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**Crossing the species barrier: Investigating vertical transmission of a
fungal endophyte from tall fescue within a novel ryegrass
association**

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
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Priscila Freitas

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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by

Priscila Freitas

Many Poaceae grasses form a symbiosis with *Epichloë* fungal endophytes. Initially a serious problem in agriculture due to their production of alkaloids toxic to livestock, endophyte strains were identified that were less toxic to livestock whilst also possessing advantageous traits, including insect deterrent alkaloids. Selection and transfer of these endophytes into elite grass cultivars has resulted in pastures with improved persistence with no, or reduced, livestock toxicosis. These novel associations are now successfully marketed in New Zealand (NZ), Australia, USA and South America. *Epichloë* species exhibit strong host specificity and the asexual form is exclusively vertically transmitted through maternal lines via the seed embryo. In New Zealand, the dominant commercially available agricultural associations are between perennial ryegrass and *E. festucae* var. *lolii* strains that rely on the production of peramine, epoxy-janthitrems and ergovaline for their insect deterrent properties. However, AgResearch Ltd. and PGG Wrightson Seeds Ltd. have developed a novel association between a tall-fescue-derived endophyte (designated strain AR501) and a perennial ryegrass host. This brings a new set of secondary metabolites to the New Zealand agriculture scenario based on perennial ryegrass pastures, namely the loline alkaloids. An association formed between this fungus and perennial ryegrass would provide the grass sward excellent protection against ASW and grass grub, without creating any animal health problems. However, this process is far from perfect because of endophyte transmission failures. Possible reasons for these failures include endophyte and host genotype incompatibility, environmental factors and crop management regimes. This study was an investigation of two of the factors that may affect the vertical transmission of AR501 and the maintenance of endophyte viability in stored seeds.

A controlled environment was used to determine the influence of temperature on endophyte transmission frequency, on fungal biomass and on concentrations of loline alkaloids, in tall fescue

and perennial ryegrass. Seedlings of both associations were arranged in a controlled environment at four temperature regimes ranging from 6-25°C. After three and six weeks, endophyte transmission frequencies and endophyte biomass were quantified. After three weeks plants from both associations were transferred from a 12/6°C to a 25/16°C environment and their endophyte biomass re-calculated. For perennial ryegrass, the endophyte transmission frequency was significantly higher in plants kept at a cold (day/night 12/6°C) temperature regime compared to the warm (day/night 25/16°C) regime. This was not observed in tall fescue. The endophyte biomass concentrations of both associations increased when after three weeks plants were transferred from the cold to warm temperature regime. To better understand how the endophyte and its host behaved under different temperature regimes, the cardinal temperature for AR501 and the host was determined.

The cardinal temperatures for AR501 were higher in perennial ryegrass (T_b of -1.82°C, T_{opt} of 25.26°C and T_m of 54.94°C) than in tall fescue (T_b of -0.04°C, T_{opt} of 15.90°C and T_m of 29.04°C). Cardinal temperatures were also higher for perennial ryegrass than for tall fescue. However the induction of thermodormancy in tall fescue seeds interfered with the calculation of cardinal temperatures for the endophyte and tall fescue host, and it is probable that the optimal temperature for AR501 in the tall fescue host may be higher than determined in this study.

Storage factors such as temperature, seed moisture content and length of storage can affect the viability of both seed and endophyte. Seeds of both associations were stored for 12 months under four storage temperature regimes (5°C, 10°C, 20°C and 30°C) and at two seed moisture contents (10% and 14%) to investigate whether temperature and/or seed moisture content would affect endophyte viability in stored seeds. In both associations, endophyte in seeds stored at 30°C and 14% SMC had died after 12 months of storage, but in seeds stored in moisture proof packaging at lower temperature (5 or 10°C), endophyte viability was maintained throughout the 12 months. In addition, the thermal time model was used to predict time for AR501 viability in the stored seeds. The prediction was that AR501 viability would remain above 70% for 13 months longer in tall fescue seeds than in perennial ryegrass seeds if they were stored at low temperature ($\leq 10^\circ\text{C}$) and at 10% SMC.

The effect of seed crop management factors, such as nitrogen (N) fertiliser, fungicide and plant growth regulator application on AR501 infection frequency in perennial ryegrass and Italian ryegrass was also investigated. None of the three seed production management factors affected the transmission of AR501 from plant to seed in perennial ryegrass, but did so in Italian ryegrass. This requires further investigation.

Keywords: Cool-season grasses, *Epichloë*, AR501, endophyte, transmission, temperature, lolines, peramine, seed storage, infection frequency, mycelial concentration, endophyte viability, cardinal temperature, compatibility, host-specificity, nitrogen, fungicide, plant growth regulator, ELISA, TPIB.

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Preface

This thesis consists of six chapters.

Chapter 1 is a general introduction relevant to the topic of the research which concludes with the research aims.

Chapter 2 is a study of the influence of temperature on the concentration of AR501 endophyte mycelia, endophyte infection frequency and concentration of insect deterrent alkaloids.

Chapter 3 determines cardinal temperatures for AR501 endophyte within perennial ryegrass and tall fescue, as well as cardinal temperatures for both perennial ryegrass and tall fescue.

Chapter 4 provides an understanding of the effects of temperature, seed moisture content and length of storage on the longevity of AR501 endophyte within perennial ryegrass and tall fescue seeds. In addition, the thermal time requirement for AR501 endophyte viability in stored seed of both grasses is calculated as a predictor of endophyte survival.

Chapter 5 is an investigation of the effect of seed production management treatments (such as nitrogen, fungicide and/or plant growth regulator application) on the transmission frequency of AR501 endophyte in perennial and Italian ryegrass, as well as a study of endophyte viability from seed harvested in plants previously exposed to these management factors and stored under ambient conditions for one year.

Chapter 6 presents a general discussion and the conclusions from this study. Suggestions for future research are highlighted.

Chapter 1

Introduction

An endophyte is defined as an organism that lives inside a plant; "endo" is derived from the Greek word "endon" which means within, and "phyte" is derived from the Greek word "phyton" which means plant (Chanway, 1996). Fungi and bacteria are the most common endophytic microorganisms reported in the literature, although other organisms such as other plants and viruses can also have endophytic lifestyles (Card et al., 2016). These endophytes can form mutualistic symbiotic interactions with plants, providing benefits to the host such as protection from pests and enhanced nutrient uptake leading to growth promotion, yield increase and reduction of disease symptoms caused by plant pathogens (Card et al., 2015a; Clay & Schardl, 2002). Fungal endophytes of the genus *Epichloë* form a unique symbiotic interaction with hosts from the grass sub-family Pooideae, (Clay & Schardl, 2002; Saikkonen et al., 2015), where the fungi live internally and asymptotically within the tissues of their host (Wilson, 1995; Young et al., 2013).

1.1 Biology of grasses and associations with *Epichloë* endophytes

The grass family Poaceae (previously Gramineae) consists of warm and cool-season perennial and annual grasses, of which there are approximately 10,000 species worldwide (Kuldau & Bacon, 2008; Stebbins, 1981). Many of these species are economically important: cereals provide a major source of carbohydrates and proteins for human consumption, while grasses provide forage for livestock, particularly for sheep and cattle. Additionally the family includes fine leafed turf grasses that are utilised for recreational and conservation purposes (Kuldau & Bacon, 2008). In New Zealand (NZ), perennial ryegrass (*Lolium perenne* L.) is the dominant pasture grass species for grazing animals and covers the greatest amount of land used in the country for pastoral agriculture (Valentine & Kemp, 2007). Other important New Zealand forage grasses include annual/Italian ryegrass (*Lolium multiflorum* Lam.) and tall fescue (*Festuca arundinacea* Schreb. = *Schedonorus arundinaceus* and *Lolium arundinaceum*), although these species are grown less frequently than perennial ryegrass (Easton et al., 1994).

1.1.1 Perennial ryegrass (*Lolium perenne* L.)

Perennial ryegrass is the most important sown forage grass species in the world's temperate regions (Wilkins, 1991). In New Zealand, it was introduced by English immigrants around 1880 (Stewart, 2006) and since then, different ecotypes have been developed in different regions of the country,

according to particular environmental conditions and farming practices (Thom et al., 1998). Perennial ryegrass is a compact grass, with dark green leaves initially folded (i.e. enveloped) within the pseudostem. The species is winter-active with poor growth in hot and dry summers, but recovers fast following cooler temperatures (Kemp et al., 1999) and is therefore suited to the cool maritime climate experienced by much of New Zealand. The optimum temperatures for plant growth range for 5°C to 18°C. Perennial ryegrass plants are susceptible to insect pests such as Argentine stem weevil (*Listronotus bonariensis*), a major pest in New Zealand pastures and grass grub (*Costelytra zealandica*) and also to fungal diseases including crown rust (caused by the pathogen *Puccinia coronata*) (Kemp et al., 1999).

1.1.2 Tall fescue (*Festuca arundinacea* Schreb. = *Schedonorus arundinaceus* and *Lolium arundinaceum*)

Tall fescue is native to Europe and was introduced to New Zealand in the 19th century where the species is now widely distributed (Easton et al., 1994). Tall fescue is a deep-rooted perennial grass with coarse large dark- green leaves. The optimum temperature for plant growth is 26°C, with growth continuing into the mid-30°C. In New Zealand, tall fescue is often used as an alternative to perennial ryegrass in areas that experience hot summers with dry soils due its greater tolerance to drought, and deterrence to insect pests such as grass grub and Argentine stem weevil (Kemp et al., 1999; Milne et al., 1997).

1.2 Endophyte life cycle

Many members of the sub-family Pooideae (family Poaceae) form symbiotic associations with fungal endophytes of the genus *Epichloë* (family Clavicipitaceae) and their asexual morphs, previously known as *Neotyphodium* (Leuchtman et al., 2014). The asexual *Epichloë* species are true endophytes for all of their lifecycle. They are found only within the plant's tissues and have lost the power of contagion, being exclusively vertically transmitted through plant seeds (Majewska-Sawka & Nakashima, 2004; Philipson & Christey, 1986; Zhang et al., 2016) (Figure 1.1). In contrast some sexual *Epichloë* species can form stromata on host reproductive tillers, and suppress seed formation by causing the disease known as choke (Leuchtman & Schardl, 1998). The spores between stroma of opposite endophyte mating types are then required to be transferred by a specific fly vector of the genus *Phorbia* (Kohlmeyer & Kohlmeyer, 1974) to complete the sexual phase (Bultman & Leuchtman, 2008; Clay & Schardl, 2002; White, 1988) (Figure 1.1).

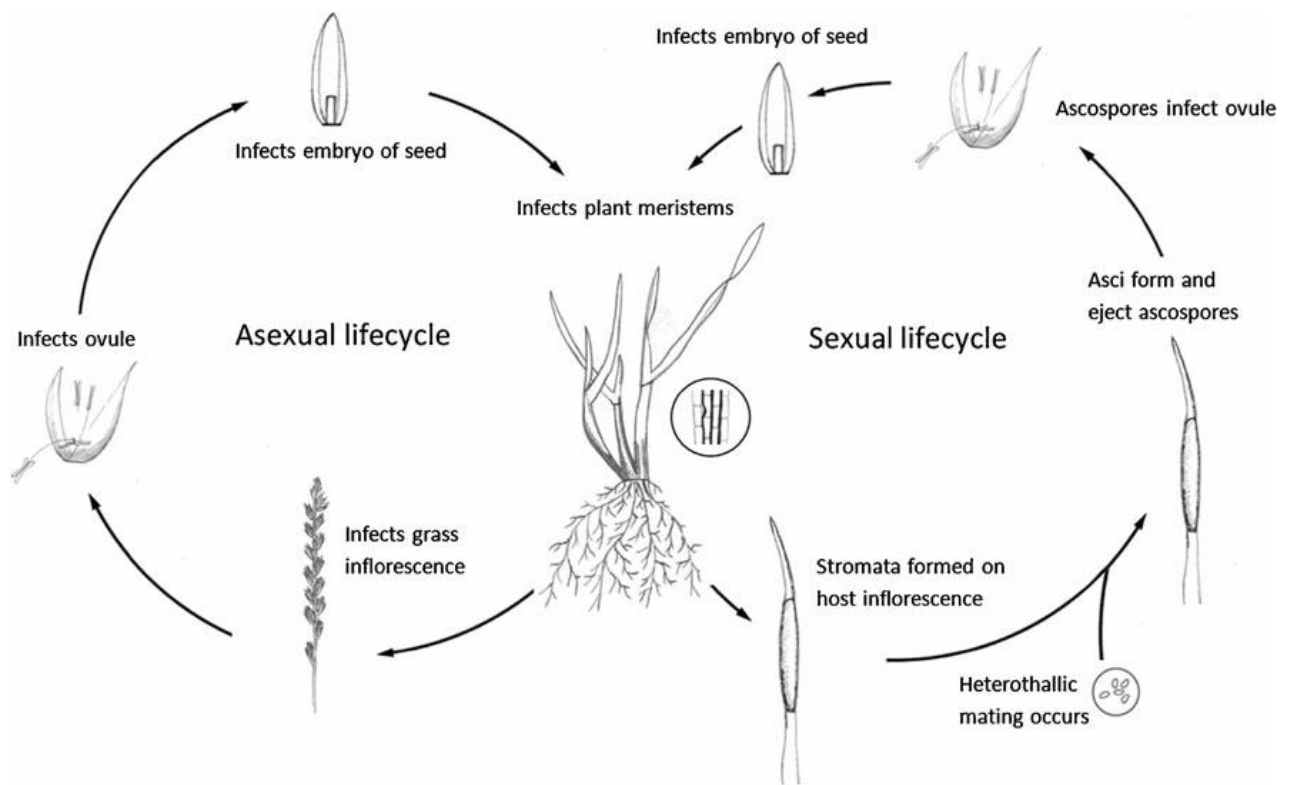


Figure 1.1 Asexual and sexual lifecycle of *Epichloë* endophytes (Johnson et al., 2013).

1.3 *Epichloë* endophyte growth in planta

Epichloë endophytes grow systemically throughout the tissues of their host, only colonising the intercellular spaces, with their hyphae parallel to the long axis of the plant cells (Figure 1.2) (Christensen et al., 2008). The endophyte colonises the floret through the base of the ovary and colonises the intercellular spaces of many maternal flower tissues including the lodicules, stamens and stigmas; however, fungal colonisation of pollen grains has not been observed (Freeman, 1904; Johnson et al., 2013). Once inside the flower tissues, the fungus colonises the nucellus tissue that surrounds the megagametophyte (Philipson & Christey, 1986; Sampson, 1933) and here, where the grass embryo sac will develop after fertilisation, the fungus grows with a high degree of hyphal branching (Christensen et al., 2008). At seed maturity, hyphae are widespread within the remnants of this nucellus tissue, lying between the seed coat and aleurone layer; an area that has been termed the 'infection layer' (Freeman, 1904). At embryo maturity, hyphae are present in the plumule apex and when the seed germinates, hyphae colonise the apical meristem, then extend into the leaf primordial and axillary buds and the vertical transmission cycle is complete (Christensen et al., 2008; Hume et al., 2013; Majewska-Sawka & Nakashima, 2004).

The growth of these endophytes *in planta* is fully synchronised with their host, with grass leaves only colonised while they are elongating (Christensen et al., 2008). In comparison to fungal pathogens, hyphae of *Epichloë* do not penetrate the host's cells or produce feeding structures such as haustoria (Freeman, 1904; Philipson & Christey, 1986; Schardl et al., 2004). The distribution and concentration of hyphae in leaves is influenced by the host grass, with leaf sheath material being more heavily colonised than leaf blades (Christensen & Voisey, 2007; Christensen et al., 2008; Clay & Schardl, 2002). Once the leaf tissue matures, hyphae stop extending and branching, but continue to be viable throughout the life of the leaf. Hyphae therefore stay metabolically active, accumulating lipids and producing certain secondary metabolites through ongoing absorption of nutrients (Christensen et al., 2001).

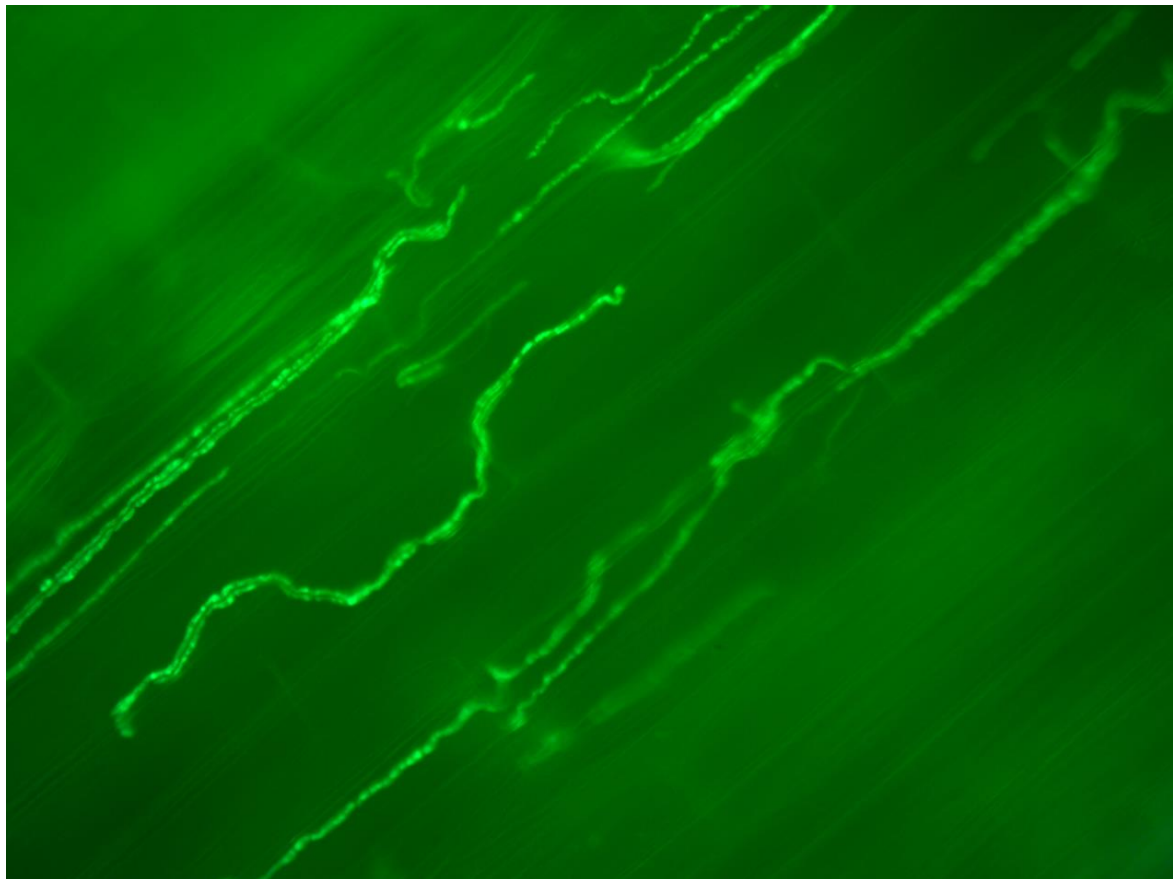


Figure 1.2 GFP- transformed endophyte hyphae colonising the intercellular spaces of a plant.

1.4 History of grass-endophyte products in New Zealand

Although asexual *Epichloë* endophytes form host-specific associations with many members of the sub-family Poöideae, the two grass species that have attracted the most attention, in terms of this

fungal association are perennial ryegrass and tall fescue (Easton, 2007). Historically this was due to the fact that some grass-endophyte associations, particularly the widespread common-toxic strain found in New Zealand pastures in the 1980s (Johnson et al., 2013), produced secondary metabolites (specifically alkaloidal compounds) that caused animal health problems and productivity losses in grazing livestock. These metabolites include lolitrem-B in perennial ryegrass which is responsible for the disease ryegrass staggers (Fletcher & Harvey, 1981; Gallagher et al., 1984) and ergot alkaloids in tall fescue, which can cause heat stress and fescue-foot syndrome (Bacon et al., 1977). When these disorders were linked to the presence of *Epichloë* endophytes in these grasses, the elimination of these fungi was the obvious solution. However, it was soon realised that *Epichloë* endophytes can also offer various benefits to their host plants, such as protection from certain biotic and abiotic stresses, improving field persistence (Latch, 1993; Malinowski & Belesky, 2000; Popay & Bonos, 2008). Further research identified other alkaloids produced by these endophyte associations in perennial ryegrass and tall fescue, namely peramine and lolines respectively that acted as insect deterrents (Popay & Hume, 2011; Rowan et al., 1986; Schardl et al., 2007). Therefore, elimination of these endophytes from these grasses resulted in reduced grass production and persistence (Fletcher, 2012). Strains of asexual *Epichloë* endophyte were imported into New Zealand, and have been now identified and characterised, some are less or non-toxic to grazing livestock whilst providing the plant with protection from insect predation (Card et al., 2014a; Prestidge et al., 1985; Young et al., 2013). These selected beneficial endophytes have been incorporated into elite cultivars of pastures grasses to improve production and persistence without harming the health and performance of the grazing animals (Fletcher, 2012).

Artificial inoculation of selected endophyte strains into elite grass cultivars has formed the basis of exploiting grass endophytes commercially (Easton, 2007). Most *Epichloë* species express a high degree of host-specificity, and an artificially created association can be stable (Karimi et al., 2012). However the most successful are usually associations transferred across grasses that are related (Leuchtman & Clay, 1993). For example, strains of *Epichloë festucae* var. *lolii* forms host specific associations with *L. perenne* and therefore strains isolated from its original host generally form more compatible associations with cultivars of *L. perenne* than any other grass species. Those few associations that are formed with hosts outside of this grass species are generally termed incompatible associations, as the plant's development is inhibited and/or distorted in some manner (Scott, 2001) or the fungal growth is inhibited to the point where endophyte-free tillers are formed and the endophyte ultimately is removed from the host over time (S. Card, personal communication 2017). Some of these incompatibility issues have been overcome with plant breeding strategies, for instance with the development of marker-assisted selection for endophyte compatibility in perennial

ryegrass (Faville et al., 2007). The use of novel grass-endophytes, defined as the association created through the inoculation of a selected endophyte strain into non-host grass germplasm, has met with commercial success and a range of grass endophyte products are now used in the pastoral industry (Table 1.1) (Johnson et al., 2013). The adoption of endophyte technology in NZ has contributed millions of dollars to the New Zealand pastoral economy since their release (Caradus et al., 2013).

1.5 *Epichloë* alkaloids and bioactivity

Alkaloidal groups associated with *Epichloë* endophytes have been intensively studied and the bioactivity, biochemistry and molecular genetics surrounding these compounds are becoming well understood (Schardl et al., 2004). The five main classes of alkaloids are janthitrems and lolitrems (indole diterpenes), ergot alkaloids, lolines (pyrrolizidines) and peramine (a pyrrolopyrazine). Epoxy-janthitrems (Figure 1.3), an indole diterpene, are produced by the commercial endophyte strain AR37 and protect against ASW, African black beetle (*Heteronychus arator*) and root aphid (*Aploneura lentisci*). However these compounds also occasionally cause staggers in sheep under some circumstances (Johnson et al., 2013; Popay & Bonos, 2008). Lolitrem-B, (Figure 1.3), is a tremorgenic mycotoxin found in endophyte infected perennial ryegrass (Gallagher et al., 1984). Peramine (Figure 1.3) is a pyrrolopyrazine alkaloid found in endophyte-infected perennial ryegrass, which deters ASW from feeding on infected plants and does not cause animal health problems (DiMenna et al., 1992; Rowan, 1993). Lolines (Figure 1.3) are pyrrolopyrazine alkaloids found most notably in endophyte associations with tall fescue and meadow fescue (Bylin, 2014). Lolines have potent insecticidal activity and feeding deterrence properties, and are not toxic for animals. These alkaloids are therefore highly desirable in pasture management (Johnson et al., 2013; Schardl et al., 2007). Ergovaline (Figure 1.3) has been associated with the poor performance of animals grazing in tall fescue infected with *E. coenophiala*, causing fescue toxicosis, the effects of which include reduced animal fertility, weight gain, heat stress and death (Bacon, 1993). As well as having these livestock toxicity properties, ergovaline has also been implicated in insect deterrence, particularly towards African black beetle (Prestidge et al., 1994).

Table 1.1 Endophyte strains and their key properties. Adapted from (Johnson et al., 2013)

Commercial or common name	Fungal species	Notable alkaloids produced	Key traits	Key regions of use
Common-toxic	<i>E. festucae</i> var. <i>lolii</i>	Lolitrems Peramine Ergovaline	Ryegrass staggers; negative impacts on animal health. Good ASW & black beetle resistance	Ryegrass pastures and turf NZ, Australia and South America
Common-toxic	<i>E. coenophiala</i>	Peramine Ergovaline Lolines	Fescue toxicosis, Broad spectrum insect resistance	Tall fescue pastures and USA turf
Common-type	<i>E. uncinatum</i>	Lolines	Broad spectrum insect resistance	Meadow fescue pastures USA, Europe
Endosafe	<i>E. festucae</i> var. <i>lolii</i>	Peramine Ergovaline	No ryegrass staggers, Good ASW resistance	Ryegrass pastures NZ
MaxQ	<i>E. coenophiala</i> strain AR542 and AR584 (MaxQII)	Lolines Peramine	No fescue toxicosis. Broad spectrum insect resistance	Tall fescue pastures USA
MaxP	<i>E. coenophiala</i> strain AR542 and AR584	Lolines Peramine	No fescue toxicosis. Broad spectrum insect resistance	Tall fescue pastures NZ and Australia
AR1	<i>E. festucae</i> var. <i>lolii</i>	Peramine	No ryegrass staggers, Good ASW resistance	Ryegrass pastures NZ, Australia and South America
Endo5	<i>E. festucae</i> var. <i>lolii</i>	Peramine Ergovaline	Good ASW and black beetle resistance. No ryegrass staggers	Ryegrass pastures Australia
NEA2	Mix of <i>E. festucae</i> var. <i>lolii</i> strains	Lolitrems Peramine Ergovaline	Good black beetle resistance	Ryegrass pastures NZ and Australia
AR37	<i>Epichloë</i> sp.	Epoxy-janthitrems	Broad spectrum insect pest resistance; Excellent animal performance but some ryegrass staggers	Ryegrass pastures NZ and Australia
Avanex	<i>E. coenophiala</i> strain AR601	Ergovaline Lolines	Bird and wildlife deterrent	Tall fescue pastures, Airports
Avanex	<i>E. lolii</i> strain AR94/95	Peramine Ergovaline Lolitrems B (only for AR95)	Bird and wildlife deterrent	Ryegrass Sport fields, recreational parks
GruboutU2	<i>E. uncinatum</i>	Lolines	Broad spectrum insect resistance	Festulolium pastures NZ

ASW=Argentine stem weevil; AR=AgResearch endophyte; NEA= New Zealand Agriseeds endophyte

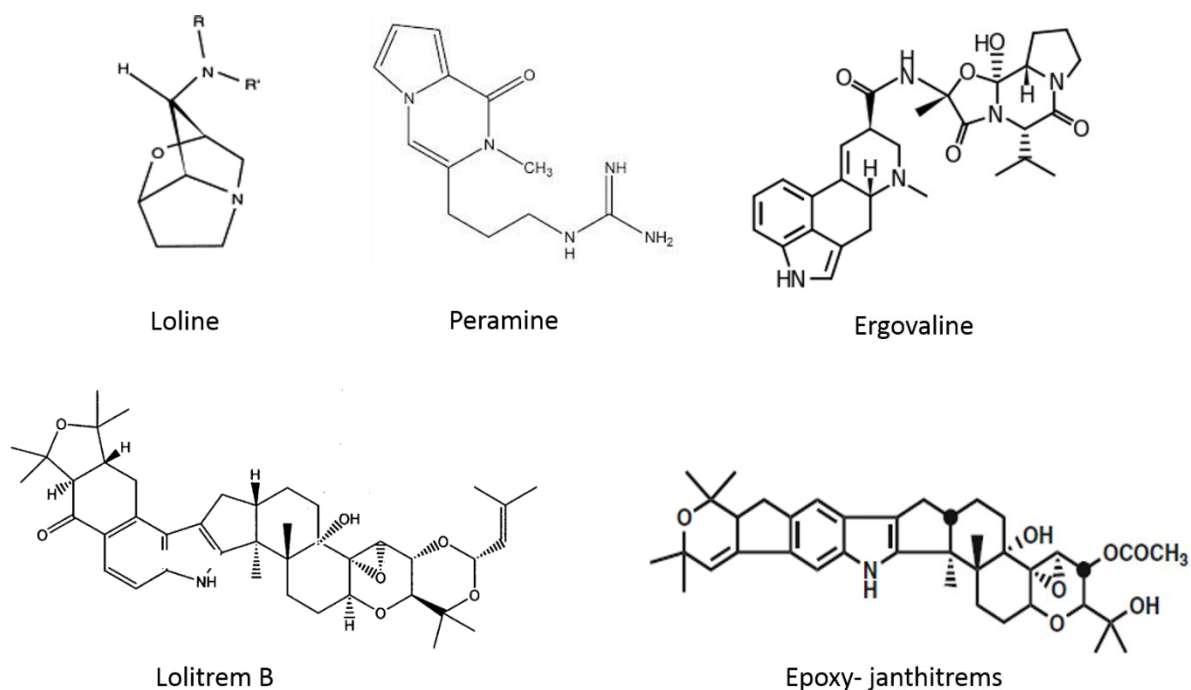


Figure 1.3 Structures of the four major alkaloids produced by epichloid fungal endophytes.

1.6 *Epichloë* FaTG-3 strain AR501

The development of the insect deterrent properties of novel perennial ryegrass-endophyte associations above that currently achieved by the current peramine producing *E. festucae* var. *lolii* strains is one aim of the Forage Improvement Group at AgResearch Ltd. (S. Card, personal communication 2013). To achieve this, a novel association was developed between a tall fescue endophyte (designated strain AR501) from species group FaTG-3 (*F. arundinacea* taxonomic group-3) which contains hybrid endophytes formed between *Epichloë baconii* × *E. typhina* (Christensen et al., 1993). This taxonomic grouping is known to characteristically produce peramine and loline alkaloids but not ergovaline. An association formed between this fungus and perennial ryegrass would provide the grass sward excellent protection against ASW and grass grub, without creating any animal health problems (Popay et al., 2003; Prestidge et al., 1994). The AR501 strain was originally isolated from a Mediterranean tall fescue plant collected in southern Spain and has since been introduced into elite perennial ryegrass cultivars in breeding programmes at AgResearch Ltd. and PGG Wrightson Seeds Ltd. (S. Card, personal communication 2013). However, two problematic issues currently face the efficacy and subsequent marketability of some artificially created

associations such as AR501 as a product; endophyte transmission to seed and endophyte storage in seed (Card et al., 2014b; Hume et al., 2013).

1.7 Problems with transmission

A high transmission efficiency is required for commercial endophyte products going to market, meaning a high proportion of seeds must be infected with the desired endophyte strain (Rolston & Agee, 2007). The current New Zealand industry target is a minimum 70% infection level; anything less than that is not acceptable (Hume & Barker, 2005). Failure in endophyte transmission has been documented for many epichloid endophyte-grass associations, both artificial and wild-type, with no specific factor identified as being responsible (Gundel et al., 2008; Ju, 2011). These transmission failures can occur in the pre-zygotic phase, where the fungus fails to successfully infect the plant during tillering and flowering, or in the post-zygotic phase when the fungus dies in the seed (Figure 1.4). Endophyte infection frequency and grass-endophyte symbiosis may also be affected by external environmental factors such as temperature (Ju et al., 2006); seed crop management, including irrigation and fertiliser, and genetic factors (i.e. host-endophyte genotype compatibility) (Gundel et al., 2011).

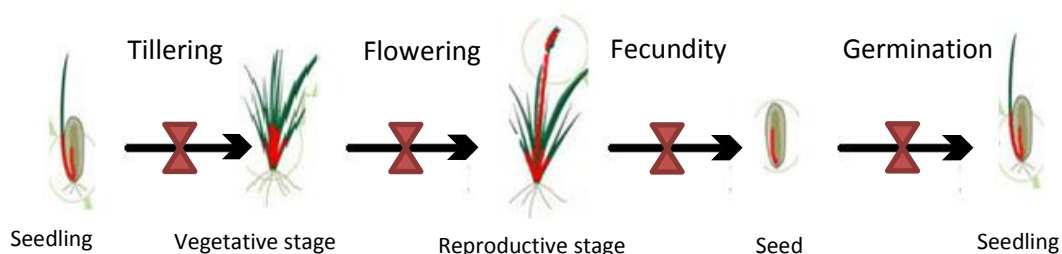


Figure 1.4 Pre-zygotic and post-zygotic phases (tillering, flowering, and seed development) in the host life cycle during which endophyte failures in vertical transmission can occur. Adapted from Gundel et al. (2011) and <http://www.grasslanz.com/>.

Delivering seed with the required level of viable endophyte to market is a challenge for the pasture seed industries. After processing, seed may be stored for periods of a few weeks to up to two years. In general, endophyte viability declines faster than that of the seed, resulting in seed which will germinate but without endophyte, reducing the value of the product. The storage factors that determine the longevity of seed and endophyte viability include temperature, relative humidity, seed moisture content and the duration of storage (Hill & Roach, 2009; Rolston & Agee, 2007;

Rolston et al., 1986; Welty et al., 1987) and these will be addressed in Chapter 4. For the benefit of the pasture grass industry it is important to investigate and understand the relationship between the host and its associated endophyte, and the factors that can affect the endophyte during vertical transmission, as well as to investigate and understand methods to maintain endophyte viability in seed.

1.8 Temperature

Temperature is an important environmental abiotic factor that has a major influence on many biological systems. Temperature influences the development and growth rate of plants, affecting plant photosynthesis, respiration and nutrient uptake (McKenzie et al., 1999). Each plant species has a specific range of temperature for its development, represented as cardinal temperatures (minimum, optimum and maximum) which will be addressed in Chapter 3. The stages of plant development are also affected by temperature; for instance, some plant species require a certain period of exposure to cold temperature to switch from vegetative (node and leaf appearance) to reproductive growth (flowers, seeds) (Hatfield & Prueger, 2015; McKenzie et al., 1999).

Temperature also has a major impact on fungal growth (Cooke & Whipps, 1993) including endophytes of the family Clavicipitaceae. Fungal species, like plants, also have specific cardinal temperatures for growth, and this will be addressed in Chapter 3. Ju et al. (2006) suggested that temperature variation had a major effect on *Epichloë coenophiala* infection frequency and concentration in the plant host. Variation in temperature can also influence the concentration of alkaloids produced by endophyte associations (Brosi et al., 2011). In addition, temperature also has an important role in seed storage as it can determine the longevity of seed and endophyte viability (Rolston et al., 1986; Welty et al., 1987). The effect of temperature on endophyte infection frequency, concentration of endophyte mycelia and concentration of insect deterrent alkaloids will be addressed in Chapter 2 and Chapter 3.

1.9 Methods of endophyte detection

Several methods are available and routinely used for identification and detection of epichloid fungal endophytes in seeds and the vegetative plant (Johnson et al., 2013; Siegel et al., 1987). Methods that involve bright-field microscopy include the seed squash and the leaf sheath peel technique (Clark et al., 1983; Latch et al., 1987), where epichloid hyphae are generally stained with a dye such as aniline blue followed by visualisation (Bacon & White Jr, 1994; Card et al., 2011; Latch & Christensen, 1985; Saha et al., 1988). The seed squash technique is commonly used to determine endophyte presence in freshly harvested seeds but cannot determine endophyte viability (Card et

al., 2011). As an alternative, immunological techniques include the tissue print-immunoblot (TPIB) technique, that uses a monoclonal antibody to detect endophyte viability in plant tissue (Gwinn et al., 1991; Simpson et al., 2012), and enzyme-linked immunosorbent assay (ELISA) (Reddick & Collins, 1988), which can determine endophyte mycelial and alkaloids concentration. The ELISA method provides high sensitivity and it is a rapid method for detection of viable endophyte, but occasionally it yields false positive results (Briggs et al., 2007; Welty et al., 1986). The identification of alkaloids in seed or plant tissue using high-performance liquid or gas chromatography (HPLC), is also an alternative tool to detect endophyte presence. Polymerase chain reaction (PCR) is routinely used for endophyte detection and quantification in fungal biomass (Groppe et al., 1995) and plant tissue (Doss & Welty, 1995), providing quick and sensitive results for presence/absence of targeted endophyte DNA. Additionally, there are several other endophyte detection methods performed regularly including direct fungal isolation onto artificial media (such as potato dextrose agar) from plant tissue (Bacon & White Jr, 1994), and the use of fluorescent tools such as green fluorescence protein (*gfp*) followed by microscopy (Card et al., 2013).

1.10 Research Aims

This research is an investigation of the factors which may influence vertical transmission and survival of *E. FaTG-3* strain AR501 in perennial ryegrass and in its original tall fescue host. Objective 1 provides a detailed understanding of the distribution of the endophyte (strain AR501) in the grass host throughout its lifecycle, and the influence of certain abiotic factors such as temperature, nitrogen (N) and various field management factors on hyphal distribution and vertical transmission frequency.

Objective 2 aims to investigate the effect of environmental conditions such as temperature and seed moisture content on endophyte longevity in stored seed.

Chapter 2

The effect of temperature on the development of a tall fescue *Epichloë* endophyte in both tall fescue and perennial ryegrass

2.1 Introduction

Many members of the grass family Poaceae form symbiotic associations with fungal endophytes of the genus *Epichloë* (family Clavicipitaceae) including the asexual morphs previously known as *Neotyphodium* (Leuchtman et al., 2014; Schardl, 1996). These asexual *Epichloë* species have lost the power of contagion, being exclusively vertically transmitted through seeds (Majewska-Sawka & Nakashima, 2004; Philipson & Christey, 1986). In New Zealand (NZ), perennial ryegrass (*Lolium perenne* L.) is the dominant pasture grass species cultivated for grazing animals and covers the greatest amount of land used in the country for pastoral agriculture (Valentine & Kemp, 2007). Tall fescue (*Festuca arundinacea* Schreb. = *Schedonorus arundinaceus* and *Lolium arundinaceum*) is generally utilised in dryland regions that experience hot dry summers, as the species is more persistent than ryegrass under these conditions (Easton et al., 1994). The novel associations artificially developed between selected *Epichloë* strains and these forage grasses can offer benefits to the grass host, such as improved field persistence through protection against insect pests (Malinowski & Belesky, 2000; Popay & Hume, 2011). One aim of the Forage Improvement Section at AgResearch Ltd is to broaden the insect deterrent properties of novel perennial ryegrass/endophyte associations above that currently achieved by the current peramine and epoxy-janthitrems producing *Epichloë festucae* var. *lolii* strains (S. Card, personal communication 2013). To achieve this, a novel association was developed between a tall fescue endophyte from species group FATG-3 (Christensen et al., 1993) and perennial ryegrass.

A high frequency of viable endophyte infection in seed is desired for commercial endophyte products going to market (Rolston & Agee, 2007), although this can be difficult to achieve. Failure in vertical endophyte transmission has been documented for many epichloid endophyte-grass associations, including novel and wild-type associations, with no single factor responsible (Gundel et al., 2008; Ju, 2011). Gundel et al. (2011) suggested that external environmental factors as well as genetic factors can contribute to incompatibility issues between these endophytic fungi and their grass hosts that subsequently influence the endophyte's transmission efficacy. Temperature has been reported as a major determinant in the development of systemic infection by *Fusarium verticillioides* (Fv) (Sacc.) Nirenb. in maize (*Zea mays* subsp. *mays* L.). This endophytic species was

located higher up in stalk internodes in plants receiving a temperature regime of 30°C during the vegetative growth stage than in plants exposed to cooler temperature regimes (Murillo-Williams & Munkvold, 2008). Ju et al. (2006) considered temperature to be one of the most important environmental factors that can affect the frequency of epichloid endophyte vertical transmission. These authors showed that cardinal minimum temperature for *Epichloë coenophiala* (formerly *Neotyphodium coenophialum*, Leuchtman et al. (2014)) and its tall fescue host differed considerably, being 10.3°C for the endophyte strain AR584 and 5.2°C for its tall fescue (cv. Jesup) host.

This study investigated the effects of temperature on the transmission of *Epichloë* FaTG-3, strain AR501 (Christensen et al., 1993) and made comparisons between the frequency observed in its original tall fescue host and that observed in perennial ryegrass, a novel association. The hypothesis was that transmission success of the same fungal endophyte would not differ between the original tall fescue and the novel perennial ryegrass host.

2.2 Material and Methods

The two grass cultivars used in this study were tall fescue line T9886, cv. Flecha (a summer dormant Mediterranean cultivar) and a tetraploid perennial ryegrass line KLp 903, derived from crosses of the cultivars Banquet, Banquet II and Bealey. Tall fescue seeds, harvested in 2012, were supplied by the Margot Forde Germplasm Centre (MFGPC), New Zealand's national gene-bank of grassland plants, and contained a viable endophyte infection frequency of 99% (S. Card et al., personal communication 2013). Perennial ryegrass seeds harvested in 2011 were supplied by PGG Wrightson Seeds Ltd., and 87% of seeds contained viable endophyte (pilot study, data not shown). Both seed lines were infected with the same strain of epichloid endophyte, namely *Epichloë* FaTG-3, strain AR501. This taxonomic grouping is known to characteristically produce peramine and loline alkaloids with no production of ergovaline (Popay et al., 2003; Prestidge et al., 1994).

Seeds were sown into 24 cell plastic trays (196 cm³ per cell) containing seedling mix (120 L Southland peat, 80 L pumice) with the following fertiliser additions per cubic meter: 4 kg Osmocote® exact mini (16% N, 3.5% P, 9.1% K), 8 kg dolomite lime and 2 kg Hydroflo® (granular wetting agent manufactured by Everris Australia Ltd.). Two seeds were sown in each cell at a depth of 1 cm. The experiment was set up in October 2013 using a randomised block design with four blocks and 14 trays in each block. Trays were placed in a heated glasshouse (approximately 20°C) and watered as required until the seedlings had emerged. Two weeks after sowing, seedlings were thinned to one seedling per cell by hand, and trays were then randomly assigned to treatment groups. Treatments

consisted of three harvest dates, 0, 3 and 6 weeks after the plants were placed into four controlled temperature regimes (Table 2.1)

Table 2.1 Temperature regime for tall fescue and perennial ryegrass plants during the six week experimental period.

Treatment groups	Temperature regime (day/night) for weeks 1-3	Temperature regime (day/night) for weeks 4-6
I	12/6°C	25/16°C
II	25/16°C	12/6°C
III	12/6°C	12/6°C
IV	25/16°C	25/16°C

The two lower temperatures were based on the usual temperature range experienced in Canterbury, New Zealand during the weeks following seed sowing. In Canterbury pasture grasses are sown in autumn (March-May) when the average air temperatures is 12°C and soil temperatures (to a depth of approximately 10 cm) are likely to range from 8-17°C (Hampton et al., 1987).

Seedlings were placed in two walk-in growth chambers (Conviron, PGV36, Canada) with a photoperiod length of 16 h light/8 h dark, one with a temperature regime set at 25/16°C: day/night (warm) and the other at 12/6°C: day/night (cool). The light intensity was 244 and 214 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the growth chambers set at 12/6°C and 25/16°C, respectively. At week zero (just before seedlings were placed into the growth chambers), two trays from each block (one tray containing tall fescue plants and one tray containing perennial ryegrass plants) were randomly selected and harvested. Seedlings were removed from cell trays and the pseudostem and leaf tissues were cut above the roots, then freeze dried using a MicroModulyo bench top freeze dryer (ThermoSavant, USA) and ground using a coffee grinder (Breville, China) before being stored at -20°C. Six samples from each tray were selected for determining endophyte mycelial concentration using an enzyme-linked immunosorbent assay (ELISA) developed by AgResearch Ltd. (Faville et al., 2015). The remaining trays containing seedlings were then transferred back into the growth chambers.

After three weeks, eight trays from each of the two temperature regimes were randomly selected and six plants per tray were harvested as described previously. The viable endophyte infection frequency of AR501 in the vegetative plants (two tillers per plant) was assessed using the tissue print-immunoblot (TPIB) technique (Simpson et al., 2012). Plants (including the tillers previously assessed by TPIB) were freeze- dried, ground and the endophyte mycelial concentration was determined by ELISA as described previously. At this stage, eight of the sixteen remaining trays from the 25/16°C temperature regime were placed into the 12/6°C temperature regime, and eight of the

sixteen remaining trays from the 12/6°C temperature regime were placed into the 25/16°C temperature regime (Table 2.1). Plants were left to grow for a further three weeks before six plants per tray were harvested and assessed for viable endophyte using the TPIB method. Plants (including tillers previously assessed by TPIB) were freeze dried, ground and endophyte mycelial concentration determined by ELISA. Viable endophyte infection frequency was not determined for plants from week 0 as these were too small for analysis. In addition, the concentration of the alkaloids peramine and loline was also determined for each treatment at the week 6 harvest. Samples were analysed for peramine using an ELISA which was developed by AgResearch Ltd. The plate coating conjugate, and the polyclonal sheep anti-peramine antibody were originally described by Garthwaite et al. (1994). The immunoassay described has since been reformatted and all other reagents, buffers and the protocol have been replaced (L. Briggs, personal communication 2014). For detection of loline alkaloids, samples were analysed using an ELISA method confidential to AgResearch Ltd. (L. Briggs, personal communication 2016).

In total 336 plants were harvested for the three harvest dates (week 0, week 3 and week 6). Statistical analysis was performed using GenStat (VSN International 2013. *GenStat for Windows 16th Edition*. VSN International, Hemel Hempstead, UK). Analysis of variance (ANOVA) and Fisher's unprotected test of least significant difference (LSD; $P < 0.05$) were performed to compare treatment effects. Loline (N-acetyllooline and N-formylloline) and peramine alkaloids concentrations in both perennial and tall fescue hosts were each regressed against endophyte concentration or biomass to investigate potential relationships. Analysis of covariance, which fitted parallel lines through the scatter plots (one point per treatment) for the two hosts, was used to correlate alkaloid and endophyte concentrations within hosts, after adjusting for differences in overall mean values between hosts.

2.3 Results

2.3.1 Concentration of endophyte mycelia

At week 0, the concentration of AR501 mycelia did not significantly differ ($P = 0.16$) between the tall fescue (4.23 mg/g) and perennial ryegrass (1.24 mg/g) host populations that had previously been placed at 20°C (data not shown). At week 3, at both the cool (12/6°C: day/night) and warm (25/6°C: day/night) temperature regimes (from now on those will be referred to as cool and warm), there were significant ($P < 0.05$) differences in the mycelial concentrations of AR501 between the two different grass host species with tall fescue recording a higher concentration (2.27 mg/g under the cool temperature regime and 1.45 mg/g under the warm regime) of endophyte mycelia than

perennial ryegrass (1.07 mg/g under the cool temperature regime and 0.57 mg/g under the warm regime) (Figure 2.1). However, there was no statistical difference ($P=0.053$) observed between the mycelial concentrations of AR501 within a host species, i.e. there was no difference in mycelial concentrations between the perennial ryegrass populations at either temperature regime, cool vs. warm, and the same trend was observed for tall fescue (Figure 2.1).

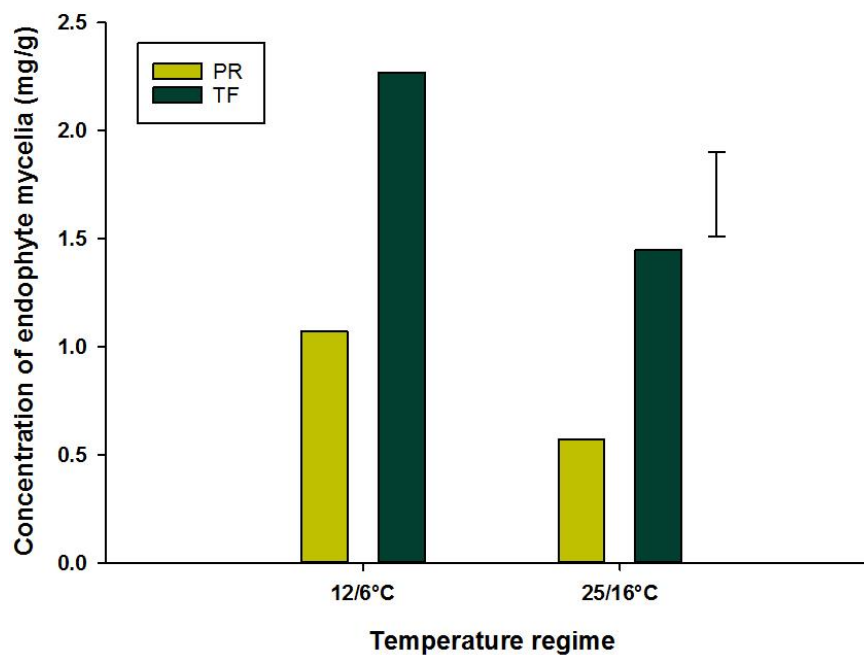


Figure 2.1 Mycelial concentration (mg/g) of AR501 at week 3 harvest in the perennial ryegrass (PR) and tall fescue (TF) hosts. Vertical bar indicates the value for LSD (at the 0.05 level of probability) for each temperature regime.

At week 6 (the third harvest date) the mycelial concentration of AR501 did not significantly differ between the two plant species at the constant cool temperature (Figure 2.2). However, the mycelial concentration was significantly higher ($P<0.05$) in tall fescue than perennial ryegrass when grown under the warm temperature regime (Figure 2.2). When plants were moved from the warm temperature regime to the cool temperature regime, the mycelial concentration did not differ significantly between grass host species. However, for both grass species, mycelial concentration of AR501 increased significantly ($P<0.01$) when plants were transferred from the cool temperature regime to the warm regime (Figure 2.2). In this circumstance the AR501 mycelial concentration in tall fescue was significantly ($P<0.05$) higher (10.82 mg/g) than in perennial ryegrass (5.18 mg/g).

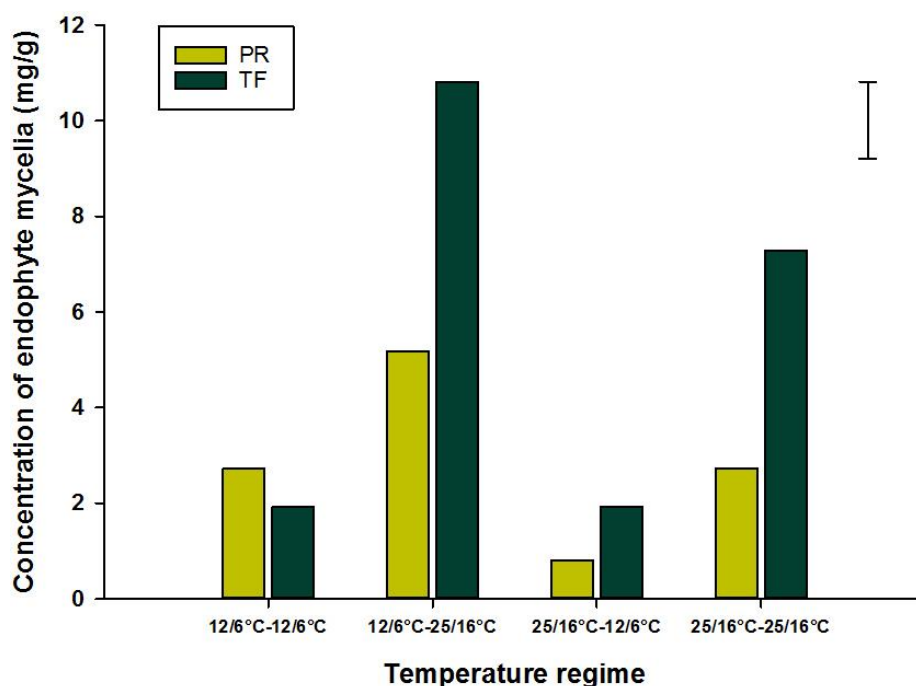


Figure 2.2 Mycelial concentration (mg/g) of AR501 at week 6 harvest in the perennial ryegrass (PR) and tall fescue (TF) hosts. Vertical bar indicates the value for LSD (at the 0.05 level of probability) for each temperature regime.

2.3.2 Endophyte infection frequency

At week 0 the endophyte infection frequency was not determined as the plants were too small (immature) for immunoblot analyses. At week 3 (the second harvest date), there was a significant difference ($P < 0.05$) in the endophyte infection frequency of AR501 within perennial ryegrass plants, between the cool and warm temperature regimes (Table 2.2). However, the endophyte infection frequency of AR501 within tall fescue was not significantly ($P > 0.05$) different between the cool and warm temperature regimes, with 100% infection frequency recorded for both (Table 2.2).

Table 2.2 Effect of temperature regime on AR501 infection frequency (%) in perennial ryegrass and tall fescue at the second harvest date (week 3).

Temperature regime (day/night)	Endophyte transmission frequency (%) in perennial ryegrass	Endophyte transmission frequency (%) in tall fescue
12/6°C	88.3	100
25/16°C	33.3	100
Significant effects	*	NS

NS=Not Significant; *= $P < 0.05$

At week 6 (the third harvest date) the endophyte infection frequency did not differ significantly ($P>0.05$) between the two plant species when the temperature regime was either cool or warm constantly (Figure 2.3). When plants were moved from cool to warm, endophyte infection frequency was also not significantly different between the host grass species. However, when plants were moved from warm to cool, the endophyte infection frequency was significantly higher ($P<0.05$) in tall fescue than in perennial ryegrass (Figure 2.3).

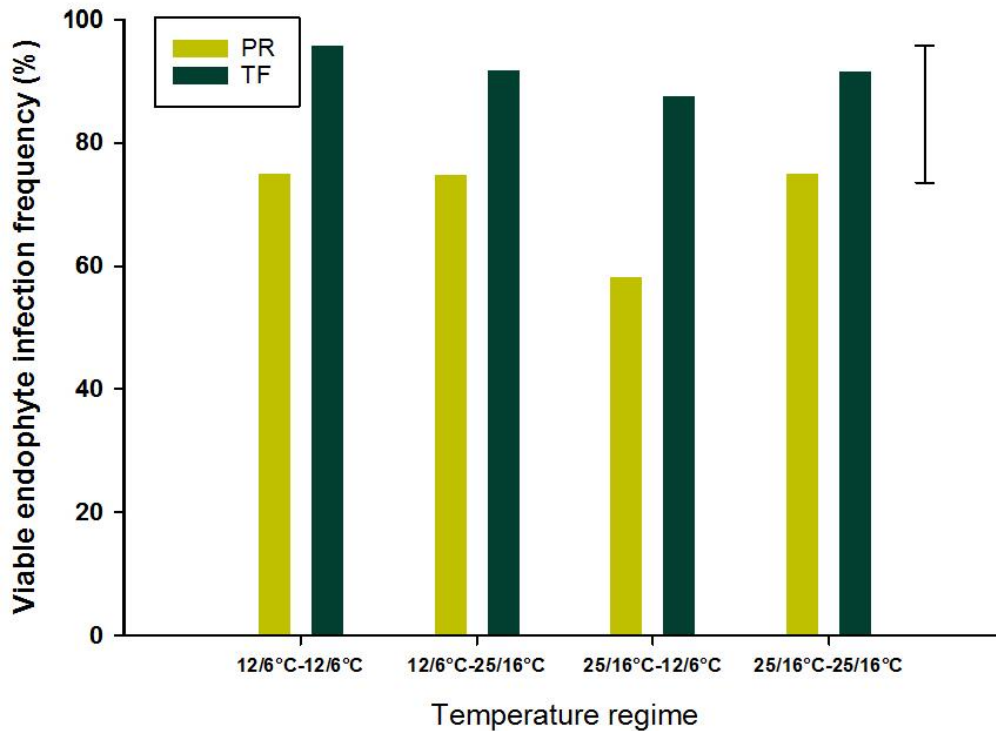


Figure 2.3 Viable endophyte infection frequency of AR501 within perennial ryegrass (PR) and tall fescue (TF) plants at week 6 under different temperature regimes. Vertical bar indicates the value for the LSD (at the 0.05 level of probability) for each temperature regime.

2.3.3 Concentration of insect deterrent alkaloids

At week 6 (the third harvest date) the concentration of peramine did not differ significantly between grass species under the cool constant temperature (Figure 2.4). However, peramine concentration was significantly ($P<0.05$) higher in tall fescue (11.02 $\mu\text{g/g}$) than in perennial ryegrass (4.26 $\mu\text{g/g}$) when they were grown under warm constant temperature (Figure 2.4). When plants were transferred from a warm to a cool temperature regime the concentration of peramine was not significantly different between the grass species. However, when plants were transferred from a

cool to a warm temperature regime, the concentration of peramine was again significantly ($P < 0.05$) higher in tall fescue (17.28 $\mu\text{g/g}$) than in perennial ryegrass (12.03 $\mu\text{g/g}$) (Figure 2.4). For both grass species, the highest concentration of peramine was observed when plants were transferred from the cool to the warm temperature regime (Figure 2.4).

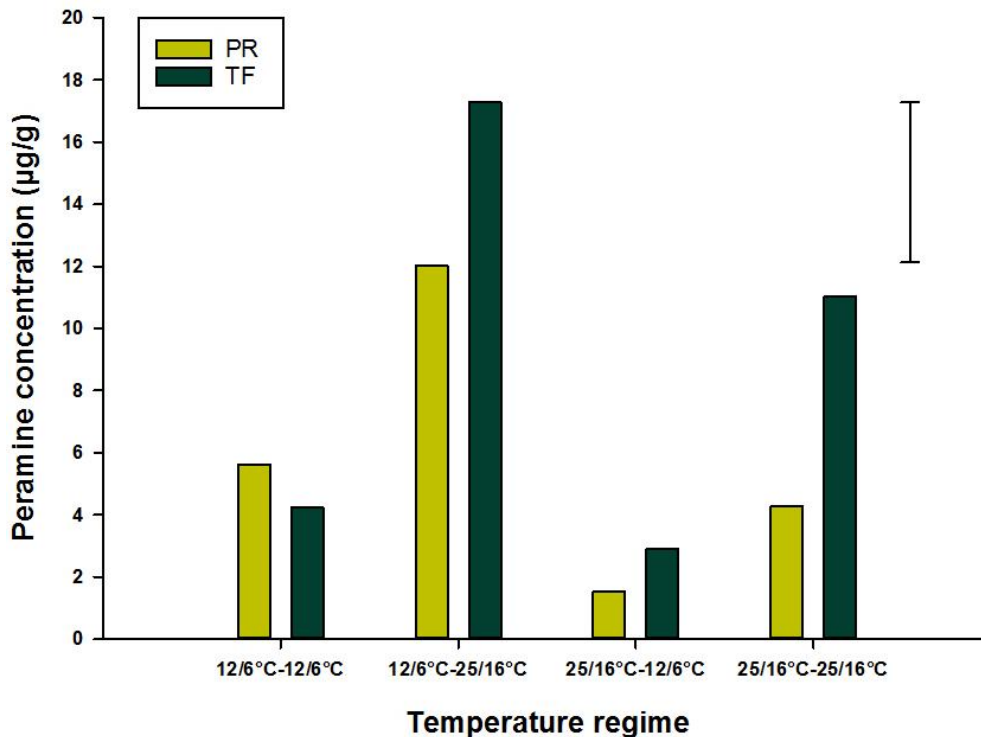


Figure 2.4 Concentration of peramine ($\mu\text{g/g}$) at week 6 harvest in perennial ryegrass (PR) and tall fescue (TF) plants, both infected with AR501. Vertical bar indicates the value for the LSD (at the 0.05 level of probability) for each temperature regime.

At week 6 the concentration of loline alkaloids was significantly ($P < 0.05$) higher in tall fescue (1.12 and 1.70 $\mu\text{g/g}$) than in perennial ryegrass (0.17 and 0.63 $\mu\text{g/g}$) at the cool and warm constant temperatures respectively (Figure 2.5). When plants were transferred from either the cool to the warm regime or vice versa, the concentration of loline alkaloids was again significantly ($P < 0.05$) higher in tall fescue (1.19 and 2.2 $\mu\text{g/g}$ respectively) than in perennial ryegrass (0.17 and 1.34 $\mu\text{g/g}$ respectively) (Figure 2.5). For both plant species, the concentration of lolines increased significantly ($P < 0.05$) when plants were transferred from the cool to the warm temperature regime but decreased when plants were transferred from the warm to the cool temperature regime.

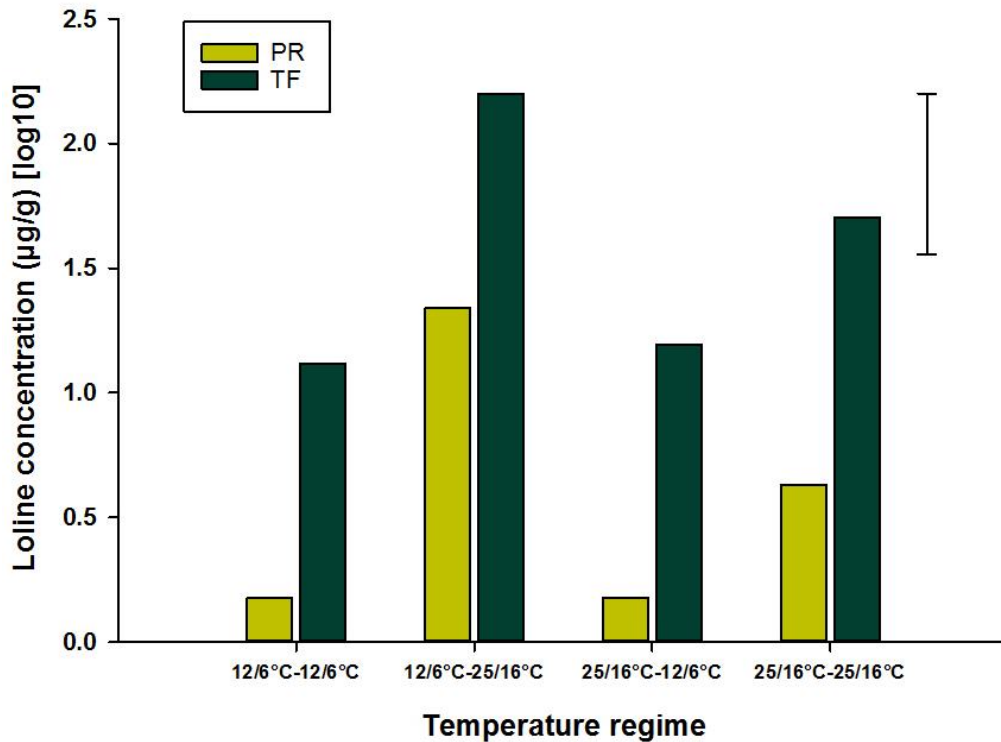
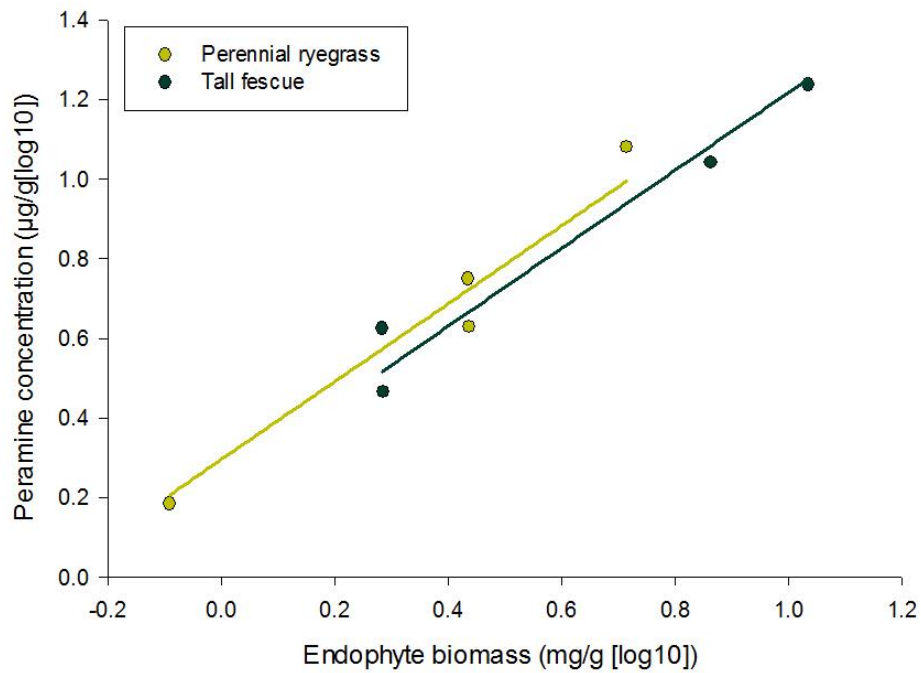


Figure 2.5 Concentration of loline alkaloids ($\mu\text{g/g}$) [\log_{10}] at week 6 harvest in perennial ryegrass (PR) and tall fescue (TF) plants, both infected with AR501. Vertical bar indicates the value for the LSD (at the 0.05 level of probability) for each temperature regime.

Correlation between endophyte biomass and alkaloid concentration

Peramine and loline alkaloid concentrations increased when endophyte biomass increased (Figure 2.6), with the tall fescue endophyte association producing greater amounts of both alkaloids (1.04 and 1.70 mg/g [\log_{10}] of peramine and loline alkaloids, respectively) when endophyte biomass reached its maximum levels. The common slope of the parallel regression lines was significantly ($P=0.012$ and $P<0.001$) positive for both loline and peramine alkaloids, respectively.

(a) Peramine



(b) Loline alkaloids

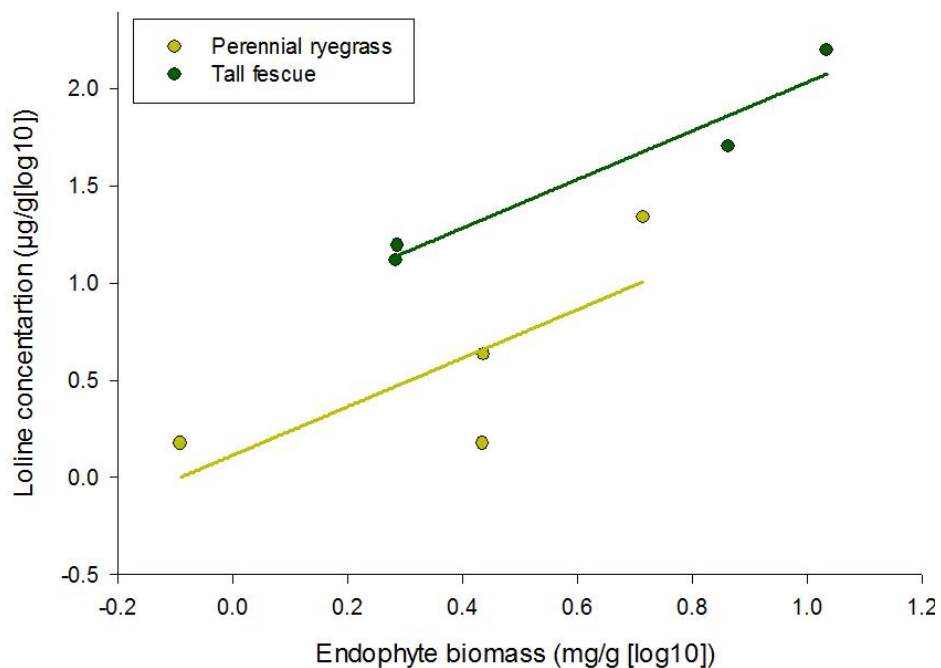


Figure 2.6 Correlation between alkaloid concentration, (a) peramine and (b) lolines, and endophyte biomass in the perennial ryegrass and tall fescue hosts. Equations for fitted curves were (a) tall fescue, $y = 0.979x + 0.2391$ and perennial ryegrass, $y = 0.979x + 0.2955$ and (b) tall fescue, $y = 1.25x + 0.7832$ and perennial ryegrass, $y = 1.25x + 0.1143$. The parallel lines were fitted using analysis of covariance. Note that the scale on the y axis differs for each alkaloid.

2.4 Discussion

This study demonstrated that the transmission of AR501 in the tall fescue host was higher than in the perennial ryegrass host, and that temperature had a dramatic effect on the concentration of AR501 endophyte mycelia and production of the alkaloids, peramine and lolines, *in vivo* within tall fescue and perennial ryegrass host plants.

2.4.1 Concentration of endophyte mycelia

The concentration of endophyte mycelia did not differ significantly between plant hosts during the initial phase (week 0) of this experiment, but the average endophyte concentration in tall fescue was more than double that in perennial ryegrass. This is likely to be because tall fescue is the original host species of AR501, while the association with perennial ryegrass was artificially developed (i.e. AR501 was inoculated into a perennial ryegrass line). The majority of *Epichloë* species display a high degree of host-specificity (Johnson et al., 2013; Karimi et al., 2012). Some artificial associations can be stable and compatible (Karimi et al., 2012) and the most successful of these associations are the ones transferred across related grasses (Leuchtman & Clay, 1993). However, failure in an association can occur, and can be related to resistance of the non-host towards the endophyte (Koga et al., 1993; Leuchtman, 1992). *Epichloë* species generally have temperature preferences, and these are intrinsically linked to their host plant.

A decrease in endophyte mycelia in plants harvested at week three from both the cool and warm temperature regimes was observed when compared to plants harvested at week zero which had been grown at 20°C constant. This indicates that the decreased concentration of endophyte mycelia was related to stress caused by the temperature regime change. Breen (1992) has also demonstrated a difference in the concentration of endophyte in plants grown under different temperature regimes, where the concentration of *E. festucae* var. *lolii* endophyte in perennial ryegrass was lower in plants growing at a constant temperature of 7 or 28°C than in plants growing at a constant temperature of 14 or 21°C.

Bacon and Siegel (1988) showed that endophyte resides within plant meristematic tissues during vegetative growth and that during a period of temperature stress, the endophyte can become dormant and its mycelium can potentially disintegrate in leaf sheaths. In the present study, the results of endophyte concentration at week six are in agreement with those obtained by Bacon and Siegel (1988). This suggests that during the first three weeks, the fungus was restricted to the plant meristematic tissue and the concentration of endophyte *in planta* was low in response to the stress caused by the variation of temperature in both regimes. During the final three weeks, as the

association had become established, the endophyte had enough time to colonise new tillers and therefore the concentration had increased by the week six harvest. At this time, the concentration of endophyte had increased significantly in plants moved from cool to warm temperature in both host species. It is likely that the first three weeks of low temperature were crucial for endophyte establishment, and at this stage, the endophyte appears to be better adapted to the high temperature, allowing for a higher concentration of endophyte mycelia. Ju et al. (2006) reported that the cardinal minimum temperatures for endophyte and the tall fescue host differed considerably, being 10.3°C for the endophyte *E. coenophiala* (AR584) and 5.2°C for tall fescue. Tall fescue is slow to establish at soil temperatures <10°C, but once established, can withstand higher air temperatures. Perennial ryegrass growth under hot temperatures and dry summers is very poor but it recovers quickly following cool temperatures (Kemp et al., 1999). In New Zealand, pasture seeds are usually sown in early autumn when maximum air temperatures are >20°C across both islands, and 10 cm soil temperatures are likely to range from 12-17°C in March, 8-14°C in April and 4- 11°C in May (Hampton et al., 1987). The optimum temperature for growth of perennial ryegrass is around 18°C (Kemp et al., 1999), although it is usually quick to establish even at low soil temperatures (Moot et al., 2000). Epichloid endophyte survival and dissemination depend on plant host success (Johnson et al., 2013). It would therefore be advantageous to have the plant host growing under optimal temperature conditions to stimulate a high concentration of endophyte mycelia, but this will not usually occur in farm systems.

2.4.2 Frequency of endophyte infection

A temperature stress response was possibly observed in the recorded endophyte frequencies in perennial ryegrass, but was not observed in tall fescue at the week three harvest. The low frequency of endophyte infection observed in perennial ryegrass under the warm temperature regime could be explained by the fact that perennial ryegrass does not respond well under hot temperatures during its establishment stage (Kemp et al., 1999).

The temperature regimes did not affect the endophyte infection frequency in either perennial ryegrass or tall fescue at the week six harvest, although, at both harvests, the frequency of endophyte was greater in tall fescue than perennial ryegrass. These results are consistent with those of Wilke et al. (2007) who showed that temperature regimes of high (between 24°C to 30°C), average (between 16°C to 22°C) and low (between 12°C to 20°C) did not affect the frequency of the systemic infection or transmission of *F. verticillioides* from seed to seedling in maize plants.

In this present work, these results were not unexpected as AR501, originally from a tall fescue host, is likely to express host specificity (Christensen, 1995; Leuchtman & Clay, 1993). As noted previously, it is likely that the three weeks following the week three harvest, provided the endophyte with the opportunity to colonise new tillers and thus endophyte frequency was increased. The TPIB technique (Simpson et al., 2012) used to assess the endophyte frequency, detects endophyte presence or absence in plant tiller tissue, and is different to the ELISA technique (Faville et al., 2015) used to estimate the concentration of endophyte mycelia. The combination of the results indicates that when the endophyte is already present in plant tissue, the change of temperature does not interfere with endophyte frequency.

2.4.3 Concentration of alkaloids

Peramine and loline alkaloid concentration increased as the endophyte concentration increased when plants were moved from lower to higher temperature, and the concentration of both alkaloids was positively and significantly correlated to endophyte concentration. These results support a previous study (Ryan et al., 2015) where concentration of endophyte and alkaloids in perennial ryegrass plants infected either with *Epichloë festucae* var. *lolii* strain AR37 or strain AR42 (previously Lp-19) were positively affected by higher temperature regimes. Associations receiving a high temperature regime of 20/10°C (day/night) had higher alkaloid and endophyte concentration than associations receiving a lower temperature regime of 10/10°C (day/night) (Ryan et al., 2015).

2.5 Conclusion

Overall, the results of this experiment support the hypothesis that temperature is one of the factors that affects the transmission of *Epichloë* strain AR501 from seed to vegetative tillers within both this selected perennial ryegrass breeding line and its original tall fescue host. In both associations, the endophyte frequency, endophyte biomass concentration and alkaloid concentrations depended on temperature and the AR501 endophyte changed its behaviour under different temperature regimes.

For autumn sowing, soil temperatures are usually declining but the lower temperatures will probably not affect the transmission of the endophyte from the seed to the seedling. Low temperatures during sowing would however affect the concentration of the endophytes which may be a disadvantage for autumn sowings. However for spring sowings, when the soil temperatures are higher (Hampton et al., 1987), the concentration of endophyte would also likely be higher. To better understand the effect of temperature on endophyte transmission, determination of the cardinal temperatures (minimal, optimal and maximal) for the endophyte is crucial, and this is addressed in the following chapter.

Chapter 3

Cardinal temperatures for an *Epichloë* endophyte colonising tall fescue and perennial ryegrass

3.1 Introduction

Many New Zealand cultivars of perennial ryegrass and tall fescue are intentionally infected with agriculturally beneficial epichloid fungal endophytes, thus forming a new mutualistic symbiosis (Johnson et al., 2013). These selected fungal strains are asexual and therefore rely on the vertical transmission pathway, via seed, for their dispersal (Gundel et al., 2011). Endophyte survival and transmission is therefore highly dependent on the success of the plant host (Johnson et al., 2013). Endophyte transmission failure has been observed in some grass-endophyte associations, with no single factor identified as being responsible (Gundel et al., 2008; Ju, 2011). However, genetic factors, (i.e. host-endophyte genotype compatibility), environmental variations and seed crop management (including irrigation and fertiliser) may also influence the grass-endophyte symbiosis and endophyte infection frequency (Gundel et al., 2011).

Temperature has a major influence on epichloid fungal endophyte transmission (Chapter 2). Ju et al. (2006) suggested that variations in endophyte infection frequency and concentration in the plant host, were related to seasonal temperature variations. Bacon and Siegel (1988) reported that endophyte presence in tall fescue seeds and vegetative tillers had decreased following a hot/dry summer and cold winter. Although some research has been carried out on the effects of temperature on the endophyte transmission, there is still very little scientific understanding of how temperature influences the grass-endophyte symbiosis. Temperature may also have an important impact on grass seed germination (Moot et al., 2000). Lu et al. (2008) reported that tall fescue seed germination under an alternating temperature regime of warm/cool was lower than that at a constant mean temperature.

Epichloë endophytes grow systemically throughout the tissues of their host, colonising the intercellular spaces, and their growth *in planta* is fully synchronised with the host (Christensen et al., 2008). These endophytes depend on the host for nutrients, shelter and water (Malinowski & Belesky, 2000). Thus if temperature affects growth of the grass host, the endophyte would be directly affected. An understanding of the effect of temperature on the growth and development of both the endophyte and its grass host is required.

Cardinal temperatures define the range of temperatures under which plant growth and development occur (Black et al., 2006). Cardinal temperatures are base or minimum (T_b) and maximum (T_m) below or above which growth and development is limited; and optimum temperature (T_{opt}) where development occurs over the shortest duration (Angus et al., 1981). In a study by Ju et al. (2006), the cardinal minimum temperature for the endophyte strain AR584 and the tall fescue host were shown to differ considerably, being 10.3°C for AR584 and 5.2°C for tall fescue. The determination of optimal temperature for endophyte development rate and comparing this with that of the plant is important for understanding the association between host and endophyte. Quantifying the cardinal temperatures for both the endophyte and its plant host is important as it will allow prediction of the best sowing conditions to achieve successful pasture establishment and maximum endophyte survival. Estimating the cardinal temperatures for many common temperate pasture grasses using mathematical models has been well described (Black et al., 2006; Lonati et al., 2009; Moot et al., 2000). The bilinear model has been commonly used for tall fescue and perennial ryegrass (Lonati et al., 2009; Moot et al., 2000). To estimate T_b , the linear model is the most common model used, due to its accuracy and simplicity (Andreucci et al., 2016). This model describes development rate as a function of temperature (Angus et al., 1981).

This study was undertaken to determine cardinal temperatures for *Epichloë* FaTG-3, strain AR501 within perennial ryegrass and tall fescue, as well as cardinal temperatures for both perennial ryegrass and tall fescue. The hypothesis was that the cardinal temperature for the endophyte would not differ from those of the tall fescue and the perennial ryegrass hosts.

3.2 Materials and Methods

Seeds of tall fescue line T9886 and perennial ryegrass line Klp 903 were used in this study. Line T9886 (cultivar Flecha a summer dormant Mediterranean tall fescue cultivar) was supplied by the Margot Forde Germplasm Centre (MFGPC), New Zealand's national gene-bank of grassland plants and line Klp 903 (derived from crosses of cultivars Banquet, Banquet II and Bealey) was supplied by PGG Wrightson Seeds Ltd. Both seed lines were infected with the same strain of epichloid endophyte, namely *Epichloë* FaTG-3, strain AR501. The tall fescue line was harvested in 2012 while the perennial ryegrass line was harvested in 2011. In June 2013 following the receipt of both lines, germination percentage was assessed using the growing medium test (ISTA, 2016) and viable endophyte infection frequency was assessed using the tissue-print immunoblot (TPIB) procedure (Simpson et al., 2012).

The germination response of the two grass lines to different temperatures was evaluated using specialist facilities only available at Massey University, Palmerston North, New Zealand from 22nd of July to 25th of August 2014. Seeds were germinated on a 76 x 76 cm two-way temperature gradient plate (Grant Instruments Ltd., UK) to determine cardinal temperatures (Figure 3.1). The square aluminium plate was heated on one side and cooled on the other, resulting in a continuous and homogeneous temperature gradient along its length (Murdoch et al., 1989) (Figure 3.1a). Treatments were constant temperatures ranging from 2 to 29°C across the gradient plate at 3°C increments (10 constants=10 treatments). A single layer of K-24 Versapack (Anchor Paper Company, USA) saturated in water was placed on the aluminium plate surface and two layers of Steel Blue Seed Germination Blotters (Anchor Paper Company, USA), saturated with water, were placed on the top and allowed to equilibrate to the 10 constant temperatures. Two thermocouple thermometers linked to a data-logger (Cole-Parmer Instrument Company, USA) were used to record the temperature every hour throughout the experiment to confirm the consistency of the temperatures across the entire aluminium plate. A plastic grid frame was placed on the filter paper to contain the seed groups in each treatment, and glass plates were used to cover the frame to prevent moisture loss. Twenty five seeds each of tall fescue and perennial ryegrass, were placed in a straight line on the filter paper in each plastic grid square of the aluminium plate per temperature (Figure 3.1b). The experiment was arranged as a randomised split plot design with temperature as the main plot and each grass species as subplots. There were four replicates for each treatment. Seed germination was defined as when the emerged radicle was the same length or greater than the seed. Seedlings that had germinated were counted and removed daily for a total of 34 days and a maximum of 16 seedlings per treatment were immediately transplanted into trays containing potting mix (sand: peat, 40:60) and placed in a greenhouse where the temperature ranged from 15 to 20°C. After eight weeks, all the plants were assessed for the presence of AR501 using the TPIB procedure (Simpson et al., 2012) by assessing two tillers per plant.

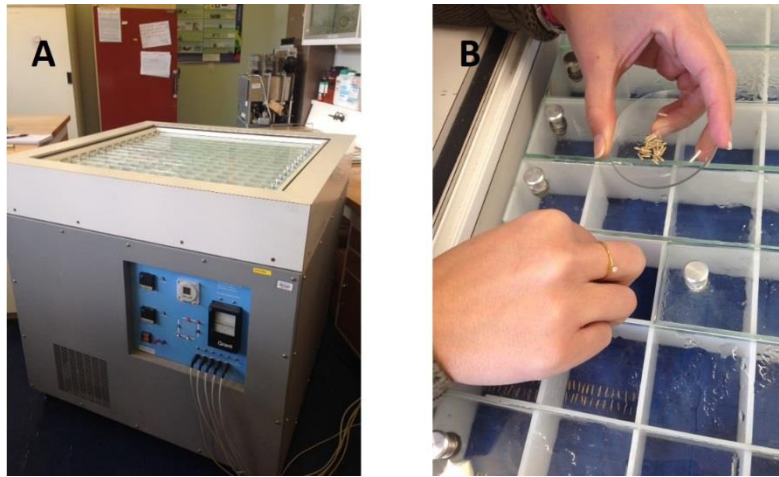


Figure 3.1 View of (A) the Grant temperature gradient plate and (B) seeds placed in line on filter paper in a plastic grid square of the plate.

3.2.1 Models and data analysis

Germinated seeds were used to calculate the accumulative germination percentage and two tillers per plant grown from germinated seeds were used to calculate the accumulative viable endophyte infection frequency. A Gompertz curve (Equation 1) described the germination percentage and endophyte infection against days for each replicate to calculate 75% final germination and 70% endophyte infection.

$$\text{Equation 1: } Y = A + C \cdot \exp[-\exp(-B \cdot (t - M))]$$

where Y is the cumulative percentage of seeds germinated or endophyte infection at time t (days), A is the lower asymptote, C the final germination percentage or final endophyte viability percentage, M is a time scale (lag related) constant and B is the rate of increase (Roche et al., 1997). The direction of the response was set to the right (C > 0 when B > 0). The calculation of days to 75% final germination percentage (t75) and 70% final endophyte infection (t70), was done using Equation 2.1 and Equation 2.2 (Black et al., 2006). The reciprocal of time to 75% final germination is the germination rate (1/day) and the reciprocal of time to 70% final endophyte frequency is the endophyte development rate (1/day).

$$\text{Equation 2.1: } t_{75} = M - \ln[-\ln(75/100)]/B$$

$$\text{Equation 2.2: } t_{70} = M - \ln[-\ln(70/100)]/B$$

The germination rate and endophyte development rate from each grass host were plotted against temperature, and the broken stick and linear models were fitted to determine the cardinal temperatures.

Linear model

The linear model (Equation 2.3) describes development rate (D) as a function of temperature (T), where 'a' is the intercept and 'b' is the slope of the regression line. Tb can be calculated through Equation 2.4 (Angus et al., 1981).

$$\text{Equation 2.3 } D(T) = a + b * T$$

$$\text{Equation 2.4 } T_b = -a/b$$

Bilinear or broken stick model

The bilinear, or broken-stick, model is the split-line regression procedure available on GenStat (VSN International 2013. *GenStat for Windows 16th Edition*. VSN International, Hemel Hempstead, UK) and the intercepts are given by the output. The T_{opt} was the x-value of the breakpoint between the two lines. T_b and T_m are the points where the two regression lines intercept the x-axis.

The models were fitted and data analysed with GenStat. Analysis of variance (ANOVA) and Fisher's unprotected test of least significant difference (LSD; P<0.05) were performed to compare temperature treatment effects on final germination and endophyte viability results. The bilinear models estimated cardinal temperatures through statistical differentials (Kempthorne & Folks, 1971). Differences in mean estimates of cardinal temperatures between lines were analysed using the t-test.

3.3 Results

3.3.1 Germination percentage and endophyte infection frequency

The standard germination percentage recorded after sample receipt was 98% and 90% for tall fescue line T9886 and perennial ryegrass line KLp 903, respectively. On the temperature gradient plate the tall fescue seed germination increased from 38% at 2°C to 73% at 5°C, reaching a maximum germination of 92-93% at 8°C to 14°C (Figure 3.2a). However, the germination past 14°C declined with further increases in temperature, falling to 6% at the highest temperature (29°C; Figure 3.2a). For perennial ryegrass, the germination at the lowest temperature of 2°C was 34%. This almost doubled to 62% when the temperature increased to 5°C and then substantially increased to 89% at 8°C and plateaued at > 90% for temperatures above 11°C (Figure 3.2b).

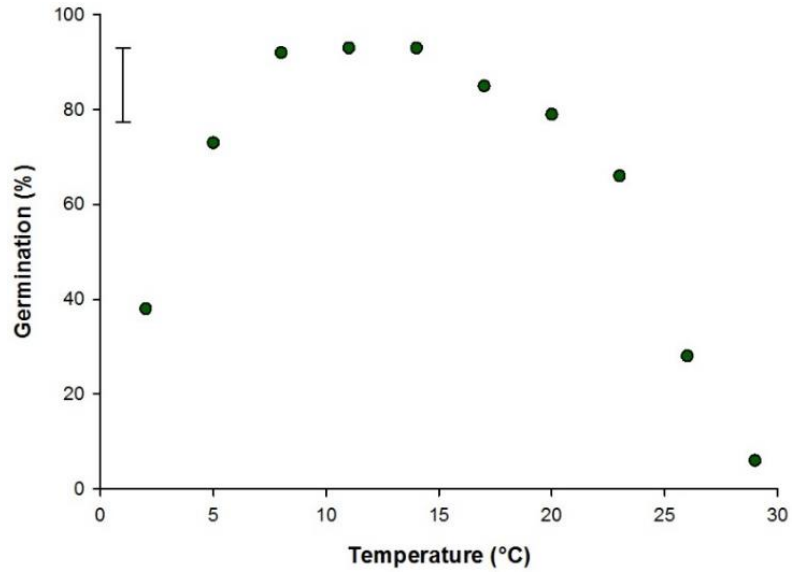
Seed germination percentage at the five lowest temperatures (2, 5, 8, 11 and 14°C) did not differ between tall fescue and perennial ryegrass, but differed significantly ($P<0.05$) at temperatures above 14°C (Table 3.1). Final germination percentage of the perennial ryegrass line was significantly ($P<0.05$) higher than that of the tall fescue line from 17°C to 29°C, as there was a rapid decline in tall fescue germination above 14°C (Table 3.1).

Table 3.1 Germination percentage of perennial ryegrass and tall fescue at 10 constant temperatures.

Temperature (°C)	Germination (%) for perennial ryegrass	Germination (%) for tall fescue	LSD values
2	34	38	25.98
5	62	73	16.74
8	89	92	6.09
11	100	93	9.00
14	97	93	5.19
17	99	85	8.22*
20	97	79	15.15*
23	97	66	27.68*
26	94	28	29.63*
29	98	6	15.59*

*= $P<0.05$

(a) Tall fescue line T9886



(b) Perennial ryegrass line KLp 903

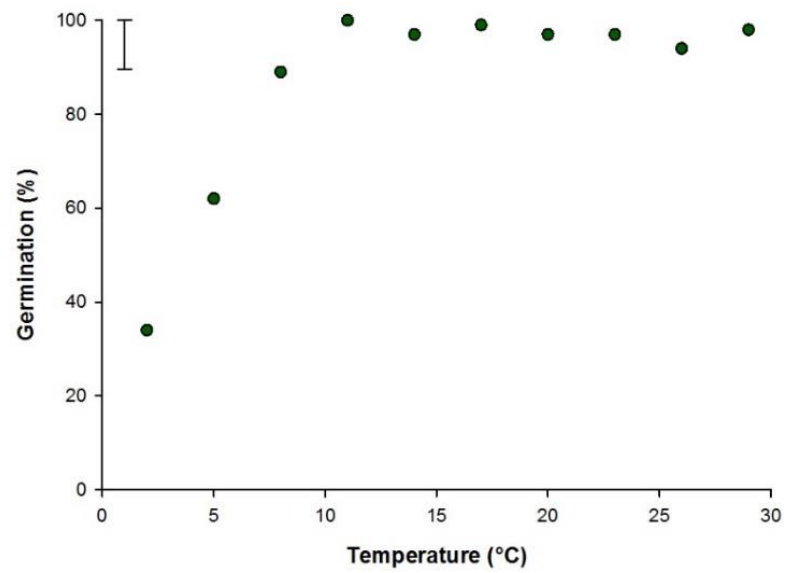


Figure 3.2 Final germination percentage (%) of seeds across a temperature gradient from 2-29°C (a) tall fescue, line T9886 and (b) perennial ryegrass, line KLp903. Vertical bars indicate the LSD (at the 0.05 level of probability) for each temperature point.

The viable endophyte infection frequency recorded after sample receipt was 99% for the tall fescue line and 87% for the perennial ryegrass line. On the temperature gradient plate the viable endophyte infection frequency in the tall fescue line was significantly higher ($P < 0.05$) than in the perennial ryegrass line at all temperatures except 14°C and 17°C. However, within both tall fescue and perennial ryegrass, the viable endophyte infection frequency did not differ among the constant temperatures (Figure 3.3).

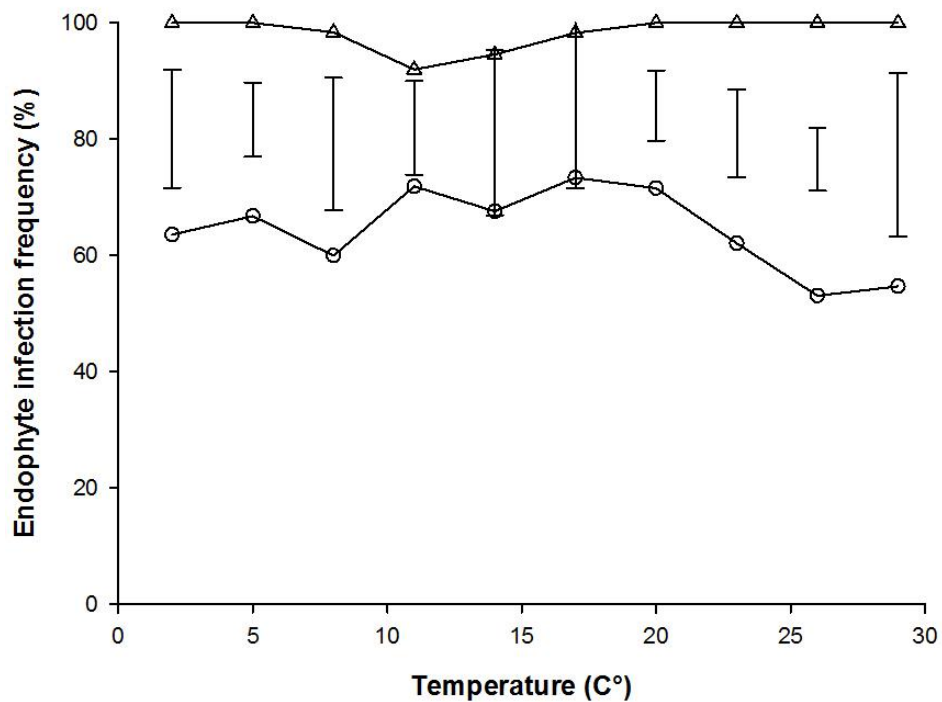


Figure 3.3 Effect of temperature on the viable endophyte infection frequency of AR501 within tall fescue (Δ) and perennial ryegrass (\circ) across a temperature gradient from 2-29°C. Vertical bars indicate the LSD (at the 0.05 level of probability) for each temperature point.

3.3.2 Seed germination rate and models

The germination rate (1/days to 75% final germination) for the tall fescue line increased linearly from 2°C to 15.75°C and then decreased as temperature increased to 29°C (Figure 3.4). The germination rate for the perennial ryegrass line increased linearly as temperature increased from 2°C to 25.19°C and then decreased as temperature increased to 29°C (Figure 3.5).

Statistical models were fitted to germination rate data obtained for each line for the complete temperature range (2 to 29°C). The broken stick model estimated a T_b of 0.62°C, T_{opt} of 15.75°C and T_m of 30.32°C for tall fescue (Figure 3.4) and a T_b of 3.27°C, T_{opt} of 25.19°C and T_m of 34.42°C for perennial ryegrass (Figure 3.5). Adjustments were made by the linear model due to a change in germination development rate near the T_b . Therefore, T_b as estimated by the linear model was -1.75°C for tall fescue and -0.09°C for perennial ryegrass (Figure 3.4 and 3.5).

3.3.3 Endophyte development frequency and models

The AR501 development rate (1/days to 70% final endophyte infection) in tall fescue increased linearly from 2°C to approximately 16°C and then decreased as temperature increased to 29°C (Figure 3.6). In perennial ryegrass, AR501 development rate increased linearly as temperature increased from 2°C to approximately 26°C and then decreased as temperature increased to 29°C (Figure 3.7).

Statistical models were fitted to the endophyte development data obtained for both hosts for the complete temperature range (2 to 29°C). The broken stick model estimated a T_b of 0.63°C, T_{opt} of 15.90°C and T_m of 29.04°C for tall fescue (Figure 3.6) and a T_b of 3.24°C, T_{opt} of 25.26°C and T_m of 54.94°C for perennial ryegrass (Figure 3.7). Adjustments were made by the linear model due to a change in endophyte development rate near the T_b . Therefore T_b estimated by the linear model was -0.04°C for tall fescue and -1.82°C for perennial ryegrass (Figure 3.6 and 3.7).

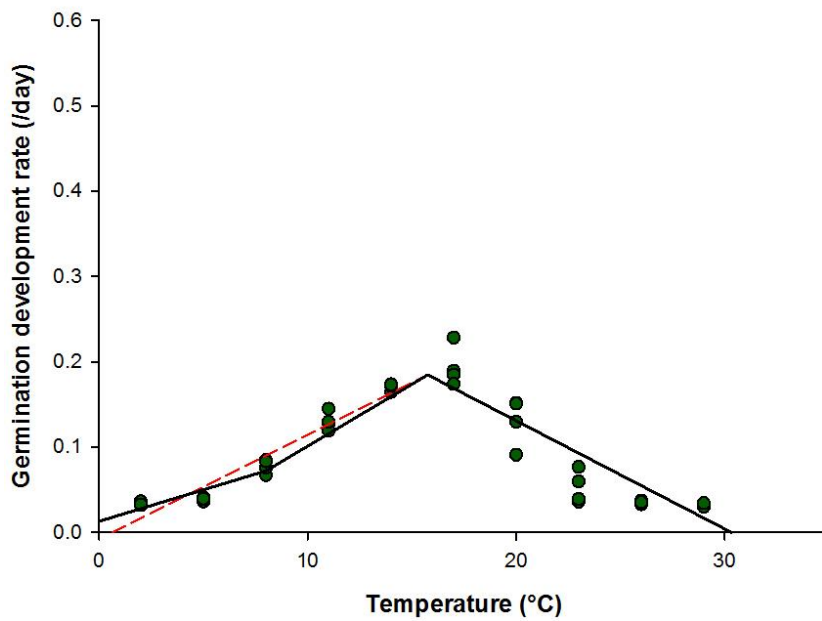


Figure 3.4 Germination rate for tall fescue incubated at different temperatures. Points are observed data; solid black line is for the linear model; dashed red line is for the broken stick model.

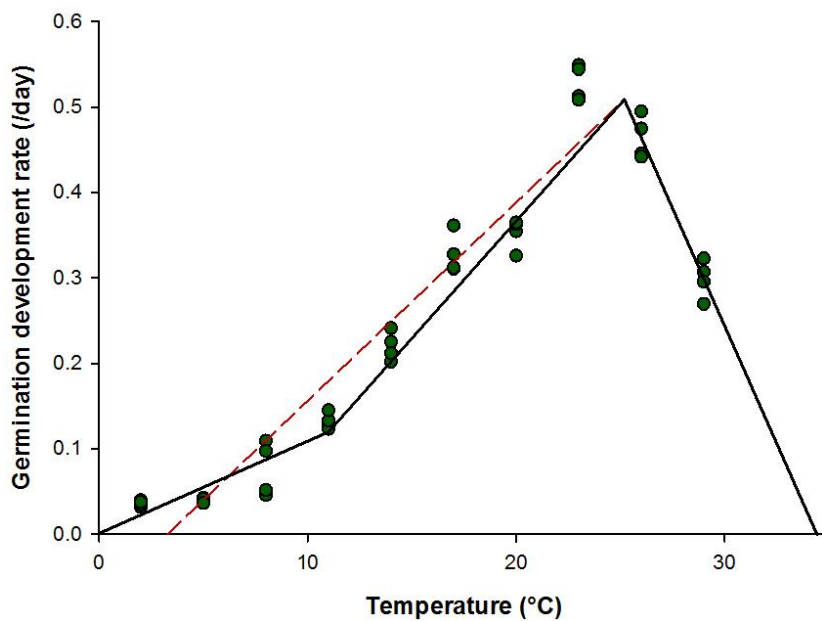


Figure 3.5 Germination rate for perennial ryegrass incubated at different temperatures. Points are observed data; solid black line is for the linear model; dashed red line is for the broken stick model.

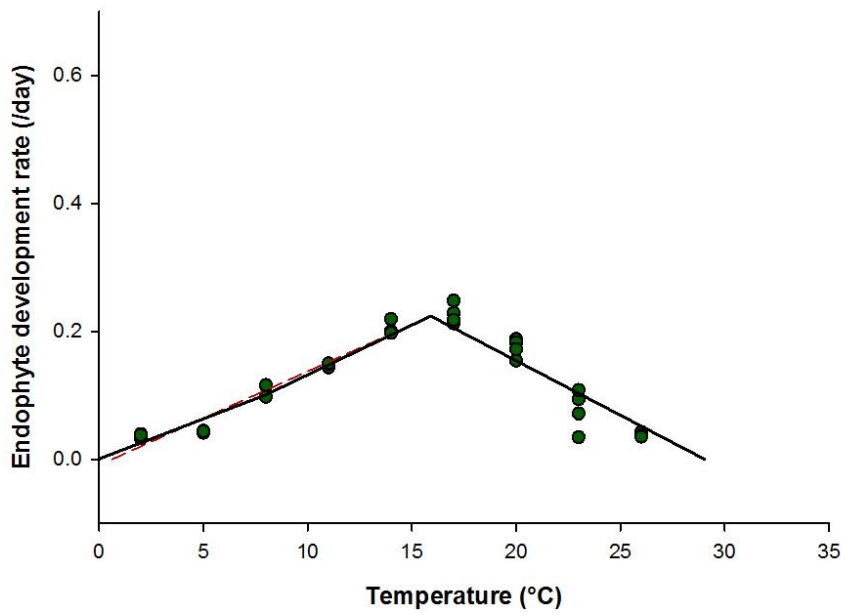


Figure 3.6 AR501 endophyte development rate in tall fescue incubated at different temperatures. Points are observed data; solid black line is for the linear model; dashed red line is for the broken stick model.

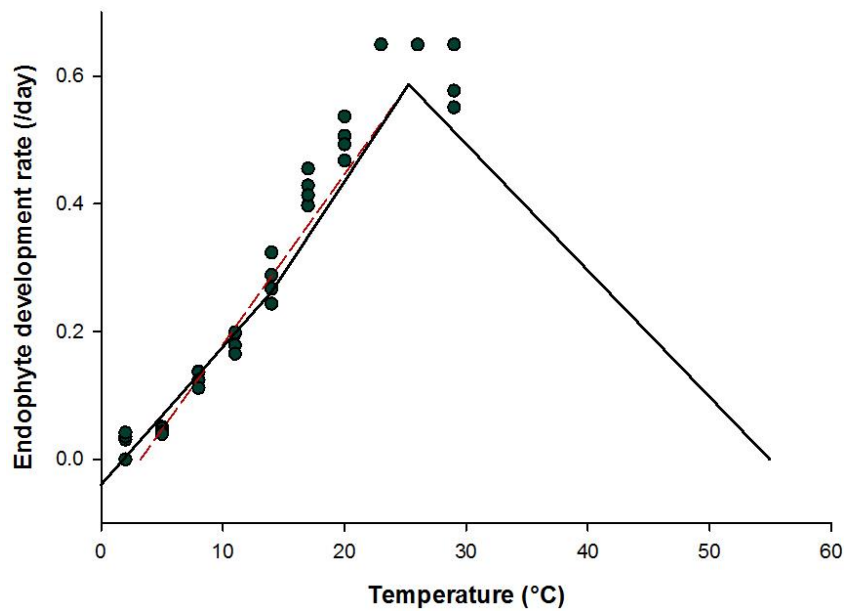


Figure 3.7 AR501 endophyte development rate in perennial ryegrass incubated at different temperatures. Points are observed data; solid black line is for the linear model; dashed red line is for the broken stick model.

3.4 Discussion

This study demonstrated that the cardinal temperatures for AR501 differed between hosts, being higher in perennial ryegrass than in tall fescue. The cardinal temperatures estimated for both hosts, were consistent with that of the endophyte, being higher for perennial ryegrass than for tall fescue.

3.4.1 Germination and endophyte infection frequency

The germination response to the temperature regimes of the perennial ryegrass line used in this study was similar to that previously reported by Charlton et al. (1986) in that from 8°C to 29°C germination was over 90%. The exception was at 2°C and 5°C where in the present experiment germination was 34% and 62% respectively. Charlton et al. (1986) reported a germination of 90% or greater at 5°C for two different cultivars of perennial ryegrass. The germination percentage difference at the lower temperatures could be explained by the fact that the cultivar used in this present study and cultivars used by Charlton et al. (1986) differed in their ploidy; the former perennial ryegrass cultivars were tetraploid and the latter was a diploid. Hill et al. (1985) suggested that pasture grass seed germination can be potentially affected by ploidy. Seeds from tetraploid cultivars are larger than seeds from diploid cultivars of the same species and usually germinate faster (Murali, 1997). Sugiyama (1998) reported that perennial ryegrass tetraploid cultivars had lower cold tolerance than diploid cultivars and this supports the explanation of the difference response at low temperature between the present study and that of Charlton et al. (1986).

The germination response of tall fescue in the present experiment differed markedly from the results of Charlton et al. (1986). These authors found that tall fescue seed germination did not differ among temperatures ranging from 5°C to 30°C, with the mean germination being 87%. However for cv. Flecha the maximum germination (90%) was achieved only between 8°C and 14°C, decreasing markedly with further increases in temperature. This response to high temperature has also been reported for cv. Flecha by Monks et al. (2009) and Zhang et al. (2013), while Lonati et al. (2009) reported a similar response in seeds of *Festuca rubra* and *Festuca ovina*.

As tall fescue seeds that had not germinated at temperatures of 26° and 29°C by the end of the experiment appeared to be still firm (i.e. fresh ungerminated; ISTA 2016), and therefore not dead, they were removed from the gradient plate and taken back to Lincoln University with the intention of testing them for viability using the tetrazolium test (ISTA 2016). The seeds were left on a laboratory bench at ambient temperature overnight, and by the next morning, most of them had germinated (radicle protruding through the seed coat). This indicates that the seeds had become thermodormant, a secondary dormancy induced by exposure to high temperatures. Unfortunately,

non-germinated seeds from the 20° and 23°C treatment were not retained. However, it is likely that the germination decline (Figure 3.2a) at these two temperatures was also due to thermodormancy. It is therefore apparent that if the thermodormancy had been broken, the final germination response to temperature for tall fescue (Figure 3.2a) would have been similar to that for perennial ryegrass (Figure 3.2b).

Thermodormancy has been reported in different grass species and it is an important adaptation mechanism in case of seed exposure to hot dry environments such as in Australia and the Mediterranean (Monks et al., 2009). Knight (1965) reported that Crimson clover (*Trifolium incarnatum* L.) seeds at temperatures ranging from 22°C to 32°C did not germinate, but when they were moved to 20°C, the majority of the dormant seeds germinated. A similar result was observed by Corbineau et al. (1993) who reported thermodormancy in domestic oat seeds. Thermodormancy occurred in this present study, due to the fact that the tall fescue cultivar used, namely Flecha, is of Mediterranean origin and is classified as a winter active and summer dormant cultivar. The tall fescue cultivar used by Charlton et al. (1986) was Grasslands Roa, which is a continental summer active cultivar (Brock, 1983) and therefore, thermodormancy did not occur. It is therefore important to understand how the germination response of individual cultivars would respond to temperature.

In the present study, the results for the perennial ryegrass germination rate are consistent with the previous work of Charlton et al. (1986), who expressed germination rate as the number of days required to reach 75% germination of viable seeds. For perennial ryegrass they reported that for temperatures between 15°C and 30°C this was around 5 days, but at 5°C it was 23 days. The corresponding data for tall fescue were around 9 days and 65 days respectively. However these tall fescue results differed from those in the present work, as temperatures required to reach 75% germination were lower (11°C to 20°C) and the germination rate decreased sharply from 20°C to 29°C. These results are supported by previous studies as Zhang et al. (2013) reported that tall fescue (cv. Flecha) germination was only 1% at 35°C and Monks et al. (2009) reported a decrease in tall fescue germination rate above 20°C.

3.4.2 Cardinal temperature for perennial ryegrass and tall fescue host

The bilinear model provided a fit (R^2 75.9 and 80.1 for perennial ryegrass and tall fescue respectively) to the data from both seed lines. However, it had some limitations that produced problems in the germination rate near the base temperature. The bilinear model estimated a base temperature of 3.27°C for perennial ryegrass and 0.62°C for tall fescue but this was an underestimation for perennial ryegrass as germination occurred at 2°C and the final germination

was around 35% at 2°C for both seed lines. Due to this underestimation by the broken stick model, adjustments by the linear model were therefore required (Figure 3.4 and 3.5). By using the linear model, the base temperature estimated was closer to the actual germination response. In this study, the estimated base temperatures were lower than previously reported. A base temperature range from 1.9°C to 3.2°C has been previously reported for perennial ryegrass (Black et al., 2006; Monks et al., 2009; Moot et al., 2000). Monks et al. (2009) reported a base temperature of 4.6°C for tall fescue cv. Flecha while Zhang et al. (2013) reported a base temperature of between 5.1 and 11.4°C. In this study, the base temperature estimated for tall fescue was -1.75°C. It is likely that the base temperatures were overestimated by the previous authors, because the minimum temperatures they used for seed germination were from 4 to 5°C, while in the present study, the minimum temperature was 2°C.

The optimum temperatures of 15.75°C for tall fescue and 25.19°C for perennial ryegrass estimated by the bilinear model were consistent with previous work. Monks et al. (2009) reported an optimum temperature range of 10-30°C for perennial ryegrass and 17°C for Flecha, while Zhang et al. (2013) reported a range of 18-26°C for Flecha.

The maximum temperature of 30.32°C for tall fescue estimated by the bilinear model was lower than the maximum temperature estimated by Monks et al. (2009), while the maximum temperature of 34.42°C for perennial ryegrass estimated by the bilinear model in the present work, was consistent with previous reports. Monks et al. (2009) reported a maximum temperature of 35.8°C for Flecha and ~35°C for perennial ryegrass. It is likely that in this study maximum temperature for tall fescue was underestimated, because the maximum constant temperature treatment for seed germination was 29°C, while for Monks et al. (2009) it was 35°C.

3.4.3 *Epichloë* AR501 infection frequency and cardinal temperatures

The final endophyte infection frequency in the germinated seeds was not affected by temperature within the plant species, but the final endophyte infection frequency was higher in tall fescue compared to perennial ryegrass. These results were not unexpected as tall fescue, as the original host of AR501, is likely to express host specificity (Christensen, 1995; Karimi et al., 2012).

The bilinear model provided a fit (R^2 96.8 and 89.0 for perennial ryegrass and tall fescue respectively) to the data from both seed lines. However, it had some limitations that created problems in the endophyte development frequency estimation near the base temperature. The bilinear model underestimated the base temperature for AR501 of 3.24°C for perennial ryegrass and 0.63°C for tall fescue, while the final endophyte infection frequency was around 63% and 100% at

2°C in the perennial ryegrass and tall fescue lines respectively. Due to the underestimation by the broken stick model, adjustments made by the linear model were required (Figure 3.6 and 3.7). By using the linear model, the base temperature estimated was more accurate. In this study, the base temperatures for AR501 endophyte were lower than those previously reported for other endophytes. Ju et al. (2006) reported a cardinal minimum temperature of 10.3°C for *E. coenophiala* (AR584). However it is likely that this cardinal minimum temperature was underestimated because the minimum temperature used by Ju et al. (2006) was 10°C, while the minimum temperature used for AR501 was 2°C.

The optimal temperature of 25.26°C for AR501 in perennial ryegrass as estimated by the bilinear model was consistent with previous work, but the optimal temperature of 15.90°C for AR501 endophyte in tall fescue was lower. Li et al. (2008) reported optimal temperatures of 25°C for *E. gansuense* (previously known as *Neotyphodium gansuense*) and *E. coenophiala* and a range of 20-25°C for *E. festucae*. However, in this previous work the endophytes were grown in potato dextrose agar (PDA), and their growth was not influenced by the plant host.

In general, the cardinal temperatures for AR501 endophyte in perennial ryegrass (comparison using the t-tests) were higher than in tall fescue. Its likely that the optimal temperature of AR501 in tall fescue is driven by the host, and this was consistent with the optimal temperature estimated for tall fescue. However, if the thermodormancy in tall fescue seeds had not occurred, it would be possible that the optimal and maximum temperature for AR501 would be have been higher than those determined in this study. Further studies with more focus on thermodormancy are therefore suggested. It is clear that endophyte survival relies on plant host success (Johnson et al., 2013).

3.5 Conclusion

In this experiment the *Epichloë* FaTG-3, strain AR501 infection frequency increased linearly as temperature rose from the base temperature until it reached the optimal temperature. Cardinal temperatures for perennial ryegrass were higher than for tall fescue as previously reported. Cardinal temperatures for the endophyte in association with perennial ryegrass were higher than in the tall fescue association, showing that endophyte survival and transmission rely on plant host performance.

Chapter 4

The effect of temperature and seed moisture content on the viability of AR501 in stored grass seeds

4.1 Introduction

In New Zealand (NZ), commercial cultivars of tall fescue and perennial ryegrass are often intentionally infected with selected asexual fungal endophytes of the genus *Epichloë* that form host-specific, mutualistic associations with their grass hosts (Johnson et al., 2013; Schardl, 2010; Young et al., 2013). These fungi increase the competitive ability of their grass hosts primarily through the production of secondary metabolites that deter a wide range of invertebrate pests (Popay & Hume, 2011). Asexual *Epichloë* species spend all of their lifecycle within the intracellular spaces of their host plant and are exclusively vertically transmitted through their host's seed (Gundel et al., 2011; Philipson & Christey, 1986) relying heavily on the plant's reproductive success for their own dissemination. From an agricultural perspective, due to this phase in their lifecycle, it is crucial that endophyte viability is maintained in the seed during storage to ensure that the benefits of this novel endophyte technology are delivered to the end user (Canals et al., 2008; Rolston & Agee, 2007). During storage endophyte viability declines faster than that of the seed (Hill & Roach, 2009; Hume et al., 2011) resulting in seed that still germinates but contains no live endophyte, thus reducing the value of the final seed product (Rolston & Agee, 2007).

The storage factors that determine the longevity of seed and endophyte viability include temperature, relative humidity, seed moisture content and the duration of storage (Rolston et al., 1986; Welty et al., 1987). These factors, particularly seed moisture content (SMC) have a profound impact on the viability of *Epichloë* within the seed, although the exact mechanism(s) that lead to endophyte decay have yet to be established (Hume et al., 2013). Rolston et al. (1986) concluded that to maintain endophyte viability in perennial ryegrass seed lots for more than 12 months, seed should be stored at less than 5°C in moisture proof containers, or at ambient temperature provided the SMC remained below 11%. To maximise the longevity of endophyte viability in stored seed, germplasm centres, particularly the Margot Forde Germplasm Centre in NZ, operate their cold stores at a low temperature of 0°C and humidity of 30% (Card et al., 2015b).

Thermal time accumulation is a common approach to express the relationship between temperature and the growth/development of an organism (Andreucci et al., 2016; Bonhomme, 2000) with the

calculation based on the mean temperature minus the base temperature, or threshold temperature, below which development does not occur (Moot et al., 2000). To calculate thermal time accumulation, it is crucial that cardinal temperatures (described in Chapter 3) are first estimated. Thermal time requirements for seed germination and emergence for perennial ryegrass and tall fescue have been reported by a number of authors (Monks et al., 2009; Moot et al., 2000; Zhang et al., 2013), but, the requirements for endophyte in seed storage have not been investigated. This information would be valuable for developing new endophyte-seed storage strategies.

This study was undertaken to determine the effect of different storage conditions on the longevity of *Epichloë* FaTG-3 strain AR501 within perennial ryegrass (a novel association) and tall fescue (the original host species) seeds. The hypothesis was that storage temperature and seed moisture content would negatively affect endophyte viability in seed of both grass species over one year of storage. In addition to that, the thermal time requirement for AR501 viability in stored seed of both grass species was calculated as a predictor of endophyte survival.

4.2 Materials and methods

Seeds of two grass species were used in this study, namely tall fescue (*Festuca arundinacea* Schreb.), line T10254 of cultivar 'Flecha' (a summer dormant Mediterranean cultivar) and a tetraploid perennial ryegrass (*Lolium perenne* L.), line KR 1509 (derived from crosses of cultivars Banquet, Banquet II and Bealey). Both seed lines were infected with the same strain of epichloid endophyte, namely *Epichloë* FaTG-3, strain AR501. Tall fescue seeds which were supplied by the Margot Forde Germplasm Centre, NZ's national gene-bank of grassland plants, were harvested in 2015, and had a germination of 89% with 99% of this germinated seed carrying viable endophyte (K. Stewart, personal communication 2015). Perennial ryegrass seed, supplied by PGG Wrightson Seeds Ltd., was also harvested in 2015 and had a 93% germination with 87% of these seeds carrying viable endophyte (A. Stewart, personal communication 2015). In Canterbury (NZ) the SMC of harvested grass seed usually ranges from 12-14% (P. Rolston personal communication 2016). To achieve the required SMC for this experiment, half of each seed line was transferred to a refrigerator set at high relative humidity (>70%) and the other half to a cold storage room set at 25% relative humidity for one week to allow the SMC to equilibrate with the moisture content of the surrounding air (Justice & Bass, 1978). A seed moisture test (ISTA 2016) was subsequently conducted on both seed lines. For seeds stored in the cold room, SMC was 7.98% for tall fescue and 9.92% for perennial ryegrass. For seeds stored in the refrigerator, SMC was 8.90% for tall fescue and 11.85% for perennial ryegrass. To achieve the 10% and 14% SMCs the volume of water required to then be added to the seed was calculated using equation 1 (ISTA 2007):

Equation 1: Volume of water (mL)/ 10 g seed = $[(M1 - M2) \div (100 - M2)] \times 1000$

where M1 is the SMC after wetting and M2 is the SMC before wetting. The calculated volumes of water were then added to the seeds which were placed into a sealed container overnight to allow equilibration. A further seed moisture test was conducted to confirm that the required SMC had been achieved. Fifty seeds from each grass line were packaged into individual heat sealed aluminium foil bags (80 mm × 90 mm, supplied by Egmont Seed Company Ltd., NZ) and assigned to treatments. These treatments consisted of four temperature regimes, 5°C, 10°C, 20°C and 30°C and two seed moisture contents (SMC), 10% and 14%.

The experiment was set up in October 2015 using a split plot design with four blocks. Main plots were sampling months and subplots were seed lines and SMC. Sixteen aluminium foil bags from each treatment for each seed line were removed from storage four times at three month intervals over one year. In total, 64 bags containing seeds were removed from storage every three months for assessment of seed germination and endophyte viability frequencies. All seeds from each bag were planted in trays (two seeds per cell) containing seedling mix (120 L Southland peat, 80 L pumice) with the following fertiliser additions per cubic meter: 4 kg Osmocote® exact mini (16% N, 3.5% P, 9.1% K), 8 kg dolomite lime and 2 kg Hydroflo® (granular wetting agent manufactured by Everris Australia Ltd.). Trays were placed in a glasshouse, at approximately 20°C, and watered as required. Two weeks after sowing, seedlings were thinned to one seedling per cell by hand and eight weeks after sowing, all plants were assessed (two tillers per plant) for the presence of *Epichloë* using the TPIB technique (Simpson et al., 2012). The percentage of germinated seedlings that contained viable endophyte was calculated. Analysis of variance (ANOVA) and Fisher's unprotected test of least significant difference (LSD; $P < 0.05$) were performed to compare treatment effects. Statistical analysis was performed using GenStat® (VSN International 2013. GenStat for Windows 16th Edition. VSN International Ltd., Hemel Hempstead, UK).

4.2.1 Thermal time models

Thermal time was calculated following the cardinal temperatures estimated in Chapter 3 (Table 4.1) for both seed hosts and through Equation 2.1; 2.2; 2.3 and 2.4, following modifications proposed by Andreucci et al. (2016) and Streck et al. (2008) to thermal time accumulation. Equation 2.4 was only used for perennial ryegrass as T_m for AR501 in tall fescue was underestimated due to thermodormancy. T_{b2} are base temperatures estimated by the broken stick model and T_{b3} are base temperatures used for adjustments using the linear model in Chapter 3.

Equation 2.1: $T < T_b = 0$

$$\text{Equation 2.2: } T_{\leq T_{b3}} = (T - T_b) * (T_{b3} - T_{b2}) / (T_{b3} - T_b)$$

$$\text{Equation 2.3: } T_{\leq T_{opt}} = T - T_{b2}$$

$$\text{Equation 2.4: } T_{\leq T_m} = (T_m - T) * (T_{opt} - T_b) / (T_m - T_{opt})$$

Table 4.1 Cardinal temperatures (°C) for AR501 in tall fescue and perennial ryegrass hosts.

Cardinal temperatures	Tall fescue	Perennial ryegrass
T _b	0	0
T _{b2}	0.627	3.24
T _{b3}	8	14
T _m	N/A	25.3
T _{opt}	15.9	54.9

A Gompertz curve (Equation 2.5) was fitted to the endophyte infection frequency data of both seed lines stored for 12 months at 10% and 14% SMC and to the thermal time accumulated. The curve was fitted to calculate the thermal time requirement for 50% of the seed to still be infected with endophyte.

$$\text{Equation 2.5: } Y = A + C * \exp [-\exp (-B * (t - M))]$$

where Y is the cumulative percentage of endophyte infection at time t (°Cd), A is the lower asymptote, C is the final endophyte infection frequency, M is a time scale (lag related) constant and B is the rate of increase (Roche et al., 1997). The direction of the response was set to the right (C > 0 when B > 0). The calculation of days to predict 50% endophyte infection frequency of both seed lines, irrespective of seed moisture content (t₇₀), was executed through Equation 2.6 (Black et al., 2006).

$$\text{Equation 2.6: } t_{70} = M - \ln [-\ln (50/100)] / B$$

A quadratic curve was fitted to the data for seedling emergence for the seeds stored at 14% seed moisture content, using Equations 2.7 and 2.8, for perennial ryegrass and tall fescue respectively, to predict the thermal time requirement for seedling emergence (= seed viability) to drop to 50%. The fitted data were analysed with GenStat[®] and differences in mean estimates of thermal time requirement between seed lines analysed using t-tests.

$$\text{Equation 2.7: } Y = -7E-07x^2 + 0.0009x + 97.656$$

Equation 2.8: $Y = -9E-07x^2 + 0.0025x + 91.365$

where Y is seedling emergence (50%) and x is the thermal time accumulated (°Cd).

4.3 Results

Prior to storage (i.e. time 0), there were no significant ($P < 0.05$) differences between the grass lines with respect to percentage of seedling emergence and endophyte viability (Table 4.2). Perennial ryegrass seedling emergence was 85% and 83% for seeds at 10% and 14% SMC, respectively (Table 4.2). Seedling emergence for tall fescue was 90% and 87% for seeds at 10% and 14% SMC, respectively. In perennial ryegrass, the endophyte infection frequency was 90% and 87% for seeds at 10% and 14% SMC, respectively while in tall fescue it was 100% for seeds at both 10% and 14% SMC (Table 4.2).

Table 4.2 Seedling emergence (%) and viable endophyte frequency (%) for tall fescue and perennial ryegrass seeds at 10 % and 14% SMC at time 0.

Seed moisture content	Tall fescue		Perennial ryegrass	
	Seedling emergence (%)	Endophyte (%)	Seedling emergence (%)	Endophyte (%)
10% SMC	90	100	85	90
14% SMC	87	100	83	87

Seedling emergence after storage

There was no significant ($P < 0.05$) difference in seedling emergence between the two grass lines from seeds stored at 5°C and 10°C regardless of their SMC and storage period (Figure 4.1a and b), with the percentage emergence generally being >90% over the 12 month period. For seeds stored at 20°C, there was no significant ($P < 0.05$) difference in perennial ryegrass seedling emergence regardless of their SMC and storage period (Figure 4.1c). However, after 12 months, the seedling emergence of the tall fescue line from seed stored at 10% SMC, was significantly ($P < 0.05$) greater (97%) than for seed stored at 14% SMC (61%) (Figure 4.1c). This was not observed for the other storage periods investigated. For seed stored at 30°C, there was no significant ($P < 0.05$) difference in seedling emergence for both grass lines stored at 10% SMC over the 12 month period (Figure 4.1d). However, when seeds were stored at 14% SMC, seedling emergence in perennial ryegrass was significantly ($P < 0.05$) higher than that of tall fescue. Perennial ryegrass seedling emergence at 14% SMC was close to 82% after 6 months of storage, but had decreased to 40% by 9 months (Figure 4.1c). After 12 month of storage, seedling emergence had further decreased, being close to 29% for perennial

ryegrass and close to 11% for tall fescue (Figure 4.1c). Tall fescue seed bags for the 6 and 9 months sampling went missing from the incubator and therefore data are not available.

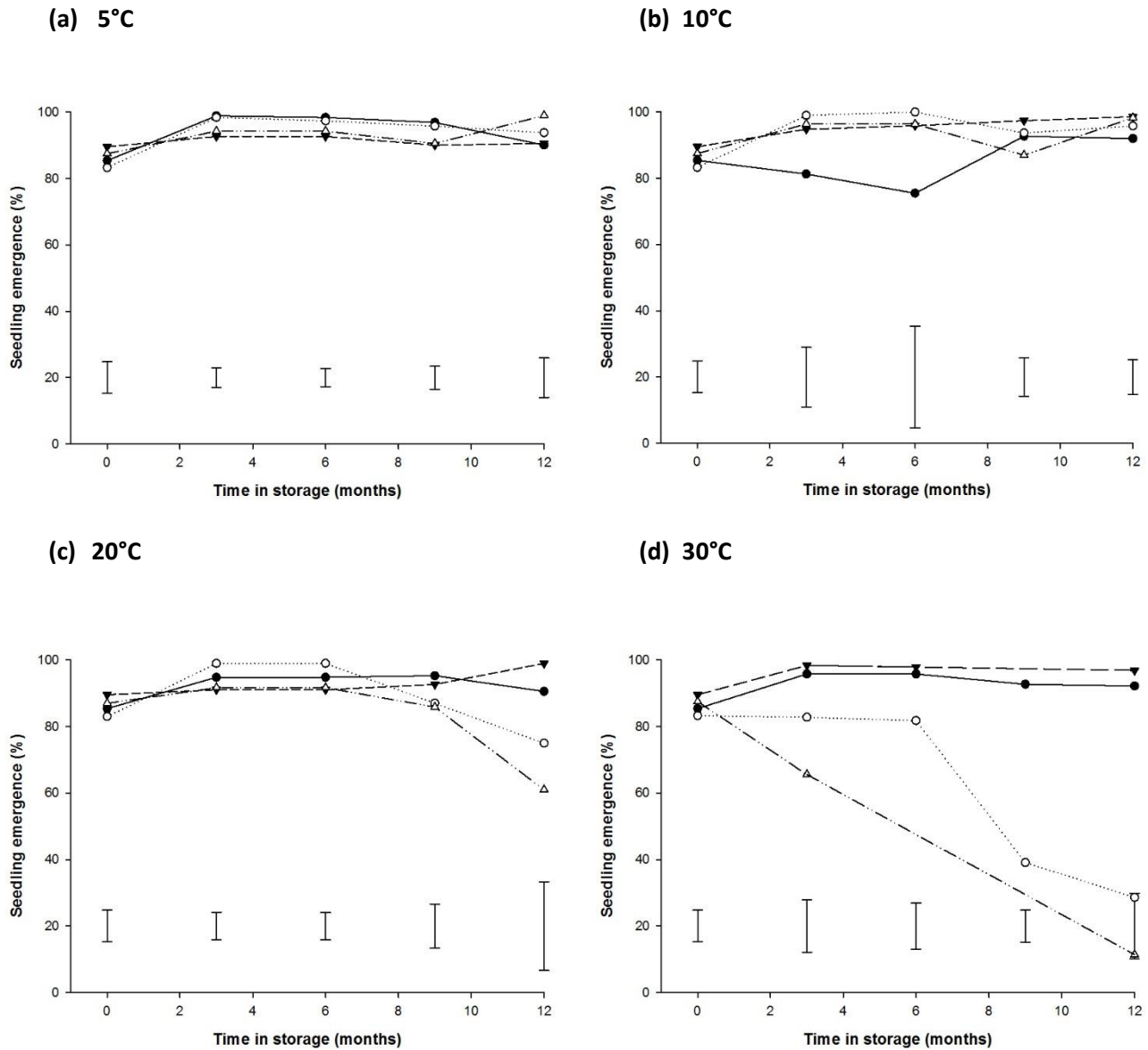


Figure 4.1 Seedling emergence for perennial ryegrass (○) and tall fescue (Δ) in seeds stored at 5°C (a), 10°C (b), 20°C (c) and 30°C (d) at 10% (solid) and 14% (open) seed moisture content for 12 months. Vertical bars indicate the LSD (at the 0.05 level of probability) for each storage time.

Endophyte viability in stored seed

Endophyte viability for the tall fescue line remained stable at nearly 100% for the entire 12 month experimental storage period at both 5°C and 10°C irrespective of SMC (Figures 4.2a and b). For the first six months of storage, for seed of both grass lines stored at 5°C and 10°C the percentage of viable endophyte in tall fescue did not differ from that in perennial ryegrass (Figures 4.2a and b). This trend changed for both assessments after 9 and 12 months storage whereby there was a significantly ($P<0.05$) greater percentage of viable endophyte in the tall fescue line compared to the perennial ryegrass for both SMCs (Figures 4.2a and b).

For both seed lines, the two warmer storage temperatures of 20 and 30°C were far less favourable to endophyte survival than the cooler temperatures of 5 and 10°C (Figure 4.2). For example, at 20°C neither of the seed lines at either SMC harboured an endophyte percentage (%) above the industry set threshold of 70% after nine months storage (Figure 4.2c) and this percentage dropped even more significantly for seed stored at 30°C with only the tall fescue seed line at 10% SMC lasting for three months above this threshold (Figure 4.2d). At 5 and 10°C, the host was more important than SMC with respect to endophyte survival, while for the higher storage temperatures of 20 and 30°C the SMC was more influential (Figure 4.2). After six months storage at 20°C, there was a greater percentage of viable endophyte (80%) in the perennial ryegrass seed that had been stored at 10% SMC compared to those seeds stored at 14% SMC (42%) and the trend was similar for tall fescue with a greater percentage of viable endophyte found in seed stored at 10% SMC (100%) than 14% SMC (80%) (Figure 4.2c).

In perennial ryegrass, the percentage of viable endophyte was significantly ($P<0.05$) higher in seeds stored at 10% SMC than in seeds stored at 14% SMC after six months storage at 20°C but after 12 months storage no viable endophyte was recorded in seedlings of either grass line (Figure 4.2c). For seeds stored at 30°C, the endophyte infection did not differ significantly ($P<0.05$) between species and SMC for each of the assessment times over the 12 months (Figure 4.2d). Neither of the seed lines at either SMC contained any viable endophyte at six months storage at 30°C.

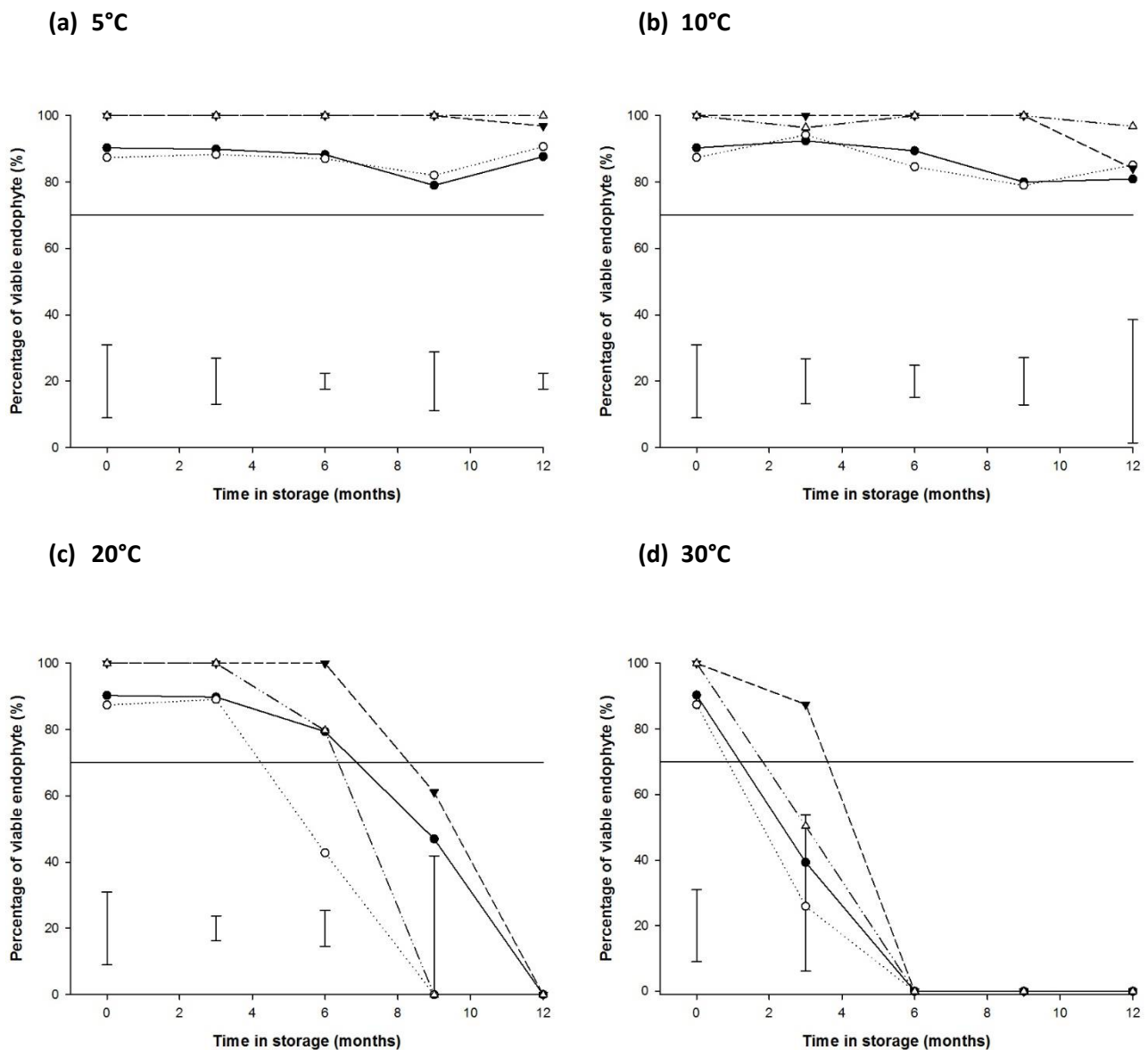


Figure 4.2 Percentage of viable endophyte for perennial ryegrass (○) and tall fescue (Δ) in seeds stored at 5°C (a), 10°C (b), 20°C (c) and 30°C (d) at 10% (solid) and 14% (open) seed moisture content for 12 months. Vertical bars indicate the LSD (at the 0.05 level of probability) for each storage time. Horizontal line indicates the 70% industry threshold.

4.3.2 Thermal time requirements for seed and endophyte survival

Thermal time requirement to reach 50% emergence (assumed to equate to 50% seed viability) was 8918°Cd for perennial ryegrass seeds stored at 14% SMC, and 8381°Cd for tall fescue seeds stored at

the same SMC (Figure 4.3). Seeds of both plant species stored at 10% SMC, did not lose viability during the 12 months storage and therefore a curve was not fitted to the data.

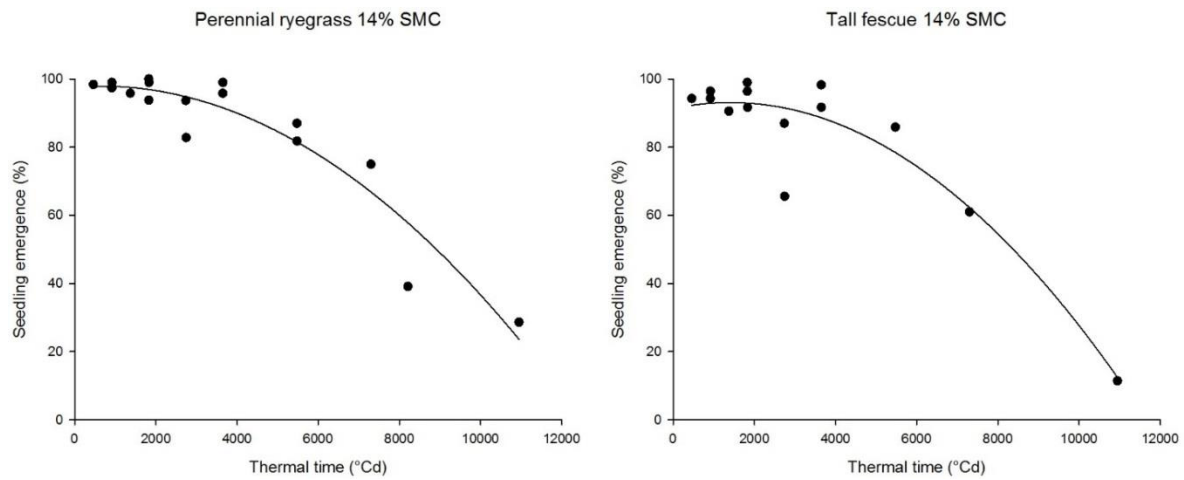


Figure 4.3 Seedling emergence of perennial ryegrass and tall fescue seeds stored at 14% seed moisture content plotted against thermal time.

The thermal time requirement for the endophyte viability to drop to 50% was 3195°Cd for perennial ryegrass seeds stored at 10% SMC, and 3198°Cd for seeds stored at 14% SMC (Figure 4.4). For tall fescue seeds stored at 10% SMC, it was 5094°Cd while for tall fescue seeds stored at 14% SMC it was 4380°Cd (Figure 4.4). In perennial ryegrass, there was no significant difference in thermal time requirement to reach 50% endophyte viability between seeds stored at 10% SMC and 14% SMC. However for tall fescue seeds stored at 10% SMC, the thermal time requirement was higher than in seeds stored at 14% SMC.

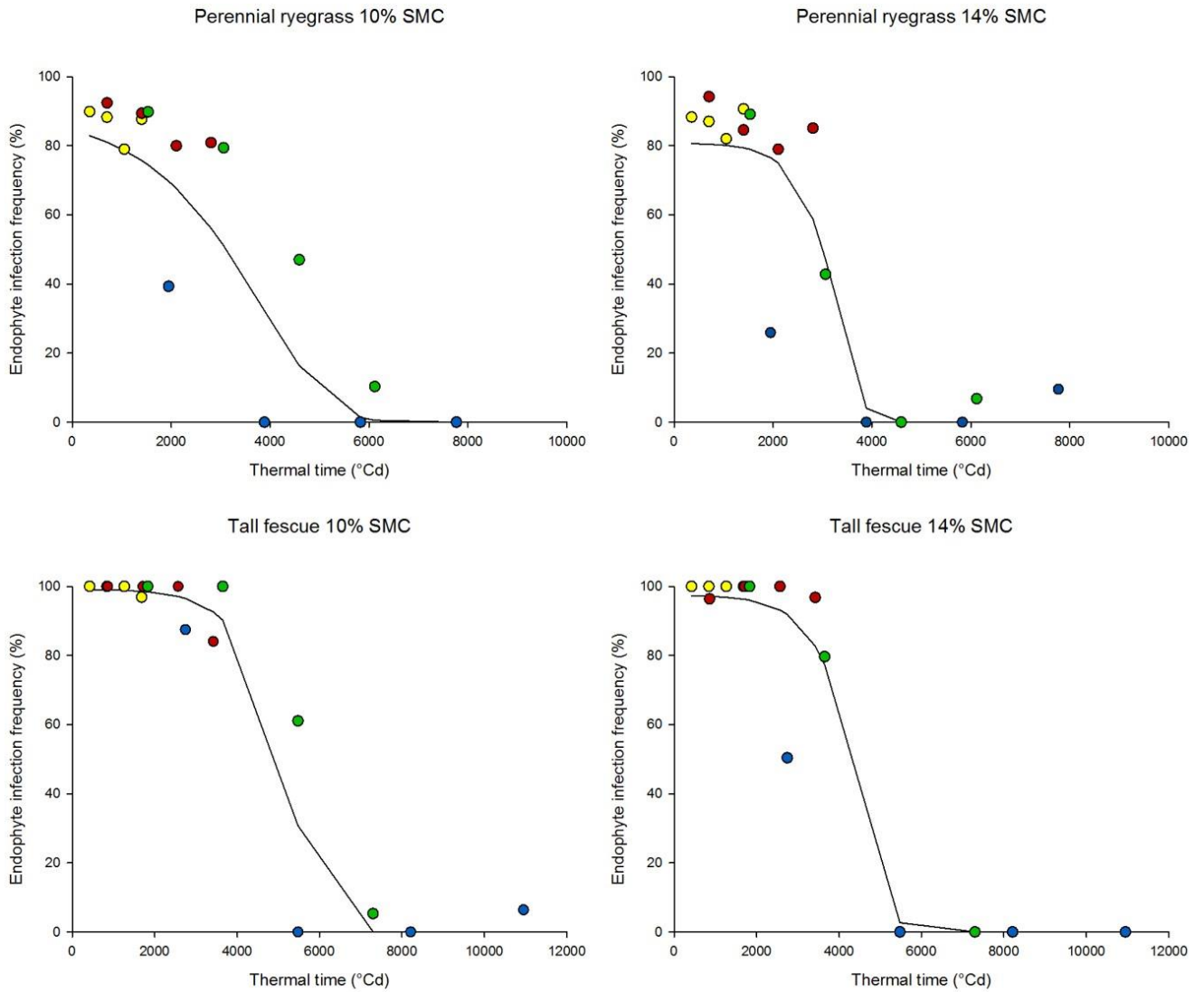


Figure 4.4 AR501 infection frequency in perennial ryegrass and tall fescue seeds stored at 10% and 14% seed moisture content plotted against thermal time. Yellow circles are for seeds stored at 5°C, red circles are for 10°C, green circles are for 20°C and blue circles are for 30°C.

4.4 Discussion

This study demonstrated that high temperature, seed moisture content and length of storage negatively influenced seed germination and AR501 viability in stored perennial ryegrass and tall fescue seeds. These results are in agreement with those previously obtained by Gundel et al. (2009), Hill and Roach (2009), Hume et al. (2011), Rolston et al. (1986) and Welty et al. (1987), all of whom reported that endophyte viability declined faster than seed viability and that loss of both seed and endophyte viability was accelerated by higher temperatures and/or higher seed moisture content.

In this present study, the two seed moisture content treatments did not affect grass seedling emergence or AR501 viability when seeds were stored at 5°C and 10°C for 12 months. On average, for these storage environments, seedling emergence was above 75% and endophyte viability above 80% throughout the 12 months of storage. Welty et al. (1987) reported that tall fescue and perennial ryegrass seeds with a 10% seed moisture content and stored at 10°C maintained their germination, but that endophyte viability decreased faster in tall fescue than in perennial ryegrass. The results of this present work are in line with those by Welty et al. (1987) for seedling emergence, but not for endophyte viability, which in the present study, did not decrease over the 12 months of storage. This difference is likely to be associated with endophyte species. Welty et al. (1987) used perennial ryegrass infected with *E. lolii* and tall fescue infected with *E. coenophialum* and in generally the rate of loss of *E. coenophialum* was higher than that of *E. lolii*. In contrast, in this present work, while two endophyte associations were investigated, there was only one endophyte strain. While AR501 is originally from tall fescue and it was inoculated into a perennial ryegrass line, a difference in viability after storage for 12 months was not expected. Previous viability differences reported have involved either different species (Welty et al., 1987) or strains of the same endophyte (Hill & Roach, 2009).

For storage at 20°C and at both seed moisture contents, seedling emergence remained high for 9 months, but decreased slightly after that. On the other hand, AR501 viability in both grass species was held at a high level for only 6 months, and then declined rapidly with further storage. These results differed from those by Welty et al. (1987), who reported an endophyte viability in perennial ryegrass and tall fescue seeds stored at 20°C of 40% and 53% respectively after 12 months of storage. They are however consistent with those of Rolston et al. (1986), who showed a rapid decline in endophyte viability in perennial ryegrass stored in ambient conditions, where temperature ranged from 5-25°C throughout the 12 months of storage. As previously mentioned, these differences could be due to the fact that endophyte viability in storage can also vary between strains of endophyte (Welty et al., 1987).

For the 30°C storage, seedling emergence was above 90% for seeds of both species held at 10% seed moisture content; however a gradual decline occurred for seeds held at 14% SMC over the 12 months of storage. Viable endophyte infection frequency in contrast, declined markedly within the first 3 months of storage, and was completely lost after the 12 months of storage. It is likely that there was an interaction between the high temperature and the seed moisture content which affected seedling emergence for both species. Endophyte viability, however, appeared to be affected predominately by storage temperature with SMC having a relatively smaller influence. These results are in agreement with those by Welty et al. (1987) who showed high levels of seed

germination for seeds held at 10% moisture content and stored at 30°C. These authors also suggested that when seed moisture content exceeds 10%, temperature and moisture content will interact and will influence seed and endophyte survival.

In New Zealand and Australia, for seeds containing novel endophyte to be traded, an endophyte viability of >70% is the agreed industry standard (Hume & Barker, 2005). Therefore, for commercial purposes, the results of this experiment have important implications for seed storage management. The use of thermal time models allows prediction for time of storage, for which the viable endophyte in seed would be still exceeding the commercial threshold. For instance, at low temperature ($\leq 10^{\circ}\text{C}$) and for seeds stored at 10% SMC, the model predicted that AR501 viability would remain above 70% for 13 months longer in tall fescue seeds than in perennial ryegrass seeds. When seeds were stored at 14% SMC, endophyte viability was predicted to be above 70% for nearly 8 months longer in tall fescue seeds than in perennial ryegrass seeds. Thermal time requirement for seedling emergence to drop to 50% of the starting point was not significantly different between species when seeds were stored at 14% SMC. In ideal storage conditions (i.e. low temperature and low relative humidity) the time predicted for seedling emergence to drop to 50% of the starting point was approximately after 5 years of storage. Thermal time requirements for tall fescue and perennial ryegrass from sowing to germination has been reported previously (Monks et al., 2009; Zhang et al., 2013), but thermal time requirement in storage conditions has not been investigated before. In Canterbury (NZ), under normal harvest practices, the SMC for perennial ryegrass seeds is around 12-14% (P. Rolston personal communication, 2016) and to achieve a SMC of 10%, seeds have to be artificially dried and stored at a low RH of 38% or stored in moisture proof bags (i.e. aluminium foil-polyethylene laminate bags). However, these post-harvest practices have higher costs and therefore in the longer term, a plant breeding solution such as improvement in the robustness of the new association, and intensive selection, to improve endophyte-host plant genotype viability in storage, would be desirable.

In general, in this study, viable endophyte infection frequency for an endophyte isolated from tall fescue was higher in tall fescue than in perennial ryegrass across all storage temperatures and seed moisture contents. The number of degree days for AR501 infection frequency to drop 50% of the starting point was also greater in tall fescue than in perennial ryegrass. This result was not unexpected as tall fescue is the original host for AR501, and specific plants and endophyte associations express variations in compatibility (Hill & Roach, 2009; Johnson et al., 2003).

4.5 Conclusion

Overall, the results of this experiment did not entirely support the hypothesis that different temperatures storage conditions and seed moisture content would negatively affect the seedling emergence and AR501 viability in perennial ryegrass and tall fescue seeds stored for one year. In both associations, endophyte in seeds stored at high seed moisture (14%) content and at the highest temperature (30°C) had died after 12 months of storage. Storage at lower temperature (5 or 10°C) maintained endophyte viability throughout the 12 months without a major interaction with seed moisture content. In addition to that, the thermal time model would be a tool for predicting endophyte survival in different temperatures at different SMC.

To maintain viable endophyte in stored seed at a high level, it is crucial to take the management of storage into account. For instance, temperature and or/humidity controlled storage facilities and or/ moisture proof packaging could be used to avoid endophyte loss in case of seeds transported to warmer and humid regions (Hume et al., 2011). Rolston et al. (1986) showed that seed of <11.3% SMC stored in moisture proof packaging, maintained viable endophyte for at least 6 months when stored in ambient conditions. When seeds are held at appropriate low temperature and relative humidity, seed viability and specific endophytes can be potentially maintained for a long period (Hume et al., 2011). Protection of the integrity of novel endophyte technology and longevity during seed storage has commercial appeal and is highly desired by seed companies that develop or license forage cultivars containing these novel endophytes (Hill & Roach, 2009).

Chapter 5

The effect of seed production management factors on AR501 transmission and subsequent viability under storage within perennial ryegrass and Italian ryegrass seeds

5.1 Introduction

Forage grass cultivars in New Zealand (NZ) are often intentionally infected with asexual *Epichloë* endophytes (Johnson et al., 2013). These beneficial fungal species form symptomless, mutualistic associations with their plant hosts and are found only within the plant's tissues, being completely reliant on their host for nutrients, protection and dissemination (Johnson et al., 2013; Schardl et al., 2004). These endophytes are exclusively vertically transmitted through seeds (Gundel et al., 2011) and incorporated into elite grass cultivars as they produce secondary metabolites that act as feeding deterrents to a number of insect pests (Latch, 1993; Popay & Bonos, 2008). In order to achieve efficacy in the field, grass seed products artificially infected with these selected endophytes need to contain a high frequency of viable endophyte that is true-to-type (Rolston & Agee, 2007). To achieve this high level of product quality it is imperative for seed producers and seed companies to fully understand the impacts of grass seed management practises utilised within the industry and adapt or remove practices that have the potential to negatively impede vertical endophyte transmission.

In NZ, the average seed yield for perennial (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) is 1700 kg/ha and 1750 kg/ha, respectively (Chynoweth et al., 2015). Seed yields have increased, on average, by 36 kg/ha/year and 46 kg/ha/year for perennial and Italian ryegrass, respectively, in the last decade due to the adoption of new technologies for seed crop management. These technologies include the introduction and adoption of a plant growth regulator (PGR), improved fungicides with greater efficacy, and adoption of enhanced management protocols for nitrogen fertiliser use and improved irrigation management (Chynoweth et al., 2015). For example, trinexapac-ethyl (sold as Moddus® in NZ), a PGR or plant exogenous hormone, is now used by more than 90% of grass seed growers (Chynoweth et al., 2010a) to prevent lodging of the seed crop by shortening the stem of reproductive tillers and allowing improved floret site utilisation, therefore substantially increasing seed yield (Chastain et al., 2003; Rolston et al., 2010). However, the effect of PGR on endophyte transmission and viability has not been thoroughly investigated, although Rolston and Agee (2007) have suggested this chemical is innocuous in this respect. Nitrogen (N) fertiliser is important for grass seed production (Kemp et al., 1999) and its application has a significant impact on seed yields (Chynoweth et al., 2010a; Hampton et al., 1987). N is required by the plant for fertile

tiller production and thus inflorescence numbers (Rolston et al., 1998). The optimum rate for N application in Canterbury is 185 kg/ha, usually applied in spring and split into three separate applications to avoid volatilisation losses and/or leaching (Chynoweth et al., 2010a; Rolston et al., 2008). The impact of N fertiliser on endophyte transmission is poorly understood. Stewart (1986) showed that application of 100 kg N/ha to perennial ryegrass at the spikelet initiation stage (early September) reduced endophyte concentration in the harvested seed, and suggested that this response may have occurred because after the N application, the increase in tillering was greater than endophyte growth, resulting in lower endophyte transmission. Rasmussen et al. (2007) reported that endophyte and alkaloid concentration in perennial ryegrass was reduced by 40% when plants received a high N application of more than 200 kg/ha compared to plants that received a low N application of 50 kg/ha.

Fungicides are also commonly used in grass seed crops in NZ to control stem rust (caused by the pathogen *Puccinia graminis*) (Rolston et al., 2002) and blind seed disease (caused by *Gloeotinia temulenta*) (Chynoweth et al., 2012). Stem rust is a foliar disease that can reduce nutrient translocation during seed head development while blind seed disease is seed borne where the fungus responsible can enter the open flower and kill the developing seed embryo (Neill & Hyde, 1943). Plant protection products labelled for the control of both these diseases include the triazole (demethylation inhibitors or sterol biosynthesis inhibitors) and strobilurin (QoI inhibitors) group of fungicides (Rolston et al., 2009). These fungicides are generally applied to grass seed crops two or three times during the period from seed head emergence to the early stage of flowering (Rolston et al., 2009). The effect of a range of fungicides on endophyte survival was reported by Harvey et al. (1982), who observed a reduction in the proportion of viable endophyte in perennial ryegrass seed after the application of the triazole fungicide, propiconazole, at a high rate. However, Rolston et al. (2002) showed that many triazole and strobilurin fungicides applied at moderate rates did not affect the transfer of *Epichloe festucae* var. *lolii*, strain AR1 in perennial ryegrass from mother plants to seed.

This study was undertaken to determine whether N, fungicide and/or PGR application would affect the transmission frequency of *Epichloë* FaTG-3, strain AR501 in perennial and/or Italian ryegrass. The hypotheses were that (i) seed production management factors would not affect the endophyte transmission frequency in both grass species and (ii) endophyte viability from seed harvested from plants previously exposed to these management factors would decrease in seeds stored under ambient conditions for one year.

5.2 Materials and Methods

5.2.1 Seed production management factors

Plants of a tetraploid perennial ryegrass line KLP 903 (derived from crosses of the cultivars Banquet, Banquet II and Bealey) from plots sown in April 2012 and a tetraploid Italian ryegrass line KLM 501 (derived from crosses of the cultivars Feast II, Concord and Cordura) from plots sown in September 2012, intentionally infected with *Epichloë* FaTG-3, strain AR501 were used in this experiment. Plants were hand transplanted to a field plot at AgResearch farm, Lincoln, NZ in September 2013. The area had been previously established with subterranean clover (*Trifolium subterraneum* L.) for two years prior to this experiment, and had been fallowed for six months before the introduction of the ryegrass plants. The experiment was set up as a randomised block design with four blocks and eight plots. Each 1.5 m² plot contained six plants (three perennial and three Italian ryegrass) and in total the experiment had 192 plants (96 plants of both perennial and Italian ryegrass).

The treatments included combinations of N (urea at 46% N), fungicides, namely Amistar® (a.i. 250 g/L azoxystrobin, Syngenta Crop Protection Ltd.) and Proline® (a.i. 250 g/L prothioconazole, Bayer Crop Science Ltd.) and a PGR, Moddus® (a.i. 250 g/L trinexapac-ethyl, Syngenta Crop Protection Ltd.) (Table 5.1). The first N treatment of 30 kg/ha was applied by hand at the beginning of October 2013. The higher N rate of 150 kg/ha was split into three applications to avoid the potential for volatilisation losses and/or leaching (Chynoweth et al., 2010b). The first 30 kg/ha was applied on the 9th October, the second on the 30th October, when 70 kg/ha was applied, and the third application of 50 kg/ha in mid-November. Moddus was applied through a motorised boom sprayer with 200 L of water/ha, using 110°.04 fan nozzles at 300 Kpa of pressure on the 21st November 2013, when the second node was observed (Zadoks Growth Stage (GS) 32; Zadoks et al. (1974)). The fungicide treatments were split into two applications, the first at late head emergence (GS 59), which was at the beginning of December 2013, and the second at the end of flowering (GS 69), which was close to the end of December 2013.

Table 5.1 Treatments, consisting of various combinations of fungicides, a plant growth regulator and nitrogen fertiliser applied to perennial and Italian ryegrass seed crops to investigate their impact on endophyte transmission.

Treatment number	Nitrogen application rate (kg/ha)	Fungicide application rate (L/ha)		Plant growth regulator application rate (L/ha)
		Amistar	Proline	
1	30	0.60	0	0
2	150	0.60	0	0
3	30	0.60	0.75	0
4	150	0.60	0.75	0
5	30	0.60	0	1.6
6	150	0.60	0	1.6
7	30	0.60	0.75	1.6
8	150	0.60	0.75	1.6

Seed was hand harvested from individual plants from late January to the beginning of February 2014, air dried at ambient temperature (20°C), hand threshed and stored in brown paper bags and kept in a moisture-proof plastic container at 4°C and 25% relative humidity (RH). Two months after threshing, 24 seeds from each plant were hand sown into trays containing seedling mix (120 L Southland peat, 80 L pumice) with the following fertiliser additions per cubic meter: 4 kg Osmocote® exact mini (16% N, 3.5% P, 9.1% K), 8 kg dolomite lime and 2 kg Hydroflo® (granular wetting agent manufactured by Everris Australia Ltd.). Trays were placed in a glasshouse at approximately 20°C and watered as required. Six weeks after sowing two randomly chosen tillers per plant were assessed to determine their endophyte infection status using the TPIB technique (Simpson et al., 2012). The remaining seeds, from endophyte infected plants only from each plot, were bulked (stored together in the same brown paper bag) as described earlier and used in the endophyte storage trial (see below). Statistical analysis was performed using GenStat (VSN International 2013. *GenStat for Windows* 16th Edition. VSN International, Hemel Hempstead, UK). Analysis of variance (ANOVA) and Fisher's unprotected test of least significant difference (LSD; $P < 0.05$) were performed to compare the main treatment effects of seed production management practices and their interactions with endophyte transmission frequency.

5.2.2 Endophyte storage trial

After nine months in storage, seeds from the bulked sample described earlier were packaged in brown paper bags and stored for one further year at 20°C and 44% relative humidity. The experiment was set up as a split plot design with four blocks and the layout was replicated from the field trial. Twenty-four seeds from each sample were planted every three months, using the method previously described, so that in total seeds were planted four times at an interval of three months during one year. The endophyte infection status of two tillers per plant was assessed six weeks after sowing

using the TPIB technique as mentioned previously. ANOVA enabled examination of main effects and interactions between the three factors. The unprotected least significant difference (LSD; $P < 0.05$) test was used to compare means where appropriate. An exponential decay curve was fitted to endophyte infection frequency data from both plant species to predict the decline in endophyte infection frequency per month.

5.3 Results

5.3.1 Seed production management factors

None of the three seed production management factors affected the endophyte infection frequency in perennial ryegrass (Table 5.2) while only one factor, increasing N from 30 to 150 kg/ha, significantly ($P < 0.05$) affected the endophyte infection frequency in Italian ryegrass (Table 5.2). For this grass species, the plants that received the higher N application rate had a 14% higher endophyte infection frequency compared to plants receiving the lower N application (Table 5.2).

Table 5.2 Main effects of nitrogen fertiliser, a plant growth regulator and fungicides applied to perennial and Italian ryegrass crops on endophyte transmission from mother plant to seed.

Treatments	Percentage of endophyte infected seedlings grown from seed originally harvested from plants that were exposed to various combinations of agricultural chemicals	
	Perennial ryegrass	Italian ryegrass
30 kg N/ha	82.2	70.8
150 kg N/ha	88.4	84.9
LSD (5%)	8.3	11.1
Significance	NS	*
Without Moddus	84.2	82.2
Moddus	86.4	73.6
LSD (5%)	8.3	11.1
Significance	NS	NS
Amistar	86.9	73.5
Amistar plus Proline	83.7	82.3
LSD (5%)	8.3	11.1
Significance	NS	NS

NS=Not significant; *= $P < 0.05$; N=Nitrogen

Examination of the interactions between the three seed product management factors showed that there was no significant interaction among them for perennial ryegrass (Table 5.3). For Italian ryegrass, there was a significant interaction for N versus Amistar plus Moddus (Table 5.4). Here, the endophyte infection frequency was significantly ($P<0.05$) higher in plants that received 150 kg N/ha compared to plants that received 30 kg N /ha (Table 5.4). This was not observed in the absence of Moddus or for the combinations of Moddus plus Amistar plus Proline (Table 5.4).

Table 5.3 Interaction among various combinations of fungicides, a plant growth regulator and nitrogen fertiliser applied to a perennial ryegrass crop on endophyte transmission (%) from mother plant to seed.

Endophyte transmission (%) from mother plant to seed				
Treatments	Moddus plus Amistar	Moddus plus Amistar plus Proline	Amistar	Amistar plus Proline
30 kg N/ha	88.1	84.2	81.2	75.5
150 kg N/ha	89.6	83.7	88.8	91.5
Significant effects	NS	NS	NS	NS

NS=Not Significant; *= $P<0.05$; N=Nitrogen

Table 5.4 Interaction among various combinations of fungicides, a plant growth regulator and nitrogen fertiliser applied to an Italian ryegrass crop on endophyte transmission (%) from mother plant to seed.

Endophyte transmission (%) from mother plant to seed				
Treatments	Moddus plus Amistar	Moddus plus Amistar plus Proline	Amistar	Amistar plus Proline
30 kg N/ha	39.5	82.7	79.7	81.5
150 kg N/ha	89.8	82.5	85.1	82.4
Significant effects	*	NS	NS	NS

NS=Not Significant; *= $P<0.05$; N=Nitrogen

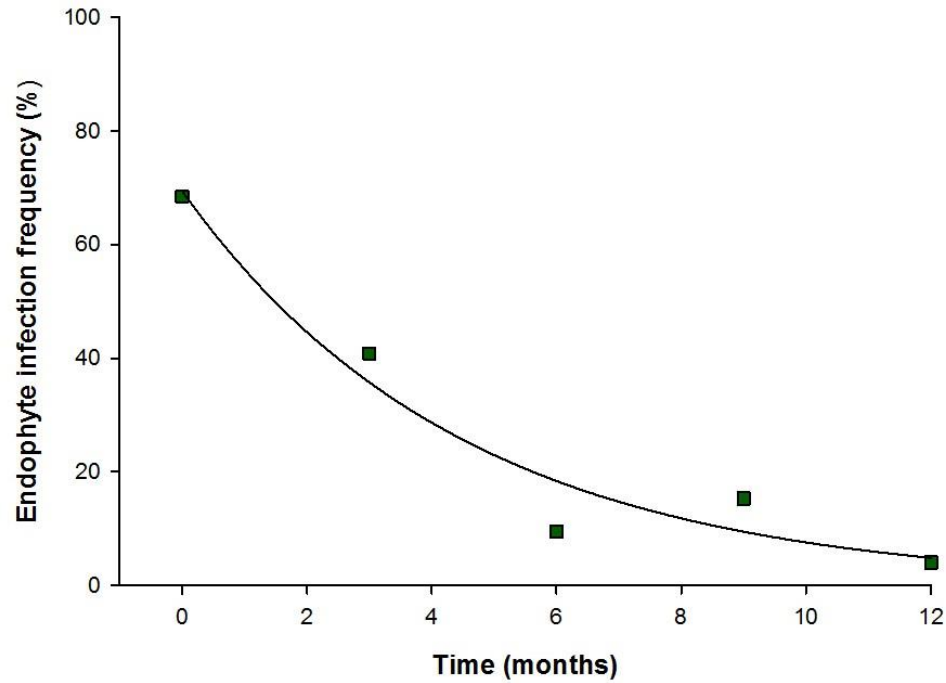
5.3.2 Endophyte storage trial

Freshly harvested seed had a viable endophyte infection frequency of 76.4% and 84.6% for perennial and Italian ryegrass, respectively. Over the course of the storage trial, the decline in viable endophyte followed a similar trend for both grass species. At the start of the experiment (time 0 as depicted in Figure 5.1), the decline was more prominent in Italian ryegrass with a decrease of 24% (to a viable

endophyte infection frequency of 60.6%) compared to a decrease of 7.9 % for perennial ryegrass (to a viable endophyte infection frequency of 68.5%) (Figure 5.1). The infection frequency continued to decline in both grass species until the end of the experiment (after 12 months in storage) when the final infection frequencies were 4% and 5.5% for perennial ryegrass and Italian ryegrass, respectively (Figure 5.1).

Exponential decay curves, used to predict the decline in endophyte infection frequency per month, were developed for perennial ryegrass ($Y = 51.7e^{-0.183x}$, $R^2 = 0.63$) and Italian ryegrass ($Y = 65.9e^{-0.221x}$, $R^2 = 0.86$) where x is the number of months of storage and fitted to the graphs in Figure 5.1.

(a) Perennial ryegrass



(b) Italian ryegrass

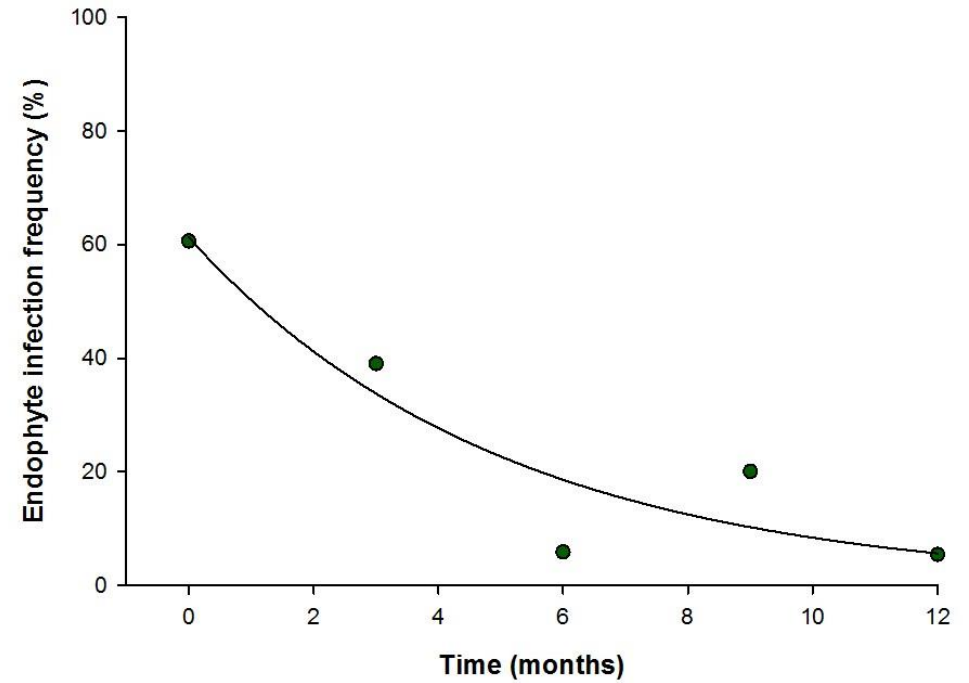


Figure 5.1 Viable endophyte infection frequency in seeds of (a) perennial ryegrass and (b) Italian ryegrass stored for 12 months at 20°C and 44.3% relative humidity. Equations for fitted curves were developed for (a) perennial ryegrass, $Y = 65.9e^{-0.221x}$, $R^2 = 0.86$ and (b) Italian ryegrass, $Y = 51.7e^{-0.183x}$, $R^2 = 0.63$.

5.4 Discussion

The majority of the seed production management factors assessed in this study did not affect the vertical transmission of *Epichloë* FaTG-3, strain AR501 from mother plants to seed in either perennial or Italian ryegrass. This included the PGR Moddus, the two fungicides Amistar and Proline, and two rates of nitrogen application. This information is of great value to the grass seed industry in NZ as maximising endophyte seed transmission is a priority (Hume & Barker, 2005). An industry agreed standard of >70% endophyte infection frequency is applied to commercial endophytes in NZ, and if seed lots do not meet this standard, this results in a financial loss to seed growers or to the seed companies (P. Rolston personal communication, 2016).

Although the amount of nitrogen applied to perennial ryegrass did not affect the endophyte transmission frequency, this study showed that increasing the nitrogen application rate from 30 kg/ha to 150 kg/ha did increase the endophyte infection frequency in the progeny of Italian ryegrass plants from 71 to 85%. This was an interesting result suggesting that the plant host has an important role to play in transmission as the same endophyte strain, AR501, was utilised in both grass species. This result has not been documented in the past as previous research showed that nitrogen application rates and timing do not impact vertical endophyte transmission in novel grass-endophyte associations such as with perennial ryegrass infected with AR1 or AR37 (P. Rolston and S. Card, personal communication 2016). A study conducted at multiple locations in Canterbury that investigated whether high winter rates of nitrogen could disrupt vertical endophyte transmission found no such evidence for AR37 in three cultivars of perennial ryegrass, namely 'Commando', 'Ohau' and 'Samson' (Rolston, Kelly and Card, unpublished).

High amounts of nitrogen fertiliser have been linked to suppression of endophyte mycelial growth and alkaloid production *in planta*. For example, Stewart (1986) reported a lower alkaloid content in perennial ryegrass plants infected with *Epichloë festucae* var. *lolii* (formerly *Neotyphodium lolii*, Leuchtman et al. (2014)) receiving 100 kg N/ha compared to those receiving 0 or 40 kg N/ha while Rasmussen et al. (2007) reported a 40% reduction in endophyte and alkaloid concentration in perennial ryegrass plants receiving more than 200 kg N/ha compared with those receiving 50 kg N/ha. Contrary to these observations, Belesky et al. (1988) reported that tall fescue plants receiving 336 kg N/ha produced increased amounts of the alkaloid ergovaline compared to those receiving 134 kg N/ha. Lyons et al. (1986) suggested that higher concentrations of nitrogen would be favourable for plant development and therefore would possibly favour synthesis of endophyte alkaloids. Increased host plant growth in response to nitrogen application (e.g. more tillers; Stewart (1986)) could provide more nutrients for endophyte growth, increasing endophyte mycelial biomass and alkaloid quantity. Lane et al. (1997) showed that ergovaline alkaloids increased in perennial ryegrass plants that had

received a high nitrogen application, although these results were associated with water stress. An increase in alkaloid concentration in perennial ryegrass plants has been reported by Hunt et al. (2005). They found that nitrogen application at ambient CO₂ levels did not affect alkaloid concentration, but when the nitrogen was applied at high CO₂ levels, alkaloid concentration increased. These authors suggested that elevated CO₂ and high nitrogen availability had a positive effect on peramine and ergovaline alkaloid production.

Most of the combinations of seed production management factors did not affect the viable endophyte infection frequency in either plant species. However, an exception occurred for the combination of Amistar, low nitrogen rate and plant growth regulator application on Italian ryegrass plants. In this combination AR501 infection frequency was lower compared to the plants that had received the combination with the higher nitrogen rate. The reason for this is not clear but it is possible that an interaction occurred between the low nitrogen rate and Amistar. If this is the reason it was unexpected, as Amistar is considered to be endophyte safe (Rolston et al., 2002) at least for perennial ryegrass plants. Recently a study by Kelly et al. (2016), showed that fungicide stacking, using combinations of fungicides to achieve greater disease outcomes, with a number of triazole fungicides, including Proline, did not adversely affect AR37 endophyte transmission to seed in perennial ryegrass cultivar 'Base'. Further research is needed in order to verify whether the combination of Amistar and nitrogen, or only Amistar, affected the endophyte in Italian ryegrass plants.

The viable endophyte infection frequency in seed of both grass species stored at room temperature (20°C) was affected by the length of storage. At 20°C/44.3% RH, seeds of Italian ryegrass and perennial ryegrass lost respectively 19% and 20% of viable endophyte per month of storage (Figure 5.2a and 5.2b). These results are in agreement with those reported by Hume et al. (2011), who showed a decline in endophyte viability in perennial ryegrass seeds stored under ambient conditions in Queensland (Australia) throughout one year, where the mean annual air temperature was 20.1°C. In this present study, the seed moisture content was not measured. However, as the average relative humidity in the storage room was 44.3%, SMC would have been close to 10.7% according to (Justice & Bass, 1978). This storage response was similar to that reported in Chapter 4 (Figure 4.2c). These results also match those observed in earlier studies by Rolston et al. (1986) who suggested that seed moisture contents above 11% can potentially affect endophyte survival. Welty et al. (1987) also reported the greatest reduction in endophyte viability in seeds stored at 20°C when SMC was close to 10%. Hume et al. (2013) reported that for harvested seeds stored in ambient conditions near to the point of sale, endophyte viability rapidly declined to below 70%. Therefore, to keep the target infection level for a marketed product, it is crucial to control temperature and seed moisture content in stored seeds.

5.5 Conclusion

Overall, the results of this experiment support the hypothesis that seed production management factors such as N availability, fungicide application and PGR application do not affect the transmission of *Epichloë* FaTG-3, strain AR501, from plant to seed within perennial ryegrass. In both endophyte-plant associations there was a trend for a decrease in endophyte infection frequency when plants received a lower nitrogen application, but this was only significant in Italian ryegrass. The significant reduction in endophyte transmission in Italian ryegrass resulting from the interaction of low N vs PGR vs Amistar fungicide is unexplained. It may have been an anomaly, but the fact that this response occurred in all replicates suggests not. Storage of harvested seed in ambient conditions (20°C/44.3% RH) resulted in a fast decline in AR501 in both seed species. Therefore, to maintain the level of endophyte above 70% in seed stored in ambient conditions for one year, it is crucial that the seed moisture content is 10% or below and temperature must be below 10°C, i.e. controlled environment storage (Rolston et al., 1986). Further studies are needed in order to better understand the interaction of seed production management effects on endophyte viability.

Chapter 6

General discussion

The objective of this research was to investigate the factors which could influence the vertical transmission and survival of *Epichloë* endophyte strain AR501 in its original host species, tall fescue and within a new host species, perennial ryegrass. This latter association, termed a novel association as it was artificially developed, was created to provide broad insect deterrence via the loline alkaloids that are not found in associations between perennial ryegrass and its host-specific *Epichloë* endophytes (i.e. *E. festucae* var. *lolii*). The aim for the New Zealand seed industry is to deliver a seed product to the market with a minimum of 70% viable endophyte (Rolston & Agee, 2007). However, this has been a challenge to accomplish for the industry, due to failures in endophyte transmission, for which no specific factor has been identified as being responsible (Gundel et al., 2008). This particular scenario is made more complex as the original host of AR501 is tall fescue and moving these host-specific endophytes across host species (e.g. from tall fescue to perennial ryegrass) can result in many compatibility issues (Karimi et al., 2012; Leuchtman & Clay, 1993). This study was an investigation of the factors that may affect the infection frequency of AR501, and of the maintenance of endophyte viability in stored seeds. The following sections provide a brief general summary of the research outcomes and discuss possible future research directions.

6.1 Summary of outcomes and discussion

Environmental factors such as temperature have a major influence on fungal growth (Cooke & Whipps, 1993), including that of epichloid fungal endophytes. Previous research has shown that temperature variations can significantly influence the development of *Epichloë* endophytes *in planta* affecting their infection frequency and overall biomass within the vegetative and reproductive plant tissues (i.e. seed) (Bacon & Siegel, 1988; Ju et al., 2006) and *in vitro*. The results of this study (see Chapter 2 and Chapter 3) clearly demonstrated that temperature was one of the factors that affected the infection frequency of AR501 within both the selected perennial ryegrass breeding line and in its original tall fescue host. In particular, this study found that temperature significantly affected the concentration of AR501 endophyte mycelia and the production of alkaloids, peramine and lolines *in vivo* within the tall fescue and perennial ryegrass host plants.

The exposure of the grass-endophyte association to various controlled temperature regimes during the plant establishment stage had a major impact on the mycelial concentration of the endophyte. As shown in Chapter 2, during the first three weeks of the plant's vegetative growth the endophyte mycelia decreased compared to the concentration of mycelia during the seed emergence stage,

when seeds emerged at a temperature of 20°C. When plants were transferred from 20°C to a warmer (25/16°C day/night) or a cooler (12/6°C day/night) temperature regime, the endophyte became dormant as a result of stress caused by the temperature variation. However, with subsequent exposure to a warm temperature for a further three weeks, it became apparent that the endophyte became acclimated to the temperature stress and was able to colonise new tillers, therefore increasing endophyte infection frequency and allowing for a higher concentration of mycelia in the host. Endophyte infection frequency on the other hand, was not affected by the different temperature regimes in any stage of the plant development, indicating that when the endophyte is already present in the plant tissue, any change in temperature will not affect the endophyte frequency. This is consistent with what was subsequently found in Chapter 3, where seeds of both hosts were germinated under 10 temperature regimes, from 2 to 29°C (at 3°C increments) and the endophyte infection frequency of the germinated seedlings was not affected by temperature (Figure 3.3). The seed germination, however, was affected by the temperature regimes (Figure 3.2). While perennial ryegrass germinated well at high temperature that of tall fescue was reduced dramatically by temperatures ranging from 17 to 29°C. However the non-germinated tall fescue seeds were still viable, indicating that these temperatures had induced thermodormancy.

Fungal endophytes, like their hosts and all biological organisms, have a specific cardinal temperature for growth. The determination of the cardinal temperature for AR501 was crucial for understanding how the endophyte and its host behaved under different temperature regimes, thus allowing predictions for best sowing conditions to achieve successful pasture establishment and maximal endophyte survival. Endophyte infection frequency increased linearly as temperature rose from the base temperature until the optimal temperature was reached. The cardinal temperatures for AR501 differed between hosts, being higher in perennial ryegrass than in tall fescue (Figure 3.6 and 3.7). Cardinal temperature was also higher for perennial ryegrass than for tall fescue (Figure 3.4 and 3.5). However it is important to note that the occurrence of thermodormancy in tall fescue seeds would have interfered with the calculation of cardinal temperatures for the endophyte and tall fescue host, and it is probable that the optimal temperature for AR501 in the tall fescue host may be higher than determined in this study. The seed germination response to various temperatures is also influenced by the cultivar and the ploidy of the plant (Hill et al., 1985). As discussed in Chapter 3, a differential response in the germination of tetraploid and diploid perennial ryegrass seeds can occur. Therefore, it is important to understand how the germination of individual cultivars may respond to temperature.

Results from Chapter 2 and Chapter 3 show that to achieve successful pasture establishment and maximal endophyte survival, it is important to consider the temperature thresholds for development and growth of the endophyte and its host and their relevance for sowing time. For autumn sowing,

while soil temperatures are decreasing as sowing is delayed, the lower temperatures will probably not affect the transmission of the endophyte from the seed to the seedling. Low temperatures during sowing would however affect the concentration of the endophytes which may be a disadvantage for later autumn sowings. Each endophyte strain has an individual preference for growth temperatures, and the same endophyte may behave differently within a different host. It is clear that as the *Epichloë* endophytes depend on the host for nutrients, shelter and water, their survival and dissemination depend on the plant host success (Johnson et al., 2013; Malinowski & Belesky, 2000). Once the endophyte has successfully completed its vertical transmission, post-harvest management must be such that the endophyte survives in the seed.

From the time of crop harvest to the delivery of a product to the market, harvested seeds may be stored for at least 6 months. During storage, seed and endophyte viability can decline, with that of the endophyte declining faster than that of the seed (Hill & Roach, 2009), reducing the value of the final seed product (Rolston & Agee, 2007). Storage factors such as temperature, seed moisture content and length of storage can affect the viability of both seed and endophyte (Rolston et al., 1986; Welty et al., 1987). The results of the storage experiment (Chapter 4) clearly demonstrated that high temperature, seed moisture content and length of storage negatively influenced seed germination and AR501 viability in stored perennial ryegrass and tall fescue seeds. In this experiment, seeds were stored for 12 months under four storage temperature regimes (5°C, 10°C, 20°C and 30°C) and at two seed moisture contents (10% and 14%). In both associations, endophyte in seeds stored at 30°C and 14% SMC had died after 12 months of storage, but in seeds stored in moisture proof packaging at lower temperature (5 or 10°C), endophyte viability was maintained throughout the 12 months, with no interactions with SMC. The results of this study were similar to those previously reported by Gundel et al. (2009), Hill and Roach (2009), Hume et al. (2011), Rolston et al. (1986) and Welty et al. (1987), all of whom reported that endophyte viability declined faster than seed viability. In addition, the thermal time requirement for AR501 viability in the stored seeds was calculated as a predictor of endophyte survival. This was the first study to use a thermal time model to predict time of storage, and thus it provided valuable information for the development of new strategies for endophyte- seed associations in storage. Using this tool, it was possible to predict how long endophyte may remain viable and meet the required industry threshold (70% viable endophyte) during seed storage. For instance, at low temperature ($\leq 10^{\circ}\text{C}$) and for seeds stored at 10% SMC, the model predicted that AR501 viability would remain above 70% for 13 months longer in tall fescue seeds than in perennial ryegrass seeds. To use this tool it is crucial that cardinal temperatures for the endophyte in the host are first estimated. Results from both Chapter 4 (Figure 4.2c) and Chapter 5, (where harvested seed was stored in ambient conditions (20°C/44.3% RH)) both showed a fast decline in AR501 viability in perennial ryegrass and Italian ryegrass seeds, supporting

previous work with others endophytes reported by Hume et al. (2011) and Rolston et al. (1986). To maintain the level of endophyte above 70% in seed stored in ambient conditions for one year, it is crucial that the seed moisture content is 10% or below and temperature must be below 10°C, i.e. controlled environment storage (Rolston et al., 1986) is required.

It has been suggested that endophyte infection frequency, endophyte concentration in harvested seeds and alkaloids concentration in the plant host, may be affected by seed crop management factors, such as nitrogen (N) fertiliser (Belesky et al., 1988; Rasmussen et al., 2007; Stewart, 1986) and fungicide application (Harvey et al., 1982). However the impact of these management factors on endophyte transmission is poorly understood. In Chapter 5 it was demonstrated that none of the three seed production management factors investigated (N application rate, fungicide application and plant growth regulator application) affected the transmission of AR501 from plant to seed in perennial ryegrass. However in both perennial ryegrass and Italian ryegrass, there was a trend for a decrease in endophyte infection frequency when plants received lower N applications, but this was significant only in Italian ryegrass. The significant reduction in endophyte transmission in Italian ryegrass resulting from the interaction of low N vs PGR vs Amistar fungicide, cannot be explained (Table 5.4). It may have been an anomaly, but the fact that this response occurred in all replicates of this treatment suggests not.

6.2 Future research

In this study, the concentration of AR501 endophyte mycelia, the production of alkaloids within tall fescue and perennial ryegrass host plants and viable endophyte in stored seeds were all affected by temperature. Endophyte infection frequency in Italian ryegrass was negatively affected by the combination of Amistar, a low nitrogen application rate and a plant growth regulator application. However, in this research there were some aspects not addressed that require further investigation:

- In this study, temperature stress response was observed for the endophyte transmission within the plant hosts. Global climate change has been well discussed in the literature over the past decade. Surface temperatures are predicted to increase by up to 3.6°C by the next century (IPCC Climate Change 2007) and these global changes are known to impact ecosystems such as plants and other organisms (Compant et al., 2010). To overcome the difficulties of temperature change likely to be produced in the near future, including the *Epichloë* endophyte's symbiosis with its host, an investigation of new endophyte strains with either cold or heat- tolerance obtained via selection or mutation is required.
- In this study the occurrence of thermodormancy in tall fescue seeds influenced the calculation of optimal and maximal temperature for AR501. This experiment, which used a

thermogradient plate to provide the range of temperatures for germination, needs to be repeated. As the thermodormancy was heat induced, using a dormancy breaking pre-treatment (ISTA 2016) is not an option. After the pre-determined numbers of days allocated for germination to occur, non-germinated seeds will need to be checked to determine that they are “fresh ungerminated” (ISTA 2016) and assessed for viability using a tetrazolium test (ISTA 2016). The addition of the numbers of viable (but dormant) seeds to the number which did germinate will provide the data for the seed optimal and maximal temperature calculations. However, as the tetrazolium test is destructive to the seed, there would be no endophyte data. To overcome this issue, splitting the non-germinated seeds in two samples would be required, one sample for the tetrazolium test and the other one for the growing medium test (ISTA, 2016), to determine germination percentage based on seedling emergence followed by the tissue-print immunoblot (TPIB) procedure (Simpson et al., 2012) to detect viable endophyte infection frequency.

- In this study, the combination of the seed production management factors Amistar fungicide, a low nitrogen application rate and plant growth regulator application, impacted negatively on AR501 infection frequency in Italian ryegrass seeds. These results were unexpected as Amistar is considered to be endophyte safe (Kelly et al., 2016; Rolston et al., 2002). Rolston and Agee (2007) have suggested the plant growth regulator use does not affect endophyte transmission and viability, but this has not been thoroughly investigated. Therefore, future studies are required in order to verify whether these results can be repeated, and if so whether the combination of Amistar, nitrogen and plant growth regulator, or only Amistar was the cause of the endophyte reduction in Italian ryegrass.
- As part of this study, a comparison of the transmission of AR501 with GFP (green fluorescence protein) from the maternal plant to seeds in two perennial ryegrass cultivars and one tall fescue cultivar was planned (Appendix A). The transformation of the AR501 strain with eGFP expression was successful, as GFP was expressed in the transformed AR501 mycelium in culture and *in planta*. The aim of this study was to track the endophyte during four phases of plant development (i.e. vegetative tillers, reproductive tillers, flowers and seeds), and the endophyte was successfully transmitted from seedlings to vegetative tillers. However, unfortunately plants did not receive the correct environmental triggers required to allow them to become reproductive, and therefore flowers and seeds were not produced. The reason for that is not clear, but it could have been due to a lack of light during the period of time that plants were growing in the glasshouse, after having been vernalized. The most challenging part of this study was successfully done (i.e. transformation of AR501 with GFP expression). Therefore, there is an opportunity to use this tool to continue the tracking of

AR501 throughout these four phases of plant development to investigate seed dissemination and long-term survival of the transformed endophyte.

Appendix A

Genetic transformation of fescue endophyte strain AR501 to express the green fluorescent protein gene (GFP) and visualization in host plants

This study was undertaken to compare the transmission of *Epichloë* FaTG-3, strain AR501 expressing GFP (green fluorescence protein) from the maternal plant to seeds in two perennial ryegrass cultivars and one tall fescue cultivar. The hypothesis was that AR501 would transmit from maternal plant to seed at the same frequency within perennial ryegrass as it does in tall fescue.

A.1 Material and Methods

Preparation of protoplast and transformation

Epichloë FaTG-3, strain AR501 was isolated from leaf sheaths of a tall fescue cultivar from the AgResearch Ltd plant collection and propagated on potato dextrose agar (PDA, Difco™ Becton, USA). Flasks with 50 ml of Minimal media (Kulkarni & Nielsen, 1986) were prepared and inoculated with 150µl of macerated mycelium of AR501 grown on PDA plates. Incubation was performed in a 20°C room for 1 week with gentle agitation (140 rpm).

Preparation of AR501 protoplasts was conducted using a protocol modified from that originally described by Fleetwood et al. (2007) and Young et al. (1998). Mycelium was harvested by filtration using Steritop GP Express Plus® 0.22µm, USA, 250 mL a solution of 450 mg lysis enzyme (Sigma, lysing enzyme from *Trichoderma harzianum*, L1412) and 30 mL OM Buffer (1.2M MgSO₄·7H₂O, 10mM Na₂HPO₄, pH 5.8; Young et al. (1998)) was used to digest the cell walls overnight at 30°C with shaking (100 rpm). The protoplast sample was then filtered through three layers of sterile mira cloth and 2x 1 mL of ST Buffer (0.6M Sorbitol, 100mM Tris-HCL pH 8.0; Young et al. (1998)) was added to the protoplast sample which was then centrifuged at 2800 rpm for 20 minutes. The protoplast sample from the interface formed was harvested using a 1 mL wide open mouth pipette tip. A solution of STC Buffer (1M Sorbitol, 50mM Tris-HCL pH 8.0, CaCl₂·6H₂O) was added up to 10 ml to the protoplast sample which was then centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded, 5 ml of STC Buffer was added and the pellet was suspended by centrifuging at 3500 rpm for 5 minutes. This step was repeated. Finally the protoplast was re-suspended carefully in 500 µl of STC Buffer and was adjusted to 1x10⁸ protoplast per ml.

Transformation of strain AR501 was done using 15 µl of plasmid, kindly provided by AgResearch Ltd., using the method of Vollmer and Yanofsky (1986) and Oliver et al. (1987) with modifications. The protoplast was co-transformed with the plasmids pTEFEGFP, where eGFP (enhanced green fluorescence protein) was fused to the pTEF promoter from *Aureobasidium pullulans* (Vanden et al., 1997) and to the pII99 promoter conferring resistance against geneticin (Namiki et al., 2001). Mycelium growth from regenerating protoplast was observed using an Olympus BX50 (Olympus, Japan) fluorescence microscope and photograph taken using an Olympus Colorview III camera (Olympus, Japan) with AnalySIS B image processing software. Fluorescent colonies were selected and sub-cultured on PDA plus 150 µl/ml geneticin.

Plant inoculation and fluorescence detection *in planta*

Fifty seedlings from two groups of perennial ryegrass seeds (line FLp 1103) of high and low transmitting endophyte supplied by PGG Wrightson Seeds Ltd, and 30 seedlings of tall fescue cultivar Flecha (line T9423) supplied by the Margot Forde Germplasm Centre were used. The tall fescue line was endophyte-free. On the 13th and 14th of May, five-day-old seedlings were inoculated with eGFP AR501 endophyte by inserting a minute section of fungal mycelium into the apical meristem as described by Latch and Christensen (1985). Eight weeks after sowing, individual plants were assessed for the endophyte presence using a technique described by Latch and Christensen (1985). The plant tissue was observed by using a fluorescence microscope. Plants containing eGFP AR501 were then selected and kept in a glasshouse until further assessments.

Tracking hyphal colonisation through primary and secondary vegetative tillers

On the 29th of October, two plants from each line containing the eGFP were randomly selected and plants were divided by hand into ramets composed of a single tiller. Four tillers per plant were then transplanted into black plastic planter bags (PB ¾) with potting mix (sand: peat, 40:60) and kept in a PC2 glasshouse. Plants were checked daily and watered as required. The experiment was set up as a completely randomised design with two repetitions of the three treatments for a total of 24 plants (Table 1). The treatments were three groups of plants, perennial ryegrass (HT and LT) and tall fescue; and the endophyte was to be tracked at four stages of plant development, *VIZ* 1-vegetative tillers, 2-reproductive tillers, 3-flowers and 4-seeds. Each individual tiller was tagged with coloured tags (white, blue, green and double green) to facilitate tracking during assessment. In total every plant had 4 tagged tillers.

Table A.1 Total of positives plants inoculated with eGFP AR501.

Plant host	Line or cultivar	Number of genotypes	Number of plants (clones)/each genotype
Perennial ryegrass	HT	2	4
	LT	2	4
Tall fescue	Flecha	2	4

HT= high endophyte transmitted; LT= low endophyte transmitted.

On the 7th of December, one month after establishment in the PC2 glasshouse, 24 plants were assessed for eGFP endophyte presence. Individual primary vegetative tillers from each plant were assessed as described previously and examined using bright field microscopy with a BX50 fluorescent microscope and a 40× UPLANFLN objective with a 0.75 numerical aperture. Images were then taken as previously described. All individual secondary tillers produced from endophyte-positive primary tillers were assessed for endophyte presence as previously described.

Plants were then shifted to a cold room (6°C), for approximately 40 days. The cold treatment was required to artificially induce reproductive growth (vernalization) and then plants were then transferred back to the glasshouse. At this stage, plants were expected to become reproductive, but unfortunately this did not occur for the majority of the plants, and therefore reproductive tillers, flowers and seeds could not be assessed.

A.2 Results and discussion

AR501 transformation and fluorescence detection *in planta*

The transformation of the endophyte strain, AR501 with eGFP expression was successful. Six fluorescent colonies were detected and sub cultured on PDA plus 150 µl/ml geneticin. The most fluorescent colony (Figure A.1) was sub-cultured and this particular isolate was then selected and used for inoculating grass seedlings. From the 50 perennial ryegrass seedlings inoculated with AR501 eGFP, five plants from the high endophyte transmitted line were positive and two plants from the low endophyte transmitted line were positive (Figure A.2a). From the 30 tall fescue seedlings inoculated with AR501 eGFP, eight plants were positive (Figure A.2b).

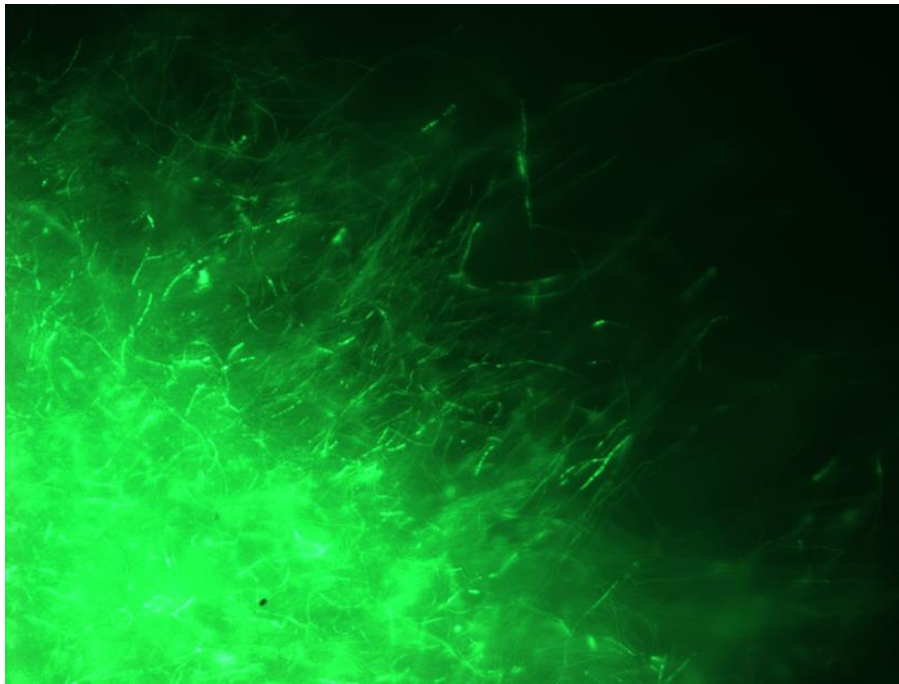


Figure A.1 GPF-transformant of AR501 in culture visualized by fluorescence microscopy.

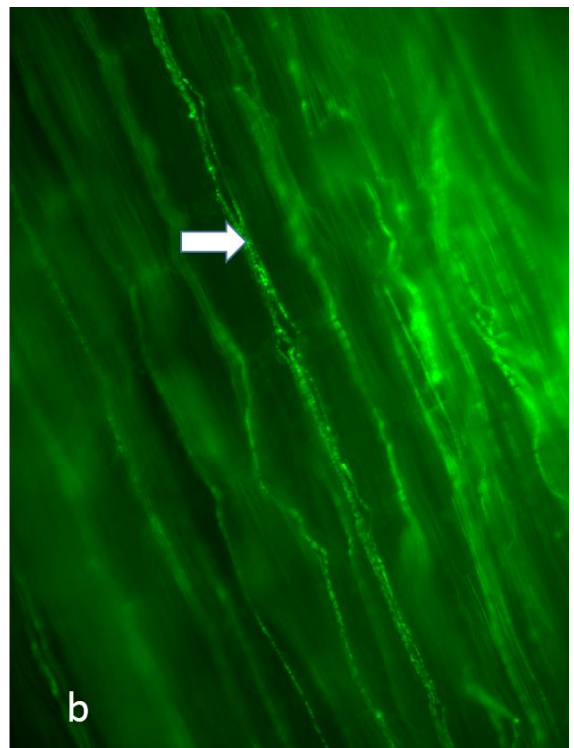
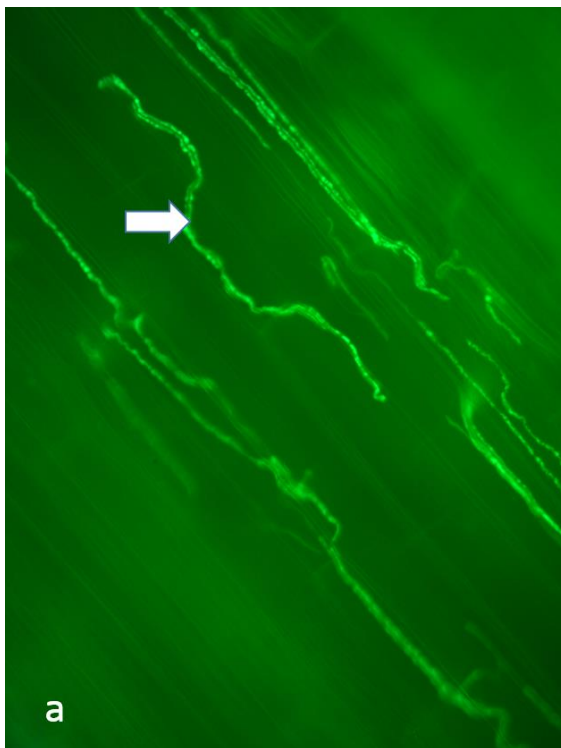


Figure A.2 GPF-transformant of AR501 in (a) perennial ryegrass and (b) tall fescue leaf sheaths. Arrows indicates fungal hyphal.

Tracking hyphal colonisation through vegetative tillers

From the 96 vegetative tillers (32 tillers from each plant line) assessed for endophyte presence, 100% of the tillers were infected. All the secondary vegetative tillers assessed were also 100% infected with AR501 expressing eGFP.

This study demonstrated that the GFP was highly expressed in the transformed AR501 mycelium in culture and in planta. The AR501 transformants (Figure A.1) were easily detected by the fluorescence microscope, and were successfully inoculated into the three hosts. Fungal mycelium was observed inside the leaf sheath, and hyphae parallel to the long axis were located in the intercellular spaces of the plant cells (Figure A.2b). Transmission failure of AR501 from seedling to the primary vegetative tillers did not occur, and endophyte infection frequency did not differ within the hosts lines. Similar results were observed in secondary vegetative tillers; there was no failure in the transmission from primary tillers to the secondary tillers. This suggests that the endophyte transmission to reproductive tillers could be potentially high. It is important to note that the three plant lines were growing under the same conditions and external effects or environmental variations (i.e. temperature variation) were not observed during plant development. Temperature has been previously shown to impact AR501 infection frequency in perennial ryegrass and tall fescue as reported in Chapter 2 and Chapter 3.

Tracking the endophyte in four phases of plant development (i.e. vegetative tiller, reproductive tillers, flowers and seeds), was one of the aims of this experiment, but, plants did not become reproductive. The reason for that is not clear, but it could be due to the lack of sufficient light during the period (February to May), when plants were growing in the glasshouse, after being vernalized. In autumn 2014, the average temperature was 13.7°C ($\pm 0.5^\circ\text{C}$) and the day light length was ~ 445 hours (NIWA). For the grass plant, photoperiod and temperature are the principal factors that control the transition from vegetative to reproductive growth (Aamlid et al., 1998). Seed dissemination and long-term survival of the transformed endophytes therefore remains to be assessed.

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