions plants were wintered the following season outdoors to allow full and prolonged vernalisation. Under these optimal vernalising conditions the P1-infected plants still did not flower while the cottony P41-infected plants did (Fig.3.8). To examine this further clones infected with P1 and cottony P41 endophyte were artificially vernalised before exposure to long days to induce floral development. Dissections were made to examine the development of the floral meristems. Differences were observable in the development of ridges on the floral meristems at day 0 of exposure to long day induction, by day 14 ridges were evident on the P1-infected grass meristems but they had a retarded development when compared to the cottony P41-infected grass meristems (Results 3.2.4). This result shows that in P1-infected plants although floral structures do not emerge, become pollinated and produce seed, inflorescence meristems are initiated but are retarded in their development.

Chapter 5 Conclusion

There is no doubt that endophytic fungi of the genus *Neotyphodium* are constantly undergoing change. This change may be at the genetic level or it may involve an alteration of the phenotype of the fungus without mutation. Changes can affect cells at the organism level where they become susceptible to evolutionary (micro-evolutionary) selection. Deleterious changes are eliminated from the population where they affect the ability of the organism to reproduce or to compete ecologically. Where an organism such as the *Neotyphodium* fungus is intimately involved with a host symbiont any change in the fungus may affect not only the fitness of the fungus in its environmental niche within the host plant but also the fitness of the host itself. In this way any change in either the fungus or the host plant may affect the symbiosis as a whole.

Neotyphodium endophytes are utilised in pastoral industries where they are essentially invisible at an organismal level. It is not possible to distinguish infected perennial ryegrass plants from uninfected in a pasture simply by looking at the morphology of the plant. Here we see a spontaneous change in a *Neotyphodium* fungus leading to a gross change in host plant morphology. The fundamental change is in the fungus alone.

Wild-type *Neotyphodium* endophytes growing *in planta* normally produce a range of secondary metabolites, specifically alkaloids. The levels produced for any given association vary but generally fall within a predictable range. The mucoid P1 fungus we examine here produces markedly reduced levels of two such alkaloids, peramine and ergovaline and none has been observed of a third, lolitrem B.

The gross morphological effect of the mucoid P1 and mucoid P41 fungi on host plants is a reduction in the size of both above and below ground plant vegetative tissues. In addition to this infected plants have lost the ability to produce floral tillers and therefore do not produce seed. It is this more than anything else that puts this variation in the fungus into the realms of a deleterious change. Because *Neotyphodium* endophytes have no sexual cycle and cannot transmit horizontally, they rely on the exploitation of the host sexual cycle for continuance and dispersal. A change in the fungus such as this where the result is effectively a sterility of the symbiotum results in an ecological dead-end and would have merely an ephemeral presence within the population. The persistence of this symbiotum can only be maintained artificially in the laboratory environment in the form of ongoing clonal replication.

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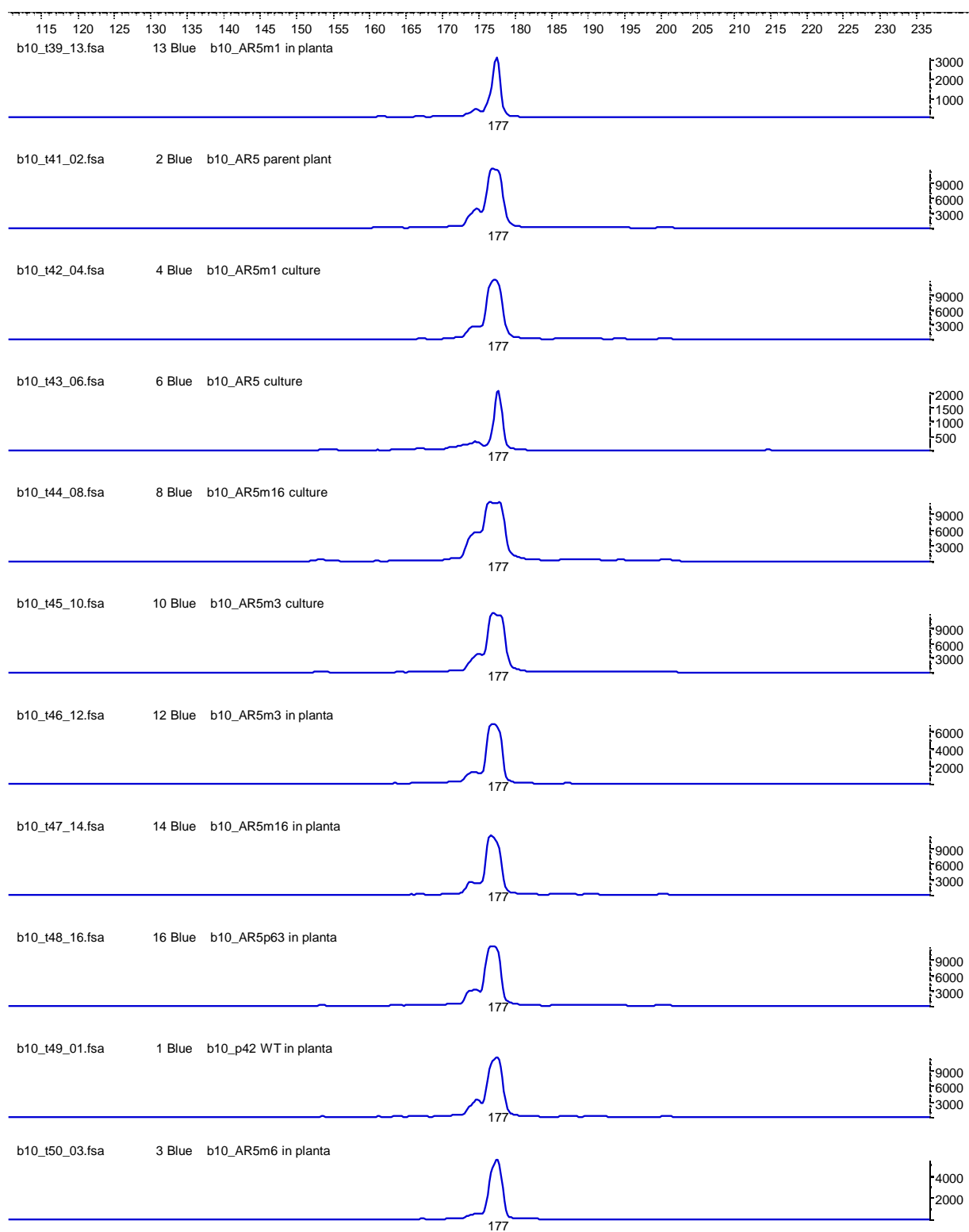
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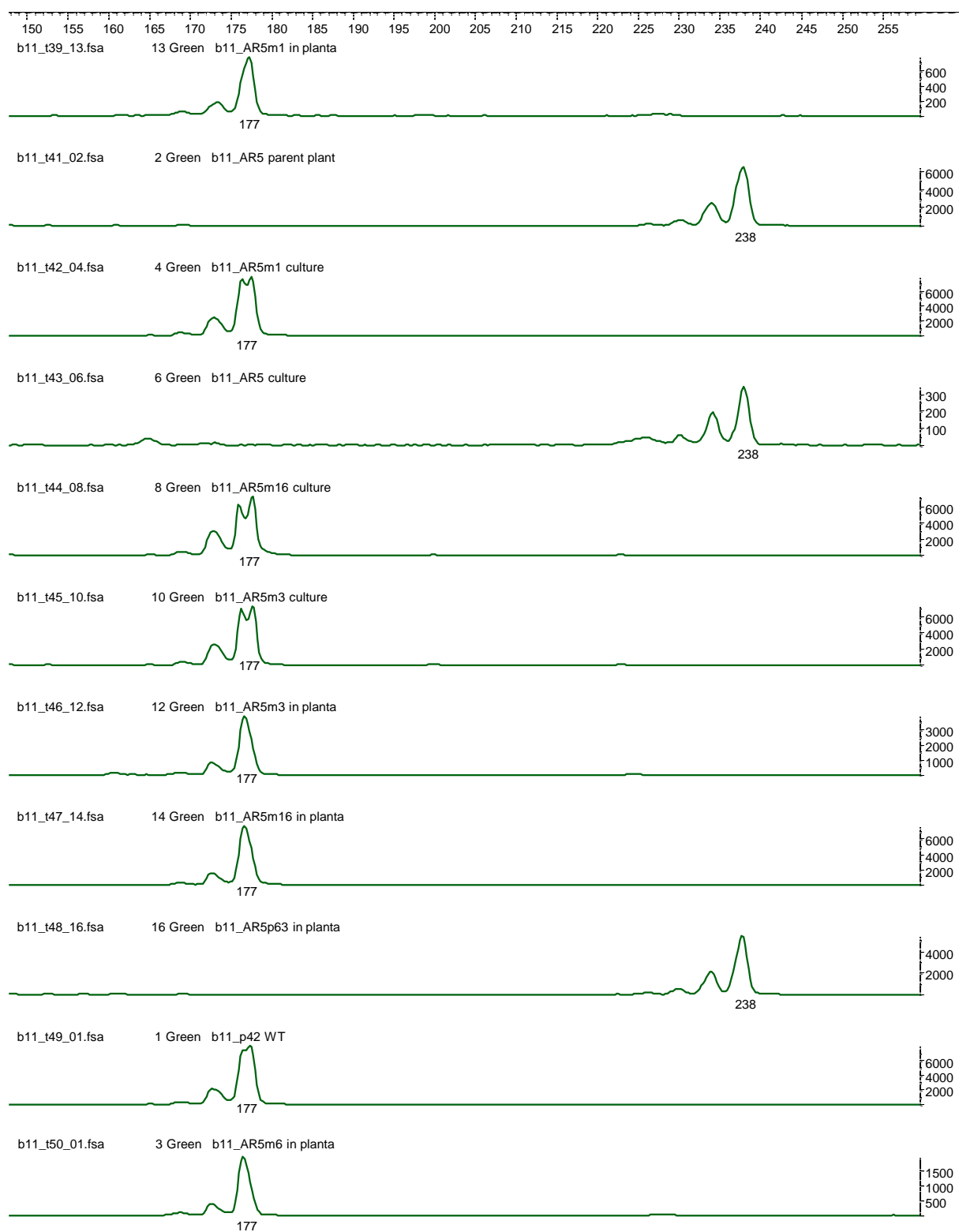
Appendix I

SSR traces – B10 allele



Appendix I

SSR trace – B11



Appendix II – Mass spectrometry data

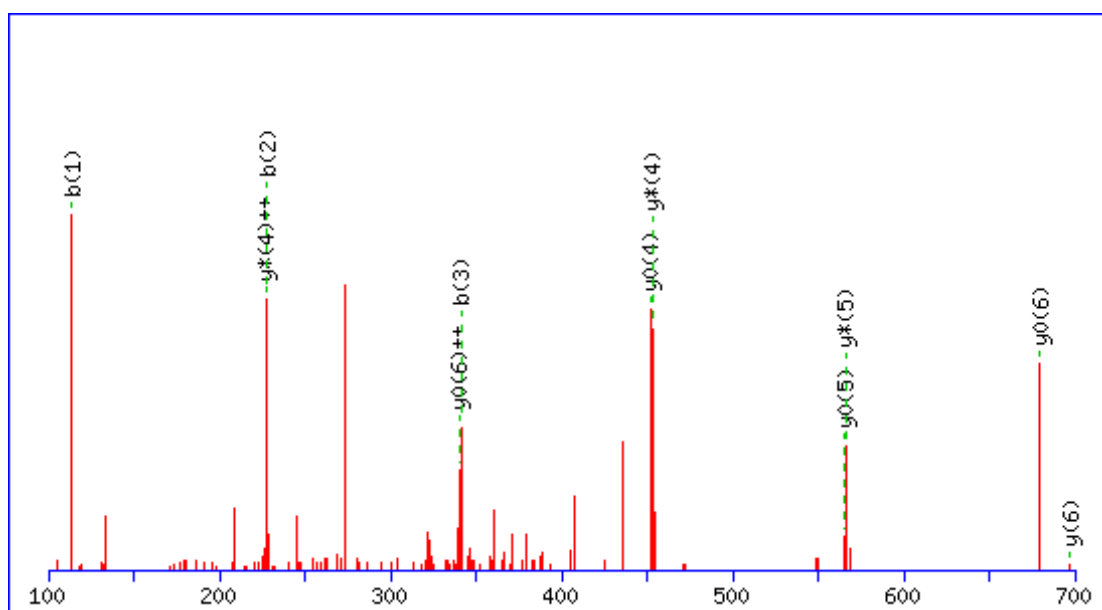
MS/MS Fragmentation of **LNLSHNL**

Found in **gi|51535346**, putative Phytosulfokine receptor precursor [*Oryza sativa* Japonica Group]

Match to Query 5: 809.620921 from(405.817737,2+)

File: SC29881.wiff, Sample: Simpson_P1 (sample number 1), Elution: 39.704 to 39.754 min, Period: 1, Cycle(s): 2618-2620 (Experiment 2)

From data file C:\Temp\SC29881.wiff



Monoisotopic mass of neutral peptide Mr(calc): 809.439529

Fixed modifications: Carbamidomethyl (C)

Ions Score: 46 **Expect:** 1.6

Matches (Bold Red): 14/56 fragment ions using 15 most intense peaks

#	b	b ⁺⁺	b*	b ^{*++}	b ⁰	b ⁰⁺⁺	Seq.	y	y ⁺⁺	y*	y ^{*++}	y ⁰	y ⁰⁺⁺	#
1	114.0913 40	57.54930 8					L							7
2	228.1342 67	114.5707 71	211.1077 18	106.0574 97			N	697.3627 63	349.1850 20	680.3362 14	340.6717 45	679.3521 98	340.1797 37	6
3	341.2183 31	171.1128 03	324.1917 82	162.5995 29			L	583.3198 36	292.1635 56	566.2932 87	283.6502 82	565.3092 71	283.1582 74	5
4	428.2503 59	214.6288 17	411.2238 10	206.1155 43	410.2397 94	205.6235 35	S	470.2357 72	235.6215 24	453.2092 23	227.1082 49	452.2252 07	226.6162 41	4
5	565.3092 71	283.1582 74	548.2827 22	274.6449 99	547.2987 06	274.1529 91	H	383.2037 44	192.1055 10	366.1771 95	183.5922 35			3
6	679.3521 98	340.1797 37	662.3256 49	331.6664 62	661.3416 33	331.1744 54	N	246.1448 32	123.5760 54	229.1182 83	115.0627 79			2
7							L	132.1019 05	66.55459 0					1

Appendix II – Mass spectrometry data

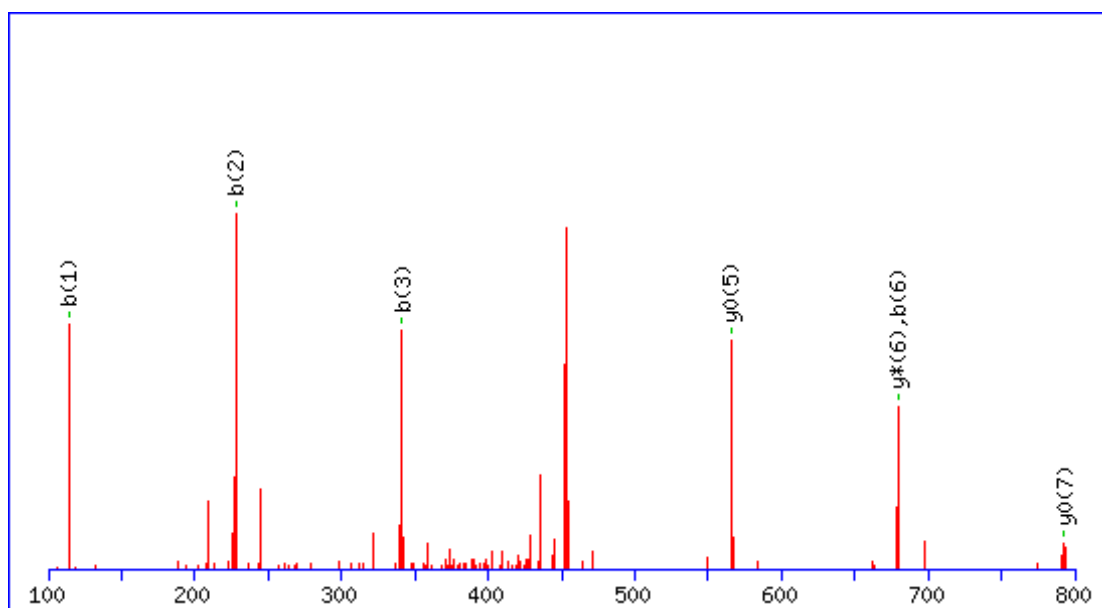
MS/MS Fragmentation of **LNLSHNLL**

Found in **gi|51535346**, putative Phytosulfokine receptor precursor [*Oryza sativa* Japonica Group]

Match to Query 73: 922.696023 from(462.355288,2+)

File: SC29881.wiff, Sample: Simpson_P1 (sample number 1), Elution: 41.529 to 41.579 min, Period: 1, Cycle(s): 2664-2666 (Experiment 2)

From data file C:\Temp\SC29881.wiff



Monoisotopic mass of neutral peptide Mr(calc): 922.523590

Fixed modifications: Carbamidomethyl (C)

Ions Score: 60 Expect: 0.051

Matches (Bold Red): 9/64 fragment ions using 9 most intense peaks

#	b	b ⁺⁺	b*	b* ⁺⁺	b ⁰	b ⁰⁺⁺	Seq.	y	y ⁺⁺	y*	y* ⁺⁺	y ⁰	y ⁰⁺⁺	#
1	114.0913 40	57.54930 8					L							8
2	228.1342 67	114.5707 71	211.1077 18	106.0574 97			N	810.4468 27	405.7270 52	793.4202 78	397.2137 77	792.4362 62	396.7217 69	7
3	341.2183 31	171.1128 03	324.1917 82	162.5995 29			L	696.4039 00	348.7055 88	679.3773 51	340.1923 14	678.3933 35	339.7003 06	6
4	428.2503 59	214.6288 17	411.2238 10	206.1155 43	410.2397 94	205.6235 35	S	583.3198 36	292.1635 56	566.2932 87	283.6502 82	565.3092 71	283.1582 74	5
5	565.3092 71	283.1582 74	548.2827 22	274.6449 99	547.2987 06	274.1529 91	H	496.2878 08	248.6475 42	479.2612 59	240.1342 67			4
6	679.3521 98	340.1797 37	662.3256 49	331.6664 62	661.3416 33	331.1744 54	N	359.2288 96	180.1180 86	342.2023 47	171.6048 11			3
7	792.4362 62	396.7217 69	775.4097 13	388.2084 94	774.4256 97	387.7164 86	L	245.1859 69	123.0966 23					2
8							L	132.1019 05	66.55459 0					1