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**Viability of endophytic fungus in different perennial  
ryegrass (*Lolium perenne*) varieties kept in different  
storage conditions**

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

at

Massey University, Manawatū, New Zealand

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2017

Abstract of a thesis submitted in partial fulfilment of the requirements for the degree of Master of Science.

**Viability of endophytic fungus in different perennial ryegrass (*Lolium perenne*) varieties kept in different storage conditions**

by

Elizabeth Rose Kitson

*Epichloë* endophytes form symbiotic relationships with cool-season grasses of the Pooideae family and are known to synthesise a range of bio-protective alkaloids. These alkaloids can provide the grass host with benefits for greater survival including; deterrence of herbivorous pests, increased persistence, better livestock health and protection from abiotic stressors. The commercialisation of novel endophytes is on the increase, and it is important to ensure the survival of the endophyte is maintained so their benefits can be realised.

This study examined the effects of different storage conditions on the viability of three commercial novel endophytes (AR1, AR37 and NEA2/6) and one pre-commercial novel endophyte (815). The different storage conditions were the top of a warehouse, the bottom of a warehouse and a temperature and humidity controlled cool store to simulate current commercial seed storage environments.

The viability of different endophytes decreases independently of grass seed germination ( $p = NS$ ) however there are many factors influencing the endophyte survival. Over the one year storage period there were significant interactions between endophyte x ploidy (host), endophyte x location and endophyte x ploidy (host) x location. The pre-commercial endophyte, 815, had the largest reduction in viable endophyte when stored outside of the controlled cool store dropping 70 percentage points at the top of the warehouse, compared with AR37 (12 percentage points), AR1 (16 percentage points), and NEA2/6 (46 percentage points) ( $p < .001$ ,  $LSD = 15.9$ ). In the cool store there was no significant decrease in any of the treatments.

As more novel endophyte/grass combinations are released for commercial sale it is important to test each for compatibility and performance post-storage. The results of this study recommend controlled low-temperature, low-humidity storage to maintain endophyte viability.

**Keywords:** Endophyte, *Epichloë*, perennial ryegrass, *Lolium perenne*, storage

## Acknowledgements

I would like to thank many people for their help and support during the time it has taken for me to complete this thesis.

- Pedro Evans, we 'put up with each other' for 8 years at DLF Seeds and Pedro offered honest opinion and guidance on not just the thesis, but, everything work related, and, sometimes not so work related. Even after I left DLF he was always just a phone call away when I had questions or just needed a sounding board. Sadly Pedro passed away near the completion of this thesis, it hasn't been easy without him.
- My wife, Deanna, for the 'gentle' nagging and support. Thanks for pretending to care about endophytes even though I know you really couldn't care less and 'just wanted me to finish the bloody thing'. It hasn't been an easy ride so thanks for sticking by me when the going got tough.
- My parents, Chris and Anthea, who supported me through the whole process and tried to take an interest in endophytes.
- My brothers, Tom and Harry, and extended family who wanted to ask me about what I was doing and even though I knew at times you regretted asking (the glazed over look gave it away) you still continued to ask. In particular Ainslie, Geoff, Lisa, Malcolm and Gen, Tim and Julie, Nana and Gran for always just asking how it was going.
- My supervisor, Michael McManus, who unfortunately passed away before I had finished. Thanks for taking an interest and providing useful ideas when I was just getting started.
- Cory Matthew, thank you for stepping in to get me back on track. It can't be easy coming in to supervise a student when they are already underway but you have done a great job and been enthusiastic about seeing me over the 'finish line'.
- Warren Williams, my co-supervisor, who has always been there in the shadows prodding me along and offering advice, even after all these years!
- My old workmates at DLF Seeds, Gavin and Garth, and my newer workmates at Plant and Food Research, Richard, Lester, Peter, Adam, Monika, Miriam and Linley, thanks for being sounding boards, proofreaders, stats helpers, asking me how things were going and, at times, the not so gentle encouragement to complete.
- DLF Seeds, thank you for the financial support and the resources to do the project. DLF Seeds gave me a great foundation to begin my career, as well as many life skills and I thank them for the opportunities given to me. In particular, Klaus Nielsen, who knew I could do it and did all he could from half a world away to keep me on track.

**For Pedro**

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## Chapter 1 Introduction

Around the world many species from the *Poaceae* family have formed unique relationships with endophytic fungi from the *Epichloë* species (formerly *Neotyphodium*). The history of *Epichloë* endophytes in pasture grasses in New Zealand is a complex story. *Epichloë* endophytes were inadvertently imported by European settlers in perennial ryegrass (*Lolium perenne* L.). In the mid-1800s forests were cleared to make way for pastoral based farming (Moot et al. 2010).

Perennial ryegrass was chosen as the main pasture species as it is an easily established, highly fertile, nutritious and tolerant grass (Hunt and Easton 1989, McKenzie 2014). Endophyte was first recorded in perennial ryegrass in 1940 (Neill 1940) however its functions were not well understood. It was not until 1981 when Fletcher and Harvey were able to link endophyte concentration to the severity of ryegrass staggers in sheep. Gallagher et al. (1981) were able confirm the correlation by identifying an alkaloid produced by the endophyte, lolitrem B, which is a tremorgenic neurotoxin.

Since this important discovery in New Zealand pastures in 1981 (Fletcher 2009) their development has continued to increase significantly. The focus of endophyte research and development in New Zealand has been on grasses from the *Poeae* tribe, including, but not limited to, tall fescue (*Festuca arundinacea*); meadow fescue (*Festuca pratensis*); Italian ryegrass (*Lolium multiflorum*) and the previously mentioned perennial ryegrass (*L. perenne*) and their relationship with asexual *Epichloë* endophytes. Although there is a wide range of both *Epichloë* endophytes and *poideae* grasses there appears to be a large degree of host specificity (Schardl and Clay 1997).

In New Zealand most ryegrass and fescue pastures in contain endophyte and over 80% of ryegrass sold contains endophyte (Fletcher 2009). The focus of the symbiotic endophyte/grass relationship has been to increase agronomic advantages whilst mitigating the detrimental effects on grazing stock (Clay and Schardl 2002, Hume et al. 2013). Endophyte strains, many isolated from wild populations, are inoculated into commercial varieties of perennial ryegrass and fescue. These 'novel' endophytes generally provide a greater deterrence to insect pests (Pennell and Ball 1999, Popay et al. 2003, Popay et al. 2009, Popay and Thom 2009), have less animal health issues (Fletcher and Easton 1997, Fletcher 1999) and also have the ability to provide increased tolerance to abiotic stressors (Bluett et al. 2005b). The asexual *Epichloë* species produce alkaloids which can provide resistance, or tolerance, against a large range of insect pests (Popay and Rowan 1994). However, alkaloids produced can also have detrimental effects to animal health such as ryegrass staggers, heat stress and fescue toxicosis (Fletcher and Easton 1997).

There are 4 main alkaloids associated with the asexual *Epichloë* species; aminopyrrolidines, pyrrolopyrazines, indole diterpenes and the ergot alkaloids. Different strains of endophyte produce different types, and levels, of the alkaloid mycotoxins; the anti-insect compounds lolines, janthitrems and peramine and the anti-mammalian compounds ergovaline and lolitrem-B. Current research is attempting to isolate or create an endophyte which produces the anti-insect compounds without the anti-mammalian ones. Some of the issues associated with this are; the ability to inoculate the endophyte into productive commercial ryegrass varieties, the transmission of the endophyte from generation to generation of the grass and the survival of endophyte in stored seed.

Inoculation of endophyte strains into new hosts allows for the discovery and manipulation of *Epichloë* species from original hosts, often other temperate grass species, into commercial, productive ryegrass and fescue varieties. There are ongoing difficulties with this as it is a technical process with often limited positive results. As few as 2% of ryegrass and/or fescue plants can be successfully infected with the asexual *Epichloë* endophyte during the inoculation process (Larsen, pers. comm. 2012). It is believed the low rate could be due to the incompatibility of the relationship between the *Epichloë* species and their specific poideae host (Clay and Schardl 2002).

The transmission of endophyte from generation to generation can also be a limiting factor in producing large amounts of commercial seed containing a novel endophyte. Endophytes grow between the cells of the plant host. As the meristems divide within the plant the endophyte grows up with the elongating cells. To successfully produce seed containing endophyte it is essential that the endophyte grows with each meristem that forms (Easton et al. 2009). Wilson and Easton (1997) have reported that in a fescue-endophyte association they observed a small amount of transmission failure at all meristem levels – tiller, panicle branch and spikelets on branches.

Another major limiting factor of the production and development of endophyte-containing commercial pasture grasses is storage. Endophytes are a living organism therefore if they are not provided ideal conditions their survival is affected. It is best to store ryegrass seed, containing endophyte, in cool conditions with a low humidity (Rolston et al. 1986, Tian et al. 2013a). Ideally it would be best to sow all endophyte-containing seed in the year of harvest however if this is not possible moving seed stocks to a controlled environment after spring sales is best practice. The survival of endophyte in different lines of grass are not fully understood as the performance of such grasses differs with differing endophyte strains.

## Chapter 2 Literature Review

### 2.1 Introduction to endophytes

In temperate regions around the world many cool-season grasses are grown as forage for grazing animals. There are more than 100 species of grass that may be infected with a fungal strain of the *Epichloë* species (Hume et al. 2016). Perennial ryegrass (*L. Perenne*) is the main sown grass species in New Zealand. Bluett et al. (2005b) reported that around 7 million hectares of ryegrass based pasture is grown, and most of this is infected with a fungal endophyte. Endophytic fungi infect a variety of cool season grasses including, but not limited to, the species *Lolium* (Craven et al. 2001, Antunes et al. 2008), *Bromus* (Antunes et al. 2008), *Festuca* (Christensen 1995, Hill and Roach 2009), and *Triticum* (Marshall et al. 1999), though the focus in New Zealand is mainly on the perennial cool season grass *Lolium perenne* with increasing interest in the *Festuca* (*arundinacea* and *pratense*) species (Milne, DLF Seeds Ltd, pers. Comm.) The other commonly sown *Lolium* species, Italian ryegrass (*L. multiflorum*), and annual ryegrass (*L. multiflorum* var. *westerwoldicum*), also have mounting interest, but being shorter –rotation grasses the importance and economical benefit is reduced.

#### 2.1.1 Taxonomy

Endophyte is the term given to any organism that lives in a plant; the Greek ‘endo’ meaning within and ‘phyte’ meaning plant (Fletcher et al. 1990, Wilson 1995). Endophytes live asymptotically in many plant species although this review will be solely on the fungi living in the space between cells of pastoral grasses (Faeth 2002).

Endophytes belong to the Balansieae tribe of the Clavicipitaceae family. Members of the Clavicipitaceae family are known pathogens of a wide range of hosts including grasses, sedges, other ascomycetes and insects. These fungi are found in both tropical and temperate regions throughout the world (Glenn et al. 1996). One genus (*Epichloë*) has spawned a diverse range of asexual forms that have stemmed interest in association with cool-season grasses in the subfamily Pooideae (Clay and Schardl 2002).

The classification of the asexual fungal endophytes has been debated and renamed several times. Initially thought of as part of the *Epichloë* genus the asexual anamorphs were reclassified as *Acremonium* in 1982; *Neotyphodium* in 1996 before returning to part of the *Epichloë* genus in 2014. The *Acremonium* genus was added to the Clavicipitaceae family by Morgan-Jones and Gams (1982) so the asexual species could be classified separately from the existing sexually reproductive *Epichloë*

genus. *Acremonium* was reclassified to *Neotyphodium* by Glenn et al. (1996) to cater for heterogeneity within the genus. Clay and Schardl (2002) suggest that the *Neotyphodium* endophytes are interspecific hybrids of the sexually reproductive *Epichloë* species. Antunes et al. (2008) agrees saying that *Neotyphodium* has derived from *Epichloë* but differs in that it does not produce spores. These spores, of the *Epichloë* species, infect cool season grasses and affect the inflorescence with a ‘choke’ which results in seed abortion (Clay and Schardl 2002). Lane et al. (2000) described the relationship between the asexual *Epichloë* endophytes and their sexual counterparts as “a trapped pathogen whose genome and associated functions have been expropriated for the benefit of the host plant”. Glenn et al. (1996) looked closely at the molecular data to determine the nomenclatural change but concluded the project stating there were limitations with the naming of the whole *Epichloë* genus, and more detailed morphological studies were required to distinguish between the diverse *Epichloë* ascomycetes.

In 2014 Leuchtman et al. (2014), realigned the nomenclature of the *Epichloë* species stating that rule changes in the International Code of Nomenclature for algae, fungi and plants need to provide a single name to be used for each fungal species. Thus, as *Neotyphodium* are asexual anamorphs of the *Epichloë* species a realignment of the fungal group into a single genus would increase the understanding of grass endophyte relationships. Returning the asexual endophyte forms (*Neotyphodium*) to the *Epichloë* species allows the evolutionary history and host interactions of the broad species to be better understood going into the future. A number of agriculturally important grass hosts, their endophytes and the endophyte nomenclatural changes are listed in Table 1.

**Table 1. Nomenclatural changes to agriculturally important grass/endophyte symbioses, as described by Leuchtman et al. (2014)**

Host Grass	Endophyte	Previous name
Perennial Ryegrass ( <i>Lolium perenne</i> )	<i>Epichloë festucae</i> var. <i>lolii</i>	<i>Neotyphodium lolii</i> (Glenn et al. 1996)
Tall Fescue ( <i>Festuca arundinacea</i> )	<i>Epichloë coenophiala</i>	<i>Neotyphodium coenophialum</i> (Glenn et al. 1996)
Italian Ryegrass ( <i>Lolium Multiflorum</i> )	<i>Epichloë occultans</i>	<i>Neotyphodium occultans</i> (Moon et al. 2000)
Meadow Fescue ( <i>Festuca Pratensis</i> )	<i>Epichloë uncinata</i>	<i>Neotyphodium uncinatum</i> (Glenn et al. 1996)
	<i>Epichloë siegelii</i>	<i>Neotyphodium siegelii</i> (Craven et al. 2001)

## 2.1.2 Biology and development

Endophytes survive in the embryos of seeds. Upon germination of the seed the endophytic hyphae within the intercellular space of the embryo resume growing and extend into the leaf primordia and axillary buds. This starts at the shoot apical meristem, the location where new shoots develop (Christensen et al. 2008).

The hyphae of the fungal endophyte, *Epichloë*, spread systematically throughout the plant. In leaves the hyphae are aligned parallel with the longitudinal leaf axis. Christensen and Voisey (2007) reported that growth of the hyphae is synchronised with that of the elongating shoots and leaves, including that of the inflorescences which begin their growth at the top of the shoot apical meristem. Hyphae growth ceases as the leaf growth stops and remains present for the life of the leaf. Despite the plant and the endophyte having different growth methods remarkably their growth and distribution is synchronised. It was previously proposed that since the leaf grows by addition and subsequent expansion of new cells at its base whereas fungi normally extend by adding new material to their tips (Schmid and Christensen 1999). The implication of this suggests that the hyphae must slide along in the spaces between the cells as the plant grows. Christensen et al. (2008) proved that endophytic hyphae actually grow in the leaves by intercalary division and extension rather than tip growth. The hyphae attach themselves to the grass host cell walls and as the plant grows the filaments stretch. This process makes more sense as the plant and the endophyte are growing together 'as one' (Tanaka et al. 2012). It is also supported by the observation that the hyphae are orientated parallel to the longitudinal leaf axis. Occasionally there are circumstances where some tillers are free of endophyte, despite the plant containing endophyte. This can be explained by the intercalary extension process; if hyphae fail to attach themselves to the axillary buds whilst they are still in the apical meristem zone the tiller will forever remain endophyte free (Christensen and Voisey 2007). This is discussed further in Appendix 1.

The apical meristem zone is the 'nursery' of plant organs and has a segmental structure with a bud associated with each segment capable of developing into a vegetative daughter tiller.

Transformation of the growing point occurs when reproductive tillers are formed. The colonisation of the vegetative tillers and the inflorescence when flowering occurs are both necessary for the endophyte to establish itself in the embryo (George 2009). The cycle is then repeated so long as the endophyte remains alive in either the plant or the seed. This process of asexual reproduction, or, vertical transmission, is demonstrated by Figure 1. As mentioned in the previous paragraph there are times when endophyte fails to colonise in some tillers, this will result in the seed produced from that inflorescence being endophyte free. Why this occurs is not well understood.



Diagram courtesy of Grasslanz technology

**Figure 1. The life cycle of the asexual *Epichloë* species present in a grass plant.**

Antunes et al. (2008) found that the endophytic fungal hyphae occur in all plant parts although they appear to have the greatest density in the crown and stem of the grass. It is this point where the cell division takes place and where the endophyte is under constant development (Christensen and Voisey 2007). The concentration of the hyphae decreases as it grows through into the plant leaves. There is also a small amount of endophyte found in the root system (Patchett et al. 2011). Endophyte found in the roots appears to be at a lower concentration than the rest of the plant, suggesting that the colonisation process differs from that of the vegetative growth. An explanation of this would be that there is no evolutionary need for the endophyte to be in the roots – it depends solely on seed dispersal for its own survival.

## 2.2 Alkaloids

Endophytes produce secondary metabolites known as alkaloids which, depending on the alkaloid/s produced, have differing effects in association with the host grass (Easton 2007).



There are a range of endophyte species and strains which synthesize different alkaloids available, as shown in Table 2.

**Table 2. Commercially available endophytes in New Zealand as of 1/12/16.**

Source: (Specialty-Seeds 2015)

Strain	Market name	Owner – commercial licensee
<i>Epichloë festucae var. lolii</i>	AR1	Grasslanz Technology
	AR6/Endo 5	Grasslanz Technology
	AR37	Grasslanz Technology
	NEA2/6	Agriseeds Ltd
	Wild-type (standard)	-
	Edge (815)	DLF Seeds A/S
<i>Epichloë coenophiala</i>	Max P	Grasslanz Technology
	Protek (E647)	DLF Seeds A/S
<i>Epichloë uncinata</i>	U2	Cropmark Seeds Ltd
<i>Epichloë siegelii</i>	HAPPE	DLF Seeds A/S

As the research and development of the endophyte/grass symbiosis continues to be explored there are many more in the pipeline with 22 different strains currently registered with the Intellectual Property Office of New Zealand (IPONZ 2016).

Currently there are four classifications of alkaloids, representing 5 main secondary metabolites (named in brackets), which have been identified and studied intensively in the *Epichloë* species; aminopyrrolidines (loline), pyrrolopyrazines (peramine), indole diterpenes (lolitrem B, epoxy-janthitrem) and the ergot alkaloids (ergovaline). There are other secondary metabolites that have been classified into these groups but these 5 have had the most research done on them.

There are a wide range of different endophyte strains that have different alkaloid profiles for example; Standard, or wild type, endophyte produces peramine, ergovaline and lolitrem B (Popay and Thom, 2009). In 2000 the endophyte strain AR1, of the *Epichloë festucae var. lolii* species, was released to the market. AR1 produces peramine which acts as some deterrence from ASW without being detrimental to stock (Popay and Thom 2009). Another *Epichloë festucae var. lolii* variety, AR37, was released in 2006 and produces epoxyjanthitrem. This is illustrated in Table 3.

**Table 3. Alkaloid profiles of the *Epichloë festucae* var. *lolii* endophyte strains AR1, AR37, NEA2/6, 815 (Edge) and standard (SE). (adapted from McKenzie (2014)).**

	Lolitrems B	Ergovaline	Peramine	Janthitrem	Loline
AR1	Nil	Nil	High	Nil	Nil
AR37	Nil	Nil	Nil	High	Nil
NEA2/6	Low-Moderate	Low-Moderate	Moderate	Nil	Nil
815	Nil	Trace	High	Nil	Nil
Standard	High	High	High	Nil	Nil

“Novel” endophyte technology is now firmly entrenched in pastoral farming in New Zealand. Fletcher (2009) stated that over 80% of ryegrass seed sold in New Zealand is infected with novel endophyte. It is estimated that 20-30% of all grass species host systemic endophytes. Faeth and Hamilton (2006) found endophytes to be very diverse and abundant, particularly in cool-season pooid grass species. The largest grass family to host endophytes is the Poaceae family (Rasmussen et al. 2007). The Poaceae family includes the popular sown pasture species Perennial Ryegrass (*Lolium perenne*) and Tall Fescue (*Festuca arundinacea*).

Perennial ryegrass is the most popular grass forage grazed by dairy cows in New Zealand. It is typically infected with endophyte, commonly *Epichloë festucae* var. *lolii*, of which there are many different strains. In older pastures the endophyte strain is known as ‘wild type’ or ‘standard endophyte’. The association between perennial ryegrass and the wild type endophyte can be detrimental to stock as moderate amounts of the alkaloid ergovaline are produced.

Endophyte strains found in grass species can be isolated and inoculated into different host populations (Latch and Christensen 1985). There are currently 9 endophyte strains available on the New Zealand market. These are available in a wide range of grass cultivars suited to most climates. The inoculation process is a complex one. Latch and Christensen (1985) described the process and showed it is possible to infect seedlings with endophytic fungi originally isolated from both within their own grass species and, to some degree, from different grass hosts.

Different grass species have different ‘common’ endophytes including *Epichloë festucae* var. *lolii* from perennial ryegrass (*Lolium perenne*) (Siegel et al. 1990, Schardl 1994); *Epichloë uncinata* from meadow fescue (*Festuca pratensis*) (Gams et al. 1990); *Epichloë occultans* from annual ryegrass (*Lolium multiflorum*) (Moon et al. 2000); *Epichloë siegelii* from meadow fescue (*Festuca pratensis*)

(Craven et al. 2001) and *Epichloë coenophiala* from tall fescue (*Festuca arundinacea*) (Morgan-Jones and Gams 1982, Christensen and Latch 1991) Of the listed endophyte strains all, apart from *Epichloë festucae* var. *lolii* are interspecific hybrids (Clay and Schardl 2002).

Over the last 20 years there has been increased interest in selecting endophytic strains that provide resistance from invertebrates but aren't associated with the toxicosis of grazing animals and using them to inoculate commercial cultivars (Christensen 1995). As more endophyte strains are isolated and used to inoculate commercial varieties of pastoral grasses there becomes more and more issues to contend with to produce safe, viable, productive grass-endophyte combinations.

Under licencing agreements, in New Zealand and Australia, there must be greater than (or equal to) 70% viable endophyte in seed being sold as containing endophyte (Hume et al. 2011).

### 2.2.1 Aminopyrrolizidines

Aminopyrrolizidines can be synthesized by many *Epichloë* species and are better known as the lolines. Lolines can be synthesized in meadow fescue and tall fescue (Ball and Tapper 1999) and ryegrass (Evans and Kitson, unpub. data). Chemically there are six loline alkaloids; N-formyl loline 6 (NFL), N-acetyl loline (NAL), N-acetyl norloline (NANL), N-methyl loline (NML), norloline (NOL) and N-formyl norloline (NFNL). The primary biologically active loline compounds found in *Epichloë* species are NFL, NAL and NANL (Patchett 2007). Lolines are of interest because they provide broad spectrum insect deterrence but have been found to be non-toxic to livestock (Bush et al. 1993). Some 'new' novel endophytes claiming to produce lolines have been inoculated into festuloliums but there are still no pure ryegrasses with loline alkaloids produced available commercially (Evans pers. Comm.).

Commercially lolines are produced in the tall fescue endophytes Max P and Protek, by the species *Epichloë coenophiala*, and in the pasture mix "Barrier combo" which contains festuloliums – A meadow fescue x Italian ryegrass hybrid. These festuloliums (meadow fescue/ryegrass interspecies crosses) are infected with the endophyte U2 which belongs to the species *Epichloë uncinata* (George 2009). Popay et al. (2009) found that meadow fescues containing a strain of *Epichloë uncinata* endophyte that produced loline had reduced damage to tillers by ASW larvae, though it was not confirmed if this was through deterrence or toxicity. Although this was a controlled pot experiment there was a strong correlation between loline concentration and the growth, development and survival of ASW however, subsequent field trials would need to be conducted to determine whether other external factors influence the alkaloid concentration effects. Loline alkaloids have also been linked to the deterrence of aphids (Christensen et al. 1993) but agronomically aphids are not considered a major pasture pest in New Zealand. Loline alkaloids are a very important metabolite to

explore for use in the New Zealand market due to their insect deterrence but non-toxicity to grazing animals (discussed in Chapter 2.3.1).

Although recognized as a primary alkaloid Ball et al. (2006) suggest that loline may actually be one of the wide range of biologically active secondary metabolites produced by different endophyte strains. There is debate as to whether or not loline alkaloids may have allelopathic properties. It is known that lolines deter feeding on grasses by invertebrates (Popay et al. 2009) but Petroski et al. (1990) note that loline alkaloids have been reported to reduce the germination of both monocot and dicot seeds. Therefore the presence of loline alkaloids may in fact enhance the competitive abilities of loline-containing endophyte-infected grasses by slowing the establishment of competitors in a sward. This study, however, was done completely in vitro using loline solutions rather than real endophytes. A recent study by Cripps et al. (2013) also found that the amount of white clover biomass grown in soils pre-conditioned with tall fescue or meadow fescue infected with endophytes containing lolines was reduced compared with the same tall fescue or meadow fescue cultivars with no endophyte infection. This could potentially be explained by some level of allelopathy though this would need to be looked at further, or it could be due to more-robust growth by the endophyte-infected grasses. The soil in the Cripps et al. (2013) study was 'conditioned' for 11 months prior to the white clover being sown. It would be worth looking at whether this allelopathic response has a similar effect when grass and clover are sown at the same time, as sowing a grass/clover mixture is common practice in New Zealand.

### 2.2.2 Pyrrolopyrazines

Pyrrolopyrazines include the anti-insect alkaloid peramine. Peramine is synthesized in some strains of *Epichloë festucae* var. *lolii* (demonstrated in Table 3) and is also synthesized in *Epichloë coenophiala*, the main tall fescue endophyte. Peramine is a broad insect deterrent (Clay and Scharl 2002) and has been shown to strongly deter Argentine Stem Weevil (ASW), an insect that puts a lot of pressure on pastures throughout New Zealand (Popay et al. 1999). Rowan et al. (1990) found the feeding of both adult and larval ASW could be reduced, in both a food-choice and non-choice test, with levels of peramine at 0.1µg/g and 10µg/g respectively. The commercially available endophyte, AR1, has been found to reach these levels (Tian et al. 2013b, McKenzie 2014) whereas another endophyte, NEA2/6, was found at levels between 5.3 - 9.3 µg/g in the McKenzie (2014) study and averaging 5µg/g across four clonal replications in the study of Tian et al. (2013b) suggesting some deterrence, more so at a larval stage.

The strong deterrence against ASW, coupled with the fact peramine has no known negative effect on grazing stock (Bush et al. 1997) makes peramine another important metabolite worth more investigation.

### 2.2.3 Indole-diterpenes

The Indole-diterpene group includes the alkaloid Lolitrem B. Lolitrem B can be synthesised in some *Epichloë festucae* var. *lolii* endophyte strains. It is the causative agent responsible for ryegrass staggers; a neurotoxic disorder which affects grazing stock (Fletcher et al. 1999). Lolitrem B is most common in the wild type endophyte strain found in New Zealand. It has significant anti-insect and anti-mammalian properties which are likely to be an evolutionary trait for its survival in the wild. George (2009) reports that Lolitrem B is the least common of the alkaloids found in endophyte-grass associations which corresponds with work done by Siegel et al. (1990) where Lolitrem B was found in just 10% of the 35 endophyte-infected host grasses tested.

Epoxy-janthitrems are another alkaloid in the indole-diterpene group. They are only known to be synthesized in one strain of *Epichloë festucae* var. *lolii* – AR37. Janthitrems are known tremorgenic toxins which have been shown to have negative effects on both insects and mammals (George 2009) however the strain in AR37 - 10-*epi*-11,12-epoxyjanthitrem G generally has less severe and less frequent negative effects on grazing stock than endophytes synthesizing other indole diterpenes (Hume et al. 2016). Popay and Thom (2009) found that the AR37 strain, which produces only the Epoxy-janthitrems and none of the other alkaloids, provided a broad spectrum control of many insect pests. This is discussed further in chapter 2.3.1.

### 2.2.4 Ergot Alkaloids

A wide range of *Epichloë* endophyte species synthesize the alkaloid ergovaline, a member of the ergot alkaloid group (Rowan et al. 1990). Other ergot alkaloids such as ergine, lysergic acid, several other precursors and derivatives of these can also be found in low concentrations in endophyte grass associations (George 2009). Ergovaline is produced in both ryegrass and fescue associations and is responsible for the commonly known problem fescue toxicosis (Popay et al. 1999).

Ergot alkaloids are toxic to grazing stock with symptoms of toxicity including; reduced weight gain, increased body temperature, restricted blood flow, poor reproduction and a reduction in milk production (Bush et al. 1997). Fletcher et al. (1999) also reported heat stress in sheep having consumed grass containing endophyte producing the alkaloid ergovaline. Serum prolactin concentrations are increased by stock grazing ergovaline infected pastures. High prolactin levels can result in reduced fertility (Popay et al. 1999). Ergovaline is most commonly found in the wild type

endophyte although low levels can be found in NEA2/6 and Endo5 (Popay and Gerard 2007, McKenzie 2014) and trace amounts in 815 (Evans, pers. Comm). The ergot alkaloids are also a deterrent to some invertebrates (Fletcher 2009). Popay et al. (1999) found that different cultivars of ryegrass affected with ergovaline deterred Black Beetle (*Heteronychus arator*). Hume et al. (2009) found, too, that cultivars infected with an endophyte that produced ergovaline (wild type, AR37 and Endo5) were damaged the least by Black Beetle compared to endophyte free pastures. With cultivars infected by the endophyte AR1, which doesn't produce any of the alkaloid ergovaline, there was a wide range of damage scores across different AR1 infected cultivars.

### 2.2.5 Alkaloid Functions

There is a wide variation in alkaloids produced depending on the grass species, cultivar, abiotic environmental conditions and even the ploidy of the host species (Clay and Schardl 2002). Levels of alkaloids, singly or in combination, within endophyte infected grasses have been found to have a large variation (Siegel et al. 1990). Easton (1999) reported that although the production of the alkaloids is solely a property of the endophyte fungus the plant is able to exercise some control of how much the endophyte grows and therefore affect the production rates of the alkaloid metabolites. van Zijll de Jong et al. (2008) agree saying that although the wide range of alkaloid profiles can be attributed to the endophyte genotype the genetic variability of the grass host can also influence the profile and concentration levels of alkaloids somewhat. So although the development of the endophyte and the control of the alkaloids are mainly controlled by the endophyte strain itself there is large influence over how they behave and produce alkaloids depending on the genotype of the host grass (Eerens et al. 1998, Easton 2007).

Patchett et al. (2008) found that lolines can be better controlled by a plant and this particular alkaloid can be redirected within the plant to a potential 'attack' site. The study was conducted with grass grub larvae and found the concentrations of the loline alkaloid was increased in the root zone, and respectively decreased in the crown, when the plant was under threat by the root-feeding insect suggesting a translocation of alkaloids within the plant.

Fletcher et al. (2006) found that all associations of diploid ryegrass cultivars with AR1 have had higher peramine concentrations than tetraploid ryegrass cultivars by an average of 5 ppm – coming in at under the threshold required for effective deterrence of ASW (Popay and Wyatt 1995). As well as differences in grass species, cultivar and ploidy have an impact on alkaloid production, and the content in the plant also varies due to time (season) and other environmental factors, as well as leaf age and tissue type (Ball et al. 2006). Rasmussen et al. (2007) agrees stating that the production and concentration of endophyte alkaloids in plant tissues vary considerably in the field with season,

weather and management. Factors such as cultivar or nitrogen supply could alter the production of alkaloids either by altering the concentration of fungus or by altering the rate of alkaloid production per unit endophyte. In the same study it was discovered that 'high sugar' cultivars substantially reduced, by up to 50%, the concentration of alkaloids expressed compared with a 'low sugar' grass cultivar. This result requires further exploration as, previously discussed, host cultivar is known to influence the concentration of alkaloids (Clay and Schardl 2002). Rasmussen et al. (2007) also found that a high nitrogen environment (9 mM N) also substantially reduced the concentration, by up to 40%, of both endophyte and alkaloids compared to a low (2.25 mM N) nitrogen treatment. Again further exploration is required to determine consistent results across a wider range of nitrogen treatments, host cultivars and endophyte strains as Cheplick and Cho (2003) have found that host genotype, of both the endophyte and the host grass, strongly influences the concentration and distribution of endophytic hyphae within the leaves.

There can be large seasonal differences in alkaloid concentrations. Easton (1999) found that water deficit increased the concentrations of both ergovaline and lolitrem B in both field and growth cabinet work. Easton et al. (2002) found that in infected plants, growing in the same conditions, large differences were measured in the levels of Lolitrem B and peramine depending on the stage of the season. In the same study there was also a large variation in alkaloid production depending on the host grass genotype. Rasmussen et al. (2007) looked into this further and found that host grasses of the same cultivar infected with different strains of endophyte, and including an endophyte free option, were biochemically very different from each other, beyond differences in alkaloid production. This leads to the theory that the host-endophyte association is a very complex one and goes beyond just the production of alkaloids. The increase in the focus on alkaloids is important because they potentially contain mechanisms that can increase the production of pasture grasses resulting in an increased profit in the agricultural field (Clay and Schardl 2002). Knowing the endophyte content, and the maximum synthesis of alkaloids produced by different endophyte x host interactions is important as the diversity of seasonal differences/locations across New Zealand farming environments is extensive and potential for detrimental effects to stock could have major repercussions.

### **2.3 Benefits of endophytes**

As the endophytes transmit vertically via the seed they are heritable components of the mutualistic relationship, providing protection to their host grass (An et al. 1993, Faeth 2002). The symbiosis is that the host grass provides the endophyte with nutrients, protection and somewhere to live (Christensen and Voisey 2009) in return for the endophyte providing protection through the



production of secondary metabolites which provide benefits for the grass host including; Resistance to herbivorous insect pests (Pennell and Ball 1999, Popay et al. 2003, Popay et al. 2009, Popay and Thom 2009, Thom et al. 2014), increased persistence and productivity of pastoral grasses (Cunningham et al. 1993, Bluett et al. 2005b, Ball et al. 2006, Hume et al. 2009), and a greater tolerance to abiotic stressors such as drought (Eerens et al. 1998, Miranda et al. 2011). There are other benefits to pastoral productivity including; a potential increase in animal performance (Bluett et al. 2005a, Fletcher and Sutherland 2009, Thom et al. 2012) an increase in the overall health of livestock (Fletcher 1999, Fletcher and Sutherland 2009) and reduced weed ingress (Tozer et al. 2007, Hume et al. 2016).

### 2.3.1 Herbivorous insect pests

Resistance to herbivorous insect pests is mostly due to the synthesis of alkaloids by the endophytic fungus. Easton et al. (2001) compiled a list of major discoveries of endophyte effects on pasture invertebrates which included a wide range of deterrents provided by alkaloids against many economically damaging insect pests such as Argentine Stem Weevil (*Listronous bonariensis*), Black Beetle (*Heteronychus arator*) and Pasture Mealy Bug (*Balanococcus poae*). Since 2001 there have been subsequent studies on other pastoral insect pests including Grass grub (*Costelytra zealandica*), Porina (*Wiseana spp*) and Root Aphid (*Aploneura lentisci*) (Jensen and Popay 2004, Popay and Gerard 2007, Popay 2009, Popay and Thom 2009, Popay et al. 2012).

Jackson et al. (2002) suggested that the effects of insect pests on the pastoral industry were estimated to be upwards of \$600 million per annum, up from the \$46-\$200million in 1991 (Easton 1999). An Integrated Pest Management strategy has been adopted including the introduction of natural enemies, insect diseases and plant endophytes.

Throughout the 1980s and 1990s the effect of endophyte on Argentine Stem Weevil (ASW) was studied a great deal. ASW is found in pastures throughout New Zealand, and, when uncontrolled, can have a significant economic impact (Prestidge et al. 1992). Adult weevils feed on the leaf sheaths of grass, which doesn't have a significant impact, however, the larvae bore into the stem and can kill the plant (Prestidge and Gallagher 1988). Different alkaloids are responsible for the deterrence of ASW in different ways; Peramine reduces ASW through adult weevils laying fewer eggs, thus, fewer larvae are hatched to cause the plant damage (Easton et al. 2001); Lolitrem B reduces larvae growth and development, but has no effect on adults (Dymock et al. 1989); and Ergovaline was found to deter the adult ASW (Popay et al. 1990). Easton et al. (2001) lists 'AR1-infected ryegrass is as resistant to ASW as wild-type' (Popay et al. 1999) as a 'major discovery' despite AR1 only having the peramine alkaloid and wild-type having peramine, lolitrem B and ergovaline. The other ASW/alkaloid



research prior to the Popay et al. (1999) paper suggests that the wild type, having a range of alkaloids, would in fact confer a higher resistance against ASW. Popay et al. (1999) even go so far as to say that the ASW damage was low throughout the trial and although there was statistical significance the correlation coefficients were low.

In 2001 the 'novel' endophyte, AR1 was released to market. As mentioned previously AR1 contains peramine which is said to deter feeding of adult ASW. In the years 1993-98 a parasitoid, *Microctonus hyperodae*, was introduced and released to target ASW (McNeill et al. 2002). This, combined with the release of AR1 has significantly reduced the impact of the ASW by reducing adult feeding, egg laying and larval damage (Jackson et al. 2002). However the reduction caused by the combined effort of the endophyte and the parasitoid cannot be quantified for each 'control method'.

Black Beetle is a major agronomical pest in the northern parts of New Zealand. Adult black beetle are not particularly damaging to established pasture but can affect newly sown pasture by chewing out the base of emerging tillers. The black beetle larvae feed on roots close to the soil surface causing the crown of the grass to die through drought stress or through pulling by grazing animals (Popay and Baltus 2001). Popay and Baltus (2001) conducted both pot and field trials to try and determine whether AR1 had an effect on black beetle. They found that, in both trials, compared with endophyte-free grasses the black beetle damage was significantly reduced on AR1 grasses. The study also showed that the wild type endophyte grasses had significantly less damage than the AR1 grasses. This is in keeping with a study by Ball et al. (1997) that concluded black beetle was only affected by the ergovaline alkaloid but peramine (the only alkaloid in AR1) had no effect. As AR1 does not contain any ergot alkaloids the suggestion that AR1 provides some deterrence to black beetle is misleading. The most probable reason for Popay and Baltus (2001) to have found significant differences between the AR1 and nil-endophyte grasses is that AR1 provides some protection against other insect pests and abiotic stressors, which were not measured in their experiment.

In 2006 another 'novel' endophyte, AR37, was introduced to the market (Milne 2007). AR37 contains epoxy-janthitrems but none of the common alkaloids. Popay and Thom (2009) conducted a field trial evaluating how one variety, Commando, infected with three different endophytes; AR1, AR37 and standard (or wild-type) endophyte along with a line containing no endophyte (nil), performed against insect pests. The paddock-scale trial was able to confirm results found in small plot trials and pot experiments previously conducted. The Black Beetle feeding was suppressed in the AR37 and SE treatments and was moderately limited in the AR1 treatment. This suggests that it is not only ergovaline that deters black beetle, but epoxy-janthitrems do too.

Pasture mealy bug is endemic to New Zealand and is often found on native grasses and tussocks. It has adapted to introduced pasture species such as perennial ryegrass and tall fescue, and can be found throughout New Zealand (Charles et al. 2009). Although no serious infestations have been reported pasture mealy bug infestation has been associated with pastures under stress. Pennell and Ball (1999) found that the presence of an endophyte in tall fescue provides some protection from pasture mealy bug infection. However this study did not take into account other factors; it was previously stated that endophyte can offer some protection from abiotic stressors and pasture mealy bug is often only associated with pastures under stress. Is the endophyte protecting its host plant from pasture mealy bug or providing tolerance to other stressors therefore the pasture mealy bug is less likely to attack? If pasture can be well managed so that it is not under stress the effect of pasture mealy bug will be minimal.

The grass grub is native to New Zealand and is considered a serious pest to pastures (Dymock and Ford 1989). The most damage occurs when the grub is in its larval state, during autumn and early winter. The larvae feed on the roots of the grass plants, and during the cooler months there is little pasture growth to reduce the impact. The damage to the roots causes pastures to be 'pulled' by grazing animals and in severely infested pastures the grass can be rolled off the surface, like ready-lawn. In the field trial by Popay and Thom (2009) treatments containing AR37 were found to have lower grass grub populations, however this was not statistically significant to treatments containing AR1 or wild-type endophyte. The observed trend of better resistance to this insect pest within the AR37 treatment could be because of increased tolerance to other insects and not actually a resistance to the grass grub larvae (Popay and Tapper 2007). In an unreplicated field trial Popay et al. (2003) found that grass grub larvae populations were significantly reduced in a meadow fescue pasture containing loline-producing *Neotyphodium uncinatum* compared to an endophyte-free meadow fescue pasture. A replicated pot trial was set up to try and confirm the findings of the field trial. It was found that grass grubs lost weight, or gained less weight, on treatments containing endophyte, than the corresponding endophyte-free controls. The levels of the alkaloid, loline, were not measured and there is theory that deterrence is only observed when lolines are above a certain level (Patchett 2007). Patchett (2007) suggests that attack by grass grubs actually increases the loline concentrations thus suppressing feeding further. It was also found that seasonal distribution of loline concentration was higher in late autumn – when grass grubs cause the most damage to pasture (Patchett et al. 2011). Loline producing endophytes are the study of much research at present as they are only naturalised in fescue/meadow fescue grasses.

Porina are a major pest throughout New Zealand. They complete one life-cycle annually but can lay up to 1700 eggs which they disperse as they fly (Popay et al. 2012) resulting in large infestations across a wide area. Larvae feed on pasture, stripping it completely down to ground level resulting in an influx of weeds, as well as the obvious loss of productive pasture (Popay et al. 2012). In two pot trial experiments Jensen and Popay (2004) found that Porina larval survival was reduced in the treatments containing AR37. In one of the experiments, where a choice of feed was offered, the AR37 treatment was observed to have some feeding but the survival, and weight of the porina larvae declined. This suggests that AR37 is not only a deterrent but also has some degree of toxicity. The experimental results provided the basis for a field trial to be established. Two paddock-scale replicated trials containing AR1, AR37 and endophyte-free grasses of the same genotype of Italian ryegrass (in one trial) and perennial ryegrass (at the second trial) were established. The AR37 treatments showed a reduction in larval feeding, compared with the AR1 and endophyte-free treatments, however across both sites the larvae numbers were low (Popay et al. 2012). There was significant evidence of other insect species and this was taken into consideration however it would be difficult to quantify the damage done by each individual species within the sward.

Root aphid is known to inhabit the roots of both tall fescue and ryegrass throughout New Zealand (Popay 2009). In a field trial Pennell et al. (2005) suggested root aphid numbers were suppressed in pasture containing AR37, the data presented however suggests no significant difference between pastures containing AR37, wild-type or nil endophyte. The AR1 treatment, however, had a significantly larger number of root aphids present. Although the root aphid was not thought to have any significant economic impact (Jensen and Popay 2004, Pennell et al. 2005), Popay and Gerard (2007) found that pasture productivity is reduced by root aphid infection. In a replicated pot trial AR37 was shown to reduce root aphid populations compared with AR1 and endophyte-free pastures. There was no significant difference in root aphid populations between AR37 and wild-type endophyte pastures (Popay and Gerard 2007). In a paddock-scale trial these results were confirmed; AR37 was found to have the lowest number of root aphids, but, again, was not significantly different to wild-type endophyte (Popay and Thom 2009). Throughout the trial, which spanned 3 years, the numbers of root aphid increased the least on the AR37 and wild-type endophyte infected pastures. There were a number of other insect pests recorded in the trial. It is possible that the combination of all of the insects, together with climate conditions and also grass host genotype made the data for each individual insect subjective. This being said it is more realistic to have other influences when conducting a paddock-scale trial.

Hume et al. (2009) believe there is considerable evidence suggesting that the performance of ryegrasses in New Zealand can be improved with infection of fungal endophytes as they offer protection from insect pests. Some dramatic effects have been recorded however across the literature there has been a lot of variation suggesting there are other factors at play and perhaps even the possibility insects are developing some form of resistance to endophytes (Hume et al. 2016).

### 2.3.2 Persistence and Productivity of Pasture

30 years ago it was found that having an endophyte may increase the yield of pasture grass. Latch and Christensen (1985) found that infected Nui perennial ryegrass plants yielded 38% more dry matter than their uninfected counterparts. Easton (1999) and Rattray (2003) agree with this in their reviews of endophyte studies however both conclude results are inconsistent across different conditions; climate, host genotype, insect pressures. Easton (1999) cites some unpublished data (Easton and Rolston, Easton and Hume) that has shown growth cabinet and glasshouse based experiments have not proven any positive growth or stress tolerance effects due to having an endophyte. This research suggests endophytes do not directly increase pasture grass productivity, but may indirectly increase productivity through a reduction in pest pressure, increase in drought tolerance, some other mechanism or a combination of characteristics.

Cripps et al. (2013) suggests grass persistence is enhanced through the selection and deliberate use of fungal endophytes which provide benefits to the pasture, particularly pest resistance. The selection of insect controlling endophytes thus in turn is increasing the persistence of the pasture. Easton et al. (2001), similarly, suggests the persistence of ryegrass can be attributed to three main things; the endophyte protects the plant from insect pests, and from over-grazing by vertebrate animals and perhaps the promotion of tolerance to moisture stress although the evidence supporting these statements is somewhat lacking.

Cunningham et al. (1993) too, agrees with the suggestion that endophyte can enhance drought resistance in some grass hosts, but adds to it, saying that endophyte also helps improve seedling vigour in perennial ryegrass. The Cunningham et al. (1993) review is mainly based on experiments conducted in Australia, but given the harsher climate, compared with New Zealand, it is relevant. Several experiments were cited finding perennial ryegrass plants, of different cultivars, were more likely to survive drought conditions if they contained an endophyte. In one of the studies reviewed it was found that seedling establishment of perennial ryegrass containing endophyte was higher compared with those having a lower infection rate.

Further controlled experiments may better determine the mechanisms controlling the perceived increase in productivity and persistence of pastures.

### 2.3.3 Livestock health

In the early 1980s endophyte in ryegrass was conclusively linked with the stock health issue, ryegrass staggers (Fletcher and Harvey 1981). Before 1980 the role of endophyte had been largely ignored in research on ryegrass though now most of this research can be applied to the association of ryegrass and endophyte (Easton 1999). Despite the stock health problems; staggers, heat stress and increased serum prolactin levels, endophyte was also shown to deter the insect pest Argentine Stem Weevil (Fletcher 2009). The challenge was then to find an endophyte that didn't cause harm to the stock but still deterred the insect pests. Recent studies have focussed on the utilisation of novel endophytes to do just this; produce alkaloids that aren't detrimental to stock health but still enhance the performance of the host grass (Hill and Roach 2009).

It is important to mention, here, that the common endophyte strain found in tall fescue; *Epichloë coenophiala*, produces a vasoconstrictor alkaloid, ergovaline, which at higher concentrations in herbage, is responsible for animals developing tissue necroses such as fescue foot.

Non-toxic endophytes have had a significant impact on the livestock industry; there are now better summer/autumn animal growth rates, the presence of dags and flystrike has decreased and there has been a reduction in ryegrass staggers (Fletcher 1999).

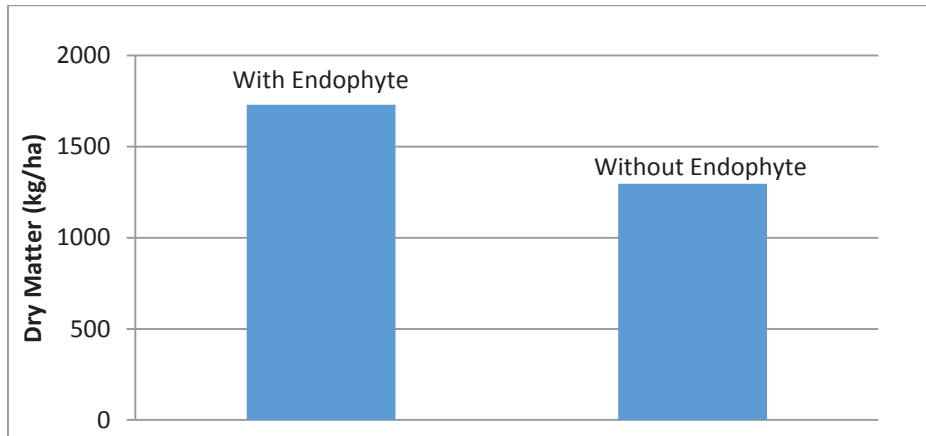
Animal production can also be influenced by endophyte. Bluett et al. (2005a) found an increase in milk production of cows grazing AR1 infected grass compared with those grazing wild type endophyte infected grass.

### 2.3.4 Abiotic stressors

The relationship between the cool season grass and the *Epichloë* endophyte is unique in that unlike the majority of plant/microbial symbioses, which are usually based on the acquisition of nutrients, it is in fact based on the protection of the host grass from abiotic and biotic stressors (Clay and Schardl 2002).

There has been some debate over whether an endophyte can improve the yield of forage grasses containing an endophyte. Evans and Kitson (2011, unpub data) found large differences in a range of grasses containing an endophyte compared to their nil endophyte counterparts over a 5 month period. Five different grasses, of differing species and ploidy, infected with the same species of endophyte (*Neotyphodium siegelii*), were pooled together against their endophyte-free

counterparts. In a replicated trial, sown in Canterbury, New Zealand, it was found that those with endophyte produced significantly more dry matter (kg/ha) than those that were endophyte-free. This is shown in Figure 2.



**Figure 2. Combined autumn dry matter production (kg/ha) of the five endophyte containing grasses versus their respective nil endophyte counterparts LSD ( $p=0.05$ ) 254. (Evans and Kitson, unpublished data, 2011).**

Hesse et al. (2005) found that having an endophyte in grass changed the plant growth for better persistence in drought conditions. The root dry weight and the root/shoot ratios tended to be higher when the grass was infected by endophyte. Conversely, Eerens et al. (1998) ran a glasshouse experiment which found that endophyte-free grass treatments had higher mean root and shoot weights. They believe this could be explained by some level of parasitism between the endophyte and its grass host.

As different endophyte strains produce different levels of endophyte, and present differently in different genotypic grass hosts, potentially there could be one alkaloid, or a series of alkaloids, in combination with certain lines of grass that affect different plant growth processes (Cheplick and Cho 2003). Siegel and Bush (1996) have suggested that the accumulation of the alkaloid loline could alter the osmotic potential of the grass host thus improving drought tolerance.

Endophyte species existence is completely dependent on the vertical transmission from plant to seed therefore there must be some benefit to the host grass or the longer term survival of the mutualistic association would be unlikely (Easton 2007).

## 2.4 Inoculation and Detection

The most commonly used method to inoculate grasses with a novel endophyte is the slitting method. This involves inserting endophyte mycelium from pure cultures, grown on agar, into the meristemic region at the junction of the mesocotyl and coleoptile of very young seedlings. Slits are made in the young seedlings by a very fine scalpel or by injection (Latch and Christensen 1985). Typically inoculations are the most successful when endophyte isolates are inoculated between closely related grass species. However Christensen (1995) isolated 18 different *Epichloë* strains from a range of hosts and tested the compatibility of introducing different strains to both natural and non-natural host grasses. Of the 54 associations inoculated by Latch and Christensen (1985) using the slitting method, 51 were compatible, however from this 7 more proved incompatible as the seedling grew. There were also some associations where endophyte-free tillers were produced. The endophyte-free tillers are rarely formed when endophytes are in association with their natural host grass. Easton (2007) agrees with Christensen (1995) that the inoculation process is a tricky one; sometimes the endophyte doesn't survive, and sometimes the endophyte does survive past the inoculation process but fails to transmit into new shoots and/or seed. Christensen and Voisey (2007) found in some endophyte-grass associations that endophyte-free tillers could actually be growing alongside endophyte-containing tillers within the same plant.

The associations between host and endophyte are very complex. It is not known how the grass host and the endophyte recognise each other, or why some interactions either accept or reject the endophyte at a molecular level (Malinowski and Belesky 2006). Stability and transmission from generation to generation of each endophyte strain-cultivar combination needs to be evaluated thoroughly before commercial release in order to determine the host-endophyte compatibility (Tian et al. 2013a). Failure is common and if not properly evaluated can be an expensive lesson.

Endophyte genotype strongly influences interactive effects with the host genotype including, but not limited to; variability in endophyte colony morphology, in the rate of synthesis or final concentration of alkaloids and differences in physiological effects, and these interactions can impact infection status (Hesse et al. 2005).

Schardl et al. (2008) suggests that grass hosts tend to benefit more from endophytes that have adapted to similar grass hosts but will benefit less, or even suffer deleterious effects, from endophytes introduced to them that have adapted in distantly related host grasses. Evolutionally endophyte infected grasses have out-competed uninfected grasses thus resulting in positive natural selection. Although there is the issue that the benefits of evolutionary adaptation may result in endophyte inoculations failing when grasses and endophytes (and those in combination) are introduced to differing ecological environments (Malinowski and Belesky 2006). Human selection

and dispersion of endophyte infected grasses has also influenced how the symbiotic relationship has adapted. Easton (2007) believes that plant breeding activity will actually enhance the fitness of selected endophytes by developing grass hosts that are already adapted to new associations. Desired alkaloids will also be controlled better with improved technological ability to exploit both host and endophytic genetics. Contrary to this (Simpson and Mace 2012) suggest that synthetic symbioses can produce both wanted and unwanted outcomes. There is the possibility of spontaneous change in *Epichloë* species, which can occur in both natural and artificial associations.

## 2.5 Storage of endophytes

The relationship between endophyte and grass host is a complex one. Different combinations of endophyte strains and grass genotypes influence the behaviour and survival of the living fungus. There have been many studies done on the survival of endophytic fungus in stored seed however with the development of both commercial grasses and the discovery of new endophytes these can differ greatly between combinations. Hill and Roach (2009) discuss the point that different plant/endophyte combinations express variation in compatibility and that endophyte survival during seed storage could be controlled by the plant genetics, the endophyte genetics or a combination of the two. In a storage experiment on endophyte viability in tall fescue they found endophyte survival depended on both the host grass and the endophyte but interestingly the endophyte isolated from a Mediterranean tall fescue host survived best in a cultivar bred with Mediterranean germplasm. Possibly the most influential endophyte storage research was done by Rolston et al. (1986) and is still continuing as a long-term project thirty years later. In this study it was found that endophyte viability decreases at a faster rate to that of seed germination, so in the commercial world 'good' seed could still be grown without the added advantage of having a live endophyte within it. The loss of the viable endophyte in seed reduces its value as the benefits of the endophyte are lost when it dies (Tian et al. 2013a). Twelve months into the Rolston et al. (1986) project there was no significant decrease in seed germination despite significant drops in endophyte viability. The 1986 study found that ryegrass containing the endophyte *Epichloë festucae* var. *lolii* (or in those days known as *Acremonium lolii*) varied in decline depending on several factors; seed moisture content, bag type and temperature and humidity.

In the Rolston et al. (1986) study it was found that seed moisture content (SMC) is in equilibrium with relative humidity (%RH). The physiological mechanisms influencing the decline of endophyte viability are not clear (Hume et al. 2013). The viable endophyte rapidly declines as the SMC goes





months (Ratray 2003). Easton et al. (2001) also found that humidity was a larger influence than temperature with reductions in endophyte viability after just a few months if humidity is too high. Seed has successfully been stored, and maintained endophyte viability, for 15 years at 5°C, 50%RH and 11%SMC. More recently Tian et al. (2013a) ran an experiment on the effects of storage temperature on seed germination and endophyte viability and found that endophyte genotype may influence seed survival and/or subsequent germination under stressful conditions, such as increased temperature. In the same experiment it was found that storage at cooler temperatures (-20°C to 4°C) was optimal for retaining both the viability of the seed and the endophyte. However there were no significant differences between the seed stored at -20°C or 4°C so from an economic point of view it would be preferred to store the seed at 4°C.

Hume et al. (2013) go so far as to suggest that the whole supply chain, from seed company to farmer, treat the endophyte infected seed as a high-value, perishable product and that measures are taken to ensure the quality.

This thesis investigates the last-mentioned issue, loss of viable *Epichloë* endophyte in seed during storage, and accordingly the objectives of the thesis were:

1. To determine whether different endophyte/genotype combinations lose viability at different rates over time.
2. To determine whether different endophytes lose viability at different rates in different plant genotypes.
3. To determine how different storage conditions affect the viability of endophyte in different endophyte/genotype combinations.
4. To determine how germination rates and endophyte viability rates compare in different storage locations over time.

## **Chapter 3 Materials and Methods**

### **3.1 Genotype/Endophyte varieties**

In September 2012 twenty cores, 15cm in diameter, were taken from separate one year old existing grass/endophyte plots at Yaldhurst, Christchurch, and planted in isolations within a ryecorn crop. The grass/endo combinations were: Tetramax 815, Indiana 815, Trojan NEA2/6, Bealey NEA2/6, Alto AR1, Alto AR37, Halo AR1 and Halo AR37. Seed from these was harvested between the 8<sup>th</sup> and 22<sup>nd</sup> of January 2013 when seed was shedding when 'tapped' into a hand. Seed was threshed and cleaned by hand in early February and placed into paper bags in a 4°C/35%RH fridge for 1 week.

### **3.2 Storage**

Initial germination and endophyte viability tests were conducted before the treatments were 'blind' coded by a third party. Samples of each treatment (n = 9, now labelled A-H) were weighed out into 100g lots and placed into hand sown polyethylene bags that were 25cm<sup>2</sup>. Three randomly selected bags of each treatment were placed into 3 different locations; cool store, top of the warehouse and ground level (or bottom) of the warehouse in a completely randomised design.

### **3.3 Germination tests**

Germination testing was performed by counting 100 seeds of each genotype/endophyte treatment on to moist tissue paper. Four replications of each treatment were done. The tissue paper was kept at room temperature for 14 days. After seed had been divided into treatment bags 33 seeds were counted out of each treatment and the same method was used. Seed viability was assessed as a percentage of seeds that had germinated over the 14 day period.

### **3.4 Endophyte viability tests**

Grow-out tiller tests were done by sowing 100 seeds of each grass/endophyte combination into a tray of Yates Black Magic seed raising mix. Trays were placed in a tunnel house and watered as necessary. The plants were grown for 8 weeks then were tested for presence of endophyte using a phytoscreen immunoblot kit (Agrinostics, Georgia, USA; cat. #ENDO797-3) following the manufacturer's instructions. Initial endophyte viability tests were conducted before the seed lots

were divided into separate replicates. In June and November 2013 and March 2014 17 seeds from each of the 72 seedlots were grown to test for presence of endophyte using the same method.

### **3.5 Measurement and Analysis**

Temperature and humidity were recorded at 6 hour intervals in both uncontrolled environments with a Hobo® U10-003 data logger.

In March 2014, after the final sampling, grass/endophyte combinations were matched up with the corresponding 'blind' codes.

Temperature and humidity data were collected and analysed using Hoboware®.

Endophyte viability and germination data were analysed by a residual maximum likelihood (REML) model using Genstat 17.

## Chapter 4 Results

### 4.1 Temperature and Humidity

Temperatures and Humidity were recorded 4 times a day by data loggers placed in the same position as the seed lots. In total there were 1510 recordings taken. The graphs from the data loggers show that both the temperature and humidity at the top of the warehouse were more extreme than the temperature and humidity at the bottom of the warehouse. Table 4 shows the differences in extremities of both temperature and humidity at the top and bottom of a warehouse.

**Table 4. Comparison of temperature and relative humidity in 2 different locations in a 1 year period.**

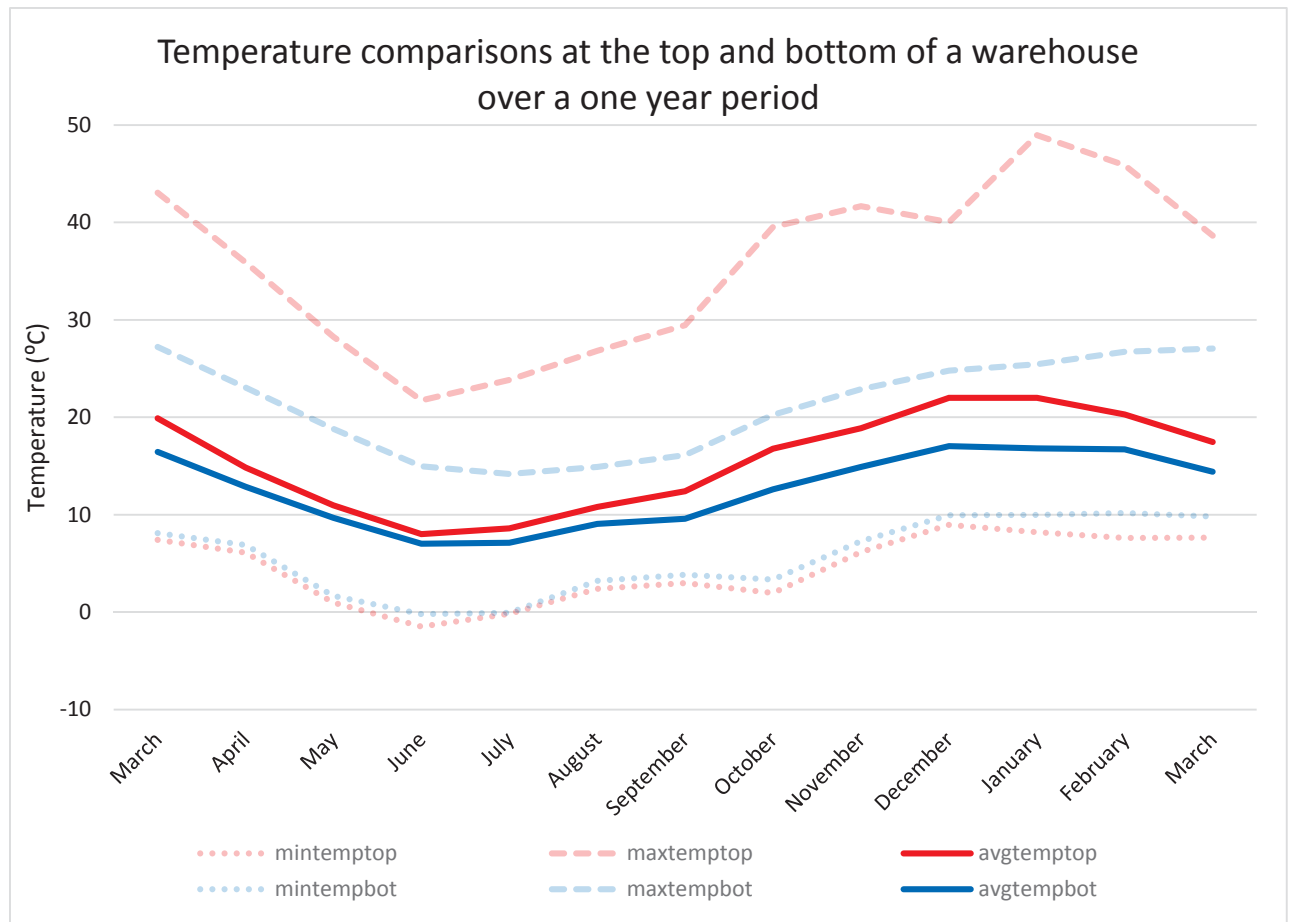
	Top	Bottom
<b>Temperature (°C)</b>		
Maximum	48.97	27.24
Minimum	-1.47	-0.2
Average	15.43	12.48
<b>Humidity (RH)</b>		
Maximum	94.96%	97.75%
Minimum	11.44%	39.81%
Average	64.08%	77.51%

The cool store was kept at a constant 5°C and 35%RH. The fluctuations in temperature and humidity at the top of the warehouse were more extreme, not just overall but on a day-to-day basis (refer to appendix 2 and 3). The top of the warehouse was on average almost 3°C higher although the humidity, on average, was over 13% lower.

The average temperature fluctuates similarly in both the top of the warehouse and the bottom of the warehouse across the year. In the summer period (December/January) the differences are at their largest with the difference between the two at just under 5°C, compared with a difference of between 1 and 2°C in the autumn and winter months (fig. 4). The cool store temperature remains constant 5°C throughout the year.

The range in temperature was larger at the top of the warehouse compared to the bottom of the warehouse. The top of the warehouse ranged from a difference of 23° (-2° to 21°C) in mid-winter to 41° (8° - 49°C) in the height of summer. The bottom of the warehouse had less drastic differences,

ranging from a difference of 12° (3 - 15°C) in mid-winter to 19° (8 - 27°C) in late summer. The average temperature in both the top of the warehouse and the bottom of the warehouse (Figure 4) show that the seasonal fluctuations are very similar in both locations. These fluctuations are typical of the Canterbury environment where cool nights are often followed by mild days.

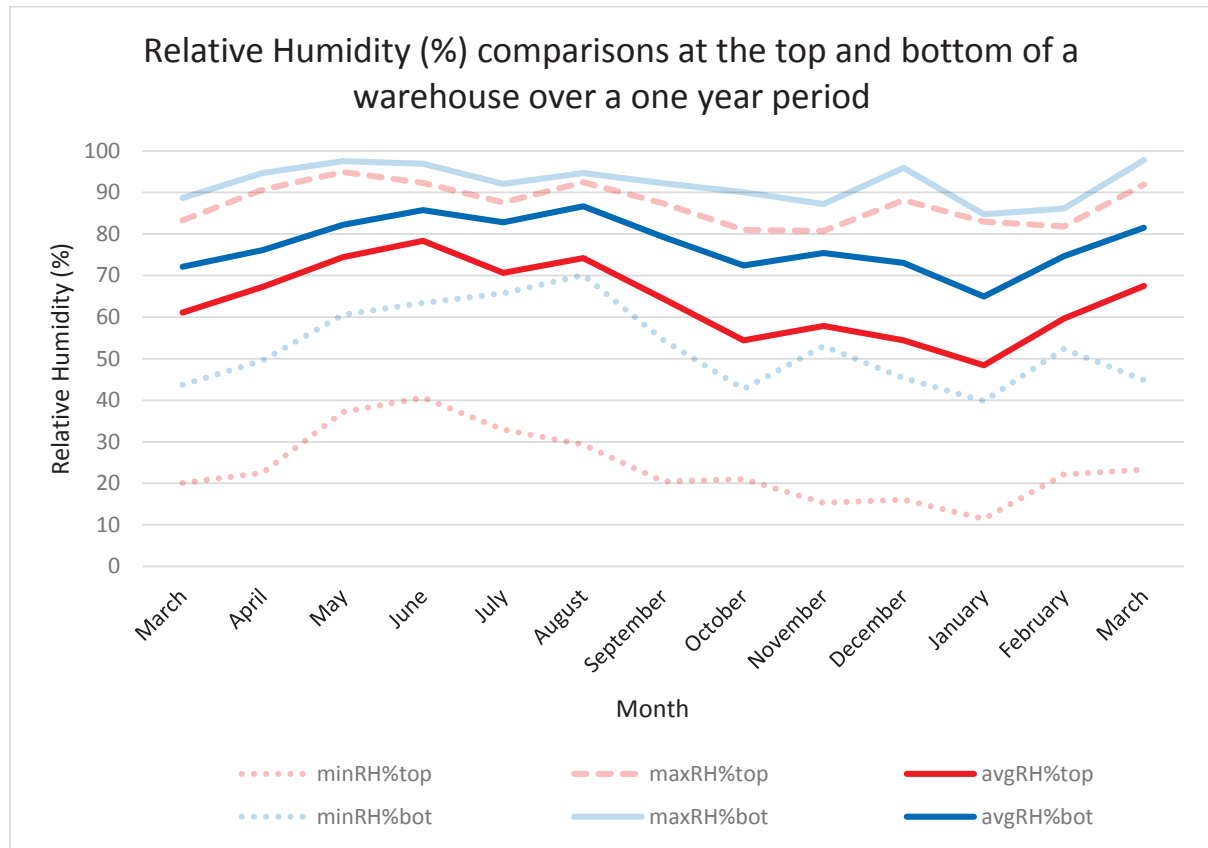


**Figure 4. Temperature comparisons at the top and bottom of a warehouse over a one year period.**

As with the fluctuations in temperature, the relative humidity has larger fluctuation at the top of the warehouse to that at the bottom of the warehouse (figure 5) The RH% ranged from 52% (40 – 92%), in August, to 72% (16 – 88%) in mid-summer. At the bottom of the warehouse the RH% ranged from 24% (70 – 94%), in August, to 51% (45 – 96%), in December. There is a correlation between relative humidity and temperature we can deduce from the above figures that, seasonally, as the temperature decreases the RH% increases, and as the temperature increases, the RH% decreases.

The seasonal fluctuations in the relative humidity are also similar between the top and bottom locations. The bottom of the warehouse has a higher average relative humidity across the one-year

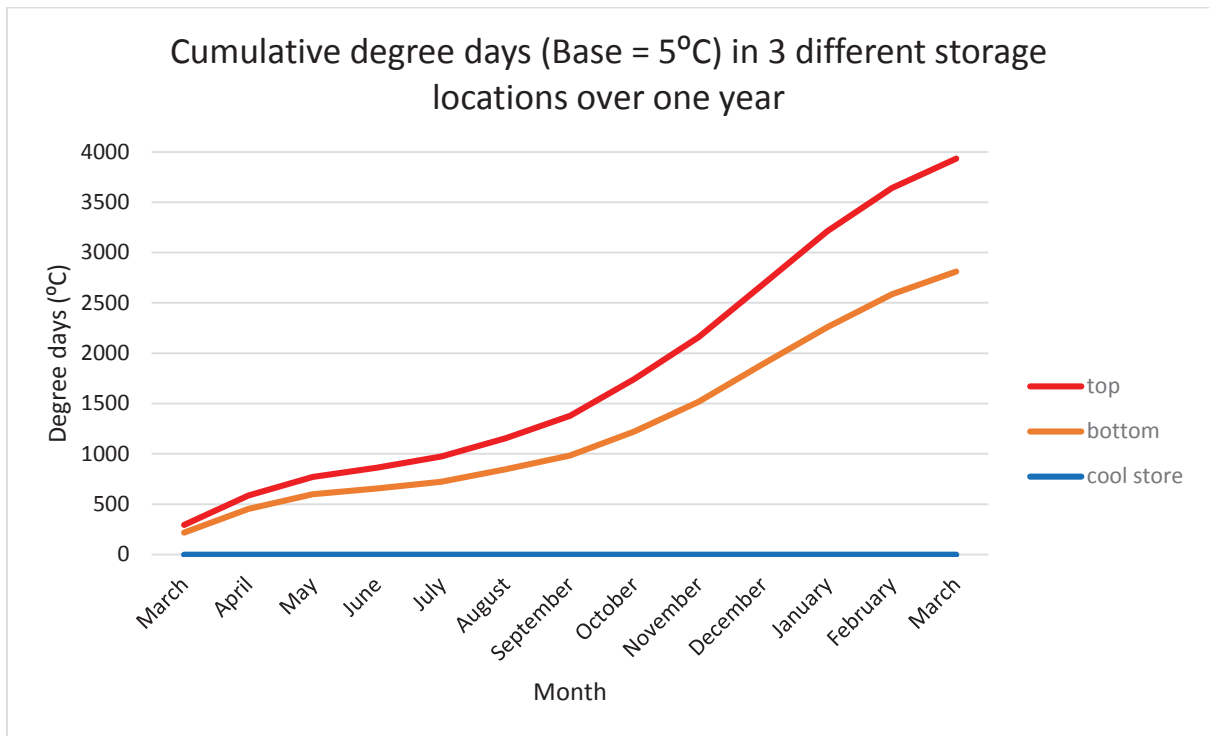
period than the top of the warehouse. Again the cool store is constant throughout the year, set at 35%RH.



**Figure 5. Relative Humidity comparisons at the top and bottom of a warehouse over a one year period.**

Degree days accumulate much faster in the top of the warehouse than the bottom of the warehouse. This is depicted in figure 6 where accumulation was calculated from a base of 5°C. As the summer months approach the top of the warehouse accumulates degree days much faster, peaking in January with an addition of 160.03 more degree days than the degree day accumulation at the bottom of the warehouse.

The degree day accumulation appears not to correspond to the germination rates however there could be some correlation with endophyte viability. In 6 of the 8 genotype/endophyte combinations (with the remaining two having the same endophyte) the endophyte viability declined more at the top of the warehouse than at the bottom of the warehouse.

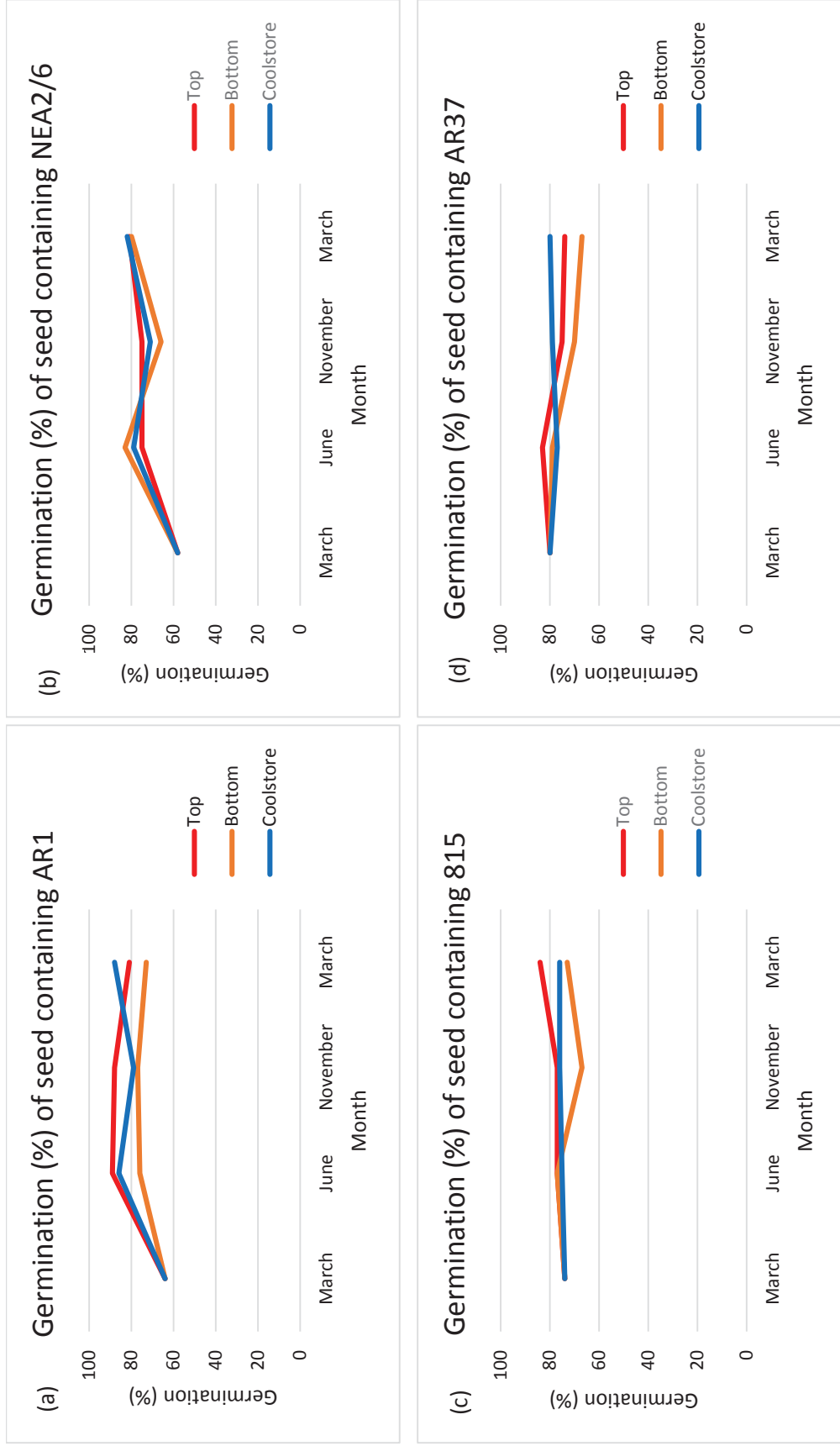


**Figure 6. The cumulative degree days (using a base of 5°C) in three different storage locations over one year.**

## 4.2 Germination

The germination rates of all the genotype/endophyte combinations stayed relatively constant over the one year period. Figure 6 (a, b, c and d) shows the germination rates of the different genotypes as influenced by which endophyte they contained. There were minor fluctuations in the germination rates at different times in the year, and the different genotypes but these were insignificant.





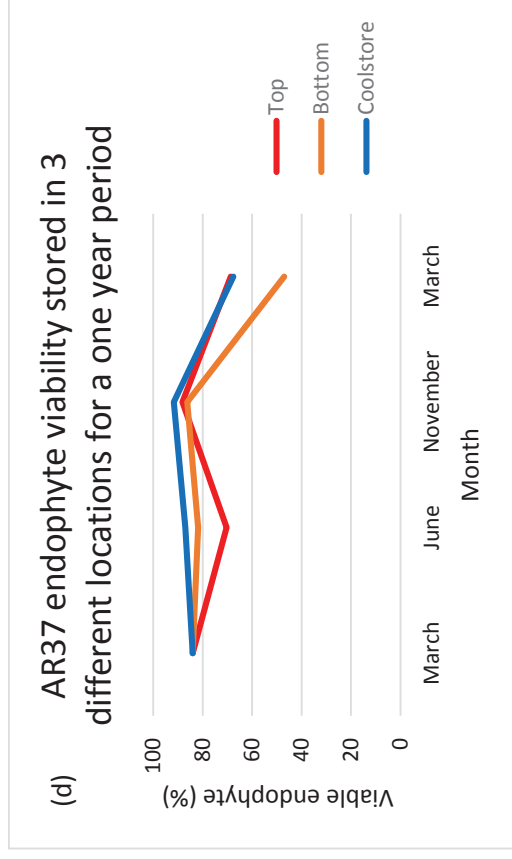
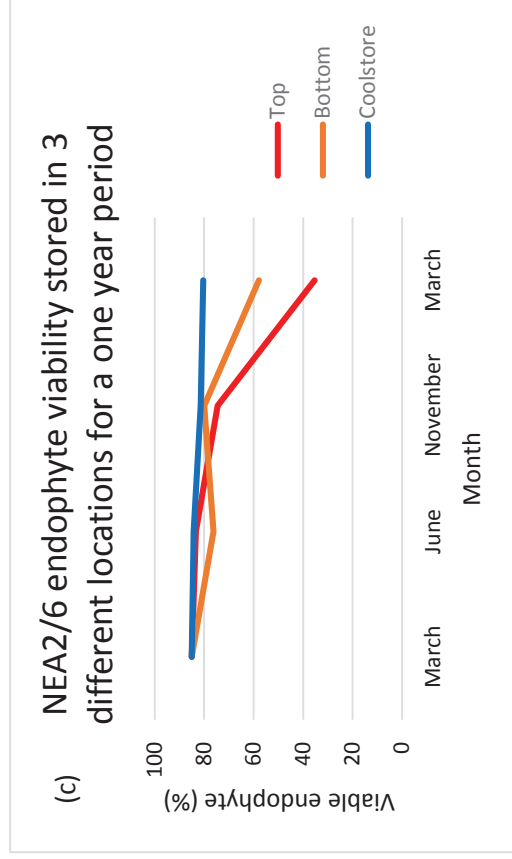
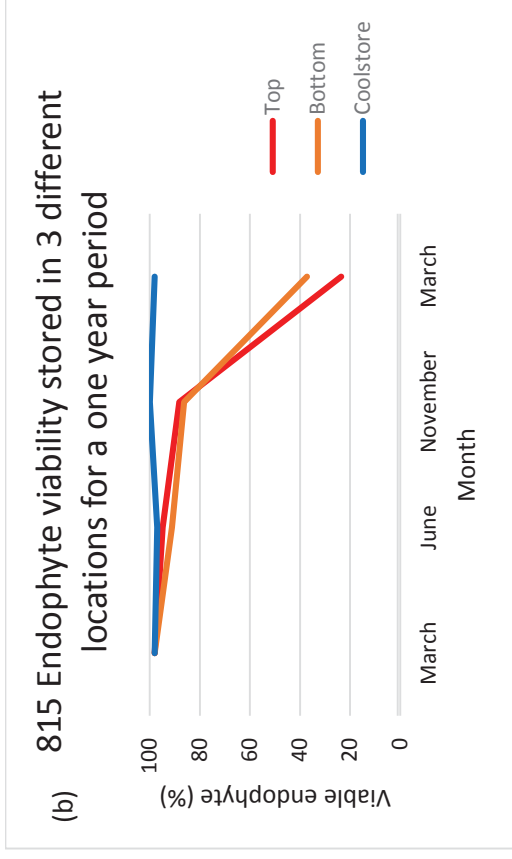
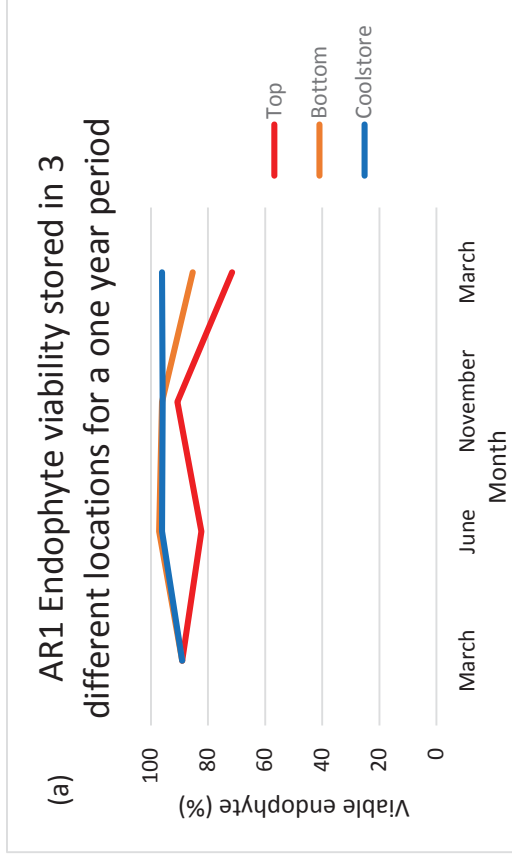
**Figure 7. Germination (%) of seed containing 4 different endophytes, in two ryegrass genotypes each, stored in 3 different locations for a one year period. P = NS, LSD = 9.7**

### 4.3 Endophyte viability

Endophyte viability declines at different rates across time, location and ploidy. In a Residual Maximum Likelihood (REML) model, using time as the random model, the results suggest there is a significant difference ( $p < .001$ , LSD = 11.62) in endophyte viability between different strains of endophyte in different locations over time (refer to appendix 2).

Figure 7 (a, b, c and d) show the endophyte viability of each endophyte in the three different storage locations. In the AR1, NEA2/6 and 815 treatments the endophyte declines more so at the top of the warehouse, followed by the bottom of the warehouse and insignificantly in the cool store. The AR37 fluctuates differently throughout the year and also in the different locations. This will be discussed further in 5.1.

The AR1, NEA2/6 and 815 endophytes all decline significantly over the one year period in the top of the warehouse decreasing by 16, 46 and 70 percentage points respectively ( $p < .001$ , LSD = 15.9). The AR37 endophyte only dropped 12 percentage points at the top of the warehouse. In the bottom of the warehouse the AR37, NEA2/6 and 815 treatments declined 34, 23 and 57 percentage points respectively ( $p = 0.002$ , LSD = 20.3). The AR1 endophyte treatment declined by 4 percentage points making it relatively stable in the conditions. In the cool store the AR1, NEA2/6 and 815 endophytes stayed stable, appearing to 'gain' endophyte over the time period (discussed in 5.1). AR37 decreased in viability however this was statistically non-significant though if tested in the commercial sense could have an impact as it falls below the 70% threshold required by licencing agreements.



**Figure 8. Endophyte viability of 4 endophytes, in two ryegrass genotypes each, stored in 3 different locations for a one year period.**

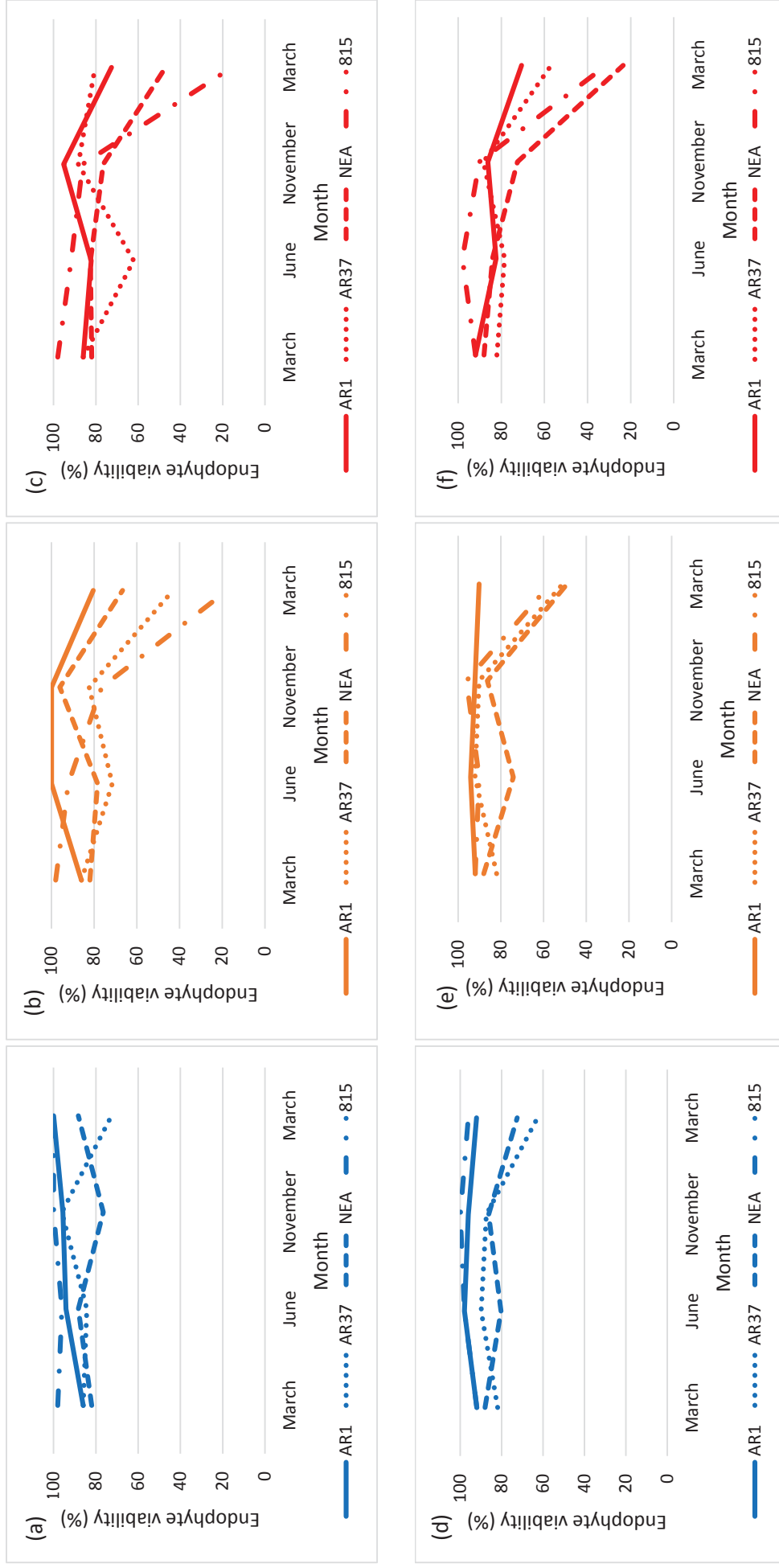
(P<.001. LSD = 11.62, standard errors: Time = 5.819, Location = 5.773, Endophyte = 5.756)

#### 4.4 Ploidy

In the REML model (appendix 2) the results suggest that endophyte viability difference is significant across time, location and ploidy ( $p = 0.005$ ,  $LSD = 16.43$ ). These results, however, do not necessarily indicate a ploidy effect as the cultivar also differs across ploidy so it could be more of a cultivar effect rather than a ploidy one, or a combination of both.

Figure 8 illustrates the differences between the tetraploid/endophyte combinations and their diploid/endophyte combination counterparts. In the combinations containing NEA2/6 the diploid cultivar appears to lose endophyte viability more so than the tetraploid cultivar across the three storage conditions. Conversely in the combinations containing 815, the tetraploid cultivar appears to lose endophyte viability more so than the diploid cultivar in both the bottom of the warehouse and the top of the warehouse. In the cool store both combinations containing 815 are stable across the time period.

The AR1 treatments are relatively stable, dropping slightly in the top of the warehouse but this is across both the tetraploid and diploid cultivars. The genotypes for diploid and tetraploid combinations with AR1 and AR37 are the same; Alto (2n) and Halo (4n). The significant drop in the AR37 treatments in all storage conditions, bar the tetraploid in the top of the warehouse, suggest it isn't a ploidy relationship but a cultivar one. This is discussed further in Section 5.1.



**Figure 9. Endophyte viability of different endophyte/genotype combinations in three different storage conditions over one year.**

(P = 0.005, LSD = 16.43, Standard errors: Time = 8.23, Location = 8.198, Endophyte = 8.187, Ploidy = 8.209)

a, b and c are tetraploid combinations in a cool store, bottom of a warehouse and top of a warehouse respectively. d, e and f are diploid combinations in a cool store, bottom of a warehouse and top of a warehouse respectively

## Chapter 5 Discussion

### 5.1 Endophyte viability in different hosts

Endophyte strain and plant cultivar both affect the rate of endophyte viability. The findings of this experiment are in line with that of Tian et al (2013) who found that the strain of the endophyte did influence the viability within a single genotype. There appears to be a significant interaction with the ploidy of the ryegrasses, however this is probably due to the fact that different cultivars represent different ploidy. To see if there is a statistically significant result with ploidy it would be necessary to run the experiment with the same cultivars containing different endophytes (discussed in Section 5.3).

The trends seem to be similar in that endophyte declines over the course of a year outside of the cool store however the rates of decline differ. In all the treatments, except the two AR37 treatments, the trend was that the endophyte declined more so at the top of the warehouse. Although there is no reason identified for the AR37 to perform differently it would be expected that the endophyte viability decreases further in the harsher environment with the greater fluctuations in both humidity and temperature.

The fact that the AR37 performed differently to the AR1 strains in the same plant genotypes suggests there is an interaction between endophyte strain and host genotype. AR1 appears to be the most stable endophyte strain, dropping the least in both the cool store and the bottom of the warehouse and not significantly less than the AR37 strain at the top of the warehouse. This is consistent with data of Hume et al (2011) who found that some ryegrass varieties infected with AR1 have maintained viable endophyte, in controlled storage, for up to 14 years, however, those stored in ambient conditions in 2 New Zealand sites and 1 Australian site declined at a far greater rate than indicated in this present study. Tian et al (2013) found that SE, AR1 and AR37 had the highest viability rates during seed storage compared with other novel endophytes, including NEA2/6. The AR1 stored well in ambient conditions and maintained a viability of >90% after storage for 14 months.

Although both Hume et al (2011) and Tian et al (2013) only used one plant cultivar a study by Hill and Roach (2009) found that across 3 fescue genotypes all infected with the same endophyte strain (AR542) the storage did effect the viability. They suggest that endophyte survival in different hosts is related to plant host genetics and is a heritable trait. They found AR542 survived best in the host plant most similar to that that the endophyte was isolated from; both being of Mediterranean

heritage. Although there is no actual determination of this it is interesting to note that both Indiana and Tetramax performed poorly in ambient New Zealand conditions and they were in fact bred in Europe. The 815 line survived the least in these host grasses which may be attributed to the warmer summers that the grass hosts were not adapted to.

There were significant relationships between grass host and endophyte viability however it was not possible to test all the endophyte varieties in each particular grass host. Each endophyte strain was in two grass hosts, a diploid and a tetraploid. Although ploidy was shown to have a significant effect ( $P = 0.005$ ) this could actually be attributed to the genotype. There is a large degree of interpretational difficulty as different endophytes were in different host genotypes and it is difficult to tease out the difference between a genotype effect and/or a ploidy effect. The AR37 in the tetraploid line had significantly less viability in seed stored at the bottom of the warehouse than the top. Although the top of the warehouse accumulated degree days much faster than the bottom of the warehouse the average RH% was higher at the bottom of the warehouse suggesting it is not just the temperature influencing endophyte viability. The AR37 viability in the diploid line (Alto) however is significantly reduced in all three storage locations across the one year period. This reinforces that there is most likely an endophyte/genotype, or potentially an endophyte/ploidy interaction although with only two treatments of each endophyte and ploidy it is difficult to draw a conclusion in this study.

Degree days give some indication that if cumulative temperature influences endophyte viability in stored seed it would be expected that endophyte viability would last longer in the bottom of the warehouse than the top. It is difficult to extract this information without testing endophyte viability at more regular intervals e.g. monthly, particularly after November. The November endophyte viabilities are not significantly different, in any genotype/endophyte combination, to the June viabilities suggesting the crucial degree day accumulations is greater than 2000°C days.

In the raw data a lot of the treatments seemed to decline in viability in the June endophyte tests then increase again in the November treatments. Christensen and Voisey (2009) found that fewer hyphae are produced in winter and perhaps the concentration was too low to be registered on an immuno-blot test. The testing procedure in this study did not account for outside temperature influences in growing the seedlings or during the testing of the endophyte viability. In future growing and testing should be done at a standardised temperature.

The seed stored in this study was of much smaller quantities than those that are stored commercially. Although the bag type and storage technique was the same there was not as much

bulk to the treatments which could affect the overall outcome as seed may dry, heat or chill faster than commercial quantities may.

## **5.2 Endophyte viability in different storage conditions**

All endophyte varieties decreased in endophyte viability over the summer period outside of the cool-store environment. The endophyte viability was stable post-harvest (rates of decline were minimal and not significant) but began to decline as the temperature increased and the humidity began to fluctuate more. Consistent with other studies (Hume et al, 2011; Tian et al 2013) endophyte viability declines at a far greater rate than germination.

Within the different storage locations there was some significance between endophyte viabilities. At both the top and bottom of the warehouse the endophyte 815 was significantly worse than the other three endophytes at maintaining viability, regardless of plant genotype host. This suggests that it is just the endophyte that is affected by temperature however more experiments could be done to find a grass genotype which is better suited to maintaining 815. Inoculating 815 into a locally bred grass host would be the best option to test this further, or even better, in to the same grass hosts that would contain AR1, AR37 or NEA2/6.

Figures 8 and 9 show quite clearly that endophyte viability is largely influenced by summer storage conditions. The 'drop' in endophyte viability ranged from 70% to 10% outside of the cool-store environment over a 1 year period whereas inside the cool-store the endophyte viability was only minimally reduced, ranging from 21% to 1%. This was consistent with previous studies by Rolston et al (1986), Hume et al (2011) and Tian et al (2013). In all 3 previously mentioned studies it was determined that an increase in temperature and/or relative humidity resulted in the endophyte viability declining. The tetraploid containing AR37 (Halo) reacted differently to the other tetraploid/endophyte combinations in this study, and the results suggest it is more likely a higher RH%, than higher temperature, which affects the endophyte viability in this particular combination.

The best storage conditions for endophyte infected seed, regardless of both cultivar type and endophyte type are in a cool store remains important for the release of 815 in the coming years.



### 5.3 Recommendations and future research

In future all endophyte containing seed should be kept in the cool store over the summer period. Seed kept post-harvest for spring sowing will maintain its viability, provided the winter is not too warm, until sowing but anything leftover should be placed in cool storage and maintained at 4°C and 35%RH until use the following autumn, or spring if required.

New cultivars infected with endophyte should be assessed for their compatibility with the host grass before being released to market. Seed companies and farmers alike depend on good quality seed with a good quality viable endophyte. Tian et al (2013) found that accelerated aging tests, in particular 80%RH for 7 days or 100%RH for 4 days, are suggested for simulating the natural aging process relevant to those cultivars containing novel endophyte commercially. There is also the potential for further research on endophyte viability on different humidity levels but at constant temperatures.

It would be interesting to have a few genotypes infected with all the endophytes, e.g. Alto with AR1, AR37 and NEA2/6 as well as 815, so that more information can be derived to see if there are any determining trends. This could allow seed companies to predict how genotype/endophyte combinations would store. The study by Hill and Roach (2009) shed some light on endophyte survival in hosts potentially being a heritable trait. This is of particular interest to plant breeders because if there was the ability to breed in/screen for the genetic material required to be compatible with a novel endophyte, this could cut down on both time and trialling of host/endophyte relationships.

Testing in different climates would also be of interest. Hume et al (2011) found a linear response to endophyte viability in regards to region. Although this study found no significant differences between the top and bottom of the same warehouse different regions in which seed is stored may affect things differently. For example, testing in a seed store in both the North Island of New Zealand and in Australia could produce far different results from testing in one region (Canterbury), as a result of different thermal time accumulation profiles.

Rolston et al (1986) looked into different bag types for storage of seed. As new polymers are created (this testing was done almost 30 years ago) it would be interesting to see if there is an efficient way of maintaining seed and endophyte viability commercially in a bag which reduces the effects of temperature and humidity without increased cost to the supplier.

As the differences in the seed lines containing AR37 suggest viability is influenced more so by RH% than temperature it would be a good idea to test the same genotype/endophyte in a range of humidity controlled environments where temperature could be maintained. Rolston et al. (1986)

reported that the RH% was in equilibrium with seed moisture content (SMC). In this study the SMC was not measured but could be a reason for the difference in the tetraploid AR37 treatment behaving differently.

The statistics model used – REML with time as the random effect calculates the p values and LSDs in a different way to a standard generalised linear model (GLM) where all effects are fixed. The REML model adjusts (increases) LSDs for random effect means compared with those for fixed effects means, based on the concept that for successive samples from the same population over time, the mean will vary by random chance because of variability in the population being sampled, so the measured samples might be an optimistic indication of the sample variability. For this reason the REML model with factors treated as random effects where appropriate is considered less likely to wrongly find a statistically significant difference when no biological difference exists.

## **Chapter 6 Conclusions**

- Storage at both temperature and higher humidity over a one-year period decrease the viability of endophyte in most genotype/endophyte associations.
- As in previous studies the data show that a cool, dry environment for seed containing endophyte is important to maintain endophyte survival. This study shows how important it is to keep the to-be-released endophyte 815 in controlled conditions so endophyte viability can be retained.
- Endophyte viability declines outside of the cool store regardless of position in the warehouse. There is a trend that it declines more in more extreme conditions though this was more evident in the tetraploid than diploid series of tests. Whether RH% or temperature have a more significant effect on genotype/endophyte combinations can vary between combinations.
- Germination and endophyte viability decline at different rates. Germination is stable regardless of cultivar or storage conditions whereas endophyte has a wide range of factors including cultivar choice, endophyte strain, ploidy and storage conditions (whether temperature and/or humidity) influencing viability.
- Endophyte viability declines at different rates in different genotypes.

## Appendices

### Appendix 1. Other work attempted

The experiment reported in Chapters 3 – 6 was set up at short notice after an earlier experiment had to be abandoned for regulatory compliance reasons outside the control of the author. Here a brief overview of the original experiment is presented.

Compatibility and transmission of the endophyte *Epichloë siegelii* in a pre-commercial perennial ryegrass (*Lolium perenne*) cultivar.

#### Introduction to the project

In recent times in New Zealand there has been a great deal of interest in the inoculation of novel endophytes into commercially viable grass cultivars. The novel endophytes selected would produce alkaloids that deter insect pests but be non-toxic to grazing animals.

The endophyte *Epichloë siegelii* was discovered in 1999 in a US plant introduction collection maintained in Pullman, Washington, United States (Accession # P.I. 237707). Its native host is *Festuca pratensis* however it was successfully transferred to *Lolium perenne*, as well as other non-native hosts *Festuca arundinacea* and *Lolium multiflorum*. The discovery of the ability to successfully inoculate to a range of grass species hosts provides a wide scope of potential in the pastoral industry. The endophyte was named by the acronym 'HAPPE' or High Animal and Pasture Performance Endophyte (Hignight and Rush 2004).

The cultivar inoculated was a diploid perennial ryegrass with *some* meadow fescue parentage resulting from 25% of the parentage descending from Revolution Enhanced® Ryegrass. The cultivar, at the time of the project, was called 06Px22, and has since been released to the market as Bronte.

An area of interest is the compatibility of novel endophytes into non-native hosts. As discussed in Section 2.5 Inoculation and Detection, the inoculation process is complex and has low success, more so, outside its native host the endophyte is more likely to have transmission failure – that is; some tillers will not contain the endophyte thus the reproductive shoots will produce endophyte-free

seed. Christensen and Voisey (2007) found that there were varying degrees of compatibility – some plants failed to be inoculated, some were inoculated but the endophyte failed to grow into any of the shoots, some had endophyte in some tillers but not others and some grew into tillers but failed to transmit to the seed (therefore the next generation).

The aims of this project were to look at the compatibility of *Epichloë siegelii* with the perennial ryegrass, 06px22, and assess where, if anywhere, in the reproductive chain the transmission became incompatible.

### Materials and Methods

06Px22 was inoculated with HAPPE by the 'slitting' method (Latch and Christensen 1985). This process was done within the organisation in Denmark. The first generation was kept in Denmark and sown as individual spaced plants so seed could be collected.

100g of seed was sent to New Zealand and contained approximately 85% endophyte (as tested by the internal laboratory in Denmark). 88g was sown into trials throughout New Zealand, leaving 12g for a small compatibility project.

In April twenty 144-cell propagation trays were filled with Yates Black Magic seed raising mix and 1 seed was planted in each cell using tweezers. Trays were placed in a plastic tunnel house and watered and trimmed as necessary. Plants were trimmed regularly to promote tillering as the endophyte viability test is somewhat destructive but the plant survival for the rest of the project was required. After 12 weeks of growing *most* plants had more than one tiller available therefore could be sampled for endophyte.

Presence of endophyte was detected using a phytoscreen immunoblot kit (Agrinostics, Georgia, USA; cat. #ENDO797-3) following the manufacturer's instructions. Plants that came back with a negative result were discarded. Plants that tested positive for endophyte were sown outside in August at 0.5m spacings.

Plants grew outside for two months before being sampled again to see if all tillers were positive for endophyte. Five tillers from each plant were taken and, again, screened for endophyte presence using a phytoscreen immunoblot kit.

It was during this process that plants were all sprayed out with Round Up due to a technicality with the importation of the seed.

## Results

Preliminary results indicated either ALL tillers were positive or ALL were negative for endophyte presence. This is shown in the figure below. 15 plants were checked on this particular immunoblot kit and the positive control indicates that in the first 2 plants all five tillers are negative, whereas in the other 13 plants all five tillers are positive.

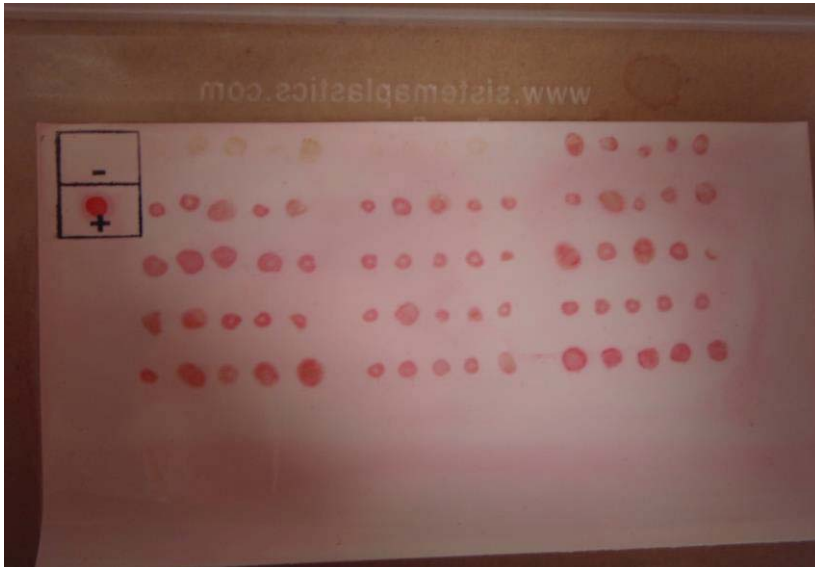


Figure: Phytoscreen immunoblot kit showing groups of five tillers from 15 different plants, the first two plants being endophyte-free and the remaining thirteen being positive.

## Discussion

The intentions of the project were to grow the plants and let them produce seed. The seed would then be collected and grown for testing. This process would allow us to see if the transmission was breaking down at the reproductive stage of the grass – as preliminary results suggest the breakdown isn't at the vegetative stage. If the thesis had run its course there are two other potential 'breakdown' points – during the reproductive stage, or during the seed storage stage. As the full thesis shows, there are large differences of endophyte survival in stored ryegrass seed.

The HAPPE endophyte is now available in New Zealand and there doesn't appear to be a transmission issue in Bronte cultivar. As Bronte has a small amount of meadow fescue parentage this could be influencing the compatibility for the better. It will be interesting to see if the compatibility can be maintained in ryegrass lines that have been bred in New Zealand as pure ryegrasses.

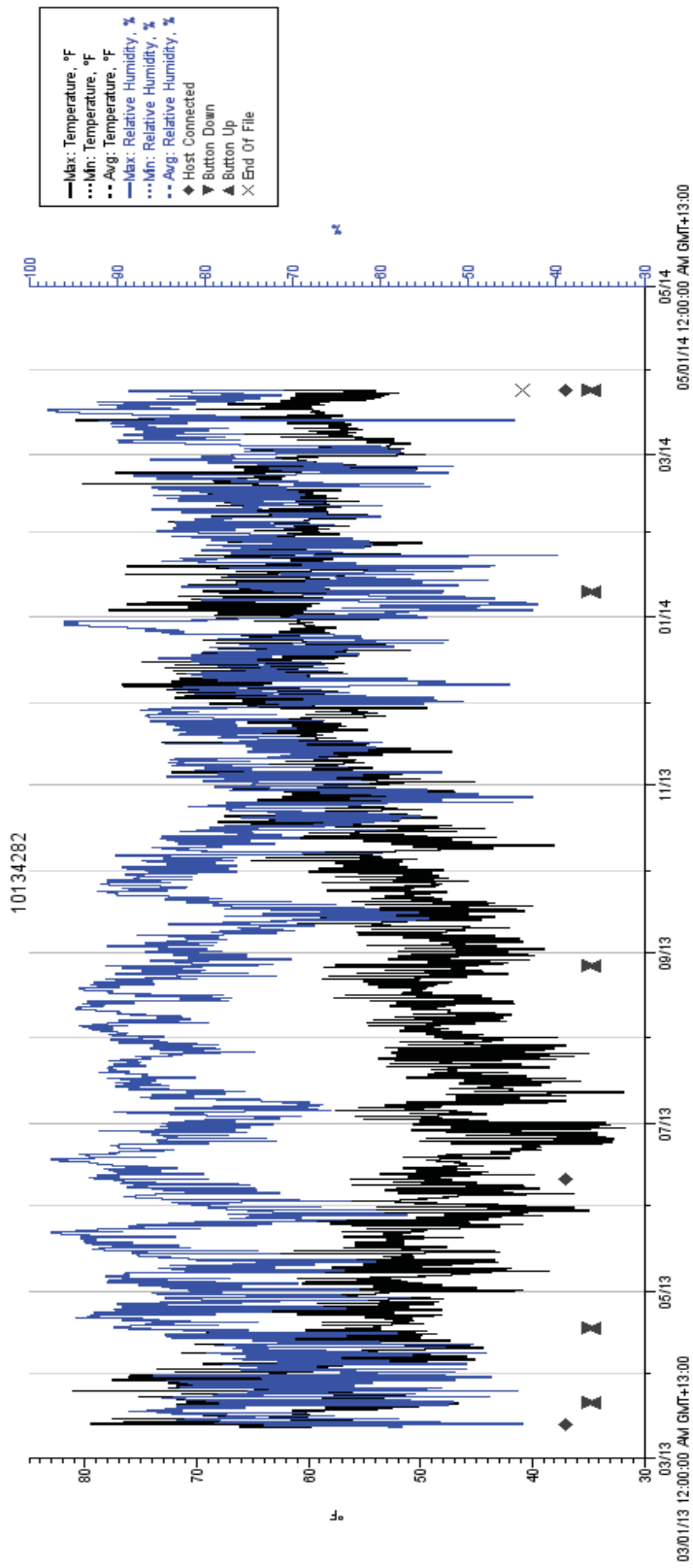
## Appendix 2. REML variance components analysis results

	Wald statistic	n.d.f <sup>1</sup>	F statistic	d.d.f <sup>2</sup>	F pr
Time	321.33	3	106.68	128.0	<.001
Location	74.29	2	37.14	68.4	<.001
Time_Location	103.65	6	17.19	137.5	<.001
Location_Endo	99.83	9	11.09	65.9	<.001
Location_Ploidy	2.31	3	0.77	65.9	0.515
Time_Location_Endo	188.86	27	6.96	141.3	<.001
Time_Location_Ploidy	17.07	9	1.89	141.0	0.059
Location_Endo_Ploidy	24.28	9	2.70	65.9	0.010
Time_Location_Endo_Ploidy	54.48	27	2.01	141.3	0.005

<sup>1</sup>Numerator degrees of freedom

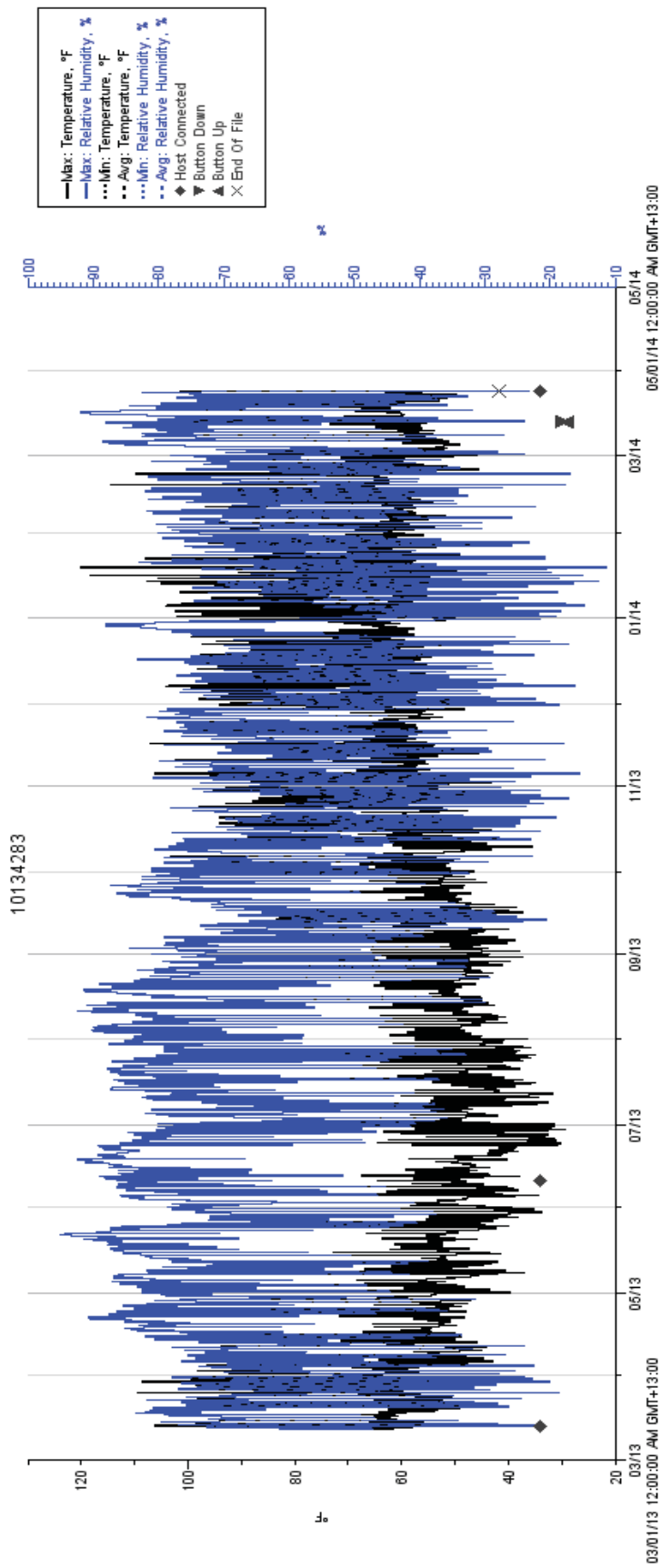
<sup>2</sup> Denominator degrees of freedom

### Appendix 3. Temperature and Humidity data from bottom of warehouse





# Appendix 4. Temperature and Humidity data from top of warehouse



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