Effect of abiotic stress on the success of the vertical transmission and survival during seed storage of two novel endophytes in perennial ryegrass

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A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at

Lincoln University,
Lincoln, New Zealand

By

Maria Elena Duter Nisivoccia

November 2018

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree in any university, and that to the best of my knowledge, contains no copy or paraphrased material published or written by any other person, except where due reference is made in the text of this thesis. All the results presented in this thesis are solely my work, unless stated otherwise. This thesis is less than 100,000 words in length, exclusive of tables, figures and appendices. Maria Elena Duter Nisivoccia

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DEDICATION

I would like to dedicate this research to my beloved family, Ricardo, Santino and Felipe. To my unconditionally supportive husband, Ricardo Felitti, who sacrificed his own career to support mine, who played the role of mum and dad, who always gave me a positive word to keep going. Without your support, help and love, this day would never have arrived. I Love you.

To my two other loves, my adorable sons, Santino and Felipe, thanks for being supportive, patient and understanding, sacrificing mummy time, holidays and coping with mum's stress. Hope you two one day will find what you love to do, and you will pursue it with your heart as I did.

Love you, Mamá.

Abstract

Effect of abiotic stress on the success of the vertical transmission and survival during seed storage of two novel endophytes in perennial ryegrass

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Epichloë fungal endophytes, which live in symbiosis with perennial ryegrass (*Lolium perenne* L.) are vertically transmitted only, totally depending on the success of the host plant for their dissemination and survival. New Zealand (NZ) leads the world in terms of adoption of endophyte technology. These novel associations are very successful in farming practices, as the endophytes provide bio-control through the production of secondary metabolites. Alkaloids like ergovaline, lolines, peramine and epoxy-janthitrems control at least six major pasture pests that decrease the quality and persistence of NZ grasslands.

Both endophyte and host plant grow in synchronization via a unique intercalary hyphal extension mechanism. However this relationship is far from being perfect as the endophyte can be lost at any stage of the host plant life cycle, either during the pre or post zygotic stage. This research aimed to identify abiotic factors affecting transmission during the development of the perennial ryegrass seed crop and survival of the endophytes in seed during storage, and used two commercial novel endophytes strains, AR1 and AR37 and three perennial ryegrass cultivars, 'Prospect', 'One -50' and 'Samson'.

Waterlogging (WL) was imposed during three plant development stages (GS 30, GS59 and GS 70) to investigate any effect on the vertical transmission of the endophyte. WL had no significant effect on ryegrass seed germination percentage, or on endophyte transmission. In both cultivars and with both endophytes, the transmission exceeded 70%. Germination did not differ between the cultivars or between endophyte strains. For the growth parameters measured, WL had no significant effect on dry matter (DM) production, number of reproductive tillers per plant, seed yield per plant or thousand seed weight (TSW). However, TSW differed significantly between cultivars and between the endophyte strains independently of the WL treatments of the experiment.

The effects of combinations of three rates of nitrogen (0, 150, 250 kg $ha^{-1}yr^{-1}$) with three rates of potassium (0, 200, 400 kg $ha^{-1}yr^{-1}$) applied during crop development on the transmission of AR1

and AR37 endophyte strains and the effect on plant growth parameters were investigated. The grand mean transmission was 87%. There were no significance differences among treatments for transmission rate, or between the two cultivars or endophyte strains. However there was a significant linear response ($p = 0.05$) for transmission between endophyte strains to the applied N only. With no N, strain AR37 transmitted 8.7% more than AR1 in cultivar 'Prospect'. At 150 and 250 kg N $ha^{-1}yr^{-1}$, there were no differences either between cultivars (cv) or endophyte strains.

N and K application rates had no significant effect on seed germination between endophyte strains. However, there was a significant difference in germination between cultivars, with 'Prospect' having a 5.6% higher germination than 'One-50'. The grand mean of germination was very low (73%), indicating poor quality.

K significantly increased DM production, but there was no significant difference between the biomass produced when 200 kg or 400 kg $ha^{-1}yr^{-1}$of K were used. The production of DM between endophyte strains did not differ. Both fertilisers increased the number of reproductive tillers produced for both cultivars, but not between endophyte strains. Cultivar 'Prospect' produced 47 more reproductive tillers than 'One-50'. Both fertilisers significantly increased seed yield.

Furthermore, the effect of temperature and relative humidity during seed storage on the survival of these endophytic associations was determined. Combinations of four temperatures and four relative humidity levels, created and controlled using saturated salts solutions in sealed containers, were used to determine the endophyte survival in perennial ryegrass infected with strain AR37 during 418 days of storage. The accumulated thermal unit approach (ATU) was used to identify the "best before time" to maintain viable endophyte ≥ 70%. At 4°C endophyte viability remained above 70% for 1800 ATUs at 24%, 40% and 50% RH (relative humidity). At 20°C, endophyte viability at all four % RH remained above the industry threshold for 2500 ATU. At 30°C, by 1500 ATU endophyte viability in seeds stored at 40%, 50% and 70% RH had fallen below the 70% threshold and for those seeds stored at 24% RH, this point was reached at 2500 ATU. Maintaining endophyte viability above 70% was possible at three different temperatures (4°C, 15°C and 20°C) and relative humidity levels of both 24% and 40% when the accumulated thermal units were below 2000. At 70% RH, the endophyte maintained its viability for only up to 1000 ATUs. At 30°C and 24% RH, the endophyte maintained its viability for 45 days, being the only safe storage combination at that high temperature (2250 ATUs). Ambient temperature with higher relative humidity levels increased SMC and as a result negatively affecting

endophyte viability when SMC >10 %. For all storage conditions, endophyte viability was always lost before seed viability.

In addition, the effect of different concentrations of carbon dioxide (CO_2) during storage on seed and endophyte viability was investigated. Controlled environments were created by capturing seed respiration at 5°C, 20°C and 30°C in sealed containers and by the use of ascorbic acid dust (AnaeroGen™) which caused anoxia and elevated the CO_2 levels in sealed containers at 20°C. Seeds from cultivar Samson containing the novel AR37 endophyte strain and the same cultivar endophyte free (E-) were used in this experiment. The endophyte viability and the seed germination were assessed after short periods of storage (1, 2, 4, 8, 16 d) and up to 32 days; in airtight glass vials.

Seed respiration rate increased with temperature and seeds containing endophyte (E+) had a higher respiration rate than seeds without endophyte (E-). Seeds stored at higher temperatures (30°C), with higher accumulative CO_2 concentrations due to respiration of seeds, lost the endophytes at a faster rate than seed stored at lower temperatures (5°C). The time of storage had a significant negative effect on endophyte survival. The effect of temperature was significant on endophyte survival between seed stored at 30°C compared with the ones at 5°C or 20°C. At equal temperature regimes there was no significant difference found between seed lots E+ or E-. Overall, there was no significant difference between seeds infected with AR37 and endophyte free seeds after 32 days of storage.
Where anoxic conditions with high concentrations of CO_2 were created, both time of storage and high concentrations of CO_2 in an airtight container negatively affected endophyte viability.
Overall high CO_2 concentrations and lack of O_2 did not reduce the germination rate of either E+ or E- seeds stored for 32 days.

Keywords: *Epichloë festucae* var. *lolii*, vertical transmission, *Lolium perenne*, perennial ryegrass, AR1, AR37, endophyte viability, germination, abiotic factor, water stress, waterlogging, nitrogen, potassium, temperature, relative humidity, storage salts, carbon dioxide, anoxic conditions, O_2 depletion, endophyte survival, storage, TPIB.

Acknowledgements

I would like to take this opportunity to thank all those who have contributed in one way or another to the completion of this research.

I would firstly like to thank Dr John Caradus and Grasslanz Technology Ltd for the opportunity of being part of their research team through this study, for the financial support granted to me for this PhD study, as well as supporting me at conferences and workshops.

My sincere thanks go to my main supervisor, Prof John Hampton for his guidance, support and valuable advice during this project. John, I definitely liked your supervisory style; trusting me and leaving enough space for me to explore problems encountered in the research, at the same time offering invaluable guidance and advice. My sincere thanks also go to my co-supervisor, Associate Professor Rainer Hofmann who was always positive and encouraging, and advisor Dr Phil Rolston during the first year of my study.

I would like to thank my advisor Associate Prof Craig Bunt. This study would have not been as smooth and pressure free without your support, patience and enthusiasm. Special thanks to him for allowing me to grow as a research scientist. His encouragement, criticism, support but most important his friendship, kept me motivated throughout my study and will always been greatly appreciated. I could not have imagined having a better advisor at both academic and personal level, for which I am extremely grateful.

I would like to thank my advisor Dr Stuart Card. Special thanks to him for being such a patient person open to ideas and for encouraging and helping me to shape my interests, never allowing me to give up. Thank you for your precious advice and continuous support.

I would like to thank Dr David Hume for his time and always being open for discussion and analysis. His attitude towards research inspired me to work tirelessly through all the ups and downs of this study. He provided great guidance and support, especially during the last stages of my studies.

I also sincerely thank biometrician Mr David Saville for his statistical help with data analysis, his patience and friendship during these four years. Dean O'Connell and Anish Shah for their friendly help in providing their insightful comments, suggestions and support during my studies. To my office mate and friend, Dr Marsha Ormskirk, for being always so considerate, supportive

patient and most of all for being my friend. You have been amazing during all these years. Dr Josefina Narciso, a great friend who provided moral support while studying. Thanks for your time, your friendship and endless laughs during tough times. Great thanks to all the Bio-Protection Research Centre staff for their assistance with laboratory facilities, materials and helpful hand every time when needed.

I am particularly grateful for the generous assistance and support that was given by the "Nursery team", Brent Richards and Leona Meachen. Thank you for supporting all my glasshouse experiments and making the time at the nursery fun and enjoyable, especially during those hot summers. I also thank Dave Jack and Dan Dash for your help with the materials at the Field Research Centre.

I would like to thank everyone at Information Technology Services (IT), in particular Tracy Boereboom, Andrew Du Plooy, Andrew Frapwell and Behiniwal Gurvinder for their endless support during these years, and the teaching and learning team with their valuable training which made my work much easier.

I would like to give a special thanks to everyone at AgResearch Grasslands (AgR) and the AgR Lincoln Farm. Special thanks to Anthony Hilditch, Lee Southerland, Jason Trethewey, Angela Ravagnani and Ivan Baird for the technical support and time spent with me at the AgR farm in Lincoln throughout my studies.

I would like to extend my gratitude to Anouck de Bonth and Jan Sprosen for all your kind help in the lab developing thousands of blots. Also a big thank you to the ladies at the AgR Lincoln office that made every blot go on time to be developed in Ruakura.

My sincere thanks to Dr Linda Johnson, Mike Christensen, Dr Lester Fletcher and Dr Ian Harvey for my first discussions about my research topic. I was inspired by their endless passion.

I want to show my gratitude to all PGG Wrightson seeds staff. A big thanks to Murray Kelly and Michael Morris for the helpful suggestions and discussions during this project. Your kindness and support will be always remembered. Dr Alan Stewart, for being a friend all these years, always being positive and excited about my studies. Your inspiring thoughts have been a great inspiration to me.

A special shout-out to my fellow postgraduate students at the Bio-Protection Research Centre for their help and joyful company during my study.

I would like to thank Dr John Hunt and Jonathan Nunez from Manaaki Whenua Landcare Research for the patience, guidance and enthusiasm given during my last experiment in this research. I wish l had met you before, however, the intensity of what l had learnt during this short period of time was priceless.

Thanks to South Island Section of The Australasian Plant Pathology Society for awarding me a travel grant to attend the Symposium on endophytes in grasses in Salamanca, Spain in 2018.

A big thanks to all the friends and families here in New Zealand and in Uruguay, and those around the world for their support during these time.

To my mum and dad (wish you could be with me today), thanks for the foundation of my education and introducing me to the love of study.

Table of Contents

List of Abreviations

List of abbreviations commonly used in the text.

a.i.	Active ingredient
AgR	AgResearch
ANOVA	Analysis of variance
ATU	Accumulated Thermal Units
BCA	Biocontrol agent
C	Celsius
©	Copyright
cm	Centimetres
CO_2	Carbon dioxide
CV	Coefficient of variation
cv	Cultivar
d	Days
diff	Difference
DM	Dry matter
DW	Dry weight
E.	Epichloë
E+	Viable endophyte
E-	Non-viable endophyte
FD	Field dressed

ES+	Viable endophyte survival
FD	Field dressed
G	Germination
GS	Growth stage
GTL	Grasslanz Technology Ltd.
g	grams
ha	Hectare
h	Hour/s
ISTA	International Seed Testing Association
K	Potassium
KCL	Potassium chloride
Kg	Kilograms
KNO_3	Potassium nitrate
L	Litres
Log	Logaritmic
LSD	Least significant difference
M	Million
MC	Moisture content
MD	Machine dressed
ME	Metabolisable energy
ml	Millilitres

m^2	Square metres
mm	Millimetres
N	Nitrogen
ns	Not significant
n◦	Number
NZ	New Zealand
NZPBRA	New Zealand Plant Breeding and Research Association
O	Organic media
P	Phosphorus
Pers. comm.	Personal communication
Pl	Plant
®	Registered
Resp	Respiration
RH	Relative humidity
SEM	Standard error of the mean
Sig	Significance
SMC	Seed moisture content
std	Standard
t	Time
T	Tonne
™	Trade mark

TPIB	Tissue Print Immune Blot
Trt	Treatment
TSW	Thousand seed weight
μ	Micro
W	weight
WL	Waterlogging
WW	Wet weight
yr	year
(*)	Significant
°	Degree
%	Percentage

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Preface

This thesis consists of six chapters. Chapters 2 - 5 are studies of factors that may affect the transmission and survival of the commercial endophyte *Epichloë festucae* var. *lolii*, strain AR37 and strain AR1 which form host specific associations with perennial ryegrass (*Lolium perenne* L).

Chapter 1 is a review of literature relevant to the research topic and outlines the research aims.

Chapter 2 determines the effect of waterlogging during three stages in plant development, on the transmission of two commercial endophyte strains, AR1 and AR37, within their perennial ryegrass host.

Chapter 3 describes a study investigating the influence of different levels of nitrogen and potassium fertilizer on the transmission of two commercial endophyte strains, AR1 and AR37, within their perennial ryegrass host.

Chapter 4 describes a study of the influence of different environmental conditions (temperature and relative humidity) on the survival during seed storage of the AR37 endophyte strain of perennial ryegrass.

Chapter 5 provides an understanding of the effects of high levels of carbon dioxide (CO_2) on perennial ryegrass seed and on the survival in seeds of *E. festucae* var. *lolii* strain AR37.

Chapter 6 is a general discussion and provides conclusions. Future research is outlined.

CHAPTER 1

Literature review

1.1 Introduction

This review briefly describes the relevance of agriculture for the New Zealand (NZ) economy. The significance of perennial ryegrass (*Lolium perenne* L) is discussed, as it is one of the most important perennial agricultural grasses that is widely-sown in moderate-to-high-rain fall temperate zones of the world, including NZ. Detailed information about endophytic fungi from the genus *Epichloë* which form symbiotic relationships with cool season grasses is provided. Specifically, the significance of *Epichloë* endophytes in their ryegrass host, including life cycle, modes of action and benefits of the bioactive secondary metabolites that they produce.

1.2 Importance of the primary industry

New Zealand has a small open economy that operates on free market principles. Over half of New Zealand's total land area of 267,710 km^2 is dedicated to pasture and arable production (Statistics N.Z, 2017) and therefore it is not surprising that the country has a highly efficient export-oriented agricultural sector. The primary sector is directly accountable for around 6.2 % of real gross domestic product (GDP) and contributes just over half of New Zealand's total export earnings. The New Zealand animal farming systems are mainly pasture based, which is unique among developed countries. There are 38.4 million (M) head of livestock (27.4 M sheep, 3.6 M beef cattle, 6.5 M dairy cattle and 0.9 M deer) (Statistics N.Z, 2017). These animals feed off quality pastures predominantly composed of ryegrass and clover mixtures.

1.3 Poaceae family

The Poaceae family, the fifth largest family of flowering plants, is monocotyledonous and is commonly known as grasses. There are approximately 10,000 species worldwide (Kuldau & Bacon, 2008; Soreng et al., 2017) .They include warm and cool-season annual and perennial grasses. They are the most economically important plant family, providing foods, fuel, building materials, and the grasses of natural grasslands and cultivated pastures. These grasses have contributed to the development of humankind as there are several important species that serve as

essential food crops, including cereals like maize, wheat and barley, forage for livestock, turf and lawn for recreational purposes as well as grasses for soil conservation and recovery of degraded landscapes amongst others. The following review concentrates on the *Lolium* genus, ryegrass species, specifically perennial ryegrass (Naylor, 1960).

1.3.1 Perennial ryegrass (*Lolium perenne* L.)

New Zealand developed perennial ryegrass cultivars have been bred from lines originating largely from seed introduced from the United Kingdom (UK) by British immigrants along with other pasture species before 1820. Continued introductions of grass germplasm occurred as part of the general trade with Britain. Small quantities may also have come from other European countries like the Netherlands, Denmark, France and other countries around the Mediterranean; by 1912 most perennial ryegrass seed used in NZ was locally produced and harvested (Stewart, 2006).

This species is best suited to environments with fertile soils with irrigation or annual rainfalls of ≥700mm (Chynoweth et al., 2012). However, it is relatively intolerant to prolonged drought and high temperatures, and therefore restricted to cool temperate regions. It has a high forage quality with an average metabolisable energy density (ME) of 11.5 MJ kg^{-1} dry matter (DM) and crude protein (CP) levels of 18-30%. It is frequently sown with white clover to improve pasture quality, reducing the need for nitrogen fertiliser and increasing the feed value. Yields of mixed perennial ryegrass-white clover pastures are less than when perennial ryegrass is grown as a monoculture with the addition of 350 to 500 kg N ha^{-1} yr^{-1} (Lowe et al., 2011).

Perennial ryegrass in New Zealand and in most other cool temperate regions of the world has become the most widely and preferred pasture grass species sown to cover feed demands for primary production in forage livestock systems, due to its nutritional quality, high digestibility and palatability, high yields, tolerance to continuous grazing, persistence and freezing tolerance among other qualities (Wilkins, 1991). Because of its high quality the grass is highly suited for lactating cows, but is suitable for all classes of livestock, especially those with high nutritional feed requirements under forage systems. The grass is used primarily for pasture and silage, but it can also be used for hay. Perennial ryegrass plays a major role on pastoral farms in New Zealand as the species can establish fast and produces high yields under a wide range of

environments, soil types and management systems. However the major limitation to the species is its requirements for moist fertile soils which are required for persistence. Its survival under dryland or in areas that have a high occurrence of drought is limited (Charlton & Stewart, 1999). The New Zealand seed industry in 2016 produced nearly 28,000 hectares (ha) of certified seed (Halford, pers. comm, 2017); with approximately 18,000 ha (64%) of this being certified ryegrass seed. Perennial ryegrass (*Lolium perenne* L.) was the dominant ryegrass species with more than 11,300 ha (63%) of seed production, followed by Italian ryegrass (*Lolium multiflorum*) at < 5,000 ha (27%) and hybrid ryegrass (*Lolium hybridum)* at 1,940 ha (10 %). Total production from gate to the industry was >25,700 tonnes (T) (FAR 2017).The total value of sales generated by the New Zealand seed industry was $540 M in 2015, with the value of grass seeds worth $78 M (BERL, 2015) a slight drop from the $84.5 M from ryegrass in 2012 (Hampton et al., 2012) most likely due to a reduction in demand from the dairy industry.

Seed yield of all ryegrass types has increased since 1992 (Chynoweth et al., 2015) with average yields reaching 1,700 kg/ha for perennial, 1,750 kg/ha for Italian and 1,730 kg/ha for hybrid ryegrass (mean of three years 2011-2013) due to changes in agronomic practices and the use of new fungicides (Rolston et al., 2009), plant growth regulators (PGRs) (Rolston et al., 2007), improved understanding of the use of nitrogen fertilisers (Chynoweth et al., 2010; Rolston et al., 2008) and an increased ability of growers to apply irrigation to remove water stress effects (Chynoweth et al., 2012) among others.

1.4 Biological control

Introduction

Increasing concerns about air and water pollution caused by chemicals and their negative impact on our environment, and the demand from society for healthy foods with less chemical residues, has driven research to seek more environmentally friendly alternatives. Biological control or biocontrol of plant pests and plant pathogens is an environmentally safe approach to reduce damage using living organisms, and therefore reducing chemical use, especially in agriculture.

1.4.1 Definition of biological control

The term "biological control" was first used by Harry Scott Smith in 1919 in Riverside, California, more specifically as a “biological method’ to refer to insect pest control (Smith, 1919). However the practice has been used for centuries. The control of *Icerya purchasi* Mask on Citrus in California by an Australian beetle *Rodolia cardianlis* (Muls.) was a good example in 1888 (Fleschner, 1959).

There are many definitions of biological control of insect pests and plant diseases. Biological control can be defined as the use of living organisms (agents), bio-products, microbial antagonists and endophytes to depress the population of a pest, or a method of controlling pests such as insects, mites, weeds and plant pathogens using other organisms (Flint et al., 1998). It is regarded as an environmentally friendly method to suppress plant diseases and is considered the best alternative to or a supplemental method for reducing chemical application in agriculture (Emmert & Handelsman, 1999). More recently Holmes et al. (2016) defined biological control as “the reduction of pest populations by natural enemies and typically involves an active human role”, and using biological control as “a component of an integrated pest management strategy (IPM), regarded as a systems approach to IPM”.

1.4.2 Pest occurrence and damage on perennial ryegrass pastures

In New Zealand, perennial ryegrass in a new environment was subject to a new ecosystem with a series of insect pests to which it did not coevolve resistance in its region of origin. These include many New Zealand, Australian, Asian, South American and South African insect pests. One of the problems that the NZ pasture sector faces is the many insect pests that can reduce pasture persistence, productivity and quality, resulting in direct and indirect revenue losses to the pastoral industries.

There are six major insect pests that directly affect ryegrass performance and production throughout the country (Cunningham et al., 1993; Popay & Ball, 1998). These include three native and three introduced species. They have all adapted to pasture plants and become significant pests.

The Argentine stem weevil (ASW) *Listronotus bonariensis*, a native species of South America (Williams et al., 1994), which arrived in New Zealand before 1927, has become a major pest, especially of ryegrass (Prestidge et al., 1985). The weevil can be found throughout NZ and generally produces two or three generations a year. The damage is produced during two stages of development. The adult stage reduces seedling establishment by defoliation and cutting of stems. The insects lay their eggs in the stems of the grass tillers, and the emerging larvae kill plants by burrowing into these tillers, eating out the central part (Jensen et al., 2009; Popay et al., 1995; Prestidge & Gallagher, 1988; Ruppert et al., 2017). This insect causes yield losses of up to 30 % and economic losses estimated at $160 million per annum (Prestidge et al., 1991). Even though the introduction of the parasitoid, *Microctonus hyperodae* in 1991 reduced the damage caused by this pest, recent studies carried out in the North Island showed that the damage is again increasing (Goldson et al., 2015; Popay et al., 2011).

The New Zealand grass grub (*Costelytra giveni)* is one of the largest subterranean pasture pests endemic to New Zealand. It is a root-feeding insect originally from native New Zealand tussock grasslands that has adapted well to agricultural pastures. The pest can cause deterioration in productivity, and loss in DM production and persistence of the swards. Under normal climatic conditions the insect's life cycle typically takes one year to complete. It is the larvae feeding on the roots of ryegrass that causes the most severe damage during the autumn months (Jackson & Townsend, 1993). Grass grub is estimated to infect 9 million hectares of pasture throughout New Zealand, and impacts on the profitability of farmers in the dairy, beef and sheep industries because of losses in production, the increased need for early pasture renewals and a reduction in stock capacity in paddocks (Zydenbos et al., 2011).

Another serious endemic insect pest is Porina (*Wiseana* spp). Adult moths are light to dark brown, with a hairy body; the eclosion and mating occurs on the ground. Both male and female moths fly on the evening of emergence. In New Zealand they fly between spring and autumn depending on the species. The female moths commence to release their eggs 2 to 30 minutes after mating as they crawl and fly. Both adults are short-lived and do not feed. Porina eggs take 3 to 5 weeks to incubate and hatch. After eight or nine months the caterpillars reach their full size of up to 70 mm in length, completing the lifecycle (Pottinger, 1968). The grey-green caterpillars initially live on the soil surface, but after about six weeks, they tunnel underground living in burrows and emerging at night to graze on foliage of ryegrass, white clover and many other pasture species, denuding leaves and tillers to ground level and damaging growing points,

compromising pasture persistence. The economic impact is due to the loss of pasture creating a deficit that the farmer has to overcome, and the cost of insecticide (with or without withholding requirements) to control the larvae infection (Greenall, 1940; Harris & Brock, 1972; Rastrick & Upritchard, 1968).

The pasture mealybug (*Balanococcus poae*) is a further endemic pest species of importance naturally found on many native grasses and tussocks of the Poaceae family that has adapted well to a number of introduced grass species, particularly perennial ryegrass. The species has been identified in several regions of New Zealand including Canterbury, Hawke's Bay, Marlborough and the Manawatū (Charles et al., 2009). The neonate nymphs crawl to the tip of the tillers and then move down the blade to feed, sucking the sap from the plant tissue beneath the protective tiller sheath. These small, pink, oval-shaped insects surround themselves with clumps of white wax, which provides them with a protective layer in the soil (Pennell & Ball, 1999; Pennell et al., 2005; Pennell et al., 2004).

The African black beetle (*Heteronychus arator*), another major pest of grasses, was first recorded in New Zealand in 1937, having been introduced from South Africa from where it is native (Blank, 1985; CABI & EPPO, 2000; Jenkins, 1965). Adult beetles are on average 15 mm long, reddish-brown in colour, changing to a shiny black as they mature. The adult insects feed at the base of grass tillers, causing damage to grass seedlings, while the larvae eat grass roots in summer (Helson, 1969).

The root aphid (*Aploneura lentisci*) originates in the Mediterranean region and like the pasture mealybug, sucks grass sap and produces a white wax. These insects are found throughout New Zealand and contribute to weakening ryegrass pastures, diminishing plant growth (Popay & Gerard, 2007).

1.5 Resistant cultivars

Many breeding programs have their focus on identifying sources of resistance to different pests and diseases. Resistant cultivars can play an important role in reducing crop losses caused by pests and diseases. For ryegrass in NZ, there has been almost no successful breeding for insect control. Other pasture species have not had much breeding for insect control either (Stewart, A pers. comm., 2018). White clover (*Trifolium repens* L.) however has had some breeding for

cyanogenesis glucosides which control some slugs and results suggest positive fitness effects (Kooyers et al., 2014). Additionally research has demonstrated resistance to multiple herbicides in Italian ryegrass (*Lolium multiflorum*) (Liu et al., 2014), reducing the effectiveness of chemicals.

As forage plants are exposed to various insect pests and could be susceptible to different diseases at any time throughout the growing season, chemical control is usually unsustainable. Additionally with the increased use of irrigation and climate change producing unexpected rainfalls, chemicals need to be applied more than once to be effective as plant protectors after the rain. The combination of these unsustainable practices with the public concern and awareness regarding chemical residues on food products and environmental contamination with agrochemicals is restricting the widespread use of chemicals to control pasture plant pests and diseases. Therefore, biological control is an alternative plant protection strategy, additional to the other mechanisms of plant defense against insect herbivores that plants have already developed (War et al., 2012).

1.6 Endophytes

The use of the term endophyte is broad and has evolved from the original definition of "any organism occurring within plant tissues" (De Bary, 1866). This referred uniquely to an organism that lives inside a plant. Many microbes live within plant tissues without causing obvious damaging effects or producing any symptoms of disease. These micro-organisms are now referred to as endophytes; literally meaning within the plant. However there has been disagreement over the use of the term endophyte, with the suggestion that the word implies a mutualistic relationship that may not exist, and that other words may be better (Wennstrom, 1994). Endophytes can develop all or part of their life cycle within the host, causing asymptomatic infections (Wilson, 1995). The term includes many microorganisms. Fungi and bacteria are the most common endophytes studied, although other plants and viruses can have endophytic life cycles (Card et al., 2016).

1.6.1 *Epichloë* endophytes and their importance as biocontrol agents (BCAs).

Epichloë fungal endophytes (family Clavicipitaceae), previously known as *Neotyphodium* (Glenn et al., 1996; Leuchtmann et al., 2014), have been well documented with regards to their

close symbiosis with many species of cool season grasses (Schardl, 1996; White, 1988, 1993; White & Chambless, 1991). These fungi are obligate symbionts while their associated grasses are facultative hosts. The plant provides the fungus with a reliable source of nutrients and water, a protected niche where there is relatively little competition from other microorganisms, and provides an effective means for dispersal via the plant seed (Kuldau & Bacon, 2008). In return, the fungus provides the plant with biotic and abiotic stress protection. These beneficial traits can include deterrence from grazing herbivores, including certain mammals, insects and nematodes via the production of a number of secondary metabolites. Endophytes with this deterrence capacity against insects have shown to increase pasture persistence and increase productivity (Clay, 1987; Easton et al., 2009; Lowe et al., 2008; Raman et al., 2012; Thom et al., 2012). These fungi have also been implicated in tolerance to drought stress, herbicides and heavy metals (Barker et al., 1997; Easton et al., 2001; Malinowski & Belesky, 2000; Popay et al., 1999). The most intensively studied endophyte-grass associations are those important to agriculture, primarily the asexual *Epichloë* morphs and the major pasture grass species belonging to the *Festuca* and *Lolium* genera within the tribe *Poeae* (Clay & Schardl, 2002; Schardl, 1996, 2001).

1.6.2 Secondary metabolites and toxicity

Wild-type *Epichloë*-grass associations found in New Zealand pastures in the early 1980s were not always desirable in agricultural systems due to their production of certain secondary metabolites. These were intensely studied because of unidentified animal toxicity issues (Fletcher & Harvey, 1981; Fletcher et al., 1999; Gallagher et al., 1982; Guerre, 2015). The secondary metabolites were later identified as alkaloids, and were found to be detrimental to livestock causing health problems and productivity losses (Johnson et al., 2013). These compounds include the ergot alkaloids (ergovaline) in tall fescue (*Festuca arundinacea* Schreb.) which causes fescue toxicosis resulting in heat stress of cattle and sheep and fescue foot syndromes (Bacon et al., 1977; Tor-Agbidye et al., 2001; Wang et al., 2004). The indole diterpenes, a class of tremorgenic alkaloids that includes the lolitrems, were found to be responsible for ryegrass staggers, a neurological disorder in cattle, sheep, horses, deer and alpacas (DiMenna et al., 2012; Fletcher & Harvey, 1981; Fletcher et al., 1999; Gallagher et al., 1984; Mackintosh & Orr, 1993; Philippe, 2016).

Aiming to control and even eradicate the problem of ryegrass staggers, preliminary research in New Zealand looked at producing endophyte-free ryegrass cultivars. However soon after sowing this material it was observed that the grasses either perished or had low performance issues due to decimation by insect pests, particularly ASW. It was subsequently realised that these *Epichloë* endophytes were required in order to deter these pests (Latch, 1993). Later the discovery that peramine was found to be responsible for ASW deterrence, created a new window of opportunity for research and commercialisation (Rowan et al., 1990; Rowan & Gaynor, 1986; Rowan et al., 1986). This alkaloid forms the basis for the insect protection given by the highly successful endophyte product AR1, based on a strain of *Epichloë festucae* var. *lolii* developed by AgR scientists (a Crown Research Institute in New Zealand) and marketed by Grasslanz Technology Ltd.(Caradus et al., 2013; Thom et al., 2013).

For the insect pests affecting pastures, the endophyte fungus infection has become the only practical and economical method of preventing pasture yield losses and increasing ryegrass persistence (Jensen & Popay, 2004; Popay et al., 2012; Popay & Hume, 2011). Seed lots with high infection of endophytes have provided better persistence under insect damage aggravated by drought conditions; research indicated that pastures with a low proportion of endophyte-infected ryegrass suffered severe pasture damage in summer and early autumn due to ASW selectively killing endophyte free ryegrasses as the adults feed on them (Popay et al., 1999; Popay et al., 2003). Also adults lay more eggs on plants without endophyte. Larvae which hatch from them bore into the tillers and cause death by damaging the growing points. Larvae are able to transfer from tiller to tiller until they are fully developed and pupate in the soil before the adult stage. Pastures with a high incidence of endophyte are able to prevent stem weevil populations from increasing, thereby increasing ryegrass yield and improve persistence.

Over the last few decades endophyte research in New Zealand has concentrated on the beneficial traits that *Epichloë* can offer agriculture and the aviation industry (Pennell et al., 2017; Pennell et al., 2010). These novel grass-endophyte associations are now one of the most successful bio-control products known worldwide within any agricultural context (Bush et al., 1997; Fletcher et al., 1991; Popay & Gerard, 2007; Popay et al., 2005).

1.6.3 The endophyte life cycle

Asexual *Epichloë* species are seed transmissible (exclusively vertically transmitted) and are true endophytes for their complete lifecycle, therefore totally dependent on the success of the host plant for their dissemination and survival (Majewska-Sawka & Nakashima, 2004; Philipson & Christey, 1986a; Zhang et al., 2017), (Figure 1-1). Many sexual *Epichloë* spp., however can also transmit horizontally, whereby epiphyllous hyphae can form on the outside of reproductive tillers forming a stroma, manifesting in the disease known as "choke" (Figure 1-2). This disease can prevent the host from flowering and therefore supressing seed formation, compromising seed yield (Gonthier et al., 2008; Leuchtmann & Schardl, 1998; Leyronas & Raynal, 2008; Schardl, 1996; White & Chambless, 1991). Following heterothallic mating, once spores of opposite endophyte mating types unite by a specific vector, the subsequently formed ascospores can then complete the horizontal lifecycle by infecting grass ovules (Leyronas & Raynal, 2008).

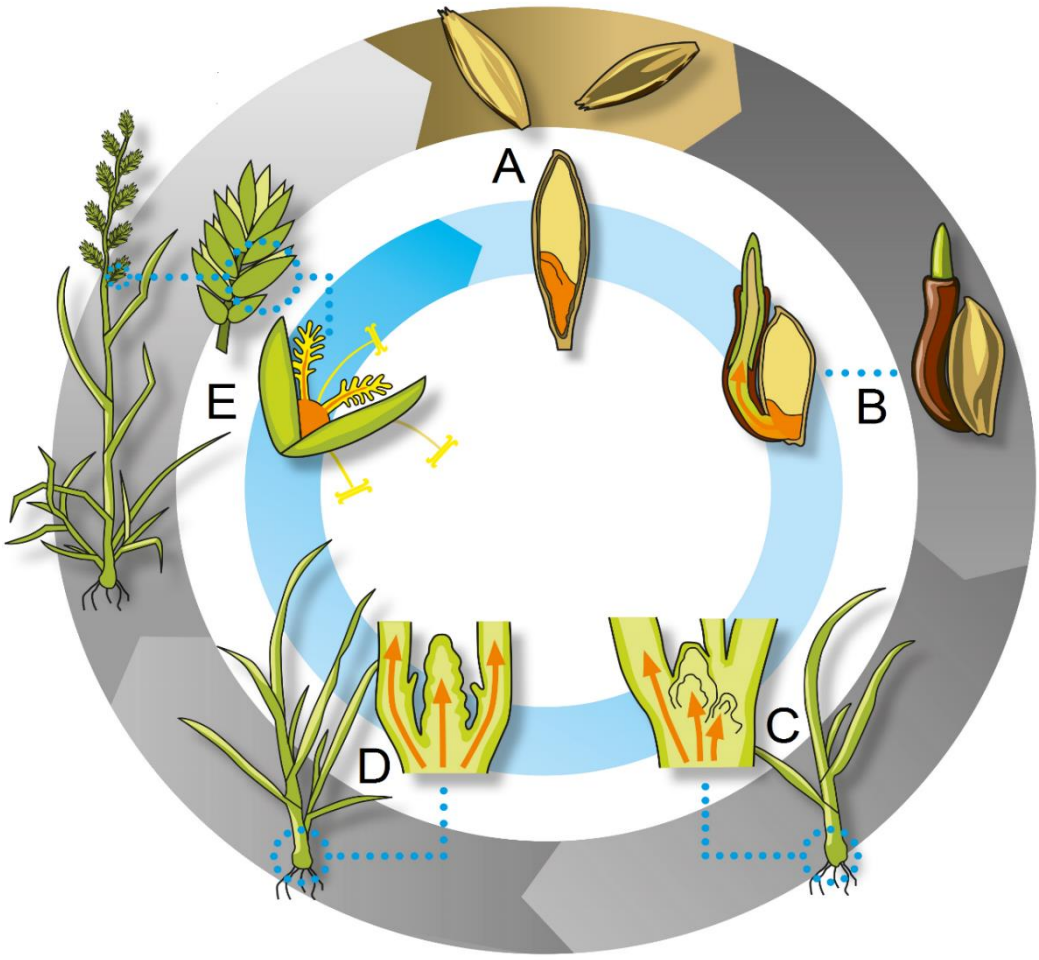

Figure 1-1 Lifecycle of *Epichloë* endophytes, the inner arrows show the endophyte vertical transmission cycle (asexual). The outer arrows show the plant (grass) reproductive (vegetative and

sexual) cycle. Infected mature seeds (A), during germination (B), hyphae (orange) within the embryo. The axillary buds are colonised, enabling the hyphae to colonise the shoot apex of the new daughter tillers (C) and from there through hyphal tip growth, to the aerial plant structures (tillers). The vegetative shoot apex may differentiate into a reproductive inflorescence primordium (D). Hyphae colonise the developing reproductive tissues including the ovaries (E). After fertilisation, hyphae in the ovary penetrate the embryo and into other seed structures. Figure provided by Christine R. Voisey, (Gagic et al., 2018).

Figure 1-2 Choke disease on *Dactylis* spp, Collected in Castilla de Leon, Spain 2018.

At the flowering stage, the fungus infects the floret through the base of the ovary and colonises the nutritive nucellus tissues that surround the mega-gametophyte (Philipson & Christey, 1986b; Sampson, 1933; White et al., 1993; Zhang et al., 2017). After fertilisation the fungus is left in the nucellus remnants (a thin layer of crushed cells) (Card et al., 2011) and at seed maturity the hyphae are located in the main components of the embryo, between the seed coat

(pericarp) and the aleurone layer and between the cells of the scutellum (Majewska-Sawka & Nakashima, 2004) (Figure 1-3).

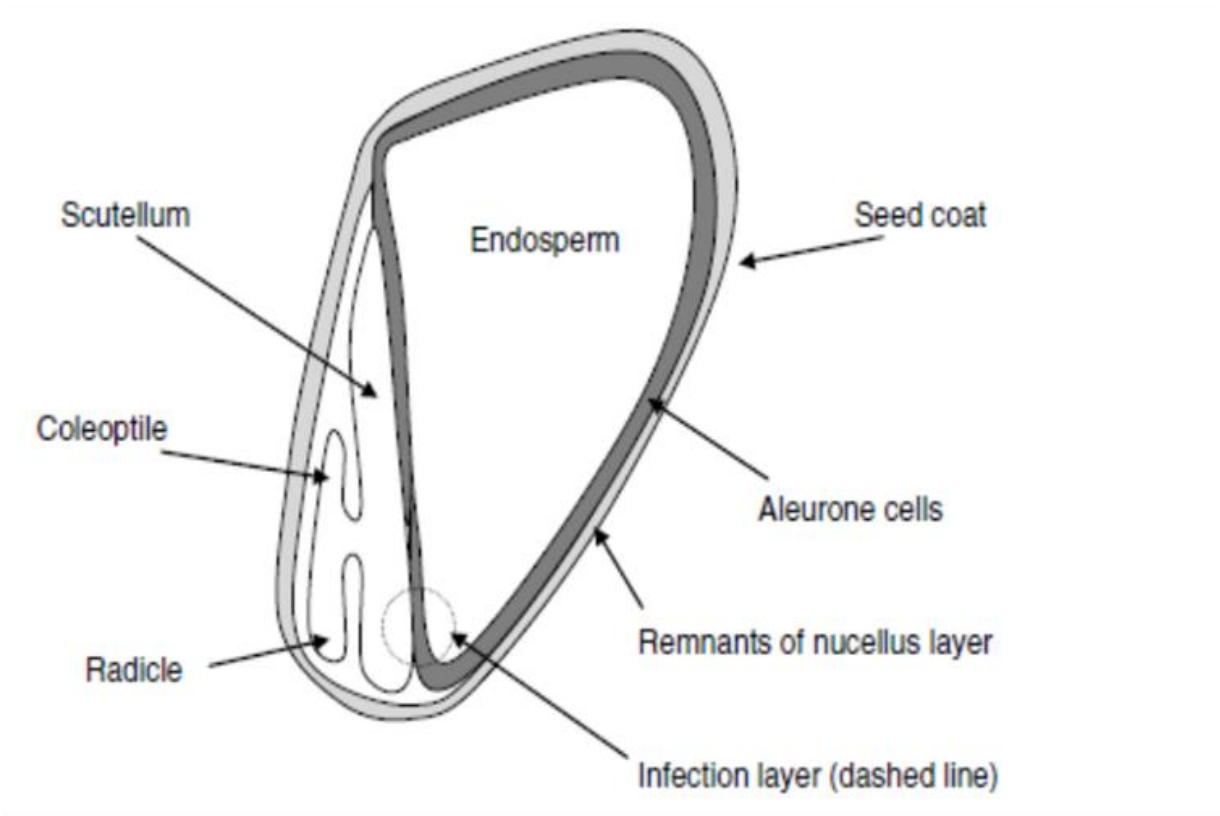

Figure 1-3 Cross section of a mature grass seed showing the location of the nucellus and the aleurone cells and the unique structure (scutellum) present only in seed of grasses (Zhang et al., 2017).

On seed germination, the fungi become metabolically active from their dormant state within the embryo tissues and start to systematically colonise plant tissues starting with the shoot apical meristem, a very active area of cell formation (Figure 1-4). Within the seedlings, the endophyte colonises the leaf primordia and axillary buds, advancing systematically throughout the above ground parts of the plant. During colonization and elongation of the leaves the hyphae align parallel with the longitudinal leaf axis and remain confined to the intercellular spaces. Hyphae are attached to these enlarging cells, allowing the fungus to extend as the host cells do. Both endophyte and host grow in synchronisation with the fungus developing *in planta* by a unique mechanism known as intercalary hyphal extension (Christensen et al., 2008; Tanaka et al., 2012). Hyphae eventually colonise the reproductive tillers and the florets, so that the vertical transmission cycle is then complete.

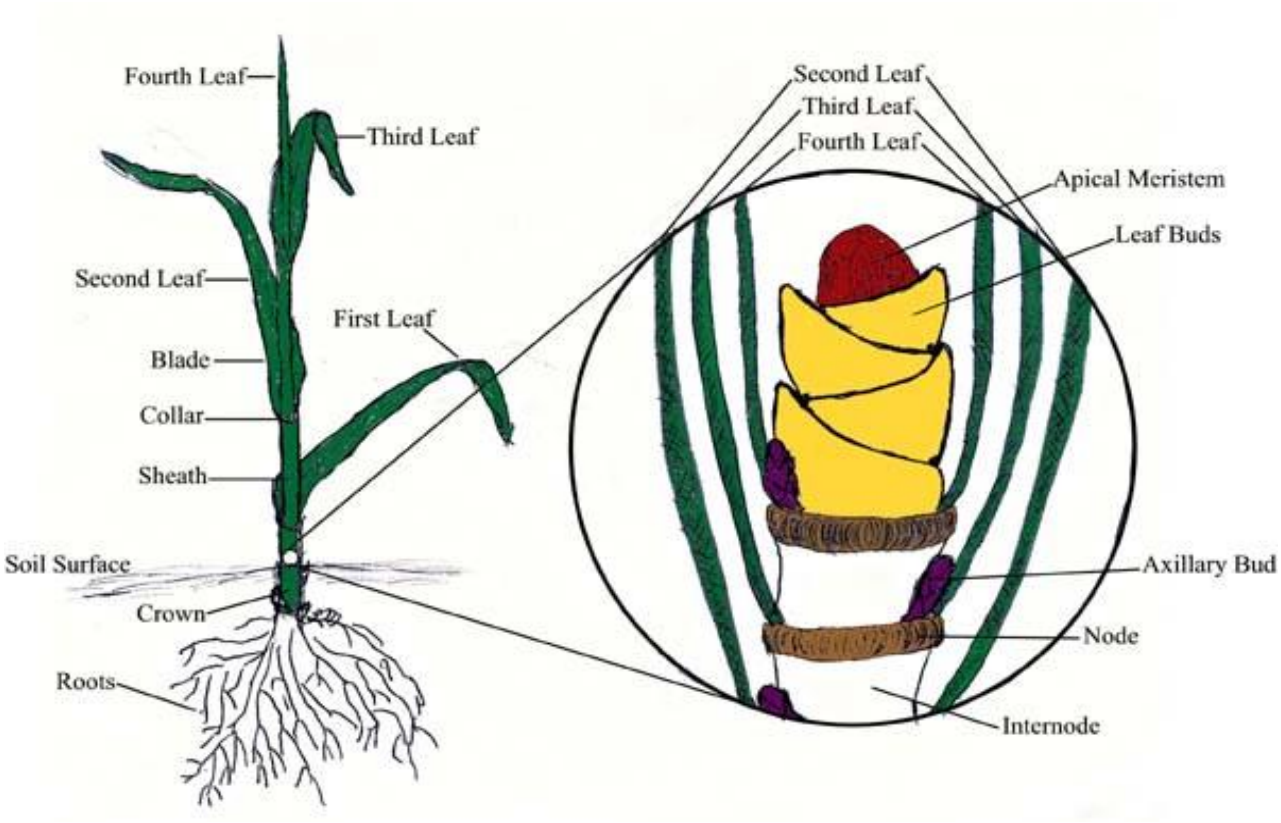

Figure 1-4 Grass tiller growth at the 3.5 leaf tiller stage (Manske, 2001).

1.7 **Seed industry perspectives**

Varietal genetic purity and physical purity during seed production, seed processing and storage are controlled by seed certification through international rules and guidelines from the Organisation for Economic Co-operation and Development (OECD, 2018). However there is no such international scheme for grass endophytes. Maintaining the quality (high levels of seeds infected with viable endophyte and desirable alkaloid profile) of a novel endophytic component in seed relies on the commercial seed companies that produce, market and deliver the seeds to the end user. Endophytes are a high value perishable product requiring the correct seed production management, harvesting, processing, conditioning and care during storage that will ensure that seeds containing viable endophyte can be delivered to the farmers for the establishing of new pastures. Currently the New Zealand seed industry has set a voluntary 70% threshold for endophyte infected seeds for commercial purposes (classed as high endophyte), with anything below that level classed as nil or low endophyte (≤ 5%) (Hume & Barker, 2008). There is a need for a high standard for delivery of high quality seeds with high rates of infected lines with live endophyte for commercialization (Rolston & Agee, 2007).

1.8 Vertical transmission failures

Vertical transmission of *Epichloë* endophytes from an infected mother plant to the next generation depends on a connection between the fungus and host during their life cycles. The endophyte must produce hyphal growth into the developing embryos (Christensen et al., 2008; Philipson & Christey, 1986a), and remain alive in the resultant seed to disperse and therefore be transmitted. The vertical transmission cycle of these asexual endophytes is however far from perfect. Vertical transmission failures can either occur during the pre or post-zygotic stages of the host plant's life cycle (Gundel et al., 2008; Gundel et al., 2011). Pre-zygotic failure occurs when the endophyte fails to infect seedlings or colonize tillers, spikes or panicles, or spikelets and ovaries during flowering (Liu et al., 2017; Ravel et al., 1997b) where infected plants produce a portion of non-infected offspring. Post-zygotic failure occurs when the endophyte dies in mature seeds, affecting the success of their transmission by producing an endophyte free offspring (Welty et al., 1987).Failures in the vertical transmission pathway of commercially available endophyte strains can create major issues for the seed and pasture grass industries. The causes of these failures are not clear, as many factors may be involved and no one defined factor is responsible (Gundel et al., 2008).

To date, genetic and environmental factors like temperature and water availability have been implicated; as an example studies on tall fescue endophyte associations showed that temperature has a major effect on the fluctuation of endophyte frequency in certain plant tissues. Specifically the minimum cardinal temperature for plant growth was observed to be significantly lower than that for its endophyte, *Epichloë coenophiala* (Ju et al., 2006). Other ecological studies have shown that wild *Lolium* populations growing in environments where there were water restrictions and periods of drought gave rise to higher levels of *Epichloë* endophyte infection compared to plant populations growing under less stressful situations. This suggest that water availability may also play an important role in the transmission of these endophyte species (Lewis et al., 2006). More recent studies have shown variation in symbiont frequencies in native grass populations was mainly explained by endophyte transmission rate, being genetically regulated at the host population level (Gibert & Hazard, 2013).

1.9 Standard endophyte: "wild type"

The dominant grass in most pastures present on NZ farms is perennial ryegrass, and if the NZ wild type strain of *Epichloë festucae* var. *lolii* is present in these pastures it is detrimental to

livestock. This endophyte is a naturally occurring organism that transmits vertically from generation to generation through seed. Wild type endophyte has also been known as the common toxic or standard endophyte (Lowe et al., 2008). Ryegrass infected with the wild type endophyte produces lolitrems (Lolitrem B), ergot alkaloids (ergovaline) and peramine. However due to the wide chemical and genetic diversity observed in this species and other *Epichloë* species, it was possible to obtain strains of the fungus that expressed nil or reduced mammalian toxicity (Fletcher, 2012; Young et al., 2013).

1.9.1 Novel-grass endophyte associations

The *Epichloë* endophyte strains incorporated in commercial products by AgR Ltd. are selected via a comprehensive discovery pipeline (de Bonth et al., 2015). Strains are selected according to their genetic uniqueness and for their beneficial chemical profiles. Selected strains are then isolated from their original host plant, grown in culture and artificially inoculated into elite grass cultivars (Card et al., 2014). Transferring endophytes from their host grass species to a new grass host species is usually restricted by the host-specificity expressed by these fungi (Scott, 2001). Research has shown that incompatibility after inoculation is common, showing damaged tissue in the host as well as fungi hyphal death (Christensen, 1995). A number of commercially successful grass-endophyte products are now marketed for either grazing livestock or wild life deterrence (Table 1-1).

Table 1-1 Endophyte strains and general summary of key properties, adapted from Johnson et al. (2013).

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

Abbreviations: E: Epichloë; ASW: Argentine Steam Weevil; AR: AgR endophyte; NEA: NZ Agriseeds endophyte.

1.9.1.1 *Epichloë festucae var. lolii strain AR1*

The strain designated as AR1 does not produce lolitrems or ergot alkaloids, but does produce peramine which deters ASW, therefore helping maintain pasture persistence and productivity. AR1 is non-toxic to animals (Ball et al., 1997; Popay et al., 1995; Popay & Thom, 2009). AR1 was released by Grasslanz Technology Ltd (GTL) in 2000 (Fletcher, 1999) and has consistently delivered high vertical transmission frequencies every harvest across more than 20 different elite grass cultivars. AR1 typically can be stored for relatively long periods of time under conditions of low temperature and relative humidity (Rolston et al., 1986). AR1 had its peak uptake of 70% of the proprietary perennial ryegrass seed sold very soon after its release. It is actually licensed to 10 seed companies in New Zealand and has been exported to and evaluated in Australia and South America, with a very quick uptake in Chile and Uruguay. Nowadays with the release of other endophytes strains like AR37 to the market, the share of AR1 has declined, but it is still holds a 30% share of the proprietary perennial cultivars containing endophyte (Caradus et al., 2013).

1.9.1.2 *Epichloë festucae var. lolii strain AR37*

The endophyte *Epichloë festucae* var. *lolii* strain AR37 was released by GTL in 2007 and possesses a different chemical profile than AR1 as it does not produce peramine, lolitrem B or ergovaline which are the cause of ryegrass staggers and heat stress respectively (Fletcher, 2008). It produces a different group of compounds called epoxy-janthitrems (Ball et al., 1997; Gallagher et al., 1980; Meale et al., 2013; Tapper & Lane, 2004). These compounds can give protection against a wider range of insect pests like ASW, root aphid (Popay & Gerard, 2007), pasture mealy bug and porina (Jensen & Popay, 2004; Pennell et al., 2005; Popay et al., 2012; Popay & Gerard, 2007; Popay & Thom, 2009). This endophyte strain can also control black beetle adults, although not the larvae stage (Bryant et al., 2010). Perennial ryegrass infected with AR37 can be more persistent and productive than the same perennial ryegrass naturally infected with the wild type or the novel endophyte strain AR1 (Bluett et al., 2001; Fletcher, 1999; Hume et al., 2007; Thom et al., 2013).

1.10 **Research Aims**

This study was undertaken to determine the effect of some abiotic factors which may have an effect on the transmission during seed production and survival during seed storage of the commercial *Epichloë* festucae var. *lolii* strains AR1 and AR37 in diploid cultivars of perennial ryegrass.

The abiotic factors investigated in this research are divided in two areas of action I) factors affecting the transmission of endophyte during plant growth, and II) factors affecting the survival of endophyte during storage of seeds.

During plant growth the factors investigated were waterlogging and different application rates of nitrogen (N) and potassium (K), while during seed storage the factors studied were (a) combinations of temperature and relative humidity conditions, and (b) modification of ambient conditions by altering atmospheric gases, modifying oxygen (O_2) and carbon dioxide (CO_2) levels during storage of endophyte infected seeds in sealed containers.

Objective 1, was an investigation of the effect of stress due to waterlogging during three different growth stages (GS) in plant development (GS 30, GS 65 and GS70) of two cultivars of perennial ryegrass with *Epichloë festucae* var. *lolii* strains AR1 and AR37. Objective 2 was a study of the effect of three nitrogen (N) rates combined with three potassium (K) rates on the transmission of both endophyte strains in two cultivars of perennial ryegrass in the first year of seed production was studied.

Objective 3, was to investigate the influence of different combinations of temperature and relative humidity (% RH) on the survival of *Epichloë festucae* var. *lolii* strain AR37 during seed storage to determine which combination maintained live and transmissible endophyte for a known period of time. This will help seed store managers to plan and make predictions for their conservation of seeds containing endophytes. Combinations of different temperatures and relative humidity levels created sixteen storage conditions which may affect the survival of the *Epichloë* endophyte strain AR37 in a diploid perennial ryegrass seed host. The accumulated thermal unit (ATU) approach was used as a predictor for persistence of endophyte strain AR37 in its perennial ryegrass seed host during storage over time.

Objective 4, was to investigate the effect of different levels of gases on the survival of *E. festucae* var. *lolii* strain AR37 endophyte in seed of cultivar Samson, stored in modified ambient conditions for short periods. (i) The effect of seed respiration rate at three temperature regimes on endophyte survival after seeds were stored for 32 days in sealed containers; the respiration rate was also measured in the same seed lot without the endophyte (E-) and the effect of these storage conditions on germination were also assessed. (ii) The effects of seed stored for 32 days

at 20°C in oxygen depletion conditions ($O_2 \leq 0.1\%$) combined with elevated CO_2 levels created using acid ascorbic dust on the survival of the endophyte was studied.

CHAPTER 2

The effect of waterlogging on the transmission of two Epichloë endophyte strains in two commercial cultivars of perennial ryegrass

2.1 Introduction

Various abiotic factors have been implicated in affecting endophyte transmission rates in natural grass populations and commercial grass seed crops, including climatic factors such as temperature and water availability (Afkham & Rudgers, 2008; Canals et al., 2008; Ravel et al., 1997a; White et al., 1991). Most of the studies done on mutualistic interactions between cool season grasses and *Epichloë* fungal endophytes have compared plants with and without endophyte, the response of the hosts to the stress, and the mutualistic relationship (Hesse et al., 2003, 2005). A good summary of the research done implicating water stress in the form of drought is presented by Gundel et al. (2016). These authors were critical of some reports, and suggested that some conclusions reached were based on misleading information due to insufficient experimental studies, the short term drought stresses applied and very little diversity in the species tested. Additionally, they found only nine papers on perennial ryegrass (*Lolium perenne* L.) and all other species that do not naturally occur in arid ecosystems. Therefore the positive effects of *Epichloë* endophytes on plant tolerance to stress is still to be fully explained.

In many environments, soils can suffer water stress in the form of flooding for variable durations in most seasons. Research on most grasses has been focused on the effect of this stress on leaf and root growth rates and recovery after waterlogging (Malik et al., 2001) or yields and plant fitness (Dunbabin et al., 1997). Most studies focused on the changes in the physiology and growth of different genotypes (McFarlane et al., 2004). Despite these studies showing differences, however, there has been little research on the effects of this stress in ryegrass seed production. Moreover, no research has been done showing the effect of waterlogging on the vertical transmission of *Epichloë festucae* var. *lolii* endophyte from the host plant to the progeny in perennial ryegrass.

Perennial ryegrass pastures may be subjected to some kind of waterlogging period during winter months due to intense rainfall over short periods of time, snow melts and poorly applied irrigation in spring, where flooding may also occur (Figure 2-1).

Figure 2-1 Waterlogged paddock during August 2018, after two days of rain. Lincoln, New Zealand.

Waterlogging of soils creates a reduction of oxygen supply to the root system, compromising growth and plant performance. Waterlogging during winter and spring could inhibit the endophyte colonization of ryegrass apical meristems which is required for subsequent colonization of reproductive tillers and subsequently seeds.

The NZ Government has been providing funding to support the setting up of irrigation systems, providing a reliable supply of water for growers, aiming for sustainable crop outcomes (Ministry for Primary Industries, 2017). In NZ the irrigated area has increased dramatically in the last 15 years, and in Canterbury the irrigated land area has reached over 500,000 ha (Ministry for the Environment, 2017). In the production of perennial ryegrass seed the use of irrigation is common. However the poor use of irrigation for seed production may lead to patchy flooded areas in the paddocks, which may have an effect on endophyte transmission.

The aim of this experiment was to investigate the effect of water stress (waterlogging) during (I) vegetative growth, (II) at anthesis, and (III) at seed set, on the transmission of the fungal endophyte to its offspring in a pot experiment using individual plants. The first hypothesis was that host-endophyte interaction is affected by excessive soil moisture levels during short periods of time during plant development; this will negatively affect endophyte transmission. The second hypothesis was that *Epichloë festucae* var. *lolii* strains AR1 and AR37 have different stress thresholds; the impact of waterlogging on transmission rates will therefore differ between the two endophyte strains.

2.2 Experimental design

The experiment was set up using a split-plot design with water treatments (waterlogging) (unequally replicated) as the main-plot treatment factor, with 2 main plots for the control treatment, 3 main plots for the vegetative treatment, 2 main plots for the anthesis treatment and 1 main plot for the seed set treatment. Sub-plot treatment factors were 2 cultivars (Grasslands® 'Prospect' and 'One-50') and 2 endophyte strains (AR1, AR37). Each main plot consisted of 16 buckets and each sub-plot consisted of a group of 4 pots randomly positioned within each main plot. Each pot containing one E+ plant.

The entire experiment originally consisted of 144 pots; however, 16 plants placed at the edge of the experiment stopped growing and were discarded from the original design, leaving 128 plants; 3x16 (48 plants) for treatment 1, 2 x 16 (32 plants) for treatment 2 and 1 x 16 (16) for treatment 3. The final 2 x 16 (32 plants) were the controls (Figure 2-2).

Figure 2-2 Experimental set up as a split plot design. Each main-plot (e.g., as shown in red frame) consisted of four rows of four pots, with the four sub-plot treatments randomly assigned to the four rows (so a row of pots is a sub-plot, as shown in yellow). Sub-plot treatments were perennial ryegrass cultivars 'One-50' and 'Grasslands Prospect', each infected with either *Epichloë festucae* var. *lolii* strain AR1 or AR37.

2.3 Materials

2.3.1 Plant material

Two cultivars of perennial ryegrass, 'Grasslands® Prospect' and 'One-50' both colonised with the commercially available endophyte *Epichloë festucae* var. *lolii* strains AR1 or AR37, were used in the different experiments included in this thesis. Seeds were harvested in Canterbury, New Zealand in 2013 and sourced from PGG Wrightson Seeds Ltd, Kimihia Research Centre (Lincoln).

2.3.1.1 *Cultivar: 'Grasslands® Prospect'*

'Grasslands® Prospect' has excellent all year round growth and tiller density for persistence. It has been bred from a combination of late heading north-west Spanish perennial ryegrasses and traditional mid-heading perennial ryegrasses. From its parentage 'Prospect' is between 96 - 98% perennial. Due to the presence of a small number of tip awns, it is certified as *Lolium boucheanum.*

Heading dates or flowering time vary between ryegrasses and in New Zealand are defined relative to the cultivar Grasslands Nui (approximately 22 October in NZ). This is noted as day 0, which is when 50% of the plants have emerged seed heads. Heading dates can vary by up to 6 weeks among perennial ryegrass cultivars, and the different cultivars are grouped as early, intermediate and late heading cultivars. For 'Prospect', the flowering date is +12. Phenotypically it is characterized by a dense fine leaved habit. One of its main strengths is the reliable all year round production. Due to its high yield potential, 'Prospect' is suitable for dairy farmers looking for dense robust and excellent persistence of pastures under insect and grazing pressures. It also has a good adaptation to a wide range of environments, from hill country farming sheep and beef, to high-performance dairy systems (Agricom Ltd, 2017).

2.3.1.2 *Cultivar: 'Ceres One-50'*

'Ceres One-50' (One-50) is a cross of the elite genetics of New Zealand and north-west Spanish origin. It was bred using individual plants that were screened in the pest and rust-prone north of New Zealand. 'One-50' is a late-heading diploid perennial ryegrass of medium leaf and tiller size with exceptional production.

The flowering date is +20 days. It is a leading variety for summer, autumn and winter use and can have a total DM production of over 12-12.5 T DM ha^{-1} yr^{-1} depending on the climatic conditions and soil fertility. It has a very high rust tolerance, excellent spring quality and summer leafiness allowing it to make excellent use of either irrigation or natural rainfall (Agricom Ltd 2017).

Commercial seed lots 'Prospect' line PRA101AC and line PG12T312TY infected with *Epichloë festucae* var. *lolii* strain AR1 and AR37, respectively, and 'One-50', line PGA110AA and line PGT310CH also infected with *Epichloë festucae* var. *lolii* strain AR1 and AR37, respectively, were used in this research.

2.3.2 Soil type

New Zealand soils are classified according to the New Zealand Soil Classification System. The soils are grouped on the basis of visual or measurable properties. The classification divides them into *Orders*, then hierarchy sorted down into *Soil Series*, and further divided into *Soil Types*, which are mostly based on the texture of the topsoil. Recent soils are often free draining soils, particularly when sitting over layers of gravel that are close to the surface, and therefore tend to be prone to drought without irrigation input. The top soils are reasonably fine textured, although soil drainage will depend on the depth of the gravels (Cutler, 1983; Thomas, 1979).

The soil used in this experiment was extracted from paddock 9 of Iversen field at Lincoln University, Canterbury, New Zealand (43°38’S, 172°28’E). This soil type is classed as a Wakanui silt loam (Udic Ustochrept, USDA Soil taxonomy) (Cox, 1978). In general these soils are weakly to moderate leached soils, deep and stoneless, with 1.8 - 3.5 m of fine textured material overlying gravels (Hewitt, 2010). They have water deficits in summer and water surpluses in winter and spring (McLaren & Cameron, 1996).

2.4 Methods

The experiment was performed in three stages. I - in the seed laboratory to assess seed quality before commencing the experiment. II - In the glasshouse and tunnel house, to sow and grow the seedlings to be tested for the presence of viable endophyte (E+), and III - on the concrete yard at AgR farm, where the plants were grown in pots and treatments applied for the purpose of this experiment, AgR Farm at Lincoln, Canterbury, New Zealand (43°38'S, 172°28'E) is 11 m above sea level.

2.4.1 I Seed laboratory

In April 2014 following the receipt of the four seed lots, and prior to the start of the experiment, seed germination and endophyte viability percentage were determined. For each seed lot received, four replicates of 100 seeds were taken at random from the pure seed fraction of the seed lot for germination testing, using the top of paper method (ISTA, 2017). Each replicate of 100 seeds was placed onto a double layer of blue germination blotter (Anchor Paper Co., St Paul

MN . USA) saturated in water. The blotters with the seeds were placed into a plastic sandwich box. A lid was placed on the box which was positioned into a controlled temperature incubator (20°C ± 2°C) with a photoperiod length of 16 h light and 8 h dark. Final germination percentage was assessed at 14 days (Table 2.1).

2.4.1.1 *TPIB method*

For endophyte infection rate, 100 seeds were sown into black plastic propagation trays (with a capacity of 7.2 L per tray) containing seedling mix prepared at Lincoln University Nursery (120 L Southland peat, 80 L pumice) with the following fertiliser additions per cubic meter: 4 kg Osmocote® exact mini (16% N, 3.5% P, 9.1% K), 8 kg dolomite lime and 2 kg Hydroflo® (granular wetting agent manufactured by Everris Australia Ltd) (Richards, B. pers. comm., 2014). At 45 days after emergence, sixty seedlings were randomly selected and blotted to assess viable endophyte infection frequency (E+) using the tissue-print immunoblot (TPIB) procedure. This is an immunological technique that uses an antibody as a detector of viable endophyte in planta, using the sap of the plant. The base of a tiller was cut and pressed onto a nitrocellulose membrane to transfer the plant sap. The membrane was then processed with antibodies and colour reagent (Gwinn et al., 1991; Simpson et al., 2012). If the stain results were unclear (some tillers positive and some negative, or the appearance of a different pink stain colour on the membrane compared with the negative control (E-), histological staining was performed. All seedlings were number coded in the plastic trays for future tracing. The leaf sheath stain technique was used on any seedling for which the TPIB result was not definite. The method involved peeling the leaf sheath of the tillers, and staining the tissue with aniline blue, followed by visualisation under the microscope to detect epichloid hyphae (Bacon & White, 1994; Card et al., 2011; Clark et al., 1983; Hiatt et al., 1999; Saha et al., 1988). The results are presented in Table 2-1.

Table 2-1 Germination and viable endophyte (E+) percentage of four commercial perennial ryegrass seed lots at receipt.

Cultivar	Seed lot	Endophyte strain	Germination (%)	E+ (%)
'Prospect'	PRA101AC	AR1	93	98
	PG12T312TY	AR37	96	90
'One-50'	PGA110AA	AR1	95	94
	PGT310CH	AR37	90	89

2.4.2 II Glasshouse - production of E+ seedlings

On 28 May 2014, 200 seeds per seed lot were sown into 2 black plastic propagation trays (with a capacity of 7.2 L per tray) containing the seedling mix prepared at Lincoln University Nursery (described in 2.4.1 Seed laboratory). Trays were placed in the propagation glasshouse, at approximately 20 ± 2°C, and watered as required (Figure 2-3).

Figure 2-3 Trays containing seedlings of both cultivars infected with *Epichloë festucae* var. *lolii* strains AR1 or AR37 before being blotted for viable endophyte presence.

After 6 weeks emerged seedlings were tested for viable endophyte presence using the TPIB method. Seedlings not containing endophyte were discarded and only the positive ones were used in this experiment (Figure 2-4).

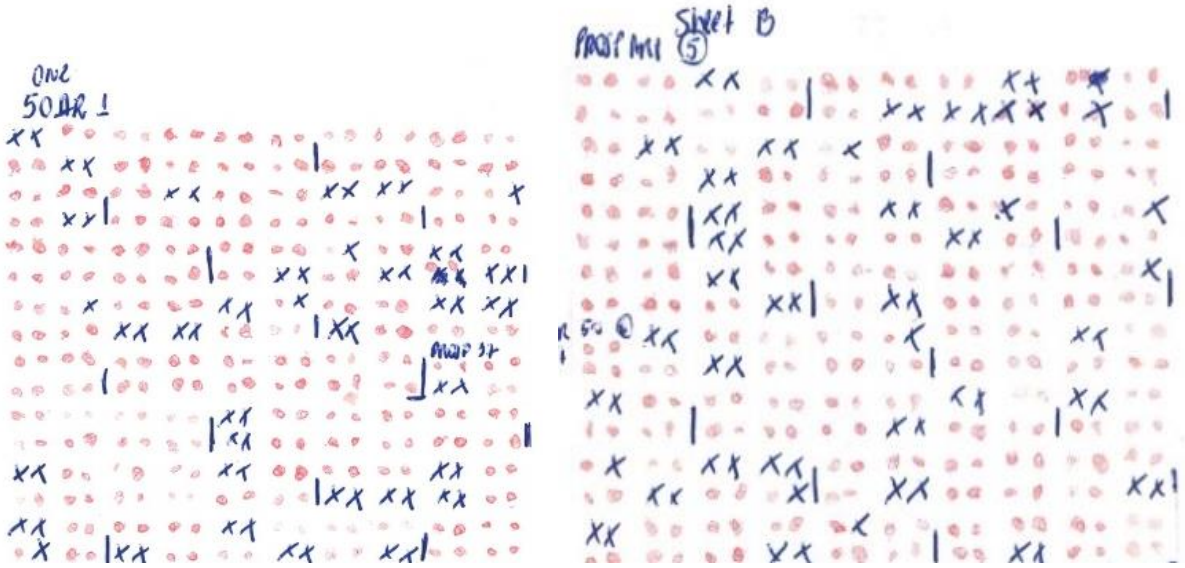

Figure 2-4 Blot results showing tillers containing viable endophyte strains AR1 or AR37 in both commercial cultivars used in this experiment. Red colour stain shows viable endophyte presence (E+), pale pink or brown colour indicates endophyte free or non-viable endophyte (E-) and missing non-germinated seeds are marked with an X.

2.4.3 III On the concrete yard at AgR farm

On 16 July 2014, trays with E+ plants were moved from the Lincoln University glasshouse to the AgR tunnel house for a week. The trial was undertaken at AgR Lincoln Farm (Boundary Road). On 23 July, E+ seedlings were transplanted from trays to individual free-draining 8 L capacity plastic pots (one seedling per pot). Each pot was filled with 8 kg of dried topsoil extracted from the Lincoln farm (see soil type section: 2.3.2). The drying of the soil was done at 80°C for 48 h until constant weights were achieved.

Pots were placed on the concrete yard. Straight after the transplanting was finished, pots were irrigated to field capacity. Field capacity was calculated using the volume method after

gravitational drainage from saturated soil (Gradwell, 1979). After 2 days seedlings were trimmed to 5 cm high using a hand battery operated shrub shear (Bosh G ASB10).

2.5 Establishment of waterlogging treatments

The waterlogging (WL) was done using plastic buckets with a 9 L capacity. The buckets were first filled with tap water and a pot containing the E+ seedling at field capacity was placed inside the bucket. Once in the buckets, to create the WL treatment, the pots were filled with water up to 2 cm from the surface (Figure 2-5). This level of water was maintained during the waterlogging period. Where evapotranspiration occurred, pots were refilled when necessary to maintain the level. The non-waterlogged pots were watered daily as required.

Figure 2-5 Red bucket with one of the pots containing a perennial ryegrass plant infected with *Epichloë festucae* var. *lolii* during treatment 1, waterlogging at vegetative stage GS 30.

2.5.1 Waterlogging treatments

The experiment consisted of four levels of waterlogging (Table 2-2): waterlogging at three different growing stages and no waterlogging (control). Plants were waterlogged for 13 days (d) and after the treatment was stopped, plants remained waterlogged for 2 more days (total waterlogging 15 d). To stop the waterlogging treatment, pots were removed from the buckets and the pots were left to drain freely again.

The timing of treatment (Table 2-2) was defined using the Zadoks growth stage (GS) description (Zadoks et al., 1974), determined for three randomly taken tillers per plant. The treatment was applied when 66% of tillers were at that stage (2 of 3 tillers had reached the GS).

Table 2-2 Treatment factors: waterlogging at different growth stages of perennial ryegrass plants infected with endophyte strains AR1 or AR37.

Treatment	Time of waterlogging	Growth stage (GS)
I	during vegetative stage	GS 30
II	during anthesis	GS 65
III	during seed set	GS 70
IV	no waterlogging	Control

2.5.2 Treatment application

On 12 November 2014, waterlogging treatment I was imposed in three randomly assigned main plots (Figure 2-5). In the meantime all other plants were watered regularly as needed. On 12 December two main plots were then waterlogged for treatment II and the same procedure was followed as previously explained. On 3 January 2015, treatment III was established. During the experiment two main plots were left as controls (treatment IV, no waterlogging) and water was applied as required.

2.6 Pot plant management

An individual ryegrass plant consists of a number of tillers that differ in age. The perennial structure allows these tillers to be grazed without compromising the status of the plant. The continuous tiller production provides perennial ryegrass with its perennial characteristic. The

plant can recover from grazing/cuttings through the production of new daughter tillers from the mother plant. To simulate grazing, during the experiment each plant was defoliated at 5 cm above ground, on 20 August, 20 September and 22 October, using a hand battery operated shrub shear (Bosh G ASB10). The final defoliation was designated as the closing date for seed production. After that date no more cuts were taken (Figure 2-6).

Maintenance N fertilizer at a rate of 65 kg urea/ha (31 kg of N) was applied three times during the experiment, 6 days after transplanting the E+ seedlings to the pots (29 July 2014) and after 60 days (23 September) and a further 60 days (24 November).

Figure 2-6 Perennial ryegrass plants after having been clipped - vegetative stage, at closing date 22 October 2014.

A plant growth regulator (PGR) was applied once during the experiment, on 30 November at GS 32. Moddus® was used (a.i. 250 g/l trinexapac ethyl) at a rate of 1.6 L ha^{-1} as a foliar application to reduce stem length and prevent lodging (Rolston, pers. comm., 2014). Fungicide use in perennial ryegrass seed production is a standard practice among seed growers. Stem rust (*Puccinia graminis*) infection is common in New Zealand, damaging plant tissue and blocking

interception of sunlight, both reducing photosynthetic area. Seed yield losses can be major if it affects seed heads and the use of fungicides has proven to increase seed yields (Hampton, 1986). One single fungicide application was performed at the end of flowering (just before treatment III, GS 69). Application of Amistar® 0.5 L ha^{-1} (a.i. 250 g/L azoxystrobin, Syngenta Crop Protection Ltd) was done on 29 December, at a rate of 200 L ha^{-1}. The sprayer used was a hand held precision gas sprayer (Tank indicates: Nitrogen-Oxygen free) using sprayer nozzles (4x) Blue TeeJet® VisiFlo® flat spray tips at 110 degree spray angle and spaced 50 cm apart. The application was done at 50 cm height above the plants with a pressure of 2 bar.

2.7 Seed moisture content, method and formula

The seed moisture content (SMC) is the amount of free water in the seed. It is usually expressed as a percentage on a wet weight basis. A seed moisture test (ISTA, 2017) was conducted on all seed lots before starting the experiment and with the remaining seed at the end of it, using a Sanyo drying oven (0°- 200°C). Four grams of seeds (weights were measured using a balance Ohaus, Adventurer ± 0.001) were placed in lightweight weighing containers (tins). The seed moisture determination was done in two replicates, with precise weighing (up to three decimal places). The open tins were placed in the forced air oven for 2 hours (h), the oven was maintained at a temperature of 130° ± 2°°C (high temperature method; ISTA 2017). After the 2 h, the tins with the seeds were removed from the oven, the lid was replaced and the tin placed into a desiccator for 10 minutes. Dried seeds were re-weighed using the same balance. The moisture content as a percentage by weight (fresh weight basis) was calculated to one decimal place, by using the following formula:

$$\% \text{ seed moisture content (mc)} = \frac{M2-M3}{M2-M1} \times 100$$

Where:

M1 = Weight of the weighing container with lid (tin) in grams

M2 = Weight of the weighing container with lid and seeds before drying

M3 = Weight of the weighing container with lid and seeds after drying

Seed moisture content was reported to the nearest 0.1%.

2.8 Harvest of the experiment

The ripening of perennial ryegrass seeds follows an apical pattern. For that reason, to avoid the loss of mature seeds, brown paper bags were used as a paper funnel (Figure 2-7). One bamboo stick longer than the plant height was buried beside each plant in the pot. A hole was made in the bottom of the paper bag, all tillers were placed through the bag, and the bag was secured to the plant by taping it to the bamboo stick. Seeds that reached physiological maturity and dropped from the spikelet were collected in the funnel daily and placed in small brown paper bags with the correspondent identification number (1 to 128).

Figure 2-7 Perennial ryegrass plants with paper funnels during harvest time. Plants without funnels had already been harvested

Seed was hand harvested between 28 January and 3 February 2015. Each plant was individually harvested and samples were placed in paper bags numerically identified. During harvest, seed

moisture content (SMC) of field dressed samples (FD) was assessed, following the method described previously, using 10 seeds per sample. FD samples averaged a SMC of 42%.

On 3 February 2015, all plant material was cut at ground level and placed individually in brown paper bags (separate from the FD seeds). Cut samples were allowed to naturally air dry before dry matter (DM) was assessed. The individual plants were placed in a brown paper bag and dried to constant mass in a fan–forced oven for 36 h at 80°C. The dry mass of the individual plants was recorded. For each plant, the number of reproductive tillers, the seed yield and the thousand seed weight (TSW) were assessed and recorded. TSW was calculated by weighing 100 pure seeds to three decimal places. The moisture content of the seed after being threshed was calculated using the method already described.

2.8.1 Management of seeds post-harvest

Seeds were air dried before being threshed, sieved and blown on a Dakota column separator (removing empty glumes and other inert matter). Seeds reached an average of 13% SMC (assessed using the moisture test described previously) in the dry and dark shed at the AgR farm. TSW was assessed on 100 pure seeds per sample weighed to three decimal places (two decimal places reported).

2.8.2 Germination test after harvest for grow out test

On April 2015, newly harvested pure seeds, were assessed for endophyte transmission through the "grow-out test". Sixty seedlings were randomly assessed, blotting all tillers per seedling (TPIB as previously described).

Germination of the pure seed samples was assessed using the organic growing media (O) method (ISTA, 2017) using 60 seeds only. Seeds were sown into black plastic trays (60 cells per propagation tray with a capacity of 45 mL per cell) containing the Lincoln University seedling mix. Trays were placed in the propagation glasshouse, at approximately 20 ± 2°C, and watered as required. Potassium nitrate (KNO_3, 0.2%) was applied to break any seed dormancy. KNO_3 was applied with a spray bottle to each seed during sowing. Trays containing seeds were placed in the Lincoln glasshouse. Seedling emergence was defined by the percentage of normal seedlings that emerged in the first 21 days after sowing. Between 6 and 8 weeks after sowing, up to 60 seedlings (when possible; if not, the maximum germinated) were blotted to determine

the infection frequency through the presence of viable endophyte strain AR1 or AR37. All tillers per plant were blotted and data were recorded as the frequency (percentage) of plants with viable endophyte (E+). Roots were checked (for normality, (ISTA, 2013)) and final germination recorded.

2.9 Statistical analysis

Data were statistically analysed using a split-plot analysis of variance (ANOVA) with four main-plot treatments (waterlogging) and a 2 x 2 factorial as sub-plot treatments (cultivars x endophyte). For the waterlogging treatment factor, three orthogonal contrasts were specified: (1) control compared to the average of three waterlogged treatments, (2) waterlogging at the vegetative stage compared to the average of the waterlogging treatments at either anthesis or seed-set, and (3) waterlogging at anthesis compared to waterlogging at seed-set. For other comparisons, the unprotected least significant difference (LSD; $P<0.05$) procedure was used (Saville, 2015; Saville & Rowarth, 2008). The ANOVAs were carried out using GenStat® (VSN International 2013. GenStat® for *Windows* 19th Edition. VSN International Ltd., Hemel Hempstead, UK).

2.10 Results

2.10.1 Effects on endophyte transmission frequency

Perennial ryegrass plants that suffered the waterlogging stress during vegetative growth, anthesis or during seed-set, did not differ significantly in viable endophyte infection frequency compared with the control treatment (see main effect means in Table 2-3). Endophyte infection of the offspring was successful, regardless of the time of the waterlogging treatment. Endophyte transmission did not differ between cultivars, or between endophyte strains. Moreover, both strains transmitted very well (over 86%) in both cultivars, with a grand mean for endophyte transmission frequency of 89% (Table 2-3). Overall the transmission frequency was high and exceeded the industry 70% threshold.

None of the two-factor and three-factor interaction contrasts were statistically significant, except for the cultivar by endophyte strain by the contrast WL at vegetative stage (GS 30) versus WL at anthesis (GS 65) and WL at seed set (GS 70), which was significant at $P < 0.1$, (Table 2-4).

Table 2-3 Main effect means of cultivar, endophyte strain and waterlogging on endophyte transmission frequency, growth parameters and seed quality. (Interaction contrasts were all not significant except in two cases, where a 3-factor interaction contrast was significant at $p < 0.10$; one involved germination % (Appendix IV) and one involved % E+, with the latter presented in Table 2-4).

	E+ (%)	**Germ (%)**	**DM (g pl^{-1})**	**Rep.tillers (n° pl^{-1})**	**Seed yield (g pl^{-1})**	**TSW (g)**
Grand mean	**89.1**	**87.0**	**20.0**	**71**	**6.1**	**1.7**
Cultivar						
One-50	87.0	85.6	20.5	69	5.8	1.66
Prospect	91.1	88.3	19.5	74	6.3	1.78
LSD (5%)	8.8	4.4	5.4	28	1.9	0.11
Sig. of diff.	**ns**	**ns**	**ns**	**ns**	**ns**	*****
Endophyte strain						
AR1	91.5	87.5	19.9	73	6.1	1.65
AR37	86.7	86.5	20.2	70	6.0	1.78
LSD (5%)	8.8	4.4	5.4	28	1.9	0.11
Sig. of diff.	**ns**	**ns**	**ns**	**ns**	**ns**	*****
Waterlogging (WL)						
Control (n=2)	90.0	86.2	18.7	70	6.1	1.73
WL GS 30 (n=3)	85.2	87.8	20.9	75	6.1	1.72
WL GS 65 (n=2)	92.7	88.0	21.5	71	6.4	1.75
WL GS 70 (n=1)	91.3	84.4	17.1	65	5.3	1.63
LSD (5%) (i)	10.9	8.3	8.2	30	2.2	0.40
Sig. diff. of contrasts						
Control vs Waterlogged	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**
WL GS 30 vs (WL GS 65 & GS 70)	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**
WL GS 65 vs WL GS 70	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

(i) There are many LSD for comparing the different treatments. The reported value is the minimum LSD, therefore, if the minimum shows no significant difference all the other ones would also do so.

Table 2-4 Viable endophyte transmission rate for perennial ryegrass hosts infected with two commercial *Epichloë festucae* var. *lolii* strains AR1 and AR37 after imposition of three waterlogging treatments during seed production. This table reports on a 10% significant 3-factor interaction contrast: cultivar x (endophyte strain) x WL (GS. 30 vs (WL GS 65 & WL GS 70)). Means with an * differ significantly from the highlighted treatment mean of 68.0.

		Viable endophyte percentage (E+ %) present in seedlings Waterlogging at			
Cultivar	Endophyte strain	**GS 30** (n=3)	**GS 65** (n=2)	**GS 70** (n=1)	**Control** (n=2)
'One 50'	AR1	94.0*	91.1*	84.9	94.3*
	AR37	**68.0**	95.9*	92.2	83.3
'Prospect'	AR1	85.9	90.2*	94.8*	96.4*
	AR37	92.9*	93.7*	93.5	86.2
LSD (5%) within WL trts.		20.4	24.9	35. 3	24.9
LSD (5%) between WL trts.		21.0	26. 5	28.1	
			28.1	23.0	

(n=) N^{o} of reps for each treatment
(→) Calculations of the different LSD between trts are shown in Appendix III

In Table 2-4, the highlighted treatment, which is the mean transmission frequency of 68% for cultivar 'One-50' infected with the endophyte strain AR37, when WL was at the vegetative stage, was unusually low. This value was significantly lower than 9 other treatment means, as shown in the table. The differences do not follow a trend, and further research is needed to confirm and explain, or refute this result.

2.10.2 Effects on germination

Waterlogging had no significant effect on germination percentage for which the grand mean was 87%. Germination did not differ between the cultivars or between endophyte strains (Table 2-3). However, there was a 3-factor interaction contrast, cultivar by endophyte strain by the WL contrast of control versus waterlogged significant at $P < 0.1$ % (Appendix IV).

2.10.3 Growth parameters

For the growth parameters measured, WL had no significant effect on DM production, number of reproductive tillers per plant, seed yield per plant or TSW. However the TSW differed significantly between cultivars and between the endophyte strains independently of the WL treatments of the experiment (Table 2-3).

2.11 Discussion and conclusions

This study demonstrated that the vertical transmission of *E. festucae* var. *lolii* strain AR1 and strain AR37 in both commercial cultivars tested, was not negatively affected by periods of waterlogging during vegetative growth (GS 30), or reproductive growth (GS 65 or GS 70). Furthermore, the experiment showed that the transmission frequency was high and over the industry threshold of 70%. Flooding during those periods of plant development is not a factor that influences vertical transmission from plant to seed. This is not in accordance with the findings of Gundel et al. (2009a) who reported that annual ryegrass plants from humid prairies showed lower endophyte transmission efficiencies compared to plants growing in other vegetation communities. However it is in accordance with the results found for tall fescue, where the differences did not vary significantly. These results may be explained similarly to Gibert and Hazard (2013) who showed that the variation found in symbiont frequencies in the native grass populations of *Festuca eskia* was principally explained by the endophyte transmission rate rather than the fitness of the plants, being the transmission a genetically based mechanism at the host population level.

The sowing of the infected seeds to initiate the experiment was done under ideal glasshouse conditions. This study confirmed that those optimal conditions allowed a high concentration of endophyte mycelia in planta, thus favouring the endophyte transmission to seeds. However, whether similar high transmission would occur in the field requires confirmation.

Even though Gundel et al. (2011) showed in *Lolium multiflorum* that plant biomass (normal– to – high) of the host was a key factor on the success of *Epichloë occultans* transmission, the temporary loss of fitness caused by the excess water during plant development during this experiment did not affect the plant biomass and none of the plant fitness variables not being able to compare the transmission frequency of *Epichloë festucae* var. *lolii* in the two perennial ryegrass cultivars in different fitness situations. Further research with longer waterlogging

periods should be performed to asses this. Hypothesis I was not supported by the data on this experiment.

Hypothesis II was that *Epichloë festucae* var. *lolii* strains AR1 and AR37 have different stress thresholds, and the impact of waterlogging on transmission rates will therefore differ between the two endophyte strains. However this hypothesis was also not supported as any difference between the strains was not significant.

It is interesting that WL had no effect on plant parameters such as seed yield. The differences found in the TSW between cultivars and between the endophyte strains independently of the WL treatment are in agreement with those reported by (Lakić et al., 2015) who showed differences in TSW between perennial ryegrass cultivars ranging from 1.8 to 2.5 g with an TSW average of 2.1 g.

Perhaps if the WL treatments had been more severe (longer) there would have been effects on plant growth (and possibly endophyte transmission to seed). However it would be very unusual for commercial grass seed crops to experience long periods of waterlogging in NZ.

Overall, the results of this experiment do not support the hypothesis that host-endophyte interaction is negatively affected by excessive soil moisture for short periods of time during plant development. In both cultivars and with both endophytes, the transmission was successful.

The results obtained also did not support the hypothesis that *Epichloë festucae* var. *lolii* strains AR1 and AR37 have different stress thresholds, so that the impact of waterlogging on transmission rates would therefore differ between the two endophyte strains.

The significant differences reported in table 4, may have occurred due to random sampling variation, as the number of seeds tested for germination was only 60, instead of four replicates of 100 (400 in total) as required by ISTA (2017). As sample size increases, the variation in test results decreases (ISTA, 2017).

It is important to note that waterlogging during earlier stages (from sowing up until GS 30) was not studied, and experiments involving water stress at earlier growth stages of perennial ryegrass grown for seed production should be considered.

CHAPTER 3

The effects of nitrogen and potassium fertiliser during ryegrass seed production on the vertical transmission of two *Epichloë* endophytes strains (AR1 and AR37) in two commercial perennial ryegrass cultivars.

3.1 Introduction

Perennial ryegrass is the most widely grass sown in NZ, and is also used in Europe, South America, South Africa and Japan. Perennial ryegrass has been through many recurrent selection breeding programmes which have focused on different production traits including dry matter production (DM), seed yield, quality and resistance and persistence to various abiotic stresses (Hendriks et al., 2017; Stewart & Hayes, 2011). Since the discovery of the beneficial novel *Epichloë* endophytes, a new factor in the breeding programmes has been added; trying to achieve high vertical transmission frequency of these novel endophytes, allowing better crop persistence and environmental benefits with a reduction in the use of pesticides (Stewart, 1987). Delivering a high level (>70%) of viable endophyte infection in seeds is a desirable quality component for the seed industry (Rolston & Agee, 2007).

Vertical transmission is often imperfect, producing unexpected proportions of infected and non-infected seeds, suggesting that crop management and or environmental conditions are the reasons for this imperfection (Canals et al., 2008; Gundel et al., 2011; Yule et al., 2013).

There are many variables that affect the input of nutrients. Nutrient applications may vary in type, amounts and also in application times during development (Bylin et al., 2014). Fertilisers provide the plant with the nutrients needed for the main physiological functions, as well as replacing the nutrients removed from the soil by plant uptake, and out of the system due to the grazing of animals.

The aim of this study was to evaluate whether a seed crop management factor, such as the amount of fertiliser applied, would affect the vertical transmission of *Epichloë festucae* var. *lolii* strains

AR1 and AR37 at an individual plant level, in perennial ryegrass host plants. Nitrogen (N) and potassium (K) are commonly applied for seed production.

Nitrogen (N) and potassium (K) are routinely used for ryegrass seed production. Frequently research has been done on the effects of the amount of nutrient applied and the time of these applications during the crop development on seed production (Cookson et al., 2000; Hampton, 1987; Hebblethwaite, 1980) but little is known about the effect of N and K on the vertical transmission of *Epichloë* endophytes in perennial ryegrass. The effect of N and K on endophyte vertical transmission is therefore still uncertain. A combination of different N and K rates was used to evaluate if there was any effect on the vertical transmission of two novel endophytes strains, AR1 and AR37. Phosphorus (P) fertiliser was not considered, as a soil test returned pH of 5.9 and an Olsen P value of 27 $\mu g\ l^{-1}$, a level adequate for perennial ryegrass seed production (Cornforth & Sinclair, 1984).

In ryegrass seed production, N is a major component in the determination of seed yield, affecting all yield components (Field-Dodgson, 1971; Knox, 1997). N fertiliser helps to enhance canopy size and persistence and avoid tiller malnutrition. N helps with the increase in cell number and therefore leaf area (Morton & Watson, 1948).
A previous study had compared the effect of N fertiliser on the host fitness, generally comparing this with the presence or absence of the fungi. An example is the significant difference noted by Ren et al. (2009) in perennial ryegrass plants, when high N was applied to E+ hosts compared with E-, as the infected plants had better shoot and root growth (> DM).
The timing of N application is important, and early spring application promotes growth, increases tillering and therefore increase light interception due to the increased leaf number (Hampton, 1987). Multi-tillering ensures good seed production.
On the other hand, excess nitrogen inputs can lead to lodging of the plant which reduces seed yield (Rolston et al., 2007).

Potassium (K) is required by all animals and plants during their life cycles for their successful development. Adequate K in plant tissue results in superior plant quality due to the improved efficiency of the photosynthesis process. Moreover, K in soil is correlated directly with tissue K (Ebdon et al., 2013). K helps to increase resistance to some diseases and improves water use efficiency. K helps to maintain a normal balance between carbohydrates and proteins in the plant. A lack of K results in retarded shoot growth and depressed chlorophyll accumulation (Stamp &

Geisler, 1980). Webster and Ebdon (2005) showed the possible benefits in enhancing perennial ryegrass tolerance to cold temperatures, when K rates are between 245 - 440 kg ha^{-1} yr^{-1}, with N rates between 340 - 440 kg $ha^{-1}yr^{-1}$.

Many studies have shown that there is a relationship and interaction between both nutrients; N fertilisation contributed to a decline in soil K, as there was more demand of the latter by the plant (George et al., 1979). No studies have shown the effect of both fertilisers on the vertical transmission of *Epichloë* endophytes in perennial ryegrass.

The hypotheses were that (i) high rates of N and/or K application would reduce the endophyte transmission frequency in both host grass cultivars studied, (ii) excessive levels of N and/or K would compromise endophyte vertical transmission due to a higher plant growth rate, while slower plant growth would enable the endophytes to infect a greater proportion of tillers and (iii) due to the existence of endophyte-host specificity, there would be different optimum fertiliser rates between the two cultivars and the two endophyte strains which would allow optimum endophyte transmission frequency in the offspring.

3.2 Design

The design was a split-plot design with 9 main-plot treatments, being a 3 (K rates; 0, 200 and 400 kg $ha^{-1}yr^{-1}$) x 3 (N rates; 0, 150 and 250 Kg $ha^{-1}yr^{-1}$) factorial laid out in 2 blocks (A & B), with 4 sub-blocks per main-plot, and 4 "sub-sub-plot" treatments being a 2 (cultivar) x 2 (endophyte) factorial (randomised within each sub-block).

3.3 Materials and methods

Perennial ryegrass seeds of cultivars 'Prospect' and 'One-50' infected with *Epichloë festucae* var. *lolii* strains AR1 and AR37 as described in Chapter 2, were used in this experiment. Seeds were sown firstly in the potting mix in the glasshouse, then the resulting seedlings assessed for viable endophyte using TPIB blotting methodology (see Chapter 2 section 2.4.1.1). Plants with no viable endophyte (E-) were discarded. Plants containing viable endophyte (E+) were used for this experiment.

The treatments consisted of different levels of N and K fertilisers. Fertiliser treatments are presented in Table 3-1.

Table 3-1 Treatments, consisting of 3 levels of nitrogen (N) and 3 levels of potassium (K) applied to perennial ryegrass cvs 'One-50' and 'Prospect' infected with the *Epichloë* novel endophyte strains AR1 or AR37.(Nil is classed as one level)

Treatment number (trt)	**Nitrogen application rate (kg ha^{-1})**	**Potassium application rate (kg ha^{-1})**
1	0	0
2	0	200
3	0	400
4	150	0
5	150	200
6	150	400
7	250	0
8	250	200
9	250	400

The N source was granular urea at 46% N, and the K source was granular potassium chloride at 50% K. The available N recommended for perennial ryegrass seed production is approximately 180-200 kg of total N ha^{-1} yr^{-1} (Chynoweth et al., 2010; Rolston et al., 2010a). Determining the N and K application rates is largely dependent on the N and K soil status. For the purposes of the experiment, the applications of 250 kg N ha^{-1} and 400 Kg K ha^{-1} during the year were classed as very high (Rolston pers. comm., 2014). S soil test, measuring in the top soil (0 - 7.5 cm), was conducted. The MAF Quick Test gave results of available K (15), Ca (10), Mg (22) and pH (5.9).

3.4 Crop management

The field trial was conducted in 2014, at the AgR Lincoln Research Farm (Boundary Road, 43°38'S, 172°28'E), NZ. The soil type in this paddock was silt loam on a sandy loam classed as Templeton-1 (*S-map*, 2010).

On 13 May, the trial site was sprayed with a pre-emerge herbicide of 4L ha^{-1} Nortron® (a.i 500 g L^{-1} ethofumesate) for annual grassweed control plus Preside (65 g ha^{-1}) with Uptake Oil (l L in

200 L of water). Broadleaf weeds were controlled with one application of Jaguar® (a.i.25g L^{-1} diflufenican and 250g L^{-1} bromoxynil).

Two square blocks of 14 m long by 14 m wide (196 m^2) were randomly allocated in the paddock, 5 m apart. Each block was divided into 9 plots (3 plots by 3 plots) of 2 m X 2 m (4 m^2) each, with a 2 m gap between them, and 2 m buffer around the edges. Using a 1 m^2 metal quadrat, each plot was divided into 4 sub-blocks. Each sub-block had 4 different plants. One plant of each cultivar, infected with strain AR1 or AR37, was randomly allocated equidistantly between other plants in the sub-block (square), Figure 3-1.

Figure 3-1 Field trial showing block B, divided in 9 "main-plots" (1 – 9) with 4 "sub-blocks" per main-plot (shown for main-plot 4), each containing 4 infected plants (individual plants being "sub-sub-plots") which were randomly allocated to the 2 endophyte strain x 2 cultivar combinations. In total, there were 144 ryegrass plants per block. Main-plots 1-3 had no N, 4-6 had 150 kg N ha^{-1} yr^{-1}, and 7-9 had 250 kg N ha^{-1} yr^{-1}. Main-plots 1, 4 & 7 had no K, 2, 5 & 8 had 200 kg K ha^{-1} yr^{-1}, and 3, 6 & 9 had 400 kg K ha^{-1} yr^{-1} (block A also had this same treatment allocation). Paddock C7, AgR Farm, Lincoln.

On 16 July 2014, trays containing E+ plant were moved from the Lincoln glasshouse where sowing took place (28 May 2014), to the AgR tunnel house, as described in Chapter 2 (see 2.4.2). On 26 of August, E+ seedlings were hand transplanted from the trays to the field, following the design previously described. On 14 September 2014 two dead plants were replaced. Each sub-block had a total of 4 different plants, 1 plant of each cultivar / endophyte strain combination,

randomly placed in each sub-block (‘One-50’ infected with AR1 (11), ‘One-50’ infected with AR37 (137), ‘Prospect’ infected with AR1 (P1) and ‘Prospect’ infected with AR37 (P37), Figure 3-2). The entire plot had a total of 16 infected perennial ryegrass plants. Each block identified as block A and block B consisted of 144 (16 x 9) infected plants. The entire experiment consisted of 288 ryegrass plants infected with viable *Epichloë* endophyte (Figure 3-1).

P7	**P1**	**P1**	**P7**
17	**11**	**11**	**17**
P1	**17**	**P7**	**17**
P7	**11**	**11**	**P1**

Figure 3-2 Example of the arrangement of the plants (sub sub-plots) in each of the 4 sub-plot in one of the 9 main plots in block A.

The control of weeds surrounding the experimental site was achieved using a John Deere ride on lawn mower (at the lowest limit the lawn mower could cut). Further weed control was done by hand.

3.5 Treatment application

Fertiliser treatments (1-9, Table 3-1), were applied in November 2014. Granular urea (with 46% N) was used as the source of nitrogen and granular pink potassium chloride (KCL) (50%) as the source of potassium. N was split within each treatment (Park et al., 2017) The first application of N was done on the 12 of November at GS 30 and the second application on the 22 of November at GS 32 (Table 3-2). The application for K was done all at once on the 12 of November at GS 31 (Table 3-3). Fertiliser was broadcast over the plots which had previously been irrigated to increase fertiliser-soil contact, and to avoid losses to the atmosphere through volatilisation of N as gaseous ammonia (Selvarajah, 1991).

Table 3-2 Calculation of N application rates used in each treatment per plot in block A and B.

N rate (kg ha^{-1})	Urea (kg ha^{-1})	g m^2	g application^{-1} m^2	g plot^{-1}	n° trt /block	trt n°
0	0	0	0	0	3	1,2,3
150	326	33	16.5	132	3	4,5,6
250	543	54	27	216	3	7,8,9

Table 3-3 Calculation of K application rates used in each treatment per plot in block A and B.

K rate (kg ha^{-1})	KCL (kg ha^{-1})	g m^2	g application^{-1} m^2	g plot^{-1}	n° trt /block	trt n°
0	0	0	0	0	3	1,4,7
200	400	40	40	160	3	2,5,8
400	800	80	80	320	3	3,6,9

On 22 of November at Zadocks GS 32, the plant growth regulator (PGR) Moddus® was applied at a rate of 1.6 L ha^{-1} (250 g L^{-1} trinexapac ethyl, Syngenta Crop Protection Ltd), as a foliar application. Application was via a motorised boom sprayer which delivered 200 L of water ha^{-1}, using 4 XR Teejet® fan nozzles at 300 Kpa at an angle of spray cover of 110°. The main objective was to reduce stem length and prevent lodging (Rolston et al., 2007). An endophyte friendly fungicide, namely Amistar® (a.i. 250 g L^{-1} azoxystrobin, Syngenta Crop Protection Ltd.) at a rate of 0.5 L ha^{-1} was applied as routine treatment for crown rust control and protection against stem rust (Rolston et al., 2002b). There were two applications, one at GS 59 on 1 of December and the other at GS 69 on 30 of December. The trial area was irrigated regularly as required.

After the seedlings had established, the trial was defoliated mechanically using a hand battery operated shrub shear (Bosh G ASB10). Further defoliations were performed on 1 of October and 20 of October. This final defoliation was designated as the closing date for seed production for the two late flowering cultivars used (Rolston & McCloy, 2006). Defoliation was above apex height (5cm above ground) to reduce the risk of apex loss, that could lead to altering the final tiller production, tiller number and subsequent seed yield.

Seed was hand harvested between the 30 of January and the 7 of February 2015. Brown paper bags were used as a paper funnel to collect mature seeds that may have fallen off the inflorescence. One bamboo stick, longer than the plant height, was buried beside each plant in the field to attach the paper funnel to the plant.

Each plant was individually harvested and seed samples were placed in paper bags numerically identified. During harvest, seed moisture content (SMC) of field dressed samples (FD) was assessed, following the method and formula described previously (see Chapter 2: Seed moisture content, method and formula), using 10 seeds per sample. FD samples averaged a SMC of 44%. On 8 February 2015, all remaining plant material was cut at ground level and placed individually in brown paper bags (separate from the FD seeds). Cut samples were allowed to naturally air dry before dry matter (DM) was assessed. The individual plants, in the brown paper bag were dried to constant mass in a fan–forced oven for 36 h at 80°C at the Field Research Centre, Lincoln University. The dry mass and the number of reproductive tillers of each individual plant was recorded. The dressed seed yield was assessed and TSW recorded.

3.6 Management of seeds post-harvest

Seeds were air dried while stored in the shed at the AgR farm, in dry and dark conditions before being threshed. Seeds were sieved and blown on a Dakota column separator (removing empty glumes and other inert matter). Seeds reached an average of 14% SMC (assessed using the moisture test described previously in Chapter 2). Machine dressed samples were stored in brown paper bags in a 5°C temperature room until tested. Seed yield was recorded.

In May 2015, newly harvested pure seeds were assessed for endophyte transmission through the "grow-out" test method. After more than six weeks of growth, sixty randomly selected seedlings were assessed, and all tillers per seedling were blotted (Germination for grow out and TPIB as previously described in Chapter 2).

3.7 Statistical analysis

Each variable was statistically analysed using an analysis of variance (ANOVA) for a split-plot design, with main plots being 2 randomised blocks of 9 main plot treatments (3K x 3N), and sub-plot treatments, being a 2 cultivar by 2 endophyte factorial. For the comparisons of the treatments, the unprotected least significant difference (LSD; $P < 0.05$) procedure was used (Saville, 2015; Saville & Rowarth, 2008). The ANOVAs were carried out using GenStat® (VSN

International 2013. GenStat® for *Windows* 19th Edition. VSN International Ltd., Hemel Hempstead, UK).

3.8 Results

3.8.1 Fertilisers (N &K) effect on endophyte transmission frequency

The transmission frequency had a grand mean of 88%. The different fertiliser treatments (Table 3-1) did not significantly affect the transmission rate when compared between the two cultivars or between the endophyte strains AR1 or AR37 (Table 3-4), (Figure 3-3). For plants with only 0, 1, or 2 germinated seedlings (out of 60), transmission frequencies were not as well determined, so were declared "missing".

A table with the full set of treatment means interactions (N x K x cv x endophyte strain interaction) for endophyte transmission frequency E+ (%) is presented in Appendix V. From this table, all lower order interaction tables can be calculated.

Table 3-4 Main effect means for the effect of N and K fertiliser applications on vertical transmission E+(%) of AR1 and AR37 strains in perennial ryegrass cultivars 'One-50' and 'Prospect'.

N fertiliser (kg $ha^{-1}yr^{-1}$)	**E+ (%)**
0	82.4
150	91.1
250	90.5
LSD (5%)	**14.3**
Sig. of linear trend	**ns**
K fertiliser (kg $ha^{-1}yr^{-1}$)	
0	86.6
200	95.5
400	81.8
LSD (5%)	**14.3**
Sig. of linear trend	**ns**
Cultivar	
'One-50'	87.2
'Prospect'	88.8
LSD (5%)	**3.5**
Sig. of diff.	**ns**
Endophyte strain	
AR1	89.1
AR37	86.9
LSD (5%)	**3.5**
Sig. of diff.	**ns**

ns: not significant at $p < 0.05$

While none of the combination rates of N and K significantly affected transmission frequency (Table 3-4 and Figure 3-3), a significant effect was evident for the application of a single nutrient.

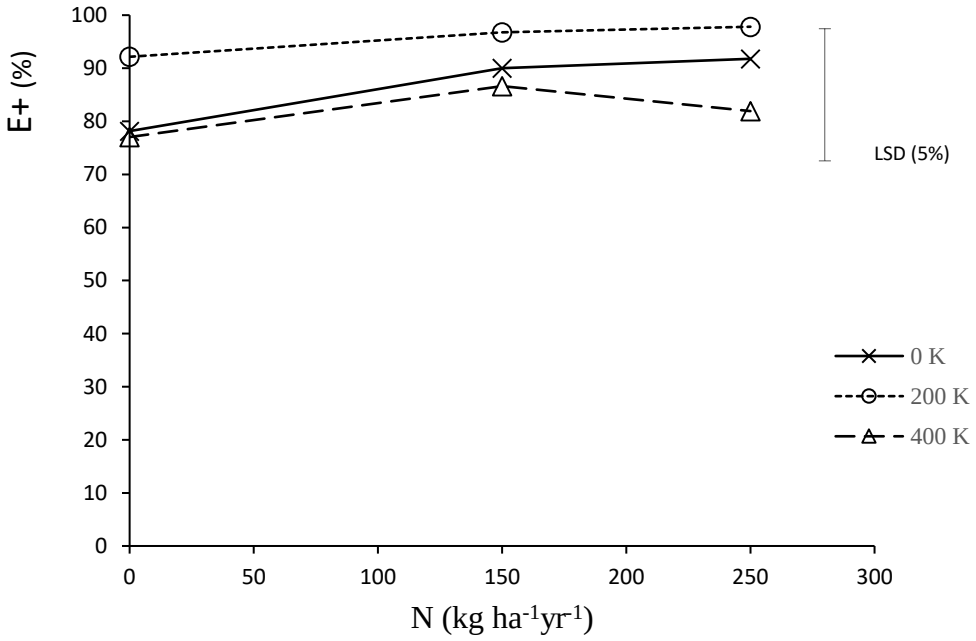

Figure 3-3 Percentage of viable endophyte (E+) present in perennial ryegrass seeds after harvest with the application of 9 combinations of N & K fertiliser during crop development for seed production. Each observed data point is the average of eight replicates. Vertical bar indicates LSD (24.9) for comparing any two data points (at 0.05 level of probability).

The only significant interactions were endophyte strain x (linear component of N) ($p < 0.05$), detailed in Figure 3-4, and more specifically in Figure 3-5 and the other significant interaction was cultivar x endophyte strain x (linear component of K) ($p < 0.01$), explained further in Figure 3-6.

3.8.1.1 *N effect on endophyte transmission*

When 150 kg N $ha^{-1}y^{-1}$ was applied, the endophyte transmission frequency in cultivar 'One-50' was 90.1% and 87.4% for AR1 and AR37 respectively. For 'Prospect' the endophyte transmission frequency was 93.2 and 93.6% for AR1 and AR37 respectively. Transmission was high in both cultivars, and increasing the N rates applied to 200 kg $ha^{-1}y^{-1}$ did not significantly change the transmission frequency with the exception of cultivar 'One-50' infected with the

strain AR1, that was significantly higher compared with the same cultivar and cv "Prospect' infected with the *Epichloë festucae* var *lolii* AR37 strain (Figure 3-4).

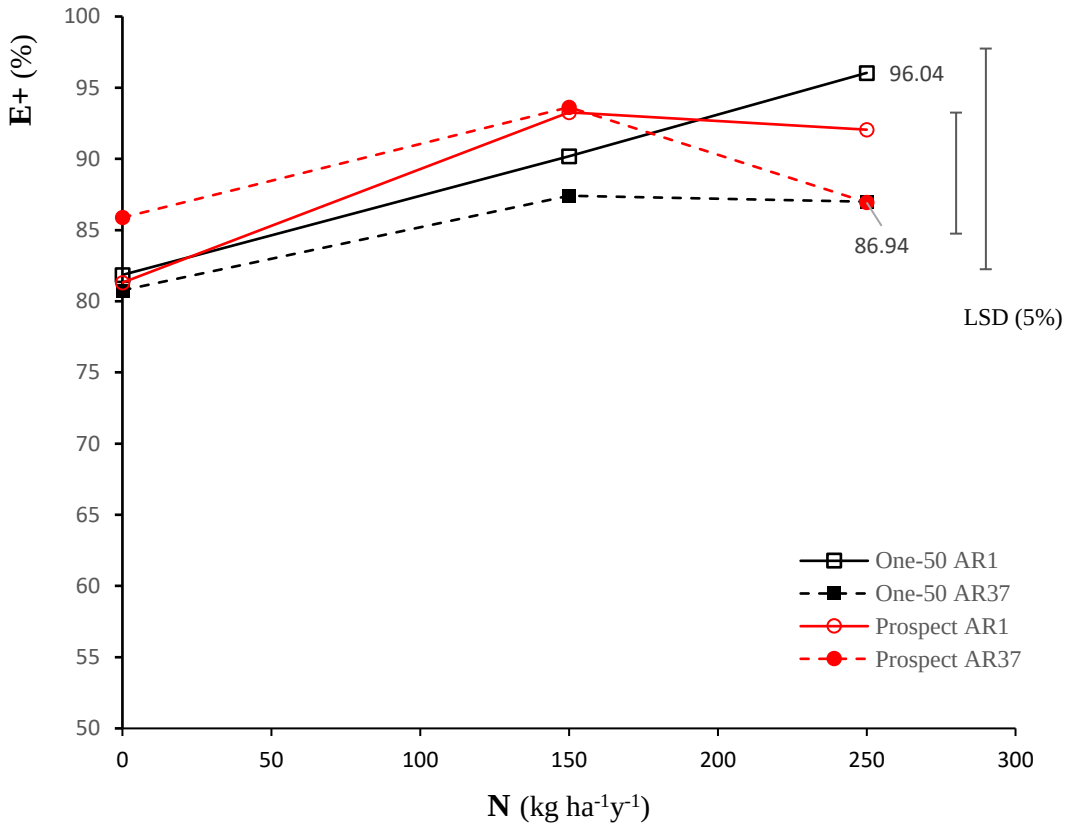

Figure 3-4 Percentage of viable endophyte (E+) present in both perennial ryegrass and for each endophyte strain (AR1 or AR37) when different rates of N were applied. Left vertical bar indicates LSD (8.5) for comparing AR1 with AR37, within the same rate of N applied. The right vertical bar indicates LSD (15.5) for all other comparisons (at 0.05 level of probability). The interaction of endophyte strain and N applied, (linear trend /slope) was significant at $p < 0.05$.

To amplify the results, and analysing the endophyte strains only (independently of the host they are infecting) with the effect on applications of N only, there was a significant difference between AR1 and AR37 endophyte strains. For nil and rates of 150 kg ha^{-1} yr^{-1}, there was no significant difference between the two endophyte strains. However when the rate of applied N was increased to 250 kg ha^{-1} yr^{-1}, strain AR1 had a 7% higher transmission than AR37 (Figure 3-5).

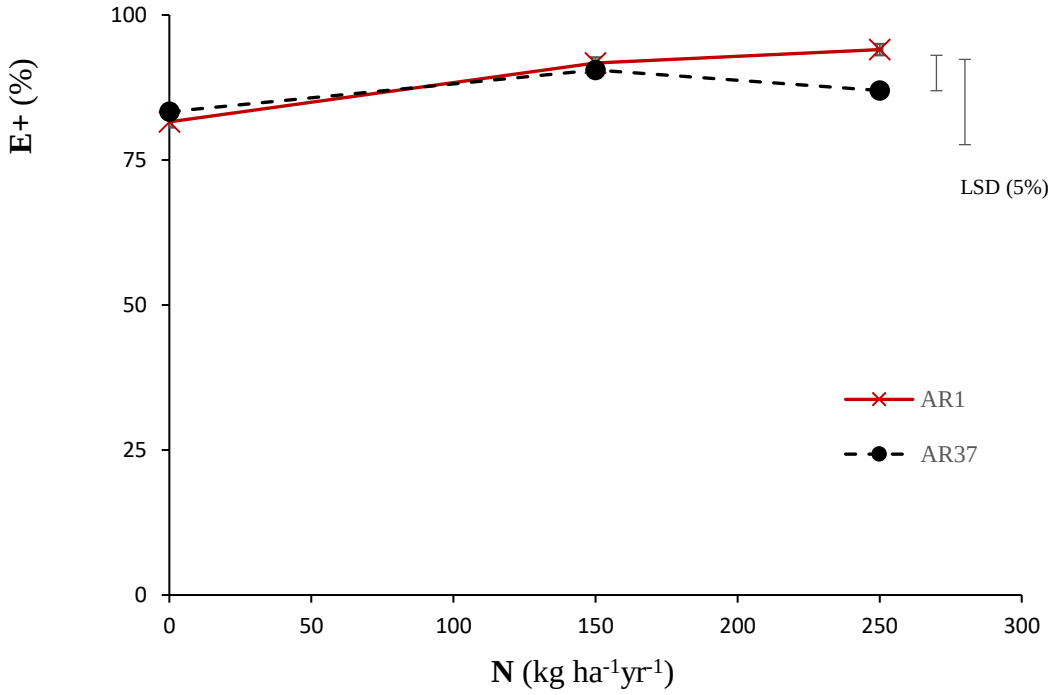

Figure 3-5 Percentage of viable endophyte (E+) present in both endophyte strain (AR1 or AR37) when different rates of N were applied. Left vertical bar indicates LSD (6.1) for comparing AR1 with AR37, within the same rate of N applied. The right vertical bar indicates LSD (14.7) for all other comparisons (at 0.05 level of probability). The interaction of endophyte strain and N applied, (linear trend /slope) was significant at $p < 0.05$.

3.8.1.2 *K effect on endophyte transmission*

Cultivar 'One-50' infected with endophyte strain AR37, showed a transmission response to K application rates. When the K rate was increased from zero to 200 kg $ha^{-1}yr^{-1}$, the transmission frequency increased by 10%, but this was not significant. This trend also occurred for AR1 in this cultivar. However when the K rate was increased to 400 kg $ha^{-1}yr^{-1}$, the transmission frequency of endophyte strain AR37 dropped significantly to 70.6% (Figure 3-6). 'One-50' infected with AR37 had a 16.6% significantly higher transmission in the control than with the highest rate of K used (400 kg ha^{-1} yr^{-1}). This did not occur for strain AR1 in the same cultivar. When the rate of K was doubled from 200 to 400 kg $ha^{-1}yr^{-1}$, there was a decrease in the transmission of this strain, but it was not statistically significant.

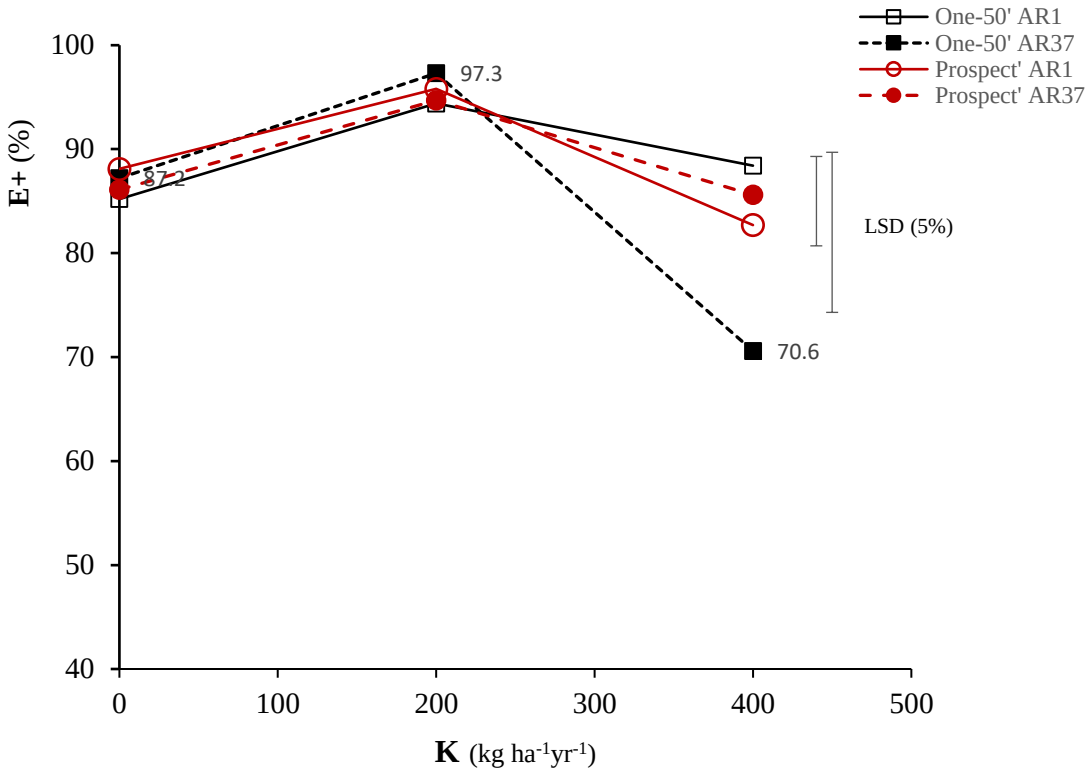

Figure 3-6 Percentage of viable endophyte (E+) present in newly harvested perennial ryegrass seeds (both cultivars infected with both endophyte strains, AR1 or AR37) with 3 rates of K (0, 200 and 400 kg $ha^{-1}yr^{-1}$). Left vertical bar indicates LSD (8.6) for comparing means with the same level of K applied and right LSD bar (15.5) for all other mean comparisons at 0.05 level of probability. The interaction of endophyte strain, cultivar and K applied (linear trend) was significant at $p < 0.01$.

Overall, with the zero and 200 kg $ha^{-1}yr^{-1}$ applications, there were no significant differences in endophyte transmission between either strains or cultivars. Each seed lot (the combination of host and endophyte strain) transmitted well, and over the industry threshold.

The significant difference was with cv 'One-50' infected with AR37, where there was a significant negative effect with the higher rate of K applied, compared with the other three seed lots.

3.8.2 Effects on germination

The newly harvested seed had a grand mean of 73.2% germination. The grand means of the treatment effects on germination are shown in Table 3-5.

Table 3-5 Main effect means of fertiliser application on the germination percentage of newly harvested seed.

N Fertiliser (kg ha^{-1} yr^{-1})	**G** (%)
0	73.5
150	76.4
250	69.7
LSD (5%)	**10.8**
Sig. of linear trend	**ns**
K Fertiliser (kg ha^{-1} yr^{-1})	
0	77.3
200	71.9
400	70.5
LSD (5%)	**10.8**
Sig. of linear trend	**ns**
Cultivar	
'One-50'	70.4
'Prospect'	76.0
LSD (5%)	**4.4**
Sig. of diff.	*****
Endophyte strain	
AR1	72.2
AR37	74.2
LSD (5%)	**4.4**
Sig. of diff.	**ns**

*Significant at $p < 0.05$

ns: not significant at $p < 0.05$

The application of the different rates of N combined with the different rates of K (trt 1-9) did not significantly affect the germination of the new harvested seeds infected with AR1 or AR37 strain. However, there was a significant difference in germination between seeds of the different cultivars. 'Prospect' had a 6.4% higher germination than 'One-50' (Table 3-5). A table of main full set of treatment means interactions (N x K x cv x endophyte strain interaction) for germination is presented in Appendix V. From this table, all lower order interaction tables can be calculated.

3.8.3 Growth parameters

The following table summarizes the main effect means of cultivar, endophyte strain and fertiliser treatments on plant dry matter production, seed yield components and seed yield.

Table 3-6 Main effect means of cultivar, endophyte strain and N and K fertilisers treatments on growth parameters and seed yield.

	DM (g)	**Rep tillers ($n°pl^{-1}$)**	**Seed yield ($g\ pl^{-1}$)**	**TSW ($g\ pl^{-1}$)**
Grand mean	**63.3**	**165**	**11.1**	**2.08**
Cultivar				
'One-50'	60.2	150	10.8	2.08
'Prospect'	66.2	179	11.4	2.06
LSD (5%)	7.4	19	1.3	0.05
Sig. of diff.	ns	*	ns	ns
Endophyte strain				
AR 1	62.8	169	11.0	2.07
AR 37	63.6	161	11.2	2.08
LSD (5%)	7.4	19	1.3	0.05
Sig. of diff.	ns	ns	ns	ns
N Fertiliser (kg ha-1yr-1)				
0	60.9	155	9.3	1.90
150	66.1	173	11.7	2.22
250	62.6	165	12.2	2.10
LSD (5%)	8.3	13	1.1	0.08
Sig. of linear trend	ns	*	*	*
K Fertiliser (kg ha-1yr-1)				
0	51.2	139	10.0	2.07
200	69.1	183	12.0	2.20
400	69.2	171	11.0	1.96
LSD (5%)	8.3	13	1.1	0.08
Sig. of linear trend	*	*	*	*

*Significant at $p < 0.05$

ns: not significant at $p < 0.05$

3.8.3.1 *Effects on DM production*

The biomass production grand mean was 63.3 g per plant. There was a significant linear response to increasing K rates on the biomass production, as well as a quadratic response between both fertilisers applied and the endophyte strains, however these two were not significant (Table 3-6).

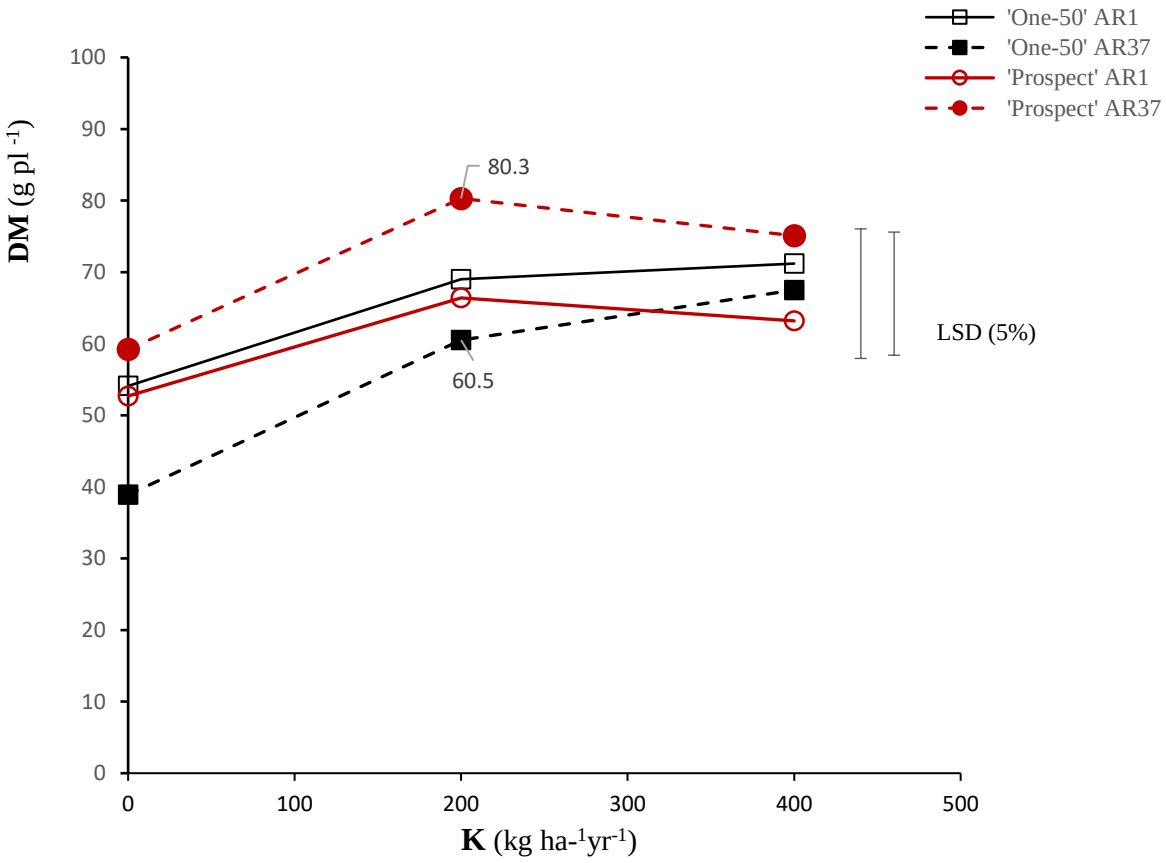

Figure 3-7 Effect of different rates of K applied during crop development for seed production on the DM (g plant $^{-1}$) production in perennial ryegrass plants. Left vertical bar indicates LSD (18.1) for comparing means with the same level of K applied and right LSD bar (17.2) for all other mean comparisons at 0.05 level of probability. The interaction of endophyte strain, cultivar and K applied (linear trend) was significant at $p < 0.01$.

The difference was noted when comparing the production with no K and 200 kg ha^{-1}yr^{-1} and also between 0 and 400 kg ha^{-1}yr^{-1}, however between 200 kg and 400 kg there was no significant difference in the DM produced.

3.8.3.2 *Effects on total number of reproductive tillers per plant*

The grand mean production of reproductive tillers, capable of producing seed and transmitting the *Epichloë* endophytes was 165 tillers per plant. Both nitrogen and potassium application significantly increased reproductive tillers per plant (Table 3-6).

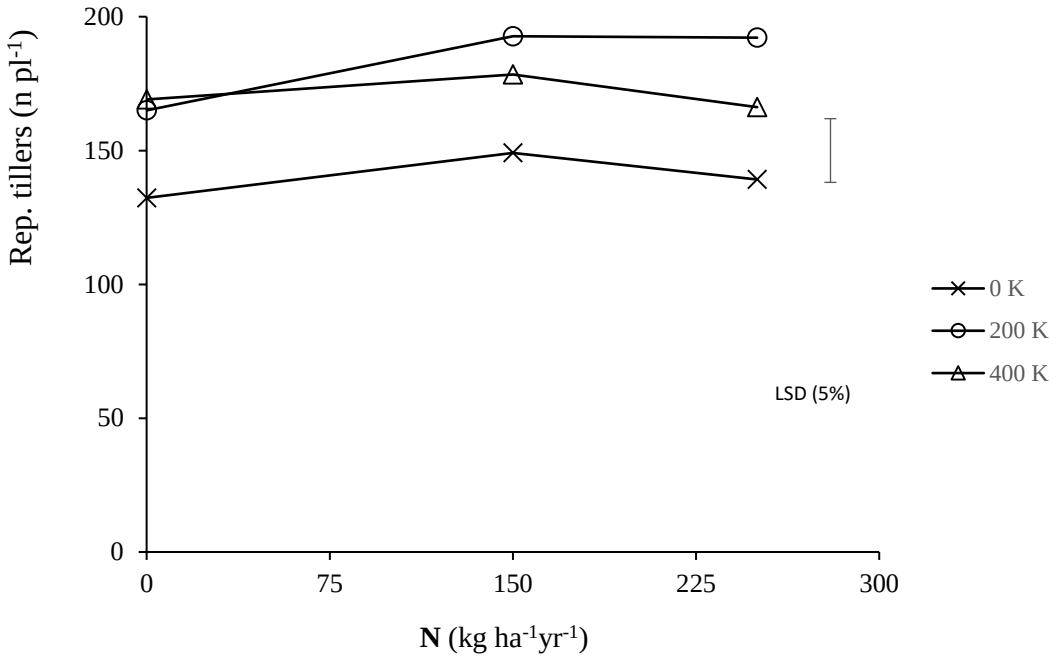

Figure 3-8 Number of reproductive (rep.) tillers per plant present in perennial ryegrass plants after 9 combinations of N & K fertiliser was applied during crop development for seed production. Vertical bar indicates LSD (23.3) for comparing any two data points (at 0.05 level of probability).

The interesting point is that K increased the number of reproductive tillers. With 150 kg N ha^{-1} yr^{-1} there was no significant difference between the two K rates. When K was applied at the higher rate (400 kg) with the higher N rate (250 kg) the number of reproductive tillers was significantly less than the 200 kg ha^{-1} yr^{-1} rate. And with 200 kg of K with the higher N rate of 250 kg ha^{-1} yr^{-1}, the increase in number of reproductive tillers was not significant. Both fertilisers showed their optimum in their intermediate rates, appearing to be the optimal at 150kg of N and 200 kg of K (Figure 3-8).

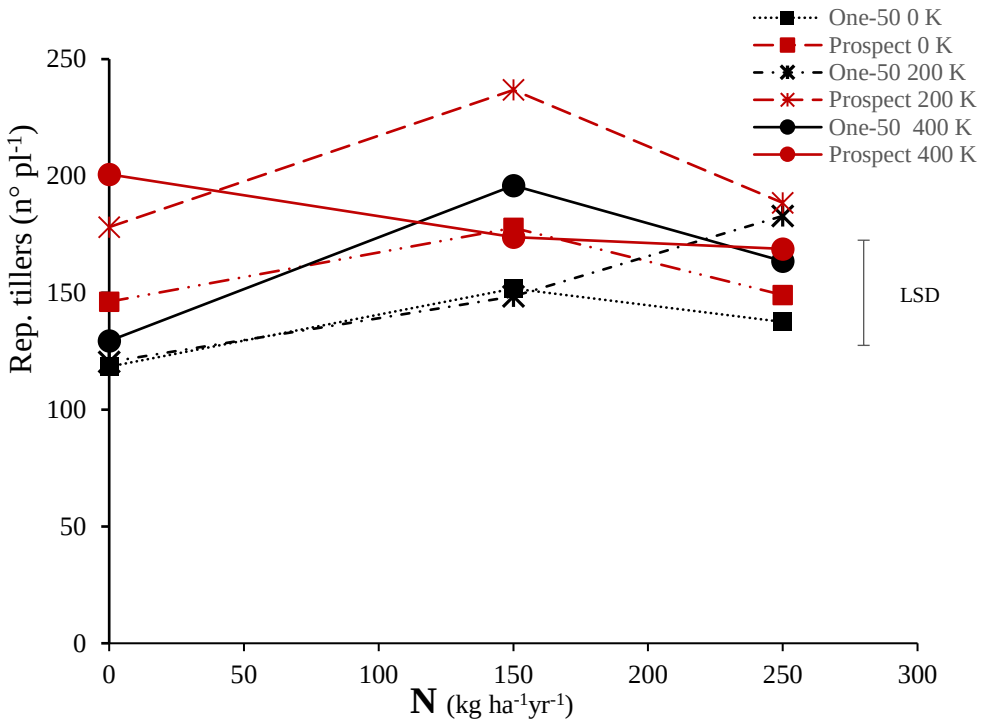

Figure 3-9 Number of reproductive (rep.) tillers per plant present in both cvs "Prospect (red-thick lines) and 'One-50' (black thin lines) perennial ryegrass plants after 9 combinations of N & K fertiliser was applied during crop development for seed production. Vertical bar indicates LSD (45.0) for comparing any two data points (at 0.05 level of probability).

There was a significant difference in reproductive tillers per plant between the cultivars but no difference between endophyte strains. 'Prospect' produced on average 29 more reproductive tillers per plant than cv 'One-50'.

3.8.3.3 *Effects on seed yield per plant*

The addition of both N and K fertilisers significantly increased seed yield per plant. The grand mean of seed yield was 11.1 g per plant. The increasing rates of N had a linear trend and the increasing rates of K had a quadratic curvature, both significant (Table 3-6).

There were no significant differences between the two higher rates of either fertiliser applied (Figure 3-10). There were also no significant differences between the seed yield of the two cultivars studied or between the two endophyte strains (Table 3-6).

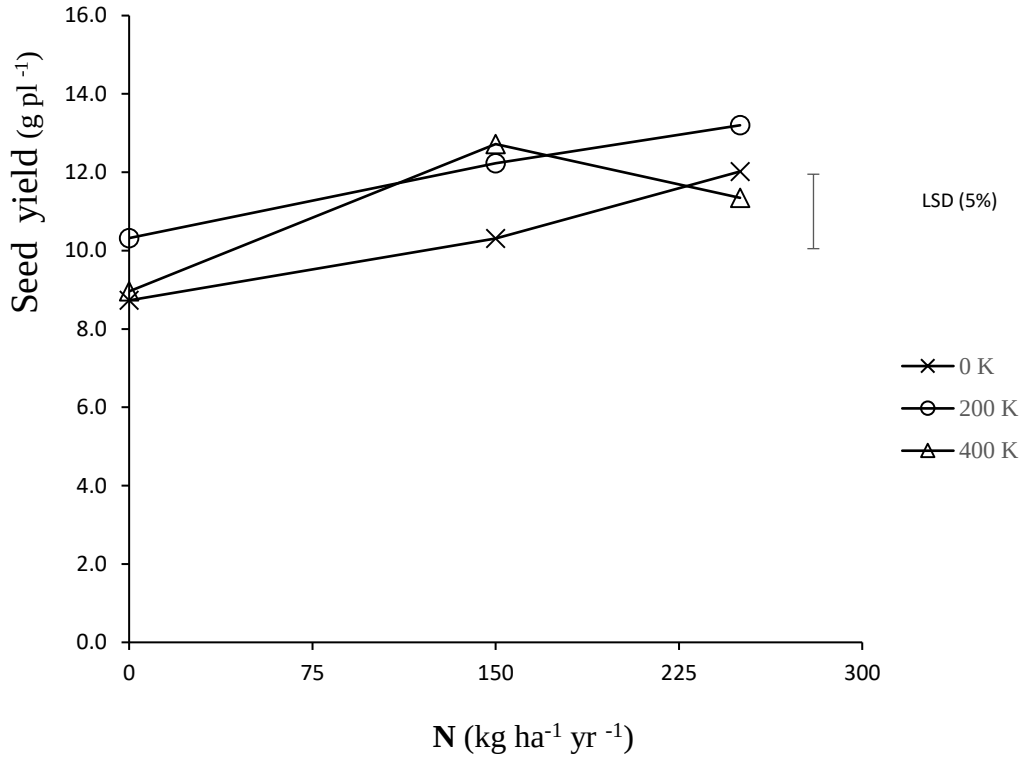

Figure 3-10 Seed yield (g) of perennial ryegrass plants with the application of 9 combinations of N and K fertiliser, during crop development for seed production. Vertical bar indicates LSD (1.9) for comparing any two data points (at 0.05 level of probability).

3.8.3.4 *Effects on thousand seed weight (TSW)of harvested seeds*

The TSW grand mean was 2.08 g. The only significant interaction was N (linear) x K (linear) with a probability $p < 0.05$ as shown in Figure 3-11.

The addition of both N and K significantly affected the TSW (Table 3-6). For both N rates applied, there was a significant increase in TSW compared with no N applied, but no difference between the 150 kg and 250 kg N ha^{-1} yr^{-1} rates. For K the effect on TSW had a different trend. The increase of K applied had only a positive significant effect for the 200 kg K $ha^{-1}yr^{-1}$ application. For 400 kg K, the effect was significantly negative compared with nil and 200 kg K ha^{-1} yr^{-1}. For both fertilisers, the intermediate application rate generated the highest seed weight. There were no significant differences between the two cultivars or the two endophyte strains (Table 3-6).

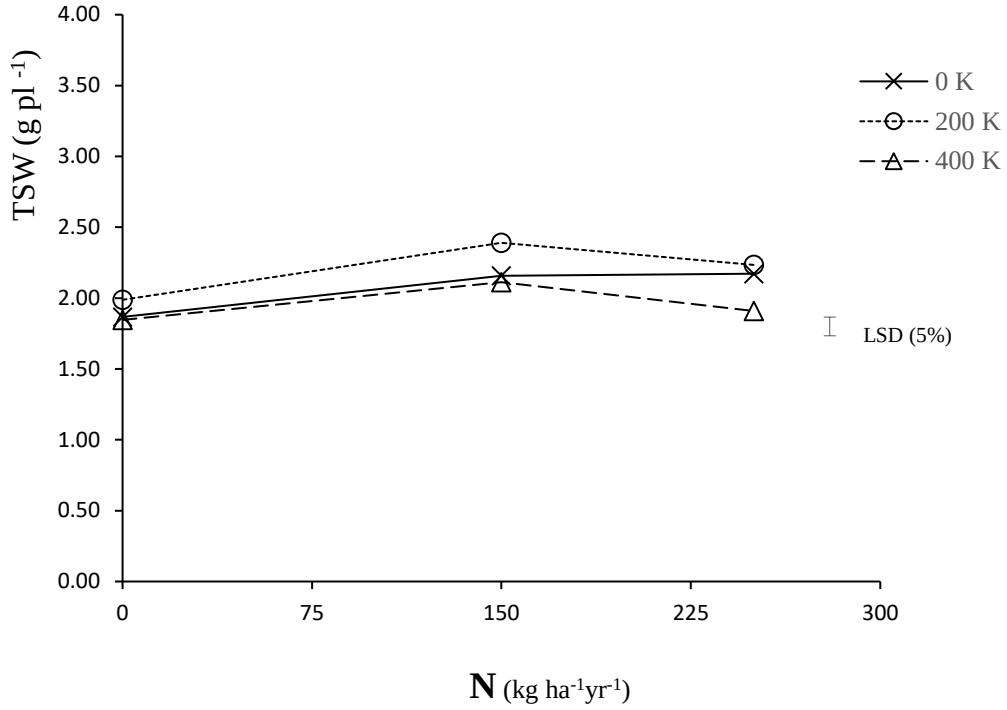

Figure 3-11 TSW of perennial ryegrass seeds with the application of 9 combinations of N and K fertiliser, during crop development for seed production. Vertical bar indicates LSD (0.133) for comparing any two data points (at 0.05 level of probability). There was a significant quadratic curvature for both N and K ($p < 0.001$).

3.9 Discussion

3.9.1 Fertiliser (N & K) effect on endophyte transmission frequency

The treatments consisted of 3 rates of N combining with 3 rates of K (0, 150, 250 and 0, 200 and 400 respectively). Transmission rate for both endophyte strains in both perennial ryegrass cultivars was high, irrespective of fertiliser treatments. The transmission frequency had a grand mean of 88%, being greater than the industry threshold of 70%, denoting a very good and high transmission. However there were some significant differences in the transmission of endophyte strains in response to single nutrients.

3.9.1.1 *N effect on endophyte transmission*

At 250 kg N $ha^{-1}yr^{-1}$ there was a difference in transmission frequency between the endophyte strains, as AR1 had a higher transmission than AR37. This result confirms the hypothesis that

there would be a difference between the two endophyte strains in the optimum fertiliser rate for endophyte transmission.

The results of this study can be partially explained and aligned with previous results. Rasmussen et al. (2007) observed that high rates of N fertiliser substantially decreased the alkaloid concentrations in the host. This could be due to either a reduction of the alkaloid production or by a reduction in the concentration of fungus in the plant tissues. Hyphae concentration in the plant tissue was not measured in the present experiment, but a reduction could explain the lower probability and or concentration of endophyte entering the seed, which would therefore affect the percentage transmission to seed. Rasmussen et al. (2007) also found that different perennial ryegrass cultivars which produced different water-soluble carbohydrates, could have reductions of up to 50% in the concentration of alkaloids produced, which explains the difference found between endophytes in different hosts associations.

Kaur et al. (2015) provided evidence for genotype-specific effects on levels of alkaloid production and Cheplick and Cho (2003) reported that both host and endophyte genotype associations strongly affect the concentration of hyphae in the plant tissue. In future studies, the concentration of hyphae in the plant should be measured, to unravel the cause of the difference in transmission as occurred for AR37.

3.9.1.2 *K effect on endophyte transmission*

Increasing the K applied from zero to 200 kg $ha^{-1}yr^{-1}$ tended to increase the endophyte transmission, however this increase was not statistically significant. At the highest K application rate, the endophyte strains differed in their response, with AR37 having a significantly lower transmission than AR1. Moreover it was significantly lower than the control with no K applied. These results support that this levels of K had a detrimental effect on the transmission of the AR37 strain in this cultivar.

As with the high rates of N fertiliser, the high rate of K was detrimental to AR37 but not to AR1. Both strains were originally collected from the Mediterranean Basin, but from different countries and certainly from different environments. These results could reflect the need to withstand different environmental conditions, like nutrient supply, in their original habitat. This may explain why AR1 has better adaptation to the host and the environment, allowing the endophyte to infect more seeds than AR37.

This experiment supported hypothesis iii, that endophyte strains have different thresholds, and different optimums of nutrient demand. It also supported the hypothesis that AR37 did have transmission problems that differ from the ones of AR1 when exposed to the same stress factors in the same host. There were no significant differences in transmission between the endophyte strains in cv 'Prospect'.

There was also a suggestion that optimum K rates may be different for each strain (at least between 200 kg and 400 kg $ha^{-1}yr^{-1}$). However future studies should be carried out using smaller differences in rates of nutrient applied, to detect those optimum levels and to support the hypothesis that different endophyte strains had different optimum requirements for fertilisers.
These results add support to the reports of host specificity, and that some strains transmit better in some hosts than in others (Gundel et al., 2011). In addition the difference observed could be due to their different alkaloid profiles (strain 'AR1' produces peramine only and strain 'AR37' lacks all three major alkaloids but produces epoxy-janthitrems.). The production of different compounds may also explain the difference in the response to nutrient inputs. This requires investigation.

3.9.2 Effect on germination

None of the fertiliser treatments had a significant effect on germination percentage. These results are aligned with the ones found by Hampton (1987).

The remaining seed in the germination test were classed as dead seeds. The low germination observed indicates poor seed quality. There was no loss of bigger and heavier seeds that could explain this quality parameter, as seeds were collected as they were maturing in the brown paper funnels. This could have been however, as a result of harvesting the seeds at an average of 44% SMC , and using a limited amount of seeds to define the SMC at harvest, instead of the protocol suggested by ISTA (2017). Seed management post-harvest is a key driver of seed quality. Harvesting immature seeds will lead to a low germination percentage. However the most likely explanation is the drying conditions. Failure to reduce SMC quickly enough would have led to storage fungi build up, and as a result, seeds were killed. However this was not determined.

3.9.3 Growth parameters

Even though the research was focused on the effect of fertiliser on transmission of the endophytes, plant growth parameters needed to be measured to understand the behaviour of the plants in response to the different treatments.

The above ground biomass production, expressed as g DM per plant, is dependent on the utilisation of solar energy by the photosynthetic tissues and factors like temperature, water supply and mineral nutrients including trace elements (Gallagher & Biscoe, 1978).

It would be expected that different rates of N and K would have an impact on the total biomass, especially with the application of N, as per the findings of Koeritz et al. (2015) in perennial ryegrass. However there was no response to application of N, although there was to K. However, there was no significant difference between 200 kg of K fertiliser and 400 kg applied. The plant could not use the highest K rate , possibly because a limitation factor of a nutrient other than N, or a limitation on the absorption of this nutrient by the plant.

Within each cultivar, the lack of significant differences encountered between the two high rates of N could be due to plants not taking up all the available soil N because it was lost from the soil through volatilization or leaching. This is in line with findings from Cookson et al. (2001) who showed that application rates above 150 kg N $ha^{-1}yr^{-1}$, were associated with a high risk of N loss, with higher rates resulting in only small benefits in seed and herbage. And as demonstrated in this experiment, on the vertical transmission of endophyte.

The lack of difference in DM production between the cultivars was as expected, because previous research had shown that both cultivars have a similar DM production, though they have differences in flowering dates (Figure 3-12).

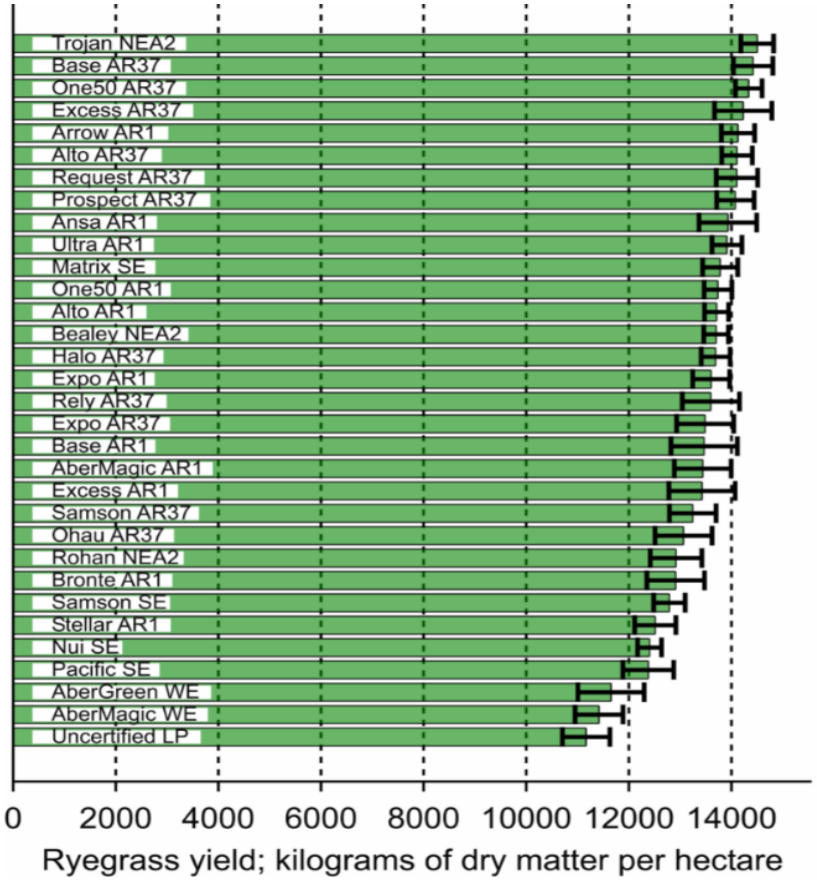

Figure 3-12 Annual herbage DM yield of perennial ryegrass cultivars in New Zealand (source: New Zealand Plant Breeding and Research Association NZPBRA 2016). Cultivars are significantly different ($p < 0.05$) where error bars do not overlap.

Grazing or cutting paddocks before ryegrass seed production is common practice as a canopy management tool, lowering the risk of lodging, although a late defoliation may result in the removal of seed heads, compromising seed yield (Hampton, 1987). Early defoliation gives the plant more time to produce more DM compared with late defoliation.

García Parisi et al. (2012) reported that grazing (cutting or topping) reduced the vertical transmission of *E. occultans* in *Lolium multiflorum* plants. On the other hand Gundel et al. (2009a) reported that defoliation did not affect endophyte transmission efficiency in the same species. However in this experiment all plants were treated the same and defoliation was not a factor. The soil N content was not tested before the experiment began. It is possible that a high N content in the soil used meant that plants could not respond to all the N added as fertilizer, but this cannot be confirmed. This should be taken into account for future similar experiments.

There was one application of plant growth regulator (PGR) to increase seed yield by preventing lodging through the reduction of stem length. Increases in seed yield of more than 50% have been obtained when PGRs were applied (Rolston et al., 2005; Rolston et al., 2014; Rolston et al.,

2007; Rolston et al., 2010b). The aim of this study was to replicate what ryegrass seed growers do and the majority of growers use a PGR. The stem shortening avoids risk of losses by lodging, also improving stem thickness. Avoiding lodging leads to a better utilisation of solar radiation through an increase in photosynthetic area of the leaves of the canopy that results in increased dry matter production and seed yield (Griffith, 2000; Hebblethwaite et al., 1978). The biomass was taken at the final harvest of the experiment only, leading to an underestimate of biomass production, as the biomass removed during the three toppings during the crop development was discarded. The yields obtained in this experiment cannot be compared with the situations in a seed field, as the sowing rate per ha differed from the real field situations. For future research, the biomass should be measured during each defoliation performed and total biomass then compared.

There were differences between the rates of N and K applied, and ryegrass cultivars, for the number of reproductive tillers produced. The 150 kg N $ha^{-1}yr^{-1}$ rate increased reproductive tillers by 11%, and the 200 kg K rate increased them by 33%. The higher rate of both N and K did not further increase reproductive tillers. Nutrient supply can affect the ability of the plant to produce tillers, and presumably the number and proportion of reproductive tillers, responsible of the vertical transmission. A possible explanation could be that the addition of potassium fertiliser which has a role in the main plant functions, like enzymes and stomatal activity and stimulating sugar transport in the plant, may have helped the plant with the N uptake thus resulting in an increase of growth, therefore more reproductive tillers (Britto & Kronzucker, 2008). These results also showed that there are optimum rates for each nutrient that these perennial ryegrass cultivars infected with *Epichloë* endophytes can assimilate, resulting in fertilisers not being assimilated by the plant and maybe lost from the soil. However, the levels of N and K existing in the soil after the experiment was finalized were not determined.

Cultivars differences are due to plant genetics. Different cultivars can have different tillering rates, final tiller numbers, tiller size and number and proportion of reproductive tillers.

Reproductive tiller number is only one seed yield component. Even though ‘Prospect’ produced significantly more reproductive tillers than ‘One-50’, seed yield did not differ between the two cultivars. There was also no difference in TSW. This suggests a difference between the cultivars in spikelet numbers and /or seeds per spikelet. These components were not assessed in this study. Both N rates increased seed yield, but only the medium K rate did so. These results are in accordance with those found by Elgersma (1990) and Rowarth et al. (1999).

Seed number was not determined in this experiment. However, it is possible that at the higher N and K rates, plants could have produced a greater amount of small seeds that seed growers would have discarded during the cleaning process, losing small but viable seeds. As the aim of this study was to replicate what the seed industry does, the same procedure was followed (small seeds were discarded). For research purposes it would have been interesting to determine the effect of those two macronutrients on seed numbers.

As N is the most limiting nutrient for grasses, it was expected that the addition of this nutrient would had a significant effect on seed production. The intermediate application rates for both N and K fertilisers generated the highest seed weight. Increasing the amount of fertiliser applied does not increase the capacity of the plants to uptake it. The relationship between the fertiliser added and TSW is in line with findings from Rolston et al. (2006) and Rolston et al. (2008). However, there was no difference between the two cultivars or the two endophyte strains which was not expected according to the genetic variations in perennial ryegrass reported by Lakić et al. (2015). However, their research was on different cultivars. This study had similar environmental conditions and factors applied to both cultivars.

3.10 **Conclusion**

The experiment did not support the first hypothesis that high rates of N and or K application would reduce the endophyte transmission frequency in both host perennial ryegrass cultivars. Moreover, the endophyte transmission frequency for both endophyte strains in both host cultivars, was statistically equal regardless of the fertiliser treatment applied.

On the other hand the second hypothesis which was that excessive levels of N and or K would compromise endophyte vertical transmission due to a higher plant growth rate, while slower plant growth would enable the endophytes to infect a greater proportion of tillers was partially supported for endophyte strain AR37 in cultivar ‘Prospect’. With no N applied, the endophyte strain AR37 transmitted 8.7% more than AR1 in cultivar ‘Prospect’. The experiment showed that there was a significant linear response ($p = 0.05$) for transmission between endophyte strains to the applied N only. This endophyte-host specificity, is very clear in the different behaviour of these two endophytes and is in accordance with findings from Rasmussen et al. (2007), who found biochemical difference in host grasses of the same cultivar infected with different endophyte strains.

Even though previous research conducted by Rowarth et al. (1999) and Hampton (1987), showed no effect of N on seed germination, there has been no research conducted on seeds containing endophyte. This research showed that N and K application rates had no significant effect on the germination of seeds containing endophyte AR1 or AR37 strains.

CHAPTER 4

Influence of temperature and relative humidity on the survival of *Epichloë festucae* var. *lolii* strain AR37 in infected seeds of perennial ryegrass during storage.

4.1 Introduction

For effective storage of seeds, the conditions under which they are stored are important for maintaining both seed germination and endophyte viability. Endophyte viability is easily lost when seed operators are not aware of the conditions required to maintain it. A rapid decline of endophyte viability during seed storage is a problem for the seed trade, because the biocontrol activity of the endophyte depends on having viable endophyte in the seed when sown. Maximising the survival of the endophyte in seed for sowing pasture is crucial.

In the commercial context, perennial ryegrass seed may often be stored for more than a year before being sold and sown. Some 78 years ago it was reported that wild type endophyte could remain viable in seed stored in NZ ambient conditions for about 24 months (Neill, 1940), but more recently Hare et al. (1990) stated that for long-term storage, to maximise endophyte viability, seeds should be stored at less or equal to 5° Celsius (°C) at a seed moisture content (SMC) less than 8 %. It is now well established that in ambient storage endophytes lose viability at faster rates than the seed, and as a consequence, leaving seeds that can still germinate, but lack the endophyte to transmit. Moreover research on *Lolium multiflorum* presented by Gundel et al. (2009b), showed that endophyte viability was lost faster than seed viability under conditions of high temperature and humidity. Previous seed storage studies on wild-type endophyte viability in perennial ryegrass have shown that storage temperature and seed moisture content (SMC) are critical (Hare et al., 1990; Rolston et al., 1986; Welty et al., 1987), because high temperatures and relative high humidity (RH) will rapidly degrade endophyte viability within ryegrass seed (Rolston et al., 2002a). However the precise combination of these factors required to produce a decline in endophyte viability below the industry threshold has not yet been investigated.

This study examined the effects of various storage conditions created by the combination of different temperatures and relative humidity levels on the survival of *Epichloë festucae* var. *lolii* strain AR37 in six perennial ryegrass seed lots. The hypothesis was that (i) the performance in storage of the novel endophyte strain AR37 contained in perennial ryegrass seed would not differ from that of the wild-type, and (ii) a combination of high temperatures and high relative humidity levels will detrimentally affect the viability of both seed and endophyte faster than low temperatures and low relative humidity levels.

Accumulated Thermal Units (ATUs) was used as a measurement unit to compare the different RH% with the time in storage at different temperatures, summarized as ATUs. ATU is a unit of measurement used to describe the cumulative effect of temperature over time. 1 ATU is equal to 1 degree Celsius (°C) for 1 day, with a base temperature of 0°C (Boyd et al., 2010; Crisp, 1981).

4.2 Experimental design

The experimental design was six replicates (= six AR37 seed lots) for each of four RH %s (20%; 40%; 60% and 80%) by four temperatures (4°C, 15°C, 20°C, 30°C; one growth chamber per temperature regime). The six seed lots were produced in Canterbury on different farms but under the same agronomic management practices (pers. comm Murray Kelly). 24 jars were placed in randomised positions in each temperature chamber. The complete experiment consisted of 96 containers. The percentage germination and viable endophyte were calculated and recorded. Endophyte viability (% E+) was recorded as a percentage of the seedlings that germinated; and plotted against time and Accumulated Thermal Units (see section 4.4.1 and Table 4-3).

4.3 Material and Methods

4.3.1 Plant material

Perennial ryegrass seeds of cultivar ‘One-50’, containing *E. festucae* var. *lolii* strain AR37 (see Chapter 2), and one seed lot containing *E. festucae* var. *lolii* strain AR1 (not included in the analysis) were used in this experiment. Six seed lots were harvested in Canterbury, New Zealand in 2013 and provided by PGG Wrightson Seeds Ltd, Kimihia Research Centre.

4.3.2 Saturated salts solutions

The use of saturated salt solutions is a simple way of producing known relative humidity levels within closed containers. Solutions maintain constant relative humidity levels in the atmosphere over them because any water solution of a non-volatile substance will have a definite water vapour pressure at a given temperature when the vapour phase is in equilibrium with the liquid (Winston & Bates, 1960) (Table 4-1). Saturated solutions with an excess of solids, also called "supersaturated solutions", maintain a very constant vapour pressure even under changing moisture conditions because a gain of water causes some of the solid to go into solution and a loss of water causes some of the dissolved material to precipitate, therefore the humidity conditions will remain unchanged (Sweetman, 1933).

An advantage in the use of saturated solutions is that they may be kept for long periods, and repeated or prolonged use does not alter their vapour pressures as long as they are handled with care.

Table 4-1 Saturated salts used in the experiment to create different RH% at different temperatures *Adapted from table in Appendix I. Relative humidity values over saturated solutions at various temperatures (Winston & Bates, 1960).

RH (%) Temp (˚C)	4˚C	15˚C	20˚C	30˚C
20	lithium iodide (LiI)	lithium iodide (LiI)	lithium iodide (LiI)	potassium acetate (KCH_3CO_2)
40	sodium iodide (NaI)	sodium iodide (NaI)	sodium iodide (NaI)	potassium carbonate (K_2CO_3)
60	magnesium nitrate ($Mg(NO_3)_2$)	sodium bromide (NaBr)	sodium bromide (NaBr)	cobalt chloride ($CoCl_2$)
80	potassium bromide (KBr)	potassium bromide (KBr)	potassium bromide (KBr)	potassium bromide (KBr)

4.4 Methods

Following receipt of the six seed lots, germination and viable endophyte frequency was assessed and subsequently a moisture test on all seed lots was conducted. Germination and moisture were assessed following the ISTA rules (ISTA, 2017). Results for germination (%), viable endophyte infection frequency (%) and SMC (%) at the beginning of the experiment are presented in Table 4-2.

Table 4-2 Germination (%), viable endophyte (E+) and SMC (%) of seed lots used in the experiment at time zero.

Seed lot	Germination(%)	Viable endophyte (E+)	SMC(%)
PGT302AC	92	89	10.50
PGT378AC	96	85	9.48
PGT311AC	93	86	9.97
PGT343CC	91	86	9.37
PGT350TC	92	96	9.35
PGT 346AC	91	84	9.33

For the germination test, prior to the start of the experiment, the top of paper (TP) method was used (ISTA, 2017). Four replicates of 100 seeds were taken at random from the pure fraction of the seed lot received (seeds were tested under a diaphanscope for identification and discarding of empty glumes). Each replicate of 100 seeds were placed onto a double layer of blue germination blotter (Anchor Paper Co., St Paul MN . USA) saturated in water. The blotters with the seeds were placed into a plastic sandwich box. A lid was placed on the box which was poistioned into a controlled temperature incubator (20°C ± 2°C) with a photoperiod lengh of 16 hrs light and 8 hrs dark. Germination was assesed at 14 days, with an extension of 7 days if needed. Seedlings were classed as normal, abnormal, fresh or dead. Seed germination was defined by the percentage of normal seedlings that emerged in the first 21 days after sowning (ISTA, 2013). Endophyte viability frequency was assessed using the TPIB method described in Chapter 2. The seed moisture content (SMC) was performed following the method described in Chapter 2.

Six grams of seed from each seed lot was placed in a 75 mL specimen container with no lid, inside a bigger polypropylene container of 500 mL capacity (Figure 4-1). The screw cap of the 500 mL container was then tightly closed creating a closed environment and a stable %RH

containing an oversaturated salt solution (weights were measured using a balance Ohaus, Adventurer ± 0.01), Within the closed containers, 5 grams of salt was placed in the bottom and 2.0 mL of water was added and an over saturated salt solution was created (salts were diluted at 40°C on a hot plate, then cooled down to room temperature).

Combinations of eight different salt solutions (lithium iodide (LiI), sodium iodide (NaI), magnesium nitrate ($Mg(NO_3)_2$), potassium bromide (KBr), sodium bromide (NaBr), potassium acetate (KCH_3CO_2), potassium carbonate (K_2CO_3) and cobalt chloride ($CoCl_2$) were used (Table 4-1). The small specimen container containing the six grams of seeds was placed over the oversaturated salt solution (Figure 4-2).

Figure 4-1 Endophyte infected seeds in a small container with an open lid, placed over the oversaturated salt solution inside a bigger container with a lid.

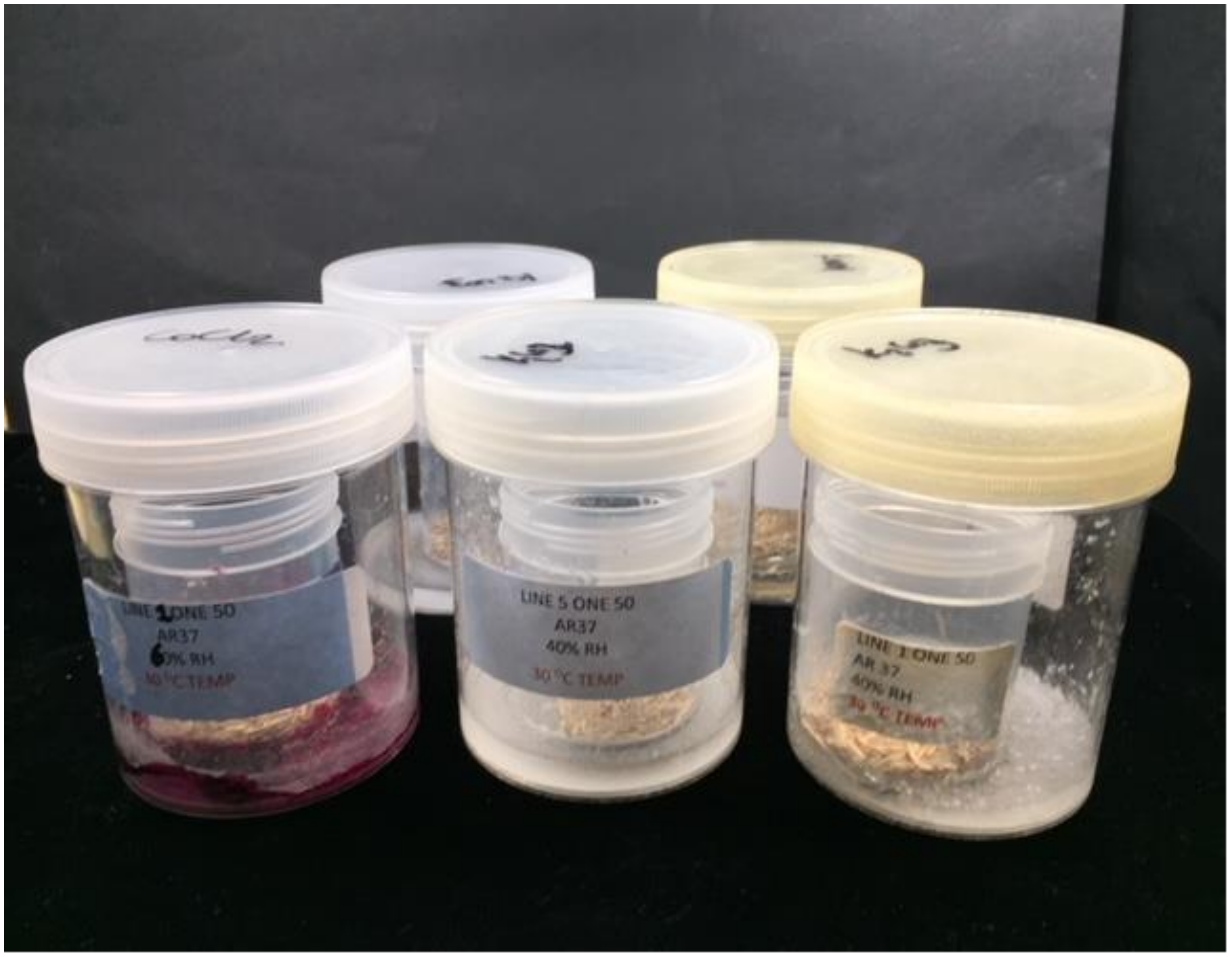

Figure 4-2 Endophyte infected seeds stored in closed containers at 30°C at the desired %RH, created using oversaturated salts.

Seeds were kept at the different levels of relative humidity (%RH) and temperature using these oversaturated salt solutions. The relative humidity-temperature relationships used in this experiment are as detailed in Appendix I (Wexler & Hasegawa, 1954; Winston & Bates, 1960).

After finalizing the experiment, the RH in each container were tested using a portable standalone hygrometer (Traceable®, Humidity/Temperature pen, (±0.5°C, ±2.5% RH), Thomas® Scientific, New Jersey, USA). A screw cap of the 500 mL capacity container was perforated to fit the data logger. After screwing the cap in the container to measure, after 16 hours the readings were recorded (Figure 4-3). The RH created were finally 24%, 40%, 50% and 70% respectively. Samples were stored at 4°C, 15°C, 20°C and 30°C, respectively. Six replicates for each storage temperature were placed randomly in the four temperature walk-in chambers located at Lincoln University. During the 418 days of storage of the experiment, there were 5 extractions of seeds to determine germination percentage and viable endophyte percentage at that point in time. Extraction of seeds to test for germination and viable endophyte (E+) was done at time zero (0)

and after 45, 130, 240, 333 and 418 days of storage. At each time point, 60 seeds were removed from each storage condition and the remaining seeds left in their containers until the next extraction date. Containers were quickly closed to re-establish the % RH created originally.

Figure 4-3 Portable hygrometer fitted in one of the storage containers, the % RH created by the salt solution was recorded after 16 hours.

The calculations of the ATU for each storage period and temperature are shown in Table 4-3.

Table 4-3 ATU calculated for the 5 different storage times used in the experiment.

ATU	45 days	130 days	240 days	333 days	418 days
4°C	180	520	960	1332	1672
15°C	675	1950	3600	4995	6270
20°C	900	2600	4800	6660	8360
30°C	1350	3900	7200	9990	12540

4.4.1 Testing after storage

After each extraction seedling emergence and germination percentage were assessed using the organic growing media (O) method using 60 seeds only (ISTA, 2017). Seeds were sown into black plastic trays (60 cells per propagation tray with a capacity of 45 mL per cell) containing seedling mix prepared at Lincoln University Nursery (see Chapter 2). Trays were placed in the propagation glasshouse, at approximately 20 ± 2°C, and watered as required. Seed emergence was assessed at 21 days after sowing, defined by seeds that produced a normal shoot twice the length of the seed. Germination was assessed after the endophyte viability test was performed; a seed was considered to have germinated if a normal shoot and root were present and they were within proportion (ISTA, 2013).

Between six and eight weeks after sowing, seedlings were assessed for the presence of viable AR37 endophyte using the immunoblot technique (TPIB) (Gwinn et al., 1991), blotting all tillers per plant. Results were presented as the percentage of plants containing viable endophyte (E+ %).

At the end of the experiment, moisture tests were performed using the remaing seeds from all treatments. Two replicates were tested using 2 grams of seed per replicate.

Both germination and viable endophyte for those six lots were plotted against time in each storage condition, temperature and relative humidity (RH) combination.

ATUs were calculated and plotted against endophyte viability. Data are the means of the six replicates.

4.5 Statistical analysis

Statistical analysis was carried out for each temperature separately using analysis of variance (ANOVA). For all three variables for the two lowest temperatures (4°C and 15°C) and for germination % for 20°C, the model terms fitted were "seed lot" and 4% RH x 5 ATUs, with orthogonal polynomial components specified for both RH% and ATU. For the two endophyte variables for the 20°C temperature and for all variables for 30°C, some of the treatments consisted only of zero values, so these treatments were omitted from the ANOVA since they had zero variability and would have violated the ANOVA assumption of homogeneity of variance. For these analyses, the model terms were "seed lot" and "treatment". For each analysis, a least significant difference (LSD; $P < 0.05$) was calculated (Saville, 2015; Saville & Rowarth, 2008). Statistical analysis was performed using GenStat® software (VSN International 2013, GenStat® *for Windows* 19th Edition, Hemel Hempstead, UK).

4.6 Results

The germination percentage recorded and viable endophyte infection frequency when the seed lots were received are presented in Table 4-2. The germination results, expressed as percentage at time zero of the six lots, were in tolerance according to the tolerance table for germination tests performed on different submitted samples done in the same or a different laboratory (ISTA, 2017) (Table 5.2).

4.6.1 Germination percentage after seed storage

There were no significant differences in germination from seeds stored at 4°C for up to 400 days for all four relative humidity levels (Figure 4-4). For seeds stored at 15°C, there was no significant difference in germination for seeds stored for up to 275 days among any of the four relative humidity levels, but after that, there was a significant decline for storage at a relative humidity of 70% (Figure 4-5).

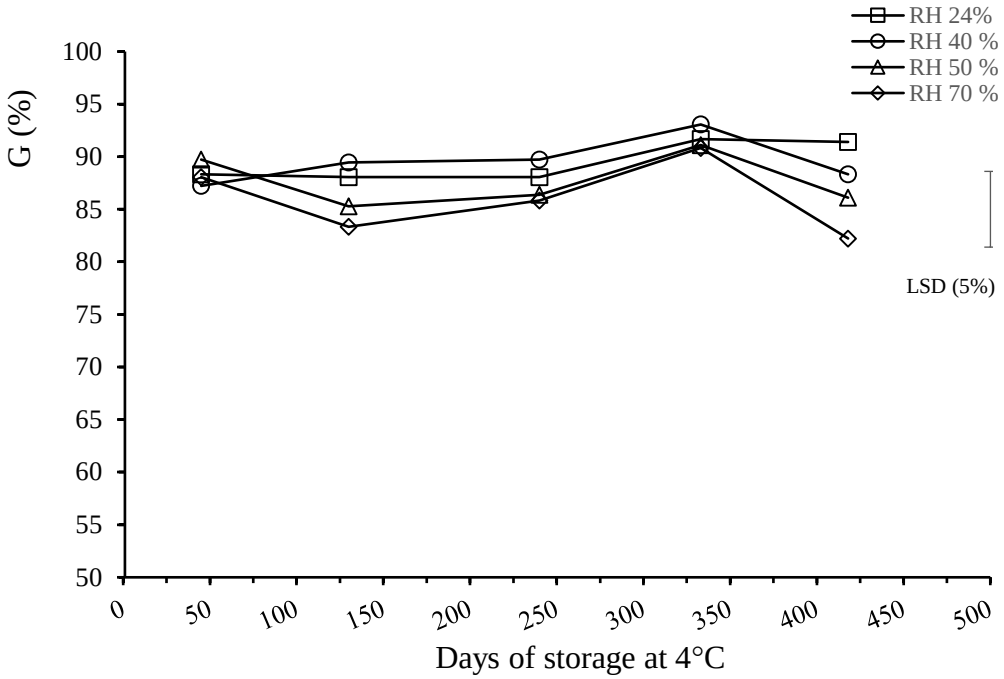

Figure 4-4 Germination (%) of endophyte infected perennial ryegrass seeds stored at 4°C at 4 different RH%. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two data points (at 0.05 level of probability).

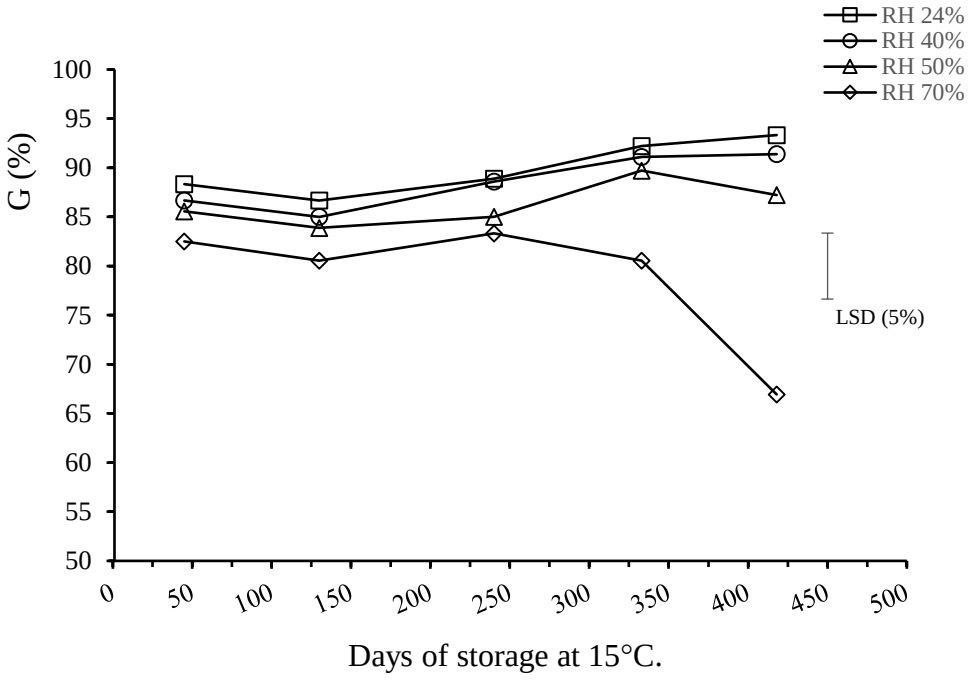

Figure 4-5 Germination (%) of endophyte infected perennial ryegrass seeds stored at 15°C at 4 different RH%. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two data points (at 0.05 level of probability).

A temperature of 20°C can be classed as ambient in Canterbury during some months of the year. Seeds stored well for a period of over 400 days in the 3 lowest relative humidity levels, but not at 70% RH where the seed lots after 230 days had a germination of 80 % and below (Figure 4-6).

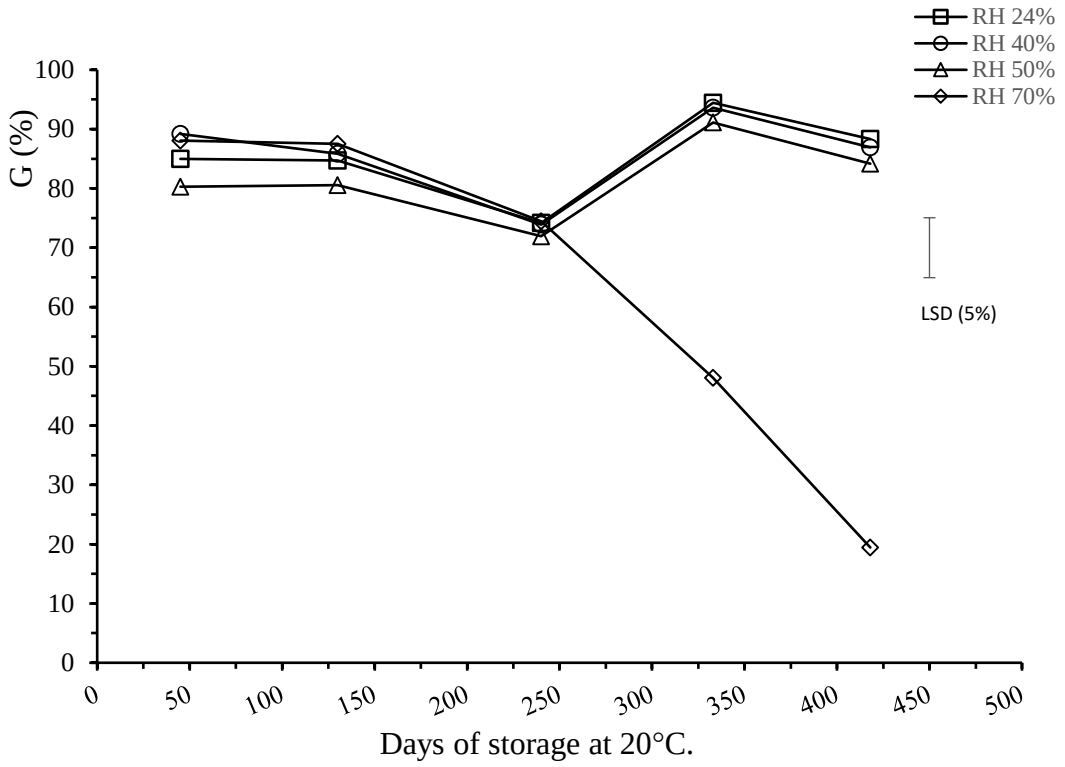

Figure 4-6 Germination (%) of endophyte infected perennial ryegrass seeds stored at 20°C at 4 different RH%. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two data points (at 0.05 level of probability).

Seeds stored at 30°C with relative humidity of 24%, 40% and 50% kept their germination for 130 days, and at 24% and 40% RH they stored well for up to 330 days; however at 50% RH the germination dropped significantly after 150 days. The germination percentage of seeds stored at 30°C and 70% RH dropped significantly after 45 days of storage, (Figure 4-7).

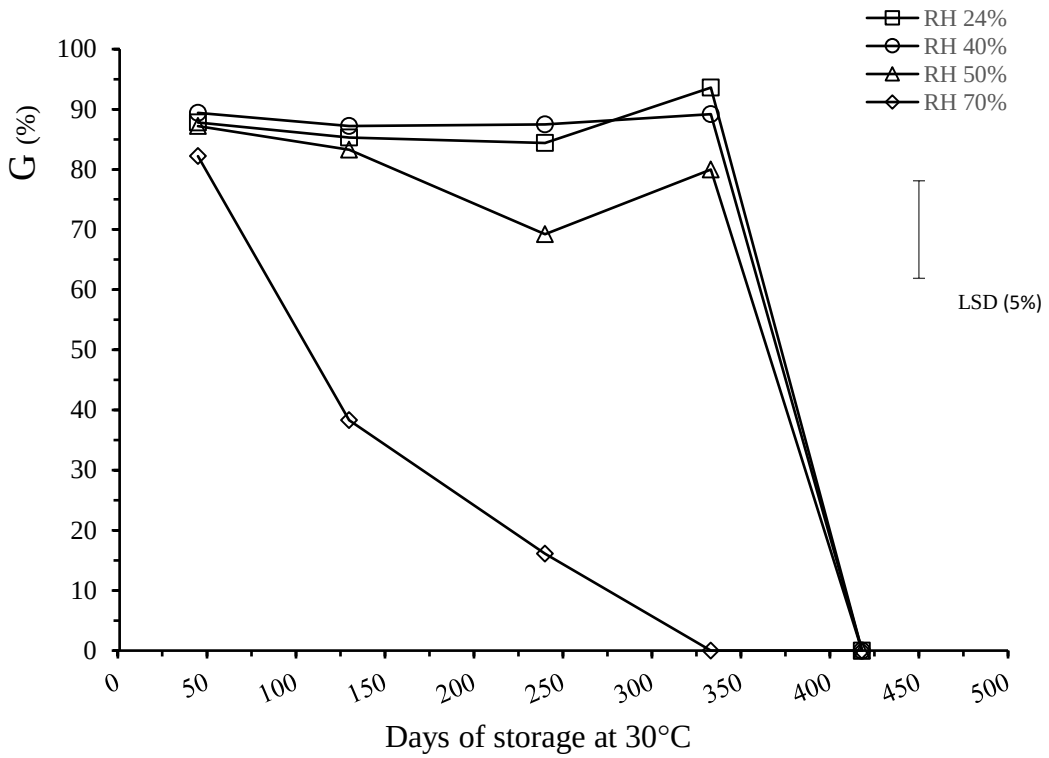

Figure 4-7 Germination (%) of endophyte infected perennial ryegrass seeds stored at 30°C at 4 different RH%. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two non-zero data points (at 0.05 level of probability).

4.6.2 Endophyte viability after seed storage

At 4°C endophyte viability at 24% RH, 40% RH and 50% RH was maintained during the 418 days of storage (nearly 14 months). However for storage at 70% RH, viability began to decline after 130 days, by 333 days had dropped below 70%, and had declined further to 57% by 418 days of storage (Figure 4-8).

When stored at 15°C the number of days the endophyte remained viable varied significantly depending on the relative humidity. At 24% RH and 40% RH endophyte stored well up to 333 days, whereas for the higher relative humidity levels of 50% and 70%, the decline of viable endophyte began after 130 days (Figure 4-9).

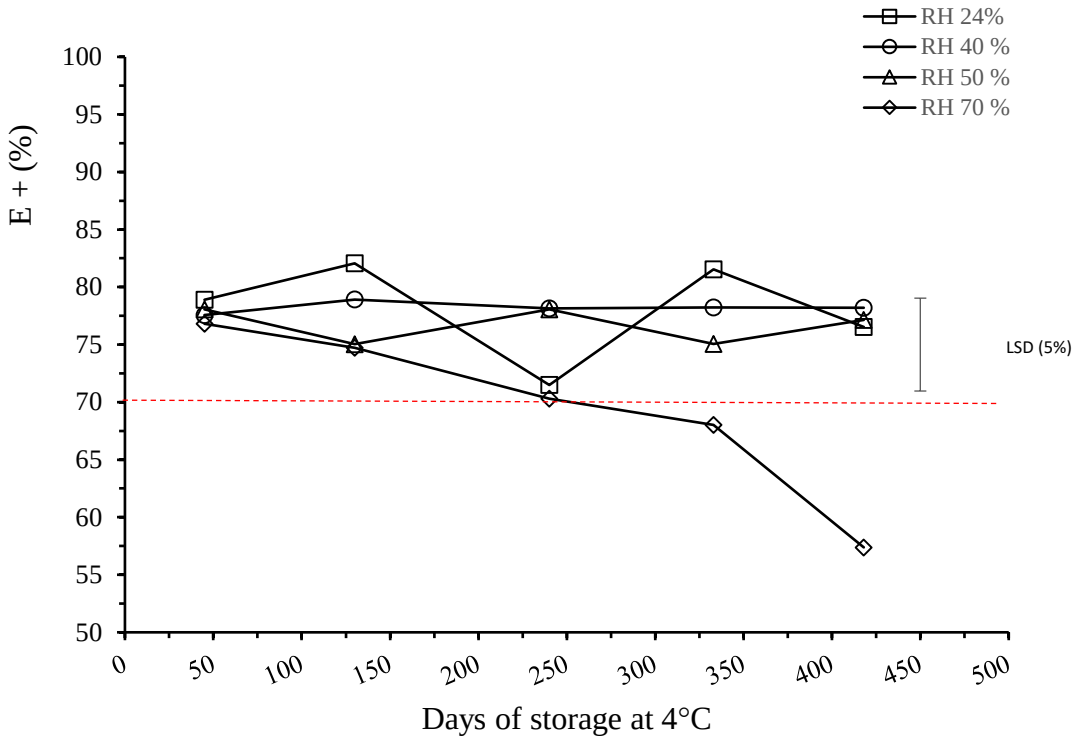

Figure 4-8 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 4°C at 4 different %RH. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

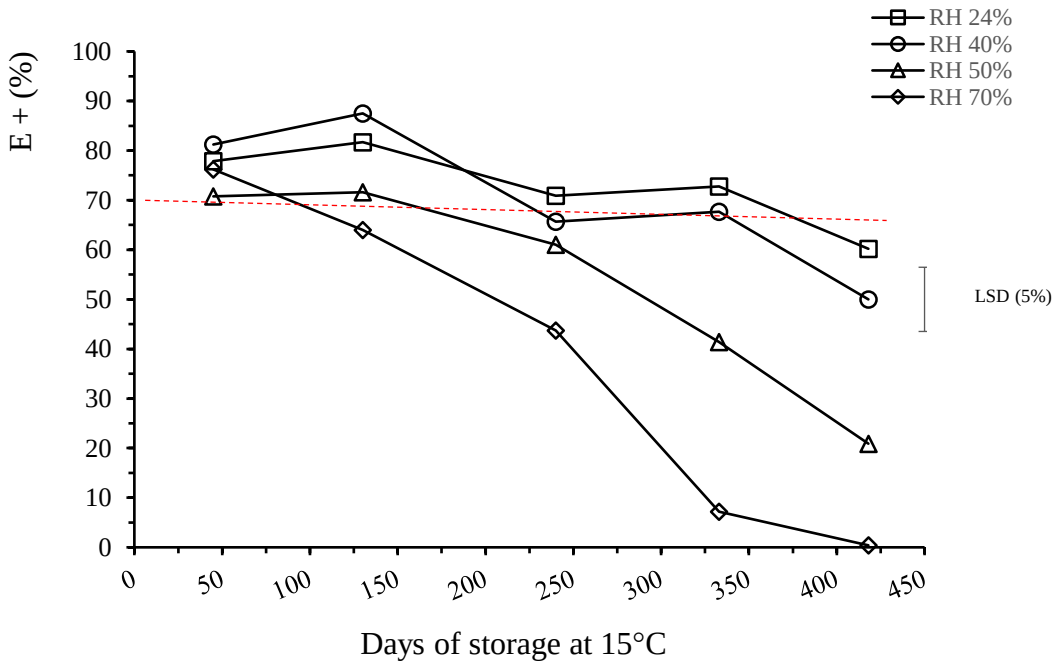

Figure 4-9 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 15°C at 4 different %RH. Each observed data point is the average of six replicates. Vertical bar indicates

LSD for comparing any two non-zero data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

For all % RH at 20°C endophyte viability was maintained for 130 days but declined very rapidly after that (Figure 4-10).

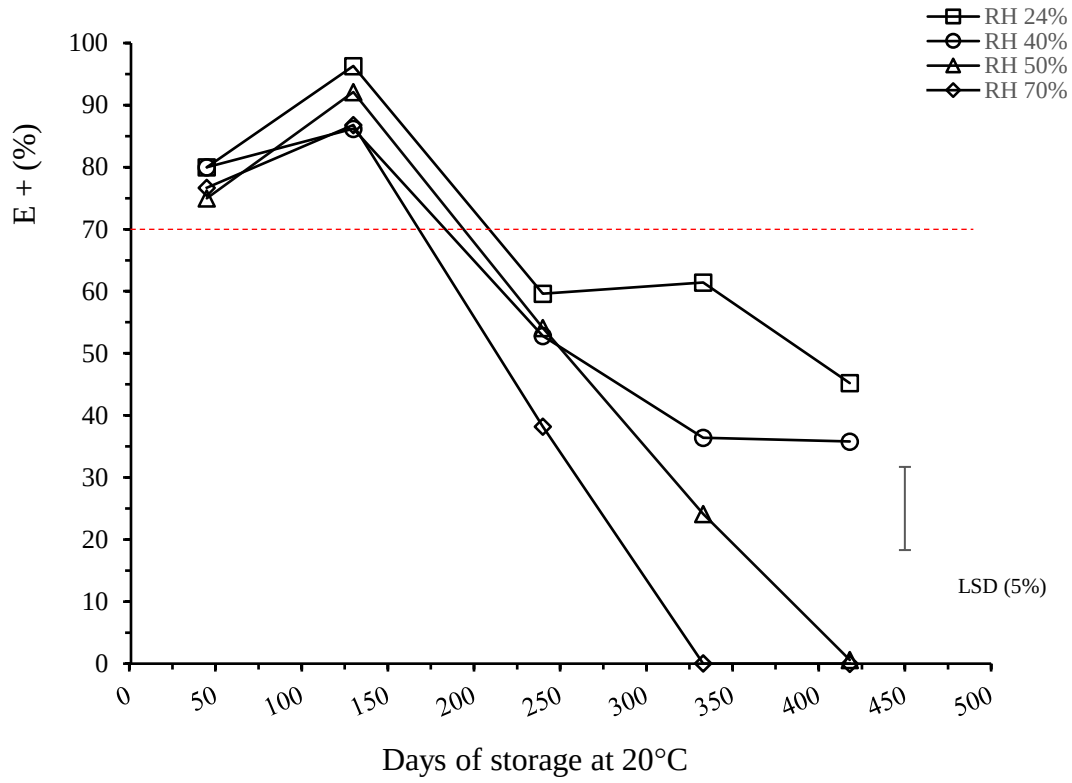

Figure 4-10 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 20°C at 4 different RH%. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two non-zero data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

At 30°C, endophyte viability had fallen to less than 70% after 45 days for the three higher %RH and even at 20% RH had fallen to 70% by 75 days of storage (Figure 4-11).

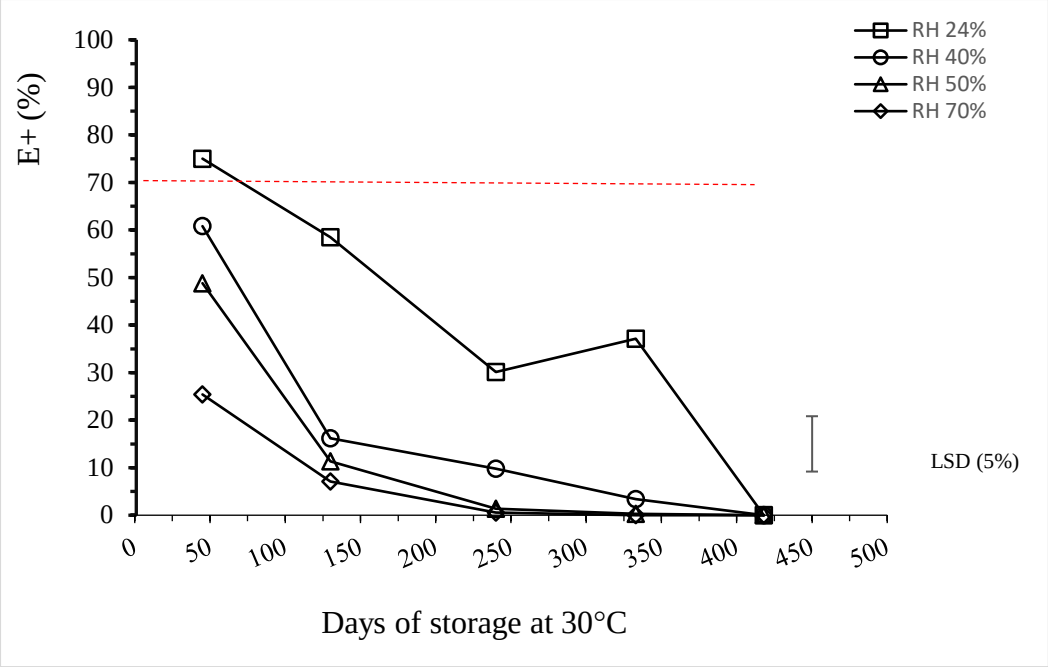

Figure 4-11 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 30°C at 4 different RH%. Each observed data point is the average of six replicates. Vertical bar indicates LSD for comparing any two non-zero data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

When the storage time was converted to accumulated thermal units (ATU), the following responses were recorded. At 4°C endophyte viability remained above 70% for 1800 ATU's at 24%, 40% and 50% RH, but at 70% RH it had dropped below 70% by 1000 ATU (Figure 4-12).

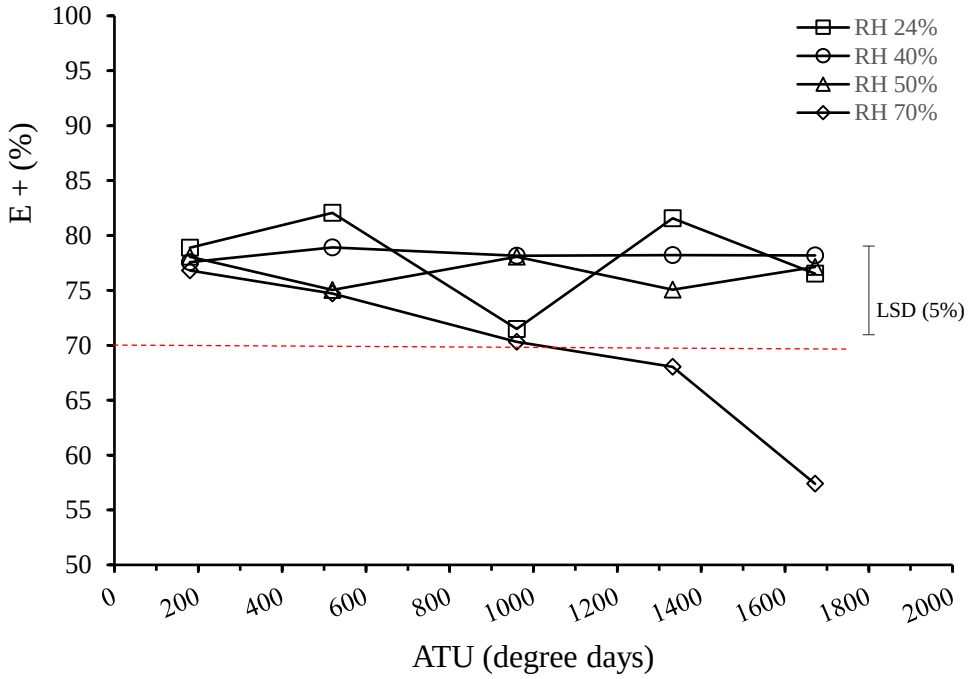

Figure 4-12 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 4°C for 1672 ATU in 4 different relative humidity levels. Each observed data point is the average of six replicates (all tillers blotted). Vertical bar indicates LSD for comparing any two data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

At 15°C, endophyte viability remained above the industry threshold for seeds stored at 24% and 40% RH for up to 4995 ATU, but for seeds stored at 50 % RH, the decline started after 2000 ATUs while for the seeds stored at 70% RH the decline started after 1500 ATUs. The latter lost endophyte viability quite rapidly (Figure 4-13).

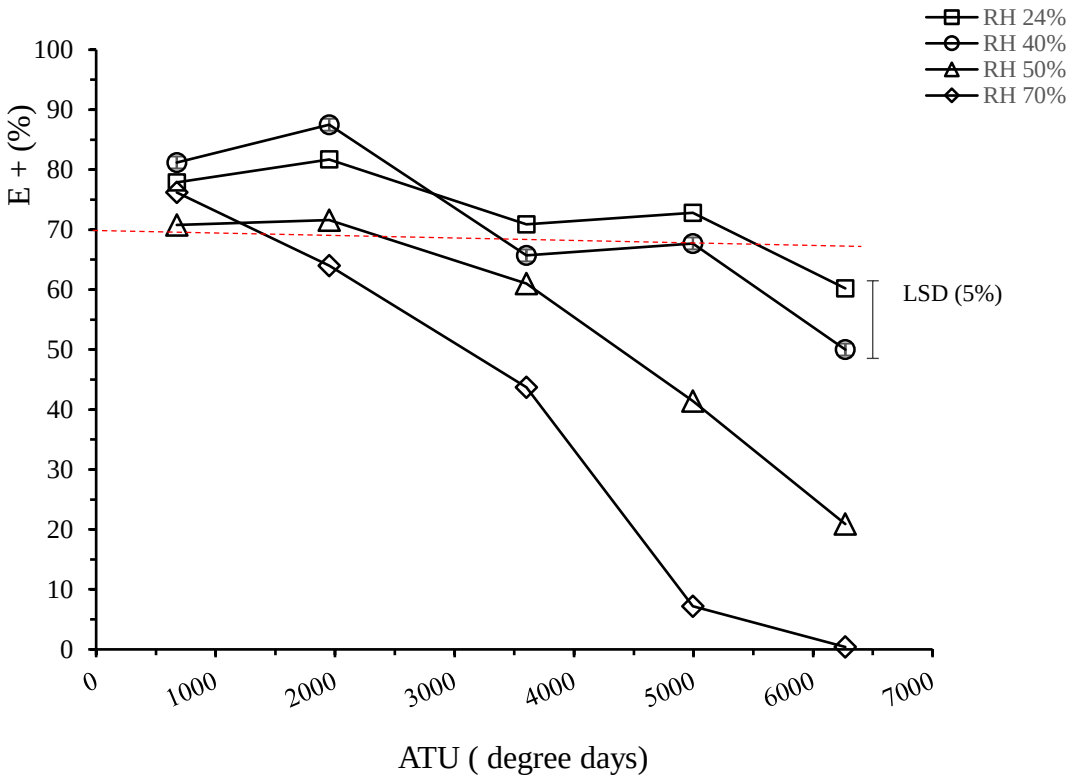

Figure 4-13 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 15°C for 6270 ATU in 4 different relative humidity levels. Each observed data point is the average of six replicates (all tillers blotted). Vertical bar indicates LSD for comparing any two non-zero data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

At 20°C, endophyte viability at all four RH % remained above the industry threshold for 2500 ATU, but had rapidly fallen below the threshold of 70% by between 3500 ATU (70% RH) and 4500 ATU (24% RH) (Figure 4-14).

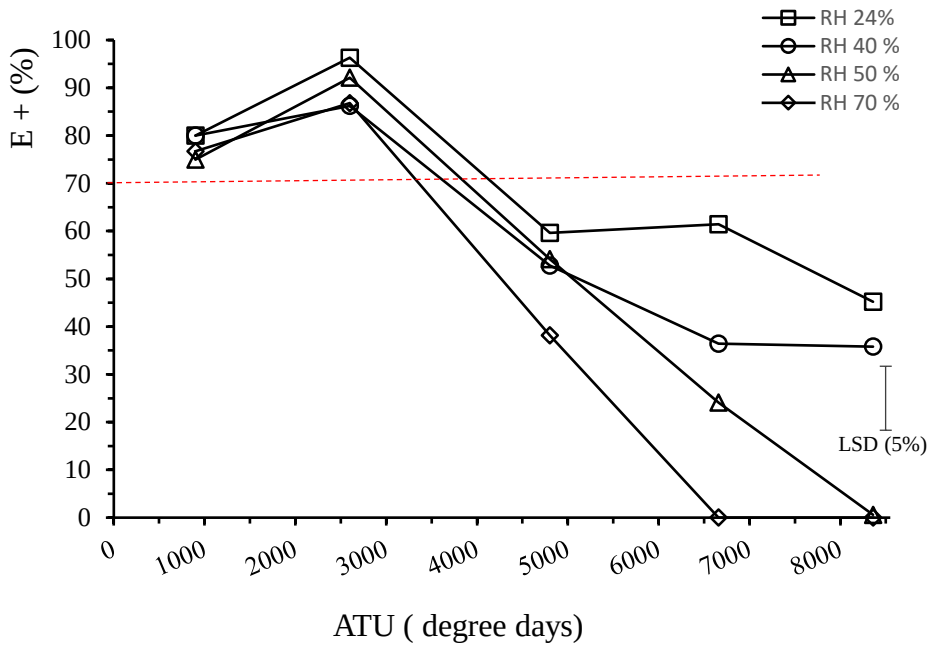

Figure 4-14 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 20°C, 8360 ATU, in 4 different relative humidity levels. Each observed data point is the average of six replicates (all tillers blotted). Vertical bar indicates LSD for comparing any two non-zero data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

At 30°C, by 1500 ATU endophyte viability in seeds stored at 40%, 50% and 70 RH% had fallen below the 70% threshold and for those seeds stored at 24% RH, this point was reached at 2500 ATU (Figure 4-15).

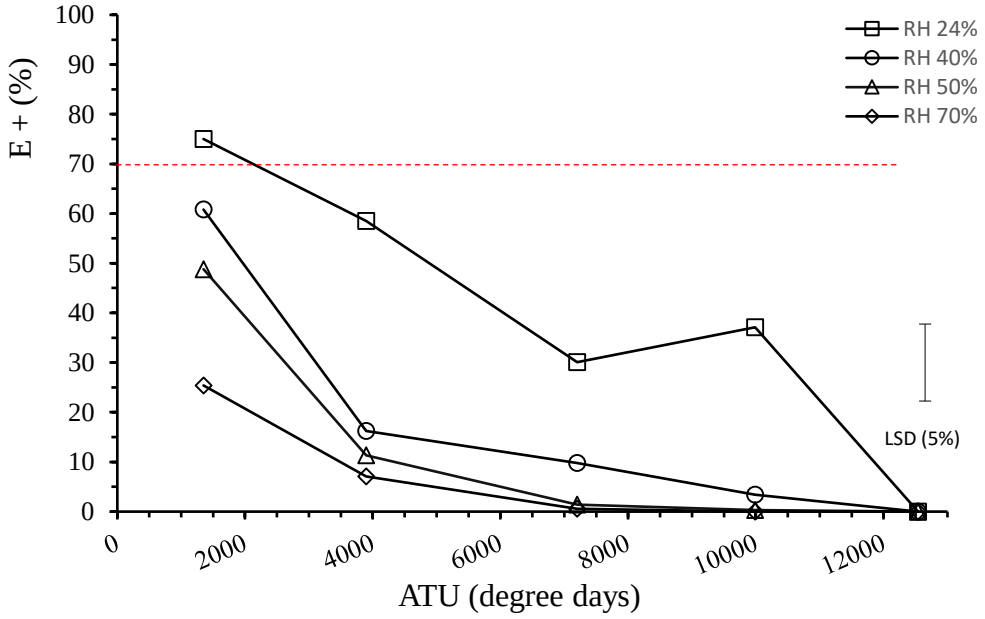

Figure 4-15 Percentage of viable endophyte (E+) present in perennial ryegrass seeds stored at 30°C for 12540 ATUs in 4 different relative humidity levels. Each observed data point is the average of

six replicates. Vertical bar indicates LSD for comparing any two non-zero data points (at 0.05 level of probability). Horizontal dashed red line indicates the 70% NZ industry threshold.

The critical ATUs required to keep endophyte viable in seeds during storage at different temperatures and % RH are summarized in Table 4-4. For 4°C endophyte viability at 24%, 40% and 50% did not fall below the industry threshold during the length of the experiment, therefore the ATUs at that temperature for those three RH are over 1800 (that was the maximum tested).

Table 4-4 ATUs for maintaining endophyte viability $\geq$ 70% under different storage conditions.

ATU to keep viable endophyte >70% at different RH (%) and temperatures (°C)				
	24	**40**	**50**	**70**
4°C	>1800	>1800	>1800	1000
15°C	3600	3600	1950	675-1000
20°C	2600	2600	2600	2600/3500
30°C	1350/2500	<1500	<1500	<1500

The SMC (%) of the seeds stored above the saturated solutions was assessed before starting the experiment (Table 4-2) and after the 418 days of storage (Figure 4-16).

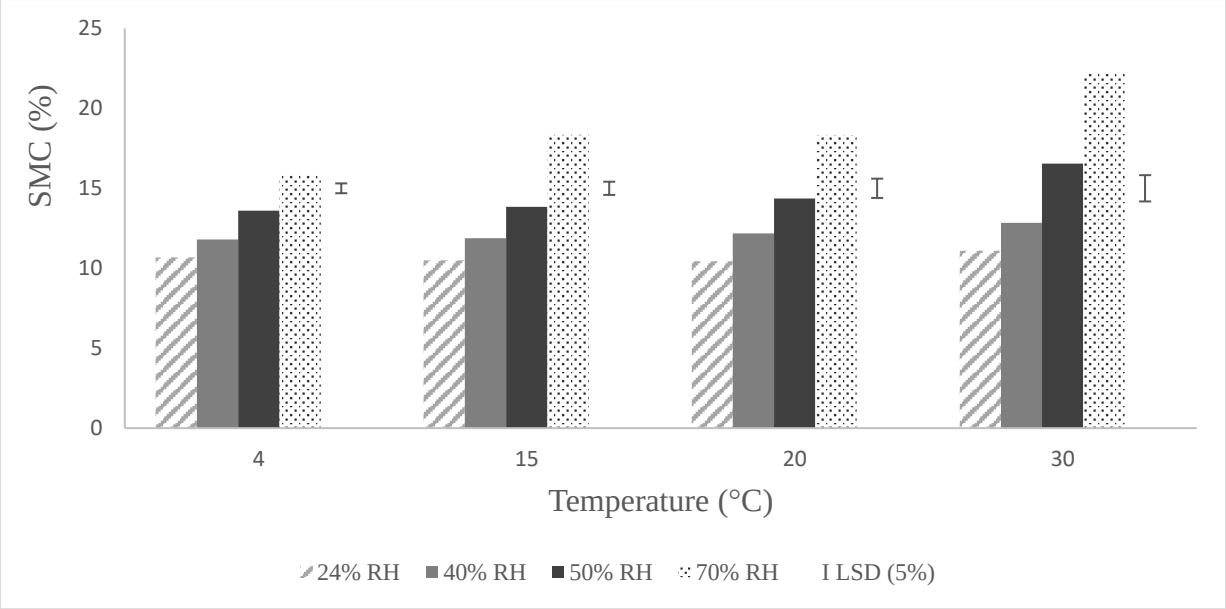

Figure 4-16 Seed moisture content (SMC %) after storage in 4 different temperatures and 4 RH% for 418 days. For each temperature, vertical bar is LSD (5%) for comparing between RH% means for each temperature chamber. LSD values form left to right are 0.617 for 4°C, 0.825 for 15°C, 1.205 for 20°C and 1.642 for 30°C respectively.

Seeds were stored for 418 days at different combinations of temperature and RH. Temperature, moisture content and time of storage interact to influence both endophyte survival and seed germination in cv 'One-50' infected with AR37. Moisture contents of seed resulting in maintaining the highest endophyte transmission and highest level of germination, were ≤ 10%. The creation of environments with high RHs clearly marked the increase of SMC in seeds in time, during the storage period. Eventhough the experiment did not record the SMC of seeds at each extraction point, it is clear that the increase was imminent and that SMCs over 10% started to be unsafe for endophyte survival (Figure 4-16).

4.7 Discussion and conclusions

Endophyte viability is more susceptible than seed viability to adverse storage conditions, particularly higher temperatures and higher RH % as reported by Gundel et al. (2009b). In addition, high SMCs negatively affect the survival of both seed and endophyte, as reported by various researchers (Hume et al., 2013; Rolston et al., 1986; Welty et al., 1987). This has important implications for the storage of seeds carrying endophytes.

Although studies reporting the importance of temperature and relative humidity on the viability of endophyte in seed during storage, and the maintenance of germination of the seed were

reported in the early 1980s (Rolston et al., 1986), there has been no research on storage of perennial ryegrass seed containing *E. festucae* var. *lolii* strain AR37. No work has been conducted to determine the time of safe storage under differing environmental conditions.

In the time between from seed harvest and seed sowing, endophyte viability may be lost during storage. For effective delivery it is important to maximise the survival of the endophyte for subsequent seed production and for pasture use.

This study gave the "shelf life" using the ATU approach. Having a "best before" or more accurately a "due by time" could help the end user to manage and handle the infected seeds before sowing, without losing endophyte viability. Mika and Bumerl (1995), in their research, found that storing seeds for more than 5 years at ambient temperatures at 5% RH did not affect endophyte viability or seed germination greatly. However these conditions are costly and expensive to achieve in commercial situations, and monitoring temperature and RH % is achievable in seed stores and doable in farm situations. Calculating the "due by" in each particular situation would improve the quality of seeds infected with the endophyte strain AR37. This study used constant temperatures that could be a disadvantage in some day to day situations, however degree days can still be calculated and therefore the ATU approach is very valid for predicting viable endophyte levels. ATU could be a simple system which can explain different thermal conditions in which seed is stored.

Germination of endophyte infected perennial ryegrass seeds remained stable during storage at 4°C in all four relative humidity levels tested. For storage at 15°C this stability continued for relative humidity levels of 24%, 40%, and 50% however at RH of 70% the seed lots dropped their germination after approximately a year (350 days) of storage.

For all storage conditions warmer storage temperatures and higher relative humidity levels were less favourable for germination of ryegrass seeds than the cooler and dryer conditions. This is in line with findings from Hume et al. (1999) where seed survival was significantly different and lower in Palmerston North than at Lincoln (NZ) were the temperatures are lower.

Conditions that favoured rapid loss of endophyte viability also favoured rapid loss of germination, while conditions that favoured maintenance of germination did not always maintain the endophyte viability, reinforcing the knowledge that endophyte viability is lost at earlier stages than germination when the seeds are exposed to conditions of high temperature and % RH.

In conclusion, endophyte viability was maintained consistently for 1800 ATU at relative humidity levels of 24%, 40% and 50%. For relative humidity levels of 70% the endophyte maintained its viability up to 1000 ATUs. At 30°C, storage at 24% RH maintained endophyte

viability for 45 days, and it was the only safe combination to store the endophyte at that high temperature (2250 ATUs).

Maintaining the endophyte viable above 70% is possible at three different temperatures (4°C, 15°C and 20°C) and relative humidity levels of both 24% and 40% when the accumulated thermal units are below 2000 (Table 4-4).

In non-moisture proof storage conditions, endophyte viability was maintained for longer with a combination of low temperature and low RH%. Higher relative humidity levels increased SMC, and as a result negatively affected endophyte viability.

For all storage conditions, endophyte viability was always lost before germination.

CHAPTER 5

Influence of respiration and levels of CO_2 on the survival of *E. lolii* strain AR37 in a cultivar of perennial ryegrass during storage.

5.1 Introduction

Plants utilise the sun's energy to make sugars, and when plants need energy, they have to metabolize their stored sugars through cellular respiration. Plants need energy to maintain homeostasis, and to perform basic functions like growth, cellular repair and nutrient uptake. After seeds mature, seeds of some species become dormant; seeds metabolize stored energy reserves very slowly during this period. Some species need very specific conditions to break dormancy. Some may require smoke, high heat, deterioration of the seed coat, elevated soil nutrients, or change in the quality of light, (Bicca Noguez Martins et al., 2016; Velijević et al., 2018). After emerging from dormancy a seed is able to germinate and will respond to more familiar growth stimulating factors such as temperature, light and moisture (Patanè et al., 2006). While seeds are not able to germinate, they cannot produce their own sugars through photosynthesis so to stay alive they need to use stored energy reserves and undergo cellular respiration**.**

Seed and endophyte longevity is largely determined by the temperature and the relative humidity during storage. The higher the temperature and the higher the relative humidity of storage of endophyte-infected seeds, the faster the deterioration and loss of viable endophytes. SMC also play an important role in seed deterioration; when SMC is higher than 10%, it negatively affects the endophyte viability in seeds (reviewed in Chapter 4).

One of the factors that can cause deterioration of seeds during storage is the exposure to oxygen (O_2) even at ambient levels. Oxygen reduces seed longevity due to oxidation of cells and membranes. It would be expected, that to maintain the longevity of the endophytes in seeds, anoxic conditions during storage would be a beneficial factor, as studies on lettuce (*Lactuca sativa*) and onion (*Allium cepa*), showed that seeds stored in anaerobic conditions were beneficial to seed longevity (Schwember & Bradford, 2011).

As the seeds respire, they take in the same amount of O_2 as the amount of CO_2 released, as per the equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \text{Equation 1}$$

Seeds in a sealed test tube produce CO_2 and consume O_2 at the same rate, while air pressure remains constant. Measuring the amount of CO_2 produced indirectly measures the amount of O_2 consumed, therefore the rate and total amount of respiration that has taken place.

This study was conducted to investigate the effects of CO_2 concentrations, air temperature and the time of storage on the survival of an *Epichloë* endophyte in perennial ryegrass seeds. To test if a reduction in oxygen levels would increase endophyte survival during storage and to define the effect of increasing CO_2 concentrations, two treatments were used.

In the first experiment, the CO_2 generated by the respiration of the seeds in airtight containers, exposed to three different temperature regimes was monitored.

In the second experiment, seeds were stored in an airtight container which had high levels of CO_2, created by depriving the O_2, using ascorbic acid, AnaeroGen™ (ai: ascorbic acid -AA).

For both experiments, seeds with and seeds free of endophyte from the same cultivar were used, to compare the effect of these abiotic factors on seeds with and without endophytes, and to differentiate the effect of both treatments on the endophyte and on the seeds.

5.2 Experiment I- Effect of respiration on endophyte survival in seed during storage

The aim of this experiment was to investigate:

(i) the effect of cumulative respiration of seeds on the viability of the novel endophyte *Epichloë festucae* var. *lolii* strain AR37.

(ii) the effect of temperature on the seed respiration rate, in a perennial ryegrass cultivar during a month of storage in an airtight container, and

(iii) the effect of storage time on the survival of the endophyte in seed.

The hypotheses were:

(i) high CO_2 concentrations produced by the cumulative respiration of seeds will have a negative host-endophyte effect,

(ii) high CO_2 concentrations in the storage container will decrease the survival of *Epichloë festucae* var. *lolii* strain AR37 in perennial ryegrass seed,

(iii) seeds with viable endophytes have a higher respiration rate than seeds without endophytes,

(iv) the increase of temperature during storage increases seed/endophyte respiration rate and

(v) longer periods of storage will decrease the viability of endophytes in the seeds.

5.3 Experimental design I

The experiment was set up using a split-plot design, with two endophyte treatments (a sub-plot treatment factor), contained in two blocks (temperature chambers located in two buildings at Lincoln University, Burns and Riddolls), with three temperatures (5°C, 20°C and 30°C) (main-plot treatments) in each block. The sampling was carried out after 6 periods of storage (a sub-plot treatment factor).

The experimental design included three replicates of 100 machine dressed (MD) perennial ryegrass seeds each, with endophyte (E+) and three replicates of 100 seeds without endophyte (E-), for each of the six storage times for each temperature chamber used (5°C, 20°C and 30°C) in each block. Temperature control rooms were used for 4 treatments (3 in Riddolls and 1 in Burns building), a test chamber and an incubator were used for the other two temperature treatments, including a 20°C chamber (Panasonic Versatile Environmental Test Chamber MLR 352H, Panasonic Healthcare Co. Ltd.1-1-1 Sakata, Oizumi-Machi, Ora-Gun, Gunma 370 - 0596, Japan) and a 30°C incubator (Contherm Biocell 1000, Contherm Scientific Ltd, 27 Cornish St, Petone, PO Box 30-605, New Zealand). Samples were tested after 1, 2, 4, 8, 16 and 32 days of storage. Thirty-six glass vials with seeds were placed in randomised positions in each temperature chamber, along with 6 empty vials with no seeds and air only (control CO_2). The entire experiment consisted of 252 vials, made up of 216 vials with 100 perennial ryegrass seeds in each (2 blocks x 3 temperatures x 3 replicates x 2 seed lots x 6 storage days) and 36 empty vials.

5.4 Materials

5.4.1 Plant material

One cultivar of perennial ryegrass Grasslands® Samson (*Lolium perenne* L. cult. *Samson*) containing *Epichloë festucae* var. *lolii* strain AR37, line SMT 301AE and the same perennial ryegrass without the endophyte, line SML710AA, (classed as low endophyte LE) were used in

both experiments. Seeds were sourced from PGG Wrightson Seeds Ltd, Kimihia Research Centre, Lincoln, New Zealand.

5.4.1.1 *Cultivar: 'Grasslands® Samson'*

Lolium perenne L. cult. *Samson*, ('Samson'), was bred by AgResearch Grasslands from a selection of plants collected in Northland, Taranaki, Hawkes Bay, Waikato, Manawatu, Canterbury and Southland. Samson is a medium tillered perennial ryegrass with a rapid and vigorous establishment, classed as a high yielding cultivar, (Agricom 2018). It has been used successfully, mainly on dairy, sheep and beef farms where farmers want a rust tolerant, reliable pasture production.

5.4.2 Storage containers

All incubations were carried out in 12.03 ml glass vials with silicone septums (Double wadded glass vacutainers, Labco Limited, Unit 3, Pont Steffan Business Park, Lampeter, Ceredigion, SA48 7HH United Kingdom). The septum in the lid of the vials allowed taking gas samples (using a fine hypodermic syringe) from them and prevent air leakage during the experiment and during sampling. The volume of the interstitial air and headspace in the vial was determined by water displacement method (Weinberg et al., 2008).

5.5 Methods

Following the receipt of the two seed lots, germination, viable endophyte frequency and seed moisture content were assessed. Both seed lots (E+ and E-) were tested for endophyte presence using TPIB and germination using the TP method (both testing methods were described in Chapter 2, section 2.4.1and section 2.4.1.1 respectively).The SMC was tested as previously described (Chapter 2 section 2.7).

5.6 Treatments

On 16 January 2018, 108 sub-samples of 100 seeds each, were randomly drawn from each seed lot and the weight recorded to 0.01g, using a balance (Ohaus, Adventurer, Parisppany, NJ 07054,

USA). The 100 MD seeds were placed in sterilised glass vials (Figure 5-1) and then hermetically stored. The seeds were incubated at three different temperatures, 5°C, 20°C and 30°C.

Figure 5-1 Each airtight 12.03 mL glass vial contained 100 pure seeds of perennial ryegrass cv 'Samson', with or without the endophyte, and was incubated at a set temperature for a specific amount of time.

At each time interval, accumulated CO_2 concentration inside the vials was measured (see section 5.2.4). The sampling dates were 18, 19, 21, and 25 of January and 2 and 18 February 2018.
For the calibration of the analyser, a Tedlar gas transfer bag was filled with gas taken from a reference cylinder of a known CO_2 concentration.

5.6.1 CO_2 concentration measurements

On each sampling date, a 1.0 mL hypodermic syringe with a fine hypodermic needle (22 gauge) (Terumo® Syringe, Terumo Corporation, Tokyo, Japan) was used to remove 0.5 mL of gas from each of the vials.

The CO_2 concentration was measured using the LI-7000 CO_2 / H_20 analyser (LI-COR, Nebraska, USA), (Figure 5-2).

Figure 5-2 CO_2 gas concentrations were measured on a LI- 7000 CO_2 / H_2O analyser.

The analyser was calibrated each time using five replicates of 0.5 ml of gas from a reference cylinder of air of a known CO_2 concentration. This was injected into a stream of CO_2-free air flowing at 0.5 l min^{-1}. The air entered the tubing after the reference cell and before the analyser cell. As the CO_2 concentration went above 10 ppm the area under the curve was integrated until it dropped back below 10 ppm (Figure 5-3).

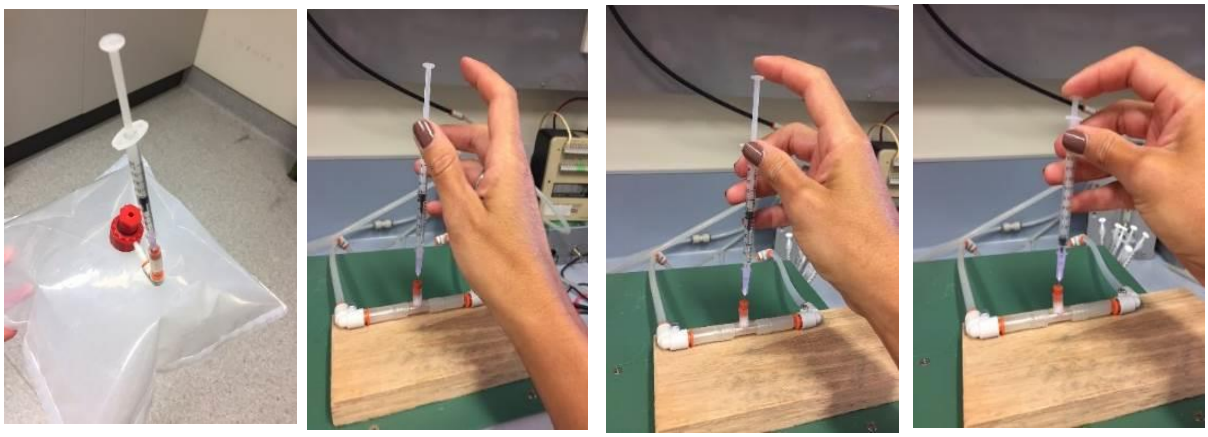

Figure 5-3 Extraction of 0.5ml of reference CO_2 sample from a Tedlar gas transfer bag to calibrate the integrated area under the curve after injection into the analyser.

To assess seed viability and therefore endophyte survival, 25 seeds were randomly selected from each vial at each sampling time and sown into black plastic propagation trays. The remaining seeds were discarded. The trays were divided into 4 quadrants and 4 samples were randomly sown in each tray. Trays contained the seedling mix as previously described in Chapter 2 (sections 2.4.1 and 2.4.2).

Trays were placed in the propagation glasshouse at 20 ± 2°C, and kept watered as required. The percentage emergence after 21 days was assessed (ISTA, 2013). After 8 weeks, normal emerged seedlings were tested for viable endophyte presence using the TPIB procedure (described in Chapter 2 section 2.4.1.1). Viable endophyte (E+ %) was recorded as a percentage of the seedlings emerged.

5.6.1.1 *Calculations*

To determine the absolute amount of CO_2 produced by the stored seeds in each vial, the concentration of gas in the empty control vials was subtracted to calculate the respiration rate.

The volume of air in the vial containing the seeds (that is less than the vials with no seeds) is required. For its determination, the gas displaced by seeds was calculated using a water displacement method (100 seeds of ryegrass occupy about 0.53 ml). Calculations are presented in Appendix VI.

5.7 Statistical analysis

Each variable was statistically analysed using an analysis of variance (ANOVA) for a split-plot design, with the main-plots being temperature chambers arranged in two randomised blocks and the sub-plots being two seed lots (E+ and E-) sampled after 6 storage times. The factorial treatment structure contained three temperature regimes x two seed lots (E+ and E-) x six storage times, with polynomial contrasts specified for the temperature and storage time factors. The ANOVAs were carried out using GenStat® (VSN International 2013. GenStat® for *Windows* 19th Edition. VSN International Ltd., Hemel Hempstead, UK). Comparisons of treatments used

the unprotected least significant difference (LSD; $P < 0.05$) procedure (Saville, 2015; Saville & Rowarth, 2008).

5.8 Results

The germination percentage, viable endophyte frequency and SMC when the seed lots were received are presented in Table 5-1.

Table 5-1 Initial seed quality parameters for perennial ryegrass seed lots, cv Samson, used in the experiment

Seed lot	Germination (%)	E+ (%)	SMC (%)
SMT 301AE	96.0	86.0	9.8
SML710AA	97.0	0.0	9.9

5.8.1 Viable endophyte survival after storage

There were significant differences in endophyte survival between the infected seeds stored at the different temperatures (5°C, 20°C and 30°C) for all six storage times tested. The grand mean of the survival of endophyte after storage was 76.8 % denoting differences between the viability before (time zero) and after the experiment (32 d) (Table 5-2).

Endophyte viability had dropped significantly compared with the viability when the experiment was set up, however, the survival of the AR37 strain was maintained better at 5°C that at 20°C or 30°C, and viability fell by around 0.33% per day over the 32 days of storage (Figure 5-4).

Table 5-2 Main effect, means for the effect of temperature and time of storage with high CO_2 concentrations on the survival of endophyte AR37 strain in the perennial ryegrass cultivar 'Samson' after 32 d of storage.

Temperature (°C)	**E+ (%)**
5	**83.4**
20	**72.3**
30	**74.8**
LSD (5%)	**3.0**
Sig. of linear trend	****
Storage time (d)	
1	**80.9**
2	**79.4**
4	**78.6**
8	**75.7**
16	**76.2**
32	**70.3**
LSD (5%)	**1.6**
Sig. of linear trend	*****

*Significant at p< 0.05

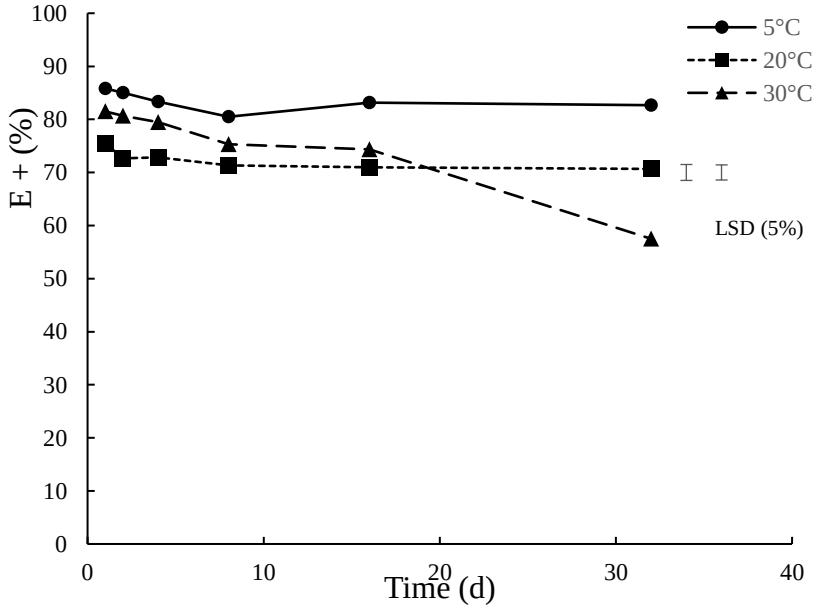

Figure 5-4 Viable endophyte percentage of seeds stored in accumulative CO_2 concentrations due to seed respiration at 3 temperatures 5°C, 20°C and 30°C. Right vertical bar indicates LSD (2.85) for

comparing any two data points for the same temperature and left vertical bar indicates LSD (3.02) for all other comparisons at 0.05 level of probability.

There were four significant interaction contrasts. These were: temperature (linear) x storage time (linear) 0.1 % significant; temperature (quadratic) x storage time (linear) 0.1 % significant, temperature (linear) x storage time (quadratic) 1% significant and temperature (quadratic) x storage time (quadratic) 5% significant.

Figure 5-5 shows the blotting performed after the first extraction of 25 seeds at day 1 and on the second day of storage in the incubator at a temperature of 30°C.

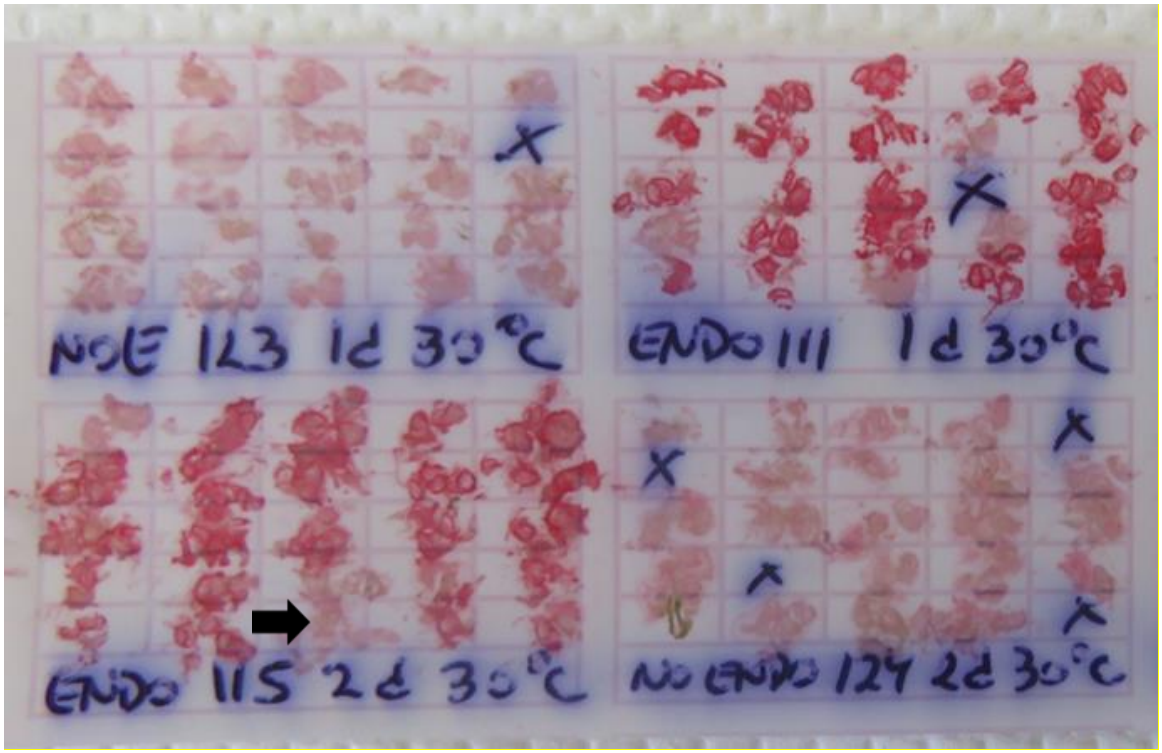

Figure 5-5 Ryegrass tillers without endophyte (E-, top left), and tillers with the endophyte (E+, top right) after 1 day of storage at 30°C. Tillers with viable endophyte (bottom left) and tillers with no endophyte (right) after 2 d of airtight storage at 30°C. The black arrow indicates tillers with no viable endophyte. The cross (X) indicates dead seeds.

5.8.2 Respiration rate of seeds during storage and the effect on the viable endophyte

There was also a significant difference among the respiration rates of the seeds stored at different temperatures (Figure 5-6) and also differences between E+ and E- seeds (Figure 5-7).

Respiration rate did not differ for seeds stored at 20°C or 30°C, but was significantly lower for seeds stored at 5°C (Figure 5-6).

Respiration rate declined with increasing time of storage at 5°C and 20°C but not at 30°C (Figure 5-6). At each assessment, respiration for E- was significantly lower than for E+ seeds at all three temperatures (Figure 5-7).

The grand mean of respiration of the 100 seeds was 1.83 Log CO_2 mg $g^{-1}h^{-1}$.

Table 5-3 Main effect, means for the effect of temperature and time of storage on the respiration rate of perennial ryegrass seeds cultivar 'Samson' after 32 d of storage.

Temperature (°C)	**Log CO_2 (**mg $g^{-1}h^{-1}$)
5	**1.138**
20	**2.129**
30	**2.240**
LSD (5%)	**0.054**
Sig. of linear trend	*******
Storage time (d)	
1	**2.138**
2	**2.012**
4	**2.005**
8	**1.775**
16	**1.604**
32	**1.481**
LSD (5%)	**0.019**
Sig. of linear trend.	*******
Endophyte strain	
E-	**1.748**
E+ (AR37)	**1.923**
LSD (5%)	**0.011**
Sig. of diff.	*******

*Significant at $p < 0.05$

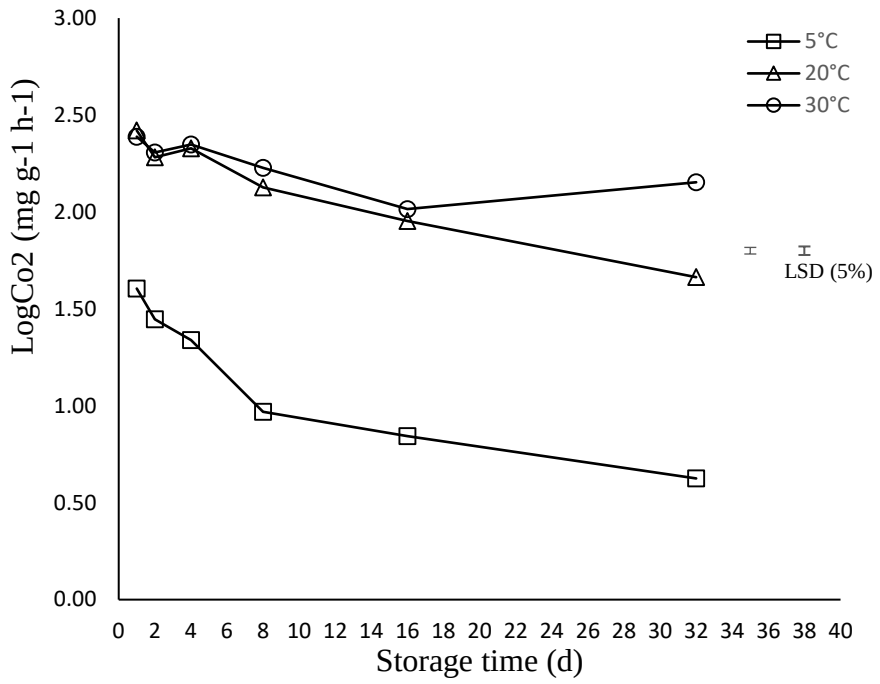

Figure 5-6 Respiration rate of 100 seeds stored at 3 different temperatures for a period of 32 days in airtight containers. Left vertical bar indicates LSD (0.034) for comparing any two data points for the same temperature, right vertical bar indicates LSD (0.044) for all other comparisons at 0.05 level of probability.

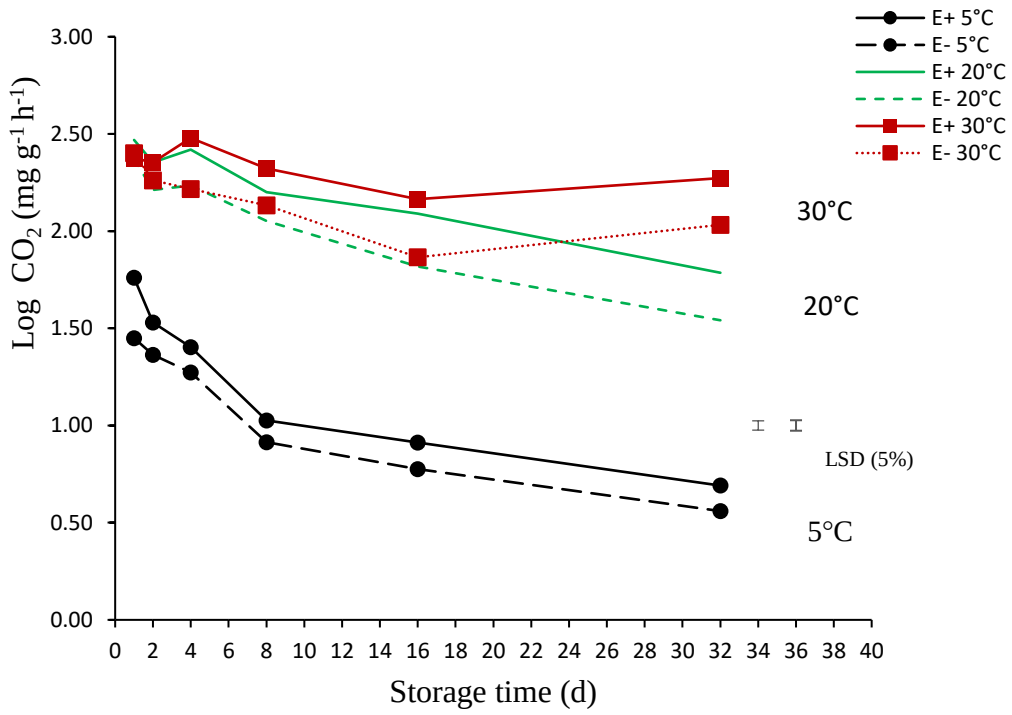

Figure 5-7 Respiration rate of perennial ryegrass seeds, with (E+) and without endophyte (E-), when stored at 3 different temperatures for 32 days in airtight containers. Left vertical bar indicates LSD

(0.048) for comparing means with the same level of temperature and right LSD bar (0.054) for all other mean comparisons at 0.05 level of probability.

5.8.3 Germination percentage after seed storage

Germination results were variable (Figure 5-9), presumably because of the small number of seeds tested at each sampling time. However, there were no significant differences between the germination percentage of the E+ and the E- seeds at any assessment time (Table 5-4) and (Figure 5-10). The grand mean for germination was 94.6%.

Table 5-4 Main effect means for the effect of temperature and time of storage on the germination percentage of perennial ryegrass seeds cultivar 'Samson' E+ and E- after 32 d of storage.

Temperature (°C)	Germination (%)
5	95.7
20	95.7
30	92.5
LSD (5%)	3.46
Sig. of linear trend	ns
Storage time (d)	
1	96.3
2	94.7
4	94.7
8	93.2
16	94.8
32	94.2
LSD (5%)	1.49
Sig. of linear trend.	*
Endophyte strain	
E-	94.5
E+ (AR37)	94.8
LSD (5%)	0.86
Sig. of diff.	ns

*Significant at $p < 0.05$

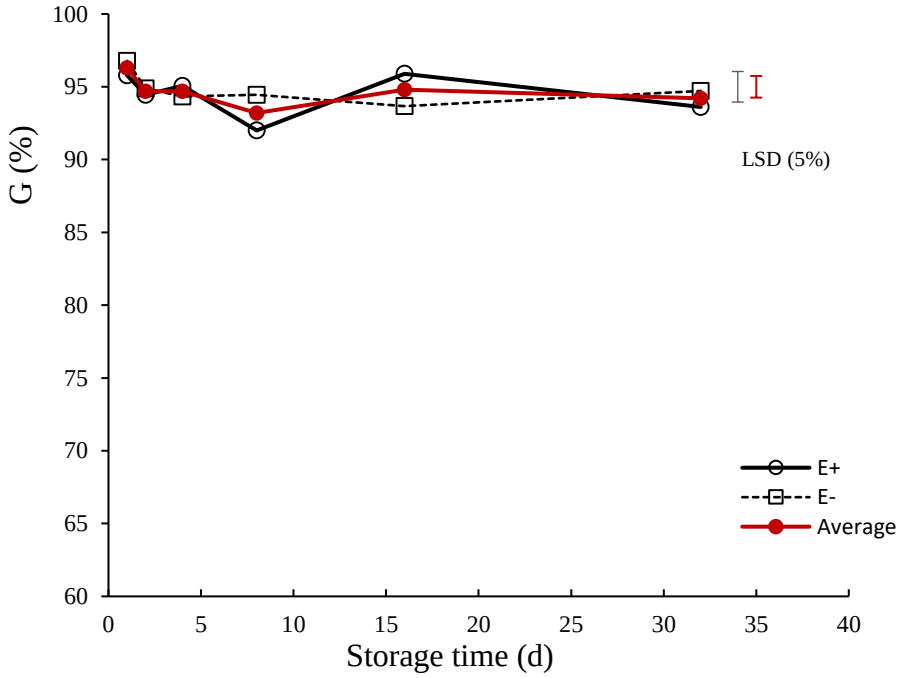

Figure 5-8 Average germination percentage of 25 seeds of each seed lot after storage in high CO_2 concentration in an airtight container. Left vertical bar indicates LSD (2.10) for comparisons between E+ and E-, right red vertical bar indicates LSD (1.49) for comparison within the average germination, at 0.05 level of probability.

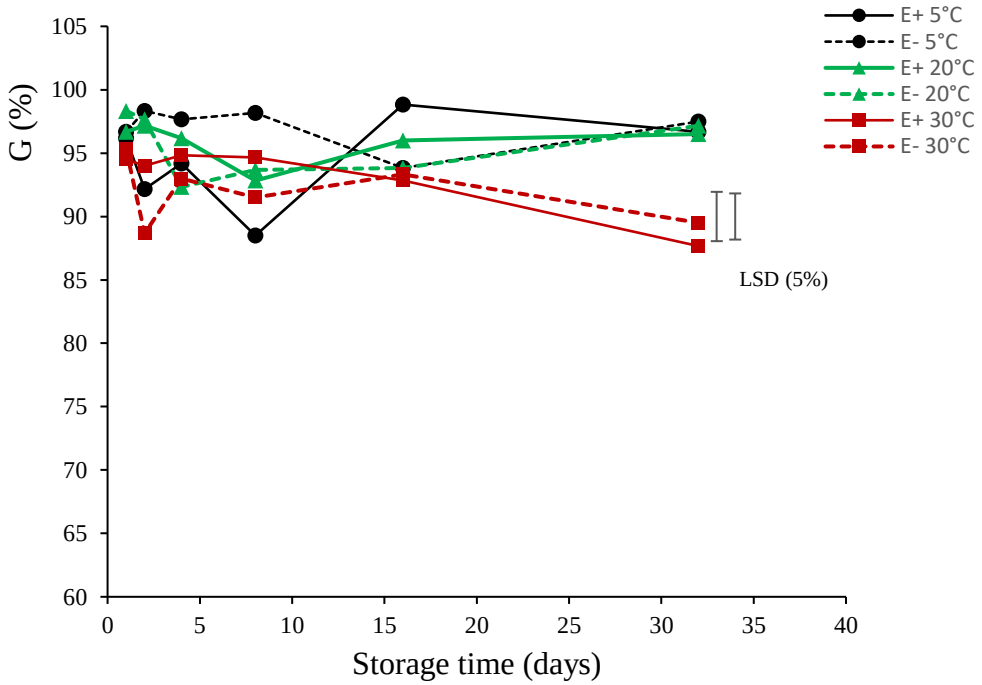

Figure 5-9 Germination percentage of 25 seeds of each seed lot after stored in high CO_2 concentration in an airtight container. Right vertical bar indicates LSD (3.64) for comparisons of the same level of

temperature and left vertical bar indicates LSD (3.89) for all other comparisons at 0.05 level of probability.

The results supported the hypothesis that high CO_2 concentrations produced by the cumulative respiration of seeds will have a negative host-endophyte effect, secondly that the high CO_2 concentrations will have a detrimental effect on the survival of the endophyte in the seed and thirdly, that E+ seeds would have a higher respiration rate than E- seeds.

5.9 Experiment II: Effect of high CO_2 concentration and oxygen (O_2) deprivation on endophyte survival in seeds during storage.

The concentration of gases (CO_2 and O_2) during storage may affect the endophyte and the host seed in different ways. Sealed glass vials containing ascorbic acid were used to store endophyte-infected perennial ryegrass seeds. Ascorbic acid in dust form (AnaeroGen™, Thermo Fisher Scientific, Oxoid Ltd, Basingstoke, United Kingdom) reacted with the oxygen in the vial, creating an O_2 deprived ambient (anoxic) atmosphere with the production of high levels of CO_2.

This experiment investigated the survival of the *Epichloë* endophyte strain AR37 during storage for short periods under anoxic conditions induced using AnaeroGen™.

The aim of this experiment was to investigate:

i. the effect of cumulative CO2 concentrations created by the use of AnaeroGen™ on the viability of the novel endophyte *Epichloë festucae* var. *lolii* strain AR37
ii. the effect of storage time on the survival of the endophyte in the seed.
iii. the effect of anoxia on endophyte survival and seed longevity.

The hypotheses were:

(i) high CO_2 concentrations (refers to levels greater than 400 ppm) during a short period of storage at 20°C, will reduce the endophyte survival in perennial ryegrass seed.
(ii) the time of storage will decrease the viability of the endophyte
(iii) the anoxic conditions created by the ascorbic acid, will not affect germination % and therefore seed survival.
(iv) seeds infected with the *Epichloë* endophyte strain AR37 will have a different germination % than endophyte free seeds.

5.10 Experimental design II

The experiment was set up using a completely randomised block design in a 20°C chamber (Panasonic Versatile Environmental Test Chamber MLR 352H, Panasonic Healthcare Co., Ltd.1-1-1 Sakata, Oizumi-Machi, Ora-Gun, Gunma 370-0596, Japan). Three replicate sealed vials with AnaeroGen™ for each of two endophyte seed lots (E+ and E-) of perennial ryegrass cv 'Samson' were sampled after each of 5 storage times (2, 4, 8, 16 and 32 days). The treatments also included two unsealed control vials (one for E+ and one for E-) for each storage time. Each vial contained 100 MD seeds. At each sampling time, 25 seeds were randomly removed from each designated vial for assessment. In total the experiment had 40 vials, made up of 30 sealed vials with AnaeroGen™ and 10 unsealed vials [two seed lots (E+ and E-) x five storage times x (three sealed replicates + one unsealed control)].

5.11 Materials and methods

5.11.1 Plant material and storage containers

Perennial ryegrass seeds cv 'Samson' as described previously (with and without, the *Epichloë* endophyte strain AR37) were used. Airtight glass vials with septa with a volume of 16.5 ml, were used as containers. AnaeroGen™ was added to create high levels of CO_2 and anoxic conditions.

5.12 Treatments

Each of the 40 vials had 0.5 g of AnaeroGen™, a 4.5 g ball of cotton wool and 100 perennial ryegrass seeds (with an average weight of 0.289 g), previously weighed, on top of the cotton wool (Figure 5-4). Half a gram of AnaeroGen™ was weighed and placed in each of the glass vials. The ascorbic acid had activated carbon in it, which reacted on contact with air. The atmospheric oxygen in the vial was rapidly absorbed with the simultaneous generation of carbon dioxide by an exothermic reaction (Beerens, 1998; Brazier & Hall, 1994). There was no need to add water, which helped to maintain the SMC of the stored seeds. AnaeroGen™ will reduce the oxygen level in the vial to below 0.1% within 2.5 hours. The resulting carbon dioxide level should rise to between 7% and 15% within 1 d.

Figure 5-10 Single glass vial, containing the three components, from the bottom, ascorbic acid (AnaeroGen™), cotton wool and 100 ryegrass seeds (left). Vials containing 100 seeds just before being placed in the 20°C temperature treatment chamber (right). Control samples have no lids, as the lid was screwed on 30 minutes before sampling the control CO_2 levels.

The airspace (volume) that remained in the vial (13.4 ml), after the three components were added (3.1 ml), was calculated by the water displacement method (Appendix VI, Figure 1).

On each sampling date the CO_2 concentration was measured following the protocol described in section 5.4.1.

Calculations were done following the protocol explained in section 5.4.1.1.

At each time interval, accumulated CO_2 concentration inside the vials was measured (see section 5.4.1). The sampling dates were 19, 21, and 25 of January and 2 and 18 February 2018.

For the calibration of the analyser, a Tedlar gas transfer bag was filled with gas taken from a reference cylinder of a known CO_2 concentration.

To assess seed viability and endophyte survival, 25 seeds were randomly selected from each vial at each sampling time and tested following the same sowing protocols described in section 5.5.1. The remaining seeds were discarded.

5.13 Statistical analysis

Each variable was statistically analysed using an analysis of variance (ANOVA) for a completely randomized design. The factorial treatment structure was two seed lots (E+ and E-) x one temperature regime (20°C) x five storage times (2, 4, 8, 16 and 32 d), with polynomial contrasts specified for the storage time factor. The ANOVAs were carried out using GenStat® (VSN International 2013. GenStat® for *Windows* 19th Edition. VSN International Ltd., Hemel Hempstead, UK). For the comparisons of the treatments, the unprotected least significant difference (LSD; $P < 0.05$) procedure was used (Saville, 2015; Saville & Rowarth, 2008).

5.14 Results

The viable endophyte frequency, germination percentage and SMC when the seed lots were received are presented in Table 5-1.

5.14.1 Viable endophyte survival after seed storage

There were significant differences in endophyte survival between the infected seeds stored at 20°C for all 5 storage times tested in both treatments (high CO_2 and anoxic conditions and the control), Table 5-5.

Time of storage and increasing CO_2 concentrations in anoxic conditions had a significant negative effect on the endophyte survival of the infected seeds stored in airtight containers. The grand mean for the survival of endophyte in seed after storage was 71.8%.

There was a 0.1% significant interaction between storage time (linear) x anoxic conditions (linear).

Table 5-5 Main effect means for the effect of time of storage and modified ambient air (elevated CO2 concentrations with anoxic atmosphere) on the survival of endophyte AR37 strain in the perennial ryegrass cultivar 'Samson' after 32 d of storage.

Storage time (d)	**E+ (%)**
2	**82.2**
4	**85.0**
8	**68.8**
16	**72.2**
32	**50.5**
LSD (5%)	**10.51**
Sig. of linear trend	*******
Treatment	
High CO_2 + Anoxic	**68.5**
Control (ambient)	**81.6**
LSD (5%)	**7.67**
Sig. of linear trend	******

*Significant at $p < 0.05$

After storage the percentage of viable endophyte in the control was 81.6%, while the seeds in the treatment (high CO_2 and anoxic conditions), had a survival below the industry threshold of only 68.5% (Figure 5-11).

The high CO_2 concentrations and the anoxic conditions were significantly detrimental for the survival of the endophyte.

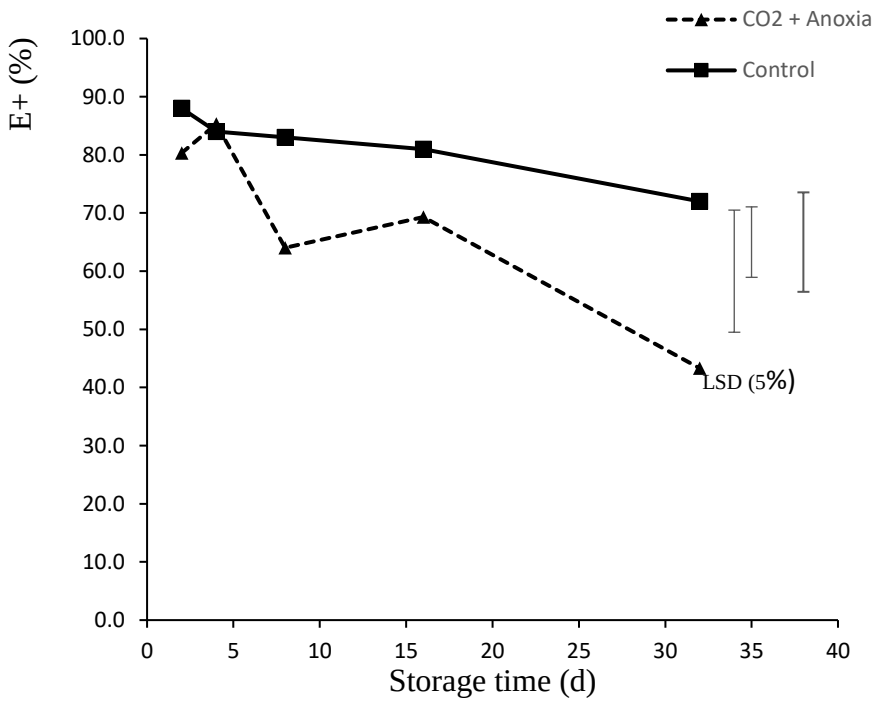

Figure 5-11 Viable endophyte percentage after seeds were stored in high CO_2 concentration and in anoxic conditions, compared with control seeds (ambient conditions). Both were stored at 20°C in an airtight container. The number of control samples and samples under anoxic conditions were unequal, therefore there are three LSD values. Left vertical bar indicates LSD (21.0) for comparing any two control data points, middle vertical bar indicates LSD (12.1) for comparing any two anoxic condition means and right vertical bar indicates LSD (17.1) for comparing a control with an anoxia condition mean at 0.05 level of probability.

5.14.2 Germination percentage after seed storage

The time of storage and the treatment (high concentrations of CO_2 and anoxic conditions) in airtight containers had no significant effect on the germination of both seed lots (E+ and E-). After 32 days of storage, the germination was 95.3% for E+ and 96.4% for E-, with a grand mean of 95.8% (Figure 5-12).

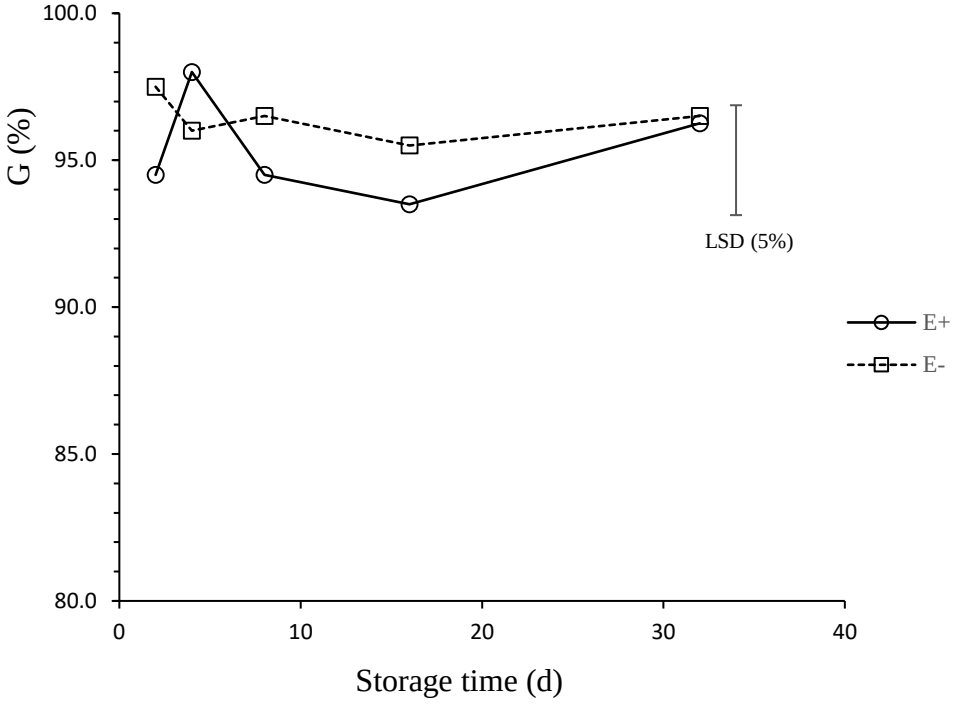

Figure 5-12 Germination percentage of 25 seeds of each seed lot after storage in high CO_2 concentration and in anoxic conditions in an airtight container. Vertical bar indicates LSD (3.74) for comparing any two data points (at 0.05 level of probability).

5.14.3 CO_2 concentration during seed storage

The ascorbic acid in contact with the O_2 created a high CO_2 concentration in the airtight containers. The time of storage and the presence of endophyte in seed, both had a very significant effect on the amount of CO_2 measured in the vials. The concentration of CO_2 increased as time increased (Figure 5-13). However it is predicted that with longer storage times this trend would end in a plateau or decrease as there would be no more chemical reaction in the glass vials. Vials containing seeds E+ had significantly greater concentrations of CO_2 than vials containing seeds E-.

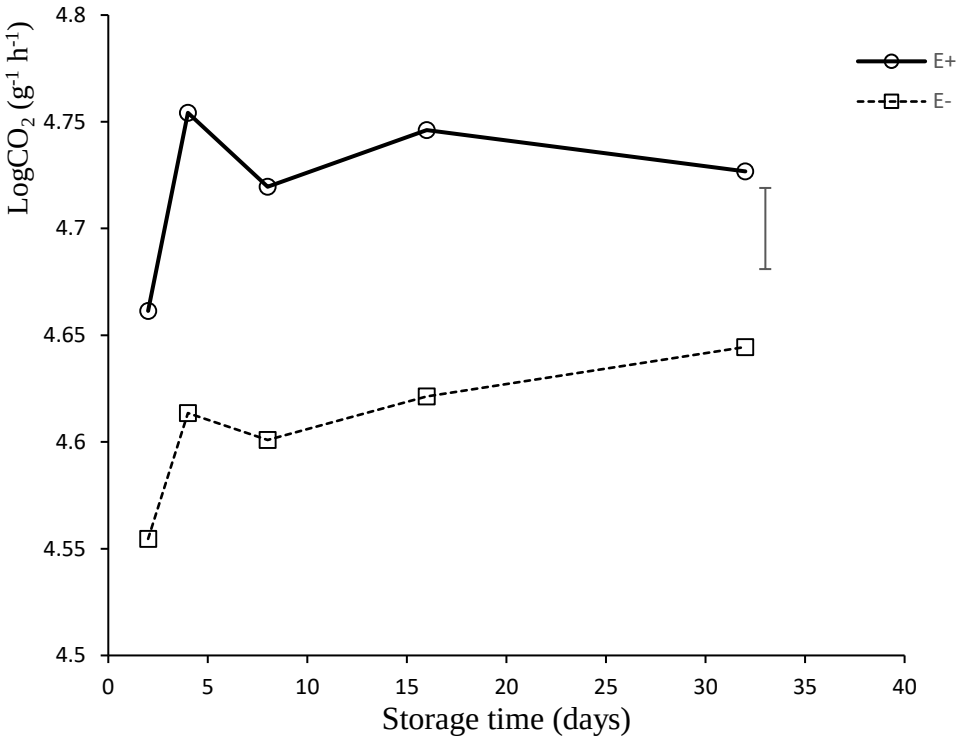

Figure 5-13 CO_2 concentration following respiration of 100 seeds stored at 20°C for a total period of 32 days in airtight containers. Vertical bar indicates LSD (0.039) for comparing any two data points (at 0.05 level of probability).

5.15 **Discussion**

The use of airtight storage containers modified the atmosphere where seeds were stored in various ways (Navarro, 2012). Firstly this created an atmosphere different from ambient, secondly this modification allowed the CO_2 product of the respiration of the seeds to accumulate within the container, and thirdly those gases could be captured and measured to observe the effect on the endophyte hosted by the perennial ryegrass seed.

The increase of time created increases of accumulated CO_2 following respiration of seeds inside the airtight glass vials. This is in line with the study done on *Glycine max* L. by Ochandio et al. (2017), where the product of respiration increased in time, during seed storage. In the same study they also concluded that an increase in temperature increased the CO_2 production, also reflected in this study. Jian et al. (2014) also found differences in CO_2 concentrations when canola, wheat and soybean seeds were stored in airtight conditions, however, in their study they also measured the effect of different SMCs and the respiration of other living microorganism on the seed, that

were not included in this study, however this study did include the effect of the endophyte in the respiration rate of hermetically stored seeds.

In this study the amount of accumulated CO_2 in the hermetic vials over 32 d had a negative effect on the survival of the endophyte in seeds. This negative effect could be due to more than one factor.

Throughout this experiment high concentrations of CO_2 as well as high concentrations of CO_2 with the added factor of O_2 depletion (using AnaeroGen™ as a CO_2 enhancer / O_2 depleter) were detrimental to the endophyte survival. When the O_2 was depleted the levels of CO_2 were very high. Higher concentrations of CO_2 reduced the endophyte survival in seed.

To elucidate the effect of why the anoxic conditions were detrimental to endophyte survival, another experiment including O_2 depletion without high CO_2 concentrations should be considered. An explanation for the loss of viability of the *Epichloë* endophyte is still to be determined. Further studies on the biochemical changes in the seed due to exposure to high concentrations of CO_2 are required.

The effect of O_2 depletion during storage should be evaluated more closely. Vacuum systems have been used for a long time to preserve seed longevity in different seed species (Danner et al., 2011; Tauer, 1979). For endophyte infected seeds, this factor should be studied in depth to define how it affects the survival of the hyphae during storage.

While abiotic factors such as high levels of CO_2 only (experiment I) and high levels of CO_2 combined with O_2 deprivation (experiment II) had a detrimental effect on endophyte viability, the germination of the seeds was maintained and did not differ with the germination at time zero (at the beginning of the experiment). This is in line with the study of Schwember and Bradford (2011) which showed that low levels of O_2 were beneficial for maintaining the longevity of onion and lettuce seeds stored at low RH.

Hume et al. (1999) found significant differences in seed longevity when burying ryegrass seeds in the soil at different depths. Even though the research was focused on the implications of seeds survival in the seed bed and the importance for cultivation practices, they also found that seed survival at 10 cm was significantly greater than seeds stored at 3 cm deep. The reasons included various factors, and lower O_2 concentrations was one of them.

Previous research showed that increase in temperature during storage was detrimental for the longevity of perennial ryegrass seeds (Bukvić et al., 2015). In this study storage temperature was also significant and detrimental for endophyte survival when comparing between storage at 30°C and storage at 5°C and 20°C for 32 days. This storage response to temperature and time was also reported by many including Hare et al. (1990), Welty et al. (1987), Hume et al. (2011) and more recently Hume et al. (2013).

During storage of perennial ryegrass seed with or without endophyte, anaerobic conditions preserved the longevity of the seed, even with high concentrations of CO_2. On the other hand the potential of preserving the endophyte as the seed is alive was not achieved, as the viability of the endophyte was significantly reduced by these conditions and furthermore, with time of storage in airtight containers.

Using hermetic conditions has some limitations for real storage situations. Hermetically sealed containers, such as polyethylene bags for seeds infected with selected endophytes are available, but no research has been reported on the effect of O_2 or CO_2 concentrations in these bags for survival of AR37 in perennial ryegrass seed during storage in these conditions.

Another possibility is the use of vacuum systems, already proven to preserve seed longevity (Danner et al., 2011). However no research had this far been done that included endophyte survival and measured of gases within the interstitial space. The use of O_2 depleting substances like AnaeroGen™ and vacuum systems for enhancing endophyte survival during storage should be considered.

The seeds used in this experiment had a safe SMC and were clean of any external fungi that could have respired and therefore produced CO_2, so the results reported here come from only the respiration of the ryegrass seeds.

As all factors were very significant, it is likely that these experiment can be easily repeated and a larger data base with longer storage times is recommended.

5.16 **Conclusions**

5.16.1 Experiment I

The respiration rate varies with temperature and with the presence of the endophyte, negatively affecting the endophyte survival during storage. Seed respiration rate increased with temperature

and cv ‘Samson’ seeds containing endophyte (E+) AR37 strain, had a higher respiration rate than seeds without endophyte (E-). Seeds stored at higher temperatures lost the endophytes at a significantly faster rate than seed stored at lower temperatures (5°C), due to the seeds being stored in airtight containers capturing and accumulation the CO_2 generated by respiration in the container.

High CO_2 concentration and anoxic storage conditions, or both, had no significant effect on the germination of either seed lot, but storage time did.

5.16.2 Experiment II

The modification of storage conditions may affect the endophyte and the host that is carrying it (the seed) differently. When there was a deprivation of O_2, all factors studied had a significant effect on the viability of the endophyte in the seed. The time of storage in the airtight containers and the high levels of CO_2 produced in a short period of time (32 d) by the ascorbic acid negatively affected the endophyte survival. A 71.8% endophyte infection is nearly at the limit of acceptance by the seed industry in NZ and Australia to class the seed lot as endophyte infected. The storage time and high levels of CO_2 in combination with the anoxic conditions did not affect the germination of either ‘Samson’ seed lots (E+ and E-). Lack of O_2 did not affect germination of seeds stored for 32 days.

This experiment supported the hypotheses that high CO_2 concentrations (refers to levels greater than the atmospheric of 400 ppm) during a short storage time (32 d) at 20°C will reduce the endophyte survival in perennial ryegrass seed, and that the time of storage was a significant factor determining the decrease in endophyte survival. However it did not support the hypothesis that seeds infected with the *Epichloë* endophyte strain AR37 would have a different germination percentage than endophyte free seeds, but did support that anoxic conditions created by the ascorbic acid maintained germination of the seeds and potentially the endophyte as the survival of the host is a requirement for the endophyte to survive.

CHAPTER 6

General discussion

The aim of this research was to investigate abiotic factors which could possibly affect the vertical transmission and the survival during storage of two *Epichloë festucae* var. *lolii* endophytes in perennial ryegrass seed. The commercial endophytes strains AR1 and AR37 which form host specific associations with perennial ryegrass (*Lolium perenne* L) were used in this study.

The Poaceae family is the most economically important plant family, providing foods, fuel, building materials, and the grasses of natural grasslands and cultivated pastures. Perennial ryegrass in NZ and in most other cool temperate regions of the world, has become the most widely and preferred pasture grass species sown to cover feed demands for primary production in forage livestock systems, due to its nutritional quality, high digestibility and palatability, high yields, tolerance to continuous grazing, persistence and freezing tolerance. The economic losses caused by insect herbivores, accelerated the exploitation and research of novel associations intentionally infected with selected *Epichloë* endophytes, for a successful alternative of plant biocontrol protection strategy (Caradus et al., 2013; Johnson et al., 2013).

Beside the desirable alkaloid profile produced by the association, the protection and symbiosis is successful when the novel endophyte association exhibits stability in colonising the plant, in transmitting to the next generation and persisting in the host during storage. Those attributes would make a successful commercial biocontrol product with a real added value for the seed industry and for the end user.

This study aimed to investigate factors that may be interfering with the success of reaching that target, both during crop development and during seed storage.

6.1 Summary of outcomes

6.1.1 During seed developement

6.1.1.1 *Endophyte transmission after waterlogging treatments.*

Temporally flooding and waterlogging is regarded as one of the most likely events to occur due to climate change increasing the risks in low lying countries (Ahmed et al., 2013).The results of this study, did not support the hypothesis that host-endophyte interaction is negatively affected

by waterlogging of the infected plant during short periods of time during plant development and seed production. In both cultivars and with both endophytes, the transmission was successful regardless the time when the WL was imposed.

Overall, the results obtained also did not support the hypothesis that *Epichloë festucae* var. *lolii* strains AR1 and AR37 have different stress thresholds, so that the impact of waterlogging on transmission rates would therefore differ between the two endophyte strains.

It is important to mention that the seeds used in the experiment were germinated in optimum conditions, assuring good development of hyphae in the seedlings. For future research it should be considered the option of an experiment involving WL during early stages of development (from sowing up until GS 30) as this was not studied in this research.

In this experiment two important management factors may be interfering with the real farm situation and secondly with the real transmission rate results. Firstly, all mature seeds were harvested. In normal farm situations farmers will harvest when seed maturity has reached an average SMC for seeds in the spikelet and the first to mature seeds may be shed. This could have helped in getting higher transmission frequencies compared with seed producers. Secondly the discard of the lighter fraction of seeds during cleaning could have resulted in the loss of small viable seeds that could have or have not transmitted the endophyte. Even though this is common practice during machine dressing of seeds, for future research these points should be considered and addressed for more accurate results.

6.1.1.2 *Endophyte transmission after nitrogen and potassium fertiliser treatments.*

Transmission rate for both endophyte strains in both perennial ryegrass cultivars was high irrespective of the 9 fertiliser treatments. However there were some significant differences in the transmission of endophyte strains in response to single nutrients.

The genetic host specificity was evident through the difference observed in transmission when exposed to the same abiotic factors. Different endophyte strains responded in different ways. The fact that these two endophyte strains differ in transmission due to abiotic factors is not surprising, as we know that the host has a strong influence over the behaviour of the endophyte, and that hosts differ in their response as reported by Hesse et al. (2004). Reinforcing the importance not only of the acknowledgment of endophyte association mismatches reported by Saikkonen et al.

(2010), but also keeping the records of the different requirements each association may have, particularly for future management on farm and breeding programmes.

6.1.2 During seed storage

6.1.2.1 *Effect of temperature and relative humidity on the survival of endophyte in seed.*

The transmission of the endophyte to the off spring can be very successful, however, the presence of the endophyte in the seed does not assure its viability or the capability to grow in the next growing season, as the endophyte can be lost during harvest practices and during post-harvest management practices, including storage.

The longevity of infected seeds is determined by various abiotic factors, temperature and relative humidity, moisture content and time have been the most studied ones (Hume et al., 2013; Rolston et al., 1986; Welty et al., 1987)

NZ and Australia seed industry agreed in delivering a novel endophyte product with no less than 70% of viable endophyte, therefore there is an evident interest on the knowhow of when the threshold is reached and even more if it can be predicted.

This study was undertaken to determine the causes of the loss of the endophyte in stored perennial ryegrass seeds when seeds were exposed to different temperatures and relative humidity levels that may be responsible for not reaching the industry threshold in the trade world.

The ATU approach was used as a tool to find an easy way to calculate the best before date of endophyte viability in different storage conditions. E+ storability or the ability of the endophyte in to maintain its viability (grade) and therefore its biocontrol potential after storage using a mathematical system that includes time and temperature is of great advantage. However, it would be interesting and useful to relate moisture content and relative humidity levels in the equation. Future studies are recommended.

Endophyte viability was maintained consistently during 1800 ATU at relative humidity levels of 20%, 40% and 60%. For relative humidity levels of 80% the endophyte maintained its viability up to 1000 ATUs. At 30°C, storage at 20% RH maintained endophyte viability for 45 days, and it was the only safe combination to store the endophyte at that high temperature (2250 ATUs).

Maintaining the endophyte viability above 70% is possible at three different temperatures (4°C, 15°C and 20°C) and relative humidity levels of both 20% and 40% when the accumulated thermal units are below 2000.

In non-moisture proof storage conditions, endophyte viability was maintained for longer with a combination of low temperature and low RH%, being in agreement with previously results obtained by Hare et al. (1990) and Rolston et al. (1986), and that higher relative humidity levels increased SMC, and as a result negatively affecting endophyte viability when SMC was over 10%, in accordance with the research done by Welty et al. (1987) who reported a decline in germination and endophyte viability when SMC was over 10%.

For all storage conditions, endophyte viability was always lost before germination meaning that endophyte viability declines at a faster rate than seed does (Rolston et al., 1986).

Hill and Roach (2009) and Tian et al. (2013) focused on the survival of the endophyte as controlled by the plant and the endophyte genetics; however in this experiment the study was performed using one cultivar and one endophyte strain (AR37) but the times of survival can be compared with other studies where the trend is the same but the timing varies depending on the associations being studied.

It is still unclear as pointed out by Hume (pers. comm., 2013), what are the mechanisms involved in the decline or even loss of endophyte when exposed to high temperatures and high relative humidity levels.

6.1.2.2 *Effect of CO_2 due to respiration of seeds and CO_2 and anoxic conditions on the survival of the endophyte AR37 strain.*

In hermetic conditions, the high concentrations of CO_2 created by both mechanisms (seed respiration and use of AnaeroGen™) significantly reduced the endophyte survival after 32 d of storage. This experiment gave an approach for future research on the effect of abiotic factors like oxygen and carbon dioxide concentrations on *Epichloë festucae* var *lolii* infected seed during storage. Measuring the extent of the effect of high concentrations of CO_2, either by respiration or by addition, on the deterioration of the endophyte survival in time is experimentally useful and promising for future experiments.

The results of this study showed that germination was not affected by the modified ambient conditions used. This fact opens the window for future research on using this method for sterilizing infected seeds, without affecting the germination. Analysing the chemical changes in

the seed tissue before and after the modification of the ambient conditions may link to the answer of what affects the endophyte hyphae in the seed.

Additional experiments measuring gases in seed stores and in different storage containers is recommended to analyse the possibilities of using modified ambient conditions in the future.

This study only focused on AR37 in cv 'Samson', but further studies including other endophytes and cultivars is recommended, especially because of the specificity that exists between host-endophyte in grasses. Future research is needed on the applicable aspects of ambient conditions modification for use in the seed industry for the storage of seeds infected with *Epichloë* endophytes.

Appendix I

Table of relative humidity values when using saturated solutions at different temperatures (Winston & Bates, 1960).

TABLE I. Relative humidity values over saturated solutions at various temperatures

Temperature °C	2°	5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
Compound											
NH_4Cl	....	....	79.0	79.5	79.5	78.0	77.5	....	74.0	....	71.5
NH_4HPO_4 (monophosphate)	....	....	....	....	93.0	93.0	92.0	....	91.0	....	....
NH_4NO_3	....	....	75.0	70.0	65.5	62.5	59.5	55.0	53.0	50.5	48.0
$NH_4NO_3 + NaNO_3$	....	....	58.0	55.0	52.0	50.0	47.0	44.5	42.0	39.5	36.5
$NH_4NO_3 + AgNO_3$	....	....	70.5	68.0	65.1	61.5	58.0	55.0	52.0	48.5	44.5
	0°										
$(NH_4)_2SO_4$	83.5	82.5	80.5	81.0	80.5	80.0	80.0	79.5	79.0	79.0	78.0
$CaCl_2 \cdot 6H_2O$[1]	....	40.0	38.0	35.0	32.5	29.5	....	....	....	....	....
$CaHPO_4 \cdot 2H_2O$[2]	....	....	97.5	99.5	95.0	97.0	95.0	....	95.5	....	95.0
$CaH_4(PO_4)_2 \cdot H_2O$[3]	....	....	98.0	99.0	94.0	96.0	93.5	....	94.5	....	94.5
	0°										
$Ca(NO_3)_2 \cdot 4H_2O$[4]	66.0	....	....	56.0	55.5	50.5	47.0	....	(36.0)	....	....
				18°							
$CoCl_2$[5]	....	....	....	72.5	67.0	....	62.0	....	56.5	....	....
Cr_2O_3[6]	....	....	....	45.5	(39.0)	....	44.5	....	45.0	....	45.5
			12°								
Glucose	(60.0)	....	57.0	....	55.0	55.0	.. .	55.0	....	....	....
	0°										
$LiCl \cdot H_2O$[7]	14.5	14.0	13.5	13.0	12.5	12.0	11.5	11.5	11.0	11.0	11.0
$Pb(NO_3)_2$[8]	....	....	98.0	97.0	97.0	95.5	95.0	94.5	94.5	94.0	92.5
$Pb(NO_3)_2 + NH_4NO_3$	....	....	66.5	62.0	58.0	55.0	52.5	49.5	47.0	44.5	41.5
	0°										
$MgCl_2 \cdot 6H_2O$	35.0	34.5	34.0	34.0	33.0	32.5	32.5	32.5	32.0	31.5	31.5
$Mg(NO_3)_2 \cdot 6H_2O$	60.5	59.0	58.0	56.0	55.0	53.0	52.0	50.5	49.0	47.5	46.0
P_2O_5	00.0	....	00.0	00.0	00.0	00.0	00.0	....	00.0	....	00.0
KAc	(23.5)	....	(21.0)	....	20.0	22.5	22.0	....	20.0	....	....
KBr	....	....	86.0	....	84.0	80.0	82.0	....	79.5	....	79.0
$K_2CO_3 \cdot 2H_2O$[9]	47.0	....	47.0	44.0	44.0	43.0	43.5	....	40.0	....	....
KCl	(88.0)	....	88.0	86.5	85.0	85.0	84.5	83.0	82.0	81.0	80.5
K_2CrO_4	....	....	....	....	88.0	....	86.5	....	85.5	....	....
$K_2Cr_2O_7$	....	....	....	....	98.0	98.0	97.5	96.5	96.0	....	95.5
KH_2PO_4	....	....	98.0	99.0	96.5	96.0	93.5	....	93.0	....	92.5
$KF \cdot 2H_2O$[10]	....	....	....	....	....	30.5	27.4	....	23.0	....	20.5
	0°										
KNO_3[11]	97.5	96.5	96.0	95.5	93.5	92.5	91.0	89.5	88.0	86.5	85.0
KNO_2	....	....	....	....	48.5	....	47.0	....	46.0	....	....
K_2SO_4	99.0	98.5	98.5	99.0	98.0	97.5	96.5	96.0	96.5	96.0	96.0
KCNS[12]	....	....	52.0	50.0	47.0	46.5	43.5	41.5	41.0	38.0	36.5
K Tartrate[13]	....	....	....	75.0	75.0	75.0	74.0	....	74.0	....	....
KNa Tartrate[14]	....	....	87.5	....	87.0	87.0	87.0	....	86.0	....	....
Pyrocatechol	....	....	....	99.0	95.5	93.5	92.5	90.5	88.0	85.0	81.5
Resorcinol	....	....	....	95.0	87.0	85.0	82.5	79.5	76.0	72.5	68.5
$AgNO_3$	....	....	88.0	86.0	84.0	82.0	80.0	78.0	76.5	74.5	72.0
$AgNO_3 + Pb(NO_3)_2$	....	....	84.0	83.0	80.5	78.5	76.5	75.0	73.0	71.0	69.0
$NaAc \cdot 3H_2O$	....	....	....	....	75.0	73.0	71.5	....	67.0	....	....
$NaBr \cdot 2H_2O$[15]	....	....	63.0	61.0	59.0	57.5	56.0	54.5	53.0	51.5	49.5
$Na_2CO_3 \cdot 10H_2O$[16]	....	....	(99.0)	....	92.0	87.0	87.0	....	....	....	....
	0°										
NaCl	75.0	75.0	76.5	76.0	76.0	75.5	75.5	75.5	75.0	75.0	74.5
$NaCl + KCl$	....	....	....	....	70.0	71.5	71.0	....	....	....	....
$Na_2CrO_4 \cdot 4H_2O$	....	....	....	....	....	....	64.5	....	62.0	....	58.0
$Na_2Cr_2O_7 \cdot H_2O$[17]	60.5	59.5	60.0	56.5	54.5	53.0	52.5	51.0	50.0	48.5	47.0
NaOH	(5.5)	....	(5.5)	....	5.5	7.0	(4.0)	....	(1.5)	....	....
NaI	....	....	....	....	38.0	38.0	36.0	....	32.5	....	29.0
$NaNO_3$	....	....	77.5	76.5	76.0	74.0	72.5	71.0	70 5	68.5	67.5
$NaNO_2$	....	....	....	....	65.5	64.0	63.0	....	61.5	....	60.0
$Na_2SO_4 \cdot 10H_2O$	....	....	....	....	(93.0)	(93 0)	....	(87.0)	(88.5)	....	(88.5)
Na tartrate[18]	....	....	....	94.0	92.0	92.0	92.0	....	92.0	....	....
Urea	....	....	81.5	80.0	8.0	76.0	73.3	....	68.5	....	62.5
$ZnCl_2 \cdot 1\frac{1}{2}H_2O$[19]	(10.0)	(10.0)	(10.0)	(10.0)	10.0	....	(10.0)	....	(10.0)	....	10.0
$ZnSO_4 \cdot 7H_2O$[20]	....	94.5	....	....	....	88.5	....	....	84.0	....	....

[1] May be unstable around 20° C.
[2] May be a transition point between 15° and 20°.
[3] Calcium monophosphate.
[4] Good up to 42°.
[5] Uncertain around 20°, large temperature coefficient.
[6] Wink and Sears (1950) have lower values (40%) at 30° and 40°.
[7] $LiCl.XH_2O \longrightarrow LiCl.H_2O$ at 12.5°.
[8] Solution slightly acid due to hydrolysis, but stable.
[9] Should be granulated then moistened; powdered form is unstable.
[10] $KF.2H_2O \longrightarrow KF$ at 50°.
[11] Large temperature coefficient.
[12] Very toxic.
[13] Potassium tartrate. Excellent.
[14] Potassium sodium tartrate (Rochelle Salts). Good only below 40°.
[15] Good up to 55°, large temperature coefficient.
[16] $Na_2CO_3.10H_2O \ldots\ldots > Na_2CO_3.H_2O$ at 32°.
[17] Very good; small temperature coefficient.
[18] This is also listed with one water of hydration, instead of two; good only below 40°.
[19] Unstable around 20°.
[20] Good below 10°.

Appendix II

Detailed main effect means for viable endophyte transmission frequency after waterlogging treatments.

(Chapter 2- section 2.10.1)

Main effect means for viable endophyte transmission rate (%) for two perennial ryegrass cultivars, containing endophyte strains AR1 or AR37 after four water treatments

Cultivar	**E+ (%)**
'One 50'	87.0
'Prospect'	91.1
LSD (5%)	**8.8**
Sig. of linear trend	**ns**
Endophyte strain	
AR1	91.5
AR37	86.7
LSD (5%)	**8.8**
Sig. of linear trend	**ns**
Waterlogging trt Trt.I	
GS 30 (n=3)	85.2
Trt.II GS.65 (n=2)	92.7
Trt.III GS 70 (n=1)	91.3
Trt.IV control (n=2)	90.0
LSD (5%)	**13.7**
Minimum least significant difference within waterlogging trts (e.g: Trt I vs Trt III = **13.7**) shows no significant difference between treatments.	

Appendix III

(Chapter 2.-section 2.10.2)

LSD Calculation

There are different LSD values for the ANOVA analysis of the variables affected by the imposition of the water stress, due to the experiment being unbalanced. Some treatments had 1 replicate (n =1) like the waterlogging at seed set, some treatments had 2 (n = 2) like the control and WL at reproductive stage and another had 3 (n=3) when WL was done at anthesis.

The calculations of the LSD depending on what was compared against were:

n= 2 vs 2 (when comparing 2 replicates versus 2 replicates) LSD = $\sqrt{S^2\ \left[\frac{1}{2}+\frac{1}{2}\right]} *$ t value=

= S * 1.0 * t value

n= 1 vs 3 (comparing 1 replicate versus 3 replicates) LSD = $\sqrt{S^2\ \left[\frac{1}{1}+\frac{1}{3}\right]} *$ t value =

= S * 1.155 * t value

The smallest LSD = LSD for n=2 vs 3, involving $\sqrt{\left[\frac{1}{2}+\frac{1}{3}\right]}$ in the formula.

GenStat prints LSD for n=1 vs n=3 (max – min), involving $\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}$.

To adjust to an LSD for n=2 vs 3 then this LSD is multiplied by

$\frac{\sqrt{\left[\frac{1}{2}+\frac{1}{3}\right]}}{\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}} = 0.79057.$ This is the multiplier for all LSD printouts.

Eg2: Endophyte (%).

Minimum LSD (2 vs 3) = 13.74 * 0.79057 = 10.86 (rounding to 10.9)

Maximum LSD compared to minimum LSD

The calculation of the multiplier is as follows:

$\frac{\sqrt{\left[\frac{1}{1}+\frac{1}{2}\right]}}{\sqrt{\left[\frac{1}{2}+\frac{1}{3}\right]}}$= 1.34164, therefore the maximum LSD is 1.34 times as big as minimum LSD.

Calculations of LSD for Treatment means table (Table 4, Chapter 2).

Calculation within WL treatments

Trt	WL trts GS 30 n=3	WL trt GS 59 n=2	WL trt GS 70 n=1	No WL Control n=2
LSD (5%)	20.4 (max rep)	24.9	35.3 (min rep)	24.9

GenStat gives LSD (5%) (n=1 vs n=1) = 35.26

To calculate LSD for n=2= $\frac{LSD(n=1)}{\sqrt{[2]}} = \frac{35.26}{\sqrt{[2]}} = 24.9$

Calculation between WL treatments

GenStat gives LSD (5%) (n=1 vs n=3) = 26.53

LSD (5%) (n=1 vs n=2) = $26.53 * \frac{\sqrt{\left[\frac{1}{1}+\frac{1}{2}\right]}}{\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}}$= 28.1 (black arrow)

(n=2 vs n=2) =$26.53 * \frac{\sqrt{\left[\frac{1}{2}+\frac{1}{2}\right]}}{\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}}$ = 23.0 (red arrow)

(n=2 vs n=3) =$26.53 * \frac{\sqrt{\left[\frac{1}{2}+\frac{1}{3}\right]}}{\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}}$ = 21.0 (green arrow)

Appendix IV

LSD Calculation

Effect of waterlogging treatments on germination percentage (%) of perennial ryegrass seeds infected with two commercial *Epichloë festucae* var. *lolii* stains AR1 and AR37

Cultivar	Endophyte strain	GS 30 (n=3)	GS.65 (n=2)	GS 70 (n=1)	Control (n=2)	Mean WL
'One 50'	AR1	86.5	85.8	83.8	89.6 *	**85.4**
	AR37	86.3	89.4*	77.1	80.6	**84.2**
'Prospect'	AR1	88.9*	90.8*	90.4	83.1	**90.0**
	AR37	89.0*	85.8	86.3	91.3*	**87.0**
LSD (5%) within WL trts.		10.3	12.6	17.8	12.6	p = 0.073
LSD (5%) between WL trts		8.3	10.5			

WL: Waterlogging

There are different LSD values for the ANOVA analysis of the variables affected by the imposition of the water stress, due to the experiment being unbalanced. Some treatments had 1 replicate (n =1) like the waterlogging at seed set, some treatments had 2 (n = 2) like the control and WL at reproductive stage and another had 3 (n=3) when WL was done at anthesis. Means with an * differ significantly from the highlighted treatment mean (77.1%) at p < 0.1%.

GenStat prints LSD for n=1 vs n=3 (max – min), involving $\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}$.

To adjust to an LSD for n=2 vs 3 then this LSD is multiplied by

$\frac{\sqrt{\left[\frac{1}{2}+\frac{1}{3}\right]}}{\sqrt{\left[\frac{1}{1}+\frac{1}{3}\right]}} = 0.79057.$ This is the multiplier for all LSD printouts.

LSD (n=1 vs n=3) = 10.521 (blue arrows), LSD (n=2 vs n=3) = 10.521*0.79057= 8.3 (red arrows), this is the smallest LSD. If there is no significant difference with the smallest LSD, it would not be for the other higher LSDs.

Appendix V

(Chapter 3 - section 3.9.1)

Treatment means for endophyte transmission frequency E+ (%), (K x N x cultivar x endophyte strain interaction table). From this table all lower order interaction tables can be calculated.

		Cultivar			
		'One-50'		**'Prospect'**	
K (kg $ha^{-1}yr^{-1)}$	**N** (kg $ha^{-1}yr^{-1)}$	**AR1**	**AR37**	**AR1**	**AR37**
0	0	73.0	81.4	75.5	82.7
	150	87.7	88.4	93.3	90.5
	250	94.9	91.8	95.4	85.0
200	0	88.7	98.7	93.4	87.8
	150	95.8	98.1	94.2	98.9
	250	98.7	95.0	100.0	97.5
400	0	83.9	62.1	75.1	87.1
	150	87.0	75.7	92.3	91.5
	250	94.5	74.1	80.7	78.3

LSD (5%) for means with the same level of N and K (horizontal comparisons) = 14.8
LSD (5%) for all other comparisons = 26.9

(Chapter 3 - section 3.9.2)

Treatment means for germination percentage G (%), (K x N x cultivar x endophyte strain interaction table). From this table all lower order interaction tables can be calculated.

		Cultivar			
		'One-50'		**'Prospect'**	
K (kg $ha^{-1}yr^{-1}$)	**N** (kg $ha^{-1}yr^{-1}$)	**AR1**	**AR37**	**AR1**	**AR37**
0	0	54.2	76.0	71.9	75.0
	150	86.5	86.5	83.3	86.5
	250	79.2	65.6	79.2	83.3
200	0	70.8	78.1	70.8	83.3
	150	82.3	62.5	76.0	80.2
	250	54.2	63.5	71.9	68.7
400	0	74.0	67.7	81.2	79.2
	150	70.8	69.8	58.3	74.0
	250	63.5	62.5	70.8	74.0

LSD (5%) for means with the same level of N and K (horizontal comparisons) = 18.7

LSD (5%) for all other comparisons = 23.5

(Chapter 3 – 3.9.3 -section 3.9.3.1)

Treatment means for DM (g plant^{-1}), (K x N x cultivar x endophyte strain interaction table). From this table all lower order interaction tables can be calculated.

		Cultivar			
		'One-50'		**'Prospect'**	
K (kg ha^{-1}yr^{-1})	**N** (kg ha^{-1}yr^{-1})	**AR1**	**AR37**	**AR1**	**AR37**
0	0	48.6	41.7	39.7	50.8
	150	58.9	29.5	65.3	74.2
	250	54.7	45.4	58.1	52.7
200	0	75.6	68.3	59.5	88.6
	150	42.7	69.7	71.6	87.1
	250	88.9	43.7	68.2	65.2
400	0	66.1	50.4	51.1	90.5
	150	72.1	79.5	73.9	68.4
	250	75.5	72.5	64.6	66.3

LSD (5%) for means with the same level of N and K (horizontal comparisons) = 29.8
LSD (5%) for all other comparisons = 31.4

(Chapter 3 – 3.9.3 -section 3.9.3.2)

Treatment means for number of reproductive tillers (n plant^{-1}), (K x N x cultivar x endophyte strain interaction table). From this table all lower order interaction tables can be calculated.

		Cultivar			
		'One-50'		**'Prospect'**	
K (kg ha^{-1}yr^{-1})	**N** (kg ha^{-1}yr^{-1})	**AR1**	**AR37**	**AR1**	**AR37**
0	0	122.4	114.4	138.5	153.9
	150	142.8	98.1	172.9	182.8
	250	132.6	126.1	171.1	126.9
200	0	139.0	164.6	183.9	172.4
	150	123.4	173.6	231.1	242.5
	250	262.1	129.8	193.5	183.5
400	0	171.0	104.0	166.8	234.6
	150	190.7	175.1	149.0	198.6
	250	172.0	155.1	183.1	154.4

LSD (5%) for means with the same level of N and K (horizontal comparisons) = 80.4
LSD (5%) for all other comparisons = 72.4

(Chapter 3 – 3.9.3 -section 3.9.3.3)

Treatment means for seed yield (g plant $^{-1}$), (K x N x cultivar x endophyte strain interaction table). From this table all lower order interaction tables can be calculated.

		Cultivar			
		'One-50'		**'Prospect'**	
K (kg ha^{-1}yr^{-1})	**N** (kg ha^{-1}yr^{-1})	**AR1**	**AR37**	**AR1**	**AR37**
0	0	8.39	8.07	7.26	11.20
	150	9.55	8.50	12.07	11.12
	250	9.07	16.55	11.63	10.83
200	0	11.77	11.29	8.65	9.59
	150	7.79	12.70	14.1	14.34
	250	16.41	10.94	14.09	11.37
400	0	9.14	7.79	9.82	9.09
	150	12.87	11.31	12.22	14.47
	250	11.15	11.36	11.85	11.03

LSD (5%) for means with the same level of N and K (horizontal comparisons) = 5.55
LSD (5%) for all other comparisons = 5.08

(Chapter 3 - 3.9.3- section 3.9.3.4)

Treatment means for TSW (g), (K x N x cultivar x endophyte strain interaction table). From this table all lower order interaction tables can be calculated.

		Cultivar			
		'One-50'		**'Prospect'**	
K (kg $ha^{-1}yr^{-1}$)	**N** (kg $ha^{-1}yr^{-1}$)	**AR1**	**AR37**	**AR1**	**AR37**
0	0	1.801	1.936	1.876	1.854
	150	2.088	2.116	2.270	2.158
	250	2.251	2.111	2.166	2.158
200	0	2.009	1.916	2.027	2.000
	150	2.266	2.515	2.389	2.388
	250	2.251	2.111	2.166	2.158
400	0	1.856	1.971	1.778	1.781
	150	2.128	2.129	2.11	2.081
	250	1.859	2.034	1.971	1.769

LSD (5%) for means with the same level of N and K (horizontal comparisons) = 0.209
LSD (5%) for all other comparisons = 0.200

Appendix VI

Calculation of seed respiration rated from changes in vial CO_2 concentrations over time.
(pers. comm, John Hunt & David Whitehead, 2018)

Conversion of absorption area to CO_2 concentration

Use the injection of a known calibration gas to calculate the conversion factor from the integrated area (µmol CO_2 mol^{-1} of air. seconds).

Conversion factor = Calibration gas concentration / Average integrated area =

e.g. Calibration gas = 550 µmol/mol

Average area = 182.1

Conversion factor = Calibration gas/ Average area =

550/182.1 = 3.02

e.g (Area of the sample (measurement) x Conversion factor)-(CO_2 concentration from control CO_2 vial x Conversion factor) =

Where area of the sample = 242.7

Control CO_2 = 189.0

(242.7 x 3.02) - (189.0 x 3.02) = 732.9-570.8 = 162.1 µmol/mol

Air volume

The volume of the vial (v) is to be measured in cm^3 (same as ml). If the diameter (d) is in cm and the depth (h) is in cm then:

$$v = \left(\frac{d}{2}\right)^2 \pi h$$

Alternatively, v can be measured using either a syringe of water to fill the vial (water displacement method, or by weight.

Figure 1 Volume of vial measured using water displacement (12 ml) (left). The volume of the 100 seeds took 0.5 ml of the vial (right).

Air volume to mols of air

Convert the volume of air in the vial from ml to mass (mols) using the universal gas law (also known as the general gas equation (PV=nRT). This then matches with the units used to measure CO_2. For any gas (including CO_2, O_2, N_2O) n is the number of moles of gas:

$$n = \frac{PV}{RT}$$

Where P is the atmospheric pressure in atmospheres (assume 1 atm),

V is volume (cm^3, calculated above),

T is the temperature in Kelvin (K) and

R is the universal gas constant (82.05 ml.atm / (mol.K)).

Example using 5°C:

Volume (ml) = 12.03 ml

Pressure = 1, temp = 278 K (273+5)

n = moles of air in empty vial = (1 * 12.03) / (82.05 * 278)

= 0.0005274 mols

=527.4 µmols

n = moles of air in vial with seeds = (1 * 11.5) / (82.05 * 278)

= 0.0005041 mols

= 504.1 µmols

Final calculation or respiration

Calculate the rate of respiration from the seeds.

$$R = \frac{\Delta CO_2 * MW * n}{a * t}$$

Where R = respiration rate, Δ= change in CO_2 concentration (µmol CO_2/mol air) where

$$\Delta CO2 = [CO_2]_{t2} - [CO_2]_{t1}$$

t_1 = initial concentration

t_2 = final concentration

n= is the number of mols of gas in the vial

a is the number or mass of seeds and (0.22 g)

t = time in units required (i.e. 24 hrs)

R is expressed in mg CO_2/g (1/100 seeds)/hour or day

R units	MW (molecular weight)	a	t
mg CO2/g(100seeds)/d	0.0044	Weight seeds (g)	days
mg CO2/g 100 seeds/h	0.0044	Weight seeds (g)	hours
mg CO2/g(1seed)/d	0.0044	Weight 1 seed (g)	days
mg CO2/g 1 seed/h	0.0044	Weight 1 seed (g)	hours

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