The effect of applications of different nitrogen types and potassium on seed quality and AR37 endophyte presence at different spikelet and floret positions of perennial ryegrass cv. Halo

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

Master of AgriScience

in

Seed Science and Technology

at Massey University, Palmerston North

New Zealand

Muyu Wang

2014

Abstract

Nitrogen has been considered as an important nutrient in the terrestrial system. In the seed production of ryegrasses, one of the most popular pastures used in New Zealand and other temperate-zone areas, the application of nitrogen is responsible for improving seed yield and seed quality. Novel fungal endophytes are also now commonly used in perennial ryegrass pasture systems. The effect of different forms of nitrogen on seed quality and endophyte infection frequency and alkaloid concentration including spikelet/floret positional effects is also of interest to researchers. This study was designed to determine the effects of three nitrogen forms and potassium treatments (six in total) on the seed quality (purity, thousand seed weight (TSW), and germination) and AR37 endophyte presence in the offspring seedlings of the perennial ryegrass cv. Halo at three spikelet positions (top, middle and bottom). Also the effect of two nitrogen forms (nitrate and ammonium) at different floret positions was investigated.

The two nitrogen forms (urea and nitrate) with potassium had a poorer seed quality compared with the control and all nitrogen treatments applied without potassium. Nitrogen application (any form by itself) did not affect TSW of 'Halo', but a reduction was found under urea or nitrate with potassium. Also, seed germination percentages were not affected by nitrogen type when compared with the control, but urea with potassium gave a lower germination than the three nitrogen forms alone; and nitrate with potassium was lower than just the urea treatment. In the purity test, urea applied alone had a higher pure seed percentage than the control and the other nitrogen forms applied alone, but, again, the nitrogen with potassium application had the poorest performance in the test. On the other hand, none of these seed quality parameters differed among the three spikelet positions (top, middle and bottom). Both nitrogen and potassium application and different spikelet positions did not affect endophyte content in the offspring seedlings of 'Halo'.

In the minor experiment, where seven floret positions and only two nitrogen forms (ammonium (NH_4^+) and nitrate (NO_3^-)) were compared, the individual seed weights

of Halo in florets 3, 4, and 7 under nitrate application were higher than that under ammonium. The seed weight in floret 7 was the only position lower than floret 1 and 2 when ammonium was applied. The germination percentages were not affected by the two nitrogen forms, nor were different floret positions. Further, nitrogen application also did not alter empty seed percentages (in frequency), but the basal florets produced less empty seeds. Differences in endophyte content between ammonium and nitrate applications were found only in floret position 1 where nitrate reduced endophyte. Also amongst florets under nitrate there was higher endophyte content in floret positions 2, 4 and 7.

Acknowledgements

I would firstly like to thank my supervisors, Mr Craig McGill and Mr Robert Southward, for their feedback, advice and constructive criticism throughout this study.

I would also like to thank Dr Phil Rolston and all the staff at AgResearch Lincoln, Canterbury, for kindly allowing me to use seed from their trials as my experimental material and for their much appreciated assistance.

Thanks to Dr Stuart Card and all the staff at AgResearch Grasslands, Palmerston North, for kindly guiding and allowing me to carry out a major part of the experiments (endophyte testing) on their premises and for their appreciated assistance.

I would also like to thank Mrs Kay Sinclair, Institute of Agriculture and Environment, Massey University, for her assistance with the experiments.

Finally, a special thank you to my family for their support throughout this study and thanks to Minshu Xu, my girlfriend, for her patience and assistance with the experiments.

Acknowledgements

Table of contents

List of Figures

List of Tables

Chapter 1. Introduction

1.1. Research background

Nitrogen has been considered to be one of the limiting nutrients for plant growth in the terrestrial system (Agren *et al*., 2012). Over the past several decades, numerous studies have been conducted in New Zealand and elsewhere to ascertain the effect of nitrogen in seed production of perennial ryegrass (*Lolium perenne* L.). One of the major findings was that the application of nitrogen fertilizer contributed to an increase in seed yield of perennial ryegrass and other grasses in New Zealand through increasing seed yield components: thousand seed weight (TSW), number of seed heads, spikelet number per seed head, and floret number per spikelet (Brown, 1980; Hebblethwaite *et al*., 1980; Hill *et al*., 1999). Rolston *et al*. (2008) also found, consistent with Hill *et al*. (1999), that seed yield increased along with an increase in nitrogen supply but levelled off or was depressed at higher nitrogen applications, and that the optimal nitrogen application varied depending on the amount of mineral nitrogen available at any specific site. However, few studies have reported the nitrogen effect on seed quality in perennial ryegrass.

Endophytic fungi of the genus *Epichloë* (syn. *Neotyphodium*) are important in pastoral agricultural systems, because they can enhance the competitiveness of their specific host plants. It is widely known there is a mutualistic relationship between endophyte and certain pasture grasses, in particular, tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass. The asymptomatic endophyte infection is beneficial to pastures especially by providing resistance to insect pests and drought stress. This resistance is conferred by the alkaloids produced by the endophyte. The effect of nitrogen on alkaloid production in plant tissues has also studied (Stewart, 1986; Lane *et al*., 1997; Hunt *et al*., 2005; Krauss *et al*., 2007; Rasmussen *et al.*, 2007), but with

no consistent conclusions. Since a linear relationship exists between alkaloid and endophyte concentration in any given tissues (Rasmussen *et al.*, 2009), there is considerable interest on the effect of nitrogen on endophyte concentration. Furthermore endophytes are imperfectly vertically transmitted from one generation to the next; with endophyte loss occurring at stages such as: from infected plants to reproductive stems, from maternal tissue to embryo in the developing seed or from seeds to seedlings (Afkhami & Rudgers, 2008).

Spikelet positions and floret positions within a spikelet are also considered to have an effect on the seed quality of perennial ryegrass. Different floret positions seem to affect the seed weight of perennial ryegrass more than spikelet positions (Warringa *et al*., 1998a). The differences in seed weight among the floret positions may be attributed to their different sink strength. Another parameter of seed quality, germination percentage, has been seldom studied, even though seed positions are considered to affect germination percentages of *Eremopyrum distans* (Wang *et al*., 2010).

1.2. Research objectives

This research had three main objectives:

1. To identify the effect of six different nitrogen treatments on seed quality of perennial ryegrass cv. Halo including thousand seed weight (TSW), germination percentage, and seed purity.
2. To identify the effect of different spikelet and floret positions on the seed quality of 'Halo'.
3. To identify the effect of six different nitrogen treatments and the effect of different floret and spikelet positions on the endophyte transmission from seed parent to seedling.

Chapter 2. Literature reviews

2.1. Ryegrass

Ryegrasses are indigenous to Europe, Asia and North Africa, and have become the most widely grown cool-season grass in the world (Hall, 1992). They have also gained a reputation of having long growing seasons, producing high yields and high quality forage under environments that supply sufficient nutrients, water and which have appropriate grazing management applied in New Zealand and other temperate zone areas.

2.1.1. Significance of ryegrass

As a pasture the vegetative growth of ryegrass (tillering and leafiness) depends on temperature, water supply and nitrogen supply. A warm spring can encourage reproductive growth, which competes with vegetative growth, reducing its feed value. Insufficient nitrogen supply may result in a reduction of dry matter (Langer & Hill, 1991; Stewart *et al*., 2014).

As a result of their high forage quality, fast establishment, easy management and compatibility with other pastures species, ryegrasses are one of the most widely used pastures for grazing.

For seed production of ryegrass, the application of the plant growth regulator trinexapac-ethyl, fungicides that control stem and crown rust and blind seed disease,

split nitrogen treatments, and irrigation are important management methods to enhance the reproductive growth of ryegrass while reducing seed yield loss (Hill *et al*., 1999; Rolston *et al*., 2004). Ryegrasses have a low tolerance to drought conditions and, as a result, they need to grow on moist, yet well-drained soils to maximize their growth (Hall, 1992). Generally, well-drained soils are preferred for most seed crops including ryegrass. This is because ryegrass only has a moderate tolerance to wet conditions; standing water encourages growth of diseases and anaerobic conditions which is detrimental to seed production (Kernick, 1961). Well-drained soils with adequate irrigation for any dry periods particularly in summer are favoured. The application of nitrogen is also favourable to ryegrass growth. A soil test is necessary to guide any application of nitrogen because excess application of nitrogen leads to nitrate toxicity and N and sugar imbalance in animals fed in the pasture paddocks, environmental pollution from surface runoff, or leaching. Insufficient application makes the ryegrass less competitive with other grasses and weeds (Hall, 1992) and seed production is reduced (Hill *et al*., 1999). When the ryegrass is being grown for seed production to achieve a high seed yielding in addition to soil and nitrogen requirements, the plant growth regulator trinexapac-ethyl, an anti-gibberellin is also used. Trinexapac-ethyl reduces stem height resulting in the repartitioning of carbohydrates from the stem to seeds resulting in more seeds being produced. Furthermore, seed losses are reduced by alleviating the severity of ryegrass lodging both by delaying the onset of lodging as well as its extent (Rolston *et al*., 2004).

2.1.2. Ryegrass species

There are two main ryegrass species, annual ryegrass/Italian ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*L. perenne*). A hybrid of the two (*L. hybridum* syn. *L. x boucheanum*) is also used. And even within these three, there are variations. In perennial ryegrass, flowering time can be divided into three types: early,

middle/standard, and late which affects the heading date (the date when 50% of a grass population starts showing flower or seed heads in spring). The ploidy level, determined by the number of chromosome sets, can also vary in perennial and Italian ryegrass. This is either diploid (2n or two chromosome sets) or tetraploid (4n or four chromosome sets). Generally, compared with tetraploid, diploid cultivars have higher tiller density and dry matter per kilogram of feed but smaller leaves and seeds. In contrast tetraploid cultivars have larger leaves, tillers, and seeds, but fewer tillers.

Italian/annual ryegrasses are short-lived. Their life cycle can range from one year (true annuals) to two or three years depending on their genetics and the environment. Wetter summers tend to increase their lifespan. These grasses have a similar appearance to perennial ryegrass but with larger tillers and leaves, and greater winter activity (White & Hodgson, 1999).

Hybrid ryegrasses can be divided into two types, long-rotation (which resembles the perennial type more) and short-rotation (which resembles the Italian type more).

There are also other types of hybrids. *Festuloliums*, which are produced by crossing ryegrasses (*Lolium* spp.) with fescues (*Festuca* spp.), are widely used around the world because of their improved stress tolerance (Barnes *et al*., 2014). Matthew *et al*. (2012) suggests that it is unrealistic to produce a perennial ryegrass with high drought tolerance and the ways to enhance drought tolerance of the pasture are to produce hybrids that have greater rooting depth and enable introgression of germplasm with high drought tolerance. Thus crossing perennial ryegrass with meadow fescue or tall fescue, which have better root systems and greater drought tolerance (Singh, 2007), is an option to improve drought tolerance including

incorporating the highly sought-after endophyte (*N. uncinatum*) that produce loline alkaloids. These loline alkaloids have activity against a broad-spectrum of insect pests and are considered superior to currently known endophytes used in perennial ryegrass.

2.2. Perennial ryegrass

2.2.1. Vegetative description

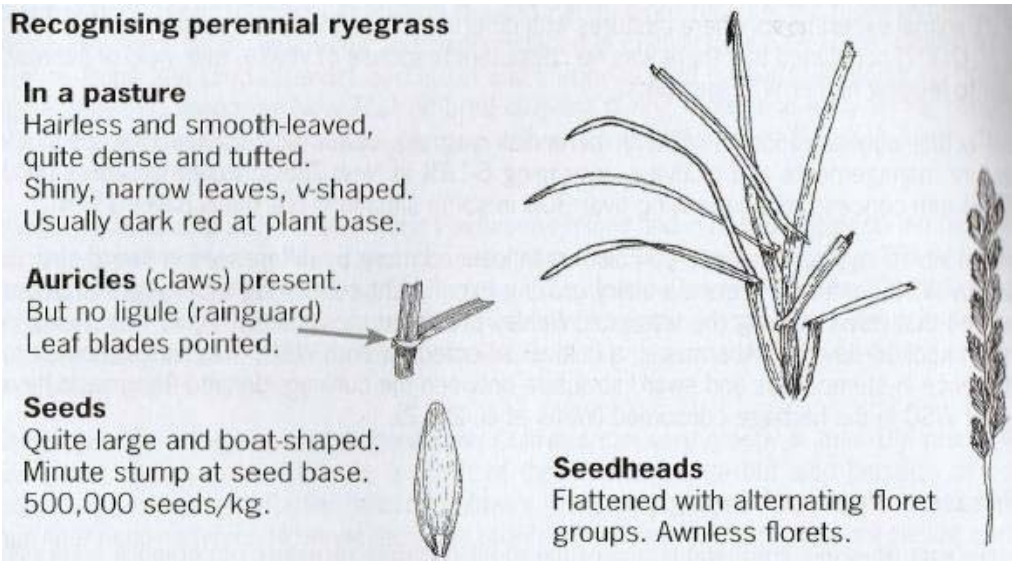
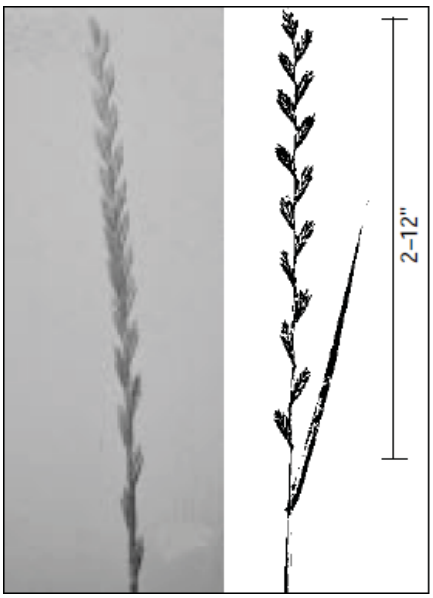


Figure 1. Components of a perennial ryegrass (Stewart *et al.*, 2014).

Perennial ryegrass is a compact grass with limited ability to spread and is sometimes referred to as a bunchgrass (Cook, n.d.). It has dark green shiny leaves on the under-surface and productive tillers (Figure 1). The ligule is short and the auricles are small. The base of the tiller is always bright red to purple. On the upper surface, the leaf blade has parallel veins shaped as corrugated ribs (Matthew, 2003). Perennial ryegrass has vigorous tillering and the stem apex and axillary buds are close to soil level (Stewart *et al.*, 2014). It is relatively shallow rooted (Kemp *et al.*, 1999).

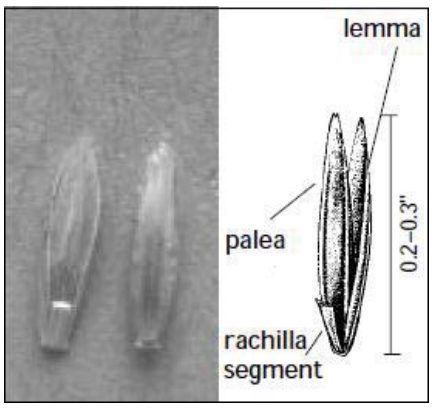
2.2.2. Reproductive description

Perennial ryegrass' inflorescence is a spike (Figure 2a). Each spike is made up of individual spikelets (Figure 2b) which are the flower units. Spikelets consist of a glume, empty bract at the base of spikelet, and florets of which there are 3 to 10 within a spikelet. A floret is characterised by a lemma (a lower bract enclosing the flower), and palea (an upper bract enclosing the flower). A floret has the potential to produce a seed which is a ripened ovule containing an embryo, nutritive tissue (mainly endosperm) and seed coat (Figure 2c).



(a)

(b)



(c)

Figure 2. The inflorescence (a), spikelet and floret (b) and seed (c) of perennial ryegrass (Hannaway *et al.*, 1999).

2.2.3. Ryegrass physiology

Perennial ryegrass generally establishes easily and rapidly, particularly if seed is of high vigour. In New Zealand, perennial ryegrass is the fastest establishing of the perennial grasses (Kemp *et al*., 1999). However, perennial ryegrass has C3 metabolism, where the Calvin cycle is the only pathway of the dark reaction process in photosynthesis meaning that only limited CO_2 can be absorbed under water stress conditions as stomata are induced to close. They are therefore susceptible to drought and high temperatures which will have a negative effect on its growth. The optimum temperature for perennial ryegrass growth is 18°C (Kemp *et al*., 1999). Higher temperatures may cause a reduction in the growth rate (Najda, 2004). This is caused by the closure of mesophyll cells under temperatures above the optimum which contributes to a reduction in internal CO_2 concentrations and, therefore, a decrease in the photosynthesis rate. Drought conditions normally prevent the growth of the pasture or even kill some plants. Turner *et al*. (2012) found that a reduction of water availability results in a reduction of leaf dry matter (DM) accumulation for perennial ryegrass (33% and 66% reduction of water availability resulted in 78% and 35% decrease of leaf DM respectively). As a consequence, the application of irrigation is important in New Zealand in areas that experience severe dry periods during summer. However, the pasture can recover rapidly from stresses such as drought and high temperature when adequate growth conditions are applied (Langer & Hill, 1991). Ryegrass can also recover easily from hard grazing and, therefore, it is widely used for grazing and is able to sustain high animal production (Kemp *et al*., 1999).

2.2.4. Ploidy level

Perennial ryegrass is naturally a diploid pasture with 14 chromosomes however several tetraploid cultivars have been developed artificially (Nair, 2004). The number of chromosomes is doubled by immersing the germinating seeds in a solution of colchicine (Langer & Hill, 1991; Nair, 2004). A tetraploid perennial ryegrass has twice

the normal diploid number of chromosomes in the cell nucleus (i.e. 28 rather than 14 chromosomes). Tetraploid ryegrass has an increased cell size resulting in a higher ratio of water soluble carbohydrate to fibre than diploid ryegrass (Balocchi & López, 2009). Therefore, the tetraploid ryegrass is more palatable to animals which improves ryegrass intake and increases animal production, but, because of the higher water soluble carbohydrate content, the actual dry matter intake is less than that of diploid ryegrass when the same amount of grass is grazed (Stewart *et al.,* 2014). With regard to the grazing management of tetraploid ryegrass**,** it is important to monitor grazing pressures, because its feed quality and palatability are favoured. Therefore tetraploid pasture is sensitive to overgrazing which may result in reduced persistence (Stewart *et al.,* 2014). In terms of the farm management for tetraploid ryegrasses, the recommended sowing rates are higher than diploid because of the larger and heavier seeds. For instance, according to Agricom (2012), the sowing rate of the diploid ryegrass cultivar 'Kingston' is recommended as 18 to 20 kg/ha while that of 'Halo' (tetraploid cultivar) is 25 to 30 kg/ha. Tetraploids are not suitable for growth in wet areas with heavy soils because treading damage will occur or farms with a high level of insect pest populations, especially Argentine stem weevil, who find tetraploid plants more attractive because of the higher carbohydrate content (Agriseeds, 2012a).

2.2.5. Cultivars

There are approximate 30 different perennial ryegrass cultivars available in the market of which the diploid perennial ryegrasses are still the major cultivars (Stewart *et al.*, 2014).

Halo is a currently released tetraploid perennial ryegrass cultivar by Agricom which has been bred through combining 'the best' tetraploid perennial genetics with strong winter growth, summer production and summer persistence in moist or irrigated

conditions, the majority of which originated from north-west Spain (Agricom, 2012). New Zealand breeders only started to use germplasm collected from Spain, France and Italy to expand the genetic resources for both diploid and tetraploid ryegrasses in New Zealand from 1986. Since 1996, the tetraploid cultivars Nevis, Quartet, Ceres Horizon, Banquet and Grasslands Sterling have been released onto the New Zealand market. Almost all were based on adapted New Zealand diploid material, although some material from Europe has also been incorporated in cultivar Sterling (Stewart, 2006). Recently, more tetraploid cultivars have been released such as Grasslands Ohau, Bealey, Halo, and Base (Stewart *et al.,* 2014).

2.2.6. Heading date

The heading date or ear emergence date is defined as the date when 50% of a grass population start showing flower or seed heads in spring; the ear emergence date of the cultivar 'Nui' is labelled as day 0 which is around 22 October in New Zealand (Stewart *et al*., 2014). Normally, based on the heading date, ryegrass cultivars can be divided into four types: early, mid-season/standard, late, and very late (Stewart & Charlton, 2006). Early is defined as more than 8 days before 'Nui'; mid-season/standard from 7 days before 'Nui' to 7 days after 'Nui'; late from 8 to 21 days after 'Nui' and very late from ≥ 22 days after Nui (Stewart & Charlton, 2006; Stewart *et al.*, 2014).

As the flowering head emerges, stems start to develop and result in an increase in fibre and a reduction of metabolisable energy (ME). ME is the energy available for use by the cow and has units of MJ/day. It is the energy used for maintenance of body systems, activity, milk production, pregnancy and weight gain (Edwards & Bryant, n.d.). Waghorn (2007) suggested that ME is an indicator of digestibility and DM intake where a high ME value is related to potentially high DM intake. It is noteworthy that, in New Zealand, ME is considered to be a better indicator of the

pastures' ability to support animal production than the simple dry matter content. Seasonal changes in forage composition also affect ME values (Waghorn, 2007). This is because chemical composition and mineral concentration in grasses vary in dry and rainy seasons and higher ME value appears in rainy seasons than that in dry seasons (Evitayani *et al*., 2004). But to a large extent, the changes in ME are due to the changes in the numbers of reproductive stems and tillers that occur as summer approaches. Pasture with a late or very late heading date indicates a delay in stem development and fibre increase and, therefore, a slower loss of ME value than with cultivars with an early or standard heading date. Accordingly, researchers and farmers consider that pastures with a late heading date always provide a higher feed quality further into late spring. Halo is characterized as a very late heading pasture whose heading date is approximately 25 days later than that of Nui (Agricom, 2013) and, therefore, it produces pasture with high feed quality and a relatively high ME value. However, the use of ME values as indicators of feed quality also has limitations, because ME cannot indicate the feed composition or if adequate nutrient components are present for animals. Feed quality can only be assessed accurately when the proportions of ME values for nutrient components are measured (Waghorn, 2007).

2.3. Tall fescue

Tall fescue (*Festuca arundinacea*) has been described as a perennial ryegrass with deep roots, large tillers and dark green leaves. It is supposed to be slow to establish (Kemp *et al*., 1999). The pasture requires high soil fertility and responds well to nitrogen application. In contrast to perennial ryegrass, it can tolerate acid, alkaline, and poorly drained soils (Charlton & Stewart, 1999). Because of better drought and heat tolerance of tall fescue than perennial ryegrass, it has been a popular alternative pasture in drier regions in New Zealand especially in summer dry. According to the flowering period, tall fescue can be divided into three types, very-

early flowering, early flowering, and mid-season flowering but it can also be recognized as broad-leaved and fine-leaved types based on the tiller density (Charlton & Stewart, 1999). A limitation to the use of tall fescue was that endophyte was not presented in tall fescue sold in New Zealand and the wild type usually contains toxic endophyte. However the identification of the non-toxic endophyte MaxP has renewed interest in using this pasture in dairy farms (Minneé *et al*., 2010).

2.4. Endophytes

2.4.1. Importance in pasture

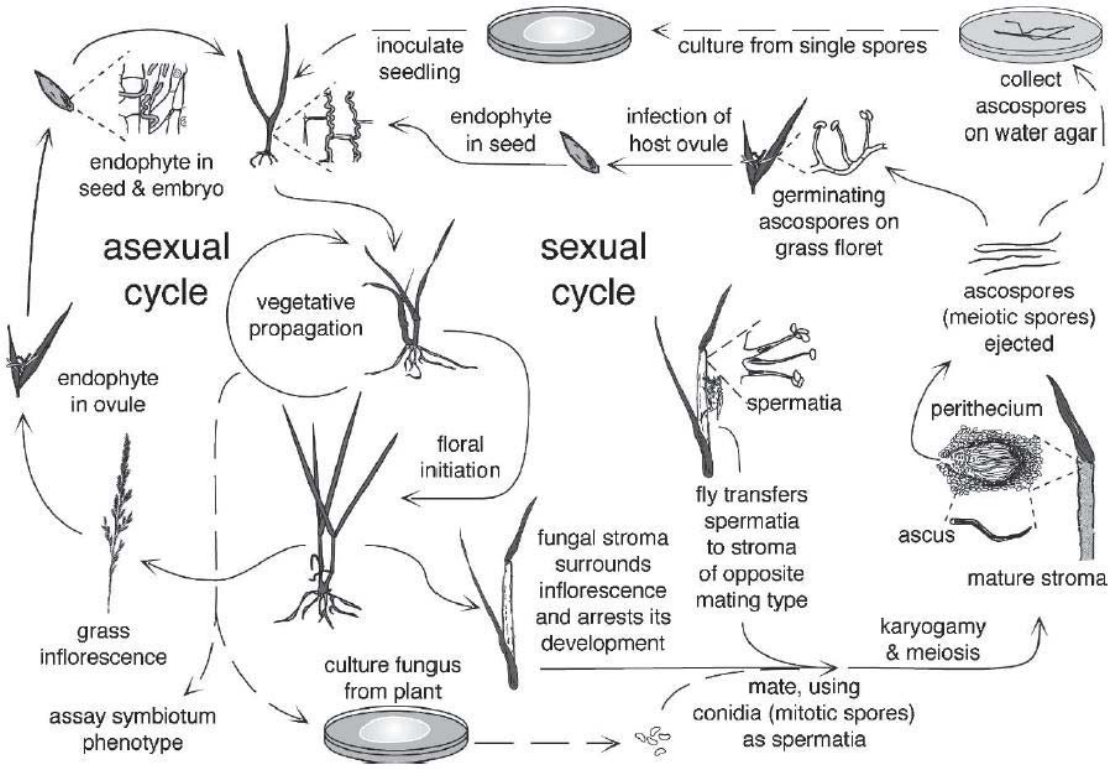


Figure 3. Life cycle of Epichloë endophyte from Schardl *et al.* (2009).

Neotyphodium endophyte is a fungus living and growing between plant cells of many grass species. The life cycle of *Neotyphodium* endophytes, which is now defined as asexual *Epichloë* spp. (Leuchtmann *et al*., 2014), is shown in Figure 3, including how it

is able to transfer from the seeds into the germinated seedling, then growing plant and ultimately into the seeds of the next generation as the plant becomes reproductive. Endophyte is attractive to farmers because of the improved performance of pastures infected with endophyte. One of the advantages endophytes confer is enhanced insect resistance thereby protecting plants under insect pressure. This resistance is conferred by certain alkaloid compounds (peramine, ergovaline, epoxy-janthitrems and lolines) produced by the endophytes. Young *et al*. (2013) reported that even though the alkaloids contribute to insect resistance of perennial ryegrass, there are some negative effects on animals. Specifically, ergovaline is a vasco-constrictor reducing livestock's weight gain as a result of elevation of both rectal temperature and respiration rate (heat stress) and can cause gangrene of extremities including feet (Bluett *et al*., 2005). Another alkaloid, lolitrem B, can trigger ryegrass staggers when the levels of lolitrem B are above a certain level 'threshold level'. The 'threshold level' can differ between animals in the same species and between species (Blythe *et al*., 2007). In contrast to ergovaline and lolitrem, peramine is safe for livestock. In the New Zealand market, there are several perennial ryegrasses that contain novel endophytes (AR37, Endo5, AR1, and NEA2+6) (Kerr *et al*., 2012). AR1 and AR37 are available with the cultivar 'Halo'.

In addition, a fungal endophyte present in tall fescue is also attractive to farmers. This endophyte, *Neotyphodium coenophialum* is known to infect most tall fescue plants. Ergovaline is produced in pastures infected with *Neotyphodium coenophialum*, which benefits pastures by providing resistance to pests but is again detrimental to animals (Clay & Schardl, 2002; Browning *et al*., 2007). Nontoxic endophytes in tall fescue have been investigated (Lacefield *et al*., 2003). Currently, the endophyte MaxP has been developed to provide protection against insects and improve drought tolerance while having no negative effect on animals (Popay & Thom, n.d.). The production of loline alkaloid by the MaxP is considered to confer a greater drought tolerance in tall fescue than those endophyte free (Nagabhyru *et al*., 2013).

2.4.2. Detection of Endophytes

Fungal endophytes live in intercellular spaces. Their detection in perennial ryegrass is normally through histochemical, immunological or molecular techniques (Brandl, 2013). Histochemical detection of endophyte is based on staining with chemical dyes, such as aniline blue or rose Bengal, after which the fungal hyphae can be observed under a light microscope (Najafabadi *et al*. 2009). The immunological technique used to detect the presence of fungal endophyte involves an enzyme-linked immunosorbent assay (ELISA) and attaching specific antibodies to fungal cell walls or metabolite while chromogen solution is also needed for a colour reaction (Koh *et al*., 2006). The detailed process for the detection of fungal endophytes is fully presented in the methods chapter. In addition, molecular methods which include DNA extraction and amplification through polymerase chain reaction (PCR) with specific primers, resulting in patterns of simple sequence repeats or microsatellites can be used (van Zijll de Jong *et al*., 2008). The molecular methods are much more complicated than the histological or immunological techniques.

2.4.3. Commercially available novel endophytes

The commercially available novel endophytes are listed in Table 1. The endophyte AR1 has become increasingly popular in New Zealand (Johnson *et al*., 2013). This is because the only alkaloid produced is peramine, but not lolitrem B or ergovaline as in the wild endophyte. This has offered improved insect tolerance and resultant milk production without causing animal health problems (Popay & Baltus, 2001; Popay & Hume, 2011). The alkaloid peramine provides tolerance to several insects including Argentine stem weevil (*Listronous bonariensis*) and pasture mealy bug (*Balanococcus poae*). Some resistance to black beetle (*Heteronychus arator*) is also achieved.

Table 1. Alkaloid profile of endophytes available in ryegrass cultivars sold in New Zealand

Endophyte strain	Alkaloid profile	References
Standard endophyte (SE)	Lolitrem B Ergovaline Peramine	Siegel *et al*. (1990) Christensen *et al*. (1993)
AR1	Peramine	Bultman *et al*. (2003)
AR37	Epoxy-janthitrems	Tapper & Lane (2004)
NEA2	Lolitrem B Ergovaline Peramine	van Zijll de Jong *et al*. (2008)
Endo5	Ergovaline Peramine	Popay & Hume (2011)

In contrast, Endo5 contains peramine and some level of ergovaline but still not lolitrem B. Endo5 perennial ryegrass has better persistence than AR1 in areas that suffer pasture attack from black beetles. The alkaloid, Lolitrem B, can cause ryegrass staggers, however, ryegrasses infected with AR1 and Endo5 do not contain Lolitrem B and therefore have high levels of freedom from ryegrass staggers.

NEA2, developed by NZ Agriseeds, is a mixture of endophyte strains and the alkaloid profile varies depending on the infected cultivar according to the proportion of endophyte strains within each particular cultivar. No ryegrass staggers for NEA2 has been reported in sheep or cattle to date.

The most recent novel endophyte, AR37 does not contain the alkaloid compounds of peramine, lolitrem B, and ergovaline. Instead, it has a set of compounds called epoxy-janthitrems (a group of indole-diterpenes related to lolitrem B) which confer resistance to a wide range of insect pests (Argentine stem weevil, adult black beetle, pasture mealy bug, root aphid (*Aploneura lentisci*) and porina (*Wiseana cervinata*) and enhance the performance of ryegrass infected with AR37. Such compounds have also been confirmed to contribute to lower frequency and severity of staggers in animals grazing AR37 under some conditions (Finch *et al*., 2013). In addition, as peramine does not confer good resistance against black beetle, ryegrasses infected

with AR1 are not recommended in regions with high levels of such insects. In northern parts of New Zealand, annual dry matter production is higher with ryegrasses infected with AR37 in contrast to those with AR1, especially during late summer and autumn. Despite the fact that the endophyte AR37 does not produce lolitrem B, it can cause ryegrass staggers but this is less frequent than wild or standard endophyte (Fletcher and Sutherland, 2009). The possible explanation for endophyte AR37 causing mild ryegrass staggers could be its production of epoxy-janthitrems. Epoxy-janthitrems are tremorgenic which may directly cause ryegrass stagger-like symptoms or alternatively these molecules may act in concert with other mycotoxins from the endophyte leading indirectly to ryegrass stagger-like symptoms (Fletcher, 2005). Even though there are risks of ryegrass staggers resulting from Halo AR37 (Johnson *et al.*, 2013), it is still strongly recommended for dairy and sheep/beef farming systems because of its outstanding ME value and high water soluble carbohydrate (WSC) value (Agriseeds, 2012b), both key drivers of animal performance (Kirkwood, 1983), and its broad-spectrum insect control in warmer northern areas.

2.5. Nutrients and nutrient cycle

2.5.1. Nitrogen

Compared with other plant nutrients, nitrogen is considered to be the limiting nutrient in the terrestrial system (Agren *et al*., 2012). There are three soluble inorganic nitrogen forms found in nitrogen fertilizers; ammonium (NH_4^+), nitrate (NO_3^-), and urea ($CO(NH_2)_2$). During plant growth, nitrogen uptake does not only come from fertiliser but also nitrogen in soils. Also, nitrogen in soils can be transformed into other nitrogen forms which are involved in the N cycle (Figure 4). As can be seen from Figure 4, ammonium and nitrate can be absorbed directly by the plants but urea needs to be transformed into ammonium first and then absorbed by plants. In the nitrogen cycle, there are four primary transformations of nitrogen,

mineralization (soil organic matter (RNH_2) or $CO(NH_2)_2$ into NH_4^+), nitrification (NH_4^+ into NO_2^- and then NO_3^-), immobilization (NO_3^- into RNH_2), and denitrification (NO_3^- into gases N_2, NO, or N_2O) (Robertson & Groffman, 2007).

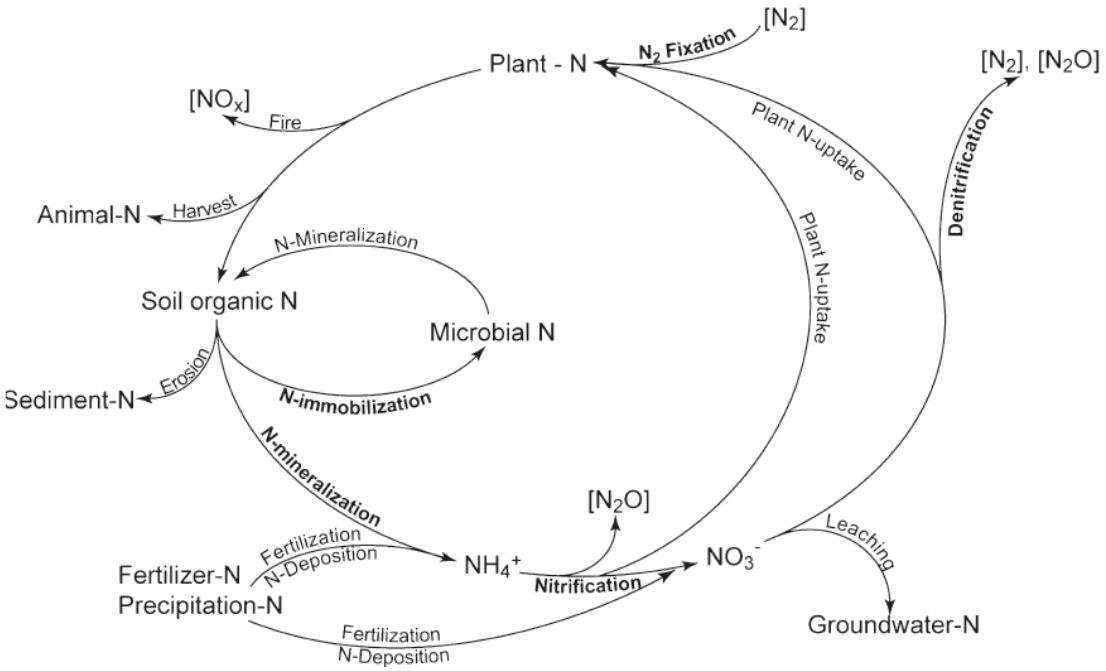


Figure 4. The utilization of nitrogen fertilizer and the soil nitrogen cycle (Robertson & Groffman, 2007).

As mineral N through mineralization can be used by plants, predicting the optimum nitrogen application is important, because excessive N inputs can accelerate nitrate leaching and soil acidification which reduce the nitrogen use efficiency (Rolston *et al.*, 2008). Based on 17 perennial ryegrass seed crop nitrogen application trials that used several cultivars, Rolston *et al*. (2008) found that the average optimum nitrogen application was 143 kg/ha with a range from 70 to 240 kg/ha. Also, the average mineral N in the top soil (0-30 cm) in the 17 trials was 41 kg/ha with most ranging from 30 to 50 kg/ha. Using a total N requirement model based on a nitrogen requirement of 185 kg/ha (spring N applied (kg/ha) = 185 - mineral N (0-30 cm) (kg/ha)) they concluded that the N predicted may either underestimate or overestimate the optimal N input in different years and at different sites around mid

and south Canterbury. The variations in optimal N inputs in the 17 trials were caused by different cultivars and different amounts of mineral N in the top soil (0-30 cm) from 2003 to 2008 (Rolston *et al.*, 2008). They suggest that an accurate N prediction depends on both cultivars and mineral N values and can be calculated using a conservative linear-plateau model when higher N inputs will not affect yields significantly or using a polynomial model when highest N input might reduce yields.

2.5.2. Potassium

Potassium is another important nutrient used by plants. K can activate enzymes, regulate stomatal activities, and stimulate transport of sugars into plants (Britto & Kronzucker, 2008). Plants with adequate K have a high drought tolerance, because the opening and closure of stomata can be completed rapidly which means the stomata have a fast response to changes in water availability (Wang *et al.*, 2013). K is an essential nutrient in plant photosynthesis as well as for transporting photoassimilate. Therefore another important role of K is that it can stimulate plant reproductive growth which is important for seed production (Wang *et al.*, 2013).

2.6. Nitrogen and its effect on endophytes

There is evidence that increased N supply leads to a reduction of alkaloids, such as lolitrem B and peramine, (estimated by quantitative PCR) in perennial ryegrass leaves or components favoured by mammalian herbivores. This could be a dilution effect (i.e. increased N supply stimulates the growth of the grass plant more than that of the fungus) (Rasmussen *et al.*, 2007). Stewart (1986) also suggested that the increased N inputs when applied at spikelet initiation reduced the alkaloid concentration (alkaloid content in a single seed) not only in the harvested seed crop, but also the subsequent seedling, mature plant, and harvested seeds from the next generation. A downward response in alkaloid concentration in ‘Grasslands Nui’ with

increasing N application has been confirmed in earlier work by Stewart (1986). The application of 100 N kg/ha gives significantly lower alkaloid concentrations compared with that of 0 and 40 N kg/ha (Table 2).

However, it is interesting that, in the same study, the application of urea fertiliser increased alkaloid concentration in perennial ryegrass suggesting alkaloids in pasture plants and seeds may be under different physiological control. Lane *et al*. (1997) reported that alkaloid concentration in pasture may both increase and decrease with high N inputs. In some other recent studies (Hunt *et al*., 2005; Krauss *et al*., 2007), in perennial ryegrass, no consistent relationship between N application and endophyte alkaloids concentration in plant tissues was found.

Table 2. Effect of nitrogen applied on endophyte infection and alkaloid concentration in seed (Stewart, 1986)

Nitrogen rate (kg N/ha)	Endophyte infection (%)	Alkaloid concentration (µg/seed)
0	64a	51a
40	71a	40a
100	68a	12b

Note: endophyte concentrations with the same letter are not significantly different

The percentage of endophyte infection was generally high but ranged from 50% to 100% in sleepy grass (*Achnatherum robustum*) infected with *Neotyphodium*. Less is known about the causes of such infection variations which may be attributed to geographic differences (Faeth *et al*., 2006). Majewska-Sawka and Nakashima (2004) found that the natural endophyte transmission percentage is close to 100% and the pathway of transmission through flowers and seeds is via sporophytic maternal tissue. The transmission of *Neotyphodium* and *Epichloë* endophyte was reported by Afkhami and Rudgers (2008) as imperfect vertical transmission. This means that the failure of endophyte presence can be caused by the failure of transmission from infected plants to seed-bearing stems, from infected seeds to seedlings, or from maternal

seed tissue to embryo. However, endophyte infection frequency directly in response to different N applications is poorly reported. Because alkaloids produced by endophyte are nitrogen-rich compounds, the supply of nitrogen may increase or decrease alkaloid content which does not definitely mean an increase or a decrease of endophyte concentration (Faeth, 2002; Rasmussen *et al*., 2009). In other words, the relationship between alkaloid and endophyte contents can be linear in given tissues such as sheaths but not in tissues such as mature leaves. Therefore, none of the studies above were able to consistently confirm whether N application altered endophyte transmission percentage in *Lolium* spp. Also there are no reports in the literature on the effect of different nitrogen forms on successful endophyte transmission frequency from seed to seedlings.

2.7. Seed quality and floret and spikelet position

2.7.1. Introduction

Ryegrass seed production has long been a focus of researchers and farmers in New Zealand. In recent years, factors, such as nitrogen application, plant growth regulators (PGR), fungicides and irrigation management, have been found to be important factors affecting seed yield and quality. The plant growth regulator, trinexapac-ethyl (TE), has been widely used from about 2000, to reduce lodging. Lodging is reduced by reducing internode height through inhibiting the synthesis of gibberellic acids (GAs). Reduced lodging has reduced the harvest loss of pasture seeds (Rolston *et al*., 2010). In addition, PGR application improves the carbon allocation to reproductive organs leading to the improvement of fertile floret site utilization which can also increase the yield of pasture seeds (Rolston *et al*., 2007; Rolston *et al*., 2004).

Similarly, the application of fungicides also increases seed yield by reducing the seed loss caused by fungal diseases. There are a number of fungal diseases causing seed

yield and quality loss in ryegrass. Stem rust is the main disease limiting ryegrass seed yield and is traditionally controlled by triazole fungicides (Rolston *et al*., 2009). In some trials with low stem rust, diseases such as leaf spot and ovularia spot reduce seed yield by reducing green leaf area (Rolston *et al*., 2004). These diseases can be controlled by epiconazole or triazole/strobilurin fungicide mixes during seed production. Other diseases, such as blind seed and ergot, also cause problems during ryegrass seed production. Blind seed is a significant problem under wet and cool conditions (Rolston *et al*., 2006). More recently, apart from triazole fungicides, strobilurin fungicides have been found to be useful in controlling various diseases (stem rust and other leaf diseases) as well as maintaining green leaf area (Rolston *et al*., 2009). However, there is still no solution for controlling ergot (Rolston *et al*., 2006).

In addition, perennial ryegrass is a heavy user of water and growth is poor during dry and hot seasons without irrigation (Hill *et al*., 1999). Rolston *et al*. (2006) applied irrigation and this contributed to a significant increase in seed yield. The increase was greatest on lighter (shallower) Canterbury soils. In another study, water deficiency was found to limit seed yield by reducing both the number of seed heads and thousand seed weight (TSW) regardless of the timing of a droughts occurrence. However the decrease in seed head number was more significant when drought occurred before anthesis while that of TSW was greater when drought occurred after anthesis (Chynoweth *et al*., 2012). As a result, irrigation is of great value in perennial ryegrass seed production by enhancing both the number of seed heads and TSW, but excessive irrigation will not only increase the cost of seed production but also exert a negative effect on seed yield because of the robust vegetative growth of the plants. For example, the seed yield of perennial cultivar ‘Grasslands Samson’ only responded to irrigation applied approximately from 150 mm to 225 mm, after which seed yield appeared to plateau (Figure 5). This can have economic implications for growers when excess irrigation is applied for no increase in yield but equally growers need to apply adequate irrigation to reach the maximal yields.

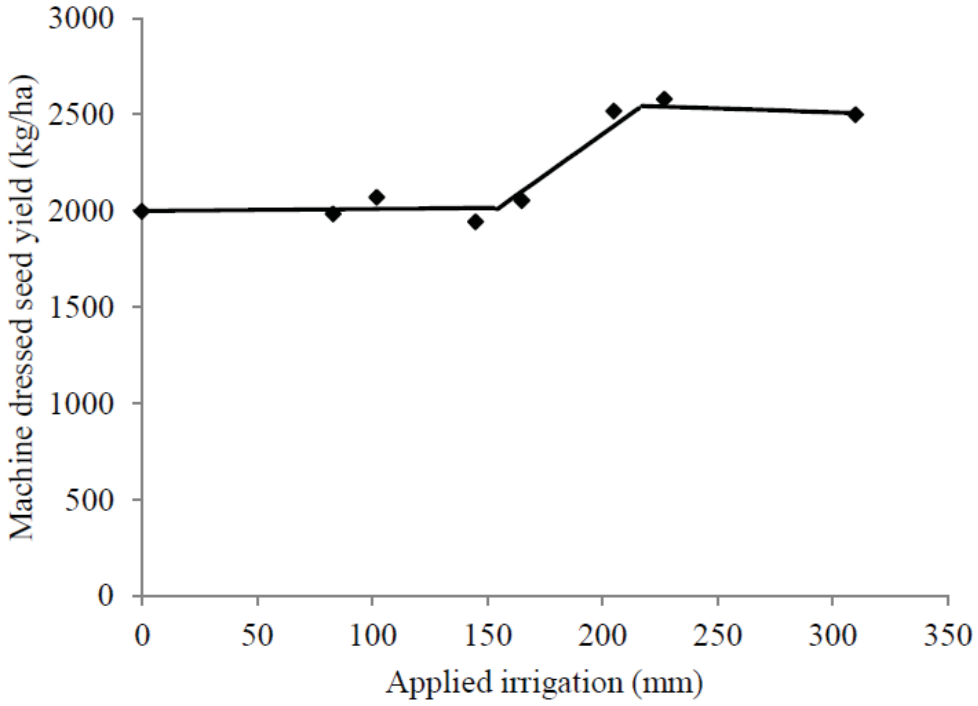


Figure 5. Perennial ryegrass 'Grasslands Samson' seed yield responses to applied irrigation (Chynoweth *et al.*, 2012).

2.7.2. Nitrogen and seed production

Since an early study (Brown, 1980), nitrogen has been thought to increase seed yield of grasses in New Zealand. Over the past 30 years, nitrogen has been the nutrient that has received an increasing amount of attention from farmers and researchers. This is it has been considered to be the most important nutrient limiting seed yield (Cookson *et al*., 2000). Actual seed yield is determined by the seed yield components. These are thousand seed weight (TSW), number of seed heads, spikelet number per seed head, and floret number per spikelet (Hebblethwaite *et al*., 1980). Even though it was known that increasing nitrogen application would increase seed yield, the optimal timing of the application remained a problem (Hampton, 1987). Also according to Hampton (1987), nitrogen application at spikelet initiation gave a higher seed yield than the application at autumn only, or a split application between

autumn and spikelet initiation, and between autumn and ear emergence. However, no difference was found between N applications at spikelet initiation and split N application in autumn, spikelet initiation, and ear emergence. It is notable that seed yield of perennial ryegrass was more correlated with seed head number than the other seed yield components (Elgersma, 1990). N application at spikelet initiation can increase seed head numbers significantly and, therefore, increase the seed yield (Hill *et al*., 1999). In addition, seed yield of perennial ryegrass did not always respond to continuously increasing N application. Yield increased significantly from 180 to 300 kg/ha when N application increased from 80 to 120 kg/ha and levelled off or sometimes was depressed under higher nitrogen applications (Rolston *et al*., 2006; Rolston *et al*., 2008). Recently, the majority of the researchers have focused on predicting the optimal amount of nitrogen applied (Rolston *et al*., 2006). Gislum *et al*. (2013) believed that the economical optimum application rate of N can be calculated based on different environments in Denmark (high productivity area (HPA) with high nitrogen application rate and environmental sensitive area (ESA) with lower advisable nitrogen rate than HPA) with the purpose of reducing N leaching. Rolston *et al*. (2013) suggested that a reduction of spring N application rate from 230 kg/ha to 150 kg/ha can achieve a higher seed yield through reducing early lodging while an interaction between PGR and N application rate and timing is important during grass seed production.

In terms of the effect of nitrogen on seed quality, Hampton (1987) found that different amounts of urea did not alter seed germination percentage of perennial ryegrass cv. Grasslands Nui. A later study by Rowarth *et al.* (1999) found that TSW was not affected by different nitrogen amounts when they were applied at anthesis, but there was a slight increase in TSW when N was added in split applications in autumn, winter, and early spring. They also concluded that TSW was positively associated with herbage nitrogen concentration but the germination percentages under standard germination test conditions were not affected by nitrogen application. In Simić *et al.*'s (2010) study, different amounts of ammonium nitrate

affected the seed quality of the tetraploid Italian ryegrass cv. Tetraflorum including seed germination and thousand seed weight (TSW) only in extremely unfavourable years. However, only a few studies (Warringa *et al*., 1998a and 1998b) have looked at the effect of different type of N fertilisers and floret/spikelet positions on seed yield components and seed quality.

2.7.3. Seed weight

In perennial ryegrass seed dry weight varies much less between similar floret positions in different spikelets than within the inflorescence. In a study by Warringa *et al*. (1998a) the average seed dry weight from individual spikelets located in the spike ranged from 1.15 mg to 1.43 mg. Within a spikelet seed weights had a greater range, from 0.71 mg in the distal floret to 1.86 mg in the proximal floret (Table 3). The variation in seed dry weight within the inflorescence depends greatly on either the ovule dry weight or its growth rate at anthesis and, to a lesser extent, the duration of the growth (Warringa *et al*., 1998a).

Table 3. Average seed dry weight of perennial ryegrass seed from different locations (proximal, central and distal floret position) within a spikelet (Warringa *et al.*, 1998a)

Seed position (floret position)	Average seed dry weight (mg)
Proximal	1.86c
Central	1.38b
Distal	0.71a

Note: Average seed weight with the same letter is not significantly different

Factors controlling inflorescence development, such as temperature, light and nutrients (nitrogen and carbohydrate), determine the ovule weight at anthesis by regulating photosynthesis thereby determining the subsequent availability of carbohydrate to the ovules and ultimately final ovule size. The final seed dry weight for different floret positions within the inflorescence where ‘seed position’ is the

same as floret position is shown in Figure 6 (Warringa *et al.*, 1998b; Atwell *et al.*, 1999). Furthermore, Atwell *et al.* (1999) believed that sink strength also plays an important role in determining the assimilate partitioning and the seed dry weight. This is consistent with earlier findings of Jenner (1980) where the removal of seeds increased the weight of remaining seeds, but relevant research on different spikelet or floret position effect of perennial ryegrass, or any other grass species, on seed weight is still scarce.

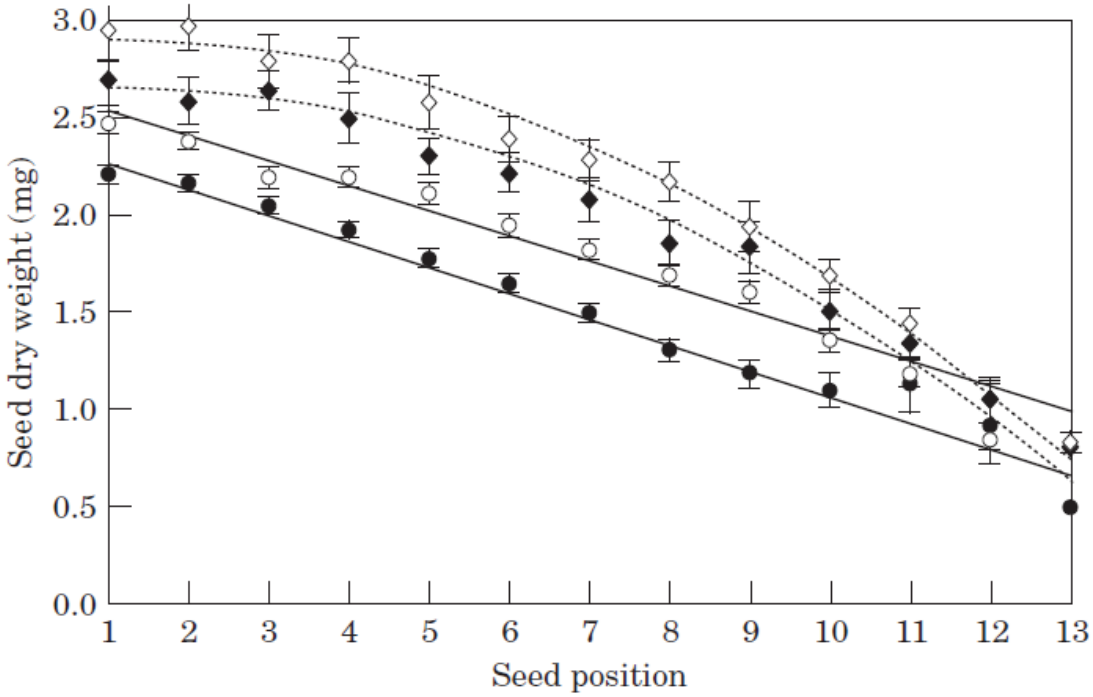


Figure 6. Effect of seed position on seed dry weight of Lolium perenne from Warringa *et al.* (1998b) where ◇ and ◆ represent a cultivar of *L. perenne* Magella while ● and ○ represent a cultivar Barlet; ◇, ○, 100% light; ◆,●, 25% light.

2.7.4. Germination

Previous research (Wang *et al.*, 2010) has been undertaken to look at the effect of the floret position within spikelet in *Eremopyrum distans* (K. Koch) Nevski. The seed position can affect the germination percentage significantly where earlier formed

seeds had a higher germination percentage than later ones. For example under a 30/15˚C germinating regime, the germination percentages of group 3 (position 3 in Figure 8) seeds were higher than that of Group 1 or 2 (positions 1 and 2 in Figure 8) regardless of light and storage effects (Figure 7) (Wang *et al*., 2010). However, there are no studies reported that look at the effect of floret and spikelet positions on seed germination of ryegrass.

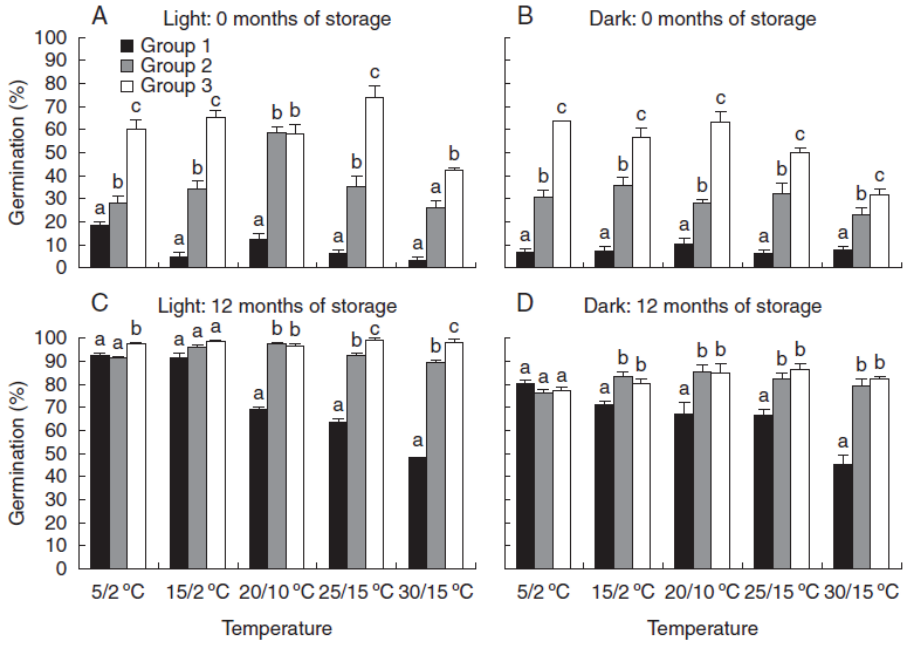


Figure 7. Germination percentages of seed of Eremopyrum distans from three floret positions (groups 1, 2 and 3) germinated under a range of light and temperature regimes and after different lengths of seed storage. Germination percentages with the same letter are not significantly different (Wang *et al.*, 2010).

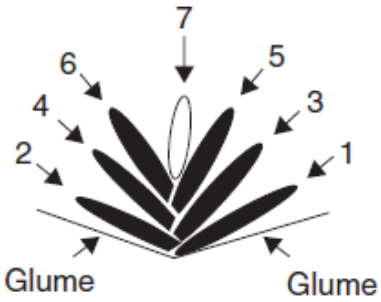


Figure 8. Floret/seed position in a spikelet (Wang *et al.*, 2010).

Chapter 3. Materials and methods

3.1. Seed production

The tetraploid perennial ryegrass cultivar 'Halo' infected with AR37 endophyte, was sown on 10 April 2012 at AgResearch Lincoln, Canterbury, New Zealand (43^{o} 38'S, 172^{o}28'E). The trial site is 11 metres above sea level, and the soil type is a 'Wakanui silt loam'. The previous field history was a crop of oats sown in autumn 2011 and grazed over the winter and spring before being sprayed out in early summer and fallowed over the remaining summer. The site was then ploughed, rolled, maxi-tilled, and harrowed. The Halo seed crop was sown with a 9 row precision plot drill with rows 15 cm apart. Before sowing, soil tests were conducted and mineral deficiencies corrected apart from N and K. Seeds were sown at a rate of 9 kg/ha in March 2012 and treated with 6 nitrogen and potassium applications: nil, urea with and without K, nitrate with and without K, and ammonium in the form of ammonium sulphate(Table 4).

Table 4. Nitrogen and potassium treatments applied to a 2012-2013 season AR37 endophyte infected perennial ryegrass cv. Halo seed crop at Lincoln, Canterbury

N forms	Split N rates/application dates (kg/ha)			Total N rate (kg/ha)	K (kg/ha)
	19-Sep	19-Oct	4-Nov		
1. nil	0	0	0	0	0
2. urea ($CO(NH_2)_2$)	30	75	75	180	0
3. urea ($CO(NH_2)_2$)	30	75	75	180	200
4. ammonium (NH_4^+)	30	75	75	180	0
5. nitrate (NO_3^-)	30	75	75	180	0
6. nitrate (NO_3^-)	30	75	75	180	200

Apart from the nitrogen/potassium applications, other management interventions, such as application of irrigation, fungicides, and plant growth regulators (PGR), were completed as required. Irrigation was applied in late spring and summer with three applications of 40 mm each. The PGR Moddus® (trinexapac-ethyl) was applied once at 1.5 l/ha at Zadocks growth stage GS 32 (average of all tillers). In addition, an endophyte-safe strobilurin fungicide Amistar (active ingredient azoxystrobin) which is specifically safe to AR37 endophyte (Chynoweth *et al*., 2012) was also applied once at late head emergence. The 'Halo' AR37 seed crop was hand harvested on 25 January, 2013 and the seed heads were stored in paper bags in the seed store at Lincoln AgResearch under ambient conditions. The typical day temperature in the store was 20-23 ˚C and night temperature 10-12 ˚C.

3.2. Seed processing

For selected seed heads which were randomly taken from the storage bags in two nitrogen treatments, 180 kg/ha NO_3^- and 180 kg/ha NH_4^+, the floret positions, including those with empty seeds, were also randomly chosen from each spikelet in a seed head and labelled from 1 to 9 in a sequence from the basal to the apical floret.

For the remaining seed heads from all treatments, the spikelet positions were divided into top, middle and bottom sections of the canopy and the seeds threshed from the seed head. The ryegrass spikes were cut using scissors to divide the seeds from the spikes into three different positions, top, middle, and bottom. The spikelets from different positions were separately rubbed by hand (Figure 9a). The seeds were then sieved in a filter to remove impurities of greater size from the seed lot (Figure 9b). Threshed seed was then cleaned using a South Dakota blower (Figure 9c) to remove empty and lighter weight seeds. Seed processing occurred from 7 to 10 May 2013. Subsequent seed quality tests and endophyte presence evaluations in seed from different spikelet positions and floret positions were conducted as separate experiments.

(a)

(b)

(c)

Figure 9. (a) Hand rubbing; (b) Metal sieves; (c) South Dakota blower.

3.3. Effect of nitrogen forms with and without potassium and spikelet positions on seed quality

The following seed quality components were assessed: purity, thousand seed weight (TSW), and germination percentage of the 'Halo' seeds. There were six nitrogen/ potassium treatments x three approximate spikelet canopy positions (top, middle, and bottom) x three replicates. To manage the workload, the experiment was designed so all treatments within a single replicate were assessed on a single day.

3.3.1. Quality Assessment Protocols

For each replicate:

3.3.1.1. Purity

The purity analysis followed the procedure in the ISTA rules for seed testing (ISTA, 2012). Pure seed of *Lolium* spp. is defined as seed with a caryopsis size at least one-third the length of the palea or piece of caryopsis larger than one-half the original size. Each replicate was assessed for pure and other (non-pure) seed, and inert matter. Pure seeds of Halo AR37 were weighed in order to calculate the percentage of pure seeds using the equation below. Other seeds included empty seeds and seeds with a size of caryopsis less than one-third the length of the palea or piece of caryopsis smaller than one-half the original size. Inert matter included ergot. Empty seed may include seed in which no embryo/endosperm development occurred and seed where limited seed fill occurred and the seed appeared empty. The other seed and inert matter weights were also determined and then calculated as a percentage of the combined weight of three components.

$$\textbf{Pure seed (\%)} = \frac{\textbf{Weight of pure seeds}}{\textbf{Total weight of three components in the seed sample}} \times 100$$

3.3.1.2. Thousand seed weight (TSW)

A random sample of a hundred seeds was taken from the pure seed fraction using the spoon method (Kruse, 2004) from each replicate of each treatment and weighed and recorded. To be specific, each replicate was poured uniformly over a tray with a side to side swinging action while the depth of the seed layer did not rise higher than the height of the vertical sides of the spoon. The spatula was pushed vertically through the seed layer down onto the tray and the spoon was pushed vertically onto the tray, adjacent to the spatula. The spoon was tilted at 45° and, while the spoon's

edge was in contact with the spatula, both were withdrawn from the tray, holding the seed in-between them. The seed was deposited into a collecting pan. The pure seed were then poured on a smooth, levelled surface and mixed thoroughly using a spatula. The seeds were spread into a long narrow line. Then 100 seeds were counted starting at one end of the line. Based on the weight of 100 seeds, the TSW of each treatment was calculated according to the following equation:

TSW (g) = mean weight of 100 seeds weight (g) (average of 8 weights) × 10

3.3.1.3. Germination

The germination method for *Lolium* spp. is top of paper (TP) (ISTA, 2012). Before germinating the seeds, 100 seeds per replicate, per treatment were sampled from one of the eight 100-seed samples per replicate, per treatment in the TSW test. Seed were then placed on two steel blue germination blotters (Anchor Paper Company, Saint Paul, Minnesota, USA) that had been soaked in 0.2% KNO_3 solution for 30 seconds then excess solution was allowed to drain off. Blotters and seeds were then placed into air tight plastic containers. The seeds were prechilled (5 °C) in cold room 1 on the third level of AgHort B building at Massey University for seven days (to alleviate any residual dormancy). Subsequently, the seeds were moved into an incubator operating at alternating temperatures and light regimes of 20 °C for 16 hours dark and 30 °C for 8 hours light. The first (interim) count occurred five days after sowing the seed and the final count was completed fourteen days after sowing.

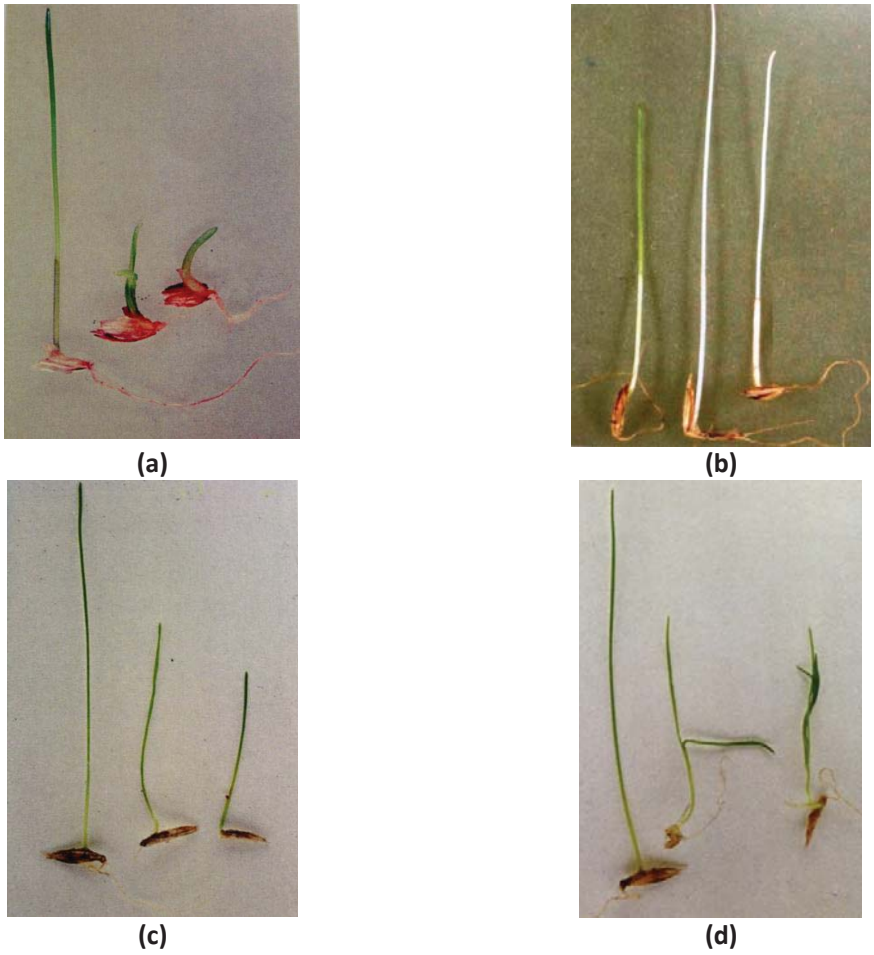


Figure 10. Examples of normal and abnormal seedlings of *L. perenne* (from Don, 2009). Note: Normal seedlings (left in (a), (b), (c), and (d)), abnormal seedlings (middle and right in (a), (b), (c), and (d)).

At the final count, seedlings were classified as normal or abnormal based on the definitions in the ISTA Handbook on Seedling Evaluation (Don, 2009). Normal seedlings of *Lolium* spp. must have the primary root, mesocotyl and coleoptile intact or with acceptable defects (Table 5). A seedling was classified as abnormal when it was deformed (Figure 10a), the colour of the seedling was yellow or white (Figure 10b), the primary root was missing (Figure 10c) or coleoptile was split from the tip for more than a third of the length (Figure 10d).

Table 5. The acceptable defects of normal seedlings of *Lolium* spp. (Don, 2009)

Seedling components	Acceptable defects for normal seedlings
primary root	Discoloured or necrotic spots
	Healed cracks and splits
	Superficial cracks and splits
mesocotyl	Discoloured or necrotic spots
	Healed cracks and splits
	Superficial cracks and splits
coleoptile	Discoloured or necrotic spots
	Loose twists
	A split for one third or less from the tip
primary leaf	Discoloured or necrotic spots
	Slightly retarded growth

3.4. Effect of nitrogen forms and floret positions on seed quality

The same seed quality components, thousand seed weight, purity and germination, were assessed for floret position as for spikelet position, but only two nitrogen treatments were assessed, 180 kg/ha of ammonium, and nitrate without potassium (treatments 4 and 5 respectively, Table 4) due to time constraints. For the germination test, seeds from the same floret position were kept separate by labelling the floret number on the blue blotter. This was to enable any effect of floret position to be identified (Figure 11). Spikelet position was also of interest. Before the experiment was set up, the number of containers needed for the germination test based on the number of pure seeds for each floret position in the two nitrogen treatments was calculated. Then the same number of 1.6 cm × 1.6 cm grids (Figure 12) were manufactured to act as an additional barrier to movement of the seed so the germination of each seed could be traced back to its individual spikelet position which was also labelled on the blue blotter (Figure 11). Blotters were then moistened by immersing them in tap water for 30 seconds, then allowing excess water to drain off, and seed positioned on the blotters.

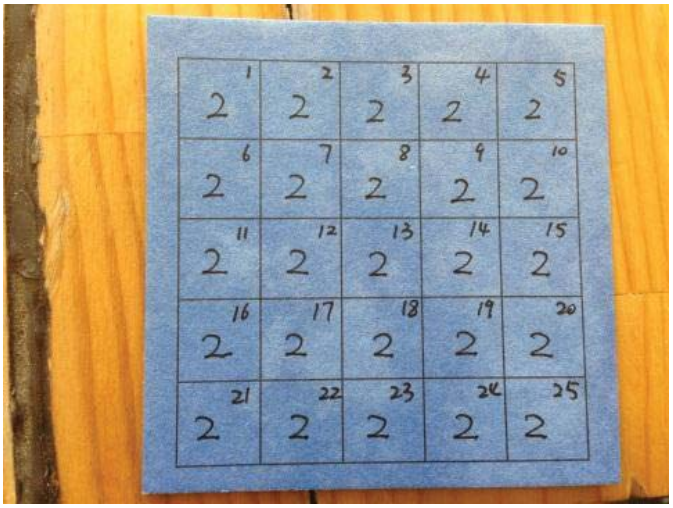


Figure 11. Germination blotter marked up to show floret number (2) and spikelet numbers (1-25).

Figure 12. Combined germination blotter and grid to prevent seeds moving from their labelled grid position on the blotter; the grid was held in place by two rubber bands.

Blotters and seed were then placed in plastic containers at alternating temperatures and light regimes of 20 °C for 16 hours dark and 30 °C for 8 hours light. Seedlings/seeds were also assessed as described in 3.2.1.3)

3.5. Effect of nitrogen forms and spikelet positions on endophyte

A range of histochemical, immunological and molecular methods have been used to detect endophyte in vegetative plant tissues and seed tissues. In this project, the immunoblot test was applied to determine the viability of endophyte. The method is a common test for endophyte present and fit for the purpose for this study.

3.5.1. Immunoblot detection of endophyte

3.5.1.1. Growing the seedlings for the assay

Cultivar 'Halo' AR37 seeds or transplanted seedlings from the germination test (if insufficient seed was available, i.e. replicate 2 of treatment 2, replicate 2 of treatment 4, and the top spikelet position of replicate 3 of treatment 5) were grown on in 54 hygiene trays with 96 seedlings in each tray (dimensions: 30 cm wide, 42 cm long and 6 cm high). Seedlings were grown in a glasshouse with a diurnal temperature range of 16 °C to 26 °C located at the Massey University Plant Growth Unit ($40^{o}22'$S, $175^{o}36'$E). Seedlings were grown in a potting medium consisting of peat (333 litres), bark (867 litres), coir fibre (200 litres), pumice (400 litres) and sterilised river sand (200 litres), plus 4 kg 3-4 month of Osmocote (N-P-K: 16-9-12 plus 2% MgO plus trace elements) (Figure 13). Each tray was filled with potting medium and a 96 station (position) stamp that fitted neatly inside the hygiene tray was used to imprint 96 holes in the medium in each tray. A seed or seedling was sown by hand into each station hole to allow up to 96 plants per tray. Additional potting medium was applied to cover the seeds in the holes and the trays were watered and placed in the glasshouse. Each tray was labelled and daily overhead watering was applied until the plant had grown three to four tillers (from 26 August to 10 October 2013) before the immunoblot test was conducted.

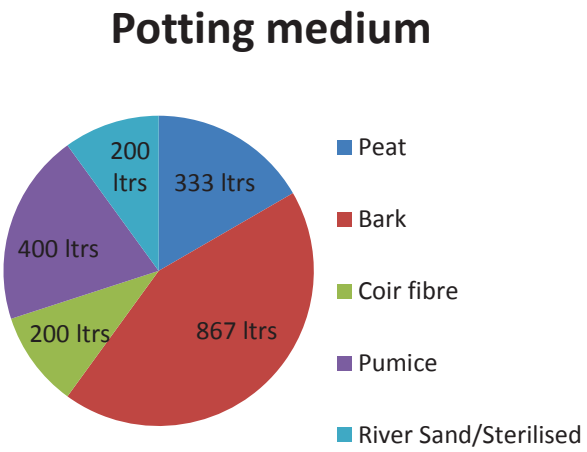


Figure 13. Proportional components of potting medium. Note: 4 kg/2000 litres 3-4 month Osmocote fertiliser was also included in the mix.

Figure 14. Top and bottom views of a stamp with 96 stations, used to sow seeds or seedlings into seedling trays for later immunoblot analysis.

3.5.1.2. Blotting the endophyte

Two tillers per plant were carefully cut at the tiller base where the sheath tissue is located. The fresh cut end of each tiller was blotted onto nitrocellulose membrane

sheets leaving circular outlines (from the compressed pseudostem leaves) of the cut ends. The blot was assessed as positively or negatively stained for endophyte.

3.5.1.3. Staining the endophyte

A blocking solution was used to reduce the background staining by blocking reactive sites where the primary or secondary antibody may bind. The amount of blocking solution required per sheet was calculated to be about 25 ml. The blocking solution was prepared by dissolving 2.42 g Tris (hydroxymethyl) methylamine, 2.92 g NaCl, 5 g non-fat milk powder, and 10 ml of 1 mol HCl before being made up to one litre using reverse osmosis (RO) water. The RO water was measured and poured into a container, then the dry ingredients were added first and then the measured volume of HCl was added to make a final volume of one litre. Finally, the pH of the blocking solution was adjusted to pH 7.5 by adding concentrated HCl. The blotted sheets with no bound protein were immersed in the blocking solution and membrane sheet, then, shaken on an orbital shaker at room temperature (approximately 20 °C) for at least 2 hours. The old dark solution was poured out and fresh blocking solution added into the container using 25 ml of blocking solution per sheet. Primary antibody (25 µl per sheet) was then added to the blocking solution and the sheets were left on the shaker overnight at 4 °C. The following day membrane sheets were rinsed three times with the fresh blocking solution and then 25 ml per sheet of fresh blocking solution was added with 6.25 µl per sheet of secondary antibody. The membrane was shaken at room temperature for at least 2 hours. Tris buffer was prepared by dissolving 24.2 g of Tris (hydroxymethyl) methylamine per litre RO water and the pH value adjusted to 8.2. The Tris buffer was then put on a shaker for a couple of minutes. Fast Red (20 mg per sheet) and Napthol (12.5 mg per sheet) were dissolved in 12.5 ml Tris buffer separately and then the two solutions were combined to produce a chromogen solution. The blocking solution was decanted off; the sheets were rinsed twice with fresh blocking solution before the chromogen solution was

poured over the membrane sheets. The membrane sheets with chromogen solution were shaken at room temperature for 15 minutes until blots developed. Finally, the sheets were rinsed three times with RO water and then rinsed with tap water. The developed sheets were placed on a paper towel and then assessed where a bright red colour indicated a positive blot and a light red colour indicated a negative blot (Simpson *et al*., 2012). After tillers were taken for the assay the remaining plant was placed outside the glasshouse at AgResearch, Palmerston North (Figure 15) to provide further tillers for re-blotting when it was not clear from the immunoblot if endophyte was present or not.

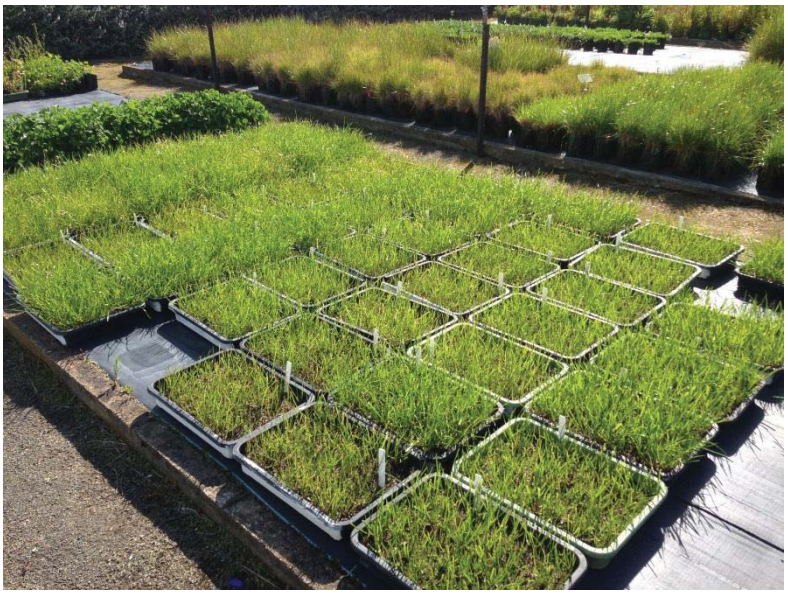


Figure 15. *Lolium* plants kept outside the glasshouse at AgResearch Grasslands, Palmerston North in case needed for endophyte reassessment.

3.6. Data analysis

Thousand seed weight, pure seed percentages (in weight), empty seed percentages (in weight), ergot percentages (in weight), germination percentages, dead seed percentages, abnormal seedling percentages and endophyte content were recorded in Microsoft Excel for nitrogen and potassium and different spikelet positions

treatments. Individual seed weight, empty seed percentages (in frequency), germination percentages, dead seed percentages, abnormal seedling percentages, and endophyte content for nitrogen and potassium and different floret positions treatments were also recorded. Percentage data do not usually have a normal distribution particularly when many of the percentage data are smaller than 20% or above 80%. In this study much of the data was above 80%, therefore, the percentage data was arcsine square-root transformed to achieve a normal distribution. The Kolmogorov-Smirnov test was then conducted to confirm the distribution of the data. For data confirmed as normally distributed, Tukey's Studentized Range (HSD) Test was done to compare different nitrogen and potassium treatments, different spikelet positions, or different floret positions. For non-normally distributed data, a non-parametric ANOVA following by multiple Bonferroni (Dunn) range test were conducted and the original data were ranked before the analysis. The Chi-square test was also done for the empty seed, germination, and endophyte presence data to compare different nitrogen forms and floret positions. Specifically, the Chi-square tests were conducted to compare both seven floret positions (only seven were compared rather than nine because the majority of the spikelets selected had only up to 7 florets) together and any two floret positions for each nitrogen form on empty seed and seed germination percentages, and endophyte presence. The data analysis was done through the statistical program SAS 9.3. (SAS Institute, Gary, North Carolina, USA)

Chapter 4. Results

Information on the details of the statistical analysis of these results is given in the appendices. This includes the results of the analysis of the thousand seed weight (TSW), germination percentage, seed purity, endophyte presence, and individual seed weight obtained from different spikelet or floret positions in response to different nitrogen applications.

4.1. Effect of nitrogen forms and spikelet positions on seed quality and endophyte presence

4.1.1. Thousand seed weight

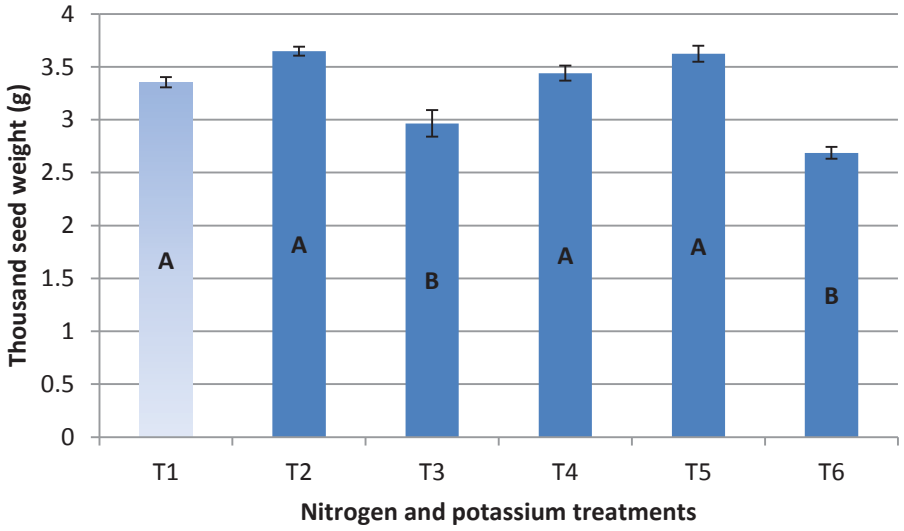


Figure 16. Mean thousand seed weight for six nitrogen and potassium treatments with the standard error (bar) for each treatment mean. Note: T1 = nil, T2 = urea, T3 = urea with K, T4 = NH_4^+, T5 = NO_3^-, T6 = NO_3^- with K.

The Kolmogorov-Smirnov test indicated that the thousand seed weight (TSW) data were normal distributed (p-value>0.150). TSW of Halo was affected by different

nitrogen and potassium applications. No difference was found among different nitrogen forms, nor was there difference in TSW between urea with potassium and nitrate with potassium. However, the TSW under a combination of nitrogen and potassium application was significantly lower than that under nitrogen applied alone (Figure 16).

A goodness of fit test (Kolmogorov-Smirnov) was applied to test the distribution of data of TSW among top, middle, and bottom spikelet positions. The data were not normally distributed even after transformation and thus were analysed using non-parametric ANOVA following by multiple Bonferroni (Dunn) range test. No difference was found among the three spikelet positions.

4.1.2. Germination percentage

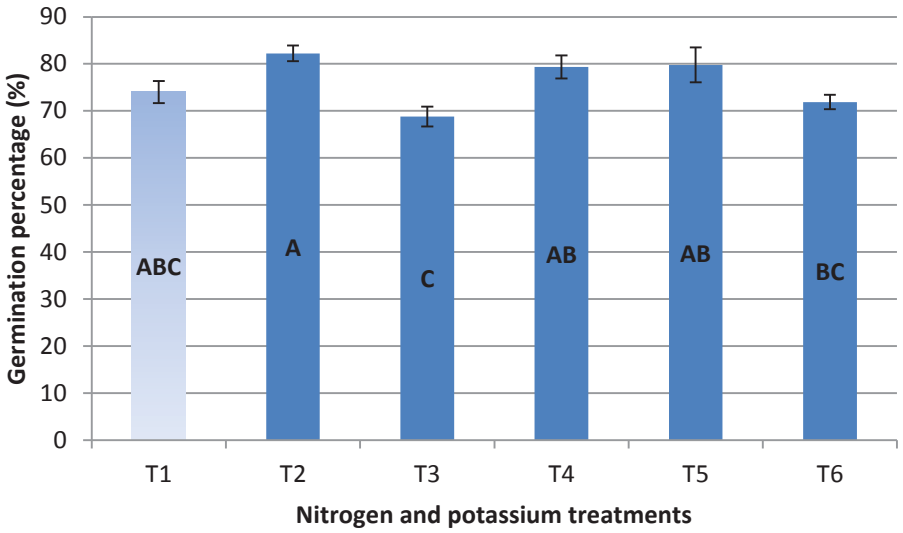


Figure 17. Mean of germination for six nitrogen and potassium treatments with the standard error (bar) for each treatment mean. Note: T1 = nil, T2 = urea, T3 = urea with K, T4 = $NH4^+$, T5 = $NO3^-$, T6 = $NO3^-$ with K.

The germination (transformed) data were found to be normally distributed through the Kolmogorov-Smirnov test (p-value>0.150). Different nitrogen and potassium

treatments affected the germination of Halo significantly. Urea germination was significantly higher than NO_3^- with K, but there was no difference among urea, ammonium, and nitrate applied alone. Nor was there any difference between urea with potassium and nitrate with potassium. The application of urea with potassium gave the lowest germination compared with that of urea, ammonium, and nitrate. There was no difference between the control and other nitrogen and potassium treatments (Figure 17).

Kolmogorov-Smirnov test of germination (transformed) for top, middle, and bottom spikelet positions determined that the data were normally distributed (p-value>0.150). There was no difference in germination among the three spikelet positions.

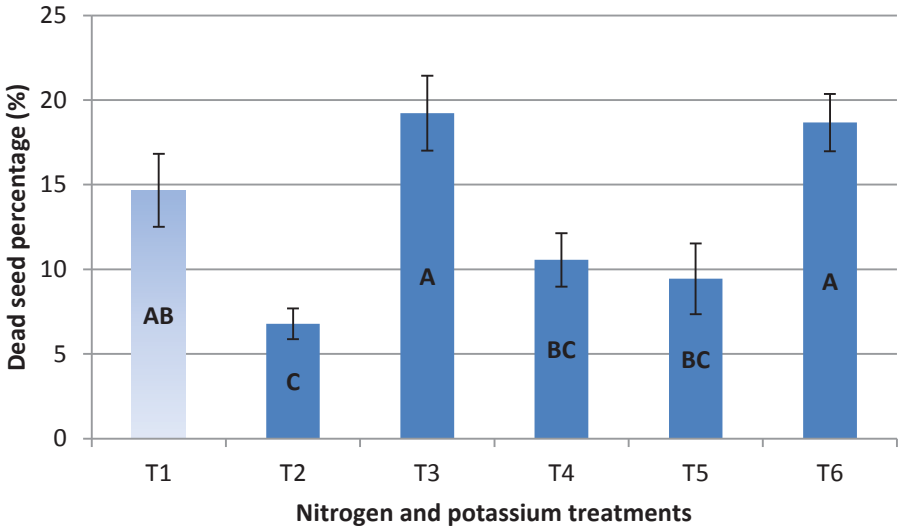


Figure 18. Mean of dead seed percentage for six nitrogen and potassium treatments with the standard error (bar) for each treatment mean. Note: T1 = nil, T2 = urea, T3 = urea with K, T4 = $NH4^+$, T5 = $NO3^-$, T6 = $NO3^-$ with K.

The distribution of dead seed data (transformed) was normal using Kolmogorov-Smirnov test (p-value>0.150). Different nitrogen and potassium treatments had an

effect on dead seed percentages of Halo. The application of nitrogen with potassium gave a higher percentage of dead seeds than nitrogen applied alone (except ammonium). There was no difference between control and the other nitrogen and potassium treatments except for urea application which gave the lowest dead seed percentage. Also, no difference was found among nitrate, ammonium, and urea applied alone (Figure 18).

The transformed dead seed percentages comparing top, middle, and bottom spikelet positions were normal distributed (p-value>0.150 using Kolmogorov-Smirnov test). There was no difference in dead seed percentages (transformed) among the three spikelet positions.

The Kolmogorov-Smirnov test showed that the transformed data of abnormal seedling percentages for both different nitrogen and potassium treatments and spikelet positions were normal distributed (p-value>0.150). No difference was found among the six nitrogen and potassium treatments, or among the top, middle, and bottom spikelets.

4.1.3. Purity

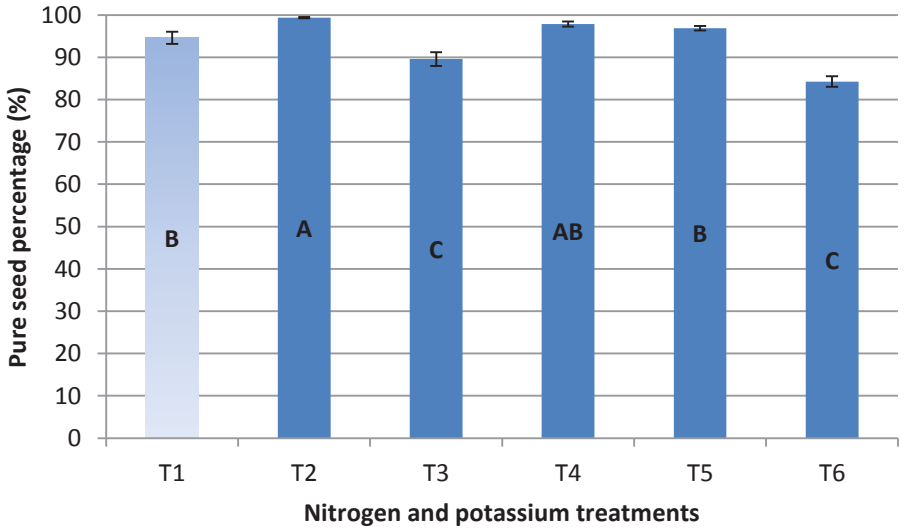


Figure 19. Mean pure seed percentage for six nitrogen and potassium treatments with the standard error (bar) for each treatment mean. Note: T1 = nil, T2 = urea, T3 = urea with K, T4 = $NH4^+$, T5 = $NO3^-$, T6 = $NO3^-$ with K.

The distribution of transformed pure seed percentage data for nitrogen and potassium treatments was normal (p-value>0.150, Kolmogorov-Smirnov test). There were significant differences among the six nitrogen and potassium treatments. Urea gave the highest pure seed percentage, but no difference was found between urea and ammonium. Nor was there any difference between control, nitrate, and ammonium pure seed percentages. The combination of nitrogen and potassium had lower pure seed percentages than nitrogen applied alone as well as control (Figure 19).

A goodness of fit test (Kolmogorov-Smirnov) was applied to test the distribution of data of pure seed percentage (transformed) among top, middle, and bottom spikelet positions, but the data were not normally distributed even after transformation and thus was analysed using a non-parametric ANOVA following by a multiple Bonferroni

(Dunn) range test. Different spikelet positions did not affect pure seed percentages.

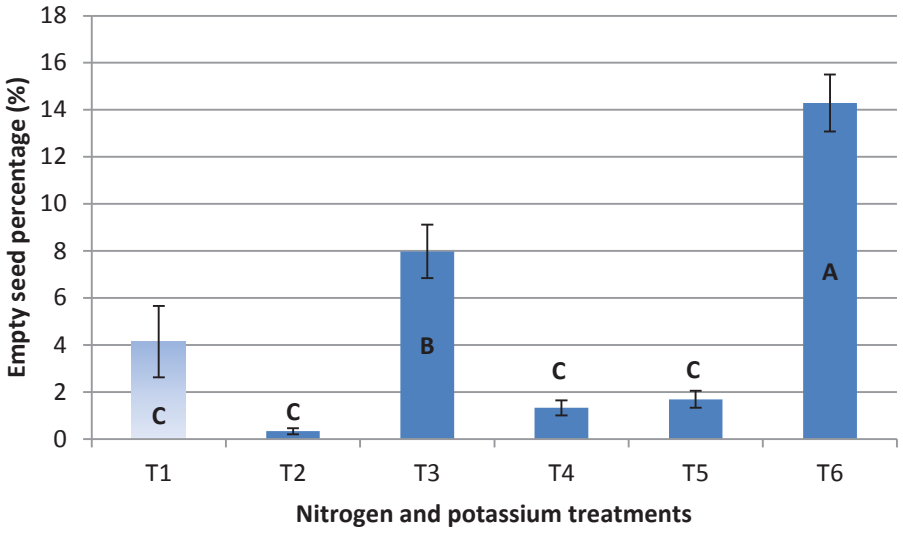


Figure 20. Mean of empty seed percentage for six nitrogen and potassium treatments with the standard error (bar) for each treatment mean. Note: T1 = nil, T2 = urea, T3 = urea with K, T4 = $NH4^+$, T5 = $NO3^-$, T6 = $NO3^-$ with K.

The empty seed data (transformed) for nitrogen and potassium treatment were normal distributed (p-value>0.150, Kolmogorov-Smirnov test). Different nitrogen forms and potassium affected empty seed percentages significantly. The application of nitrate with potassium gave the highest percentage of empty seeds, followed by urea with potassium. However, no difference of empty seed percentage was found with the application of nitrate, ammonium, urea and control (Figure 20).

A goodness of fit test (Kolmogorov-Smirnov) was applied to test the distribution of empty seed percentage data (transformed) among top, middle, and bottom spikelet positions, but the data were not normally distributed even after transformation and thus was again analysed using a non-parametric ANOVA following by a multiple Bonferroni (Dunn) range test. No difference in empty seed percentage was found among the three spikelet positions.

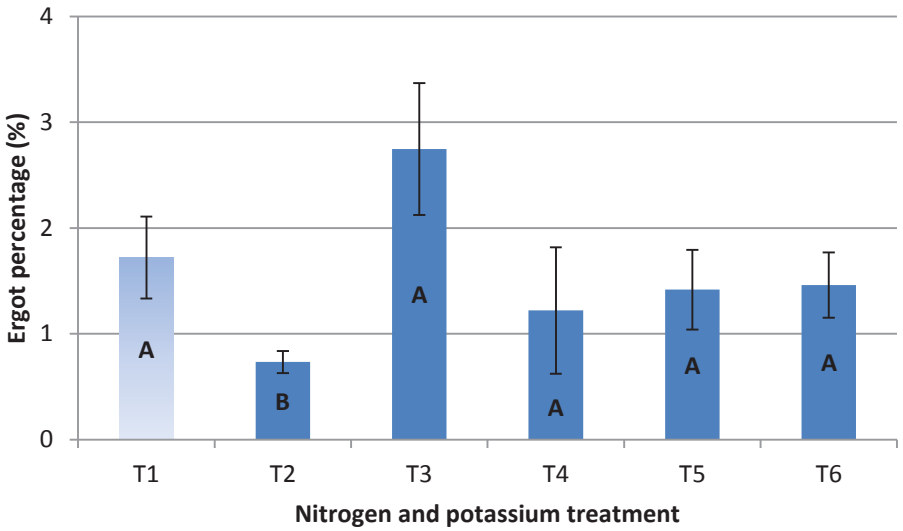


Figure 21. Mean of ergot percentage for six nitrogen and potassium treatments with the standard error (bar) for each treatment mean. Note: T1 = nil, T2 = urea, T3 = urea with K, T4 = $NH4^+$, T5 = $NO3^-$, T6 = $NO3^-$ with K.

The Kolmogorov-Smirnov test determined that the ergot percentage data (transformed) for nitrogen and potassium treatment were normal distributed. A difference in ergot percentage was only found with the application of urea (Figure 21).

A goodness of fit test (Kolmogorov-Smirnov) was applied to test the distribution of the ergot percentage data (transformed) among top, middle, and bottom spikelet positions, but the data were not normally distributed even after transformation and thus were analysed using a non-parametric ANOVA following by multiple Bonferroni (Dunn) range test. There was no difference of ergot among top, middle, and bottom spikelets.

4.1.4. Endophyte presence

The data of endophyte content (transformed) for nitrogen and potassium treatment were normal distributed (p-value>0.150, Kolmogorov-Smirnov test). There was no difference in endophyte content among different nitrogen and potassium treatments (range 69% (T2) to 78% (T4)). A goodness of fit test (Kolmogorov-Smirnov) was applied to test the distribution of data of endophyte content (transformed) among top (75%), middle (76%), and bottom (73%) spikelet positions, but the data were not normally distributed and thus analysed using non-parametric ANOVA following by multiple Bonferroni (Dunn) range test. There was no difference in endophyte content between top, middle, and bottom spikelets.

4.2. Effect of nitrogen forms and floret position on seed quality and endophyte transmission

4.2.1. Individual seed weight

Different nitrogen forms had an effect on the individual seed weight in floret positions 3, 4, and 7, but did not affect the seed weight of floret positions 1, 2, 5 and 6. Floret positions only affected the seed weight significantly, where the seed weight in floret 7 is lower than floret 1 and 2, when NH_4 was applied. There was no difference of individual seed weight among different floret positions under NO_3 application (Figure 22).

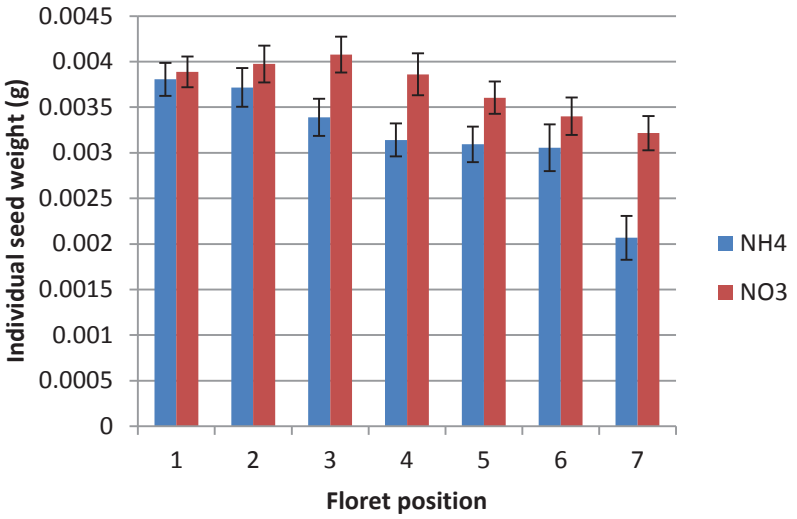


Figure 22. Mean individual seed weight of Halo under $NH4^+$ and $NO3^-$ application in seven different floret positions with standard error (bar) for each treatment mean and floret position.

4.2.2. Empty seed percentage

There was no difference in the empty seed percentage comparing NH_4^+ and NO_3^- for each floret position (p-values>0.05,Chi-square test) (Appendix 8.3.46). Also, the empty seed percentages did not differ among different floret positions under NH_4^+ application (Appendix 8.3.47). However, under NO_3^- application, different floret positions had an effect on empty seed percentages. Specifically, floret position 2 (22%) produced less empty seeds than that of floret position 4 (44%), 5 (41%), 6 (53%), and 7 (50%). Also the empty seed percentage of floret position 3 (30%) was significantly lower than that of floret position 6 (Appendix 8.3.49 - 8.3.53).

4.2.3. Germination percentage

The two nitrogen applications, NH_4^+ and NO_3^-, did not affect the germination percentage of Halo (p-value>0.05 Chi-square test). Nor was there any effect on dead

seed and abnormal seedling percentages (p-value>0.05). Also, when given nitrogen was applied, different floret positions did not affect the germination percentage, the dead seed percentage, and the abnormal seedling percentage (p-values>0.05) (Appendices 9.3.54 – 9.3.62).

4.2.4. Endophyte presence

The viable AR37 endophyte content in the seedlings grown from each seed only varied in floret 1 when comparing NH_4^+ (88%) and NO_3^- (68%) applications (Appendix 8.3.64). Nitrogen did not affect the endophyte content in the other floret positions, based on the p-values≤0.05 using the Chi-square test. Under NO_3^- application, there were significant differences only between floret position 1 (68%) and 2 (93%), 1 and 4 (94%), 1 and 7 (100%), and 3 (75%) and 4 (p-value≤0.05) (Appendices 8.3.67 - 8.3.70). No differences were found among different floret positions under NH_4^+ application.

Chapter 5. Discussion

5.1. Effect of nitrogen form and potassium and spikelet position on seed quality and endophyte presence

5.1.1. Thousand seed weight

This study found that there was a significant effect from nitrogen form and potassium on ryegrass thousand seed weight (TSW), but TSW from different spikelet positions did not differ. Specifically, the application of nitrogen alone did not increase TSW; however, a reduction of TSW was observed under a combination of nitrogen and potassium treatments (T3 and T6). Rolston *et al*. (2007) showed that total nitrogen at 33 kg/ha to 200 kg/ha was not the limiting factor in determining TSW. However, in the same study, a reduction of TSW was found from the highest N amount (250 kg N/ha). In this study the highest rate of N applied was 180 kg/ha, however nitrogen is also available from soil sources and for this site this is estimated to be 30-40 kg/ha (M.P. Rolston, pers. comm.), giving an estimated total N of 210-220 kg N/ha. This is in the range where a reduction TSW may occur (> 200 kg/ha but < 250 kg/ha), but it is unlikely that a reduction is occurring because there was no difference in TSW between control and treatments with a nitrogen only application. However, a possible explanation for the lower TSW of cv. Halo under nitrate and potassium is that the addition of potassium increased the nitrogen use efficiency which resulted in an increase of NO_3^- uptake by the pasture (Anon., 1998) to the point where TSW was being reduced. Equally, the lower TSW under the urea and potassium treatment may be attributed to the competition between transporting NH_4^+ (produced from the breakdown of urea in the soil) and K^+ into plant cells (Britto & Kronzucker, 2008), which decreased N availability for the plant and, therefore,

reduced dry matter accumulation in the seed. In addition, the type of cultivar was less likely to determine TSW in that, for some other ryegrass cultivars mentioned in the literature review chapter, TSW was not affected by nitrogen application.

The different interactions between NH_4^+ and K^+, and NO_3^- and K^+ and the existence of a threshold amount for a reduced TSW response to total nitrogen means that during ryegrass seed production, the effect of potassium included as a fertilizer in achieving the improvement of TSW will depend on the nitrogen forms and the amount of mineral nitrogen in soils. When total nitrogen is close to the threshold amount that will limit TSW, the addition of potassium may decrease TSW of ryegrass seed produced with either urea or nitrate as the nitrogen source. However, when nitrogen is well below the threshold potassium can be included with nitrate fertilizers because of the increased N use efficiency under potassium application. Addition of potassium with urea should be done with caution because of the competition for the uptake of ammonium and potassium in pastures. Moreover even though the addition of potassium reduced TSW potassium has important functions in in seed production, such as stimulating sugar partition and drought tolerance. It also contributes to stomatal opening and closure (Wang *et al.*, 2013). There for the inclusion of potassium may be necessary but its application needs to be carefully managed to minimise depression of TSW.

5.1.2. Nitrogen form and potassium and spikelet position effect on germination

Seed germination percentages of cv. Halo under different nitrogen and potassium treatments did not differ from that of the control, nor was there any difference when comparing different spikelet positions. The resultant effects of nitrogen form and potassium on germination for this study were in accordance with Simić *et al*. (2010) who found that nitrogen did not have an effect on seed germination of Italian ryegrass. However, nitrogen applied alone gave higher germination percentages than

nitrogen with potassium. The combination of urea and potassium had the lowest germination percentages. As no differences in abnormal seedling percentages was found amongst all the treatments, the germination percentage differences can be attributed to difference percentages of dead seeds. Simić *et al*. (2010) also suggested that low germination was caused by low assimilate supply limiting seeds from achieving their potential weight, which can also be an explanation for the lower germination result under the nitrogen with potassium treatment. It is notable that, in this germination test, the normal seedling percentage of nitrate with potassium did not differ from that of control. This may be attributed to the application of nitrate which is an important nutrient enhancing early embryo development (Wang *et al*., 2012). The reduction in seed germination with the inclusion of potassium in a nitrogen application suggests seed producers need to take care with the addition of potassium, although before firm conclusions can be drawn other cultivars would need to be investigated to confirm the effect was not limited to Halo.

5.1.3. Nitrogen form and potassium and spikelet position effects on seed purity

The pure seed percentages (by weight) responded to different nitrogen forms and potassium, but they did not differ whether from top, middle, or bottom spikelet positions in the standard purity test. Again, nitrogen with potassium gave the lowest pure seed percentages compared with other treatments. High seed purity appears correlated to high pure seed weight. The lower pure seed weight under nitrogen with potassium therefore gave a lower pure seed percentage in the test. In addition, this study also found that nitrogen with potassium gave relatively higher empty seed percentages (by weight) in contrast to the other treatments indicating that high seed weight accompanied with low empty seed percentages contributed to high seed purity. The percentage of ergot found was low (<3%) and was therefore not a limiting factor to seed purity.

There were significantly higher empty seed percentages (by weight) when urea with potassium and nitrate with potassium were applied but not urea, ammonium or nitrate alone suggesting that the higher empty percentage may have been the result of poor seed fill rather than lack of fertilisation or early abortion. There are two possible mechanisms. K^+ may compete with NH_4^+ for plant uptake, meaning less nitrogen was available for producing assimilates which play an important role in seed fill and resulted in higher "empty" seed percentage (by weight) (Simić *et al*., 2012). On the other hand, under the nitrate with potassium treatment, there was more nitrogen available for the pasture (refer 5.1.1.) which may have resulted in the increased production of vegetative tillers (Hebblethwaite & Ivins, 1977). Therefore, the higher empty seed percentages (by weight) were caused by the competition from the growth of vegetative tillers and seed sinks when nitrate with potassium was applied (Simić *et al*., 2012). In this study seeds were cleaned with a South Dakato blower before the purity analysis but all treatments were blown at the same setting and for the same length of time. Therefore the differences in the "empty" seed percentage may still indicate that the addition of potassium may produce more empty seeds before blowing. When the seed was cleaned the higher percentage of empty seed present in some treatments resulted in poorer separation of full and empty seed, hence more empty seed remained.

5.1.4. Seedling endophyte levels

The endophyte infection percentages in the six-week old seedlings did not differ both among nitrogen forms and potassium and among top, middle, and bottom spikelet positions. In this study, the endophyte infection in the assessed seedlings grown from the seeds obtained in Canterbury was about 75% (still above the industry minimum standard (70%) (Anon., 2013)), which was lower than that of their parents' seedlings (about 85%); a result obtained through the standard blot test in spring 2012. Given that there was no significant difference in endophyte levels between treatments or

compared with control this loss of endophyte is unlikely to be the result of application of nitrogen or potassium. The loss of endophyte may be caused by the failure of transmission from infected plants to seed-bearing stems or from infected seeds to seedling (Afkhami & Rudgers, 2008).

5.2. Effect of two nitrogen forms, NH_4^+ and NO_3^- and floret position on seed quality and endophyte presence

5.2.1. Individual seed weight

Different nitrogen forms affected individual seed weight significantly only in the middle and distal florets where nitrate gave a higher seed weight than ammonium. In addition, under ammonium application, the basal florets produced heavier seeds than the distal florets, but there was no difference with the nitrate treatment. The finding that the seed weight from basal florets was higher than distal floret under ammonium is consistent with the perennial ryegrass study of Warringa *et al*. (1998b). The difference of seed weight may be caused by the difference in sink strength (Atwell *et al.*, 1999), where the basal florets are stronger sinks than the distal florets. However, no difference in seed weight was found among floret positions under nitrate despite the reported importance of nitrate for early embryo growth (Wang *et al*., 2012) and the finding that the seed weight difference among different floret positions in this study was reduced. It may be that the application of nitrate reduces the difference in early embryo growth for each floret position. However, even though the accumulations of nitrate and ammonium are independent of each other, the uptake of NH_4^+ had an inhibiting effect on the absorption of NO_3^- (Lycklama, 1963). This means that there may have been less NO_3^- available for embryo growth under NH_4^+ application than just NO_3^- application. The flowering pattern for perennial ryegrass within a spikelet is central florets first, then basal florets, and finally distal florets, therefore there will be even less NO_3^- for basal and distal florets. The seed weight differences from floret positions 3, 4, and 7 when comparing NH_4^+ and NO_3^- may be caused by this.

Even though the two nitrogen forms had an effect on the individual seed weight at several floret positions, there was no effect of nitrogen form on overall TSW in an inflorescence. Further study on the effect of floret position, under specific nitrogen application form, on TSW should be undertaken to determine if TSW, which is one of the major of seed yield parameters, is affected by floret position or if there is an interaction effect between nitrogen and floret position.

5.2.2. Effect of NH_4^+ and NO_3^- and floret position on empty seed percentage

This study found that the empty seed percentages (in frequency) did not differ between NH_4^+ and NO_3^- which was the same as the finding (empty seed percentage by weight) from the purity test for nitrogen forms and potassium (refer section 5.1.3.). On the other hand, with NO_3^- application, the basal florets 2 and 3 produced less empty seed compared with the distal florets 4, 5, 6, 7 and 6 respectively. The increased nitrate availability may enhance the competition of assimilates and NO_3^- partition among those florets. The distal florets which had lower sink strength were likely to receive less assimilates and NO_3^- indicating a relatively weaker growth of the embryo and, therefore, produced more “empty” seeds.

5.2.3. Effect of NH_4^+ and NO_3^- and floret position on germination

Neither different nitrogen forms nor floret positions had an effect on seed germination percentages. However, when comparing any two floret positions under NH_4^+ or NO_3^- application, the chi-square test showed that some comparisons had significant results (for NH_4^+, the comparison between floret position 1 - 3 and 1 – 5; for NO_3^-, the comparison between floret position 1 - 2, 1 - 3, and 1 - 7). The reason for the difference between the overall comparison among the seven floret positions and between any two floret positions is because the two tests are using different samples sizes the variation associated with each analysis differs. In addition, the

significant results in the comparison between any two floret positions may be caused by the limited sample size which means that each individual seed contributes a high proportion of the germination percentage.

5.2.4. Seedling endophyte levels

Different nitrogen forms were found to affect endophyte content only in floret position 1. A study by Rasmussen *et al*. (2007) suggested that high nitrogen supply reduced endophyte concentration. The significant lower endophyte content under NO_3^- may be attributed to the higher affinity of this nitrogen form for embryo development (Wang *et al*., 2012) which competed with the growth of endophyte. When comparing different floret positions for endophyte presence for each nitrogen form, there was a significant difference with NO_3^- application. The relatively higher endophyte content in floret position 2, 4, and 7 may suggest that more NO_3^- is distributed into the remaining florets. However, for this study, the more distal florets (especially positions 6 and 7) gave either 100% or close to 100% endophyte infection but with quite small sample sizes and this work should be repeated using more samples.

Chapter 6. Conclusion

This study was undertaken to determine the effects of different nitrogen forms and potassium, spikelet positions, and floret positions within a spikelet on the seed quality of the tetraploid perennial ryegrass cv. Halo, and the effects on the viability of the novel endophyte AR37 in the early seedlings grown from the seeds receiving different nitrogen and potassium applications. Two experiments were conducted to complete this study, one of which was designed to determine six nitrogen and potassium treatments and three spikelet positions on the seed quality (purity, germination, and thousand seed weight) of cv. Halo as well as endophyte content and the other was focusing on two nitrogen forms and different floret positions on cv. Halo seed quality (individual seed weight, empty seed percentage, and germination) and the subsequent AR37 endophyte content in the offspring seedlings.

In the main experiment, where nitrogen forms and potassium were applied and three spikelet positions were obtained, nitrogen with potassium always gave poorer performances in purity, germination, and TSW tests than the control and the other nitrogen forms applied alone. However, the seeds obtained from top, middle, and bottom had no difference in terms of the seed quality parameters. Also, the endophyte content was not affected by either nitrogen and potassium applications or spikelet positions. The much poorer seed quality performance under nitrogen with potassium may be due to the nutrient interactions which ultimately altered the absorption of nitrogen and potassium.

In the second, smaller, experiment, where only two nitrogen forms, ammonium and nitrate, were compared and seeds from different floret positions within a spikelet were tested, the parameters of seed quality did not differ between NO_3^- and NH_4^+ except for the individual seed weights in floret positions 3, 4, and 7 where the application of nitrate gave a better performance. In general, the basal florets

produced heavier seeds (especially under ammonium application) as well as less empty seeds. The differences are likely to be caused by the difference in sink strength among floret positions. In addition, germination percentages for each floret position did not differ. Again, the AR37 endophyte content was not affected by the two nitrogen forms as well as different seed positions, even though some floret positions differed in endophyte content the variability of the data was high because the limited sample size meaning that a single floret contributed a large amount to the result. Again a follow-up experiment using a larger number of samples is warranted.

During seed production of perennial ryegrass especially for cv. Halo AR37, growers will need to decide which nitrogen fertiliser will be better in maximizing the final seed yield with high seed quality. This study suggested that a combination of 180 kg/ha nitrogen and 200 kg/ha potassium was not advisable compared with control and nitrogen applied alone. Considering one component of seed yield, TSW, nitrogen did not have an effect. Also nitrogen did not alter the germination percentage of 'Halo'. However, in order to achieve high pure percentages during a purity test, urea may be a better choice. In addition, it is not necessary for growers to be concerned that seeds harvested from different spikelet positions may determine seed yield and seed quality in the resultant seed lot; nor is there any concern regarding the effect of nitrogen application or different spikelet positions affecting the endophyte content of the seed produced. Further research can be designed to determine the effect of different nitrogen forms and potassium and spikelet positions on the other seed yield components as well as different amounts of nitrogen and potassium for achieving a better performance during the seed production of perennial ryegrass.

Chapter 7. Recommendations

In terms of the first experiment, future studies can be undertaken to determine the effect of different nitrogen forms and potassium on the seed quality of N-deficient ryegrasses grown on N-deficient soils, especially on TSW. Also, different amounts and forms of nitrogen application should be tested to determine their effect on endophyte presence and on the transmission of endophyte. This can be determined through assessing both viable endophyte content in seeds and that in the offspring seedlings together.

The smaller, second experiment, provided data which suggests a larger follow-up experiment is warranted with seeds being produced on a low nitrogen-status soil to accentuate any effect of the different forms of nitrogen added. A much larger sample size is also necessary.

Recommendations

Chapter 8. References

Afkhami, M.E., & Rudgers, J.A. (2008). Symbiosis lost: imperfect vertical transmission of fungal endophytes in grasses. *The American Naturalist*, 172(3), 405-416.

Agren, G.I., Wetterstedt, J.A., & Billberger, M.F. (2012). Nutrient limitation on terrestrial plant growth – modeling the interaction between nitrogen and phosphorus. *New Phytologist*, 194, 953-960.

Agricom. (2012). *Halo*. Retrieved from http://agricom.com.au/products/ryegrasses/perennial-ryegrass/halo/

Agricom. (2013). *Halo AR37 tetraploid perennial ryegrass*. Retrieved from http://agricom.com.au/assets/Uploads/Halo%20perennial%20ryegrass_Agricom.pdf

Agriseeds. (2012a). *Managing tetraploid perennials*. Retrieved from http://www.agriseeds.co.nz/downloads/Pasture%20management%20-%20managing%20tetraploids%202012.pdf

Agriseeds. (2012b). *Feed value of perennial ryegrass*. Retrieved from http://agriseedsnz.co.nz/downloads/Pasture%20management%20-%20feed%20value%20of%20perennial%20ryegrass2012.pdf

Anon. (1998). Potassium interactions with other nutrients. *Better Crops*, 82(3), 12-13.

Anon. (2013). *Choosing a ryegrassendophyte: summary – endophyte insect control (perennial and long rotation ryegrass).* Retrieved from http://pestweb.co.nz/wp-content/uploads/2013/06/Endophyte-Table-2013.pdf

Atwell, B., Kriedemann, P., & Turnbull, C. (1999). *Plants in action: adaptation in nature, performance in cultivation*. Melbourne, Australia: Macmillan Publishers Australia Pty Ltd.

Balocchi, O.A., & López, I.F. (2009). Herbage production, nutritive value and grazing preference of diploid and tetraploid perennial ryegrass cultivars (*Lolium perenne* L.). *Chilean Journal of Agricultural Research*, 69(3), 331-339.

References

Barnes, B., Kopecký, D., Lukaszewski, A., & Baird, J. (2014). Evaluation of turf-type interspecific hybrids of meadow fescue with perennial ryegrass for improved stress tolerance. *Crop Science*, 54, 355-365.

Bluett, S.J., Thom, E.R., Clark, D.A., & Waugh, C.D. (2005). Effect of a novel ryegrass endophyte on pasture production, dairy cow milk production and calf liveweight gain. *Australian Journal of Experimental Agriculture*, 45, 11-19.

Blythe, L., Estill, C., Males, J., & Craig, A.M. (2007). Determination of the toxic threshold of lolitrium B in cattle eating endophyte-infected perennial ryegrass. In A.J. Popay & E.R. Thom (Eds.), *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses* (pp. 399-402). Dunedin, New Zealand: New Zealand Grassland Association.

Brandl, H. (2013). Detection of fungal infection in *Lolium perenne* by Fourier transform infrared spectroscopy. *Journal of Plant Ecology*, 6(4), 265-269.

Britto, D.T., & Kronzucker, H.J. (2008). Cellular mechanisms of potassium transport in plants. *Physiologia Plantarum*, 133(4), 637-650.

Brown, K.R. (1980). Seed production in New Zealand ryegrass. *New Zealand Journal of Experimental Agriculture*, 8, 33-39.

Brown, K.R., Lill, G., McCartin, J., Jarman, D., McCloy, B., Hampton, J.G., Rolston, M.P., Stewart, A.V., & White, J. (1988). *Ryegrass seed crops*. In J.S. Rowarth (Ed.), *Management of Grass Seed Crops Seminar* (pp. 40-46). Palmerston North, New Zealand: New Zealand Grassland Association. Retrieved from http://www.grassland.org.nz/publications/nzgrassland_publication_604.pdf

Browning, R., Donnelly, J.B., Payton, T., Pandya, P., & Byars, M. (2007). Body weight gain and voluntary feed intake in meat goat does fed endophyte-infected and endophyte-free tall fescue seed. In A.J. Popay & E.R. Thom (Eds.), *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses* (pp. 427-429). Dunedin, New Zealand: New Zealand Grassland Association.

Bultman, T.L., McNeill, M.R., & Goldson, S.L. (2003). Isolate-dependent impacts of fungal endophytes in a multitrophic interaction. *Oikos*, 102, 491-496.

Charlton, J.F., & Stewart, A.V. (1999). Pasture species and cultivars used in New

Zealand - a list. *Proceedings of the New Zealand Grassland Association,* 61, 147-165.

Christensen, M.J., Leuchtmann, A., Rowan, D.D., & Tapper, B.A. (1993). Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*),meadow fescue (*F. pratensis*) and perennial ryegrass (*Lolium perenne*). *Mycological Research*, 9, 1083-1092.

Chynoweth, R.J., Rolston, M.P., Kelly, M., & Grbavac, N. (2012). Control of blind seed disease (*Gloeotinia temulenta*) in perennial ryegrass (*Lolium perenne*) seed crops and implications for endophyte transmission. *Agronomy New Zealand*, 42, 141-148.

Chynoweth, R., Rolston, M., & McCloy, B. (2012). Irrigation management of perennial ryegrass (*Lolium perenne*) seed crops. *Agronomy New Zealand*, 42, 77-85.

Clay, K., & Schardl, C. (2002). Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *The American Naturalist*, 160, 99-127.

Cook, T. (n.d.). *Perennial ryegrass Lolium perenne L.* Retrieved from http://horticulture.oregonstate.edu/system/files/PerennialRyegrass.pdf

Cookson, W.R., Rowarth, J.S., & Cameron, K.C. (2000). The response of a perennial ryegrass (*Lolium perenne* L.) seed crop to nitrogen fertilizer application in the absence of moisture stress. *Grass and Forage Science*, 55, 314-325.

Don, R. (Ed.)(2009). *ISTA handbook on seedling evaluation* (3rd ed.). Bassersdorf, Switzerland: The International Seed Testing Association (ISTA).

Edwards, G., & Bryant, R. (n.d.). *What perennial ryegrass should you sow?* Retrieved from http://researcharchive.lincoln.ac.nz/bitstream/10182/4397/1/ryegrass.pdf

Elgersma, A. (1990). Seed yield related to crop development and to yield components in nine cultivars of perennial ryegrass (*Lolium perenne* L.). *Euphytica*, 49, 141-154.

Evitayani, Warly, L., Fariani, A., Ichinohe, T., & Fujihara, T. (2004). Seasonal changes in nutritive value of some grass species. *Asian-Australasian Journal of Animal Science*, 17(12), 1663-1668.

Faeth, S.H. (2002). Are endophytic fungi defensive plant mutualists? *Oikos*, 98, 25-36.

Faeth, S.H., Gardner, D.R., Hayes, C.J., Jani, A., Wittlinger, S.K., & Jones, T.A. (2006). Temporal and spatial variation in alkaloid levels in *Achnatherum robustum*, a native grass infected with the endophyte *Neotyphodium*. *Journal of Chemical Ecology*, 32, 307-324.

Finch, S., Thom, E., Babu, J., Hawkes, A., & Waugh, C. (2013). The evaluation of fungal endophyte toxin residues in milk. *New Zealand Veterinary Journal*, 61(1), 11-17.

Fletcher, L.R. (2005). Managing ryegrass-endophyte toxicoses. In C.A. Roberts, C.P. West, & D.E. Spiers (Eds.), *Neotyphodium in cool-season grasses* (pp. 229-242). Oxford, UK: Blackwell Publishing Ltd.

Fletcher, L., & Sutherland, B. (2009). Sheep responses to grazing ryegrass with AR37. *Proceedings of the New Zealand Grassland Association*, 71, 127-132.

Gislum, R., Abel, S., & Boelt, B. (2013, September). Working within nitrogen limits Paper presented at the 5th International Herbage Seed Group Workshop, Methven, New Zealand.

Hall, M.H. (1992). *Agronomy facts 19: ryegrass*. Penn State Extension. Retrieved from http://pubs.cas.psu.edu/freepubs/pdfs/uc080.pdf

Hampton, J.G. (1987). Effect of nitrogen rate and time of application on seed yield in perennial ryegrass cv. Grasslands Nui. *New Zealand Journal of Experimental Agriculture*, 15, 9-16.

Hannaway, D., Fransen, S., Cropper, J., Teel, M., Chaney, M., Griggs, T., Halse, R., Hart, J., Cheeke, P., Hansen, D., Klinger, R., & Lane, W. (1999). *Perennial ryegrass (Lolium perenne L.)*. PNW 503, Forage Information System. Corvallis, OR: Oregon State University. Retrieved from https://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/17827/pnw503.pdf?sequence=4

Hebblethwaite, P.D., & Ivins, J.D. (1977). Nitrogen studies in *Lolium perenne* grown for seed I. Level of application. *Journal of the British Grassland Society*, 32, 195-204.

Hebblethwaite, P.D., Wright, D., & Noble, A. (1980). Some physiological aspects of seed yield in *Lolium perenne*. In P.D. Hebblethwaite (Ed.), *Seed Production* (pp.

71-90). London, UK: Butterworths.

Hill, M.J., Hampton, J.G., & Rowarth, J.S. (1999). Herbage seeds. In J. White, & J. Hodgson (Eds.), *New Zealand pasture and crop science* (pp. 83-100). Auckland, New Zealand: Oxford University press.

Hunt, G.M., Rasmussen, S., Newton, P., Parsons, J.A., & Newman , A.J. (2005). Near-term impacts of elevated CO2, nitrogen and fungal endophyte-infection on *Lolium perenne* L. growth, chemical composition and alkaloid production. *Plant Cell and Environment*, 28, 1345-1354.

ISTA (2012). *International rules for seed testing 2012.* Bassersdorf, Switzerland: International Seed Testing Association.

Jenner, C. (1980). Effects of shading or removing spikelets in wheat: testing assumptions. *Australian Journal of Plant Physiology*, 7, 113-121.

Johnson, L., Bonth, A., Briggs, L., Caradus, J., Finch, S., Fleetwood, D., Fletcher, L., Hume, D., Johnson, R., Popay, A., Tapper, B., Simpson, W., Voisey, C. & Card, S. (2013). The exploitation of *Epichloae* endophytes for agricultural benefit. *Fungal Diversity*, 60, 171-188.

Kemp, P.D., Matthew, C., & Lucas, R.J. (1999). Pasture species and cultivars. In J. White, & J. Hodgson (Eds.), *New Zealand pasture and crop science* (pp. 83-100). Auckland, New Zealand: Oxford University press.

Kernick, D. M. (1961). Ecology. In *Agricultural and horticultural seeds: their production, control and distribution* (pp. 32-61). Rome, Italy: Food and Agriculture Organization of the United Nations.

Kerr, G.A., Chapman, D.F., Thom, E.R., Matthew, C., van der Linden, A., Baird, D.B., Johnston, E., & Corkran, J.R. (2012). Evaluating perennial ryegrass cultivars: improving testing. *Proceedings of the New Zealand Grassland Association,* 74, 127-136.

Kirkwood, J. K. (1983). A limit to metabolisable energy intake in mammals and birds. *Comparative Biochemistry and Physiology*, 75A(1), 1-3.

Koh, S., Vicari, M., Ball, J.P., Rakocevic, T., Zaheer, S., Hik, D.S., & Bazely, D.R. (2006). Rapid detection of fungal endophytes in grasses for large-scale studies.

Functional Ecology, 20(4), 736-742.

Krauss, J., Harri, A.S., Bush, L., Husi, R., Bigler, L., Power, A.S., & Müller, B.C. (2007). Effects of fertilizer, fungal endophytes and plant cultivar on the performance of insect herbivores and their natural enemies. *Functional Ecology*, 21, 107-116.

Kruse, M. (Ed.) (2004). *ISTA handbook on seed sampling* (2nd ed.). Bassersdorf, Switzerland: The International Seed Testing Association (ISTA).

Lacefield, G.D., Henning, J.C., & Phillips, T.D. (2003). *Tall fescue*. Lexington, KY: University of Kentucky. Retrieved from http://www2.ca.uky.edu/agc/pubs/agr/agr59/agr59.pdf

Lane, G., Tapper, B., Davies, E., Hume, D., Latch, G., & Barker, D. (1997). Effect of growth conditions on alkaloid concentrations in perennial ryegrass naturally infected with endophyte. In: Bacon, C.W., Hill, A.C., (Eds.), *Neotyphodium/grass interactions:* Proceedings of the 3rd International Symposium on Acremonium/Grass Interactions held in Athens, Georgia, May 28-31, 1997 (pp. 179-182). New York, USA: Plenum Press.

Langer, R.H.M., & Hill, G.D. (1991). *Agricultural plants* (2nd ed.). Cambridge, UK: Cambridge University Press.

Leuchtmann, A., Bacon, C.W., Schardl, C.L., White jr, J.F., & Tadych, M. (2014). Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia,* 106, 202-215. Retrieved from http://www.mycologia.org/content/106/2/202.full.pdf+html

Lycklama, J.C. (1963). The absorption of ammonium and nitrate by perennial ryegrass. *Acta Botanica Neerlandica,* 12(4), 361-423.

Majewska-Sawka, A., & Nakashima, H. (2004). Endophyte transmission via seeds of *Lolium perenne* L.: Immunodetection of fungal antigens. *Fungal Genetics and Biology*, 41, 534-541.

Matthew, C. (2003). *Perennial ryegrass (Lolium perenne)*. Retrieved from http://pastureinfo.massey.ac.nz/grasspages/gperennialryegrass.html

Matthew, C., Linden, A.V., Hussain, S., Easton, H.S., Hatier, J.H., & Horne, D.J. (2012).

Which way forward in the quest for drought tolerance in perennial ryegrass? *Proceedings of the New Zealand Grassland Association,* 74, 195-200.

Minneé, E.M., Knight, T.L., Sutherland, T.L., Vlaming, J.B., Fletcher, L.R., & Clark, D.A. (2010). Herbage production from perennial ryegrass and tall fescue pastures under irrigation in the Canterbury and Waikato regions of New Zealand. *Proceedings of the New Zealand Grassland Association,* 72, 185-190.

Nagabhyru, P., Dinkins, R.D., Wood, C.L., Bacon, C.W., & Schardl, C.L. (2013). Tall fescue endophyte effects on tolerance to water-deficit stress. *BMC Plant Biology*, 13, 127.

Nair R.M. (2004). Developing tetraploid perennial ryegrass (*Lolium perenne* L.) populations. *New Zealand Journal of Agricultural Research*, 47, 45-49.

Najafabadi, A.S., Solouki, M., Mofid, M.R., Mohammadi, R., & Nekouei, S.M. (2009). Isolation and characterization of new *Neotyphodium* infected tall fescue in Iranian rangelands. *Australian Journal of Basic and Applied Sciences*, 3(3), 2544-2548.

Najda, H. (2004). *Perennial ryegrass seed production in western Canada*. Agri-Facts Agdex 127/20-1. Alberta Agriculture, Food and Rural Development. Retrieved from http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/agdex8345/$file/127_20_1.pdfOpenElement

Popay, A.J., & Baltus, J.G. (2001). Black beetle damage to perennial ryegrass infected with AR1 endophyte. *Proceedings of the New Zealand Grassland Association*, 63, 267-271.

Popay, A.J., & Hume, D.E. (2011). Endophyte improve ryegrass persistance by controlling insects. In C.F. Mercer (Ed.) *Pasture Persistence Symposium Grassland Research and Practice Series 15* (pp. 149-156). Dunedin, New Zealand: New Zealand Grassland Association.

Popay, A., & Thom, E. (n.d.). *Ryegrass and tall fescue endophytes in your pasture*. Retrieved from http://www.dairynz.co.nz/file/fileid/29153

Rasmussen, S., Parsons, A.J., Bassett, S., Christensen, M.J., Hume, D.E., Johnson, L.J.,

Johnson, R.D., Simpson, W.R., Stacke, C., Voisey, C.R., Xue, H., & Newman, J.A. (2007). High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. *New Phytologist*, 173, 787-797.

Rasmussen, S., Parsons, A.J., & Newman, J.A. (2009). Metabolomics analysis of the *Lolium perenne–Neotyphodium lolii* symbiosis: more than just alkaloids? *Phytochemistry Reviews*, 8(3), 535-550.

Robertson, G.P., & Groffman, P.M. (2007). Nitrogen transformation. In E.A. Paul (Ed.), *Soil microbiology, biochemistry, and ecology* (pp. 341-364). New York, NY: Springer.

Rolston, M.P., Chynoweth, R.J., & Stewart, A.V. (2006). Forage seed production: 75 years applying science and technology. *Proceedings of New Zealand Grassland Association*, 68, 15-23.

Rolston, M.P., McCloy, B.L., & Chynoweth, R.J. (2008). Predicting nitrogen requirement in perennial ryegrass seed crops. In C.R. McGill & J.S. Rowarth (Eds.) *Seed symposium: Seeds for futures.* Agronomy Society of New Zealand Special Publication No. 13 (pp. 59-65). Palmerston North, New Zealand: Agronomy Society of New Zealand.

Rolston, M.P., McCloy, B.L., Chynoweth, R.J. & Trethewey, J. (2013). Nitrogen rates for ryegrass seed crops in Canterbury, New Zealand. In Abstracts for the 5th International Herbage Seed Workshop and FAR International Seminar (pp 22-23). Methven, New Zealand.

Rolston, M.P., McCloy, B.L., Harvey, I., & Chynoweth, R.W. (2009). Ryegrass (*Lolium perenne*) seed yield response to fungicides: a summary of 12 years of field research. *New Zealand Plant Protection*, 62, 343-348.

Rolston, M.P., McCloy, B.L., & Pyke, N.B. (2004). Grass seed yields with plant growth regulators and fungicides. *Proceedings of the New Zealand Grassland Association*, 66, 127-132.

Rolston, M.P., McCloy, B.L., & Trethewey, J. (2007). Nitrogen-moddus interaction in perennial ryegrass seed production. *Agronomy New Zealand*, 37, 37-44.

Rolston, M., Trethewey, J., Chynoweth, R., & McCloy, B. (2010). Trinexapac-ethyl delays lodging and increases seed yield in perennial ryegrass seed crops. *New Zealand Journal of Agricultural Research*, 53(4), 403-406.

Rowarth, J.S., Archie, W.J., Cookson, W.R., Hampton, J.G., Sanders, C.J., Silberstein, T.B., & Young, W. (1999). The link between nitrogen application, concentration of nitrogen in herbage and seed quality in perennial ryegrass (*Lolium perenne* L). *Journal of Applied Seed Production*, 17, 1-6.

Schardl, C.L., Scott, B., Florea, S., & Zhang, D. (2009). *Epichloë* endophytes: clavicipitaceous symbionts of grasses. In H.B. Deising (ed.), *Plant relationships* (2nd ed., pp. 275-306). Berlin, Germany: Springer.

Siegel, M.R., Latch, G.C., Bush, L.P., Fannin, F.F., Rowan, D.D., Tapper, B.A., Bacon, C.W., & Johnson, M.C. (1990). Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. *Journal of Chemical Ecology*, 16, 3301-3315.

Simić, A.S., Vučković, S. M., Ćupina, B.T., Krstić, D.B., Stanisavljević, R.S., & Milivojević, M.S. (2010). Impact of management practices on Italian ryegrass seed quality. *Journal of Agricultural Sciences*, 55,131-140.

Simić, A., Vučković, S., Sokolović, D., Stanisavljević, R., Mandić, V., & Duronić, G. (2012). Response of Italian ryegrass seed crop to spring nitrogen application in the first harvest year. *African Journal of Biotechnology*, 11(26), 6826-6831.

Simpson, W., Schmid, J., Singh, J., Faville, M., & Johnson, R. (2012). A morphological change in the fungal symbiont *Neotyphodium lolii* induces dwarfing in its host plant *Lolium perenne*. *Fungal Biology*, 116, 234-240.

Singh, D. (2007). *Grasses for hay and pasture*. University of Idaho Cooperative Extension. Retrieved from http://www.extension.uidaho.edu/forage/Proceedings/2007%20Proceedings%20pdf/2007SinghGrasses%20for%20Hay%20and%20Pasture.pdf

Stewart, A. (1986). Effect on the *Lolium* endophyte of nitrogen applied to perennial ryegrass seed crops. *New Zealand Journal of Experimental Agriculture*, 14, 393-397.

Stewart, A. V. (2006). Genetic origins of perennial ryegrass (*Lolium perenne*) for New Zealand pastures. C.F. Mercer (Ed.) *Advances in pasture plant breeding: Proceedings of the 13th Australasian Plant Breeding Conference* (pp. 11-20). Dunedin, New Zealand: New Zealand Grassland Association.

Stewart, A., & Charlton, D. (2006). Pasture and Forage Plants for New Zealand (3rd ed.). Grassland Research and Practice Series. Dunedin, New Zealand: New Zealand Grassland Association.

Stewart , A., Kerr, G., Lissaman, W., & Rowarth, J. (2014). *Pasture and forage plants for New Zealand* (4th ed.). Dunedin: New Zealand Grassland Association.

Tapper, B.A., & Lane, G.A. (2004). Janthitrems found in a *Neotyphodium* endophyte of perennial ryegrass. In R. Kallenbach, C.J. Rosenkrans, & T.R. Lock (Eds.) *Proceedings of the 5th International Symposium on Neotyphodium/Grass Interactions* (p301). Fayetteville, AR

Turner, L., Holloway-Phillips, M., Rawnsley, R., Donaghy, D., & Pembleton, K. (2012). The morphological and physiological responses of perennial ryegrass (*Lolium perenne* L.), cocksfoot (*Dactylis glomerata* L.) and tall fescue (*Festuca arundinacea* Schreb.; syn. *Schedonorus phoenix* Scop.) to variable water availability. *Grass and Forage Science*, 67, 507-518.

van Zijll de Jong, E., Dobrowolski, M.P., Sandford, A., Smith, K.F., Willocks, M.J., Spangenberg, G.C., & Forster, J.W. (2008). Detection and characterisation of novel fungal endophyte genotypic variation in cultivars of perennial ryegrass (*Lolium perenne* L.). *Australian Journal of Agricultural Research*, 59, 214-221.

Waghorn, G.C. (2007). What is dietary metabolisable energy? *Proceedings of the New Zealand Grassland Association,* 69, 153-159.

Wang, A., Tan, D., Baskin, C.C., & Baskin, J.M. (2010). Effect of seed position in spikelet on life history of *Eremopyrum distans* (Poaceae) from the cold desert of north-west China. *Annals of Botany*, 106(1), 95-105.

Wang, M., Zheng, Q., Shen, Q., & Guo, S. (2013). The critical role of potassium in plant stress response. *International Journal of Molecular Science*, 14, 7370-7390.

References

Wang, Y., Hsu, P., & Tsay, Y. (2012). Uptake, allocation and signaling of nitrate. *Trends in Plant Science*, 17(8), 458-467.

Warringa, J.W., Struik, P.C., Visser, R.D., & Kreuzer, A. (1998a). The pattern of flowering, seed set, seed growth and ripening along the ear of *Lolium perenne*. *Australian Journal of Plant Physiology*, 25, 213-223.

Warringa, J.W., Visser, R.D., & Kreuzer, A.D. (1998b). Seed weight in *Lolium perenne* as affected by interactions among seeds within the inflorescence. *Annals of Botany*, 82, 835-841.

Young, C.A., Hume, D.E., & McCulley, R.L. (2013). Forages and pastures symposium: Fungal endophytes of tall fescue and perennial ryegrass: Pasture friend or foe? *Journal of Animal Science*, 91, 2379-2394.

References

Chapter 9. Appendices

9.1. Field plot number of 3 replicates in relative to 6 N treatments (N treatment numbers are referred in table 4)

N treatment Number	Plot Number		
	Rep 1	Rep 2	Rep 3
1	4	10	21
2	6	29	22
3	1	12	19
4	5	13	20
5	8	28	25
6	33	11	27

9.2. Timing of the spikelet germination experiment

Replicate 1: 19 Aug, count onto 18 KNO_3 blotters and prechilling

26 Aug, germinating and first count

4 Sep, germinating and final count

Replicate 2: 20 Aug, counting onto 18 KNO_3 blotters and prechilling

27 Aug, germinating and first count and transferring the seedlings of plot 13 and 29 to PGU glasshouse

5 Sep, germinating and final count

Replicate 3: 21 Aug, counting onto 18 KNO_3 blotters and prechilling

28 Aug, germinating and first count and transferring the seedlings of plot 25 top spikelet position to PGU glasshouse

6 Sep, germinating and final count

9.3. Statistical results

9.3.1. Thousand seed weight (TSW) test: Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	0.050403
Critical Value of Studentized Range	4.19719
Minimum Significant Difference	0.3141

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	Nitrogen
A		3.6471	9	T2
A		3.6246	9	T5
A		3.4398	9	T4
A		3.3532	9	T1
	B	2.9651	9	T3
	B	2.6869	9	T6

9.3.2. Thousand seed weight (TSW) test: Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.08321559	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.04176182	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.29244759	Pr > A-Sq	>0.250

9.3.3. Thousand seed weight test: Bonferroni (Dunn) t Tests for rankweight for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	51
Error Mean Square	235.3235
Critical Value of t	2.47551
Minimum Significant Difference	12.658

Means with the same letter are not significantly different.			
Bon Grouping	Mean	N	Spikelet position
A	32.500	18	Middle
A	28.500	18	Bottom
A	21.500	18	Top

9.3.4. Germination test (transformed): Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	0.007868
Critical Value of t	4.19719
Minimum Significant Difference	0.1241

Means with the same letter are not significantly different.					
Tukey Grouping			Mean	N	Nitrogen
A			1.13895	9	T2
A	B		1.11723	9	T5
A	B		1.10574	9	T4
A	B	C	1.03863	9	T1
	B	C	1.01302	9	T6
		C	0.97966	9	T3

9.3.5. Germination test (transformed): Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07284092	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03569568	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.27372116	Pr > A-Sq	>0.250

9.3.6. Germination test (transformed): Tukey's Studentized Range (HSD) Test for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	51
Error Mean Square	0.010921
Critical Value of t	3.41382
Minimum Significant Difference	0.0841

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Spikelet position
A	1.07719	18	Top
A	1.06693	18	Middle
A	1.05250	18	Bottom

9.3.7. Germination test (transformed): Goodness-of-Fit tests for Normal Distribution for top, middle, and bottom spikelet positions

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07617297	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.05239037	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.35916481	Pr > A-Sq	>0.250

9.3.8. Germination test (transformed) (Dead seed): Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	0.007014
Critical Value of t	4.19719
Minimum Significant Difference	0.1172

Means with the same letter are not significantly different.					
Tukey Grouping			Mean	N	Nitrogen
A			0.44897	9	T3
A			0.44407	9	T6
A	B		0.38720	9	T1
	B	C	0.32150	9	T4
	B	C	0.29519	9	T5
		C	0.25879	9	T2

9.3.9. Germination test (transformed) (Dead seed): Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07406932	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.04901398	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.34051815	Pr > A-Sq	>0.250

9.3.10. Germination test (transformed) (Dead seed): Tukey's Studentized Range (HSD) Test for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	51
Error Mean Square	0.012161
Critical Value of t	3.41382
Minimum Significant Difference	0.0887

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Spikelet position
A	0.36618	18	Bottom
A	0.35600	18	Middle
A	0.35568	18	Top

9.3.11. Germination test (transformed) (Dead seed): Goodness-of-Fit tests for Normal Distribution for top, middle, and bottom spikelet positions

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.06093932	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.02379568	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.16175753	Pr > A-Sq	>0.250

9.3.12. Germination test (transformed) (Abnormal seedling): Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	0.005221
Critical Value of t	4.19719
Minimum Significant Difference	0.1011

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.34318	9	T3
A	0.34163	9	T1
A	0.33328	9	T2
A	0.32680	9	T5
A	0.31875	9	T4
A	0.30569	9	T6

9.3.13. Germination test (transformed) (Abnormal seedling): Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07777380	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03372224	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.30564536	Pr > A-Sq	>0.250

9.3.14. Germination test (transformed) (Abnormal seedling): Tukey's Studentized Range (HSD) Test for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	51
Error Mean Square	0.004961
Critical Value of t	3.41382
Minimum Significant Difference	0.0567

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Spikelet position
A	0.33947	18	Bottom
A	0.33235	18	Middle
A	0.31284	18	Top

9.3.15. Germination test (transformed) (Abnormal seedling): Goodness-of-Fit tests for Normal Distribution for top, middle, and bottom spikelet positions

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07177053	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03570458	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.27412198	Pr > A-Sq	>0.250

9.3.16. Purity test (transformed) (pure seed): Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	0.004837
Critical Value of t	4.19719
Minimum Significant Difference	0.0973

Means with the same letter are not significantly different.					
Tukey Grouping			Mean	N	Nitrogen
A			1.50101	9	T2
A	B		1.43856	9	T4
	B		1.39952	9	T5
	B		1.35508	9	T1
		C	1.25121	9	T3
		C	1.16477	9	T6

9.3.17. Purity test (transformed) (pure seed): Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.10062155	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.08089615	Pr > W-Sq	0.205
Anderson-Darling	A-Sq	0.41848163	Pr > A-Sq	>0.250

9.3.18. Purity test (transformed) (pure seed): Bonferroni (Dunn) t Tests for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	51
Error Mean Square	255.9504
Critical Value of t	2.47551
Minimum Significant Difference	13.201

Means with the same letter are not significantly different.			
Bon Grouping	Mean	N	Spikelet position
A	29.000	18	Middle
A	27.028	18	Bottom
A	26.472	18	Top

9.3.19. Purity test (transformed) (empty seed): Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	42
Error Mean Square	0.004674
Critical Value of Studentized Range	4.22176

Comparisons significant at the 0.05 level are indicated by ***.				
Nitrogen Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
T6 - T3	0.1066	0.01039	0.20281	***
T6 - T1	0.21087	0.1117	0.31004	***
T6 - T5	0.26299	0.16678	0.35919	***
T6 - T4	0.27903	0.18282	0.37523	***
T6 - T2	0.33193	0.20929	0.45457	***
T3 - T6	-0.1066	-0.20281	-0.01039	***
T3 - T1	0.10427	0.0051	0.20344	***
T3 - T5	0.15638	0.06018	0.25259	***
T3 - T4	0.17242	0.07622	0.26863	***
T3 - T2	0.22533	0.10269	0.34797	***
T1 - T6	-0.21087	-0.31004	-0.1117	***
T1 - T3	-0.10427	-0.20344	-0.0051	***
T1 - T5	0.05212	-0.04705	0.15129	

T1 - T4	0.06816	-0.03101	0.16733	
T1 - T2	0.12106	-0.00392	0.24604	
T5 - T6	-0.26299	-0.35919	-0.16678	***
T5 - T3	-0.15638	-0.25259	-0.06018	***
T5 - T1	-0.05212	-0.15129	0.04705	
T5 - T4	0.01604	-0.08017	0.11225	
T5 - T2	0.06895	-0.0537	0.19159	
T4 - T6	-0.27903	-0.37523	-0.18282	***
T4 - T3	-0.17242	-0.26863	-0.07622	***
T4 - T1	-0.06816	-0.16733	0.03101	
T4 - T5	-0.01604	-0.11225	0.08017	
T4 - T2	0.05291	-0.06974	0.17555	
T2 - T6	-0.33193	-0.45457	-0.20929	***
T2 - T3	-0.22533	-0.34797	-0.10269	***
T2 - T1	-0.12106	-0.24604	0.00392	
T2 - T5	-0.06895	-0.19159	0.0537	
T2 - T4	-0.05291	-0.17555	0.06974	

9.3.20. Purity test (transformed) (empty seed): Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07280526	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.04014931	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.23877705	Pr > A-Sq	>0.250

9.3.21. Purity test (transformed) (empty seed): Bonferroni (Dunn) t Tests for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	45
Error Mean Square	203.958
Critical Value of t	2.48678

Comparisons significant at the 0.05 level are indicated by ***.				
Spikelet position Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Bottom-Middle	1.746	-10.624	14.117	
Bottom-Top	1.821	-10.943	14.585	
Middle-Bottom	-1.746	-14.117	10.624	
Middle-Top	0.075	-12.506	12.655	
Top-Bottom	-1.821	-14.585	10.943	
Top-Middle	-0.075	-12.655	12.506	

9.3.22. Purity test (transformed) (ergot): Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	41
Error Mean Square	0.002113
Critical Value of Studentized Range	4.22657

Comparisons significant at the 0.05 level are indicated by ***.				
Nitrogen Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
T3 - T1	0.03494	-0.03181	0.1017	
T3 - T6	0.04327	-0.02348	0.11003	
T3 - T5	0.04843	-0.01832	0.11519	
T3 - T4	0.06587	-0.00832	0.14006	
T3 - T2	0.075	0.00081	0.14919	***
T1 - T3	-0.03494	-0.1017	0.03181	
T1 - T6	0.00833	-0.05643	0.07309	
T1 - T5	0.01349	-0.05127	0.07825	
T1 - T4	0.03093	-0.04147	0.10333	
T1 - T2	0.04006	-0.03235	0.11246	
T6 - T3	-0.04327	-0.11003	0.02348	
T6 - T1	-0.00833	-0.07309	0.05643	
T6 - T5	0.00516	-0.0596	0.06992	
T6 - T4	0.0226	-0.0498	0.095	
T6 - T2	0.03173	-0.04068	0.10413	
T5 - T3	-0.04843	-0.11519	0.01832	
T5 - T1	-0.01349	-0.07825	0.05127	

T5 - T6	-0.00516	-0.06992	0.0596	
T5 - T4	0.01744	-0.05496	0.08984	
T5 - T2	0.02657	-0.04584	0.09897	
T4 - T3	-0.06587	-0.14006	0.00832	
T4 - T1	-0.03093	-0.10333	0.04147	
T4 - T6	-0.0226	-0.095	0.0498	
T4 - T5	-0.01744	-0.08984	0.05496	
T4 - T2	0.00913	-0.07019	0.08844	
T2 - T3	-0.075	-0.14919	-0.00081	***
T2 - T1	-0.04006	-0.11246	0.03235	
T2 - T6	-0.03173	-0.10413	0.04068	
T2 - T5	-0.02657	-0.09897	0.04584	
T2 - T4	-0.00913	-0.08844	0.07019	

9.3.23. Purity test (transformed) (ergot): Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.12442048	Pr > D	0.068
Cramer-von Mises	W-Sq	0.12602088	Pr > W-Sq	0.048
Anderson-Darling	A-Sq	0.82212869	Pr > A-Sq	0.033

9.3.24. Purity test (transformed) (ergot): Bonferroni (Dunn) t Tests for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	44
Error Mean Square	182.0552
Critical Value of t	2.48897

Comparisons significant at the 0.05 level are indicated by ***.				
Spikelet position Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Top-Bottom	3.429	-8.692	15.549	
Top-Middle	8.750	-2.948	20.448	
Bottom-Top	-3.429	-15.549	8.692	
Bottom-Middle	5.321	-6.969	17.612	
Middle-Top	-8.750	-20.448	2.948	
Middle-Bottom	-5.321	-17.612	6.969	

9.3.25. Endophyte presence test: Tukey's Studentized Range (HSD) Test for different nitrogen and potassium treatments

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	0.017744
Critical Value of Studentized Range	4.19719
Minimum Significant Difference	0.1864

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	1.09278	9	T4
A	1.07450	9	T1
A	1.07422	9	T5
A	1.05512	9	T3
A	1.02953	9	T6
A	0.98757	9	T2

9.3.26. Endophyte presence (transformed) test: Goodness-of-Fit tests for Normal Distribution for different nitrogen and potassium treatments

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07652401	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03812644	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.27087041	Pr > A-Sq	>0.250

9.3.27. Endophyte presence (transformed) test: Bonferroni (Dunn) t Tests for top, middle, and bottom spikelet positions

Alpha	0.05
Error Degrees of Freedom	51
Error Mean Square	255.0768
Critical Value of t	2.47551
Minimum Significant Difference	13.179

Means with the same letter are not significantly different.			
Bon Grouping	Mean	N	Spikelet position
A	28.778	18	Middle
A	28.194	18	Top
A	25.528	18	Bottom

9.3.28. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 1

Alpha	0.05
Error Degrees of Freedom	68
Error Mean Square	1.08E-06
Critical Value of Studentized Range	2.82202
Minimum Significant Difference	0.0005
Harmonic Mean of Cell Sizes	34.74286

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.003888	32	NO_3^-
A	0.003805	38	NH_4^+

9.3.29. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 1

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.073661	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.046196	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.274967	Pr > A-Sq	>0.250

9.3.30. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 2

Alpha	0.05
Error Degrees of Freedom	69
Error Mean Square	1.52E-06
Critical Value of Studentized Range	2.82128
Minimum Significant Difference	0.0006
Harmonic Mean of Cell Sizes	35.15493

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.0039744	39	NO_3^-
A	0.0037156	32	NH_4^+

9.3.31. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 2

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.07801732	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.04428502	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.29407319	Pr > A-Sq	>0.250

9.3.32. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 3

Alpha	0.05
Error Degrees of Freedom	61
Error Mean Square	1.263E-6
Critical Value of Studentized Range	2.82789
Minimum Significant Difference	0.0006
Harmonic Mean of Cell Sizes	31.11111

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.0040771	35	NO_3^-
B	0.0033893	28	NH_4^+

9.3.33. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 3

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.08389390	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.04827283	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.31847898	Pr > A-Sq	>0.250

9.3.34. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 4

Alpha	0.05
Error Degrees of Freedom	48
Error Mean Square	1.142E-6
Critical Value of Studentized Range	2.84337
Minimum Significant Difference	0.0006
Harmonic Mean of Cell Sizes	24.64

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.0038607	28	NO_3^-
B	0.0031409	22	NH_4^+

9.3.35. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 4

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.06101596	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.02466208	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.17992481	Pr > A-Sq	>0.250

9.3.36. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 5

Alpha	0.05
Error Degrees of Freedom	52
Error Mean Square	9.402E-7
Critical Value of Studentized Range	2.83772
Minimum Significant Difference	0.0005

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.0036037	27	NO_3^-
A	0.0030926	27	NH_4^+

9.3.37. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 5

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.10308995	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.05848284	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.33176443	Pr > A-Sq	>0.250

9.3.38. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 6

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	6.947E-7
Critical Value of Studentized Range	2.91262
Minimum Significant Difference	0.0007
Harmonic Mean of Cell Sizes	13.03704

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.0034000	16	NO_3^-
A	0.0030545	11	NH_4^+

9.3.39. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 6

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.11835598	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.05919451	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.42365867	Pr > A-Sq	>0.250

9.3.40. Individual seed weight test: Tukey's Studentized Range (HSD) Test for floret 7

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	4.553E-7
Critical Value of Studentized Range	2.99786
Minimum Significant Difference	0.0008
Harmonic Mean of Cell Sizes	7.222222

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Nitrogen
A	0.0032154	13	NO_3^-
B	0.0020800	5	NH_4^+

9.3.41. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for floret 7

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.09186613	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.02057977	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.18152330	Pr > A-Sq	>0.250

9.3.42. Individual seed weight test: Tukey's Studentized Range (HSD) Test for different floret positions under NO_3^- application

Alpha	0.05
Error Degrees of Freedom	156
Error Mean Square	1.11E-06
Critical Value of Studentized Range	4.34511

Comparisons significant at the 0.05 level are indicated by ***.				
Floret position Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
3-2	0.000103	-0.00066	0.00087	
3-1	0.00019	-0.00062	0.000996	
3-4	0.000216	-0.00062	0.001052	
3-5	0.000473	-0.00037	0.001318	
3-6	0.000677	-0.00032	0.001672	
3-7	0.000862	-0.00021	0.001932	
2-3	-0.0001	-0.00087	0.000665	
2-1	8.69E-05	-0.0007	0.000873	
2-4	0.000114	-0.0007	0.00093	
2-5	0.000371	-0.00045	0.001196	
2-6	0.000574	-0.0004	0.001553	
2-7	0.000759	-0.0003	0.001815	
1-3	-0.00019	-0.001	0.000616	
1-2	-8.7E-05	-0.00087	0.000699	
1-4	2.68E-05	-0.00083	0.00088	
1-5	0.000284	-0.00058	0.001145	
1-6	0.000488	-0.00052	0.001497	
1-7	0.000672	-0.00041	0.001756	
4-3	-0.00022	-0.00105	0.000619	
4-2	-0.00011	-0.00093	0.000703	
4-1	-2.7E-05	-0.00088	0.000826	

4-5	0.000257	-0.00063	0.001146	
4-6	0.000461	-0.00057	0.001494	
4-7	0.000645	-0.00046	0.001751	
5-3	-0.00047	-0.00132	0.000371	
5-2	-0.00037	-0.0012	0.000454	
5-1	-0.00028	-0.00115	0.000577	
5-4	-0.00026	-0.00115	0.000632	
5-6	0.000204	-0.00084	0.001244	
5-7	0.000388	-0.00072	0.001501	
6-3	-0.00068	-0.00167	0.000317	
6-2	-0.00057	-0.00155	0.000404	
6-1	-0.00049	-0.0015	0.000522	
6-4	-0.00046	-0.00149	0.000572	
6-5	-0.0002	-0.00124	0.000836	
6-7	0.000185	-0.00105	0.001415	
7-3	-0.00086	-0.00193	0.000209	
7-2	-0.00076	-0.00181	0.000297	
7-1	-0.00067	-0.00176	0.000412	
7-4	-0.00065	-0.00175	0.000461	
7-5	-0.00039	-0.0015	0.000724	
7-6	-0.00018	-0.00142	0.001046	

9.3.43. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for different floret positions under NO_3^- application

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.05446263	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.05964702	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.39991257	Pr > A-Sq	>0.250

9.3.44. Individual seed weight test: Tukey's Studentized Range (HSD) Test for different floret positions under NH_4^+ application

Alpha	0.05
Error Degrees of Freedom	183
Error Mean Square	1.155E-6
Critical Value of Studentized Range	4.33645

Comparisons significant at the 0.05 level are indicated by ***.				
Floret position Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
1-2	8.96E-05	-0.00069	0.000865	
1-3	0.000416	-0.00039	0.001221	
1-4	0.000664	-0.0002	0.00153	
1-5	0.000713	-0.0001	0.001526	
1-6	0.000751	-0.00036	0.001857	
1-7	0.001725	0.000188	0.003262	***
2-1	-9E-05	-0.00086	0.000686	
2-3	0.000326	-0.00051	0.001163	
2-4	0.000575	-0.00032	0.00147	
2-5	0.000623	-0.00022	0.001467	
2-6	0.000661	-0.00047	0.00179	
2-7	0.001636	8.18E-05	0.00319	***
3-1	-0.00042	-0.00122	0.000389	
3-2	-0.00033	-0.00116	0.00051	
3-4	0.000248	-0.00067	0.001169	
3-5	0.000297	-0.00057	0.001168	
3-6	0.000335	-0.00082	0.001485	
3-7	0.001309	-0.00026	0.002878	
4-1	-0.00066	-0.00153	0.000201	
4-2	-0.00057	-0.00147	0.00032	
4-3	-0.00025	-0.00117	0.000672	
4-5	4.83E-05	-0.00088	0.000976	
4-6	8.64E-05	-0.00111	0.00128	
4-7	0.001061	-0.00054	0.002662	
5-1	-0.00071	-0.00153	0.000101	
5-2	-0.00062	-0.00147	0.000221	
5-3	-0.0003	-0.00117	0.000575	
5-4	-4.8E-05	-0.00098	0.00088	
5-6	0.000038	-0.00112	0.001194	
5-7	0.001013	-0.00056	0.002586	
6-1	-0.00075	-0.00186	0.000356	
6-2	-0.00066	-0.00179	0.000468	
6-3	-0.00033	-0.00148	0.000815	
6-4	-8.6E-05	-0.00128	0.001107	
6-5	-3.8E-05	-0.00119	0.001118	
6-7	0.000975	-0.00077	0.002717	
7-1	-0.00173	-0.00326	-0.00019	***
7-2	-0.00164	-0.00319	-8.2E-05	***

7-3	-0.00131	-0.00288	0.00026	
7-4	-0.00106	-0.00266	0.00054	
7-5	-0.00101	-0.00259	0.000561	
7-6	-0.00097	-0.00272	0.000768	

9.3.45. Individual seed weight test: Goodness-of-Fit tests for Normal Distribution for different floret positions under NH_4^+ application

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Kolmogorov-Smirnov	D	0.05995013	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.08721897	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.51933801	Pr > A-Sq	>0.250

9.3.46. Empty seed test: Chi-square test: two-way frequency table for NH_4^+ vs NO_3^- application for each floret position

Floret position	DF	Chi-Square value	Prob.
1	1	0.0000	1.0000
2	1	2.3798	0.1229
3	1	2.1021	0.1471
4	1	1.4400	0.2301
5	1	0.2147	0.6431
6	1	0.2435	0.6217
7	1	0.0826	0.7738

9.3.47. Empty seed test: Chi-square test: two-way frequency table for different floret positions under NH_4^+ application

Statistic	DF	Value	Prob.
Chi-Square	6	7.9722	0.2401
Likelihood Ratio Chi-Square	6	8.0066	0.2376
Mantel-Haenszel Chi-Square	1	4.6769	0.0306
Phi Coefficient		0.1670	
Contingency Coefficient		0.1647	
Cramer's V		0.1670	

9.3.48. Empty seed test: Chi-square test: two-way frequency table for different floret positions under NO_3^- application

Statistic	DF	Value	Prob.
Chi-Square	6	12.6748	0.0485
Likelihood Ratio Chi-Square	6	12.9889	0.0432
Mantel-Haenszel Chi-Square	1	7.5855	0.0059
Phi Coefficient		0.2035	
Contingency Coefficient		0.1994	
Cramer's V		0.2035	

9.3.49. Empty seed test: Chi-square test: two-way frequency table for floret 2 and 4 under NO_3^- application

Table of floret position by seedtype			
Floret position	seedtype		
	Emptyseed	Solidseed	Total
2	11	39	50
	11.00	39.00	50.00
	22.00	78.00	
	33.33	58.21	
4	22	28	50
	22.00	28.00	50.00
	44.00	56.00	
	66.67	41.79	
Total	33	67	100
	33.00	67.00	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	5.4726	0.0193
Likelihood Ratio Chi-Square	1	5.5520	0.0185
Continuity Adj. Chi-Square	1	4.5228	0.0334
Mantel-Haenszel Chi-Square	1	5.4179	0.0199
Phi Coefficient		-0.2339	
Contingency Coefficient		0.2778	
Cramer's V		-0.2339	

9.3.50. Empty seed test: Chi-square test: two-way frequency table for floret 2 and 5 under NO_3^- application

Table of floret position by seedtype			
Floret position	seedtype		
	Emptyseed	Solidseed	Total
2	11	39	50
	11.46	40.63	52.08
	22.00	78.00	
	36.67	59.09	
5	19	27	46
	19.79	28.13	47.92
	41.30	58.70	
	63.33	40.91	
Total	30	66	96
	31.25	68.75	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	4.1557	0.0415
Likelihood Ratio Chi-Square	1	4.1867	0.0407
Continuity Adj. Chi-Square	1	3.3057	0.0690
Mantel-Haenszel Chi-Square	1	4.1124	0.0426
Phi Coefficient		-0.2081	
Contingency Coefficient		0.2037	
Cramer's V		-0.2081	

9.3.51. Empty seed test: Chi-square test: two-way frequency table for floret 2 and 6 under NO_3^- application

Table of floret position by seedtype			
Floret position	seedtype		
	Emptyseed	Solidseed	Total
2	11	39	50
	13.10	46.43	59.52
	22.00	78.00	
	37.93	70.91	
6	18	16	34
	21.43	19.05	40.48
	52.94	47.06	
	62.07	29.09	
Total	29	55	84
	31.25	68.75	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	8.5712	0.0034
Likelihood Ratio Chi-Square	1	8.5603	0.0034
Continuity Adj. Chi-Square	1	7.2571	0.0071
Mantel-Haenszel Chi-Square	1	8.4692	0.0036
Phi Coefficient		-0.3194	
Contingency Coefficient		0.3043	
Cramer's V		-0.3194	

9.3.52. Empty seed test: Chi-square test: two-way frequency table for floret 2 and 7 under NO_3^- application

Table of floret position by seedtype			
Floret position	seedtype		
	Emptyseed	Solidseed	Total
2	11	39	50
	14.47	51.32	65.79
	22.00	78.00	
	45.83	75.00	
7	13	13	26
	17.11	17.11	34.21
	50.00	50.00	
	54.17	25.00	
Total	24	52	76
	31.58	68.42	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	6.2067	0.0127
Likelihood Ratio Chi-Square	1	6.0611	0.0138
Continuity Adj. Chi-Square	1	4.9784	0.0257
Mantel-Haenszel Chi-Square	1	6.1250	0.0133
Phi Coefficient		-0.2858	
Contingency Coefficient		0.2748	
Cramer's V		-0.2858	

9.3.53. Empty seed test: Chi-square test: two-way frequency table for floret 3 and 6 under NO_3^- application

Table of floret position by seedtype			
Floret position	seedtype		
	Emptyseed	Solidseed	Total
3	15	35	50
	17.86	41.67	59.52
	30.00	70.00	
	45.45	68.63	
6	18	16	34
	21.43	19.05	40.48
	52.94	47.06	
	54.55	31.37	
Total	33	51	84
	39.29	60.71	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	4.4656	0.0346
Likelihood Ratio Chi-Square	1	4.4588	0.0347
Continuity Adj. Chi-Square	1	3.5555	0.0593
Mantel-Haenszel Chi-Square	1	4.4124	0.0357
Phi Coefficient		-0.2306	
Contingency Coefficient		0.2247	
Cramer's V		-0.2306	

9.3.54. Germination test (normal seedling): Chi-square test: two-way frequency table for NH_4^+ vs NO_3^- application for each floret position

Floret position	DF	Chi-Square value	Prob.
1	1	0.1369	0.7114
2	1	0.5718	0.4495
3	1	0.0286	0.8656
4	1	0.0192	0.8898
5	1	2.6703	0.1022
6	1	0.0539	0.8163
7	1	0.6785	0.4101

9.3.55. Germination test (dead seed): Chi-square test: two-way frequency table for NH_4^+ vs NO_3^- application for each floret position

Floret position	DF	Chi-Square value	Prob.
1	1	0.1863	0.6660
2	1	0.4650	0.4953
3	1	0.1212	0.7278
4	1	0.0446	0.8326
5	1	2.7000	0.1003
6	1	0.0036	0.9521
7	1	0.0554	0.8139

9.3.56. Germination test (abnormal seedling): Chi-square test: two-way frequency table for NH_4^+ vs NO_3^- application for each floret position

Floret position	DF	Chi-Square value	Prob.
1	1	0.0152	0.9018
2	1	0.0154	0.9013
3	1	0.5250	0.4687
4	1	0.0304	0.8615
5	1	0.0000	1.0000
6	1	0.0767	0.7818
7	1	0.8654	0.3522

9.3.57. Germination test (normal seedling): Chi-square test: two-way frequency table for different floret positions under NH_4^+ application

Table of situation by Ngermination			
Floret position	Ngermination		
	Normal	NoNormal	Total
1	27	11	38
	71.05	28.95	
2	16	16	32
	50.00	50.00	
3	13	15	28
	46.43	53.57	
4	13	9	22
	59.09	40.91	
5	10	17	27
	37.04	62.96	
6	6	5	11
	54.55	45.45	
7	3	2	5
	60.00	40.00	

Note: For each floret position, 1st row is frequency and 2nd row is row percentage

Statistic	DF	Value	Prob.
Chi-Square	6	8.7314	0.1893
Likelihood Ratio Chi-Square	6	8.9160	0.1784
Mantel-Haenszel Chi-Square	1	2.7440	0.0976
Phi Coefficient		0.2314	
Contingency Coefficient		0.2255	
Cramer's V		0.2314	

9.3.58. Germination test (normal seedling): Chi-square test: two-way frequency table for different floret positions under NO_3^- application

Table of situation by Ngermination			
Floret position	Ngermination		
	Normal	NoNormal	Total
1	24	8	32
	75.00	25.00	
2	16	23	39
	41.03	58.97	
3	17	18	35
	48.57	51.43	
4	16	12	28
	57.14	42.86	
5	16	11	27
	59.26	40.74	
6	8	8	16
	50.00	50.00	
7	5	8	13
	38.46	61.54	

Note: For each floret position, 1st row is frequency and 2nd row is row percentage

Statistic	DF	Value	Prob.
Chi-Square	6	10.5001	0.1051
Likelihood Ratio Chi-Square	6	10.8345	0.0936
Mantel-Haenszel Chi-Square	1	1.3354	0.2479
Phi Coefficient		0.2351	
Contingency Coefficient		0.2288	
Cramer's V		0.2351	

9.3.59. Germination test (dead seed): Chi-square test: two-way frequency table for different floret positions under NH_4^+ application

Statistic	DF	Value	Prob.
Chi-Square	6	5.9333	0.4307
Likelihood Ratio Chi-Square	6	5.9262	0.4315
Mantel-Haenszel Chi-Square	1	2.4892	0.1146
Phi Coefficient		0.1908	
Contingency Coefficient		0.1874	
Cramer's V		0.1908	

9.3.60. Germination test (dead seed): Chi-square test: two-way frequency table for different floret positions under NO_3^- application

Statistic	DF	Value	Prob.
Chi-Square	6	6.1361	0.4081
Likelihood Ratio Chi-Square	6	6.3693	0.3831
Mantel-Haenszel Chi-Square	1	0.4417	0.5063
Phi Coefficient		0.1797	
Contingency Coefficient		0.1769	
Cramer's V		0.1797	

9.3.61. Germination test (abnormal seedling): Chi-square test: two-way frequency table for different floret positions under NH_4^+ application

Statistic	DF	Value	Prob.
Chi-Square	6	6.1058	0.4114
Likelihood Ratio Chi-Square	6	6.1493	0.4067
Mantel-Haenszel Chi-Square	1	0.0485	0.8257
Phi Coefficient		0.1935	
Contingency Coefficient		0.1900	
Cramer's V		0.1935	

9.3.62. Germination test (abnormal seedling): Chi-square test: two-way frequency table for different floret positions under NO_3^- application

Statistic	DF	Value	Prob.
Chi-Square	6	3.7972	0.7041
Likelihood Ratio Chi-Square	6	4.1192	0.6606
Mantel-Haenszel Chi-Square	1	0.8353	0.3607
Phi Coefficient		0.1414	
Contingency Coefficient		0.1400	
Cramer's V		0.1414	

9.3.63. Endophyte presence test: Chi-square test: two-way frequency table for NH_4^+ vs NO_3^- for each floret position

Floret position	DF	Chi-Square value	Prob.
1	1	5.7152	0.0168
2	1	0.0000	1.0000
3	1	1.7829	0.1818
4	1	0.0897	0.7645
5	1	0.5002	0.4794
6	1	1.6154	0.2037

9.3.64. Endophyte presence test: Chi-square test: two-way frequency table for NH_4^+ and NO_3^- in floret 1

Table of N by Endophyte			
N	Endophyte		
	Endophyte	Noendophyte	Total
NH4	45	6	51
	47.37	6.32	53.68
	88.24	11.76	
	60.00	30.00	
NO3	30	14	44
	31.58	14.74	46.32
	68.18	31.82	
	40.00	70.00	
Total	75	20	95
	78.95	21.05	100.00

Note: For each nitrogen form, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	5.7152	0.0168
Likelihood Ratio Chi-Square	1	5.7954	0.0161
Continuity Adj. Chi-Square	1	4.5724	0.0325
Mantel-Haenszel Chi-Square	1	5.6551	0.0174
Phi Coefficient		0.2453	
Contingency Coefficient		0.2382	
Cramer's V		0.2453	

9.3.65. Endophyte presence test: Chi-square test: two-way frequency table for different floret positions under NH_4^+ application

Statistic	DF	Value	Prob.
Chi-Square	6	2.5521	0.8626
Likelihood Ratio Chi-Square	6	4.0804	0.6658
Mantel-Haenszel Chi-Square	1	1.1805	0.2773
Phi Coefficient		0.1267	
Contingency Coefficient		0.1267	
Cramer's V		0.1267	

9.3.66. Endophyte presence test: Chi-square test: two-way frequency table for different floret positions under NO_3^- application

Statistic	DF	Value	Prob.
Chi-Square	6	14.7959	0.0219
Likelihood Ratio Chi-Square	6	16.7340	16.7340
Mantel-Haenszel Chi-Square	1	5.1327	0.0235
Phi Coefficient		0.2762	
Contingency Coefficient		0.2762	
Cramer's V		0.2762	

9.3.67. Endophyte presence test: Chi-square test: two-way frequency table for floret 1 and 2 under NO_3^- application

Table of floret position by Endophyte			
Floret position	Endophyte		
	Endophyte	Noendophyte	Total
1	30	14	44
	41.67	19.44	61.11
	68.18	31.82	
	53.57	87.50	
2	26	2	28
	36.11	2.78	38.89
	92.86	7.14	
	46.43	12.50	
Total	56	16	72
	77.78	22.22	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	6.0278	0.0141
Likelihood Ratio Chi-Square	1	6.8246	0.0090
Continuity Adj. Chi-Square	1	4.6847	0.0304
Mantel-Haenszel Chi-Square	1	5.9441	0.0148
Phi Coefficient		-0.2893	
Contingency Coefficient		0.2779	
Cramer's V		-0.2893	

9.3.68. Endophyte presence test: Chi-square test: two-way frequency table for floret 1 and 4 under NO_3^- application

Table of floret position by Endophyte			
Floret position	Endophyte		
	Endophyte	Noendophyte	Total
1	30	14	44
	39.47	18.42	57.89
	68.18	31.82	
	50.00	87.50	
4	30	2	32
	39.47	2.63	42.11
	93.75	6.25	
	50.00	12.50	
Total	60	16	76
	78.95	21.05	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	7.2869	0.0069
Likelihood Ratio Chi-Square	1	8.2214	0.0041
Continuity Adj. Chi-Square	1	5.8298	0.0158
Mantel-Haenszel Chi-Square	1	7.1911	0.0073
Phi Coefficient		-0.3096	
Contingency Coefficient		0.2958	
Cramer's V		-0.3096	

9.3.69. Endophyte presence test: Chi-square test: two-way frequency table for floret 1 and 7 under NO_3^- application

Table of floret position by Endophyte			
Floret position	Endophyte		
	Endophyte	Noendophyte	Total
1	30	14	44
	55.56	25.93	81.48
	68.18	31.82	
	75.00	100.00	
7	10	0	10
	18.52	0.00	18.52
	100.00	0.00	
	25.00	0.00	
Total	40	14	54
	74.07	25.93	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Statistic	DF	Value	Prob.
Chi-Square	1	4.2955	0.0382
Likelihood Ratio Chi-Square	1	6.7631	0.0093
Continuity Adj. Chi-Square	1	2.7984	0.0944
Mantel-Haenszel Chi-Square	1	4.2159	0.0400
Phi Coefficient		-0.2820	
Contingency Coefficient		0.2714	
Cramer's V		-0.2820	

9.3.70. Endophyte presence test: Chi-square test: two-way frequency table for floret 3 and 4 under NO_3^- application

Table of floret position by Endophyte			
Floret position	Endophyte		
	Endophyte	Noendophyte	Total
3	24	8	32
	37.50	12.50	50.00
	75.00	25.00	
	44.44	80.00	
4	30	2	32
	46.88	3.13	50.00
	93.75	6.25	
	55.56	20.00	
Total	54	10	64
	84.38	15. 63	100.00

Note: For each floret position, 1st row is frequency, 2nd row is percentage, 3rd row is row percentage, and 4th row is column percentage

Chi-Square	1	4.2667	0.0389
Likelihood Ratio Chi-Square	1	4.5229	0.0334
Continuity Adj. Chi-Square	1	2.9630	0.0852
Mantel-Haenszel Chi-Square	1	4.2000	0.0404
Phi Coefficient		-0.2582	
Contingency Coefficient		0.2500	
Cramer's V		-0.2582	