Transmission and survival of perennial ryegrass endophyte during field based seed production.

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Agriculture in New Zealand is heavily centred on the use of perennial ryegrass (*Lolium perenne*) based pasture systems. However, like all forage plant species, perennial ryegrass is susceptible to pests and diseases. To help overcome these pests and diseases, perennial ryegrass has developed a symbiotic relationship with the specific fungal endophyte species, *Epichloë festucae* var. *lolii* (syn. *Neotyphodium lolii*). The New Zealand pasture industry uses *E. festucae* var *lolii* which colonise perennial ryegrass (*Lolium perenne*) and this interaction has been extensively researched due to the positive and negative attributes they cause. This endophyte is only transmitted vertically (i.e. from parent to seed) and the transmission process is not always absolute which can result in a decrease in endophyte percentage. One such endophyte strain known to have transmission issues is AR37. This decrease commonly occurs during the seed production process. However, it is not yet fully known why this decrease occurs.

The overall aim of this research (Objective 1) was to determine where in the seed production process the decrease in AR37 endophyte percentage in perennial ryegrass occurred. To facilitate this aim, the seed production processes of two farms in Canterbury (Farm A and Farm B) were followed during the 2017-2018 seed production season. The two farms were chosen based on their seed production history. Farm A was known to consistently produce seed with high (over 70%) endophyte levels, while Farm B was not as consistent. Both farms grew the same seed line (cv. Governor) containing the same endophyte strain (AR37).

The management practices used to produce the ryegrass seed differed between the two farms. The most notable differences occurred during fungicide application and harvest. Farm A carried out six fungicide applications whereas Farm B only carried out three. Farm B also stored around 10% of the harvested seed in a tractor trailer for the duration of the harvest period (2 days). No significant

decrease in endophyte percentage occurred for the seed stored in the trailer. This result can help assure seed companies that, if a grower must store seed under poor conditions for short periods, no loss in endophyte should occur. More research would be needed to further confirm this, such as repeating the experiment with various seed lines. No significant decrease in endophyte percentage occurred during the entirety of this experiment. However, it was a good harvest season for endophyte, in that no obvious loss in endophyte occurred compared to other seasons, so it is possible that losses may be seen during other harvest seasons. More research would be required to determine this.

Fungicides are commonly used during ryegrass seed production to reduce the effect of pathogenic fungi such as *Gloeotinia temulenta* which causes blind seed disease. However, fungicides also have the potential to harm beneficial fungi such as endophyte. Two controlled experiments were set up for objective two to determine if fungicides commonly used during ryegrass seed production had any effect on endophyte level. The first experiment showed that application of benzimidazole (Protek), tebuconazole (Folicur), prothioconazole (Proline) and azoxystrobin (Amistar) fungicide to Alto AR37 ryegrass plants did not affect endophyte level. However, in this experiment the initial >70% endophyte level of seed (from laboratory analysis) was unexpectedly below 20% (on average) when tillers were tested as a control grow out during the experiment. Therefore, an additional experiment was set up to determine why the endophyte level dropped between the laboratory test and the tiller tests carried out during the experiment. In this second experiment the endophyte level was maintained at over 90% on average, so the variables tested (potting mix, fungicides, and pots used as well as area grown) were not those responsible for the drop in endophyte detection. Different seed lots were used for both experiments which could explain this difference seen in endophyte percentage. Another possible reason is that the plants in the first controlled experiment may have been exposed to different growing conditions, such as nutrient stress or water stress, which hindered colonisation of the endophyte in emerging tillers.

The final objective involved observing the effect that temperature, humidity and storage length had on endophyte viability. It was found that as the storage length increased, to 16 weeks, the viability of the endophyte found in seed (seed endophyte) decreased by up to 66% (14% moisture), particularly at higher temperature (20.3-31.2°C) and humidity levels (65.1-71.1% RH).

No major differences were observed in the first two experiments indicating that the key factors investigated (fungicide application and harvest methods) are not the sole reason for loss of endophyte transmission. This combined with other interesting but not tested observations has provided ideas for future research (such as testing the influence of climatic conditions on endophyte

growth) and has helped to confirm that certain field environment and management factors, such as fungicide application, have no effect on endophyte viability.

Keywords: Perennial ryegrass, AR37, endophyte, seed production, fungicide, seed storage, seed squash, immunoblot, seed transmission.

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Chapter 1

Introduction

In New Zealand, the agricultural industry is heavily centred on grass-based pasture systems. However, like any plant, grasses can face a loss in production due to pests, diseases, and adverse environmental conditions such as drought. There are many strategies plants can use to reduce this loss, including forming a symbiotic relationship with another organism. One such group of organisms are endophytes which colonise the internal tissues of the plant (Schulz, 2006). These endophytes can be either bacteria or fungi and some have been used to enhance crop performance.

The New Zealand pasture industry in particular uses *Epichloë festucae* var. *lolii* (syn. *Neotyphodium lolii*) (Leuchtman *et al*., 2014) endophytes to improve the persistence of perennial ryegrass (*Lolium perenne*), which is the main pasture species sown. These endophytes colonise the perennial ryegrass and have been extensively researched due to the positive and negative effects they cause. The endophytes are referred to as clavicipitaceous endophytes (Rodriguez, White Jr, Arnold, & Redman, 2009). The term clavicipitaceous refers to their taxonomy, with these fungi being ascomycetes of the family Clavicipitaeae (Rodriguez et al., 2009). Colonisation by *E. festucae* var. *lolii* may increase the plant's tolerance to drought and insect herbivory, due to secondary metabolites produced by the endophyte (Easton, Latch, Tapper, & Ball, 2002). These secondary metabolites can however also pose animal health issues such as heat stress and ryegrass staggers (a neuro-muscular disorder) in sheep and cattle. The severity of these traits is dependent on, not only the endophyte strain, but also the ryegrass genotype (host-endophyte interaction) and the environmental conditions (Easton et al., 2002).

Asexual clavicipitaceous endophytes are transmitted via the host seed. As a result, environmental and management conditions during seed production is incredibly important for the survival of these endophytes as seed can sometimes be produced with no endophyte present despite endophyte being present in the parent plant. As with the traits previously outlined, endophyte transmission is also affected by the ryegrass genotype, the endophyte genotype and the environment (G x G x E) (Bouton et al., 2002; Hill & Roach, 2009). Since this endophyte is transmitted via the seed only, the seed production process is crucial in maintaining the necessary level of endophyte.

To better understand how endophyte is maintained in the seed, the factors affecting the colonisation and survival in the seed must first be examined. A reduction in infection of seed by the endophyte or decreased viability of the endophyte, can occur due to a variety of environmental and management factors during crop growth, such as the type of fungicide applied potentially reducing colonisation of

the seed, or the postharvest storage conditions leading to a reduction in endophyte viability. These factors have been extensively researched under laboratory conditions (Bylin, Card, Hume, Lloyd-West, & Huss-Danell, 2016; Cruz et al., 2018; Missaoui & Hill, 2015; Rolston, Archie, & Simpson, 2002; Rolston, Hare, Moore, & Christensen, 1986; Rolston, McCloy, Harvey, & Chynoweth, 2009). However, under real world field conditions more variables are present such as the weather and management practices.

This research project aimed to determine the factors which influence endophyte transmission and survival under field conditions through detailed investigation of the management practices used by two contrasting commercial growers producing the same seed line of perennial ryegrass in Canterbury, New Zealand. Further experiments were carried out to investigate the effect of fungicide application during crop growth on endophyte colonisation of the seed, and seed storage conditions on maintenance of endophyte viability in the seeds. This chapter reviews all literature relevant to the experiments carried out in order to meet the research objectives.

1.1 Fungal endophytes

While endophytes may be a very broad term, fungal endophytes are commonly classified as being either clavicipitaceous or non-clavicipitaceous endophytes (Rodriguez et al., 2009). These two types are very different, both biologically and phylogenetically, from each other and as a result behave differently with plants. Within the two types, endophytes are further split into four classes based on their interaction with the plant host (Table 1.1). Class one is the only class of clavicipitaceous endophytes whereas the other three classes are non-clavicipitaceous endophytes (Hardoim et al., 2015; Khiralla, Spina, Yagi, Mohamed, & Laurain-Mattar, 2016; Rodriguez et al., 2009). Clavicipitaceous endophytes have a narrow host range and are found in warm and cool season grass species such as perennial ryegrass and tall fescue (*Festuca arundinacea*).

Table 1.1 Criteria used to characterise clavicipitaceous and non-clavicipitaceous fungal endophytes into classes based on interaction with the plant host. From Rodriguez et al. (2009)

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Within the clavicipitaceous class one endophytes there are three types: type one, type two and type three (Clay & Schardl, 2002). These types differ largely based on the method of transmission between plant generations, being either asexual i.e. vertically transmitted from the parent plant into the offspring through seed infection, or sexual, i.e. horizontally transmitted between individual plants through ascospores. Type one endophytes are symptomatic and pathogenic fungal species which, during flowering of the grass, grow over the developing flower head to form a stroma, preventing the further development of the seed head (Khiralla et al., 2016). These stromata can be formed on most or all of the tillers found on the infected plant. If there are tillers of the plant that do not get infected by stromata, then these tillers will be free from infection, even if there are other tillers from the same plant that are infected. This means that tillers free from infection are able to produce seed whereas tillers that are infected are unable to produce seed due to the presence of the stromata (Rodriguez et al., 2009).

Type two endophytes are asymptomatic and mixed-interaction endophytes, which develop stromata on a proportion of tillers rather than all infected tillers. The endophyte will also reproduce sexually on some but not all the infected tillers, with the ascospores produced being dispersed to infect other plants (horizontal transmission) to complete the life cycle. This enables partial seed production to occur as some tillers contain endophyte but will not produce stromata, allowing vertical transmission to occur (Khiralla et al., 2016; Rodriguez et al., 2009).

Type three endophytes are the most common of the clavicipitaceous endophytes as they are able to grow within the plant without showing any visible symptoms of the infection (Rodriguez et al., 2009). Like type two endophyte, type three endophytes are asymptomatic and mixed-interaction endophytes; however, they do not reproduce sexually and instead rely on vertical transmission. They are found in most grass plants, in particularly ryegrass and tall fescue plants (Hardoim et al., 2015; Khiralla et al., 2016). As these endophytes are the focus of this research project, when the word endophyte is used alone, it is referring to type three clavicipitaceous endophytes unless otherwise stated.

1.2 Endophytes of ryegrass

The endophyte species commonly associated with perennial ryegrass is *E. festucae* var. *lolii*, which has also been previously known as *Neotyphodium lolii* (also *Acremonium lolii*) (Leuchtmann, Bacon, Schardl, White Jr, & Tadych, 2014). Both names have been used at the same time for this species but the name is specific to how the fungus reproduces. *Neotyphodium lolii* is the anamorph name for the fungus referring to the asexual stage of the fungal lifecycle which occurs in the southern hemisphere, and *E. festucae* var. *lolii* is the teleomorph name, referring to sexual reproduction. Due to a recent (2011) change in nomenclature rules regarding the naming of fungi, this species is now referred to using the sexual (teleomorph) name only so that one species has one name (Leuchtmann et al., 2014).

This endophyte species has many different strains such as NEA2, AR37 and AR1 and each endophyte strain can produce different levels and types of alkaloids, some of which are more beneficial to crop production than others (Thom, Popay, Hume, & Fletcher, 2013). Many of the alkaloids produced help increase the performance of the plant by conferring herbivory deterrence to the plant. Some alkaloids, such as ergovaline and lolitrems, may provide good insect deterrence but can also cause livestock health issues when ingested, such as heat stress and ryegrass staggers, respectively (Pennell & Rolston, 2013). Ryegrass staggers is a neuro-muscular disorder resulting in the affected livestock animal having difficulty standing and walking properly, which results in loss of productivity (Thom et al., 2013). While ryegrass staggers does not result directly in the death of the animal, it can indirectly lead to the death of the animal if for example, it falls in a waterway or in a gully (Di Menna, Finch,

Popay, & Smith, 2012). Ergovaline can cause heat stress in animals, as well as other animal health issues.

For both ergovaline and lolitrems it is the dosage which influences the severity of the effects on the animal. Endophyte strains such as NEA2 produce ergovaline. However if the grass is grazed normally (at a height of 4-5 cm) then no significant animal health effects will occur due to a low ergovaline concentration (0.03mg/kg $LW^{0.75}$/day) in the grazed herbage (Eady, Corkran, Bailey, Kerr, & Nicol, 2017; Klotz & Nicol, 2016).

Bastias et al. (2017) found that aphids had a tolerance level for alkaloids as aphids were able to metabolize the allelochemicals. However, it was hypothesised that this metabolization led to an increase in energy demands for the aphids, which lead to more feeding and so on. When the feed demand was not met, detrimental effects were seen such as a reduction in insect growth, fecundity and life-span. It was also hypothesised that once the tolerance level of the alkaloids was reached, a decrease in the amount of phloem sap ingested would occur. More research is needed to confirm this (Bastias et al., 2017).

Drought tolerance has been found to increase in plants with endophytes by way of an increase in root growth (He, Hatier, & Matthew, 2017; Kuldau & Bacon, 2008). It has been found that the rate and length of root growth of endophyte infected plants, is higher than those plants without endophyte. An increase in root growth enables the plant to take up more water from the soil during droughts and recover raster due to rapid water uptake (Malinowski & Belesky, 2019). This ability also has the potential to improve the nutrient status of the plant as there is the possibility of an increase in nutrient uptake from the soil, further emphasising the importance of endophyte pastures (Kuldau & Bacon, 2008).

Endophytes have also been reported to suppress the growth of annual grass weeds such as barley grass (*Critesion murinum*) (Tozer, Lucas, & Edwards, 2007). Tozer et al. (2007) found that the addition of the endophyte, AR542 (a tall fescue endophyte), caused a significant decrease in the amount of barley grass present. This was most likely due to the increased growth to the tall fescue plants induced by the endophyte. An increase in growth would also increase the competitive advantage of the crop, allowing it to outcompete other plants found in the pasture. This increased growth further emphasises the importance of endophyte in pastural systems.

One *E. festucae* var. *lolii* endophyte strain used widely for perennial ryegrass in New Zealand is AR37 due to the alkaloids that are produced. This endophyte strain (AR37) does not produce lolitrems or ergovalines, instead AR37 produces epoxy-janthitrems (Thom et al., 2013) which can also cause ryegrass staggers (Eady et al., 2017). Janthitrems are known to be good at deterring insects such as

Argentine stem weevil (*Listronotus bonariensis*) and black beetle (*Heteronychus arator*), and AR37 is purported to be the only ryegrass endophyte strain which provides protection against the New Zealand grass grub (*Costelytra zealandica;* syn *C. giveni*) (Coca-Abia & Romero-Samper, 2016). AR37 is currently the only strain that has been shown to produce this alkaloid. Thom et al. (2013) compared AR37 with the common endophyte strain AR1, which produces peramine and no ergovalines, lolitrems or epoxy-janthitrems in perennial ryegrass, and found that AR37 provided better protection for not only black beetle, but also for root aphid (*Rhopalosiphum rufiabdominale*) and porina (*Wiseana cervinata*). The number of grass grub present was also consistently lower in the presence of AR37 however this was not significant (Thom et al., 2013). Patchett, Chapman, Fletcher, and Gooneratne (2008) also found that when meadow fescue (*Festuca pratensis*) plants, containing the endophyte *Neotyphodium uncinatum*, were attacked by grass grub, the loline concentrations in the roots increased by up to 40%, indicating the ability to deter herbivory.

1.3 Potential applications of endophyte infected grasses

Clavicipitaceous endophytes are currently used primarily in the pastoral farming industry because of their ability to enhance ryegrass productivity by up to 30% (Popay et al., 1999). As discussed further below, new research has resulted in them also being used in other grassed areas where they can be used such as on golf greens, airfields, parks and the horticultural industry using the alkaloid ergovaline (Card, Rolston, Lloyd-West, & Hume, 2014; Pennell & Rolston, 2013).

While ryegrass and tall fescue endophytes are commonly found and developed for agricultural purposes, improvements can still be made to further increase their utilisation. A large proportion of New Zealand pasture comprises endophyte-colonised ryegrass although in regions such as Southland, endophyte-colonised ryegrass is not as commonly used (Phil Simons, personal communication). This is because grass seeds that contain endophytes are commonly more expensive than grass seeds that contain no endophyte and in Southland, since the insect pest levels there are often less severe than other parts of the country such as Waikato, the economic return is often not considered sufficient (Easton & Fletcher, 2007). Even though endophyte can be used as a preventative measure there are some farmers that are averse to the use of endophyte due to the animal health issues that can be associated with it. As the climate continues to change, it is possible that the average temperature in Southland will increase (Lawrence et al., 2016). With this increase in temperature, an increase in pasture pests such as Argentine Stem Weevil is also possible and therefore an increasing need for the benefits of endophyte strains such as NEA2 and AR37 is required. Other factors such as rainfall could also affect the benefits of endophyte, popularising it in other areas such as Taranaki as well.

Clavicipitaceous endophytes are also able to be used in the aviation industry whereby grass plants colonised by endophytes are used to surround the landing strips found at airports, such as the Christchurch airport (Pennell et al., 2017). Grass species are typically planted around these landing strips and grasses such as perennial ryegrass and tall fescue can be used. Bird strike can be an issue at airports as they forage on the invertebrates that are found in the grass swards and feed on the plants themselves. AgResearch has developed two different endophyte strains that can be used to deter birds, one of which is used with perennial ryegrass (AR95) and the other is used with tall fescue (AR601) (Pennell et al., 2017; Pennell & Rolston, 2013). These endophytes have reduced bird feeding on grass plants by up to 40% which will reduce the number of birds around airfields (Pennell & Rolston, 2013).

Bird feeding is also a major issue in horticultural crops and the use of endophyte colonised grasses in orchards and vineyards could help reduce the amount of birds found in the orchards and therefore help reduce the amount of fruit that is damaged and lost due to bird feeding (Eisemann, Werner, & O'Hare, 2011). Other endophyte strains could also be beneficial when used in orchards and vineyards as they can deter insect feeding and nematode activity (Pennell et al., 2017).

While endophytes are typically beneficial in pastures due to their insect deterrence, they have been found to negatively affect beneficial insects and impact surrounding ecosystems. Cripps and Edwards (2013) found that the application of endophytes in pastures reduced the rate at which faecal degradation occurred because of the alkaloids produced. Alkaloids such as ergovaline, were found in faecal matter after consumption of endophyte infected pasture and are the reason why faecal degradation was reduced. While more research is needed to understand the full extent of this reduction, it is clear that nutrient cycling in pasture, and ecosystems surrounding this cycle, will be negatively affected.

Lastly, another potential use for grasses infected with endophyte is to use them to control plant parasitic nematodes (Thom et al., 2013). Bacetty et al. (2009) investigated the impact that ergovaline and lolitrem alkaloids had on the nematode, *Pratylenchus scribneri* and found that both alkaloids exhibited nematocidal properties. While both alkaloid compounds are found as a result of grass endophytes it has been shown that other plant endophyte types can also exhibit nematocidal properties such as those found in bananas, rice and tomatoes (Muponda, 2014; Niere, Speijer, & Sikora, 1999). There is also the potential for grass based endophytes to be used to help control animal nematodes. For example, Muponda (2014) found using in vitro studies, that alkaloids did in fact have a negative impact on parasitic animal nematodes, particularly *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*, by reducing larvae mobility and egg hatching rate. This could

potentially help reduce drench resistance which is becoming problematic, particularly in the sheep industry (De & Sanyal, 2009).

1.4 Ryegrass endophyte life cycle

This review deals with concerns the asexual endophyte and as such the sexual stage of the lifecycle will not be covered. The asexual (vegetative) reproduction cycle of ryegrass endophytes is carried out by vertical transmission by fungal hyphae and begins with the infected plant as shown in Figure 1.1 (Cheplick & Faeth, 2009; Hettiarachchige et al., 2015). The highest concentrations of endophyte are found at the base of the young seedling and most of this will remain at the base until the plant is ready to begin its reproductive stages. However, hyphae can be found in other parts of the plant as the endophyte grows (Cheplick & Faeth, 2009). These hyphae grow parallel to the plant cells and grow in the gaps between these cells (Clay & Schardl, 2002; Hettiarachchige et al., 2015). As a general rule the endophyte hyphae growth is highly synchronised with leaf growth (Easton, 1999) until the plant reaches its reproductive stage, when the hyphae of the endophyte will continue growing upwards, into the inflorescence. In general only one endophyte strain will be found colonising an individual plant (Clay & Schardl, 2002).

Once the seed has begun to form, the hyphae of the endophyte will grow into the ovule where it will remain and develop, so that it infects the seed (Cheplick & Faeth, 2009). In mature infected seeds the endophyte will be found between the pericarp and the aleurone layer as well as between the scutellum cells (Hume, Card, & Rolston, 2013). Once the seed drops from the plant and begins to germinate, the endophyte begins to grow and will once again be found in the developing plant tissue (Cheplick & Faeth, 2009). However, during commercial seed production care must be taken to ensure the endophyte is maintained in the seed, as higher temperatures and humidity will decrease the endophyte viability as described later in this review (Thom et al., 2013).

Figure 1.1 The life cycle of clavicipitaceous endophytes, showing the sexual and asexual life cycles corresponding to horizontal and vertical reproduction, respectively. From Cheplick and Faeth (2009).

1.5 Factors affecting endophyte transmission

The relationship between the plant species/cultivar and the endophyte strain is highly important in determining the efficiency of transmission into the seed and resulting seed colonisation (Card et al., 2014; Cheplick & Faeth, 2009). If the endophyte does not form a relationship with the plant, then it will not be successfully transmitted throughout the plant and therefore into the seed. As previously stated, endophytes are found within plants and often form a symbiotic relationship but often these endophytes are specific to a single plant species or cultivar. As a result, these endophyte species are then separated further into two categories: standard endophytes and novel endophytes (Thom et al., 2013). Standard endophytes are naturally found within plants and are often native to the country in which they are found whereas novel endophytes are selected for and will then be used to specifically infect grass plants for commercial purposes. As these relationships are often not formed naturally there is a potential for the endophyte to not be stably maintained within the plant. A novel endophyte, *E. festucae* var. *lolii* strain AR37, will be the focus of this research.

Fungicides are widely used during crop production to limit the damage caused by pathogenic fungi. In some grass species fungicides are required to limit losses in seed production due to diseases such as blind seed (*Gloeotinia temulenta*) which reduces the amount of viable seed and as a result has a major economic impact (Lynch, 1952). However, if the wrong fungicide type is used (systemic fungicides) (Hill & Brown, 2000) there is the potential to negatively affect clavicipitaceous fungal endophytes colonising these plants which can result in the reduction of endophyte viability and

therefore seed transmission. Although endophytes colonising the internal tissue of grass plants are likely to not be as negatively affected by some fungicide spray applications, compared with other fungal species, systemic fungicides (such as propiconazole) have been reported to significantly lower the endophyte percentage in seed (Hill & Brown, 2000). The impact that fungicides have on endophyte viability and transmission has not been widely researched and as a result the overall effects are unknown although Hill and Brown (2000) stated that systemic fungicides resulted in a higher loss of endophyte and as a result contact fungicides should be used instead. This contrasts with the work carried out by Rolston et al. (2002) who reported no loss in endophyte as a result of systemic fungicides. Despite this the active ingredients in fungicides used by growers, are often changing (Rolston et al., 2009) and as a result, these may affect endophyte differently.

Despite the fact that systemic fungicides may cause a loss in endophyte production, seed companies such as Agriseeds still recommend that growers use a spray programme which consists of fungicides such as benzimidazole (Protek), tebuconazole (Folicur), prothioconazole (Proline) and azoxystrobin (Amistar), all of which are systemic fungicides. These fungicides are applied around two months before harvest so it is possible that endophyte colonisation of the plant tissue is sufficient to withstand the application of the fungicide as the experiment conducted by Hill and Brown (2000) only tested these fungicides on grass seedlings as a method of controlling damping off. Applying the fungicides to seedlings may mean that the endophyte hyphae would be quite young/new and less extensively established and may not have been able to tolerate the fungicide. More testing is required to determine if this is in fact the case.

The weight of the seed is also seen to be important in terms of endophyte colonisation and transmission in seed production. However, like the effect of fungicide application, this has not been widely researched. Within a single seed line there can be a range of seed weights, with the percentage of endophyte colonisation of heavier seeds often being higher than for the smaller seeds (Card et al., 2014). It has been hypothesised that the colonisation of heavier seeds was higher since larger seeds are likely to contain more carbohydrates, nutrients and provide increased protection to the fungus. However, more research is needed to determine this (Card et al., 2014).

Seed weight is dependent on the position at which the seed is found on the seed head as the basal spikes are found to be the heaviest (Holmes, 2016). The seed weight then decreases further up the seed head with the distal spikes being the lightest. Within these spikes more seeds of varying weights are found with the weight decreasing from the basal seeds to the distal seeds (Holmes, 2016). The lighter the seed, the lower the carbohydrates found within, which is likely the reason as to why the endophyte colonisation is lower within the lighter seeds (Warringa, De Visser, & Kreuzer, 1998). It is

also possible that because the heavier seeds are found closer to the stem, the endophyte has a higher chance of colonising these first, however this has yet to be proven.

The overall seed weight of a seed crop can also be determined based on the type of harvesting method. Harvesting methods can alter the overall endophyte percentage of the crop such as when the crop is field dressed the seed line will contain higher levels of light seed, therefore potentially decreasing the overall endophyte percentage. If however, the crop is machine dressed then this will decrease the amount of light seed, potentially increasing the endophyte percentage of the seed lot due to the lack of light seed (Card et al., 2014).

1.6 Factors affecting endophyte maintenance within the seed.

Seed storage temperature and seed moisture, have been considered to be the most important factors in terms of maintenance of viable endophyte during seed production (Hume, Schmid, Rolston, Vijayan, & Hickey, 2011) although it is the combination of these two factors which proves to be of upmost importance. Kitson (2017) found that the combination of fluctuating humidity and increasing temperature, reduced endophyte viability significantly, supporting work carried out by Gundel, Martínez-Ghersa, Garibaldi, and Ghersa (2009), Rolston et al. (1986) and Freitas (2017).

Seed exposed to high temperatures (above 20°C) as well as high levels of humidity (75%), was reported to negatively affect the viability of *Neotyphodium occultans* in Italian ryegrass (*Lolium multiflorum*) seed with no viable endophyte present after 400 hours, as shown in Figure 1.2 (Gundel et al., 2009). At 40°C and 5% relative humidity the viable endophyte levels decreases, although not to the same extent as seen when the relative humidity is 75% and 43%. Although this study is on the effect of these parameters on the viability of *Neotyphodium occultans* and not *E. festucae* var. *lolii*, it is likely to be similarly affected. While it is unlikely that under a controlled storage environment these conditions would be reached, during the harvest process the seed moisture level can be anywhere from 11% to 14% depending on the grower. The temperature of the seed coming out of the harvester is also around 10°C hotter than the ambient temperature (Phil Rolston, personal communication). Therefore, if the seed is being harvested during periods of 30°C heat then the seed coming out of the harvester could be exposed to temperatures of up to 40°C. If the seed remains in the truck for an extended period of time under these conditions, then it is possible for this to reduce the percentage of seeds with viable endophyte. While this reduction may not be large, it is possible that it could have detrimental economic effects especially if this results in the downgrading of seed from being classed as high endophyte level (above 70%) to below this level. To prevent any reduction in viability it is important that the seed is cooled down as soon as possible, by methods such as placing the seed in an aerated grain silo.

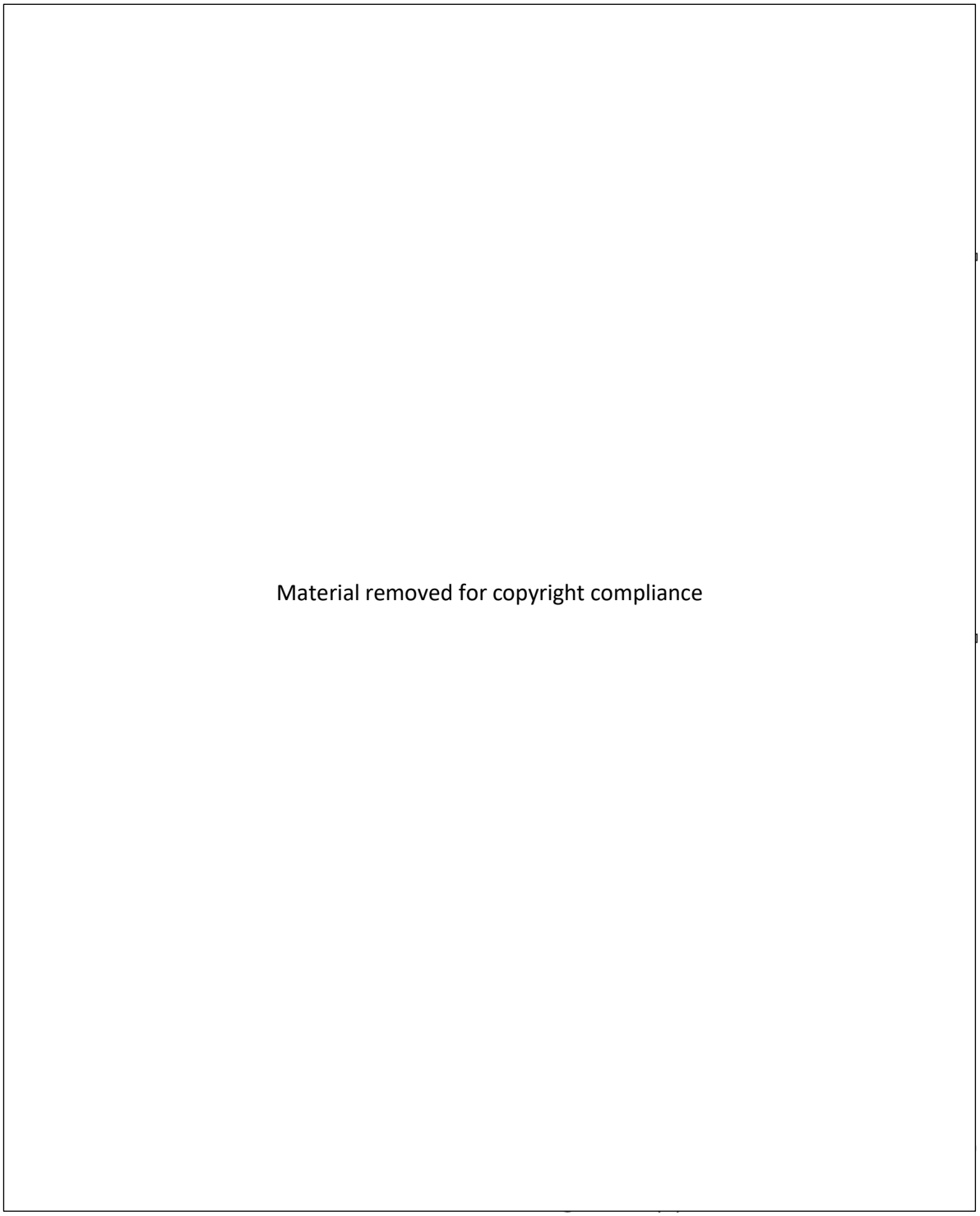

Figure 1.2 The effect of storage temperature and relative humidity on Neotyphodium occultans endophyte viability in Italian ryegrass (*Lolium multiflorum*) seeds assessed after different storage times. From Gundel, Martínez-Ghersa, Garibaldi, and Ghersa (2009).

The length of storage time of the seed, or seed age, can play a large role in the percentage of seed with viable endophyte as endophyte viability decreases over time, irrespective of seed viability. The length of time that endophyte colonised seeds can be successfully stored and for the endophyte to remain viable, will depend greatly on the conditions they are exposed to. If the endophyte-colonised seeds are exposed to unfavourable conditions, such as high relative humidity (75%) and high

temperatures (> 20°C), then the length of time that the seed can be stored and maintain viable endophyte will be significantly reduced and the endophyte may die out completely within one year (Gundel, Martínez-Ghersa, & Ghersa, 2012). If the endophyte colonised seed is exposed to favourable conditions (4°C and 30% relative humidity), the seed can be stored for up to eight years whilst maintaining high endophyte viability (Clement et al., 2008).

1.7 Endophyte detection methods

Endophytes are found in both the tillers and seeds of the plants and as such it is important that the hyphae can be detected within the seed as well as the tillers. Visualisation of seed endophyte can be achieved by carrying out a seed squash test (Section 2.1.2) and histological analysis on the stained seed tissue. While this is a standard technique it does require a high skill level and can be very time consuming. However, it does not rely on expensive consumables or specialised equipment for testing the seed for endophyte (Trento, Elias, Garay, & Zavala, 2007).

Another issue with seed endophyte visualization is that it is not possible to determine if the endophyte is alive or dead. It is possible that the endophyte was present in the seed but has subsequently died. The histological seed endophyte detection method only provides information as to whether the endophyte is present or not and not the viability status of the endophyte (Card, Tapper, Lloyd-West, & Wright, 2013). This is helpful when determining if the endophyte has had a high transmission rate, but it does not provide any additional information with regards to endophyte viability. For this the seed must be planted, grown and the new growing tissue tested for endophyte, indicating viability as the endophyte has grown into the new tissue.

Endophyte viability testing involves germinating the seed and then taking a sample of plant tissue from the grass tillers at the base of the resulting plant (i.e. the leaf sheath). The leaf sheath will contain the highest concentration of endophyte hyphae so is therefore the best part of the plant to test (Easton, 1999). A basal section of the leaf sheath is laid flat and stained to make it as easy to analyse as possible. This leaf can be stained using stains such as rose bengal or aniline blue (Jackson, Saha, & Johnson-Cicalese, 1988). Plant material can also be stained with fluorescent stains with great success, making detection easier when using histological analysis (Card et al., 2013). One of the limitations of this method is that endophyte hyphae in seed does not stain.

Testing the tillers this way enables the number of hyphae present to be counted and as a result the most effective and productive endophyte-grass relationship to be determined. It can also provide information on how the endophyte behaves over subsequent plant generations. For example, if the first generation has a large number of hyphae (e.g. 50) present, then in theory the ideal second generation should have a similar number of hyphae when tested under the same conditions. If the

second generation has a substantial decrease in hyphae (e.g. 25 hyphae), then it suggests that this endophyte-grass relationship is not suitable for production as the transmission rate decreases across generations.

As using this method for testing tillers also enables the viability of the endophyte to be determined, combining this technique with histological seed testing will provide information as to whether best management practices regarding endophyte storage and production are being carried out. It can also enable seed producers to determine how long the endophyte can be successfully stored for, as although the seed may have endophyte hyphae present if the endophyte is unviable the tillers will not have any endophyte hyphae present.

The presence of endophyte in the plant tillers can also be determined using an immunoblot detection method (Trento et al., 2007). Briefly, tillers are tested by cutting the tiller at the base of the plant, as close to the crown as possible, and then pressing this cut section onto nitrocellulose blotting paper transferring plant sap and associated endophyte mycelial proteins. The paper is then developed, by first adding blocking solution which prevents any other proteins binding to the paper (and potentially causing false results). This is then removed, and the primary antibody is added. This antibody binds to the endophyte proteins. An enzyme linked secondary antibody is then added to bind to the primary antibody and a chromogenic substance, which produces a coloured compound upon cleavage by the enzyme, is also added. The resulting development will give a visible red to light pink colour, indicating endophyte infection level. Occasionally the colour may differ depending on the dye mix used but it is generally characterised as the deeper the colour, the higher the endophyte level (Trento et al., 2007).

The immunoblot method is less labour intensive than histological analysis of tillers and does not require as high a skill level although it is more expensive than the histological seed test. This expense occurs due to the specialised equipment needed, although this cost can be minimised by processing multiple tests at once. The time required for growth of the tillers, which can be at least four weeks before they are of sufficient size to be able to be tested, also needs to be taken into account.

Molecular methods can also be used to detect the presence of endophyte within plant tissue. This can be done in a variety of ways, one of which being the kompetitve allele specific PCR (KASP) assay (Semagn, Babu, Hearne, & Olsen, 2014). This KASP assay involves taking a section of plant tissue, extracting DNA and using particular primers that are specific to certain endophyte strains in a PCR reaction to detect these strains within the plant (Semagn et al., 2014). Once the PCR mix has been created with the plant sample included in it, it can be placed in the machine and analysed. The analysis gives a yes or no answer in terms of endophyte presence based on the fluorophores released. Two fluorophores are used for each test, each one specific to the primers used. If no

endophyte is present, then amplification will not occur, and no fluorophore is released (Semagn et al., 2014).

One of the main issues with this process is that it can be relatively expensive due to the cost of the machine itself, as well as the chemicals required. This process can also be limiting in that only one endophyte strain can be detected for at any one time, resulting in more tests needed until the endophyte strain is identified. While this can be helpful when used in a breeding program as the presence of the specific endophyte can be determined, it requires knowledge of what endophyte is present as well as which primers to use (Semagn et al., 2014).

1.8 Research context and objectives

In order to produce a perennial ryegrass-based pasture containing endopyte, farmers needs to plant ryegrass seed with viable endophyte. Currently industry standards determine that perennial ryegrass seed containing >70% viable endophyte is such seed. When seed containing novel endophyte is being produced the endophyte level tends to decrease from generation to generation. If it falls below 70% then the seed producer has to class the seed as low endophtye and consequently loses potential income. As described above there are many potential reasons for this but, the magnitude and importance of these causes *in situ* are not yet well understood. Therefore, the main focus of this research project was to determine the effect of crop management practices on endophyte colonisation of perennial ryegrass plants and seeds (Objective 1). For this objective the seed production process of perennial ryegrass with AR37 endophyte on two farms was followed and samples were taken at different stages (fungicide application, cutting and harvest) to determine endophyte status. The hypothesis behind this objective was that different management conditions employed on the two farms would affect endophyte viability during harvest and farm storage.

When growing plants many variables need to be considered and controlled as much as possible. These variables include the control of diseases. Fungicides are often used to control these diseases. However, issues may occur if these fungicides also have activity against these fungal endophytes. Therefore a second objective was to determine the effect of fungicide application on AR37 endophyte colonisation of perennial ryegrass tissue/seeds and therefore endophyte viability and transmission (Objective 2). The hypothesis for this objective was that fungicide would not hinder the growth of the endophyte. In undertaking Objective 2 an unexpected low initial endophyte viability rate was observed and so an additional experiment was designed. The purpose of this additional experiment was to determine why apparent initial loss in endophyte occurred and whether it was caused by new and untested germination conditions.

Another variable in the seed production process, which was highlighted in the literature review (Section 1.6), was that ryegrass seed is harvested during the summer period and is therefore exposed to high temperatures. Seed can also sit in trucks for many hours or days prior to being transported to the drying facility, which may result in a loss of endophyte viability in the seeds. Therefore, a third objective of this study was to investigate the effect of seed moisture and age on endophyte viability (Objective 3). Specifically, a final experiment was carried out to determine the length of time seed can be stored at high temperatures and moisture levels before a significant loss in endophyte viability occurs. The hypothesis here was that only the seed with the higher moisture levels will show a greater loss in endophyte viability following storage.

1.9 Thesis format

This thesis comprises four chapters. In this first chapter, Chapter 1 Introduction, the general background information and the context and objectives of the research project have been provided. Chapter 2 describes the methodology used in each of a series of experiments to address the three objectives the study. Chapter 3 presents the results of each experiment. A discussion of the results from each experiment, and possible further studies, is presented in Chapter 4. The complete list of references is presented at the end of the thesis.

Chapter 2

Materials and Methods

2.1 Objective 1 – Determine the effect of crop management practices on endophyte colonisation of ryegrass plants and seeds

2.1.1 Field sites and sampling

Two farms that produced proprietary seed of perennial ryegrass with AR37 for Barenburg Agriseeds in Canterbury, were sampled for this objective. These farms were labelled Farm A and Farm B so that the farms remained anonymous. Farm A produced ryegrass seed with a consistently high endophyte level (above 70%). By comparison, the endophyte level in the seed produced by Farm B had historically been more variable (personal communication, Barenbrug Agriseeds production department), having a high endophyte level some years and other years lower than 70%. Both farms grew seed of the same perennial ryegrass cultivar, Governor AR37, from the same batch of basic seed (pre-commercial seed) with an initial endophyte percentage of 90%. The endophyte percentage was obtained by Agriseeds staff using a grow-out quality control test (QC test). The farms were located approximately 18 km apart, near Ashburton, and were cropping farms so had a variety of different crops growing at different times. The farm owners carried out different management processes, as outlined in Table A1, with variations regarding timing, chemical applied and harvest process.

The ryegrass seed crops on the farms were sampled using the same method. For each farm the paddock under study was bisected with four replicate transect lines laid out in a zig zag pattern across the field. From each line 100 samples were taken at roughly even distances apart along each transect (Figures 2.1 and 2.2). Replicates lines 2 and 3 for Farm B were on the same diagonal due to the shape of the paddock (Figure 2.2). Two different types of samples were taken, tiller samples (fresh plant tissue) and seed samples (whole spikes). Tiller samples were taken during the vegetative stage, while seed samples were taken once the grass crop had been cut and the harvest process had begun. Tiller samples could not be taken during the harvest process as the plant tissue did not contain enough sap to carry out an immunoblot test.

Tiller samples were taken approximately 1 week (27th November) before fungicide application began, and approximately 2 weeks (18th December) after the main fungicide applications occurred (1st and 4th December 2017, for Farm A and Farm B, respectively; Table A1.1). The tiller samples for each farm were collected on the same day to help reduce any variation. These tillers were cut as close to the base as possible using a scalpel, since the base of the plant is known to have the highest endophyte

concentration during the vegetative stage (Cheplick & Faeth, 2009). Tillers collected before fungicide application were tested as normal, cut at base and immunoblotted, whereas tillers collected after fungicide application were cut at the node closest to the base and then blotted. This was done as tillers collected after fungicide application had "dried out" so only the nodes had enough juice to be immunoblotted. Tillers were stored in plastic bags in a fridge at ~4°C and processed within 3 days to ensure the plants did not dry out.

Tillers were assessed for presence of endophyte using the immunoblot method as outlined in Section 2.1.2 and then cut at the base so that a 2 cm pseudostem tissue sample was obtained. These samples were labelled and stored in small plastic bags in a fridge at ~4°C until the immunoblot results were obtained so that they could later be analysed for confirmational presence of endophyte hyphae under the microscope (Section 2.1.2.2).

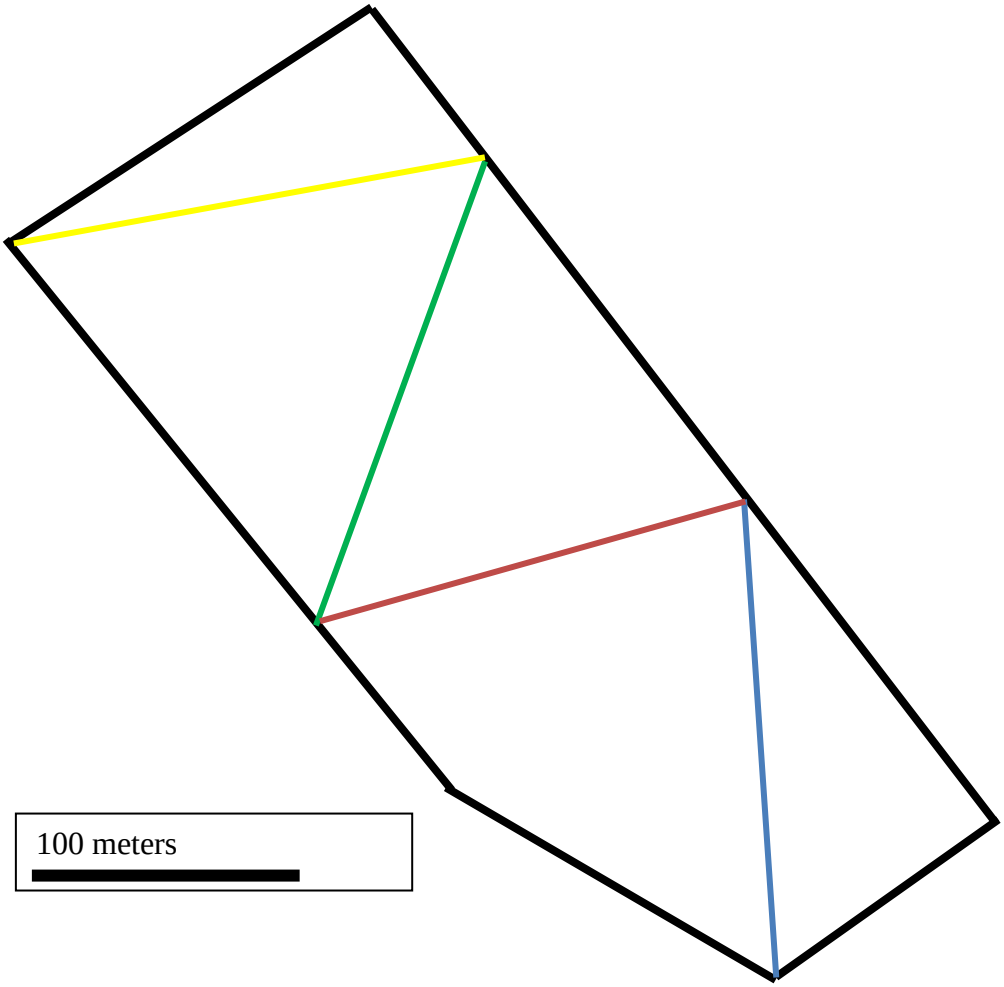

Figure 2.1 Diagram of the paddock sampling method for Farm A. Each diagonal line represents a two replicate and 100 plants were sampled along each of these lines. The blue line is replicate 1, red replicate 2, green replicate 3 and yellow replicate 4.

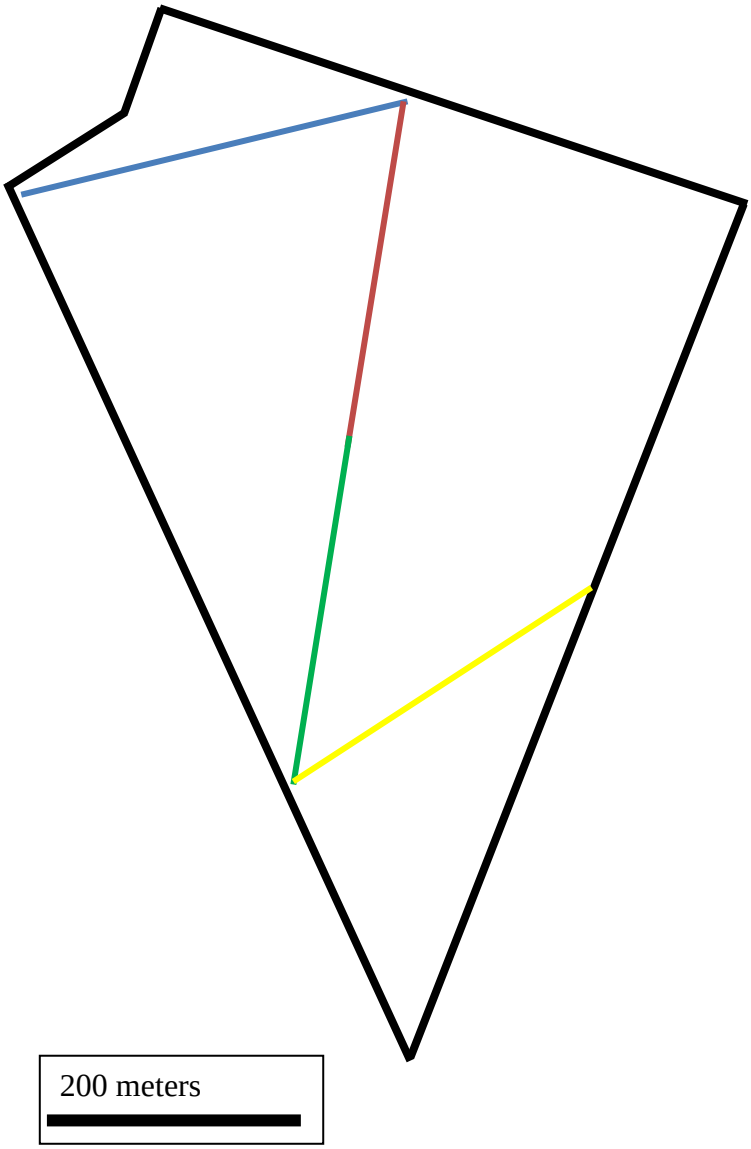

Figure 2.2 Diagram of the paddock sampling method for Farm B. Each diagonal line represents a replicate and 100 plants were sampled along each of these lines. The blue line is replicate 1, red replicate 2, green replicate 3 and yellow replicate 4.

After the fungicide had been applied and the tillers were sampled, the next sampling stages were carried out during the harvest period and seed was analysed, as immunoblots of tillers were not possible on the dry stalks. Two sets of samples were taken: 1) when the pasture had been cut, and 2) when the seed was being harvested. Each farmer cut their own pasture as outlined in Table A1. One hundred spikes were collected from each replicate (Figure 2.3), following the same sampling lines as described for the tiller samples. These spikes were stored in a fridge at ~4°C in plastic bags. The seeds were removed within 3 days of collection from each spike.

Figure 2.3 Whole spike collected before harvesting. Spikes were taped to paper and split into thirds (top, middle and bottom) before analysis.

Seeds were put into labelled, sealed plastic bags and placed into the Agriseeds seed vault at Courtenay (2-3°C, 20% RH (relative humidity)) until a seed squash and grow out test was carried out (Section 2.1.2). The seed samples used for the grow out tests were planted the day after all of the spikes had been processed to reduce the impact of external factors. The seed samples used for the seed squash assessments were processed as required, as this test only determines presence/absence of the endophyte so maintaining the viability of the endophyte was not required. During the just cut collection a further 10 seed heads per replicate were collected and taped to paper. Each spike on these seed heads were analysed to determine how far up the seed head the endophyte hyphae were colonising. Spikes were divided into three sections, the top, middle and bottom. The seeds from each spike section were then removed and these were planted to enable a grow out test to be conducted (Section 2.1.2.3).

During the harvest collection spikes were sampled on the same day as the seed was harvested. Farm B harvested over a period of 2 days due to the size of the paddock. Farm B also had rows wider than Farm A because the cut grass had been windrowed (cut and raked into a row at the same time) not just mowed. The windrows were quite large so at sampling half of the spikes were taken from the top of the rows and half from the bottom. These were collected and tested separately to see if the windrowing practice had an effect on the endophyte levels.

Additional seed samples were also taken during processing. The farms processed seed differently before sending it to the dressers due to storage facility variations. Farm A used one silo during the harvest and processing stage as all of the seed was able to fit into this silo. As a result the seed was able to be transferred into the silo within 2 hours of harvest so a seed sample was taken once the seed was removed from the silo. The silo also had a cooling fan so the seed was able to be cooled down before it was taken to the dressing facility (2 km away) in Hinds the next day.

Farm B was not able to treat all the seed the same as the harvesting process began before the transport truck arrived. Around 10% of the seed was harvested and put into a tractor trailer before the truck arrived and remained in this tractor trailer overnight until the harvest process finished. The remaining 90% was sent to an offsite drying facility (11.6 km away). This was done as the farmer was unsure how much the drier could hold and didn't want to put it in the silo in case the seed in the

tractor trailer could also be sent to the drying facility. As a result a seed sample was taken from the truck once it arrived at the drying facility as well as when it left the drying facility. A seed sample was also taken from the tractor trailer just prior to it being moved from the silo to the dressers. These samples were obtained by randomly sampling 20-30 g of seed from five different areas of the piles, and then mixing these seed samples together.

The endophyte colonisation of the seed samples were determined using two different tests: a grow out test and a seed squash test as described in Section 2.1.2. Staff at Agriseeds also carried out their own grow out tests as part of the seed production process. This gave the seed lines final endophyte percentages of 73% (Farm A, machine dressed) and 83% (Farm B, machine dressed).

2.1.2 Endophyte colonisation assessments

Endophyte colonisation was determined using two separate plant materials: tiller samples and seed samples.

Immunoblotting test

Tiller samples were blotted by pressing the freshly cut base of the tiller onto immunoblot paper (Nitrocellulose blotting membrane; MicroAnalytix) so that the sap from the plant formed a blot on the paper. Postitive and negative controls (four or five of each) were added to the margins of the paper. The positive control plants contained standard endophyte and the negative controls do not contain any endophyte.

If endophyte is present then endophyte fragments (proteins) carried by sap will be bound to the blotting membrane which, once developed (as decribed later) indicates the presence (or absence) of the endophyte in the tiller giving an endophyte percentage for that replicate. This test can be used for all *Epichloë* endophyte testing as it is not specific to particular strains. This nitrocellulose was printed with a grid (25 x 20) illustrated in Figure 2.4 and one tiller was blotted in each square, with up to 500 plants being sampled for one piece of paper.

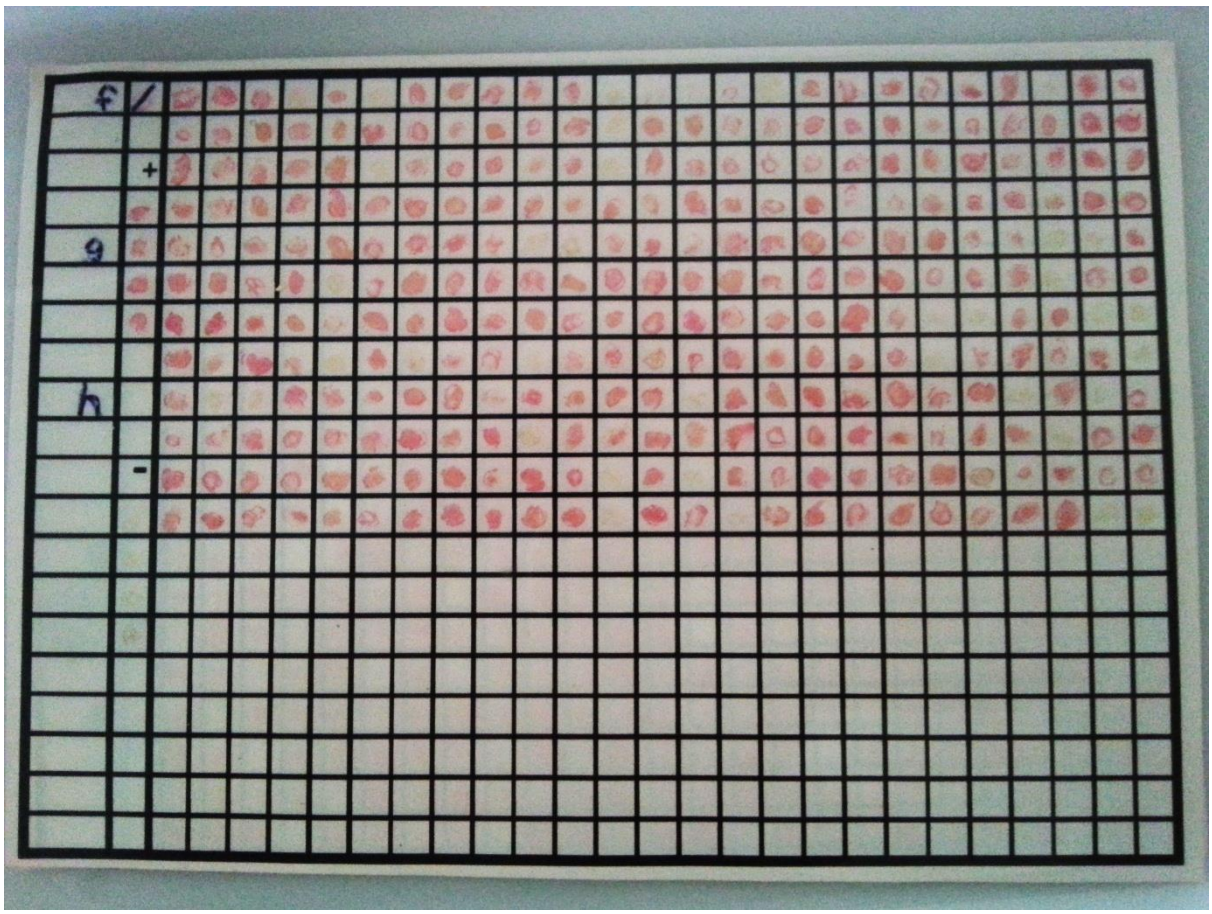

Figure 2.4 Nitrocellulose paper used for endophyte testing using the immunoblotting method. Each deep pink-coloured square means that endophyte is present in that tiller, whereas each non-coloured square is a negative result.

Once blotted the membrane was developed as follows. The membranes were placed in a plastic container (3 cm × 18 cm × 11 cm), two sheets per container and just covered with the blocking solution (~50 mL; Appendix 2) then placed on a shaker for 30 minutes at 50-100 rpm at room temperature. This blocking solution binds to any part of the nitrocellulose that does not already have a protein attached. This prevents the antibodies from binding to parts of the membrane other than the blot, and therefore prevents false results from occuring. This blocking solution was then removed and an additional 40 mL of blocking solution containing 100 µL of the primary antibody (sourced from Cropmark Seeds, Rolleston) was poured over the sheets. The container was then placed back on the shaker for 1 hour and the solution then tipped off. The sheets were then rinsed twice with blocking solution (~50 mL) the blocking solution tipped off each time, and another 50 mL of blocking solution added. The container was then placed on the shaker for 5 minutes, and the solution then tipped off. This ensured that the excess primary antibody was completely removed as the secondary antibody binds to the primary antibody. If there is excess primary antibody then the secondary antibody will bind to this and may alter the appearance of the results.

After the excess primary antibody was removed, 40 mL of blocking solution containing 15 µL of the secondary antibody (Agriseeds, proprietary information) and the container was placed back on the shaker for 1 hour. The sheets were then rinsed again as described for the primary antibody.

After this final rinse, two solutions, A and B (Appendix 2), were then mixed and poured onto the sheet in the box and shaken again for around 10-30 minutes or until the positive controls developed a dark pink/red colour. This mix binds to the secondary antibody and results in the development of the pink/red colour for the endophyte positive blots. Once the colour developed the sheets were then rinsed with tap water and the presence or absence of endophyte in each sample was visually determined based on the colour development. The presence of the endophyte in the sample was indicated by the individual blots turning red, with absence denoted by the blots remaining uncoloured, or very faintly coloured. The results were analysed using a t-test and one-way ANOVA (analysis of variance) in Minitab to compare between the two farms and the different sample times.

Histological analysis

Histological analysis was carried out on sections of the tillers and was used to establish the validity of the immunoblot as a method for detecting endophyte within the samples. Once the immunoblot results were obtained from the pre-fungicide samples, any negative tillers or tillers which had a faint positive result, were seperated as well as five randomly chosen strongly positive tillers which were used as controls. These tiller pieces were secured to microscope slides using Renoir permanent glue tape (sourced from Spotlight) and unrolled so that each outer leaf blade was flat against the slide. These were then stained using a drop of 2% analine blue (Appendix 2) and left for at least 30 minutes before a coverslip was placed on top and the leaf blades examined under a compound microscope (x40 magnificiation) to confirm the presence or absence of endophyte hyphae. This process was only carried out for the first tiller sampling (before fungicide) as the plants harvested during the second tiller sampling were too stiff and dried out so could not be rolled flat.

Grow out test

A grow out test involved germinating the seed and testing the tillers of the resulting plant at 6 weeks for presence of endophyte using the same immunoblot method as described in Section 2.1.2. This test was used to confirm the viability of the endophyte in the seed, as only viable endophyte will grow into the tillers. The seeds in each seed sample bag were mixed thoroughly to ensure that a random sample was taken and one teaspoon, approximately 250 seeds, was collected and planted in square 2 L plastic pots containing a short term potting mix (Intelligro, Christchurch). This potting mix contained no fungicides, to ensure no potentially negative effect on the growth of the endophyte within the plant.

The pot was filled to 5 cm from the top with potting mix and the seeds were sprinkled on the surface of the potting mix (250 seeds per pot) and ~0.5 cm of potting mix was sifted on top of the seeds to cover the seeds with a fine dressing they could easily emerge through. The pots were then placed in the glasshouse (20°C:25°C 12:12 hours, under lights for 12 hours) at the Agriseeds facility at Courtenay for the duration of the assessment. The pots were watered as needed, approximately every 2 days, and the plants trimmed at least four times during the growth period as needed. The plants were trimmed to ensure that they could be watered easily and that lodging did not occur (Figure 2.5).

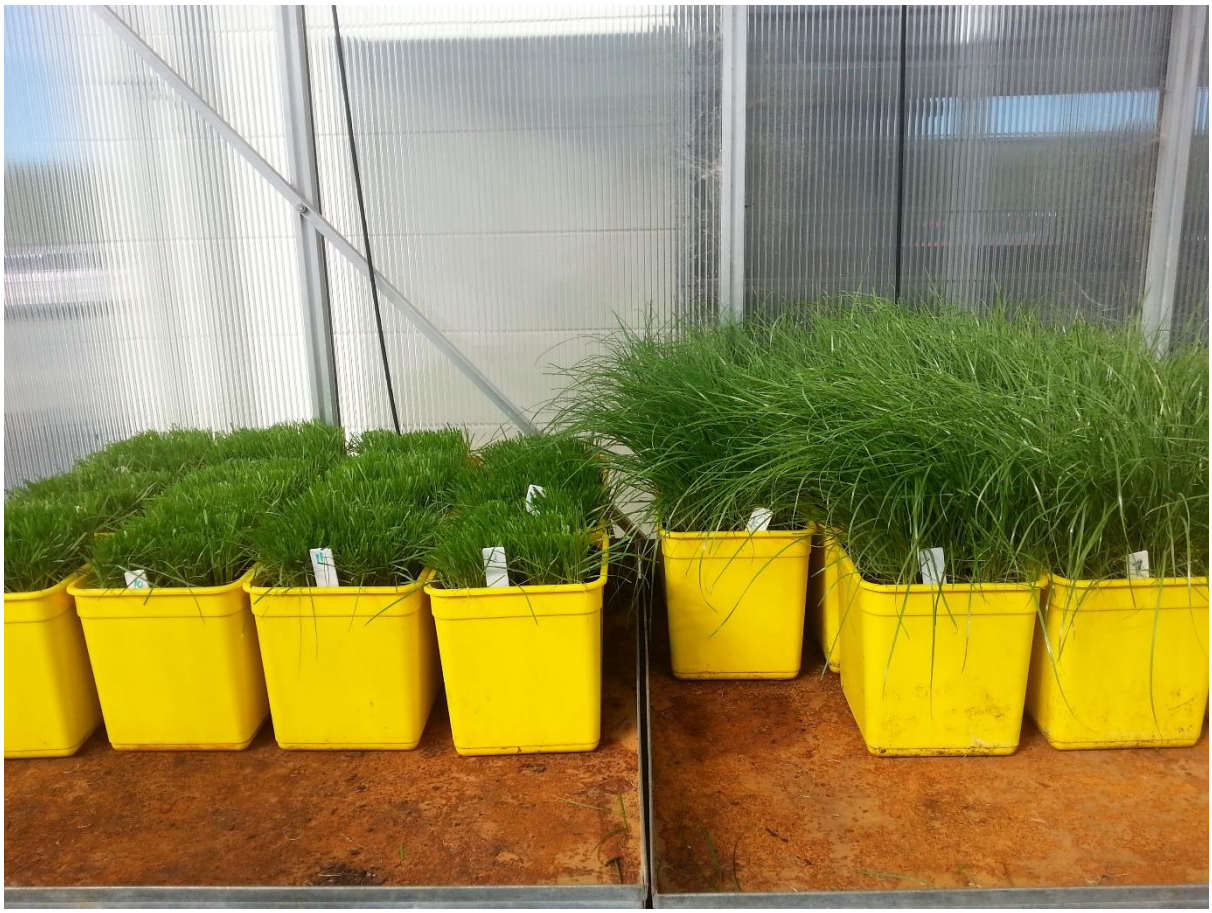

Figure 2.5 Pots containing ryegrass plants during the trimming stages. Plants on the left have been trimmed whereas plants on the right have yet to be trimmed.

Once the plants were 6 weeks old, around half of the seedlings were harvested by gently removing them from the potting mix to ensure that no damage occured to the seedlings. The seedlings were cut at the base as close to the seed as possible, through the pseudostem, and all dead material was removed. The base of the plant was then immunoblotted as outlined in Section 2.1.2.

Seed squash test

A seed squash was carried out with approximately 250 seeds from each sample. These were placed in a test tube and 10 M Nitric acid added so the acid level was double the volume of the seeds. The test

tubes were then heated at 60°C in a water bath for approximately 15 minutes until most of the seeds had floated to the top of the acid and were a pale yellow colour (Figure 2.6).

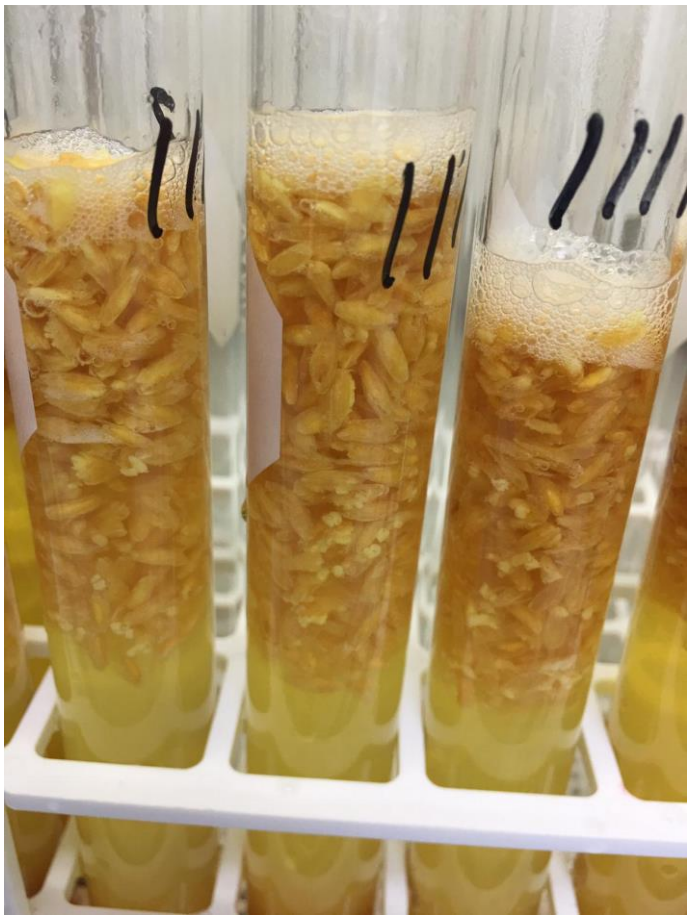

Figure 2.6 Seed that has been heated in Nitric acid for 15 minutes at 60°C in preparation to be rinsed and tested for seed endophyte.

The seeds were then rinsed under tap water using a sieve to ensure that no acid remained and the seeds were then placed into a Petri dish with sufficient water added to ensure that they didn't dry out. Each seed was removed individually and cleaned with a dissecting needle, so that all plant material other than the caryopsis, was removed (Figure 2.7). This was then placed onto a microscope slide so that it was flat and stained with a drop of 2% analine blue (Appendix 2). The slides were then left for about half an hour to ensure any endophyte hyphae was stained before the coverslip was placed on top. These were then observed under a microscope at 100x magnification for presence of endophyte hyphae (Figure 2.8). Fifty seeds were examined per replicate to determine the endophyte percentage. The number of seeds positive for endophyte hyphae out of the total number of seeds tested per replicate for each farm were determined. The seed squash test was only carried out for 12 of the 24 seed samples collected from Farm B. These seed samples were collected at three different sampling points; just cut (first), at harvest from the top of the windrow (second) and underneath the windrow (third).

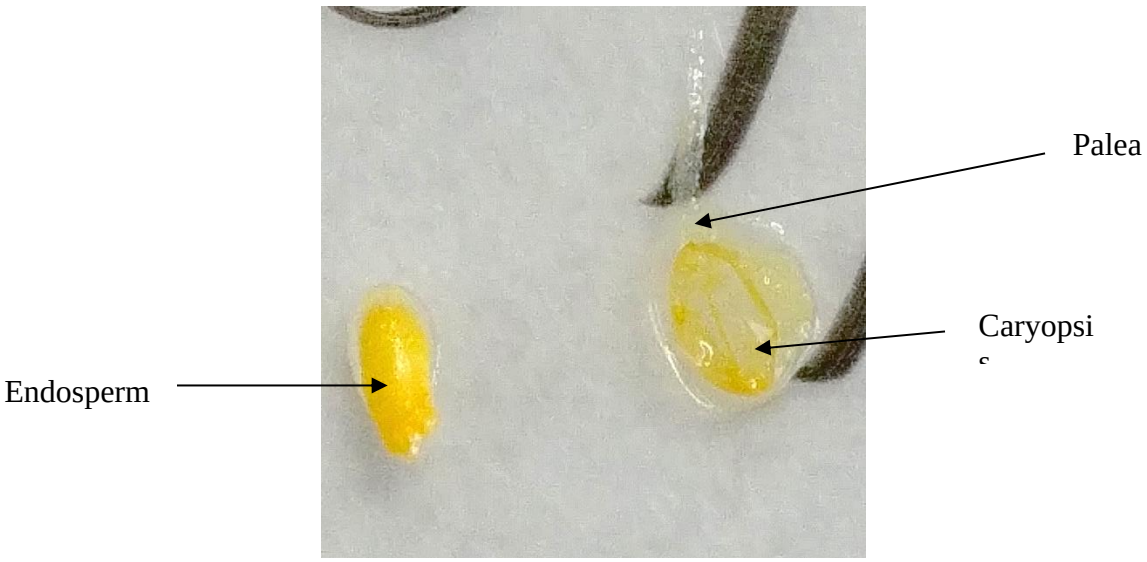

Figure 2.7 Preparation of seeds for endophyte testing using the seed squash method. One seed still contains the endosperm (left) which needs to be removed while for the other seed the endoperm has been removed (right) but with the palea still attached. These need to be removed so that only the caryopsis remains.

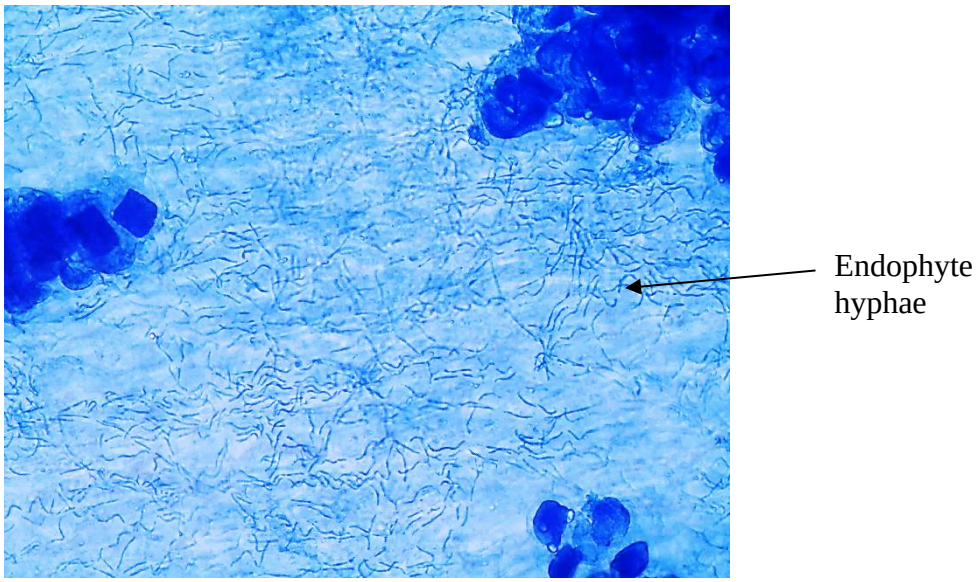

Figure 2.8 Example of a positive seed squash endophyte result showing the endophyte hyphae found within the seed.

Seed moisture test

To determine the variance in seed moisture between seed from Farm A and Farm B a seed moisture test was carried out on all seed samples collected from both farms following the method outlined by the International Seed Testing Association (ISTA) guidelines. This test determined the moisture percentage of the seed and involved placing 2 g of seed into a pre-weighed metal bowl (approx. 5 cm in diameter) of a known weight and drying the seed in an oven at 103°C for 17 hours. After drying for 17 hours, the seed and the bowl were placed within a dessicator to prevent the seeds absorbing

moisture from the air. The seed and the bowl were then weighed and the weight of the bowl was removed to give a dry weight of the seed. The seed moisture percentage was calculated using the following equation:-

Seed moisture % = (Wet seed weight – dry seed weight)/wet seed weight × 100

2.1.3 Temperature and humidity data

All minimum and maximum daily temperature data was obtained from the website AccuWeather ("AccuWeather," 2019). This data was then plotted together to show the trends. LogTag® data loggers were also used and placed in the paddocks underneath the mown grass (Figure 2.9A). The data loggers recorded the temperature and humidity of the windrow while contained inside plastic 5 L Sistema containers. The containers have cut outs on all four sides to allow the data loggers to record while being covered (Figure 2.9B). Data loggers were attached to the lid of the container with hook and loop fasteners and two data loggers were used in each paddock. The containers containing the loggers were placed underneath the mown pasture as soon as it had been cut and followed the harvested seed through the different stages and for the different lengths of times, for the two farms depending on the management practices used.

Farm A kept all harvested seed together so one data logger followed the seed from underneath the grass to in the tractor trailer, then into a silo with a cooling fan then finally to the dressing facility. The second data logger used for Farm A was kept underneath the mown pasture and then stopped recording. Farm B had different seed production processes so both data loggers were used up until the point where the seed reached the dressing facility. One data logger went to the drying facility and the other stayed on farm with the seed in the silo. The data logger that went to the drying facility followed the seed from underneath the windrow, to the harvested seed in the truck to the drying facility and finally to the dressing facility. This was used as the main data logger so when the rest of the harvested seed (10%) was transferred to the dressing facility this was the data logger used. The other data logger recorded the temperature and humidity for the windrow then harvested seed in the tractor trailer before it was then transferred to the silo.

Figure 2.9 Data loggers used to record the temperature and humidity. (A - left) Windrow with data logger underneath the mown grass. The orange flag acts as a marker to show where the data logger is. (B – right) Container with data logger inside. Containers had cut outs on all four sides to enable the data logger to record. The data logger was stuck to the lid of the container with a hook and loop fastener.

2.2 Objective 2 – Effect of fungicide application on endophyte colonisation of ryegrass tissue/seeds

2.2.1 Fungicide application treatment

A ryegrass seed line with a known high endophyte level (72%) was used for Objective 2. The seed line used (Alto AR37) was not the same as that used in the field experiment of Objective 1, but it contained the same novel endophyte, AR37, to ensure consistency with the field experiment. There were four replicates of each of the two treatments: one control and one fungicide treatment. These were arranged in a completely randomised design (two rows by four columns design) using a random number generator (fungicide 1-4, control 5-8), to minimise the effect of the environment (A3.2). The control treatment had no fungicides applied whereas for the fungicide treatment the fungicides benzimidazole (Protek), tebuconazole (Folicur), prothioconazole (Proline) and azoxystrobin (Amistar) were applied. These fungicides were the same as those applied by the growers the previous year to Governor perennial ryegrass crop. These fungicides were applied at double the recommended rate (Appendix 2) to simulate a worst-case scenario event and determine if these fungicides have any impact on the endophyte growth within the plant.

The plants for this experiment were grown by a contract grower, specialising in growing large numbers of seedlings and were planted in a large tray which contained up to 260 wells (2 × 2 cm × 5 cm depth), 13 rows by 20. All of the wells within this tray had seeds planted which ensured that there were at least 100 seedlings, as required for the experiment. The plants were grown in proprietary potting mix blend (vermiculite, nutrient mix) and were grown under the conditions standard for this grower for 6 weeks. The seedling trays were then transferred to Agriseeds and maintained outside on a gravel bed, for the remainder of the experiment. One tray of seedlings represented one replicate.

From each replicate, 100 plants were selected at random using a computerised random number simulator. Prior to fungicide application these plants were tested for the presence/absence of endophyte using the immunoblot method described in Section 2.1.2. If the plant selected to be tested had not grown, then the one next to it was used instead. From each of the 100 replicate plants one tiller was chosen at random and blotted on the nitrocellulose membrane. The tillers of each of the selected plants were then tied so that all the tillers of that plant were together (Figure 2.10). This allowed the new tiller growth after fungicide application to be distinguished from the tillers to which the fungicides were applied, as the new tiller was found outside of the tied bunch (Figure 2.10).

Figure 2.10 A whole plant tied together after tiller sampling, allowing the new tiller growth to be distinguishable. The plant on the left was not sampled and is therefore untied whereas the plant on the right was sampled and subsequently tied together.

All fungicides were applied at the same time and together within 1 week after the initial testing of endophyte colonisation of the tillers had been carried out. The same plants tested before fungicide application were retested for the presence of endophytes once the new growth was around 6 weeks old (approximately 8 weeks after fungicide application). The percentage endophyte colonisation

results before and after fungicide application were compared with the untreated control and analysed using a t-test, in Minitab, to determine the effect of the fungicide application on endophyte level in the new ryegrass tillers. These tillers were not left to develop seeds due to time constraints.

2.2.2 Potting mix treatment

Due to the low endophyte colonisation in the seedlings in the previous experiment, an additional experiment was carried out with the aim to determine the potential reason for the low endophyte level and ensure that it was not a result of practices used by the external grower. The normal potting mix used by the external grower and used in the previous experiment was different to that used by Agriseeds and was also amended with fungicides. Therefore, three factors were assessed: i) the type of potting mix by comparing the external grower's and Agriseeds potting mix, ii) the effect of fungicide, by comparing the external grower's potting mix with and without fungicide amendment, and iii) the type of tray used by comparing the tray used by the external grower with the pots used by Agriseeds. To do this, five different treatments were used, including a positive and negative control as follows:

1) the external grower's original potting mix (with fungicides), in the grower's trays,
2) the external grower's new potting mix (without fungicides), in the grower's trays,
3) Agriseeds potting mix in Agriseeds pots (2 L),
4) Agriseeds potting mix in the external grower's trays, grown at Agriseeds, and
5) Agriseeds potting mix in the external grower's trays, grown by the external grower.

Treatments were set up in this manner as the external grower also applied fungicide via the overhead sprinkler system. The treatments grown at the external growers were kept there for 6 weeks before testing. For each treatment there were four replicates and 100 plants were tested using the immunoblot method outlined in Section 2.1.2 and were tested 6 weeks after sowing. Results were analysed in Minitab using a t-test.

After experiment 2.2.1 was carried out there was an insufficient amount of seed remaining to carry out experiment 2.2.2 so Tyson NEA12 was used instead. This was chosen as it had previously returned a similarly low level of endophyte detection as was observed in experiment 2.2.1.

2.3 Objective 3 – Effect of seed moisture and age on endophyte viability

The effect of seed moisture levels at harvest on endophyte viability during storage was investigated in an experiment for Objective 3. Low quality seed was used to create 200 kg bins of seed with different moisture levels. These moisture levels had varying temperatures and humidity levels due to

respiration from the seed. Old and fresh (new) endophyte positive seed was stored within this to determine the effect on endophyte.

The initial moisture content of the seeds was 12%, with water added in varying ratios to raise the moisture of the seed to 14% and 16% before they were subsequently placed in separate bins. The seed was placed into a seed mixer (custom made for Agriseeds, Figure 2.11), and the appropriate amount of water added to achieve the desired moisture level; 4 L water/ 200 kg seed for 14% and 8 L water/200 kg seed for 16%. Seeds at 12% moisture level were used as a control.

Figure 2.11 Custom made seed mixer used to add water to increase the seed moisture of the seed used in Objective 3.

The seed moisture levels were selected to simulate the potential sub-optimal moisture content of seeds at harvest time. The seeds were then placed into large sacks inside wooden seed bins (sacks: 90 x 90 x 120 cm, seed bins: 100 x 100 x 100 cm), one for each moisture level. To ensure that the seed was at the correct moisture before any further preparation the seed moisture level was determined using the method outlined in Section 2.1.2. The bins were left in a shed at the Agriseeds facility for the duration of the experiment to simulate ambient storage.

Two seed lines were tested, being old and new seed with the old seed (Trojan AR37) being over 2 years old (2015) and the new (Governor AR37) being from the most recent season (2017). Both seed

lines had a high initial endophyte percentage, with the old seed being 92% and the new seed, 81%. For each seed line 24 muslin bags were placed in each seed pile, with four replicate seed bags removed from the seed bins at 2 weekly intervals (2, 4, 6, 8, 10 and 12 weeks). These bags had a piece of string attached so that they could be easily retrieved from the pile with as little disturbance as possible. At the end of each string a label was attached so that the seed line treatment in the bags could be determined easily and removed accordingly.

Data loggers (HAXO-8; Logtag) were also placed outside and inside the seed piles alongside the seed bags to determine the temperature and humidity conditions.

The data retrieved from the loggers at 4 weeks indicated that the temperature was lower than expected and differences were minimal so to create greater temperature variation, around 5 kg of seed from each bin, and half the remaining seed bags, were removed and placed in three separate plastic 5 L (Sistema) containers (one for each moisture level). These containers were placed in the germinator at Agriseeds and kept between 20-25°C. To ensure that there were enough seed bags to continue the experiment, the number of assessment times was reduced to (8, 12 and 16 weeks) with three replicates rather than four sampled at each assessment time (Figure A6.1).

At respective times seed bags were removed from the seed pile and endophyte viability determined using the grow out and immunoblotting test as previously described in Section 2.1.2. Once an endophyte percentage for each sample had been obtained, a two-way ANOVA test was carried out using Minitab, to determine the effect that temperature, relative humidity and storage time had on the percentage of viable endophyte. When analysing in Minitab, there were two different factors (treatment and time) and one interaction (time x treatment). Treatment refers to the moisture group (e.g. 12% moisture) and time refers to how long the seed was left before being extracted (e.g. 2 weeks). Each seed type (e.g. old seed in large box) was analysed separately.

Chapter 3

Results

3.1 Objective 1 – Determine the effect of crop management practices on endophyte colonisation of ryegrass plants and transmission to seeds

3.1.1 Endophyte level based on immunoblot and grow out tests during crop growth and harvest

The seed used was the same for both farms and had an initial endophyte percentage of 90%. This data was obtained by Agriseeds staff using a quality control grow out test (QC test).

The immunoblot results obtained before the fungicide was applied showed that there was no significant difference (t-value=0.68, *P*=0.527) between the endophyte levels in the tillers sampled from the two farms (Figure 3.1). The average endophyte percentage for Farm A before fungicide application was 89.5% (SE = 0.87) and for Farm B it was 88.5% (SE = 1.19).

After fungicide applications, the endophyte percentage in the tillers remained above 85% for both farms (Farm A 88.25%, SE = 1.84, Farm B 90.0%, SE= 1.83) and again there was no significant difference between the two farms (t-value=-0.67, *P*=0.530). The two farms were analysed separately to determine if there was a significant difference between before fungicide treatment and after. No significant difference was found for Farm A (t-value=0.61, *P*=0.572) or Farm B (t-value=-0.69, *P*=0.522).

The tiller-determined endophyte level during plant growth between farms was not significantly different. However, the endophyte percentage measured dropped significantly by the time the seed was analysed. Seed tests from when the grass was just cut were significantly lower than the tiller immunoblot result after fungicide application (Farm A t-value=2.58, *P*=0.049, Farm B t-value=3.31, *P*=0.021).

Farm A had a seed endophyte level of 79.5% (SE = 2.84) from just cut grass, and seed collected at harvest had an endophyte level of 79.3% (SE = 2.56). Farm B had a seed endophyte level of 79.8% (SE = 2.50) from just cut grass and seed collected at harvest had an endophyte level of 83.75% (SE = 1.69). Again, there was no significant difference between farms (t-value=-0.07, *P*=0.950). There was also no significant difference (t-value=0.23, *P*=0.826) in the endophyte level in the seed collected during the transport and storage process, with the seed collected from Farm A having an endophyte level of 83.8% (SE = 1.75) and Farm B with an average of 83.3% (SE = 1.29). The difference between

the harvest and storage of seed on both farms was also assessed and again no significant difference was found for Farm A (t-value=-1.45, *P*=0.207) or Farm B (t-value=0.24, *P*=0.817).

Farm B had multiple steps during the storage/transport process (Figure 3.2) and when these steps were compared to the endophyte percentage at the time of cutting and harvest it was found that there was no significant difference in endophyte level at any of these stages (f-value=1.08, SE = 2.33, *P*=0.357).

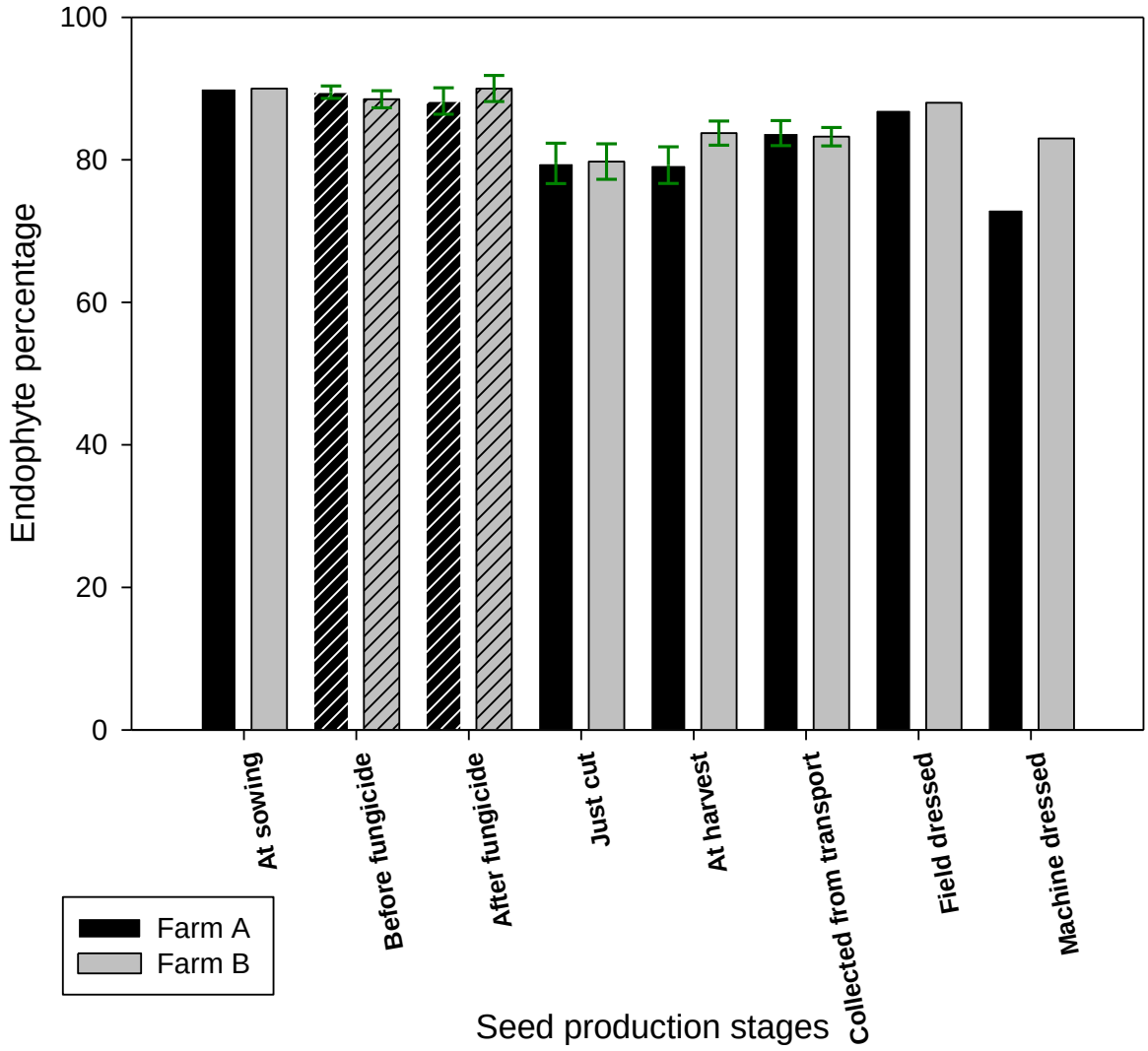

Figure 3.1 Average endophyte percentage at different seed production stages for the two farms sampled. Before and after fungicide treatments (striped bars) are assessments of endophyte level in tillers, whilst 'just cut', 'at harvest' and 'collected from transport (full colour bars) are grow-out immunoblots of seeds. At sowing, field dressed and machine dressed data were obtained by Agriseeds staff using QC protocols. Field dressed was a seed squash and machine dressed was a grow-out test. There was no significant difference in the endophyte percentage between the two farms at each assessment time, determined by t-test analysis.

When all the results were combined, across all of the seed production processes there was no significant difference between the farms (t-value=-0.46, *P*=0.645). The average for Farm A was 83.8% (SE = 3.14) and the average for Farm B was 84.8% (SE = 2.48).

The final endophyte percentage, obtained by Agriseeds staff, using both field and machine dressed samples found no significant difference between the field and machine dressed samples (f-value=3.57, *P*=0.199), despite Farm A having a field dressed endophyte level of 87.0% and a machine dressed level of 73.0%. Farm B had endophyte levels of 88.0% and 83.0%, for field and machine dressed, respectively. No significant difference was found between the machine dressed samples and the endophyte level at sowing (90.0% for both sampling points; f-value=5.76, *P*=0.138).

3.1.2 Comparison between the seed squash and grow out test

Three different sampling points from Farm B were compared using the seed squash and grow out methods (just cut, at harvest on top and at harvest underneath the windrow). The endophyte level determined using the seed squash and grow out test differed by 5% or less for seven of the twelve samples (58.0%), and only one grow out test gave a higher endophyte level than the seed squash test. A significant difference was found between the seed squash and grow out data for all samples (t-value=-2.92, *P*=0.008), with the seed squash results typically being higher than the grow out endophyte result (Table 3.1, Figure 3.2). No significant difference was found between the just cut samples grow out and seed squash results (t-value=-2.02, *P*=0.114), the on top of windrow samples (t-value=-1.79, *P*=0.134) and the underneath windrow samples (t-value=-1.49, *P*=0.196).

Table 3.1 Percentage endophyte level obtained for grow out and seed squash tests carried out for three different sampling times for Farm B.

	Endophyte level (%)								
	Just cut			On top of the windrow			Underneath the windrow		
	Seed squash	Grow out	Difference	Seed squash	Grow out	Difference	Seed squash	Grow out	Difference
Replicate 1	86.0	81.0	-5.0	86.0	87.0	+1	80.0	75.0	-5
Replicate 2	84.0	73.0	-11	88.0	84.0	-4	96.0	91.0	-5
Replicate 3	82.0	80.0	-2	86.0	80.0	-6	92.0	84.0	-6
Replicate 4	92.0	85.0	-7	90.0	86.0	-4	94.0	83.0	-11

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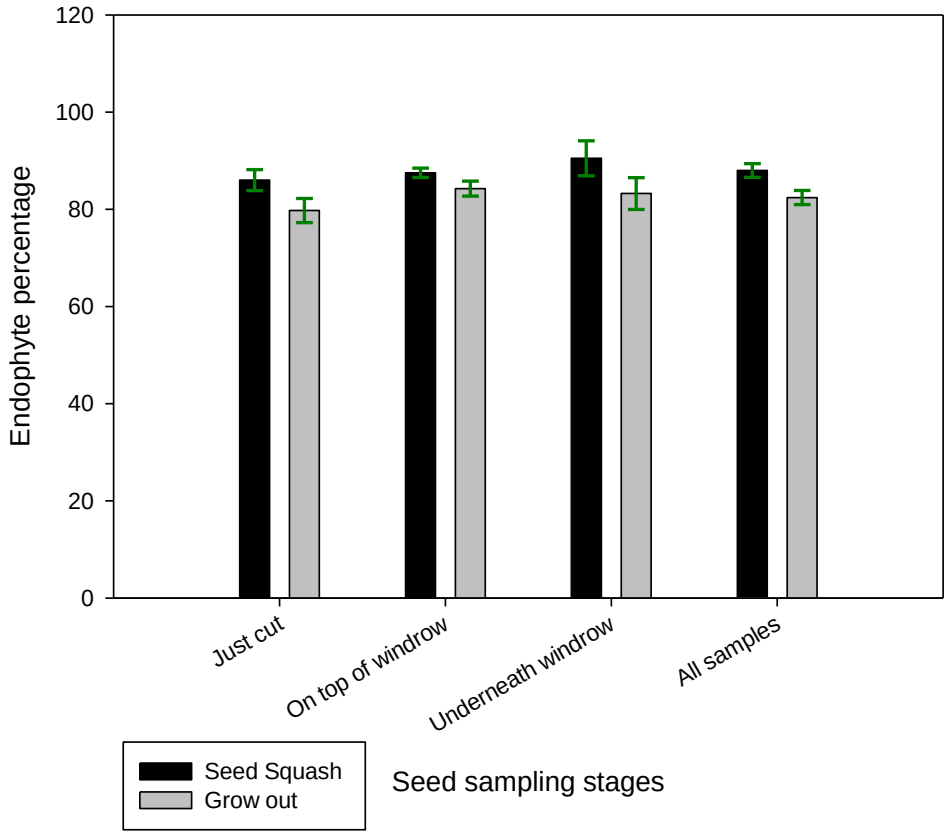


Figure 3.2 Endophyte percentage levels determined using the seed squash and grow out tests for the seed collected from Farm B. The first sample was collected when the grass had just been cut, the second sample was collected from the top of the windrow at harvest and the third from underneath the windrow at harvest. A significant difference was found between the seed squash and grow out results for all samples (t-value=-2.92, *P*=0.008).

3.1.3 Histological confirmation of immunoblot data

Histological analysis was carried out on the first vegetative tiller samples collected for immunoblots (before fungicide application) to test the efficacy of the immunoblot test. Tillers from immunoblots that were deemed to give a faint colour result, as well as all that gave negative immunoblot results, were histologically examined for the presence of endophyte hyphae (Figure 3.3).

For Farm A, there was a total of 16 faint positive immunoblot results, 14 were positive by microscopy and two were negative, and of the 43 negative immunoblot results three were positive by microscopy and 40 were negative (Table 3.2).

For Farm B, there was a total of eight faint positive immunoblot results, five were positive by microscopy and three were negative, and of 43 negative immunoblot results all 43 were negative (Table 3.2).

Table 3.2 The total number of faint positive and negative endophyte results from the immunoblot (Blot) tests which subsequently gave a positive or negative endophyte result by histological (Histo) analysis in ryegrass tillers sampled from Farms A and B.

	Farm A				Farm B			
	Faint positives		Negatives		Faint positives		Negatives	
	Blot result	Histo result	Blot result	Histo result	Blot result	Histo result	Blot result	Histo result
Positive	16	14	0	3	8	5	0	0
Negative	0	2	43	40	0	3	43	43

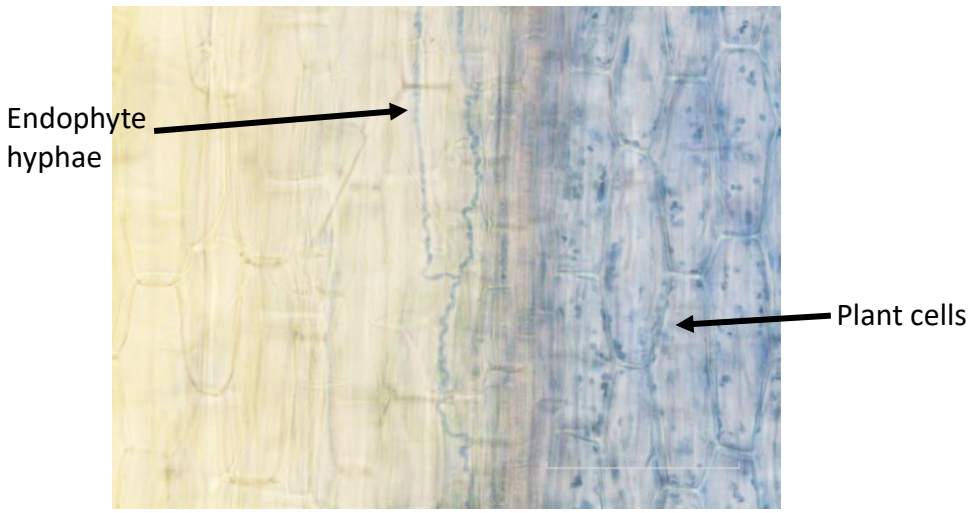


Figure 3.3 Example of a positive microscopy result. Ryegrass plant tissue containing AR37 endophyte.

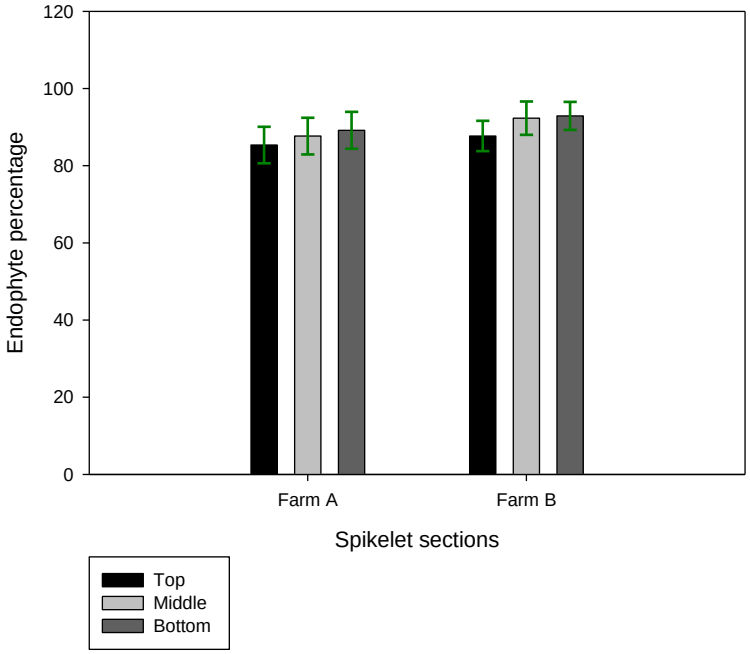
3.1.4 Whole spike endophyte assessment

The endophyte level in the seed of whole spikes was analysed (Figure 3.4A). There was no significant difference in the endophyte level between any of the samples, across both farms. There was no significant difference in the endophyte level in the seed from the top and bottom sections of the spikes from Farm A (t-value=-0.57, *P*=0.572) or between the seeds from the top and bottom sections of the spikes from Farm B (t-value=-0.97, *P*=0.335). The data from the two farms were combined and

again there was no significant difference between the endophyte level between the seeds from the top and bottom of the spike (t-value=-1.05, *P*=0.297).

There was also no significant difference (t-value=-0.38, *P*=0.704) in the endophyte level for the top spike sections from Farm A having an average of 85.4% (SE 4.74) and Farm B with an average of 87.7% (SE 3.94) (Figure 3.4B). There was also no significant difference (t-value=-0.72, *P*=0.471) in the endophyte level for the middle sections from Farm A, having an average of 87.7% (SE 4.74), and Farm B with an average of 92.3% (SE 4.31). Finally, there was also no significant difference (t-value=-0.62, *P*=0.537) in the endophyte level for the bottom section with Farm A, having an average of 89.2% (SE 4.78), and Farm B with an average of 92.9% (SE 3.62).

Graph A



Graph B

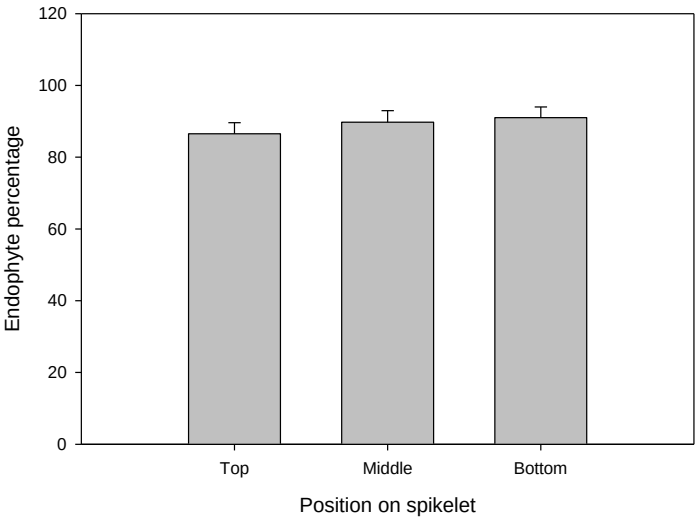


Figure 3.4 Average endophyte percentage of ryegrass plant spike collected from Farm A and Farm B and separated into three sections, top, middle and bottom determined using grow out tests. (A) Comparison between the two farms, 10 ryegrass plant spike samples each. No significant difference between endophyte level in seeds from the different sections based on t-test. (B) Combined data of both Farm A and B for a total of 20 spikes, 10 from each farm. No significant differences between endophyte level in seeds from the different sections based on-test.

3.1.5 Weather data

The average daily temperature for both farms during the seed production process was very similar, as illustrated in Figure 3.5. There was only one day where the average temperatures were not the same and this was the 14th July 2017 when Farm B had an average high of 8°C whereas Farm A had an average high of 5°C.

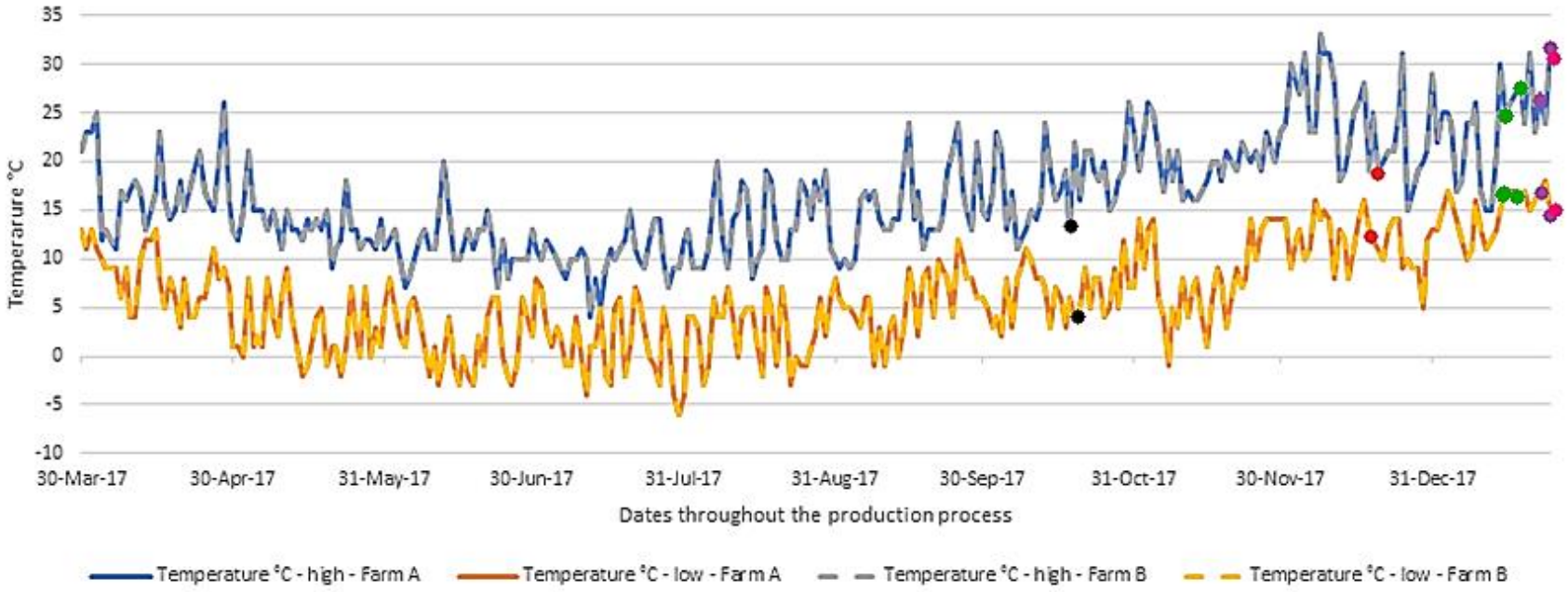


Figure 3.5 Average daily temperature for both Farm A and Farm B during the seed production process. Black dots are the pre-fungicide tiller sampling, red are post-fungicide tiller sampling, green are seed samples from when the grass was just cut, purple are seed samples at harvest time and pink are seed samples collected for Farm B from the drying facility.

3.1.6 Temperature and relative humidity data during the harvesting process

Data loggers were placed in the sampled paddock once the grass had just been cut and recorded both the temperature and the humidity. The Farm A data logger, which was only used underneath the cut grass while it was drying out, showed that the temperature reached a high of 32.5°C and a low of 14.9°C. The relative humidity was highest at 98.8% and lowest at 37.3% (Figure A4.2). The other data logger, which followed the seed throughout the whole process, recorded a temperature high of 33.2°C and a low of 13.9°C when placed underneath the cut grass. The relative humidity was highest at 95.6% and lowest at 39.0% (Figure A4.1).

For Farm A the seed was then harvested and stored in a tractor trailer for around 2 hours, where the temperature reached a high of 31.8°C and had a low of 31.4°C. The highest the relative humidity reached was 72.2% and lowest at 70.4%. The seed was then transferred to the silo which had a temperature high of 37.8°C and a low of 26.7°C, with the relative humidity reaching a high of 41.5% and a low of 36.2%. The seed was stored in the silo overnight and then transferred to the dressing facility which had a recorded temperature high of 28.4°C and a low of 20.6°C and a relative humidity high of 49.9% and a low of 46.1% (Figure A4.1).

Farm B had different seed production processes and the data logger, which remained with the seed in the silo, recorded a temperature high of 31.4°C and a low of 14.3°C while underneath the cut grass, and a relative humidity with a high of 95.7% and a low of 44.6%. The harvested seed was then transferred to a tractor trailer where it remained for approximately 2 days with the temperature remaining consistent (Figure A4.4). The highest temperature that was recorded was 33.8°C and the lowest was 32.8°C. The relative humidity was highest at 72.9% and lowest at 66.9%. When the seed was transferred to the silo the highest temperature recorded was 30.7°C and lowest at 28.3°C, and the relative humidity was recorded at a high of 71.7% and the lowest was 70.1%.

The second data logger for Farm B showed that underneath the cut grass, the temperature reached a high of 34.4°C and a low of 13.9°C and the relative humidity reached a high of 96.5% and a low of 36.4%. When the seed was then harvested, it was stored in the truck trailer for around 5 hours where the temperature reached a high of 36.5°C and remained around this temperature, with the lowest temperature recorded being 36.2°C. The relative humidity in the truck was highest at 59.1% and lowest at 54.9%. When the seed was transported to the drying facility the temperature dropped to a high of 29.8°C and a low of 27.8°C. The relative humidity increased compared to the seed stored in the truck, with a high of 65.0% and a low of 59.2%. After three days the seed was transferred to the dressing facility where the temperature reached a high of 32.5°C and dropped as low as 23.4°C. The relative humidity reached a high of 57.9% and dropped as low as 53.4% (Figure A4.3).

3.2 Objective 2 – Effect of fungicide application on endophyte colonisation of ryegrass tillers

3.2.1 Effect of fungicide on endophyte colonisation

The seed used in this experiment (Alto AR37) had an initial endophyte percentage of 72.0% (Agriseeds QC test). However, when the seed was planted, and the resulting tillers tested using the immunoblot method, just prior to fungicide application, the endophyte percentage was less than 15.0% (Table 3.3). This was an unexpected result and led to experiment described in Section 3.2.2 being conducted.

For the untreated control trays, a significant difference was found between the endophyte level in the old tillers at the second assessment, compared to the tiller tissue in the initial assessment, with the older tissue being higher (39.5% vs 13%, t-value=-4.41, *P*=0.012) (Table 3.3).

For the treated fungicide trays a significant difference was found between the endophyte level in the tillers before fungicide application and the new tiller after fungicide application (t-value=-5.36, *P*=0.006) with the endophyte level in the new tiller (40.0%, SE=4.3) being higher than in the tillers before fungicide application (14.75%, SE=1.9) (Table 3.3).

Table 3.3 The mean endophyte percentage, determined by immunoblotting, for seedlings with and without fungicide application.

	Seed endophyte percentage	Initial endophyte percentage	After fungicide treatment endophyte percentage	
			New tillers	Old tillers
Control	72.0	13.0 adg[1]	37.0 dj	39.5 hj
Fungicide	72.0	14.8 amp	40.0 qs	28.0 ms

[1] Mean values followed by the same lower-case letter (a-b, d-e, g-h, j-k, m-n, p-q, s-t) are not significantly different at *P*=0.05. Lower-case letters are compared in pairs e.g. a and b. Missing letters (b and e) indicate no significance for that comparison.

3.2.2 Effect of potting method on endophyte levels

This experiment was set up due to the low initial endophyte level in the previous experiment (Section 3.2.1). It was designed to determine whether it was the external grower's potting mix, trays, fungicides or location which influenced this apparent loss and reappearance in endophyte. Only one treatment produced seedlings with a significantly lower immunoblot result (92%, t-value=-2.78, *P*-value=0.014) and this was the external grower's new mix without fungicides (Figure 3.6).

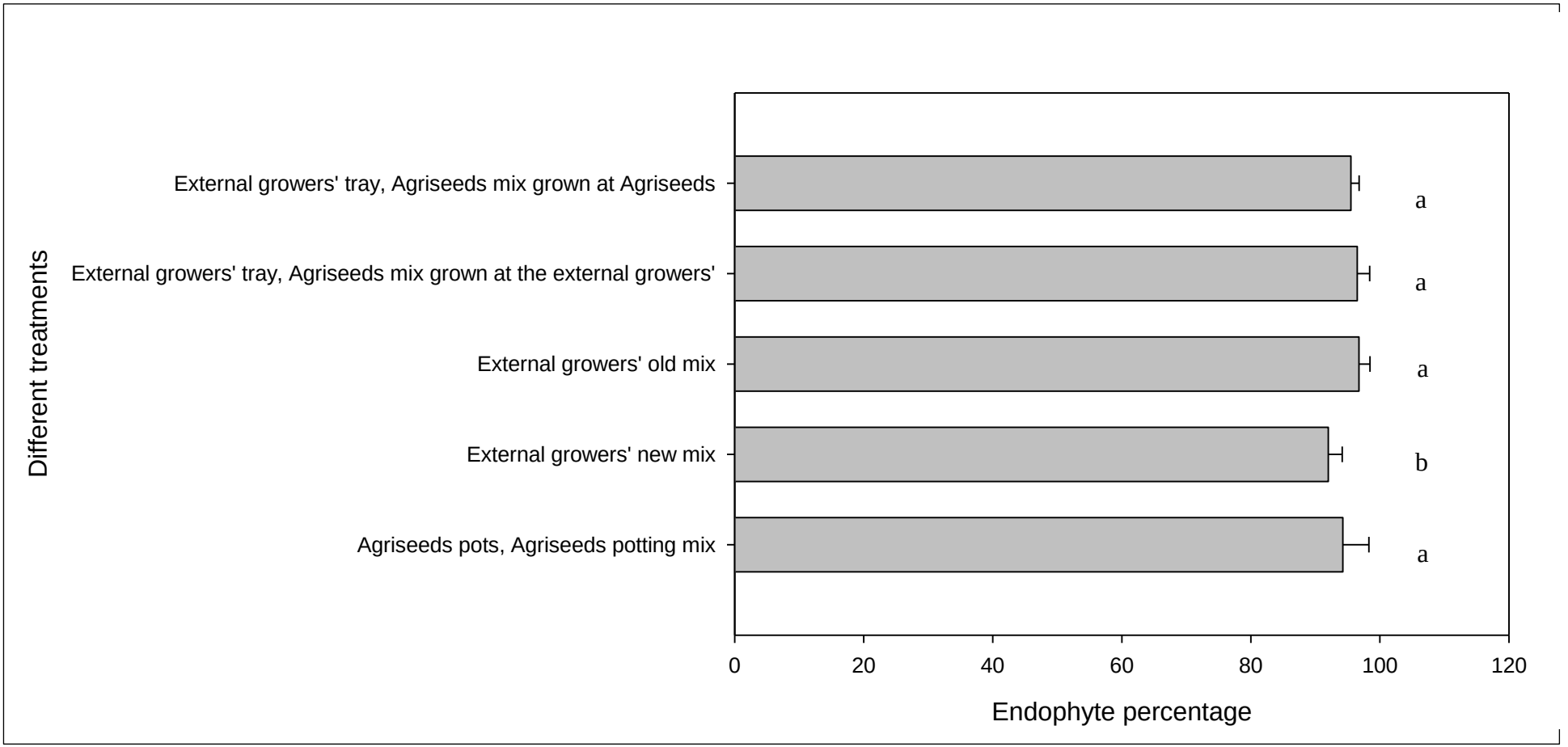


Figure 3.6 The percentage endophyte level (± 0.62) determined by immunoblot method in Tyson NEA12 ryegrass tillers grown in trays (external grower) or pots (Agriseeds) containing different growing media and grown at the Agriseeds or the external grower's facilities. The different growing media included Agriseeds potting mix, external grower's old mix (with fungicide) and external grower's new mix (without fungicide). Mean data with different letters are significantly different based on t-test at *P*=0.05.

3.3 Objective 3 – Effect of seed moisture and age on endophyte viability.

3.3.1 Seed moisture levels

The initial seed moisture of the new seed (Governor AR37, 2017) placed into the muslin bags was 12% moisture, and the old seed (Trojan AR37, 2015) was 11% moisture. Water was added to achieve three moisture levels of 12%, 14% and 16%. At the end of the experiment the old seed in the muslin bags placed in the container had seed moistures, corresponding to the nominal levels of 12%, 14% and 16%, of 12.1%, 12.6% and 13.3% while the new seed in the containers had seed moistures of 11.8%, 12.2% and 13.3%, respectively. The old seed in the muslin bags, stored in the larger boxes, had seed moistures of 12.1%, 12.9% and 14.1% while the new seed in large boxes had seed moistures of 11.6%, 12.5% and 14.0%, respectively.

3.3.2 Seed endophyte level

The initial endophyte level of the old seed was 92.0% while the new seed had an endophyte level of 81.0%, determined using grow out test. Grow out tests followed by immunoblotting were performed on the seeds collected during the course of the experiment to determine the endophyte percentage after different time intervals in the different storage treatments. Initial data indicated the 'in vivo' storage variables were having little effect so after 4 weeks the samples in the large boxes were divided into subsamples placed into smaller boxes (Section 2.3) stored at higher temperatures to try and accelerate viability differences. For each of the box sizes there were two different factors (treatment and time) and one interaction (time x treatment).

For the old seed in the larger boxes there was a significant effect of time on endophyte level (f-value=12.61, $P<0.001$). The endophyte level in the seed stored for 8 weeks was significantly lower compared with all the other weeks (2, 4, 12, 16) being 84.0% compared with 90.0-92.2% for the other assessment times. Although the endophyte percentage did decrease over time, there was no significant difference between the endophyte level and any of the other four time-lengths (2, 4, 12, and 16 weeks) (Table 3.4).

For the endophyte level of the old seed in the container, a significant interaction was observed between time and moisture level (f-value=16.33, $P<0.001$), time (f-value=115.27, $P<0.001$), and moisture treatment (f-value=31.95, $P<0.001$). The endophyte level in the seed stored at 14% seed moisture of 58.2% was significantly lower from that in seed at 12% and 16% moisture levels of 72.3% and 75.9%, respectively (Table 3.4).

There was a significant difference between the endophyte level at all assessment times (8, 12 and 16 weeks), with 48.8% being the lowest at 16 weeks, 75.1% the next lowest at 12 weeks (t-value=-11.26,

P<0.001) and 82.6% the highest at 8 weeks (t-value=-14.45, *P*<0.001) (Table 3.4). There was also a significant difference between endophyte level in the seed sampled after 12 and 8 weeks (t-value=-3.18, *P*=0.014).

Seed stored at 14% moisture for 16 weeks had a significantly lower endophyte level (26.0%) than all other treatments (Appendix 5). Endophyte levels in seed stored at 14% moisture for 8 weeks (84.7%), 12% moisture for 8 weeks (83.0%) and 16% moisture for 12 weeks (81.0%), were all significantly different from seed stored at 14% moisture for 12 weeks (64.0%) and 12% moisture for 16 weeks (53.7%) (Table 3.4).

For the new seed in bags stored in the large boxes, endophyte level was significantly affected by time (f-value=7.09, *P*<0.001). Seed stored for 8 weeks had an endophyte level of 91.3%, significantly higher than any of the other sample times at 79.7-86.0% endophyte (Table 3.4). There was no significant difference in the endophyte level between any of the other four sample times.

For the new seed stored in the container endophyte level was significantly affected by time (f-value=31.44, *P*<0.001) and the interaction between time and moisture treatment (f-value=3.32, *P*=0.033). The endophyte level in seed sampled at all three assessment times (8, 12 and 16 weeks) were significantly different from each other, with seeds sampled after 16 weeks having a significantly lower endophyte percentage of 70.0% compared with seed stored for 12 weeks of 80.8% (t-value=-5.15, *P*<0.001) and 8 weeks of 86.3% (t-value=-7.80, *P*<0.001). The endophyte level in seed stored for 8 weeks was also significantly higher than in the seed stored for 12 weeks (t-value=-2.65, *P*=0.041). For the interaction effect, the endophyte level in seeds at 12% moisture and stored for 8 weeks of 92.0% was significantly higher than for all other treatments (Figure 3.7).

Table 3.4 The mean endophyte percentage determined by grow out test followed by immunoblotting for seed stored in muslin bags placed in larger seed piles for different time periods (2, 4, 8, 12, and 16 weeks) at varying moisture percentages (12%, 14% and 16%). Two seed lines were tested with the old seed having an initial endophyte percentage of 92% while the new seed had an endophyte percentage of 81%. Mean of 100 seeds for each of the three (or four) replicates per treatment.

	Treatments															
	Old seed in large box				Old seed in container[#]				New seed in large box				New seed in container[#]			
Time (weeks)	12%	14%	16%	Overall[1]	12%	14%	16%	Overall[1]	12%	14%	16%	Overall[1]	12%	14%	16%	Overall[1]
2	94.0 a[3]	91.5 abcd	91.0 abcd	92.2x					83.0 a[3]	83.5 a	86.0 a	84.2y				
4	89.8 abcd	94.3 a	92.3 abc	92.1x					82.0 a	84.8 a	85.3 a	84.0y				
8	83.3 d	85.0 bcd	83.7 cd	84.0y	83.0 a[3]	84.7 a	80.0 ab	82.6x	91.0 a	91.7 a	91.3 a	91.3x	92.0 a[3]	84.3 ab	82.7 ab	86.3x
12	89.0 abcd	92.3 abc	92.0 abcd	91.1x	80.3 ab	64.0 c	81.0 a	75.1y	85.7 a	84.0 a	85.0 a	84.9y	81.3 ab	80.3 ab	80.7 ab	80.8y
16	92.3 abc	89.3 abcd	91.0 abcd	90.9x	53.7 c	26.0 d	66.7 bc	48.8z	83.7 a	82.0 a	79.7 a	81.8y	65.0 c	72.0 bc	73.0 bc	70.0z
Overall[2]	89.7A	90.5A	90.0A		72.3A	58.2B	75.9A		85.1A	85.2A	85.5A		79.4A	78.9A	78.8A	

[#] no seeds available for testing at 2- and 4-weeks assessment times

[1] For the overall effect of time on each seed treatment, mean values followed by the same lower-case letter (x-z) are not significantly different at *P*=0.05. [2] For the overall effect of moisture level on each seed treatment mean values followed by the same upper-case letter (A-C) are not significantly different at *P*=0.05. [3] For interaction between time and moisture treatment means values followed by the same lower-case letter (a-d) are not significantly different between treatments at *P*=0.05.

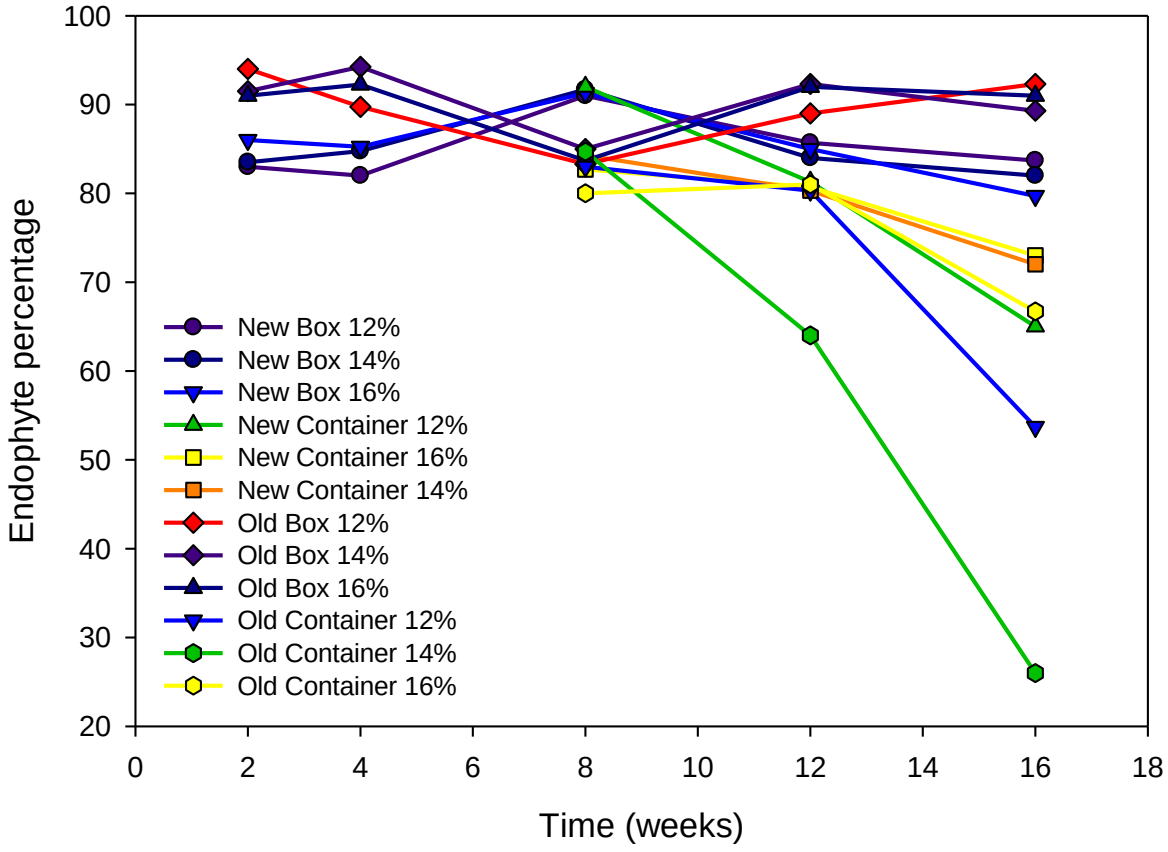


Figure 3.7 The mean endophyte percentage determined by grow out test followed by immunoblotting for seed stored in muslin bags placed in larger seed piles for different time periods (2, 4, 8, 12, and 16 weeks) at varying moisture percentages (12%, 14% and 16%). Two seed lines were tested with the old seed having an initial endophyte percentage of 92% while the new seed had an endophyte percentage of 81%. Mean of 100 seeds for each of the three (or four) replicates per treatment.

3.3.3 Temperature and relative humidity data

The results obtained from the data loggers placed in the middle of the large seed boxes (Section 2.3) showed that the outside of the seed pile with 16% moisture had a temperature high of 21.0°C and low of 8.3°C. The highest relative humidity was 76.9% and the lowest was 45.9% (Figure A4.5).

In the centre of the seed sack with 12% moisture, the highest temperature was 22.2°C and the lowest was 6.1°C (Table 3.5). The relative humidity had a high of 68.4% and low of 42.5% (Figure A4.6). The sack of seed with 14% moisture had a high of 22.2°C and a low of 6.2°C, and a relative humidity of 76.3% and a low of 45.6% (Figure A4.7). The seed at 16% moisture had a high of 22.4°C and a low of 9.0°C, and its highest relative humidity was 76.3% and lowest was 41.9% (Figure A4.8).

When the data loggers were placed within the containers of seed in the germinator (at 20-25°C), the results showed that the temperatures were higher than that of the seed in the larger boxes. For the 12% moisture container the highest temperature was 31.2°C and the lowest was 20.6°C (Table 3.5). The highest relative humidity was 68.9% and the lowest was 63.1%. The container containing the 14% moisture seed had a temperature high of 31.2°C and a low of 20.3°C. The highest relative humidity was 71.1% and the lowest was 65.1%. The container of 16% moisture seed had a lower temperature than the other two containers of seed with a high of 30.6°C and a low of 20.4°C. However, the relative humidity was higher with a high of 81.5% and a low of 69.1% (Figure A4.5).

Table 3.5 Temperature (°C) and humidity (%) recorded from data logger for seed stored in both large and small containers at varying seed moisture levels. The large seed containers were stored in an outdoor shed whereas the small seed containers were stored in a germinator (20-25°C). 16 – on top, is the data logger stored on top of the 16% moisture seed, stored in the large container.

	Large containers stored outside		Small seed containers stored in a germinator	
Seed moisture %	Temperature (Low-high)	Humidity (Low-high)	Temperature (Low-high)	Humidity (Low-high)
12	6.1-22.2°C	42.5-68.4%	20.6-31.2°C	63.1-68.9%
14	6.2-22.2°C	45.6-76.3%	20.3-31.2°C	65.1-71.1%
16	9.0-22.4°C	41.9-76.3%	20.4-30.6°C	69.1-81.5%
16 – on top	8.3-21°C	45.9-76.9%		

Chapter 4

Discussion

The overall aim of this study was to investigate two ryegrass seed production practices (Farm A and B) and try to determine if any management or environmental differences between the practices are responsible for variation in endophyte levels. This loss in endophyte can be the difference between selling seed with a high endophyte percentage and a low endophyte percentage and represents a large difference in the economic return for both seed companies and farmers estimated to be NZ $100-350/ha (DairyNZ, ND). If this loss could be reduced, then the pastoral sector would benefit greatly.

To carry out this study the production processes of two farms were followed with a particular focus on the effect that fungicide and harvest had on the endophyte transmission in the plants and seeds. Overall, no particular management practice was found to result in a large drop in endophyte percentage. As far as we are aware this is the first study to follow the seed production process of two farms over a growing season to pinpoint where in the seed production process loss in endophyte occurs. It is likely that commercial companies have carried out similar studies in house, but this data is not readily available.

Areas of this study have been investigated before (Card et al., 2014; Hill & Brown, 2000; Hill & Roach, 2009; Rolston et al., 2002; Rolston et al., 1986). However most, if not all, studies were carried out under laboratory conditions. This study is therefore informative as it can give a clearer picture of what happens under real world conditions.

4.1 Effect of management practice on endophyte viability and transmission

The field experiment was the main focus of this project as variable losses in endophyte percentage of up to 41% (Governor AR37 lines sown over a 3-year period, personal communication Agriseeds production team) have been seen in commercial production situations. The objective for this experiment was to determine the effect of crop management practices on endophyte colonisation of ryegrass plants and seeds. The hypothesis for the experiment was that there would be a significant difference in endophyte level between the two farms based on their different management practices. The main differences between the two farms being the fungicide application (amounts nad types), harvest practices (windrowing vs moving) and storage practices (external dryer vs on farm cooling). This was found to not be the case with no significant differences observed. This was not expected based on endophyte levels from the previous season, with Farm B (below 70%) having a lower endophyte percentage than Farm A (above 70%), as well as other studies (Hill & Roach, 2009;

Hume et al., 2013). However, it does mean that the practices used had no significant effect on the presence of endophyte. This is helpful for further studies as these two farms had a variety of differences within their management factors which can now be eliminated as the main variables affecting endophyte transmission.

The first main difference that occurred between the two farms, which would have affected the endophyte level based on other studies, was the fungicides used. Farm A had three more fungicide applications than Farm B and applied two different fungicides (Table A2.1). Farm A had a history of suppling high endophyte seed, so it was thought that this would have no impact on the endophyte level of the seed. This hypothesis was further supported by the results obtained by Rolston et al. (2009) who found that the fungicides that were used at that time for commercial seed production, had no effect on the endophyte level of the seed. The results from Objective 1 show that fungicide application indeed did not influence the endophyte level. Rolston et al. (2009) also stated that the fungicides used during commercial seed production were always changing so it would be imperative to test all fungicides in a similar manner to Section 2.2, to ensure that the endophyte level is not affected. To reinforce this data an experiment (Section 2.2, Objective 2) was designed to test the application of high doses of fungicide on endophyte colonisation of the tillers.

The second and third differences between the two farms were the seed, how long the harvest process occurred (harvesting and drying), and the amount of seed harvested. While the harvest itself should not have had any effect on endophyte level, the length of time of the harvest directly impacted how long before the seed was cooled down. Farm B took 2 days to harvest the seed whereas Farm A only took 1 day. Farm A was not only able to harvest all the seed in 1 day, but also able to store all the seed in one silo. This enabled all the seed to be cooled the same day, before being sent to the dressing facility. The seed harvested from Farm B was not able to be treated the same due to the amount harvested. Around 90% of the seed was transported to an off-site drying facility in two trips, one on the first day of harvest while the other was carried out on the second day. However, due to logistical issues, some (10%) of the seed harvested on the first day was stored in a tractor trailer overnight. This seed was stored in the trailer until the harvest process was finished in the hopes that it would be able to be sent to the drying facility also. This resulted in the seed being exposed to a constant high temperature (30°C) for 2 days. It was anticipated that this might affect the endophyte viability, but this was not found to be the case. This is a reassuring result for producers and seed companies as it indicates that during unanticipated high temperatures and unpredicted situations (i.e. transport issues such as breakdowns), the endophyte level will not decrease immediately. High temperatures should still be avoided as much as possible though as it is not clear how long seed can be exposed before damage occurs.

While the endophyte level was not immediately affected, it is not known what affect these elevated temperatures had on the long-term endophyte viability or fitness. It is possible that the endophyte may appear to be unharmed, but after a year of storage under optimum conditions (Section 4.3) the level of viable endophyte may decrease due to this initial mis-treatment (Gundel et al., 2012; Hill & Roach, 2009; Kitson, 2017; Missaoui & Hill, 2015). More research would need to be carried out to test this 'effect on shelf-life' hypothesis.

Results obtained by Agriseeds staff showed that the endophyte level between field dressed and machine dressed seed showed a large decrease in endophyte (Figure 3.1) with Farm A having a loss of 14% and Farm B having a loss of 5%. While this difference was not significant, it does point to a reduction in viable endophyte. This drop was the largest decrease in endophyte for Farm A when all other stages were compared, and while the seed endophyte may have remained above 70%, it was only just above this threshold. This would suggest that a future area of investigation could be focussed at the dressing facility to better understand what variables are present during seed dressing (Section 4.5). No significant difference was found for the machine dressed samples despite a visible numerical decrease occurring. It is likely that the reason for no significance is due to only having two samples, which has therefore generated a large standard error.

While no significant differences were found, the season in which this experiment was carried out appeared to be a good season and produced large amounts of high endophyte seed (Colin Eady, personal communication). More field trials would need to be conducted over multiple growing seasons to determine this, as outlined in Section 4.5.

When the entire seed spikes were compared no significant difference was found between the top third and bottom third of the spike (Figure 3.4). However, a consistent trend was observed. As previously mentioned, 2017-2018 was a good season for endophyte, so it is possible that during poorer seasons, or with greater sampling intensity, this may have become significant. The endophyte level of the top third section of the spikes was lower than the bottom section, which was expected based on the growth of endophyte (Johnson et al., 2019). Thus, only analysing the latter third of the spike would accentuate the ability to measure endophyte transmission differences and potentially provide valuable information to seed companies regarding the relative merits of different seed lots. More tests/collections should be carried out to investigate whether there is a significant difference between the top and bottom of the spike and also to determine if similar more extreme results are obtained during poorer growing seasons.

4.2 Effect of growing methods on endophyte viability

The results of the field experiment in Objective 1 showed no effect of the different fungicide applications on endophyte level, however applying all the fungicides at the same time and at higher application rates to mimic potential issues with applications may affect endophyte colonisation. Therefore, the initial objective for this experiment was to determine if there was an effect of fungicide application on endophyte colonisation of ryegrass tissue/seeds. The fungicides were therefore applied at double the rate of what would be applied in the field. However, the unexpected low endophyte level observed in potted seedlings before fungicide application meant that initial infection was so low that further loss of infection was unlikely to be significant. Thus, a change in focus to investigate the potting and germination conditions was decided upon as this was thought to be the reason for the initial dramatic loss of endophyte. This new objective was to determine if different growth methods influenced endophyte viability.

For the initial fungicide experiment the pre-fungicide endophyte level recorded was between 13.0% and 14.8%, much lower than the initial supplied seed endophyte percentage of 75% (Table 3.3).

Significant differences were found for the initial experiment, with the endophyte level post-fungicide application being higher than that pre –fungicide application (Table 3.3). As endophyte is transmitted vertically (Khiralla et al., 2016) an increase would not be possible. However, it is possible that the endophyte remained dormant at the base of the seedling, below the plant section used for the initial immunoblotting, until a point where it began to grow later in the life of the seedling (Di Menna & Waller, 1986; Johnson et al., 2019). For example, the initial tiller test could have missed the endophyte as it lay dormant in the base and only moved up into the tiller later thus appearing to be a gain in endophyte. Such a finding has large consequences on endophyte testing and viability scores as ‘dormant’ endophyte may easily be missed.

Christensen and Voisey (2007) hypothesized that when endophyte was exposed to cooler ambient temperatures fewer tillers were colonised as a result of reduced endophyte growth. Di Menna and Waller (1986) found results supporting this hypothesis with the endophyte *Acremonium lolii* in the perennial ryegrass cultivar, Ellett. They found that the mean endophyte level per mm of leaf sheath during the winter months was around half that of the endophyte in the summer months. Fuchs, Krischke, Mueller, and Krauss (2017) further emphasised this following endophyte quantity over a 3-year period. In that study it was found that each year, during the summer months endophyte concentration increased dramatically when compared to the endophyte concentration found during the winter months.

It is possible that the seedlings used in the first experiment (Objective 2) were exposed to similar climatic conditions. However, this is unlikely as the experiment was planted during October, when temperatures began to slowly increase (Figure 3.5). While the temperature may have been increasing, when the seedlings were collected there was a period of cool, rainy days which may have impacted the growth of the endophyte. The external grower also stored the seedlings in a glass house. However, the glass house was not heated so during the cooler months it would stay cool rather than warming. This may negatively impact growth of the endophyte used in these experiments, AR37, as transmission is typically higher under warmer conditions (Gagic et al., 2018).

The external grower also used fungicides in both their potting mix and in their watering system. Initially it was thought that these fungicides may have caused the loss in endophyte as they are different to those tested in Objective 2. However, this was later thought unlikely due to the endophyte level increasing at the later assessment. Nevertheless, these fungicides could have retarded the growth of the endophyte into the new tiller growth. It was, however, important to rule out this factor and ensure that these fungicides had no negative effect on the endophyte viability. While a large amount of research has been carried out, there are conflicting reports as to whether the use of fungicide results in a loss of endophyte in perennial ryegrass.

Hill and Brown (2000) reported a loss in the endophyte infected tall fescue cultivar “Jesup”, infected with *Neotyphodium coenophialum*. This loss was seen using the systemic fungicide Propiconazole, which was not used for any of the objectives of this research. However the four main fungicides (Amistar, Folicur, Proline and Protek) used for Objectives 1 and 2 (first experiment) are systemic fungicides. Hill and Brown (2000) also applied the fungicides at the seedling stage, which may have contributed to this loss as the endophyte may not have established enough in the plant, therefore potentially being more vulnerable to the fungicides. While it is not known what fungicides were used by the external grower (proprietary information), systemic fungicides may have been used, attributing to the loss observed in endophyte level.

While Hill and Brown (2000) reported a loss in endophyte with systemic fungicides, Cruz et al. (2018) found no loss in endophyte using the same fungicides used for Objectives 1 and 2. Rolston et al. (2002) also reported no loss in endophyte as a result of systemic fungicides. However the work carried out by Cruz et al. (2018) is of more relevance to this study as they tested the endophyte AR37, the same endophyte tested in this study. The active ingredients used by growers are also often changing (Rolston et al., 2009) and the work carried out by Cruz et al. (2018) was carried out in 2012, just 5 years prior to this current study.

Another possible reason for this difference in endophyte level is that the seed may have been left in the sun before it was planted. This could have occurred at the external grower’s location before

planting begun. To confirm if this was what occurred the plants could be planted in larger containers or in a field and be left to grow for around 2 months before being tested again. This would confirm if the seed itself was damaged or instead if the endophyte was affected by cooler temperatures and remained dormant.

The second experiment (Objective 2) was designed to not only look at the effect of fungicide applications, but also the other key variable, the tray type used, and where the plants were grown, at Agriseeds or by the external grower. The objective of this experiment was to determine if different growing methods had any effect on endophyte viability. Only one significant difference was found, and this was for the seedlings grown in the external grower's new potting mix, having a lower endophyte percentage compared to the other treatments (92.0% vs 96.8, Table 3.4). While it is possible that the growing conditions may have resulted in a loss in endophyte, there is also the possibility that the difference may be due to sampling errors whereby the seedlings selected for testing did not show a true representation of the overall endophyte level for this treatment. For this to be ruled out more seedlings could be tested. However, because the endophyte level was over 90%, this was not done. The seed used for this second fungicide experiment was different to the first fungicide experiment (both Objective 2) but was one that also previously had a low initial endophyte result (16%) when grown by the external grower. In this case the seedlings with the low endophyte were removed from the trays and planted out into the paddock where the endophyte level increased to 78% in later assessments (Hooman Vakilotojjar, personal communication), supporting the theory of the endophyte remaining dormant in tillers.

These results follow a similar trend to the first experiment for Objective 2 and support the theory of the endophyte lying dormant in the base of the plant. It is not known why this occurs as the second experiment for Objective 2 was planted in August, during winter, and the endophyte did not remain dormant. However, the endophyte used in the two experiments differed and may therefore respond differently to environmental conditions. It is also possible that during the period between these two experiments the external grower may have altered their growing methods, but this is proprietary information. Another possible explanation is that there may have been a combination of factors which hindered the growth of the endophyte. For example, an extended cold period may have occurred which, when combined with fungicide application, resulted in the growth of the endophyte being hindered. It is also possible that the host/endophyte relationship was not strong enough to withstand these factors (Gagic et al., 2018). While these factors alone may not affect the endophyte, together they may do enough damage to result in what appears to be a loss in endophyte.

One of the problems associated with this experiment was the way in which endophyte was detected. Immunoblot tests were carried out resulting in the destruction of the tiller so only one tiller was

tested, ensuring that there were enough tillers remaining to carry out further testing. This could have led to an inaccuracy for example if only one tiller of five contained endophyte and this tiller was tested. To remove this inaccuracy in further testing, whole plants could be sacrificed, and the basal tissue analysed as well as all surrounding tillers.

Based on these results it is not possible to conclude whether the loss in endophyte in the first experiment for Objective 2 (Section 2.1.2) was due to the external grower's methods, or from other factors, or from a combination of the two. Future work is needed to determine this but until that happens it is best to avoid the application of any additional fungicides unless absolutely clear that these do not negatively affect the endophyte, particularly while the ryegrass plant is a seedling. While the first experiment for Objective 2 was inconclusive, the second experiment was informative, with different growing methods shown to have no effect on endophyte viability. This will also pave the way for more experiments to be designed based on these results.

4.3 Effect of storage conditions on endophyte

The third and final objective was to determine if the seed moisture, length of storage and age of the seed influenced endophyte viability. It was found that the seed moisture and length of storage had varying effects on the seed endophyte level depending on the age of the seed and temperature that the seed was stored at. This section interprets the results from the different storage treatments separately.

Old seed stored in large boxes: Initially the seed at the different moisture contents were incubated in large boxes, split into two categories, old and new seed. Only the old seed assessed after 8 weeks of storage, irrespective of seed moisture level, showed any significant differences in endophyte level, having a reduced endophyte level (average 84.0%, compared to 90.9% (next lowest at 16 weeks) (Table 3.4). This low endophyte level at 8 weeks was most likely due to sampling error as the endophyte level at subsequent sampling (12 and 16 weeks) was higher and similar to the initial level. The result must be an anomaly as the endophyte used is only transmitted vertically (from plant to seed) so it would not be able to horizontally re-infect seed. Therefore, an alternative explanation must be the reason for the perceived dip in endophyte level at this assessment time.

The potential explanations for this decrease include that the seed selected to be planted for the grow-out test may have had a higher proportion of seeds without endophyte before the experiment began, or the seedlings collected during the grow-out immunoblot test may not have been a true representation of the overall immunoblot level. To remove this natural variation, more replicates would need to be undertaken, and this experiment could also be repeated with multiple different seed lots. Due to the nature of this experiment a small sample size was used (one container/box for

each moisture content). This small sample size has the potential to create a large sampling error which would be removed once additional testing is carried out.

Alternatively, conditions during the grow-out test may have inhibited the movement of the endophyte from the seed into the developing tillers. However, this is unlikely to be the cause as the grow-out tests were carried out under the same controlled conditions in the glasshouse at the Agriseeds facility and all seed collected at 8 weeks of storage were planted at the same time.

One of the other possible reasons for a loss in endophyte being observed could be due to issues with the detection of the endophyte because of the chemicals used during the development of the immunoblots. This can be an issue if the solutions have not been prepared correctly, are tainted, are out of date, or if, for some other reason, the antibodies do not bind to the nitrocellulose paper. It is highly unlikely that the latter reason was the case because the control immunoblots used gave clear positive reactions (were a strong red colour).

It is also possible that during the collection of the seed the old and new seed were mistaken for the each other, resulting in the variation seen. To ensure that this was not the case further testing would need to be carried out.

New seed stored in large boxes: No significant difference was found for the new seed in the large box (Table 3.4). However, a downward trend was observed at 16 weeks with 12% moisture having the highest endophyte level (83.7%), followed by 14% moisture (82.0%) and 16% moisture with the lowest (79.7%). This trend concurs with the work of Rolston et al. (1986) who found that as the seed moisture increased, the endophyte level dropped (Figure 4.1). The work carried out by Rolston et al. (1986) involved a different grass seed cultivar ("Nui" perennial ryegrass, *Lolium perenne*) and a different endophyte strain (*Acremonium lolii*), and involved a much longer storage period of 12 months compared with the 16 weeks used in the current study. The Rolston et al. (1986) experiment, like many others, was conducted on a small scale using only 100 grams of seed per sample. The results for the seed in Objective 3 followed a similar trend (Figure 3.7). However, the results were not as extreme for the seed in the large boxes, likely because of the lower temperature and shorter experimental timeframe.

Figure 4.1 Effect of ambient temperature (5-25°C) and seed moisture on endophyte and germination percentage on Lolium perenne seed containing Acremonium loliae endophyte after 12 months of storage (Rolston, Hare, Moore, & Christensen, 1986).

Gundel et al. (2012) observed similar results to those found for Objective 3, using another endophyte species *Neotyphodium occultans* (syn. *Epichloë occultans*) and *Lolium multiflorum* with varying levels of relative humidity (5%, 43%, and 75% RH) and temperatures (20°C and 40°C) and smaller amounts of seed (2.2 g). The endophyte strain used was collected from Argentina in an area that had not been disturbed for 30 years. In their research, the endophyte level in the seed incubated at 20°C remained high (94%, 85%, and 90% for 5%, 43% and 75% RH, respectively) across all levels of RH, until the seedlings were assessed at 18 weeks of storage to give 88%, 62%, and 89% for 5%, 43%, and 75% RH, respectively.

Seed in containers: The individual moisture levels of the old and new seed stored in the incubator followed the expected trend in that there was a significant drop in viability over time and the old fared worse than the new. However, across the three moisture levels the results were inconsistent. The expected trend was that the 16% moisture would have the lowest endophyte level at the end of the experiment however for the old seed the 14% moisture had the lowest endophyte level (26.0%) at 16 weeks and for the new seed the 12% moisture had the lowest endophyte level (Table 3.4). It is difficult to explain this inconsistency, except to say that apart from the old seed at 14% at 12 and 16 weeks the other results were not significant across moisture levels. This would suggest that the temperature and time in this experiment caused a greater loss in viability than the moisture variable.

A larger difference in moisture levels tested may have produced a greater differentiation. However, the purpose of this experiment was to simulate real world situations, in particular the conditions the seeds are exposed to at harvest time. This data would indicate that short term temperature and moisture variations are not an issue for endophyte viability.

Missaoui and Hill (2015) observed similar results with their experiment which was primarily around using accelerated aging on *Lolium arundinaceum* (tall fescue cv. Jesup AR542) seed containing the *Neotyphodium coenophialum* endophyte. The accelerated aging involved using varying saturated salt solutions to maintain varying levels of RH to which 5 g of seed placed in containers was exposed to, within incubators for 5 weeks. Missaoui and Hill (2015) found that the percentage of endophyte found in seeds stored at 50% RH for 21 days was significantly higher (98%) than seeds stored at 35% RH (33%), 75% RH (67%), and 100% RH (35%). Based on these results it is possible that the seed stored in the current study for Objective 3 were not at a high enough RH to result in any significant impact on endophyte level within a short time period, which is further implied by the decrease in endophyte level seen in the seed stored in the 20-25°C incubator. Accelerated aging, as the name would suggest, does not provide a true representation of how fast the seed and endophyte would deteriorate when stored under real-life conditions. However, it can provide researchers insight into how some endophyte strains compare when placed in sub-optimum environments.

Similar results to those found for Objective 3 were also observed by Bylin et al. (2016), when storing seed in a fridge (10°C), freezer (-20°C), and at room temperature (21°C), and by Hume et al. (2011) when storing seed in different geographical locations (Location in Queensland, Australia and Palmerston North and Kerikeri in New Zealand). Bylin et al. (2016) found that the survival of endophyte decreased faster when stored at room temperature, while Hume et al. (2011) found that endophyte viability decreased faster in the warmer climate of Queensland than it did in Palmerston North.

The temperature of the seed stored in the containers in the incubator, was higher than the seed stored in the containers outside (Table 3.5), which in turn increased the overall relative humidity of the seed. A difference was also seen in the temperatures of the seed stored in the incubator. However, the same log tag was used in all three containers, so this difference in temperature was most likely due to the differing moisture of the seed as containers were shifted weekly within the incubator.

The highest levels of RH were similar between the seeds in the boxes and in the containers (68.4 vs 68.9% RH at 12% moisture, 76.3 vs 71.1% RH at 14%, 76.3 vs 81.5% RH at 16% moisture for box vs container, respectively). The lowest relative humidities were different between the seeds in the boxes and in the containers (42.5 vs 63.1% RH for 12% moisture, 45.6 vs 65.1% RH for 14% and 41.9

vs 69.1% RH for 16% moisture, box vs container, respectively). Based on Missaoui and Hill (2015) and the log tag results, it could be assumed that the seed stored outside was around 50% RH for around 75% of the time that it was stored (16 weeks) and 75% RH for the remaining 25% of the time, whereas the seed stored in the incubator would be around 75% RH for the entire 8 weeks. Based on this, if the storage of the seed outside was to continue for another 8 weeks, then a similar drop in endophyte level to that seen in the incubator might be expected.

The results of the seeds stored in the incubator indicate that an increase in temperature had more of a detrimental effect on the old seed compared to the new seed. This could be because the fitness of the endophyte in the new seed was higher than the endophyte in the old seed and so was able to withstand the sup-optimum conditions longer. This may be due to the age of the seed or it could be a result of the host-endophyte interaction as shown by Hill and Roach (2009) who compared four different host-endophyte interactions in tall fescue plants. The fitness of the interaction between cultivar and endophyte strain has been shown to have an important effect on endophyte level in seed and potential retaining of the endophyte in the seed. Hill and Roach (2009) tested three different tall fescue cultivars ('Flecha', 'Jesup' and 'Advance') against two different endophyte strains (AR542 and AR584) with four combinations; 'Flecha' AR542, 'Jesup' AR584, 'Jesup' AR542 and 'Advance' AR542. The endophyte level in all four cultivar endophyte strain interactions were significantly different with 'Advance' AR542 having the lowest endophyte level (49.7%) and 'Flecha' AR542 having the highest (95.2%) indicating the importance of having a good host-endophyte relationship (Hill & Roach, 2009). It is also possible that substrate exhaustion occurred in the older seed as seen by (Zhang, Mace, Matthew, & Card, 2019). They found that fungal sugars were reduced in older seed, leading to a loss of endophyte. More testing would be required to determine if this occurred in Objective 3.

The purpose of Objective 3 was to determine what would happen if the growers had to store the seed under sub-optimum conditions (high temperatures and humidity) for longer than expected. It is unlikely that a grower would be placed in this situation for longer than 16 weeks, so it is likely that no significant effects would occur in regard to a decrease in endophyte level on farm but dressing facilities and distributors may store seed for a considerable time. One improvement on this research would be to recreate the external storage during the summer months as was originally conceptualised, as it is likely that the hotter summer temperatures would accentuate the endophyte viability differences as was observed in the containers.

Another improvement would be to recreate this experiment using multiple containers and boxes to ensure that each treatment has enough true replicates. Ideally each box/container would be

classified as one replicate rather than each muslin bag. Due to time, space and material constraints, this was not able to be carried out.

The overall objective of the storage experiment was to determine the effect on endophyte viability in seed if stored for extended periods of time in sheds or silos prior to being sent to the seed dressing facilities. The results of the experiment showed that the effect on the endophyte viability could differ greatly depending on the temperature of the stored seed. In an ideal situation the seed would be stored in a cool environment and in this case, based on the results from the experiment, the seed could be stored at this temperature for at least 16 weeks. It would however, be unwise to store the seed for any longer based on the results produced by Gundel et al. (2012) as an increase in time and temperature increases the risk of endophyte loss.

4.4 Endophyte assessment protocols

When the histological analysis and the immunoblot tests were compared the results were consistent (minor variations) between the two methods (Table 3.2). This was expected as a large amount of variation would indicate that one of these tests would be inaccurate when testing for endophyte. Both Hiatt, Hill, Bouton, and Stuedemann (1999) and Hahn, Huth, Schöberlein, Diepenbrock, and Weber (2003) found similar results when comparing histological analysis and tissue immunoblots. Hahn et al. (2003) also stated that the tissue immunoblot was able to identify 12 more positive tillers than the histological analysis indicating that the immunoblot method was more sensitive in detecting the presence of endophyte. This would however also depend on the microscopy analyser and quantity of tissue prepared, as it is possible for small amounts of mycelia to be missed or mycelia of other fungal species to be miss-identified as endophyte.

It is possible that any variation found between the immunoblot test and the histological analysis was due to experimental error. Other fungal hyphae can be present within the plant tissue and therefore appear on the microscope slide and these can be mistaken for endophyte hyphae if care is not taken. It is also possible that some plant material could have touched another square on the immunoblot paper, resulting in a faint result. This would then mean that the plant that was deemed to have a faint result, would in fact be negative for endophyte. These two reasons are crucial when carrying out either test as care needs to be taken to ensure that false positives do not occur.

Based on the small amounts of variation in the results between the histological analysis and the immunoblot results it can be determined that using the immunoblot test when testing for endophyte provides sufficient information to accurately determine the percentage of viable endophyte found within ryegrass plants. Further work could be carried out to determine if these results can be

replicated by different people, ensuring that there is an industry wide standard to compare results too, as well as blind testing of the antibodies used.

The significant differences between the seed squash and immunoblot results (Table 3.1) show that while the seed squash test can be used to test for endophyte, it should not be used as a sole endophyte detection method. This was expected as the seed squash test only determines if the endophyte is present or not, rather than if it is viable (Siegel, Latch, & Johnson, 1985). While there were significant differences, the difference was small (between 1% and 11%). Generally, the seed squash test is only used on freshly harvested material where it is assumed that the endophyte is viable. The findings here would agree with such a use. It is however possible that under different seasonal or harvest conditions the difference between the seed squash and immunoblot test could be much larger. More testing would be required to determine this, and perhaps such a comparison would help identify conditions that lead to viability loss.

4.5 Recommendations for future research

The overall aim of the research described in this thesis was to determine the causes for loss in endophyte during seed production. Although no direct cause was found, potential causes were ruled out. While this does not provide a clear answer, it does pave the way for future work to be carried out to examine a variety of other factors such as the endophyte viability and fitness, the climatic conditions and plant stresses.

The endophyte strain is an important factor when analysing a loss in endophyte as some host-endophyte relationships are less compatible than others, therefore making it “easier” for the endophyte to be lost (Hill & Roach, 2009). It is possible that the endophyte used for Objective 1, may have had a strong host-endophyte relationship with the ryegrass cultivar selected, which in turn would have increased both the viability and fitness of the endophyte. Further experiments could be carried out using the same methods but with various seed lines and endophyte strains to determine the importance of these factors. Another way would be to use a seed line that is known to have a strong host-endophyte relationship and compare it to a seed line that has a weaker host-endophyte relationship, or a less viable seed.

The experiment could be repeated using the seeds produced from both farms harvested for Objective 1. Although there was no significant loss in endophyte between the farms in this research, the seed from Farm B was sitting at higher temperatures for longer than the seed from Farm A and this could have affected the fitness of the endophyte in future use (Johnson et al., 2019; Kitson, 2017). This could mean that when the seed is planted and then placed under stressful conditions in the field, the endophyte colonisation of the plant and growth into the new seed may be hindered.

When growing the seed for testing endophyte level in Objective 1 the seed was grown under optimum conditions, so the fitness of the endophyte would not have been easily evaluated. The seed could also be stored for an extended period (up to a year or two) and then tested periodically to determine if the fitness of the seed was inhibited. The same seed batch could be used as a control; however, it would be important that this seed was not exposed to high temperatures after harvest. More work could also be carried out regarding field and machine dressed seed. Multiple seed lines could be tested to determine if the dressing type has any effect on the overall endophyte percentage of the seed.

The climatic conditions may also inhibit the growth of the endophyte, particularly if the endophyte and plant are grown in cooler areas or under low light levels. For Objective 2 it was possible that, because the plant and endophyte were grown during the cooler months of the year, the endophyte did not grow into the developing plant as quickly as it might have under more optimum growing conditions for the seed as transmission is greater during warmer periods (Gagic et al., 2018). To determine if this was the case, multiple treatments could be carried out using endophyte containing seed of varying fitness levels. The growth of the endophyte into the plant could be compared for seeds growing under optimum temperature and light levels compared with those under suboptimum conditions. Seed could be grown under controlled conditions (in growth cabinets), with varying temperatures and humidities. Lower temperatures should be used, initially with large differences e.g 5, 15, 25°C etc., to determine if a difference is found. If a difference is found, then the temperature range should be reduced to further pinpoint the temperature at which endophyte growth is affected.

For this experiment seed could be grown in pots similar to those used in the grow-out test with pots containing positive (with standard endophyte) and negative (without endophyte) controls and exposed to the different temperatures. If these suboptimum growing conditions resulted in a reduction in endophyte level in the growing plant, then it could indicate that cooler temperatures and/or lower light levels have a negative effect on the growth of the endophyte. While the climate in the field cannot be controlled, it can be mitigated for by planting the seed later if needed or if this is not possible, then at the very least seed companies may be able to gain some insight into the endophyte level of the seed to be sold commercially in the coming season. Seed companies would also be able to gain further insight into the potential endophyte level by testing the top third section of random spikes in fields using the methods outlined in Section 2.1.1.

It is also possible that the seed grown for Objective 2 was under other stresses such as water or nutrient stress. The results from the second experiment for Objective 2 showed that the tillers grown at Agriseeds were a much darker green than those grown at the external growers, indicating a difference in nutrient level between the potting mixes. While nutrients alone may not affect

endophyte viability, a lack of nutrients and cooler weather combined may be the tipping point for the endophyte. Drought or water stress may also affect the growth of the endophyte, and this could be controlled for during the seed production process by using irrigation (Card et al., 2014; Gagic et al., 2018; Johnson et al., 2019).

To test and ensure that these three factors are not issues, grow-out tests could be performed using a variety of different treatments such as seeds planted in growing media with very little nutrients, optimum nutrients and excessive nutrients, as well as seeds grown in growth cabinets with differing water levels. Each factor should be both tested individually and then in combination, as although alone these factors may not affect the endophyte or seed fitness, in combination with other factors may negatively influence the fitness.

As stated in Section 4.3, future work for Objective 3 would involve carrying out the same experiment, but during the summer and extending the weeks in storage by at least 16 weeks so the experiment would run for 32 weeks. To accurately determine at what stage when the endophyte level begins to drop off, seed should be removed at weekly intervals.

4.6 Concluding Discussion

The purpose of this research was to determine where in the seed production process a loss in seed endophyte occurred using the perennial ryegrass cultivar, Governor, inoculated with the novel endophyte, AR37. While none of the management practices carried out on the two farms resulted in a reduction in endophyte levels in the plant or seed, the study has ruled out multiple factors as the sole reason resulting in endophyte loss. Further work over future seasons could be carried out to determine if different climatic conditions had any effect on endophyte viability. Future work could also be carried out to ensure that no other factors during the seed production process, including seed dressing processes or harvesting methods, affected seed endophyte levels. While no set causes could be identified, it does provide information to seed companies when dealing with commercial growers and ensure that issues, such as the seed being exposed to high temperatures for 1-2 days, are likely not to negatively affect seed endophyte viability.

Although results from the experiments in Objective 2 did not in the end address the question of how fungicides affected endophyte viability and colonisation, the results did provide information on further avenues of research, particularly around endophyte/host fitness and relationships, and climatic responses of endophyte. Freitas (2017) investigated the host/endophyte relationship using the endophyte strain AR501 and the grasses, perennial ryegrass, tall fescue and Italian ryegrass. It was found that the endophyte behaved differently between the three grasses and as such the loss of endophyte also differed between the three grasses. Perhaps the most relevant interaction to occur

within the study carried out by Freitas (2017) was that within Italian ryegrass containing AR501. A combination of treatment applications were used on this grass species as well as perennial ryegrass containing AR501, including nitrogen fertilisers, fungicides and plant growth regulators. No significant loss in endophyte was observed in perennial ryegrass while a significant loss was seen in the Italian ryegrass interaction, likely due to differences in the host/endophyte relationship. This further emphasises the importance of a strong host/endophyte relationship, particularly for studies such as the experiments for Objective 2.

The experiment completed for Objective 3 was the only experiment in this research project where significant differences were seen. This experiment followed the expected trend, of endophyte viability decreasing over time, particularly at higher temperatures. The experiment should, however, be repeated to further demonstrate the effect of temperature and humidity on seed in real world conditions as this experiment was carried out during the winter period which does not reflect the conditions at harvest time.

While the results obtained from this research were not what was initially hypothesised, they have still provided valuable insight in determining which factors alone are not responsible for the loss of endophyte observed, but in combination might be important. These results will also provide valuable insight for future research and enable researchers to better determine why this loss in endophyte occurs and ways that it can be mitigated.

Appendix A

A.1 Farm management practices and timing of applications

Table A1.1 Details of the management practices and timing of application, which were applied during growth of the ryegrass crop in the two farms sampled in Objective 1.

MANAGEMENT FACTOR	FARM A – 5.4 HA	FARM B – 22.9 HA
PADDOCK PREPARATION – CULTIVATION	16th March 2017	20th March 2017 4th April 2017 6th and 7th April 2017
PADDOCK PREPARATION – FERTILISER APPLICATION (N:P:K:S:MG:CA)	Cropmaster 15 (14.8:10:10:7.4:0:0) applied at a rate of 300 kg/ha on the 16th March 2017	Lime, applied at a rate of 3 t/ha on the 14th March 2017 Superphosphate (0:9:0:11:0:20), applied at a rate of 200 kg/ha on the 16th March 2017
SOWING INFORMATION – GOVERNOR AR37	Sown at a rate of 8 kg/ha on the 30th March 2017. Sowing depth – 15 mm, row spacing – 125 mm	Sown at a rate of 9 kg/ha on the 10th of April 2017. Sowing depth was approximately 20 mm, row spacing unknown
INSECTICIDE	Diazinon 20G (Organophosphate), applied at a rate of 4 kg/ha on the 30th March. Metarex (Metaldehyde bait and Bitrex animal repellent), applied at a rate of 3 kg/ha on the 9th and 24th of April.	None applied

Table A1.1 continued

MANAGEMENT FACTOR	FARM A – 5.4 HA	FARM B – 22.9 HA
HERBICIDE	Agpro Glyphosphate (phosphono methyl glycine), applied at a rate of 2 L/ha on the 30th March. Nortron (ethofumesate), applied at a rate of 4 L/ha on the 31st March. Trimec (mecoprop), applied at a rate of 3 L/ha on the 30th May. Quantum (tribenuron Methyl), applied at a rate of 75 mL/ha on the 30th May. Puma S (fenoxaprop – p – thyl), applied at a rate of 750 mL/ha on the 21st June.	Legend (quinoxyfen), applied at a rate of 2.5 L/ha on the 15th June. Jaguar (diflufenican), applied at a rate of 1 L/ha on the 15th June. Puma S (fenoxaprop – P – pthyl), applied at a rate of 750 mL/ha on the 7th July.
PLANT GROWTH REGULATOR APPLICATION	Moddus (trinexapac-ethyl), applied at a rate of 1.5 L/ha on the 6th November	Optimus (trinexapac-ethyl), applied at a rate of 900 mL/ha on the 3rd November Optimus (trinexapac-ethyl), applied at a rate of 1 L/ha on the 10th November

Table A1.1 Continued

MANAGEMENT FACTOR	FARM A – 5.4 HA	FARM B – 22.9 HA
FUNGICIDE APPLICATION	Folicur (tebuconazole), applied at a rate of 400 mL/ha on the 6th November Versatill (clopyralid), applied at a rate of 400 mL/ha on the 6th November Proline (prothioconazole), applied at a rate of 400 mL/ha on the 1st December Amistar (azoxystrobin), applied at a rate of 500 mL/ha on the 4th December Protek (benzimidazole), applied at a rate of 500 mL/ha on the 18th December Protek (benzimidazole), applied at a rate of 500 mL/ha on the 15th January	Seguris flexi (isopyrazam), applied at a rate of 400 mL/ha on the 3rd November Proline (prothioconazole), applied at a rate of 400 mL/ha on the 4th December Amistar (azoxystrobin), applied at a rate of 500 mL/ha on the 4th December
HARVEST	Cut on the 17th January 2018 - Mown Harvested on the 24th January 2018	Cut on the 14th January 2018 – Windrowed Harvested on the 20th January 2018

A.2 Media

Blocking solution (Enough for four sheets/two boxes)

Add 2.42 g Tris methylamine, 2.92 g sodium chloride, 5 g non fat milk powder, and 10 mL of 1 M hydrochloric acid in 1000 mL of deionised water. The hydrochloric acid was added last, once everything has dissolved. The ph was then adjusted to 7.5 using concentrated hydrochloric acid.

Dye Mix

Solution A - Napthol (20 mg) was mixed with 20 mL of Tris buffer until dissolved.

Solution B- A 32 mg aliquot of Fast Red (Sigma Aldrich) was mixed with 20 mL of Tris buffer until dissolved.

Solution A and B were then combined to make the dye.

2% Analine Blue stain

Analine Blue (0.1 g) was dissolved in 10 mL distilled water. Lactic acid (20 mL) and 20 mL of distilled water was then added and stirred to mix.

Fungicide amounts

Benzimidazole (Protek) recommended rate 500 mL/ha applied at rate of 1 L/ha

Tebuconazole (Folicur) recommended rate 440 mL/ha applied at a rate of 880 mL/ha

Prothioconazole (Proline) recommended rate 400 mL/ha applied at a rate of 800 mL/ha

Azoxystrobin (Amistar) recommended rate 750 mL/ha applied at a rate of 1.5 L/ha

A.3 Plant sampling procedure for Farms A and B, and experimental design for the fungicide experiment

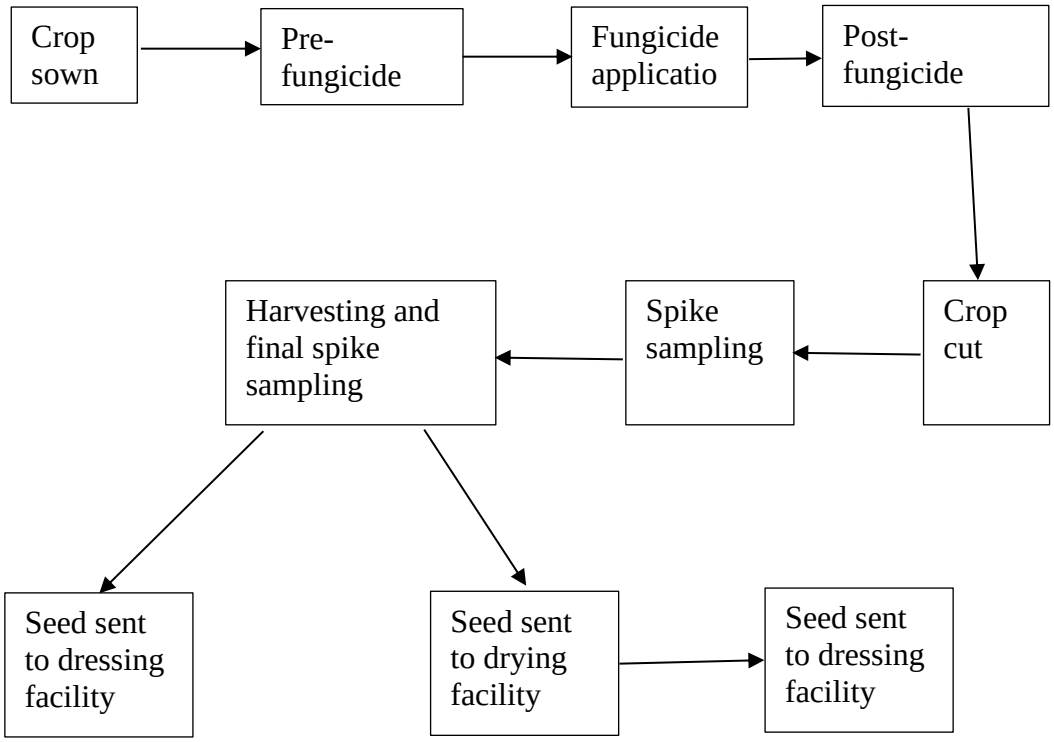


Figure A3.1 Diagram of the sampling process during the seed production process of both Farm A and Farm B.

Fungicide	Control
Fungicide	Control
Fungicide	Control
Control	Fungicide

Figure A3.2 Diagram of the tray layout for the fungicide trial where four fungicides were added to four trays only all at the same time.

A.4 Temperature and relative humidity results from the data loggers

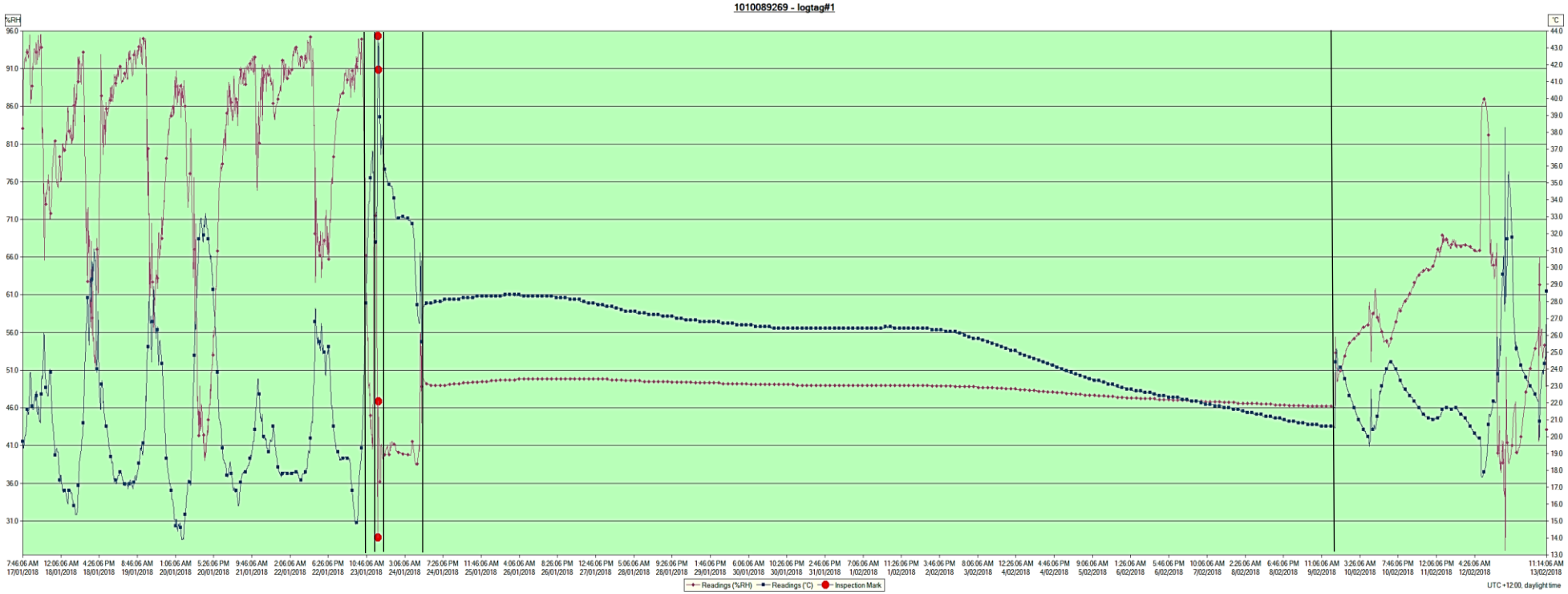


Figure A4.1 Log tag results for Farm A recording data from underneath the mown grass swath to truck and then to dressers. The first section (up to the first vertical black line) is the data from underneath the grass swath, the second section is when the data logger was not used while the third is when the data logger was placed in the middle of the seed in the tractor trailer. The fourth section is when the seed was stored in the silo and the fifth is when the seed was stored at the dressing facility.

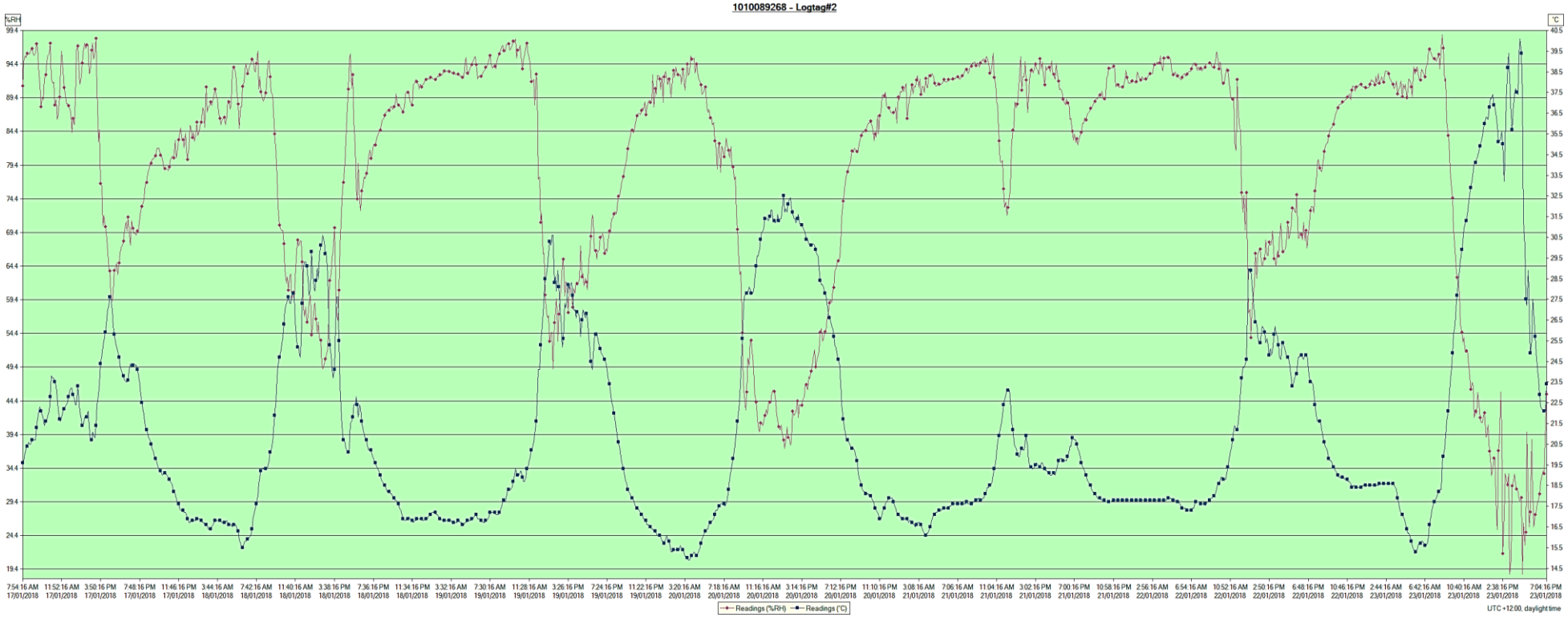


Figure A4.2 Log tag results for Farm A underneath the mown grass swath.

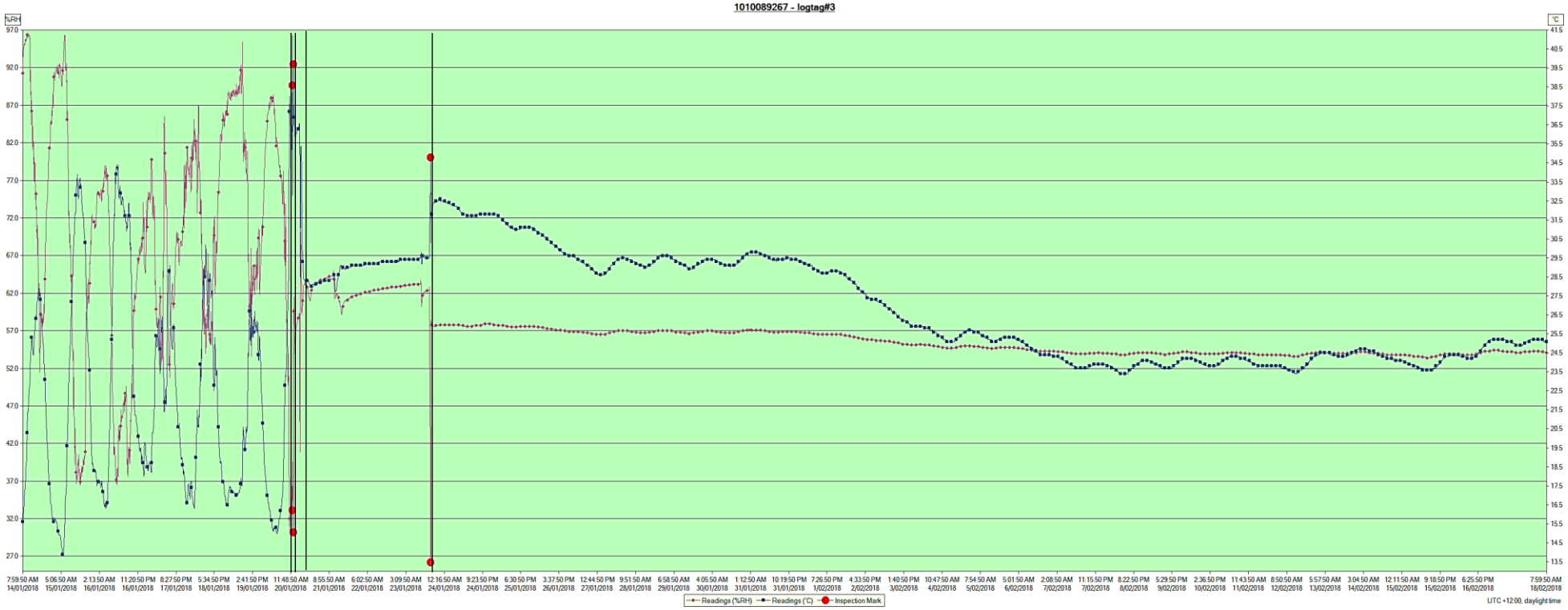


Figure A4.3 Log tag results for Farm B recording data from underneath the mown grass swath to truck and then to dressers. The first section (up to the first vertical black line) is the data from underneath the grass swath, the second section is when the data logger was not used while the third is when the data logger was placed in the middle of the seed in the truck trailer. The fourth section is when the seed was stored at the drying facility and the fifth is when the seed was stored at the dressing facility.

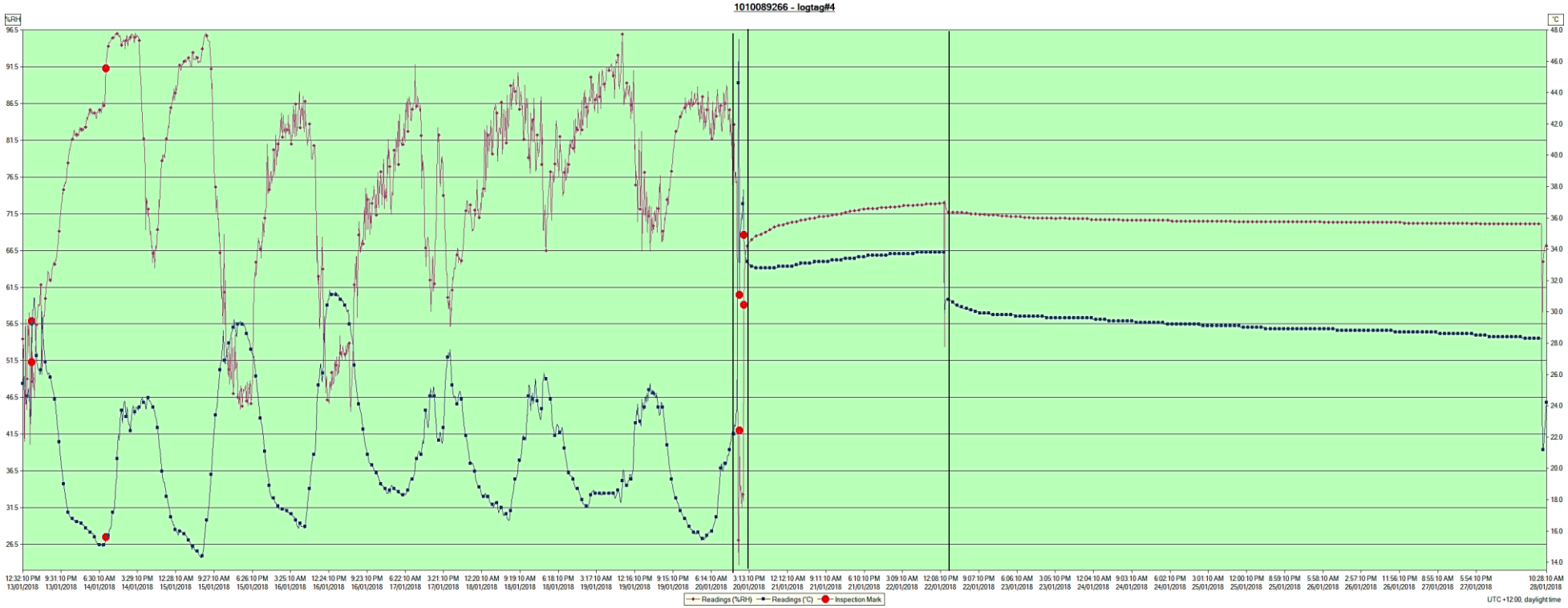


Figure A4.4 Log tag results for Farm B recording data from underneath the mown grass swath to the tractor trailer and to the seed stored in the silo. The first section (up to the first vertical black line) is the data from underneath the grass swath, the second sec section is when the data logger was not used while the third is when the data logger was placed in the middle of the seed in the tractor trailer. The fourth section is when the seed was stored in the silo.

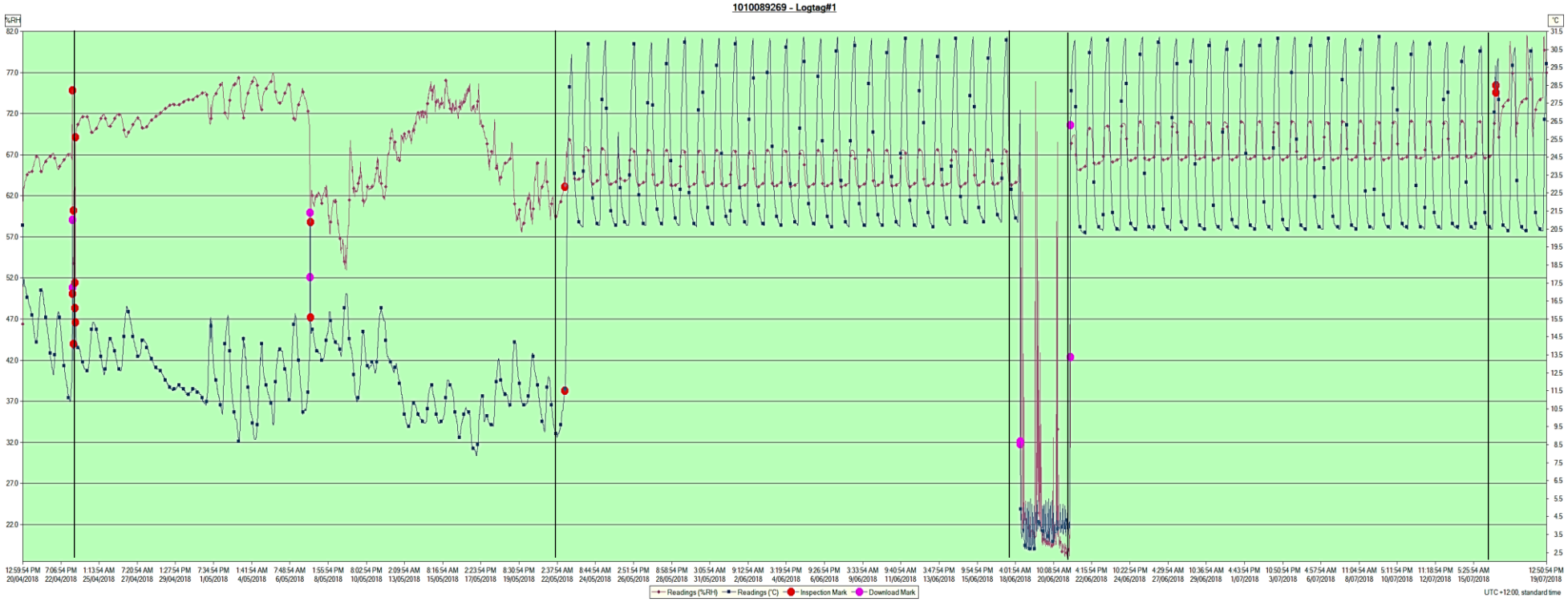


Figure A4.5 The temperature and humidity data from the log tag used first on top of the 16% moisture sack (20/04-22/05, second section), and then transferred to 12% (22/05-18/06, third section), 14% (21/06-16/07, fifth section) and 16% (16/07-19/07, sixth section) moisture containers in the germinator. The log tag was removed for an unrelated experiment for two days (18/06-21/06, fourth section).

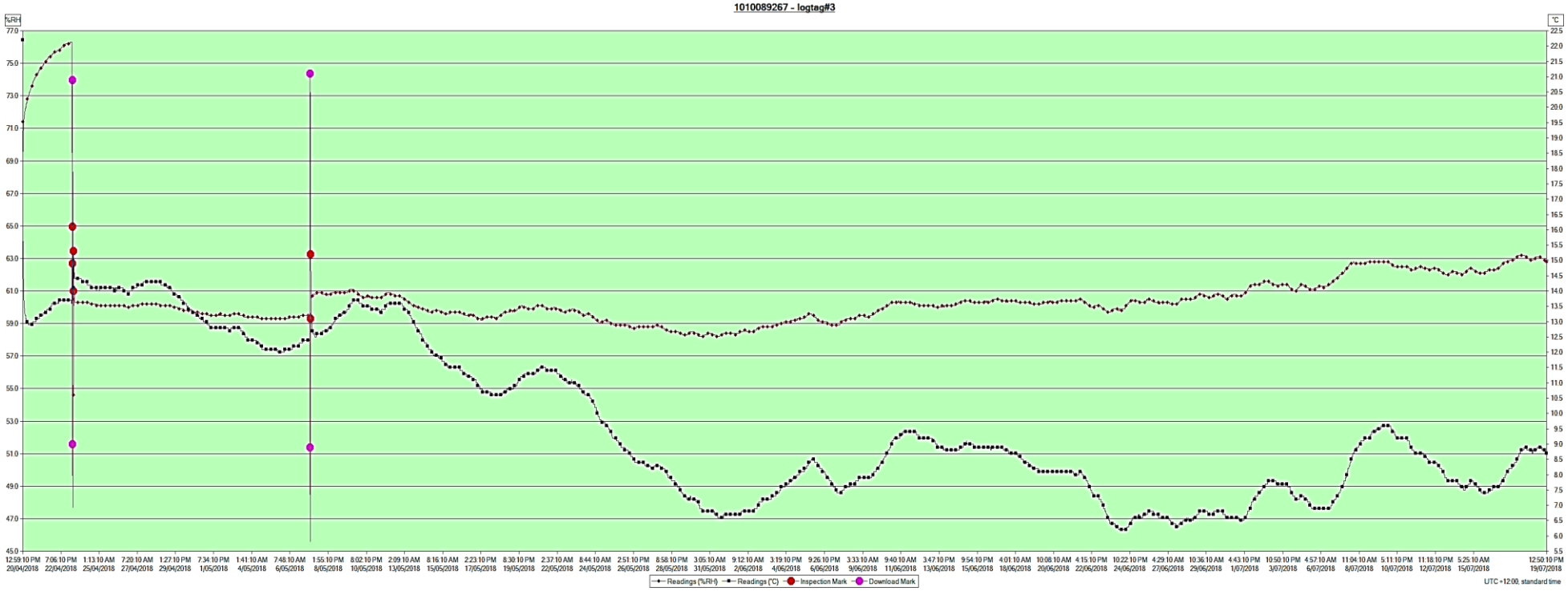


Figure A4.6 The temperature and humidity data from the log tag used in the 12% moisture sack (20/4-19/07).

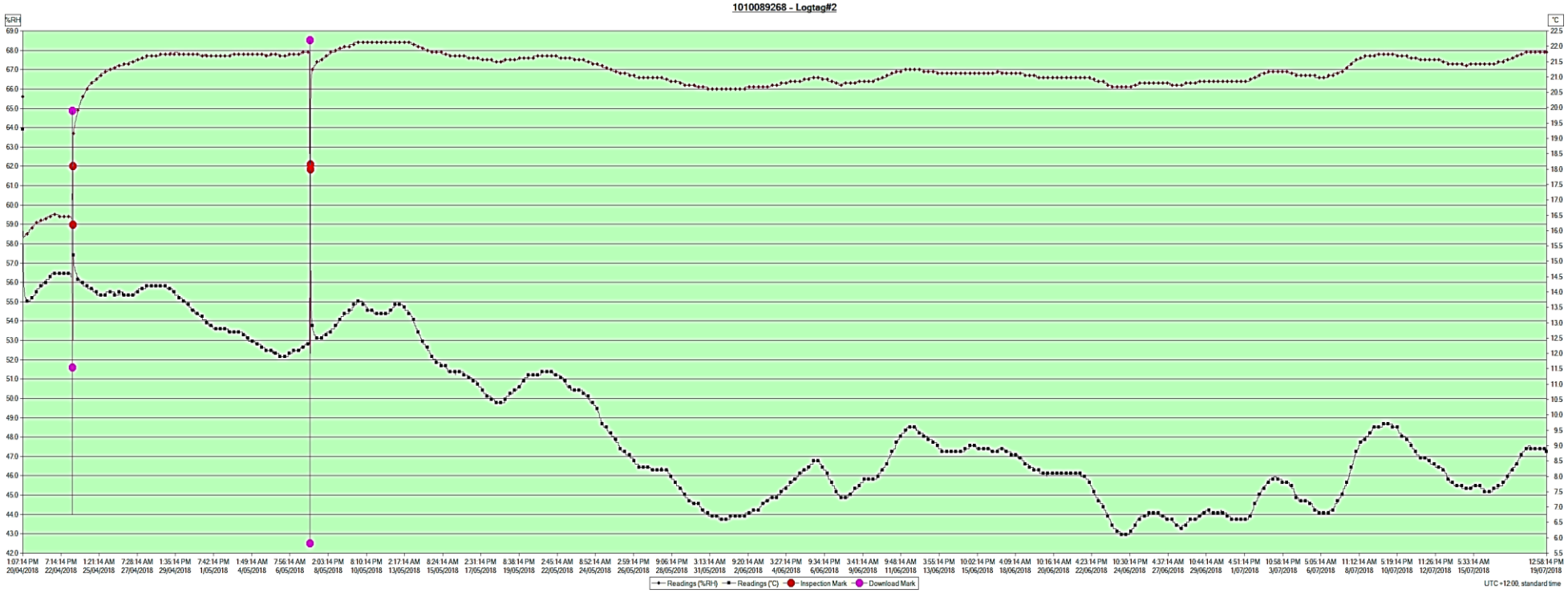


Figure A4.7 The temperature and humidity data from the log tag used in the 14% moisture sack (20/4-19/07).

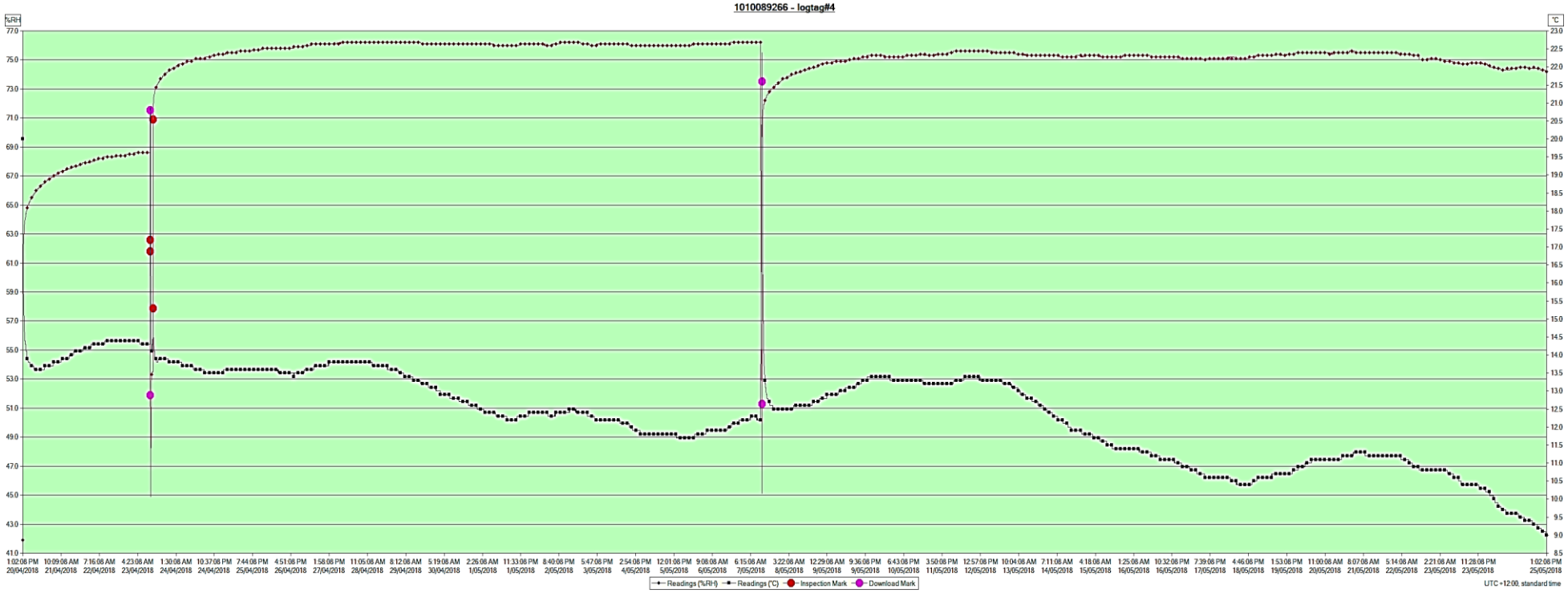


Figure A4.8 The temperature and humidity data from the log tag used in the 16% moisture sack (20/4-25/05)

A.5 ANOVA tables

Table A5.1 Two-sample T-Test result of the before fungicide endophyte percentage of both Farm A and Farm B (Section 3.1.1).

T-Value	DF	P-Value
0.68	5	0.527

Table A5.2 Two-sample T-Test result of the after-fungicide endophyte percentage of both Farm A and Farm B (Section 3.1.1).

T-Value	DF	P-Value
-0.67	5	0.530

Table A5.3 Two-sample T-Test result of the before and after fungicide, endophyte percentages for Farm A (Section 3.1.1).

T-Value	DF	P-Value
0.61	4	0.572

Table A5.4 Two-sample T-Test result of the before and after fungicide, endophyte percentages for Farm B (Section 3.1.1).

T-Value	DF	P-Value
-0.69	5	0.522

Table A5.5 Two-sample T-Test result of the just cut and after fungicide, endophyte percentages for Farm A (Section 3.1.1).

T-Value	DF	P-Value
2.58	5	0.049

Table A5.6 Two-sample T-Test result of the just cut and after fungicide, endophyte percentages for Farm B (Section 3.1.1).

T-Value	DF	P-Value
3.31	5	0.021

Table A5.7 Two-sample T-Test result of the just cut endophyte percentages for both Farm A and B (Section 3.1.1).

T-Value	DF	P-Value
-0.07	5	0.950

Table A5.8 Two-sample T-Test result of the transport and storage seed endophyte percentage for both Farm A and B (Section 3.1.1).

T-Value	DF	P-Value
0.23	6	0.826

Table A5.9 Two-sample T-Test result of the harvest and storage seed endophyte percentage for Farm A (Section 3.1.1).

T-Value	DF	P-Value
-1.45	5	0.207

Table A5.10 Two-sample T-Test result of the harvest and storage seed endophyte percentage for Farm B (Section 3.1.1).

T-Value	DF	P-Value
0.24	14	0.817

Table A5.11 One-way ANOVA result of the storage seed endophyte percentage of Farm B (Section 3.1.1).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Replicate - Seed test	2	46.83	23.42	1.08	0.357
Error	21	454.50	21.64		
Total	23	501.33			

Table A5.12 Two-sample T-Test result of all endophyte percentage samples for both Farm A and B (Section 3.1.1).

T-Value	DF	P-Value
-0.46	41	0.645

Table A5.13 One-way ANOVA result of the field and machine dressed endophyte percentage of both Farm A and Farm B (Section 3.1.1).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor – dressing type	1	90.25	90.25	3.57	0.199
Error	2	50.50	25.25		
Total	3	140.75			

Table A5.14 One-way ANOVA result of the sowing and machine dressed endophyte percentage of both Farm A and Farm B (Section 3.1.1).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor – Seed type	1	144.00	144.00	5.76	0.138
Error	2	50.00	25.00		
Total	3	194.00			

A.6 Objective 3 set up process

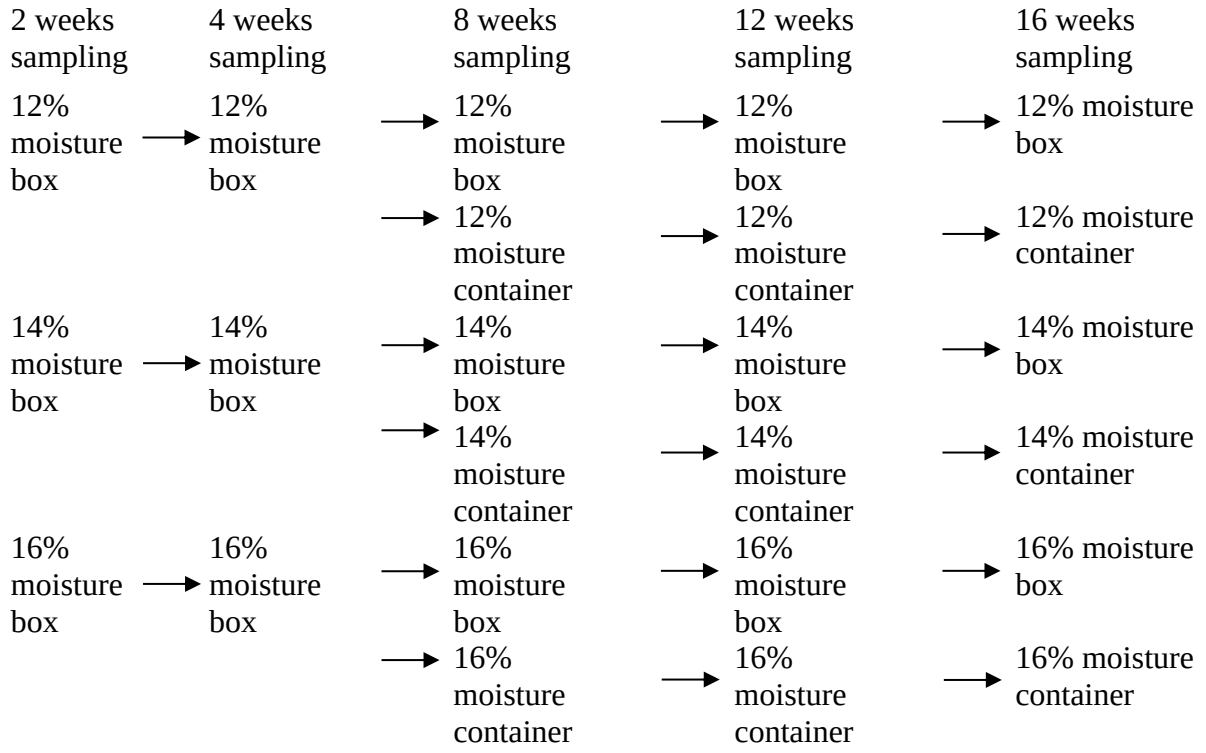


Figure A.6. 1 Set up process for Objective 3 showing the point at which seed was split into two different temperature treatments – large boxes stored outside and smaller containers stored within an incubator.

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