Fungicide control of blind seed disease (*Gloeotinia temulenta*) without affecting AR37 endophyte in ryegrass seed crops

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

Master of AgriScience

in

Seed Science and Technology

at Massey University, Palmerston North

New Zealand

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2014

Abstract

Blind seed disease (BS) is caused by the fungus *Gloeotinia temulenta* that directly affects the germination of grass seeds by killing the embryo. This disease continues to periodically affect the forage grass seed industry (Alderman, 2001). *Epichloë* fungal infection has a symbiotic association with grasses, providing beneficial traits to the plant host, having a crucial role in ensuring the persistence of grasses against biotic and abiotic threats (Mortimer and Di Menna, 1982; Popay and Rowan, 1994). This study focuses on new fungicide testing used to control BS and its effects on the transmission of the AR37 endophyte into the new seed generation. In this study, thousand seed weights, germination percentages, blind seed determinations and immunoblot detection of endophyte were carried out to assess the effects of different foliar fungicide treatments used to control blind seed (BS) and other pathogens, on the transmission of the AR37 endophyte into the developing seed of perennial and hybrid ryegrass cultivars (Samson, Horizon and PGone50). Trial one, but not trial two, was conducted on a paddock where there were abundant buried seed with BS disease to ensure a high potential for this disease to develop in the treatments plots. In trial one, germination in Samson with all fungicide treatments used was higher, and conversely BS was lower, than the control (except T12 composed of folpet). The treatments that best controlled BS in Samson were T2 (70% germination, composed by 100 g/ha prothioconazole applied at mid-flowering); T4 (72% germination, composed by 100 g/ha prothioconazole + 250 g/ha carbendazim applied at mid-flowering and mid-seed fill); T8 (73% germination, composed by 125 g/ha azoxystrobin with 189.2 g/ha tebuconazole applied twice (at mid-flowering and mid-seed fill and 250 g/ha carbendazim at mid-seed fill); and T9 (73% germination, composed by 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim applied at mid-flowering and mid-seed fill). No reduction in endophyte transmission to seed was observed with the fungicide treatments with the exception of the applications of folpet. In turn, with Horizon several fungicide combinations were able to improve the germination performance by controlling BS, however Horizon had a lower performance in terms of controlling BS. The percentage of Horizon seed with endophyte in all treatments was very low, possible reflecting the use of seed with a low percentage of viable AR37 endophyte when the grass seed crop was established some years previously. In trial two, germination, endophyte content, and seed yield between the treatments were not different. All treatments (including the

control) had a germination level between 84 to 89%. All treatments used in this trial maintained the AR37 endophyte content in the resultant seed lots. It is known that the application of some fungicides used to control a range of pathogens is detrimental to the viability of endophytes. Therefore, it is imperative that research in the quest of new treatments that control effectively BS without exerting detrimental effects on endophyte continues.

Acknowledgements

I would like to thank each person that helped the development of my thesis, Craig McGill, Robert Southward, Kay Sinclair, and Xiong Zhao He from the Institute of Agriculture & Environment, Massey University; Phil Rolston, Stuart Card, Michael Christensen, Anouck de Bonth from AgResearch Grasslands; Nik Grbavac from AsureQuality; Richard Chynoweth from the Foundation for Arable Research. I would also like to thank the people that have supported me through funding: the Foundation for Arable Research Scholarship; the Seed Tech Services Scholarship; the John Hodgson Pastoral Science Scholarship; and TR Ellet Agricultural Research Trust funding. Without the help and support of each of these sources, the completion of this thesis could not have been achieved - I appreciate it.

Also I will like to dedicate this thesis to my wife who has always supported me, and my baby Bianca, who marked the end of this study and the beginning of a new phase in my life…I love you both.

Follow your dreams, no matter how far or how long it will take you to meet them, because only then you will attain personal growth. Remember, live your dreams and not someone else's….

Eduardo Sandoval

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Chapter 1. Introduction

1.1. Background

Blind seed disease (BS), an infection caused by the fungus *Gloeotinia temulenta* (Prill. & Delacr.) M. Wilson, Noble & E. G. Gray 1954 (Calvert and Muskett, 1945; Wilson *et al.*, 1954) has been an important issue in the seed industry since the 1920s in different countries, such as the United States (Hardison, 1945), Australia (McGee, 1971), New Zealand (Greenall, 1943), England (Noble and Gray, 1945) among others. It affects about 56 species of grasses (Hyde, 1938). Many of the species affected are important turf and forage grasses such as *Lolium, Agrostis,* and *Festuca* (Alderman, 2001). BS disease affects New Zealand seed crops periodically. According to Alderman (2001), this disease is prone to occur in areas of seed production during cool seasons. It is also particularly affected by high moisture during summer, or wet weather during anthesis, and early seed fill. It is well known that *G. temulenta* directly affects the germination of ryegrass seeds (Hampton and Scott, 1981; Chynoweth *et al.*, 2012). The reduction in the incidence of the disease can be undertaken through timely use of fungicides. However, there is concern that the control of BS with fungicides may in turn cause a decrease in the transmission of desirable fungal endophytes (*Epichloë festucae* variety *lolii* syn. *Neotyphodium lolii*) to the developing seed (Harvey *et al.*, 1982). In this context, Latch and Christensen (1982), reported the use of fungicides (such as Benomyl) eliminating endophytes from infected plants.

Endophytic fungi of the genus *Epichloë* are important in pastoral agricultural systems because of their ability to increase the competitiveness of certain agronomic host grasses. This fungus is an endosymbiont that lives within a number of grass species. In particular, the agronomically important, tall fescue (*Festuca arundinacea*), and perennial ryegrass (*Lolium perenne*) have a symbiotic association (endosymbiosis) with the fungus. This asymptomatic endophyte infection provides a number of benefits to grasses. These include improved plant growth, increased resistance to invertebrate pest attack, resistance to nematodes and some fungal pathogens, decreased overgrazing, and drought tolerance. All these are contributing to the productivity of pasture in New

Zealand (Grasslanz, 2010; Mei and Flinn, 2010; Simpson *et al.*, 2012). *E. festucae* var. *lolii* live completely in the intercellular spaces within ryegrass and are vertically propagated through the vegetative structure or via seeds. Therefore, the infection is maternally transmitted (Simpson *et al.*, 2012; Johnson *et al.*, 2013).

One major impediment of the introduction of improved endophytes strains to market is the poor transmission from parent plants to the seed. Factors that can affect endophyte transmission, viability and the mutualistic association with the host include biotic and abiotic environmental factors. Lack of transmission can occur, in particular, when resources such as nutrients are lacking (Piippa *et al.*, 2006), It can also occur in relation to the following factors: management practices including the use of fungicide and fertiliser; the use of growth regulators/time of harvest; soil type; pH and moisture; harvest timing; as well as post-harvest factors (Gundel *et al.*, 2011; Ball *et al.*). In addition, grazing can alter the symbiosis dynamics of endophytes by means of decreasing seed production and the efficiency of endophyte transmission (Garcia *et al.*, 2012).

1.2. Research objectives

This project has two main objectives:

1. To identify a range of fungicide treatments that will control BS disease.

2. To determine if the application of these fungicide treatments as part of the seed production process will have a detrimental effect on the endophyte transmission from seed parent to progeny.

To be of commercial value, any fungicide treatment must control or reduce BS disease in the seed crop without having a detrimental effect on endophyte transmission. The first part of this study (trial one) will assess the seed germination and BS percentage in ryegrass seed lots obtained from seed production trials harvested at AgResearch Lincoln in the 2012-2013 season to determine the effectiveness of a range of fungicide treatments in controlling BS disease. Fungicide treatments able to control BS disease will then be assessed to determine their effect on the transmission of endophytes. Identification of fungicide treatments that will provide control of BS disease without

detrimental effect on endophyte transmission will enable the development of best practice guidelines for the forage grass seed industry. In the second part, there will be an assessment of the effects of other combination of fungicides (and also two confidential products, marked as product A and product B) on the transmission of endophytes and its viability on PGone50, a perennial ryegrass cultivar.

This project was developed from a pre-existing AgResearch Lincoln seed production trial and continued in close collaboration with staff members from both AgResearch Lincoln and Grasslands Palmerston North. Important sponsorship was provided by the Foundation for Arable Research, Seed Tech Services, the John Hodgson Pastoral Science Scholarship, and the T.R. Ellet Agricultural Research Trust.

Chapter 2. Research background

2.1. Blind seed

BS disease (caused by *G. temulenta*) was given the name by Neill and Hyde (1939), due to fact that seeds appear to be normal, but the embryo has been infected and killed and is therefore unable to germinate. Seeds require close inspection and observation to identify BS. The disease has mainly affected the ryegrass and tall fescue seed crops in Australia and New Zealand. In ryegrass, BS disease decreases the germinable seed yield during extreme conditions, such as high humidity in summer and when it is extremely wet over winter (Grant, 1982; Alderman, 1992). The epidemic history of this disease has been reported over time. In Australia, the disease greatly affected ryegrass seed yield during 1987, 1988 and 1992. In New Zealand, BS was reported to occur on fescue and ryegrass seed crops from 1992 to 1994 (Ramsey *et al.*, 1997). In fact, it has been reported that BS was one of the major diseases of importance in the first half of the last century affecting seed germination in New Zealand by as much as 99% (Greenall, 1943). Over the last few years, there are no records of seed loss beyond 50% in New Zealand but there have been some years which have certainly reduced the germination of many seed lots (N. Grbavac, personal communication, July, 2013). However, in 1994/97/99, the New Zealand seed industry was significantly affected by BS infection (Table 1), reducing the germination to below 90% (germination is required to be above 90% or certainly above 85% to fulfil contractual obligations and facilitate the ease of selling commercial seed lots) (Rolston and Archie, 2000). There was also an economic loss in Canterbury from the harvests between 2000, 2002 and 2005 as well as 2007 (Table 1) which are likely a result of BS infection on ryegrass seed lots (N. Grbavac, personal communication, February, 2014). In fact, the Foundation for Arable Research (FAR) stated that it was a combination of seed dormancy and BS (60% of non-germinated seeds) (FAR, 2007b). The disease periodically damages seed grasses in New Zealand, especially in Canterbury, South Otago and Southland, acting as an epidemic disease during extreme weather conditions (Skipp and Hampton, 1996; Harvey and Harvey, 2009). Infected seed survives through the winter. Soils with high moisture and temperatures near 2°C, are necessary to develop BS apothecia (sexual stage)(Hardison,

1945). During spring or early summer, apothecia emerge from overwintering infected seeds of perennial ryegrass. One seed can develop up to seven apothecia (Calvert and Muskett, 1945).

Table 1: Mean ryegrass germination percentage in New Zealand certified seed lots from 1994 to 2008.

Year	90 to 100%	85 to 90%	80 to 84%	70 to 79%	0 to 69%
2008	95.46	3.61	0.74	0.14	0.04
2007	64.56	15.67	6.96	6.41	6.41
2006	96.01	3.07	0.46	0.21	0.25
2005	60.28	16.73	11.23	7.50	4.26
2004	85.72	8.57	3.03	1.93	0.76
2003	83.45	9.90	3.34	1.84	1.47
2002	55.79	27.07	9.33	5.79	2.01
2001	90.37	4.88	2.07	1.66	1.01
2000	55.70	16.19	6.94	4.07	1.88
1999	66.98	7.43	1.73	0.76	0.13
1998	85.57	7.88	3.16	1.97	1.41
1997	76.86	12.42	4.99	3.22	2.51
1996	92.27	5.07	1.14	0.93	0.59
1995	82.57	7.03	4.83	3.30	2.26
1994	48.45	18.03	12.75	12.06	8.71

(Quality, 1998-1999/ 2003-2004/ 2008-2009).

The ovaries and developing seeds will be infected by BS solely by the ascospores that land on the grass flower (Alderman *et al.*, 2009; Harvey and Harvey, 2009). A successful infection of BS on perennial ryegrass depends on the availability of susceptible host tissue (when florets are open allowing an infection pathway to grass ovaries). Blind seed affects mainly ryegrass and tall fescue florets killing the developing embryo (Alderman *et al.*, 2009). Mebalds (2010) states that to model a successful prediction of the likelihood of *G. temulenta* infections, it is necessary to include: a prediction of apothecial populations; the proportion of susceptible florets available; and prevailing environmental conditions. The spore release and the apothecial production period can be extended by a combination of low temperatures and wet weather. According to Alderman (1998; Alderman *et al.*, 2009), the severity of BS attack is closely related to the increase of moist and rainy conditions during spring. High humidity promotes the

development of the disease during hot days. After seven to 10 days of infection, a large number of conidia appear on the surface of infected seeds. Once rain splash occurs, the disease is easily spread to other flowers.

2.1.1. Blind seed taxonomy

Taxonomically, *G. temulenta* is not clearly defined (Alderman, 2001). According to Wilson (1954), it was part of the *Sclerotiniaceae* family. Later, it was thought that *Gloeotinia* was part of *Symphyosirinia*, a member of the *Leotiaceae* family (Ellis, 1956; Baral, 1994). Later, Holst-Jensen et al. (1997), after performing DNA analysis on BS, partly supported earlier contentions that *G. temulenta* should be considered a member of the *Leotiaceae* family, subfamily *Hymenoscyphoideae* (Alderman, 2001).

2.1.1.1. Synonymy (Alderman, 2001)

Gloeotinia temulenta Prill. & Delacr. (Wilson *et al.*, 1954)

Phialea temulenta Prill. & Delacr. (Prillieux and Delacroix, 1892b)

Peziza (Phialea) temulenta Prill. & Delacr. (Prillieux and Delacroix, 1892a)

Ciboria (Stromatinia) temulenta Prill. & Delacr. (Prillieux and Delacroix, 1893)

Sclerotinia secalincola Rehm (Rehm, 1900)

Sclerotinia temulenta (Prill. & Delacr.) Rehm (Höhnel, 1903)

Stromatinia secalincola (Rehm) Boudier (Höhnel, 1903)

Phialea mucosa Gray (Gray, 1942)

Gloeotinia granigena (Q.) Schumacher for hosts other than Bromus (Alderman, 1997)

Endoconidium temulentum Prill. & Delacr. (Prillieux and Delacroix, 1891)

2.1.2. Location of inoculum

G. temulenta hyphae can be found inside the endosperm, embryonic tissues and throughout the caryopsis area. A large production of macroconidia appears on the surface hyphae (Matthews, 1981). Furthermore, according to Alderman (2001), hyphae are present in the inner epidermis, nucellus, and embryo sac. After nine days conidia produced between the inner epidermis and the outer integument, appear on the seed surface. In turn, the endosperm and embryo are colonised by *G. temulenta* hyphae when the infections occur after the embryo was differentiated into endosperm, scutellum, radicle, and plumule (Alderman, 2001). In the early work of Neill and Hyde (Neill and Hyde, 1939), it was thought that *G. temulenta* hyphae did not penetrate cells of the aleurone layer. However, soon after, Wilson, Noble, and Grey (1947) observed that hyphal penetration of *G. temulenta* did occur through the epithelial and aleurone layers. This observation is still supported today (N. Grbavac, personal communication, Dec, 2013), confirming that the infection occurs in the aleurone layer of the seed with mycelium being present both in and between the cells.

2.1.3. Symptoms

BS symptoms are not visible (asymptomatic) because the grass caryopsis (seed) is covered by the palea and lemma glumes, which have the same appearance as uninfected healthy seeds (Figure 1a). However, BS is easy to recognise once seeds have imbibed water overnight. Occasionally, the difference can be recognised with the lemma and palea still present (Figure 1b), but, for a proper analysis, it is necessary to remove the lemma and palea from the seed. In the lab, once the seed is exposed after being dissected, the damage on the endosperm caused by BS is clearly visible. In infected seed, the caryopsis is flecked with a red-brown coloured area and the embryo is shrunken (Figure1c) (Matthews, 1981; Mebalds and Price, 2008). The conidia are easy to see under a microscope if the seed is placed in a drop of water with the lemma and palea removed. The infection can occur at one of two growth stages. Depending on the stage at which infection occurs, one of two outcomes is possible. If the infection occurs in the early stages of flowering, the seed filling will be significantly affected, not allowing the formation of the seed. If the infection occurs later, the seeds will be lighter and slimmer and are still unable to germinate (Foy, 1927; Grant, 1982; Harvey and Harvey, 2009).

Figure 1: Healthy and blind seed infected ryegrass seeds.

1a: Comparison between healthy (uninfected) dry seed (right), and dry seed infected with BS disease (left); 1b: Comparison between healthy (uninfected) imbibed seeds (left), and imbibed seed with BS disease (right); 1c: Comparison between healthy (uninfected) imbibed seeds (two seeds on the left) and imbibed seeds with BS (three right seeds) without the lemma and palea.

2.1.4. Transmission

There are two possible mechanisms of transmission. Ascospores are produced from a stroma on the infected seed (Wilson et al., 1954). They are spread from the apothecium which releases them in response to changes in the relative humidity (high humidity). Macroconidia[♦] are produced on the surface of the seed from conidiophores that arise laterally on hyphae. Each conidiophore can produce up to 30 conidia. These conidia, which are short-lived, are responsible for secondary infection. Macroconidia in the seed

[♦] Macroconidia are usually the predominant type of conidia produced under saprophytic conditions Cole, G. & Hoch, H. (eds.) 1991. *The Fungal Spore and Disease Initiation in Plants and Animals*

are inlaid in a pinkish and slimy matrix, which later becomes a hard red-brown crust (Calvert and Muskett, 1945). They are spread through rain or by direct contact with neighbouring plants (Figure 3). Subsequently, conidia can also spread infection from inflorescence to inflorescence in the same way (Alderman, 2001; Harvey and Harvey, 2009). Thus, conidias developed in the spike (seeds) form a slimy matrix, re-infecting neighbouring flowers and developing seeds. Conidia are short lived and are responsible for secondary infections. They are budded from the conidiophores which arise laterally on the hyphae. Each conidiophore can develop up to 30 macroconidia (Wilson *et al.*, 1947).

The complete lifecycle of *G. temulenta* is described in Figure 3. In the same matrix, microconidia are also produced as part of the reproductive system of the fungus. Only from stromata (plural of stroma), which have been fertilised by microconidia of an opposite mating type, are apothecia developed (Hampton, 1987; Harvey and Harvey, 2009). The mycelium spreads through the infected seeds. The BS overwinters in infected seeds that have been dispersed from the parent plant (e.g. shed seeds). Once the seeds are on the ground, the apothecia slowly develop (Figure 2). Later, the apothecia release ascospores at the time the host plants are flowering.

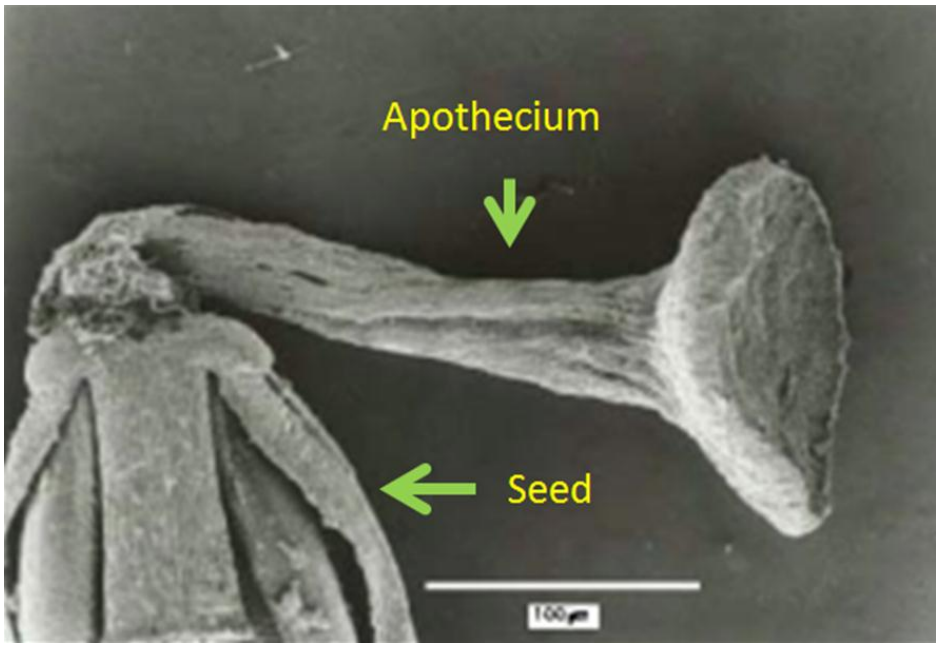


Figure 2: Scanning electron micrograph of mature apothecium of *Gloeotinia temulenta* in a ryegrass seed. Source: Alderman, 2001.

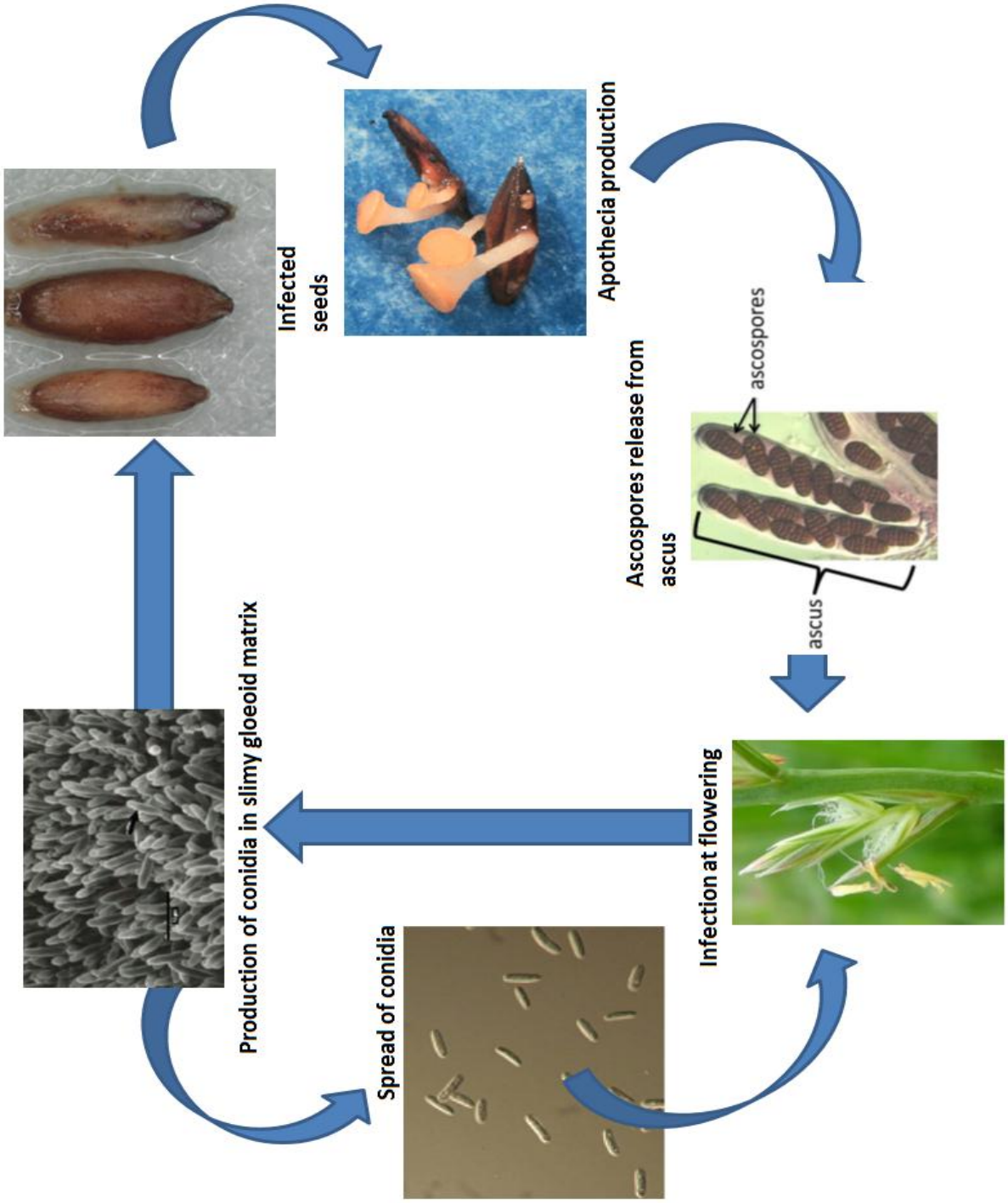


Figure 3: Life cycle of *Gloeotinia temulenta*, adapted from Harvey (2009).

Sources: BS apothecia and conidia (OSU, 2012); flowering ryegrass (Trust, 2011); ascospores (Studyblue, 2014); and conidia production (Alderman, 2001).

2.1.5. Transmission in the New Zealand environment

BS is often more severe in early flowering ryegrass cultivars i.e. those which flower at a similar time to Grasslands Nui♦. The apothecial population of BS in Victoria, Australia, reach their peak typically between November 8 and 18 (Mebalds and Price, 2010). Similar patterns are observed in Canterbury, New Zealand, with ascospores typically spread between 10:00 to 15:00 hours daily from early November to mid-December, thus coinciding with the opening of ryegrass florets in preparation for pollination (flowering occurs from mid-November to early December in early flowering cultivars)(Johnston et al., 1964). The ascospores are transported by the wind to the open florets completing the life cycle of BS (Alderman, 2001; Harvey and Harvey, 2009).

2.1.6. Yield loss and economic impact

Through time, grass seed production has been reduced by the presence of seeds infected with BS that do not germinate. This results in seed lots with a germination result below the certification or contract standards which in turn considerably reduce the seed lot value and in some countries make the seed unmarketable (Chynoweth *et al.*, 2012). For example, in 1993, BS disease infected a high number of ryegrass seed lots in New Zealand, due to favourable environmental conditions for the disease. However, in 1995 the disease incidence decreased owing to unfavourable weather conditions for the disease (Skipp and Hampton, 1996). Since this disease periodically attacks seed production in New Zealand, a focus on effective control measures for this disease is an important research priority, thus avoiding economic losses in the seed industry.

2.1.7. Management to control blind seed disease

The most common methods used to manage and control BS, are the use of nitrogen (N) fertiliser (urea), systemic fungicides and crop rotations (Blair, 1947; Hampton and Scott, 1980a; Hampton and Scott, 1981; Rolston and Falloon, 1998). The use of these

♦ Grasslands Nui, often simply referred to as Nui, was bred by the New Zealand Department of Scientific and Industrial Research (DSIR) and was released onto the commercial market in 1976 (Armstrong, 1977). Today this public cultivar is still grown and seed is sold both to New Zealand farmers and is exported in large quantities – typically 5000-7000 tonnes, worth $8–14 million per year (AsureQuality, 2009; NZ Statistics Infoshare, 2014).

management techniques has produced some excellent results, although typically it is successful only when the disease pressure is low. The principal way of controlling BS disease infection, through infected seeds, is the use of urea fertiliser (nitrogen 46%). Urea increases the density of plants, which then act as a physical barrier to ascospore propagation. The mode of action is the prevention of the movement of the ascospores from apothecial fruiting bodies growing from seed shed at ground level in previous seasons up to open florets in the reproductive canopy.

The use of benomyl was tested by McGee (1971) as a possible treatment to control BS. Complete protection of apothecial production was possible in the laboratory, however results showed the spraying of this fungicide in the field failed to prevent the disease. It was recommended by McGee (1971) that the use of benomyl (Benlate) can be used as a control of BS reinfestation from diseased seed (McGee, 1971). Later, Mebalds and Price (2008) had proved in Australia the use of benomyl as a seed treatment of BS, recommending its use in alternation with propiconazole, thus avoiding developing fungicide resistance from BS. However, benomyl is no longer in production by Du Pont and is banned in many countries for causing birth defects (Harrison, 2007).

2.1.7.1. Seed management

Infected seeds usually are lighter than uninfected seeds due to the fact that the infection occurs at flowering or prior to endosperm developing, thus are thin and light in weight (Neill and Hyde, 1939). In earlier times, burning straw and stubble in the fields after the seed harvest was an efficient control of BS in the USA. In the 1970s legislation in Oregon eliminated this practice (Hardison, 1978). During the pre-cleaning (field dressing) of the harvest, much of the lightweight seed fractions (including infected seeds) return to the field. If infected seed is left in the field during harvest, the incidence of BS will be greater the following spring if a second or subsequent seed crop is again taken from the established stand (Alderman *et al.*, 2009). Another option for controlling BS is to inactivate infected seeds that will be subsequently used for further seed multiplication. This can be achieved by storing the seed for two years before sowing, as both conidia and mycelium have a life span of less than two years (Harvey and Harvey, 2009), and will therefore die before being sown. If the presence of shed seed infected with BS is high in a paddock, the paddock must be grazed and not used for seed production.

It is possible that endophyte presence in ryegrass may help to control BS. A previous study performed on perennial ryegrass infected with endophyte demonstrated that its presence significantly increased the plant biomass when high or intermediate soil-nutrient level (resulting from nitrogen application) is available (Cheplick *et al.*, 1989). A greater number of leaves on vegetative and reproductive tillers either by genotype characteristics, urea application or some other factor may work as a BS barrier, preventing the ascospores from reaching the grass flowers (R. Chynoweth, personal communication, July, 2013).

2.1.7.2. Fungicide application

In early times, the use of fungicides applied as inflorescence or foliar sprays were ineffective in controlling BS (Corkill and Rose, 1945; Hair, 1952; Hardison, 1970). Historically, the use of sodium azide (active ingredient N_3Na) in the USA was an effective fungicide treatment but required large amounts of water to drench the fungicide through the dense vegetation of ryegrass to the soil surface (Hardison, 1978). However, while many products were used in the quest to control BS, it was reported that some of these fungicides may also affect endophyte, reducing their transmission to the developing seed (Jones *et al.*, 1983).

Later, Rolston and Falloon (1998) in partnership with the Foundation for Arable Research found that the use of carbendazim or tebuconazole are an effective control of BS. Alderman (1998) found that the application of Amistar (a.i. 250 g/l azoxystrobin) did not have any effect on BS, whereas the application of triazole (a.i. propiconazole) reduced the production of BS conidia considerably on tall fescue seeds (Rolston and Archie, 2000). Other authors (Mebalds and Price, 2008) suggest that applying fungicides on the soil surface can control BS. The alternate use of fungicides (benomyl and propiconazole) applied at flower emergence and anthesis can control BS infection, and the use of sodium azide reduces the source of primary inoculum apothecia on the soil (Mebalds and Price, 2008).

Fungicides used to control rust diseases are also used to control BS disease, such as prothioconazole and azoxystrobin(Harvey and Harvey, 2009). Usually two applications of fungicides are performed during the flowering period to prevent the disease.

However, it been reported that the use of some triazole (demethylation inhibitor) fungicides, such as epoxiconazole (Opus) and prothioconazole (Proline), may decrease the seed transmission of endophytes in ryegrass (Harvey and Harvey, 2009; Chynoweth *et al.*, 2012).

2.1.7.3. Urea application

As stated in the section 2.1.7., the application of N into the soil during spring seems to reduce the incidence of BS in perennial ryegrass seed crops. It is believed that the use of a high amount of nitrogen promotes vegetative growth before flowering, making it harder for the ascospores, originating from apothecia on the soil surface, to reach the seed heads (Hampton and Scott, 1980a; Hampton and Scott, 1980b; Hampton, 1987). However, the use of growth regulators will reduce the vertical growth of reproductive tillers, increasing the incidence of BS by creating a short, open crop of low dry matter. This encourages primary infection from apothecia at the soil surface (Hampton, 1987; De Filippi *et al.*, 1996; Chynoweth *et al.*, 2012).

According to earlier work summarised by Hampton and Scott (1980b; Hampton and Scott, 1980a), the application of N during spring to a ryegrass crop infected with BS, reduced the levels of *G. temulenta* detected and increased the germination of the seed produced (Table 2). Urea can limit the infection but only when the application occurs early in spring. Hampton and Scott (Hampton and Scott, 1980b) were unable to determine if the urea was having a suppressive effect on BS. Later, Hampton and Scott (1981) reported that N did not directly control BS. The use of high amounts of N in the field did not suppress the incidence of BS. However, they suggested that there was significant control of BS when the application was performed in spring (56 kg/ha urea), when the infection level is moderate. Therefore, they recommended spring application of urea (Hampton and Scott, 1981), but this was not always an effective control method of the BS disease. Later researchers have suggested the possibility that the use of urea in spring promotes vegetative growth, thus producing a barrier against the spread of ascospores (R. Chynoweth, personal communication, July, 2013). Nowadays, in commercial ryegrass seed crops, the use of N fertiliser in combination with the application of fungicides at the flowering stage (spring) are both used in controlling BS (Mebalds and Price, 2010).

Table 2: Means of results from U.K. trials in relation to the effects of nitrogen fertiliser on blind seed (Hampton and Scott, 1980a).

Source	Blind seed % Nitrogen	Blind seed % No nitrogen	Germination % Nitrogen	Germination % No nitrogen	Type of nitrogen
Rutherford (1956)	2.4	6.3	89.5	86.5	313kg/ha nitro chalk
Chestnutt (1958)	1.6	6.7	93.1	88.8	313kg/ha sulphate of ammonia
Stewart (1963)	4.5	13.5	81.0	68.0	226 kg per ha of an unspecified nitrogenous fertiliser

2.1.7.4. Crop rotation

It has been reported that the inoculum of BS in the field is lower when three to four year rotations of arable crops, such as cereals or root or forage crops (those other than grass that are not susceptible), follow the infected cultivar (Blair, 1947; Alderman, 2001).

2.2. *Epichloë festucae* variety *lolii* endophyte

Epichloë festucae var. *lolii* is a non-hybrid endophyte which comes from sexual progenitors that have lost the ability to generate stromata (fungal fruiting structures in which perithecia and ascospores may be produced) (Moon *et al.*, 2002). The asexual fungal *E. festucae* var. *lolii* is an endophyte that is part of the family *Clavicipitaceae*. This endophyte is found in permanent pasture (Pooideae) grasses, *Lolium* and *Festuca* from the tribe *Poeae*. Endophytes take nutrients from the host plant, conferring in return protection against biotic and abiotic threats, thus improving the grass performance. The associated endophytes in *Lolium* and *Festuca* have been extensively studied (Johnson *et al.*, 2013) because of their mutualistic association. This association (*E. festucae* and host) is asymptomatic. In the 1980s it was discovered that endophyte produce deleterious chemical compounds called alkaloids from some natural endophyte associations which were toxic to livestock (Fletcher & Harvey, 1981). The most important ergot alkaloid produced by *epichloid* endophytes is ergovaline, which causes hyperthermia (heat stress) especially in hot humid conditions, leading to poor weight gain, decrease of fertility, convulsions, gangrene and death (Bacon, 1995; Grasslanz, 2010). Some endophyte strains also produce lolitrem alkaloids in perennial ryegrass which produce

the classically observed ryegrass staggers in livestock. Ryegrass staggers is a neurological disability in deer, sheep, alpacas, horses, and cattle which causes severe incoordination and hypersensitivity to external stimuli (Fletcher and Harvey, 1981; Tor-Agbidye, 2001). Initially research was focused on finding the causal factors of ryegrass staggers followed by the causal factor related to protection of the plant from the Argentine stem weevil (ASW) (Fletcher and Harvey, 1981). Following the link between animal health issues after the release of the first commercialised endophyte (Endosafe) the link was made between the presence of ergovaline and livestock ill-thrift (heat stress) (Fletcher and Popay, 1991).

Soon after, however, Prestidge (1982) discovered that ryegrass endophytes provided protection against the Argentine stem weevil (*Listronotus bonariensis*). The ASW was introduced from Argentina into New Zealand in the early 20th century and became a significant pest in the New Zealand pasture. The resistance to pests is an important benefit of endophytes in pasture grasses. The resistance comes from the production of peramine alkaloids (Rowan, 1990). Subsequent studies (as described below) established the important role that endophytes play in ensuring the persistence of perennial ryegrass and the reputation of the adapted endophyte species in New Zealand (Easton *et al.*, 2001). Without the presence of endophytes, perennial ryegrass in many regions in New Zealand, such as Waikato and Canterbury, would not persist (Mortimer and Di Menna, 1982; Popay and Rowan, 1994). Other research established that some natural endophyte associations produced less mammalian toxicity and actually provided beneficial traits to the grass, such as an increase in drought tolerance (Latch and Christensen, 1982). The discovery of new types of secondary metabolites in the 1980s (lolines, ergovaline, lolitrem B, peramine) provided better understanding of the role of the endophyte produced alkaloids on the functional characteristics of host grasses, has had a significant effect on the development of pastoral agriculture. Plants infected with endophytes suffer less leaf dehydration under drought conditions (He *et al.*, 2013). Drought tolerance is reached after endophytes induce an increase in total dry mass, green shoot mass, an increase in the number and height of tillers, and total yield (Kane, 2011). Today, the presence of endophytes in the seed of grasses is an important element within the New Zealand pasture industry, and the use of the new strains of endophytes today contribute about $ 200 million to the New Zealand economy (Johnson *et al.*, 2013).

2.2.1. *Epichloë* alkaloids

The endophytes produce a range of alkaloids (Table 3), which confer resistance to pasture pests, but which can also cause animal health problems (Grasslanz, 2010). It is important to understand the effects that individual endophytes produce, and use this knowledge to enable their better use, for example, to produce higher vigour pastures while, at the same time, not affecting animal health. There are four important alkaloids produced by endophytes: ergot alkaloids; indole diterpenes; peramine; and lolines. The indole diterpenes are often tremorgenic mycotoxins, which produce neurotoxicoses. One class of indole diterpenes is lolitrem B which causes ryegrass staggers in livestock. Another class of indole diterpenes are the epoxy-janthitrems which are produced by the endophyte AR37 and confer protection against a wide range of insect pests, an important trait for commercial strains (Johnson *et al.*, 2013). Peramine is a pyrrolopyrazine that dissuades the larvae and adults of the ASW from feeding on ryegrass. It is only synthesized by *Epichloë* and is the most widely distributed of the bio protective metabolites present in many asexual *Epichloë* such as *E. festucae var. lolii* (Johnson *et al.*, 2013), but has no known effects on animal health. Lolines have a strong insect toxicity and insecticidal effect which so far has not shown any toxic effects on livestock. Lolines are extensively used as a component of agricultural forage only when fescues are used in New Zealand. In the USA however, endophyte strains producing lolines are commonly used. Today, there is a range of novel endophytes used in the pastoral industry compared to the historical situation of the use of just the wild-type of endophyte about three decades ago (Table 3). Their use depends on different factors such as endophyte traits, type of insects pests present, which produces resistance, paddock environment and the type of grazing animal.

Table 3: Endophyte strains and their general properties (Adapted from Johnson *et al.*, 2013).

	Fungal species	New nomenclature name (Leuchtmann *et al.*, 2014)	Notable alkaloids produced	Key traits	Key regions of use
Common-toxic	*N. lolii*	*Epichloë festucae* var. *lolii*	Lolitrems Peramine	Ryegrass staggers; negative impacts on animal health. Good ASW & black beetle resistance	Ryegrass pastures and turf in New Zealand, Australia and South America
Common-toxic	*N. coenophialum*	*Epichloë coenophiala*	Peramine Ergovaline	Fescue toxicosis. Broad spectrum insect resistance	Tall fescue pastures and turf in the USA
Common-type (wild-type)	*N. uncinatum*	*Epichloë uncinata*	Lolines	Broad spectrum insect resistance	Meadow fescue pastures in the USA and Europe
Endosafe	*N. lolii*	*Epichloë festucae* var. *lolii*	Peramine Ergovaline	No ryegrass staggers. Good ASW resistance	Ryegrass pastures in New Zealand
MaxQ	*N. coenophialum* strain AR542 and AR584 (MaxQII)	*Epichloë coenophiala*	Lolines Peramine	No fescue toxicosis. Broad spectrum insect resistance	Tall fescue pastures in the USA
MaxP	*N. coenophialum* strain AR542 and AR584	*Epichloë coenophiala*	Lolines Peramine	No fescue toxicosis. Broad spectrum insect resistance	Tall fescue pastures in New Zealand and Australia
AR1	*N. lolii*	*Epichloë festucae* var. *lolii*	Peramine	No ryegrass staggers and good ASW resistance	Ryegrass pastures in New Zealand, Australia and South America
Endo5	*N. lolii*	*Epichloë festucae* var. *lolii*	Peramine Ergovaline	Good ASW and black beetle resistance. No ryegrass staggers	Ryegrass pastures in Australia
NEA2	Mix of *N. lolii* strains	*Epichloë festucae* var. *lolii*	Lolitrems Peramine	Good black beetle resistance	Ryegrass pastures in New Zealand and Australia
AR37	*Neotyphodium sp.*	*Epichloë*	Epoxy-janthitrems	Broad spectrum insect pest resistance; Excellent animal performance but some ryegrass staggers	Ryegrass pastures in New Zealand and Australia
Avanex	*N. coenophialum* strain AR601	*Epichloë coenophiala*	Ergovaline Lolines	Bird and wildlife deterrent	Tall fescue pastures Airports
Avanex	*N. lolii* strains AR94/95	*Epichloë festucae* var. *lolii*	Peramine Ergovaline Lolitrem B (only for	Bird and wildlife deterrent	Ryegrass sport fields and recreational parks

Note: There is also presented the new nomenclature e of Neotyphodium, changing its name to *Epichloë* adopted at the 18th International Botanical Congress in Melbourne, Australia, in 2011 (Leuchtmann *et al.*, 2014).

2.2.2. The endophyte life cycle

Epichloë spp. are vertically transmitted (via seed) to new generations of the grass subfamily *Pooideae* hosts, where they spend their whole lifecycle (Chung and Schardl, 1997; Johnson *et al.*, 2013).During development of the reproductive tillers, endophytes grow synchronically with them (Figure 4), transmitting themselves to the next seed generation (Christensen *et al.*, 2008). They draw nutrients from the plant but, in return, confer an increase in stress resistance. AR37 endophyte has a purely asexual life cycle. Seeds with AR37 are transmitted vertically through the ovule (S. Card, personal communication, December, 2013).

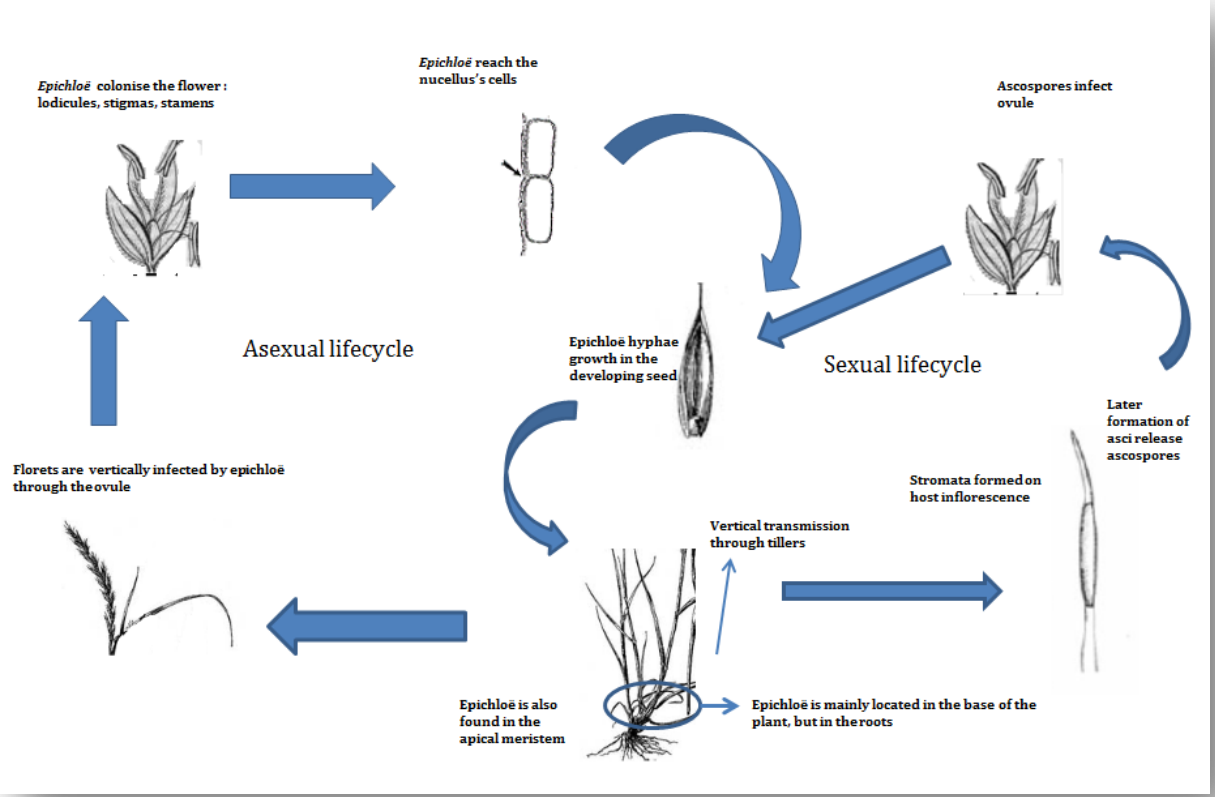


Figure 4: *Epichloë* spp. life cycle (asexual and sexual cycle).

During spring, AR37 endophytes transmit themselves to the next grass generation by growing inside the developing seed head to later, growing out of the seed, moving up through the stem.

(Adapted from Johnson et. al,, 2013).

Endophyte hyphae are located in the shoot apex from where they colonise developing leaves and axillary buds. They are not found in roots (Figure 4). Florets that are part of the maternal host are colonised as the inflorescence is maturing. Seed developing tissues such as embryo and the endosperm are infected via the ovule. In this place the fungus colonise, the apoplastic spaces of different types of flower tissues, such as the lodicules, stigmas and stamens (Figure 4). Eventually, the fungus reaches the nucellus tissues that enclose the megagametophyte (White *et al.*, 1993; Card *et al.*, 2011). In the nucellus, during the development of the embryo sac, the endophyte grows with a high level of hyphal branching. When the grass seed is mature, the endophyte is left in the nucellus residuum, with the rest of the tissue, being used as a nutrient source for the developing embryo (Figure 5). At seed maturity, endophytes are located in two places, in the nucellus tissue, located between the pericarp (seed coat) and aleurone layer, and in the scutellum/embryo tissues (Figure 5). The scutellum (a modified cotyledon that secretes enzymes) is a structure that is found only in true grasses (*Poaceae*). It transfers nutrients from the endosperm to the developing embryo (Card *et al.*, 2011).

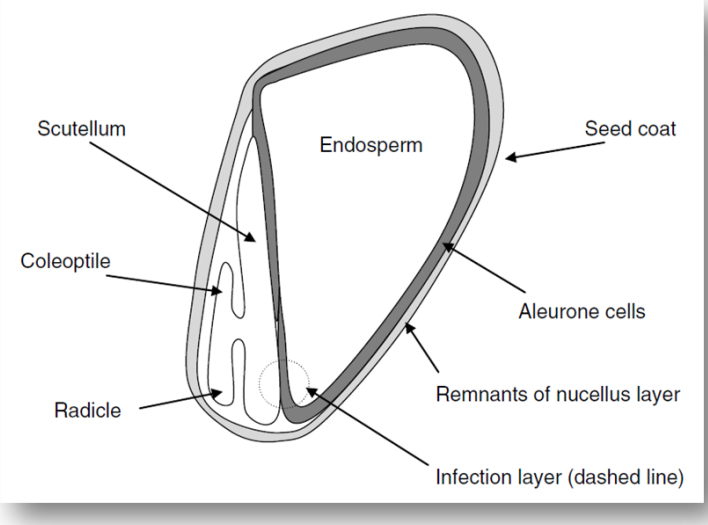


Figure 5: Cross-section of a mature grass 'seed'.

The endophyte's mycelium is located in the infection layer, between the seed coat and the aleurone cells, where it moves through the scutellum thus colonising the embryo

Source: (Card *et al.*, 2011).

Majewska-Sawka and Nakashima (2004) suggest that the fungus never colonises the young embryo and that, in fact, endophyte colonisation occurs in the late stage of seed formation. In contrast, Philipson and Christey (1986) earlier claimed that endophyte is present in the embryo from the early developmental stages in perennial ryegrass and tall fescue, before differentiation of the scutellum. Notwithstanding, both assertions confirm that the fungus is found at seed maturity in the radicle apex and plumule. Once in the seed, the endophyte waits in a dormant state until seed germination (Ngugi and Scherm, 2006). During germination, the endophyte infection occurs when the hyphae in the shoot apical meristem colonise the axillary buds and primordia in a systemic invasion, proceeding to grow into plant parts above the ground level, thus completing the vertical transmission cycle (Ngugi and Scherm, 2006; Card *et al.*, 2011). In the leaf, the endophytic fungi grow synchronically with the leaves, and when the leaves reach maturity, the hyphae stop growing, but remain metabolically active (Tan *et al.*, 2001).

2.2.3. Endophyte storage conditions

Temperature and humidity are the most important factors that determine the retention of the viability of endophytes (Hume *et al.*, 2013). Relative humidity of ≤ 60% is necessary to maintain the equilibrium seed moisture content of ryegrass at ≤ 11.3 %, and, thus, the ryegrass endophyte viability (Rolston *et al.*, 1986). A cool environment during storage increases the chances of endophyte survival (Rolston *et al.*, 2002b). Wheatley (2007), stated that *L. perenne*, endophyte survival reduces considerably with longer periods of storage (12 to 24 months). In turn, according to Canals (2008), the viability of endophyte in ryegrass can be maintained under different storage conditions for a short period (three months). The seed tested by Canals (2008) was packed in a light, porous textile material and stored in the conditions of: a)frozen at -20°C; (b) lightly frozen at 0°C; (c) refrigerated at 5°C; (d) maintained in a room at summer temperature and packed in transparent material (temperatures: 30°C/15°C; photoperiod: 12 h/12 h); and (e) maintained in a room at summer temperature and packed in opaque material (temperatures: 30°C/15°C; complete darkness). Three lots were also buried in a field. After three months of storage, the seed lots were sown. No significant differences of endophyte survival were detected across the different storage environments for the relatively brief storage period used (Canals *et al.*, 2008).

It is recommended to store ryegrass seeds between 0°C to < 15°C, with a seed moisture < 8%, or at ≤ 5°C, in aluminium laminated packets to reduce the moisture uptake by seeds (Rolston *et al.*, 2002b). The practice for high value commercial-sized lots (nucleus/breeder seed), and valuable genetic resources (grass and fungal) is the use of specialised storage conditions (e.g. low temperature and humidity). The use of aluminium-polyethylene laminated bags in the storage of perennial ryegrass at a commercial level (Hume *et al.*, 2013) can maintain the seeds up to 15 years, stored at either -15 or 0°C at 30% relative humidity (Rolston *et al.*, 2002b). Temperature and humidity conditions used for storage of genetic material are 0°C and 30% respectively (M. Rolston, personal communication, July, 2013).

Endophyte strains from AR1 and AR37 seem to have higher viability during seed storage than others endophyte strains (Tian et al., 2013). In addition, it seems that air-drying (a post-harvest treatment) affects the viability of endophytic fungus. Air-drying reduces the seed water content to lower than 13%, reducing the percentage of viable hyphae in the seed, thus adversely affecting the viability of the endophyte (Canals *et al.*, 2008; Tian *et al.*, 2013).

2.2.4. AR37 endophyte

The AR37 endophyte is a novel endophyte developed by AgResearch (Wrightson, 2012). The AR37 produces epoxy-janthitrems alkaloid which confers protection to the host plants to a much wider range of insects than AR1 (Table 3). The downside of AR37 is that occasionally they cause ryegrass staggers in livestock. This alkaloid has relatively high concentrations in perennial ryegrass, especially over summer, with a peak of above 5 mg/kg DM between December and April (Meale *et al.*, 2013). This endophyte provides to pasture tolerance against the principal pests in New Zealand, such as porina, the ASW, the mealybug, the adult black beetle and the root aphid (Table 4). In addition, the AR37 endophyte improves the grass dry matter by about 12% in New Zealand, in contrast to the same cultivars infected with other endophytes (Finch and Percival, 2011; PGG Wrightson, 2013). The transmission of AR37 into the seed is critical for the control of the ASW and other pests (Table 4).

Table 4: Most common pests in New Zealand controlled by endophytes: a) Black beetle, b) the Argentine stem weevil, c) Porina, d) the root aphid, e) the pasture mealy bug.

	Pest	Location and AR37 effects	
a)		**Black beetle** (*Heteronychus arator*) is found in the Bay of Plenty, Waikato and Northland. The larvae produce a significant damage on grass roots in summer and early autumn. Adults feeding at the base of the shoots, killing established grasses. AR37 endophyte provides better resistance to the black beetle than AR1. It controls the larvae which cause the most damage, but not the adults which can damage establishing seedlings.	
b)		**Argentine stem weevil** (*Listronous bonariensis*) can be found throughout the country. AR37 provides high resistance against this pest which is highly detrimental to pasture. Adult produce damage by feeding on the foliage but larvae causes the most significant losses. Feeding and oviposition are decreased with the presence of endophyte.	
c)		**Porina** (*Wiseana spp*.) is found the length of New Zealand; however, they are normally not as big a problem in the northern half of the North Island. A large number of Porina larvae die when AR37 ryegrass is sown in the paddock.	
d)		**Root aphid** (*Aphidoidea*) is a New Zealand wide pest. All root aphids die on contact with AR37 inoculated ryegrass in comparison with AR1 where most of the aphids survive in the field.	
e)		**Pasture mealy bug** (*Balanococcus poae*) is a New Zealand wide pest, which lives in cloudy and moist summer places, in particular. AR37 provides excellent resistance against this pest.	

Sources: (Grasslanz, 2010; Popay and Hume, 2011; Gerard *et al.*, 2013; PGG Wrightson, 2013; Specialty Seeds New Zealand Ltd, 2013).

2.2.5. Endophyte management used on pasture

Table 5 Endophytes used in New Zealand pastoral systems.

Endophyte Content	Endophyte management in pasture
Without endophyte	Grasses without endophytes provide a good animal performance but decreased insect tolerance. This type of pasture is good for areas with cool, wet summers such as Southland.
Low wild-type endophyte	It provides better animal performance than grasses with high endophyte but low grass persistence.
High wild-type endophyte	This type of endophyte persists extremely well in hard conditions. On the other hand, it produces ryegrass staggers, reduces weight gain in lambs and milk production in dairy cows.
AR1	This endophyte does not produce animal staggers, providing good animal performance, less fly strike and better weight gains and milk production than wild type endophyte (Johnson *et al.*, 2013). However, the active alkaloid is peramine, which does not provide good resistance to the black beetle (Specialty Seeds New Zealand Ltd, 2013).
AR37	AR37 has a wider range of pest resistance. It provides resistance to four of the main pasture pests in New Zealand and Australian pastures (Argentine Stem Weevil, Pasture Mealybug, Root Aphid and Black Beetle). Ryegrasses with AR37 show improved persistence, with higher tiller densities over time than the same cultivars with nil endophyte or standard endophyte (PGG Wrightson. 2013).
AR6	This endophyte has better resistance against black beetle than AR1, due to the retention of moderate levels of ergovaline in the host grasses.
Endo5	This controls a range of pasture insects including the mealy bug, the adult black beetle and the ASW. Farmers use tetraploid ryegrass with Endo5 to improve grass persistence and animal productivity. In addition, Endo5 does not produce animal staggers (lolitrem B) (Johnson *et al.*, 2013; Specialty Seeds New Zealand Ltd, 2013).
Nea2	Nea2 it is used in intensive farming systems in New Zealand. It provides persistence, grasses without staggers, and good animal performance. It has similar traits in controlling the black beetle as AR37 and provides a level of control of the mealy bug, the ASW and the root aphid (Johnson *et al.*, 2013).
GruboutU2	GruboutU2 produces alkaloids called lolines, which suppress a wide spectrum of insect attack from species such as the ASW, the porina caterpillar, the grass grub larvae, the black beetle adult and larvae, and the red headed cockchafer. They provide persistence, no staggers nor heat stress (unknown effects on sheep, deer or cattle). However, it has only been successfully infected with festuloliums, a hybrid between meadow fescue (*Schedonorus pratensis* syn. *Festuca pratensis*) and perennial ryegrass, with a high degree of meadow fescue.

Source: Adapted from Specialty Seeds New Zealand Ltd, 2013.

2.3. Perennial ryegrass (*Lolium perenne*)

Perennial ryegrass was widely dispersed in New Zealand from the late 19th century. It first was introduced from England, and then was also imported via Australia (Stewart, 2006). During 1880s, the seed harvest in the more arid Hawkes Bay and Canterbury regions became an important source of seed for the sowing of pastures. The hybridisation of ecotypes with greater traits (such as better cool yield and summer quality factors) from New Zealand and European plant material (such as hybrid ryegrass, recently reclassified as: *Lolium hybridum* syn. L. *xboucheanum*), demonstrated high performance in comparison to standard cultivars (Widdup and Ryan, 1992).

Ryegrass is characterised by being resistant to overgrazing and trampling damage (due to endophyte presence). However, some of the toxins produced by the endophytes also deter grazing ruminants (the toxicosis is considered the negative aspect of the endophytes), making the infected pastures less severely grazed (less often and not as close to ground level and the shoot apex region of tillers) than those not infected. This has ecological significance in keeping wild prairies well protected from overgrazing but is a problem in pastoral agriculture (Easton and Fletcher, 2006).

2.3.1. Plant description

Perennial ryegrass is a winter active grass that possesses a large number of tillers. It is compact with dark green leaves that are shiny underneath and folded in the sheath. It has a shallow root system. The inflorescence is a spike (Kemp *et al.*, 1999). This type of ryegrass has a yield that (depending on the environment) ranges from 10 to 25 t DM/ha/yr with higher yields obtained from high fertility and well drained soils. According to Kemp (Kemp *et al.*, 1999), perennial ryegrass has a poor performance in areas and seasons where the temperatures are too extreme (the optimum temperature range is between 5 and 18°C). It is sensitive to insect attack by the mealy bug, porina and the ASW. It is also susceptible to fungal diseases such as scald (*Rhynchosporium orthosporum*) and crown rust (*Puccinia coronata*) which are worse during wet summers.

Among perennial ryegrass cultivars, there are differences in the following areas: the concentration of endophyte present in the plant; crown rust resistance; tiller size; flowering time; and seasonal production (growth rates). These problems have led to the breeding of new cultivars that have greater productivity as well as the finding of better

ryegrass cultivars that have enhanced palatability. The discovery and development of tetraploid (4n) cultivars in perennial ryegrass has improved its palatability. However, 4n cultivars are less persistent than 2n ryegrass and, therefore, need appropriate grazing management practices (Kemp *et al.*, 1999).

2.3.2. Ploidy (tetraploid vs diploid)

4n have greener larger leaves, more extensive root systems, larger inflorescences and bigger seeds than 2n plants. They also have fewer but larger tillers and are later flowering. In addition, they are more palatable for livestock, have a higher ratio of energy content (better quality), and a higher proportion of water soluble carbohydrate to fibre because of the higher volume of soluble carbohydrates and proteins in cell contents to cell walls. However, good grazing management of 2n perennial varieties can be maintained at high energy content (> 12 MJ energy content/ kg DM) with grazing aimed to keep low residual and leafy grasses (Edwards and Bryant, 2013). In addition, 2n varieties are more persistent and less prone to being overgrazed. In contrast, 4n cultivars due to their palatability, lower tiller numbers and erect growing habits are less persistent and more susceptible to overgrazing, especially during spring, in association with treading damage and wet soil conditions.

Smith et al, (2003) and Sugiyama, (1998) report that diploid cultivars are smaller than tetraploid. In the majority of the species, 4n cultivars have slower germination than 2n (Mlyniec, 1971; Naylor, 1980). However, some studies stated that 4n cultivars germinate faster and to a higher percentage in comparison with 2n seeds under normal and pH conditions (at pH's 5, 7 and 8) respectively (Soliman, 1980; Bretagnolle *et al.*, 1995; Petit *et al.*, 1997). It been suggested that large size of 4n cultivars provide seedlings with more vigour, and faster growing than its homologous 2n (Kostoff, 1943; Hutton and Peak, 1954). In contrast, Sugiyama (1998) states that there is no significant difference in the establishment of 2n and 4n cultivars.

The higher ploidy level increases seed weight. However, germination rate varies in different plant species. A study on the grass *Dactylis glomerata* showed a higher germination rate in 4n than 2n (Bretagnolle *et al.*, 1995). In contrast, a study on

Catharanthus roseus (Hosseini, 2013), indicated that the germination rate and vigour is reduced when the ploidy levels increase (4n).

2.3.3. Ryegrass cultivars used in this project

2.3.3.1. Grasslands Samson

The Grasslands Samson♦ cultivar is a medium tillered perennial diploid ryegrass bred for persistence, disease resistance and production. Grassland Samson was released in New Zealand in the 1990s and resulted from the combination of germplasm from the Mangere ecotype (Grassland Nui and Ellett) with persistent plants collected from the Gisborne to Canterbury regions in New Zealand (Stewart, 2006; Agricom, 2012). It is described as an ideal general purpose pasture grass, due to its production of a relatively low number of reproductive tillers during late spring and summer and to its great production of dry matter during summer and autumn. Samson is a mid-season heading cultivar (+3, table 5). It has excellent performance under beef and sheep grazing and is available in the pasture industry with both AR1 and AR37 (Agricom, 2012). It is reported to have good performance in dry environments with a strong tolerance to crown rust and is also reported as ideal for irrigated or spring/summer rainfall areas.

2.3.3.2. Horizon or Ceres Horizon

Horizon was named firstly as Ceres Horizon in the 1990s (Stewart, 2006), but later was bought by PGG Wrightson, and thus had a name change to Horizon (Programme, 2012). It is a long rotation tetraploid hybrid ryegrass cultivar (*L. hybridum*) and is available with AR37 endophyte (NSW Department of Primary Industries, 2013). It is a mid-season (+4, table 5) heading 4n, being 4 days later than Nui in Canterbury, New Zealand (M. Rolston, personal communication, July, 2013). In addition, tetraploid cultivars have later flowering (3 days) than their 2n progenitors (Dhawan and Lavania, 1996). The cultivar is a prolific grower, which allows rapid return to grazing. In comparison to traditional 2n perennial ryegrass, Horizon has a high productivity and persistence with

♦ Plant Variety Rights were applied for by AgResearch Grasslands on 6 March 1995, but refused on the grounds it was not sufficiently distinct and uniform on 16 Sep 1996 (http://www.iponz.govt.nz/app/Extra/IP/Mutual/Browse.aspx?sid=635280706704913048).

improvement in animal intake and quality. The long rotation trait was bred into the cultivar by crossing it with perennial material (Hearne, 2008). The results of this cross was a higher proportion of perennial to Italian genetics (Launders *et al.*, 2010), which produced a high quality variety with greater cool season potential and persistence. Horizon has a high metabolisable energy and peak growth is maintained from spring into the summer demonstrating a high performance in farming systems (Procampo, 2013). It also produces good production in winter and spring in temperate climates with persistence in appropriate environments. This variety has high performance in the dairy industry, beef cattle production, and for the finishing for all stock types (Hearne, 2008). It is possible that Horizon, as a 4n cultivar, will be more affected by BS, due to a longer flowering period than 2n cultivars (such as Samson), and thus be more exposed to the disease (Dhawan and Lavania, 1996).

2.3.3.3. PGone50 (or Ceres One50)

This is a diploid perennial ryegrass from PGG Wrightson Ltd. PGone50 was obtained from crossing elite New Zealand and north west Spanish germplasm (Agricom, 2012). This cultivar is late heading (+20, table 5), heading approximately 20 days later than Nui (11th November). It is available in the market with low endophyte levels and also containing AR1 or AR37 endophyte (Agricom, 2012). It is reported to be highly productive in the climatic conditions of Australia, being a high producer of dry matter while reaching its best yield potential under fertile, productive conditions (Matthew *et al.*, 2012; IH Seeds Pty Ltd, 2013). Management in late spring and early summer is easier due to its late heading traits and continuing leaf production during this time. This contrasts with early to mid-heading ryegrass cultivars that require appropriate grazing management to restrict seed head production during this time. The main characteristics of PGone50 are its improved tolerance to rust, long period of high yield production, high production during summer, autumn and winter, and high quality late summer feed (IH Seeds Pty Ltd, 2013).

2.3.4. New Zealand main ryegrass seed production

The ryegrass seed production areas of in New Zealand are Canterbury and Otago (42 to 45°S). The climate is maritime in this area, which, in turn, is highly influenced by the Southern Alps, which have a height of over 2800 m and are located some 40 km or more

from the west coast. During summer, the Southern Alps affect Canterbury through north-west winds. These are warm winds that give a rain shadow effect and produce drying weather that gives an increase of about 300 mm of evapotranspiration plus the normal soil moisture deficit that usually occurs during summers (Chynoweth, 2011).

2.3.5. Ryegrass germinability

The viability of a seedlot is not the same as seed germinability. Even if a seed lot is fully viable, there can be important seed loss due to seed immaturity or dormancy. A seed may be germinable, but it does not mean that it is completely developed. There are three stages in the development of caryopsis on ryegrass. Stage one has a rapid increase in fresh and dry seed weight; meanwhile, stage two shows a constant increase in dry weight but a slower or non existent increase in fresh weight (Coolbear *et al.*, 1997). During stage three, the ripening stage, dry weight is constant and fresh weight decreases as the seed tissues lose moisture rapidly (the rate depends on the environmental conditions). Seed ripening in grass may be associated with seed loss through shedding but because by this time the seed are functionally independent of the plant, harvest can be earlier for some cultivars (Anslow, 1964; Coolbear *et al.*, 1997).

Germinable seeds in the early stages of seed development are characterised by poor vigour. According to Grabe (1956), maximum seedling growth is achieved from seeds that have reached the end of stage two, where seed is at maximum seed dry weight. However, some studies suggest that in grass seeds maximum germination ability may not be reached at the end of stage two, despite the final dry weight being reached. In grasses, physiological maturity is reached at 35 days post anthesis, but the germination at this stage is 55% (Coolbear *et al.*, 1997). On the other hand, Ellis (1992), reported that seeds continue to mature after the end of the seed-filling period. According to Anslow (1964), there is variation in the development of early germinability between early heads and the later heads (more than a week) from the same plant. Also, there is variation of seed development among individual spikes, where development is slower in the apical floret (Coolbear *et al.*, 1997). The onset of germinability can be strongly affected by environmental conditions. Climatic effects on ryegrass seed development can be similar to the effects produced by pathogens, and, therefore, are easily confused (Anslow, 1964; Coolbear *et al.*, 1997).

2.3.6. Flowering

Perennial ryegrass requires low temperatures and/or short days for the primary induction of flowering and the flower bud initiation (Laidlaw, 2004). These short days and/or low temperatures are required for the induction of flowering depending on the latitude where the ecotype or ecotypes of ancestor plants are from (Halligan *et al.*, 1993). After the induction of flowering, initiation depends subsequently on the day length (Evans, 1960). Conditions during spring or summer may vary from day to day, making it difficult to predict a flowering date (Laidlaw, 2004).

2.3.6.1. Flowering date

The timing of heading is influenced directly by the general agronomic traits of the ryegrass cultivar (Laidlaw, 2004). The ear emergence date is the time when seedheads start appearing out of the pseudostem. The flowering date is controlled genetically and is triggered when the day length is appropriate (later spring). In late flowering varieties, ear emergence is activated when the day is long enough, in late spring (Kerr, 2013). The flowering date of ryegrass is classified from the ryegrass cultivar "Grasslands Nui", which is used as a reference point, with its ear emergence set at zero (Table 6). The day zero or ear emergence date for Nui occurs between October 22 and 25 in the Canterbury region (Edwards and Bryant, 2013). This day (zero day) may vary depending on temperatures throughout the year. During a cold spring, flowering will be delayed; whereas, a warm spring may bring earlier flowering (Kerr, 2013).

Table 6: Flowering dates of permanent pasture ryegrasses cultivars used in New Zealand (Kerr, 2013).

Very early	Standard	Late	Very late
Meridian = -17	Aries HD = +2	Arrow = +10	Bealey = +25
	Bronsyn = 0	Banquet = +21	Matrix = +23
	Cannon = -1	Impact = +21	Quartet = +28
	Commando = -3	Revolution =+19	
	Extreme = 0	PGOne50= +20	
	Nui = 0		
	Samson = +3		
	Horizon = +4		

2.3.7. Harvest

There are different factors that help identify when it is the right time to harvest the seed crop. According to Anslow (1964), high seed yield with 100% germination can be obtained in the period between 20 and 26 days post anthesis (DPA). Similarly, Hill and Watkin (1975) propose that the greatest viability of ryegrass seed is reached between 14-17 DPA, but as stated earlier, ryegrass germinability rather than viability should determine the harvest date. The optimal harvest day, which is the day with the highest seed quality, depends on the cultivar and the harvest method. The right time to perform the pre-threshing is when the seed reaches its maximum germinability (Coolbear *et al.*, 1997). In addition, an effective harvest time occurs when the seeds are at 40-50% seed moisture content. When seeds reach 40% moisture, they shatter. This moisture is usually reached approximately 30 days after the first anthesis (Coolbear *et al.*, 1997).

2.4. Fungicides used in this research

In this research, fungicides from the chemical families: triazole; benzimidazole; strobilurin, pyrazole; and phtalimides were used. Previously, many studies have been done to assess the effectiveness of different fungicides in the control of fungus on ryegrass such as BS, stem rust and even endophytes among others. BS control during the first half of the last century was difficult as there was no effective fungicide to control it (Hardison, 1970). Afterward, the use of triadimefon and fenarimol (1.12 kg/ha) gave control after been applied as a soil-surface drench, and apothecia was prevented by the use of sodium azide (12 and 16 mg/0.92m^2) soil surface applied in a glasshouse (Hardison, 1978). However, according to Alderman (1997), sodium azide requires large volumes of water to drench the fungicide through a dense canopy and, therefore has not been useful for Australia with its limited water availability and large paddocks. It was then stated that large amounts (448 kg/ha) of nitrogen use (urea) in spring produces moderate control, and the use of 56 kg/ha of urea has significant control if the disease level is low (Hampton and Scott, 1980a; Hampton and Scott, 1980b; Hampton and Scott, 1981). However, later studies showed that the use of triazole or benzimidazole (recommended in moderate rate by Hardison, 1970) were effective in the control of BS (Rolston and Falloon, 1998). Still later, studies to control the detrimental BS fungus without exerting any effect on the presence of endophyte in ryegrass were carried out.

Rolston (2002a) performed a study to evaluate the effects of triazole and strobilurin (used to control stem rust) on AR1 endophyte infected harvested seeds. Results showed that these fungicides considerably increased ryegrass germination (Rolston *et al.*, 2002a; Rolston *et al.*, 2009) without having any effect on the viability of AR1. Subsequently, FAR performed a study to control BS using a plant growth regulator (trinexapac-ethyl) and different fungicides treatments (FAR, 2006; FAR, 2007a).

The use of triazole, and benzimidazole fungicides gave a higher germination rate (FAR, 2006; FAR, 2008), although, the use of strobilurin + azoles + benzimidazole; benzimidazole; and azoles did not significantly differentiate from the higher germination rates. They also suggested the crop canopy was directly helping to control BS, where crop mass-height index provided a better germination result. Meanwhile, low tiller number, low mass, and a small crop size reduced germination and increased BS incidence (FAR, 2006; FAR, 2007b; FAR, 2008). In addition, they stated that the use of trinexapac-ethyl is not related to the reduction of germination, yet concluded that the combination of foliar fungicides and canopy management helped control BS (FAR, 2006; FAR, 2007b). Later FAR stated that the use of two application dates of foliar fungicides during the ear emergence and flowering period provided better outcomes than a single application. In addition, further work has found that the use of Proline (prothioconazole) at flowering followed by prothioconazole + carbendazim or tebuconazole + carbendazim at mid-seed fill increased ryegrass germination. The endophyte transmission was reduced by the use of triazole in harvested seed (FAR, 2008). According to Mebalds and Price (2008), the application of propiconazole, flutriafol, and benomyl fungicides are most effective to control BS when applied at seed head emergence and anthesis stages of crop growth.

2.4.1. New discovery about the use of triazole fungicides

Earlier studies have reported that the use of triazole is an effective fungicide treatment to control BS (Rolston *et al.*, 2002a; FAR, 2008; Rolston *et al.*, 2009; Chynoweth *et al.*, 2012). It is important to note recent reports that speckled leaf blotch (*Septoria*) populations are becoming less sensitive to the use of triazole to control it. This effect has also been reported in Europe and now is also occurring in New Zealand (Chynoweth and Craigier, 2013). Therefore in response, higher rates of triazoles have been applied to re-

enforce the control of this pathogen (Chynoweth and Craigier, 2013). It is also important to consider new alternatives, such as the testing of new fungicide combinations, to achieve effective control of these pathogens.

Chapter 3. Methodology

Two trials were used for this research. The first trial was a fungicide trial using two ryegrass cultivars, Samson and Horizon. The aim of this trial was to determine which fungicides were effective in controlling BS disease. The second trial using the ryegrass cultivar PGone50 had the aim of evaluating the effects of various fungicide treatments on the transmission of endophyte to the new seed generation.

3.1. First trial: Blind seed incidence

The BS trial to determine the effectiveness of a range of fungicide combinations on BS was established in paddock № 11, Lincoln AgResearch farm (43° 38′ 08.18" S, 172° 28′ 08.63 E). This contained three year old plots of the cultivars Grasslands Samson, which is a 2n breed, and Horizon, a 4n breed. The endophyte levels in the Samson and Horizon plots were unknown at sowing because the trial was originally sown to investigate N responses. The paddock was divided into 12 plots and each plot split into four. The size of each plot was 2 m x 3.5 m = 7 m^2 and with 4 replicates this gave a total area of 28 m^2. The soil type in the paddock is Wakanui silt loam. Wakanui silt loam is well structured and friable, although the drainage through the profile can be slow and the soil can become waterlogged (Officer et al., 2004). The presence of BS in this paddock was highly induced. High seed loss had occurred in this paddock from previous seasons and, therefore, it was highly likely to contain high inoculum loads. To further promote BS incidence, no N was applied during spring, as the enhanced vegetative growth from N fertiliser is known to physically block ascospores produced at ground level from apothecia from reaching the spikes (Mebalds and Price, 2010; Chynoweth et al., 2012). Finally, Moddus®, a plant growth regulator (that contains 250 g/litre trinexapac-ethyl in the form of an emulsifiable concentrate) was applied at 2.0 l/ha , two to three weeks after the closing date[♦] (mid-September approximately) to reduce reproductive stem

[♦] Closing date: removing stock from a crop; no herbage is removed from a crop after closing Rowarth, J. (ed.) 1998. Practical herbage seedcrop management. Lincoln University Press: Lincoln University Press, Canterbury and Whitireia Publishing with Daphne Brasell Associates Ltd, Wellington.

height in the hope of again encouraging BS primary infections (Chynoweth,(2012). Moddus® produces a short open crop (FAR, 2007a). Despite the theoretical likelihood that trinexapac-ethyl increases BS incidence. According to W. Simpson (personal communication, December, 2013), there is no conclusive information on the effects of plant growth regulators such as trinexapac-ethyl on endophyte transmission. This appears to also be supported in previous studies (Rolston *et al.*, 2004; FAR, 2006).

To determine the effect of fungicide combinations on BS, different combinations of six fungicides (Table 7), to create 12 different treatments (included control) were applied as four replicates in a randomised block design.

Table 7 Names, active ingredient and chemical family of the fungicides used in the first trial.

Product	Active Ingredient	Chemical Family
Proline	a.i. 250 g/l prothioconazole	triazole
Protek	a.i. 500 g/l carbendazim	benzimidazole
Amistar	a.i. 250 g/l azoxystrobin	strobilurin
Folicur	a.i. 430 g/l tebuconazole	triazole
Seguris Flexi (S.F.)	a.i. 125 g/l isopyrazam	pyrazole
Folpan	a.i. 500 g/k folpet	phtalimides

There were two fungicide application dates; the first application was at late anthesis (13 December 2012); and the second, 14 days later (27 December). The fungicide combinations and application rates used in this trial are given in Table 8.

Regarding fungicide choice, prothioconazole is the only fungicide treatment that carried a label registration and protection for BS. Other fungicides that have never been tested for the control of BS were also included. Previous studies appear to implicate prothioconazole as reducing AR37 endophyte transmission (Harvey and Harvey, 2009; Chynoweth *et al.*, 2012). Isopyrazam is reported to not affect endophyte transmission, but the effectiveness of controlling BS is unknown.

Table 8 First Trial: Treatment combinations (fungicides and rates) and application dates used in the trial with the Horizon and Samson cultivars.

	First Application (Flowering, 13 Dec)	Second Application (+14 days, 27 Dec)
Treatment	**Product**	**Active Ingredient**
1	Nil (Control)	Nil (Control)
2	100 g/ha prothioconazole	0
3	0	100 g/ha prothioconazole
4	100 g/ha prothioconazole + 250 g/ha carbendazim	100 g/ha prothioconazole + 250 g/ha carbendazim
5	250 g/ha carbendazim	0
6	0	250 g/ha carbendazim
7	250 g/ha carbendazim	250 g/ha carbendazim
8	125 g/ha azoxystrobin + 189.2 g/ha tebuconazole	125 g/ha azoxystrobin + 189.2 g/ha tebuconazole + 250 g/ha carbendazim
9	100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim	100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim
10	189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim	189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g /ha carbendazim
11	189.2 g/ha tebuconazole + 250 g/ha carbendazim	189.2 g/ha tebuconazole + 250 g/ha carbendazim
12	375 g/ha folpet	375 g/ha folpet

3.1.1. Seed harvest

Spikes were harvested by hand, by cutting the spikes using a square meter deposited in the centre of each plot. Then, seed heads were placed into hessian sacks to allow drying to be completed outdoors. The seeds were separated from the spikes after being rubbed by hand prior to seed coming to Massey University, in July, 2013. The weather during the development of this trial is displayed in the Figure 6. Data was provided by the Lincoln, Broadfield Ews[♦]

[♦] Lincoln, Broadfield Ews: Provider of summary of climatological observations.

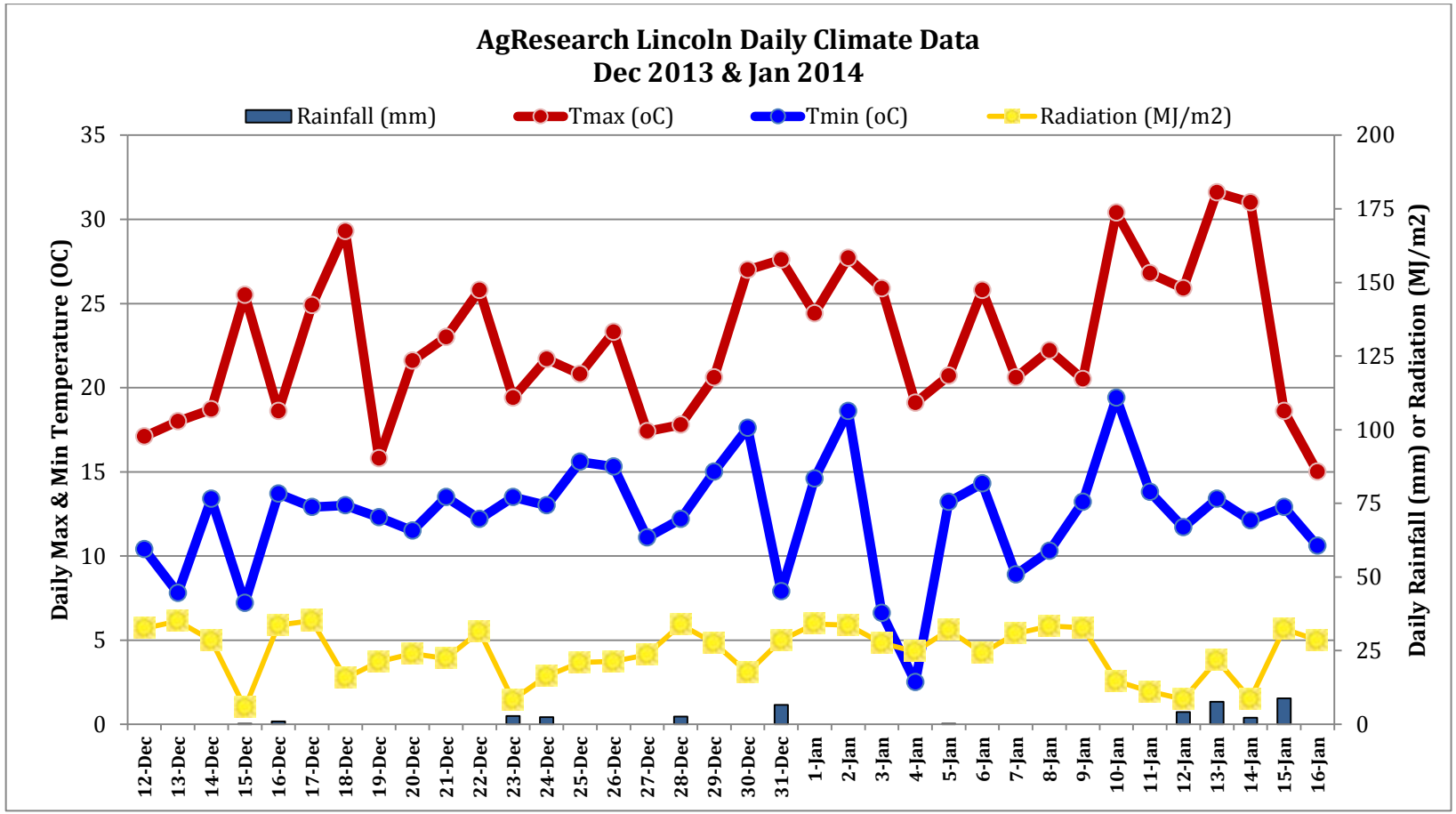


Figure 6: Climatological descriptions (rainfall, T° max and min, and radiation) from 12th December 2013 to 16th January 2014 in Canterbury, New Zealand.

3.1.2. Seed cleaning

The ryegrass spikes were rubbed by hand inside of a plastic container (Figure7a, Figure7b) to separate the seeds from the spikes. The seeds were then sieved to remove impurities of greater size from the seed lot (Figure 7c). Subsequently, the seeds were cleaned using a South Dakota seed blower (Figure 7d). This device increases the germination rate of seed samples by removing empty and light seed (FAR, 2007b). The South Dakota seed blower consists of a centrifugal blower, which possesses a column with an upward stream of controlled air. Samples are held in place by fine wire gauze at the bottom of the tube. Six grams of seed are placed on the gauze at one time. The air flow is set to carry the lighter weight seed and empty glumes upward where they are trapped, leaving the heavy fraction at the bottom (ISTA, 2013). The South Dakota seed blower has an approximate size of 48 x 56 x 86 cm without the tube, with an electronic motor of 1/2 HP single phase, and a large tube of approximately 10 cm diameter and a height of 93 cm.

Figure 7: (a) Hand rubbing; (b) Plastic container; (c) Metal sieves; (d) South Dakota seed blower.

Then, a second blowing was performed to achieve more heterogeneity of the heavy fraction. The blower was operated for five seconds per sample, with the blower regulated at the opening setting of 9.0. Each sample was blown twice for a short period each time as seed separation is more effective with this technique compared with long operating periods (Davide *et al.*, 2011). All seeds from Samson and Horizon were blown with the same settings, and the filter was cleaned between samples to avoid cross contamination of samples. Seeds were then maintained in a cold storage chamber (5°C / 60% RH) in a sealed plastic bucket.

3.1.3. Purity test

Conducting a purity test is useful as it ensures that subsequent thousand seed weight and germination testing is completed on pure seed. Pure seed includes mature florets with an obvious caryopsis containing endosperm; broken seed pieces larger than one half of their original size; and a caryopsis size of at least 1/3 of the palea length. Inert matter includes: broken seed less than half the original size; seed with a caryopsis <1/3 the length of the palea; and seeds with disease signs such as ergot. Seed other than ryegrass ("other seed") is also classified as an impurity (ISTA, 2013). The purity test was conducted on 6 g of seeds as this is the ISTA working sample (ISTA, 2013). The purity result is given as a percentage of pure seeds in the seed lot. Subsequently, pure seeds from the purity test were used in a thousand seed weight (TSW) assessment; a germination test on 100 seeds per sample (12 treatments x rep); and the endophyte testing (growing 96 seedlings from 12 treatments x rep).

3.1.4. Thousand seed weight

The thousand seed weight in each seed sample was determined by selecting and weighing eight samples of 100 seeds on a balance accurate to 0.0001g (with results calculated to four decimal places), and the average weight multiplied by 10 (ISTA, 2013). The thousand seed weight was determined using this formula (ISTA, 2013):

Weigh 100 seeds for 8 replicates, separately

Calculate the average weight of 100 seeds, and multiply by 10.
1000 seeds weight = 100 seeds weight x 10

This TSW may provide information on the effect of the fungicides on the seed weight.

3.1.5. Germination test

After the purity test, seeds were removed from the pure seed fraction to perform the germination analysis. Four reps of 100 seeds per treatment were removed and placed on double blotter sheets (Anchor Steel blue germination blotters) that had been soaked in 0.2% potassium nitrate (KNO3) and then placed into plastic containers (germination boxes) and pre-chilled at 5° for seven days. The germination boxes were then transferred to a germination chamber. The germination chamber was set at alternating temperatures of 20°C and 30°C, lit by two fluorescent tubes providing light for eight hours at 30°C and 16 hours dark at 20°C (Hume *et al.*, 2013). The germination test lasted for 14 days, where the first count was performed on the fifth day (normal seedlings removed at this time), and the last count was on the fourteenth day. The result was expressed as an average percentage of normal seedlings (Figure 8). Seeds and seedlings were assessed as stipulated in the ISTA Rules (2013). Immediately after the final count, the non-germinated seed were evaluated with a magnifying glass to assess BS incidence. BS positive seeds were determined by the presence of accumulation of conidia on the seed surface in a spore secretion, appearing as a reddish-brown crust (Alderman, 2001).

3.1.6. Blind seed test

Non-germinated seeds remaining from the germination tests were examined under a microscope, following the procedure of Matthews (1981), immediately after the seedling count for the germination test was completed. Before being evaluated for BS under the microscope, seeds were moistened with tap water. Conidia are easily seen under a microscope after dissecting a moistened seed. An infected caryopsis looks shrivelled, with a pink to reddish-brown colour (Calvert and Muskett, 1945; Blair, 1947). On the seed surface, conidia accumulate in a spore secretion (slime) with a clear or pale pink colour (Hyde, 1938) or may also have a reddish-brown crust (Calvert and Muskett, 1945). The BS test was performed to determine the presence of *G. temulenta* in both

Grasslands Samson and Horizon cultivars. This test also quantified the amount of BS in both cultivars for each of the treatments used, allowing the separation of seed that had been killed by BS from other causes of seed death. Dead seed was placed on filter paper moistened with tap water, which was placed into a petri dish. Seed were examined using a Nikon SMZ-1 at10x magnification. The lemma and palea were removed from the softened imbibed seeds to expose the caryopsis and embryo. Infected seeds showing spots of rusty red-brown copper colour with shrunken embryos were scored as infected seeds.

Figure 8: Normal seedlings and dead seeds as a result of BS. Seeds and seedlings are on a germination blotter on which 100 seeds were deposited.

3.1.7. Seeds squash test

The seed squash test is used to determine whether endophytes are present or absent in the seeds. Nevertheless, this test cannot determine the viability of the endophyte in the seed. For the test seeds were softened by soaking them with 5% (w/v) sodium hydroxide overnight (Figure 9a). The following day, seeds were washed thoroughly in

water and deposited into universal glasses that contained Garner's stain solution. Garner's stain contains 0.325g of aniline blue, 100ml water and 50ml of lactic acid at 85% (Figure 9b). Subsequently, the seeds were boiled for 10 minutes (Figure 9c), and left to cool down. The aniline blue stain used to develop the seeds on the microscope was made of one part of lactic acid, two parts of glycerol, one part of water and 0.05% aniline blue (Latch and Vaughn, 1995).

9a

9b

9c

Figure 9: (a) Seeds softened with sodium hydroxide; (b) adding Garner's stain solution; (c) Boiling seeds.

3.1.7.1. Detection of hyphae in seeds

The ryegrass seed glumes were removed (Figure 10a) and the remaining caryopsis deposited onto microscope slides with a strip of aniline blue stain (Figure 10b). The cover slip was placed on the seed while applying gentle pressure over the seeds to squash them. Five seeds were deposited on each slide. The slides were examined under a microscope at 10x or 40x stage lens objective (Figure 10c). Endophytes are observed as a convoluted stained filament which is easily located on the seed squash by looking for the large square aleurone cells. Endophytes are found between or above these cells.

10a

10b

10c

Figure 10: (a) Deglumed seeds; (b) seeds deposited in slides; (c) seeds evaluation.

3.1.8. Preparation of viable endophyte in seed assessment

In viable seed, endophytes are transmitted into the tillers of the developing plant, but when the seeds are not viable this is not possible (M. Christensen, personal

communication, October, 2013). To break seed dormancy, seed samples (48 x 96 seeds) were sown on Anchor Steel blue germination blotters and pre-chilled at 5°C with 0.2% potassium nitrate (KNO_3) for seven days at Massey University. After pre-chilling, 96 imbibed seeds were sown into seedling hygiene trays (dimensions: 30 cm wide, 42 cm long and 6 cm high). Planting was in rows of 8 x 12 to provide the seedlings with sufficient space to grow. The hygiene trays were filled with potting media (v/v 17% peat, 10% washed and sterilised river sand, 43% bark, 10% coir fibre, and 20% pumice). Final germination/emergence was recorded. The plants were grown until they reached between two to three tillers in size. This took approximately 6 weeks (beginning from the first week of October). Two to three tillers is the appropriate size to perform the tissue print immunoblot (TPIB) endophyte test. This was done in an unheated glasshouse called 'the bubble' (Figure 11a) at AgResearch Grasslands, Palmerston North, where temperatures were regulated automatically, settled between 15 to 20°C (15°C at night and 20°C during the day, with an average humidity of 54%).

11a

11b

Figure 11: (a) The bubble, AgResearch; (b) trays placed on a capillary matting.

Irrigation was also automated. The watering was set up to work once a day per three minutes/day with the dampness of the potting mix in the trays being checked daily to see if supplementary watering was required. The technique used was where the trays were placed on tables with capillary matting (Fig.11b), having an inclination slope of 10% (5.71°). With this technique, water pipes slowly deposit water into the table water container. The capillary matting, which is a synthetic absorbent material, soaks up water like a sponge. Subsequently, the potting mix in the trays absorbs water from the damp capillary matting. This technique follows the property of cohesion. The water in the

seedling tray is connected to the water in the capillary mat which is connected to the water container and all three objects act like a chain. They move an even amount of water across themselves, maintaining it as long as there is water in the water container to be drawn (Colovic, 2000).

3.1.9. Tissue print immunoblot detection of endophyte (TPIB)

The seedlings for this test were grown in hygiene trays for about 6 weeks (time necessary for plants to reach a proper size to perform this test). The Tissue Print ImmunoBlot (TPIB) test involves imprinting a cross-section of the tiller's pseudostem onto a nitrocellulose membrane (the "blotter paper").The nitrocellulose membrane (NCM) is cut to an appropriate size for the number of tillers. This is often a 10 x 10cm square divided into 100 cells of 1 x 1cm. Divisions are inscribed on the NCM using a ball-point pen (Figure 12). The tillers were cut basally at approximately 5 mm above the turf surface (to avoid contamination). It is in the basal part of the pseudostem that a higher concentration of endophyte mycelium is found. Any necrotic sheath tissue was removed before performing the transverse cut so as to avoid false positive results. The TPIB can pick up mycelia proteins found in necrotic sheath tissue and many of these may not be specific to *E. festucae* var*. lolii*. Removal of the outer sheath removes any epitopes of saprophytic fungi that could also cause a reaction when the antibody used is not highly specific (Simpson *et al.*, 2012).

The freshly cut pseudostem is then pressed against the nitrocellulose membrane paper (0.45µm length), leaving a circular outline from the fresh cut. The freshly cut surface of the tiller is pressed onto the membrane, allowing efficient use of the NCM, leaving sufficient antigen binding to the membrane to elicit an immuno response (S. Card, personal communication, August, 2013). It is important that individual blots are linked to the source plant in case a second test is needed if some of the blots from the first tillers are diffuse. A negative and positive control tiller was blotted on the membrane with plants of known endophyte status, to be used as controls (Figure 12). The membrane is then stained for the presence of endophyte proteins in tissue prints of ryegrass (Gwinn *et al.*, 1991).

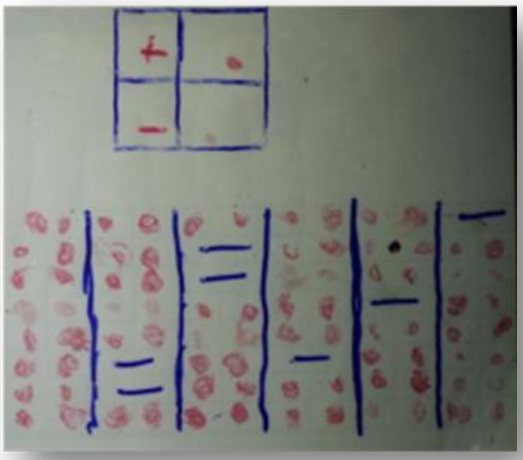


Figure 12: Imprints from tillers on the blotter paper (nitrocellulose membrane) with positive (+) and negative (-) endophyte presence.

Surfaces on the blotted sheets with no bound protein were blocked by immersion in a milk protein blocking solution (Tris (hydroxymethyl) methylamine 2.42 g, NaCl 2.92 g, Non-fat milk powder 5 g, 1 M HCl 10 ml made up to 1 l reverse osmosis (RO) water adjusted to pH 7.5). This blocks any unbound sites prior to the application of a primary antibody (Gwinn *et al.*, 1991). Then, the membranes in the blocking solution were shaken with an orbital shaker for approximately 2 h at room temperature. The blocking solutions were then decanted off the membrane and the membrane was rinsed twice with fresh blocking solution before adding 25 µl of primary antibody (rabbit antibodies, obtained from IVABS, Massey University, Palmerston North New Zealand) in 25 ml blocking solution (1:1000 dilution) (Simpson et al., 2012). The membrane was incubated in a shaker overnight at 4°C. The next day, excess primary antibody was removed by decanting and the membrane rinsed twice with fresh blocking solution. The secondary antibody (goat anti-rabbit IgG-AP, sc-2034, Santa Cruz Biotechnology, USA), 6.25 µl was added to the 25 ml blocking solution (1:4000 dilution) and shaken for 15 minutes at room temperature before being incubated for five hours at 4°C (Gwinn *et al.*, 1991). As for the first antibody, the excess of the secondary antibody was removed by decanting.

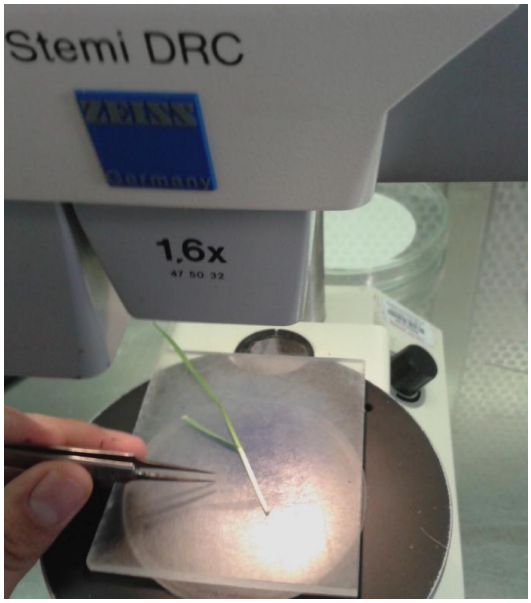
Subsequently, the membrane was rinsed twice in the fresh blocking solution. Separately, chromogens were prepared by dissolving 20 mg Fast Red -TR (Sigma F-2768) in 12.5 ml

Tris buffer (Tris (hydroxymethyl) methylamine 24.2 g in 1 l RO water adjusted to pH 8.2) and 12.5 mg of naphtholAS-MXphosphate (SigmaN4875) in 12.5 ml Tris buffer per 10 cm2 of nitrocellulose membrane. When the chromogen solution was combined, the nitrocellulose membrane was submerged in it and shaken at room temperature for 15 minutes, until the red colour developed as a control positive blot. Then, the development of colour is stopped by rinsing the nitrocellulose membrane three times in RO water. After completing these procedures, the sheets were ready to be read (Gwinn *et al.*, 1991). As Figure 12 shows, the maximum intensity of red colour on the printed paper is an indication that the tissue is highly infected by endophyte whereas a brown or lesser red colour indicates that the pseudostem does not contain endophyte (Simpson *et al.*, 2012).

3.1.10. Microscopy of leaf sheaths

Inconclusive results obtained from the tissue TPIB test were re-evaluated under a microscope. To do this, the presence of endophyte hyphae were observed after cutting and removing the outer sheath tissue of the tiller, using fine-tipped tweezers (Figure 13a). Once the outer sheath was gone and the inner sheet surface was reached, transversal strip cuts were made in the epidermis with a scalpel, obtaining two strips of the basal part of the sheet. Then, the epidermal strips were deposited onto a glass slide (the inner side of the leaf placed upwards) which had added to it a drop of aniline stain (1: 2: 1 v/v lactic acid, glycerol, water, 0.15% aniline blue) (Christensen *et al.*, 2002). The strips were then covered with a glass slip and the slide was warmed to eliminate air bubbles. The strips were analysed to confirm the presence of endophyte hyphae in the epidermal plant tissue (Figure 13b), using a Zeiss stemi DRC microscope with objectives of 1,6x and 0,8x.

13a



13b

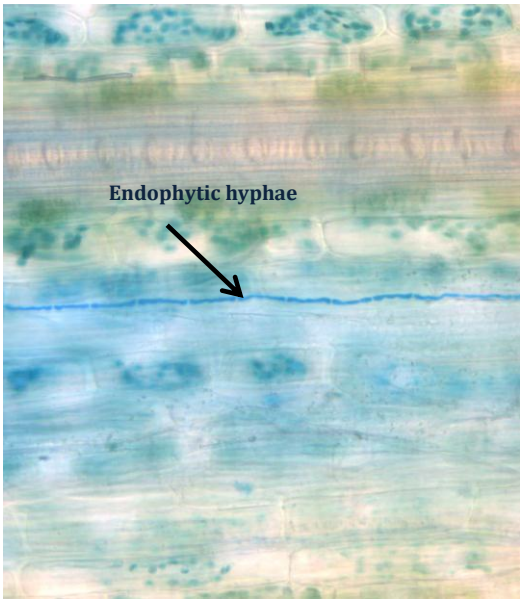


Figure 13: (a) Observation of endophytic hyphae in the leaf sheaths under microscopy; (b) AR37 endophyte hyphae present in the leaf sheath.

3.2. Second trial: Endophyte transmission

This trial was established to better test the hypothesis that some of the combinations of fungicides (used to control different pathogens such as stem rust, *Fusarium*, BS), can affect seed transmission of AR37 endophyte and the retention of viability. In this trial, seeds of a first year crop of the cultivar PGone50, a perennial 2n ryegrass from PGG Wrightson were used. The paddock used for the PGone50 production had remained in fallow for the previous three years and prior to this wheat had been grown in it. Unlike the first trial, this cultivar was not grown under conditions likely to induce BS. This cultivar contained the AR37 endophyte (*E. festucae* var. *lolii)*. The trial used eight fungicides from different chemical families applied. The fungicide treatments were applied on 24 December, 2012, at the mid-anthesis/flowering stage (R. Chynoweth, personal communication, July, 2013). The fungicides were combined in 11 treatments and a control treatment (Table 9).

The trial was replicated four times in a randomised block design, giving a total of 48 plots from which seed sample were harvested for assessment. The PGone50 paddock was windrowed for approximately seven days before seeds were extracted using a combine harvesting machine (a machine harvester that combines reaping, threshing and winnowing), on 10 February, 2013. In contrast to the first trial, seeds were then machine

dressed to improve seed purity. The production site of the trial was at Barrhill (43°41'23.35"S, 171°49'52.85"E), Canterbury New Zealand. Treatments were applied on 24 December, 2012.

Table 9: Second Trial: Treatment combinations (fungicides and rates) in the trial with the PGone50 cultivar.

Treatment	Product applied on 24/12/12	Chemical Family
1	Nil (Control)	None
2	75 g/ha prothioconazole	triazole
3	75 g/ha prothioconazole + 125 g/ha pyraclostrobin	triazole strobilurin
4	75 g/ha prothioconazole + 75 g/ha isopyrazam	triazole pyrazole
5	125 g/ha pyraclostrobin + 75 g/ha isopyrazam	strobilurin pyrazole
6	75 g/l/ha isopyrazam	pyrazole
7	Product A	confidential
8	Product B	confidential
9	375 g/k/ha folpet	phtalimides
10	375 g/ha folpet + 125 g/ha pyraclostrobin	phtalimides strobilurin
11	189.2 g/ha tebuconazole + 125 g/ha azoxystrobin	triazole strobilurin
12	125 g/ha azoxystrobin + 75 g/ha isopyrazam	strobilurin pyrazole

3.2.1. Germination test

Seed harvested in this trial was assessed for germination following the same procedure as described in section 3.1.5.

3.2.2. Preparation of endophyte assessment

Proceedings in the preparation of TIPB test evaluation were performed following the procedure described previously in section 3.1.8.

3.2.3. Tissue print immunoblot detection of endophyte

The tissue TPIB test was performed in this trial following the same procedures described in trial one, section 3.1.9.

3.2.4. Microscopy of leaf sheaths

Inconclusive results obtained from the tissue TPIB test was re-evaluated under a microscope following the procedures described previously in trial one, in section 3.1.10.

3.3. Statistics

All statistical analysis was performed with SAS 9.3 (SAS, 2009). All data was transformed (arcsine square root transformation) prior to analysis. A test for normal distribution (Kolmogorov-Smirnov) was applied to all data, where $P \geq 0.05$ was considered normal distribution, followed by the multiple Bonferroni (Dunn) t-test for multiple comparisons. Significance level was declared at $P<0.05$.

The responses analysed were control vs. fungicide treatments for: TSW, seed purity, germination, BS, and endophyte transmission. When results are significantly different, figures are presented with error bars (created mainly in Microsoft Excel plus a few interaction charts created by SAS). Excel charts have both standard error bars and letters displayed to show individual treatment variation and significance.

Chapter 4. Results

Microscopic evaluation of dead seeds with BS infection in both trials confirmed in some seed the presence of *Alternaria* spp. or *Epicoccum* spp. (saprophyte) fungi. Ergot (*Claviceps purpurea*) was also present in some seed, but was not enough to cause a significant reduction in seed purity. In addition, as reported in previous studies (FAR, 2006), the use of trinexapac-ethyl did not appear to have affected the germination results obtained in this study.

4.1. Trial one: Horizon

4.1.1. Thousand seed weight (TSW) test Horizon

The data obtained from the thousand seed weight test performed on Horizon (a long rotation hybrid perennial ryegrass (*L. hybridum*)), had a normal distribution according to Kolmogorov-Smirnov (P> 0.150). The t-test (LSD= 0.025) grouped the treatments into six groups (Figure 14), where all treatments (except T3) increased the seed weight. On the one hand, the fungicide combination composed of T2, T8, and T7, yielded heavier seed. On the other hand, the use of prothioconazole at the second application date during the mid-seed fill (T3) slightly reduced the seed weight in this cultivar (0.02 g below control) in contrast to its earlier application (T2).

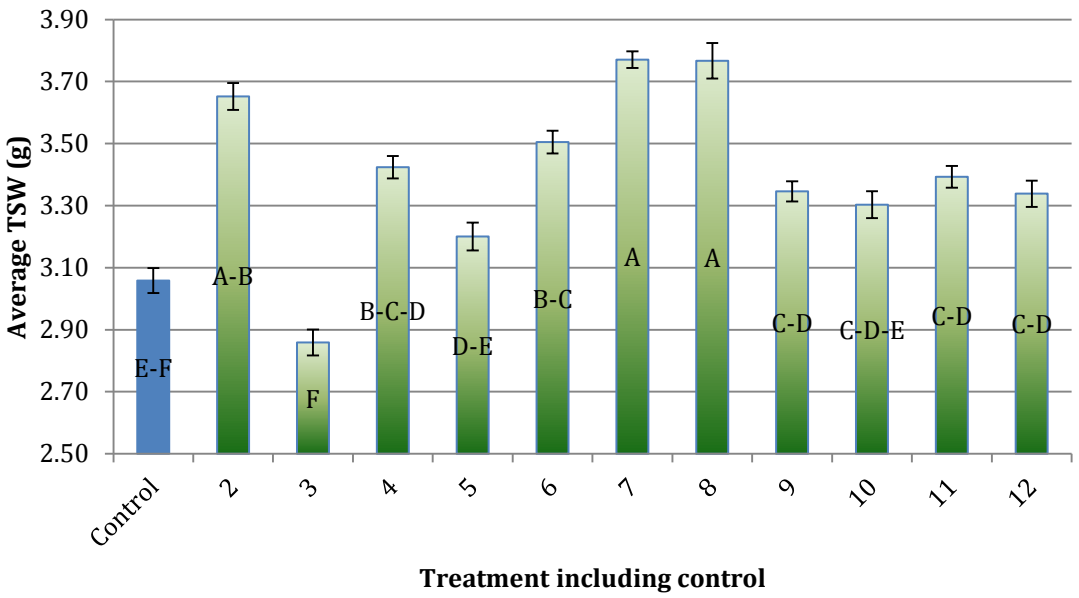


Figure 14: Thousand seed weight test: Horizon (control vs fungicide treatments at up to two applications – 13 Dec; 27 Dec). The T-test grouped the treatments in six groups from A to F. Treatment means for groups with same letter do not differ significantly.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.1.2. Purity test Horizon

Purity test data were normally distributed (P>0.250). However, no treatments were significantly different from the control treatment.

4.1.3. Germination test Horizon

Germination data from Horizon were normally distributed (P>0.150), and is presented as percentages. The t-test grouped the treatments in four groups as Figure 15 shows, relating to the germination percentage. The control treatment had a normal germination

of 20%. The t-test indicated that five out of the twelve treatments used in this trial, had a higher germination than the control. While the t-test grouped the results into four groups (Figure 15) only five treatments were greater than the control: T4, T2, T6, T7, and T9. The germination for these treatments ranged from 39% (T6) to 57% (T4). The increase in germination in T4 was about 37% above the control. Germination of treatments T3 and T5 did not differ from control. The ungerminated seed in all treatments were due to BS. Irrespective of treatment however, the germination percentage remained low with high levels of BS still present.

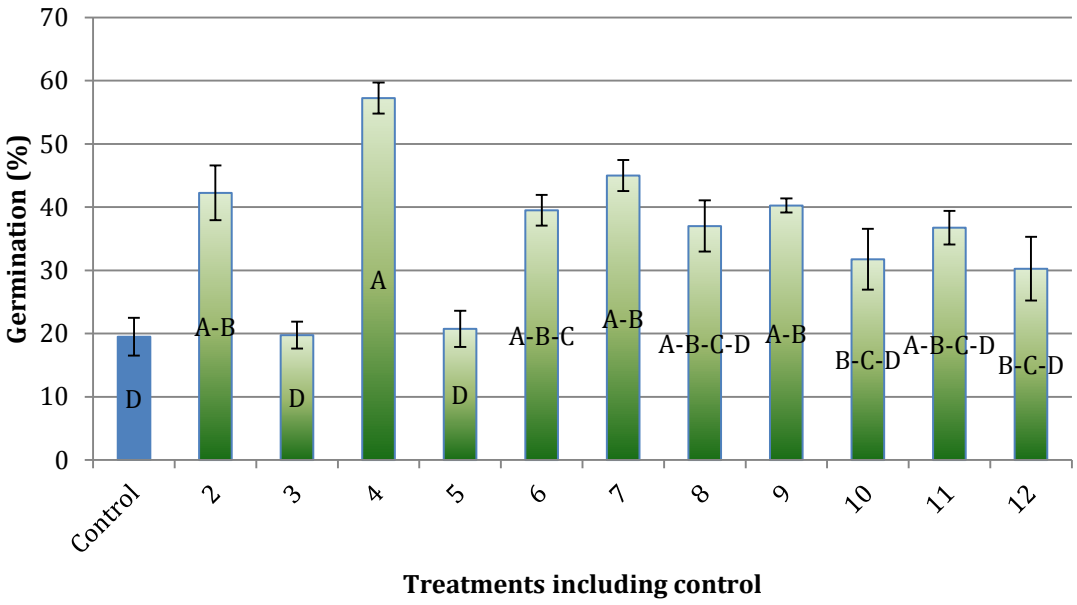


Figure 15: Germination test: Horizon (control vs fungicide treatments at up to two applications – 13 Dec; 27 Dec). The t-test grouped the treatments in four groups from A to D. Treatment means for groups with same letter did not differ significantly.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.1.4. Blind seed test Horizon

At the end of the germination test, seed that had not germinated was analysed for the percentage of BS. This data was shown to be normally distributed by the Kolmogorov-Smirnov test (P >0.150). The t-test grouped the treatments in four groups (Figure 16). The incidence of BS decreased from 11 to 38%, below the control. Group A had the highest percentage of BS present in the seed samples. Similar to the germination test, T3

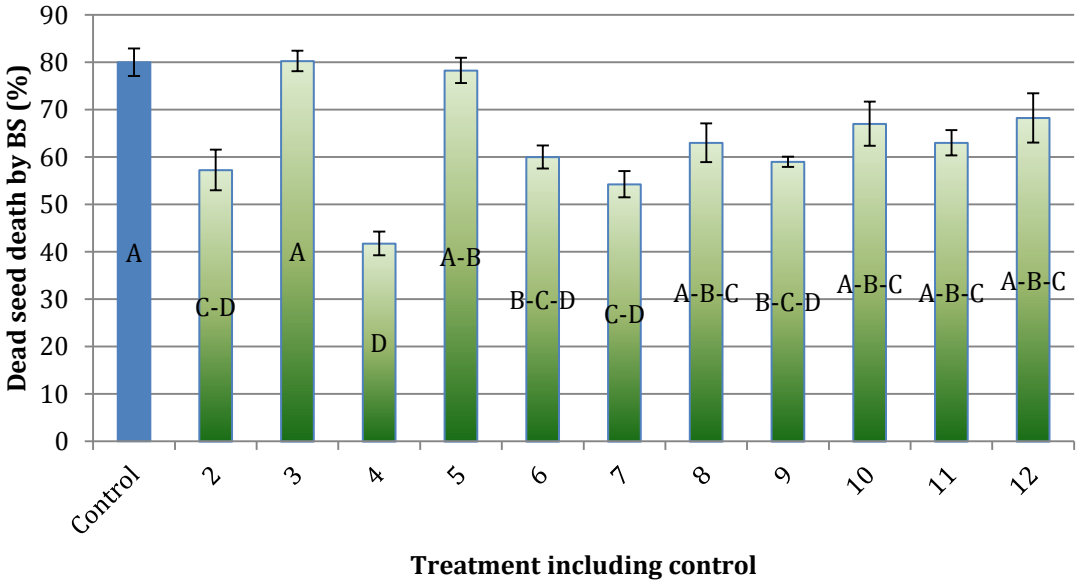


Figure 16: Treatments (control vs fungicide treatments at up to two applications – 13 Dec; 27 Dec) in BS (%) vs germination (%) (Horizon). The t-test grouped the treatments in four groups from A to D. Treatment means for groups with same letter did not differ significantly.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

and T5 did not differ in the amount of BS in comparison to the control (80% BS). Nor did treatments: T8 (63%), T10 (67%), T11 (63%), or T12 (68%) differ from the control in

their level of BS. Conversely, T2, T4, T6, T7 and T9 had a greater reduction of BS incidence in the seed samples. T4 had the lowest and less variable amount of BS. The combination of triazole (prothioconazole) and benzimidazole (carbendazim) increased the control of BS (T4), reducing the infection 38% below the level in the control. The use of prothioconazole (T3) at second application and carbendazim at anthesis application (T5) did not control BS.

4.1.5. Relationship between germination and blind seed Horizon

A regression analysis shows a direct negative linear relationship between germination and BS across all treatments (Figure 17), i.e. as more seeds are infected by BS (%), the germination percentage decreases.

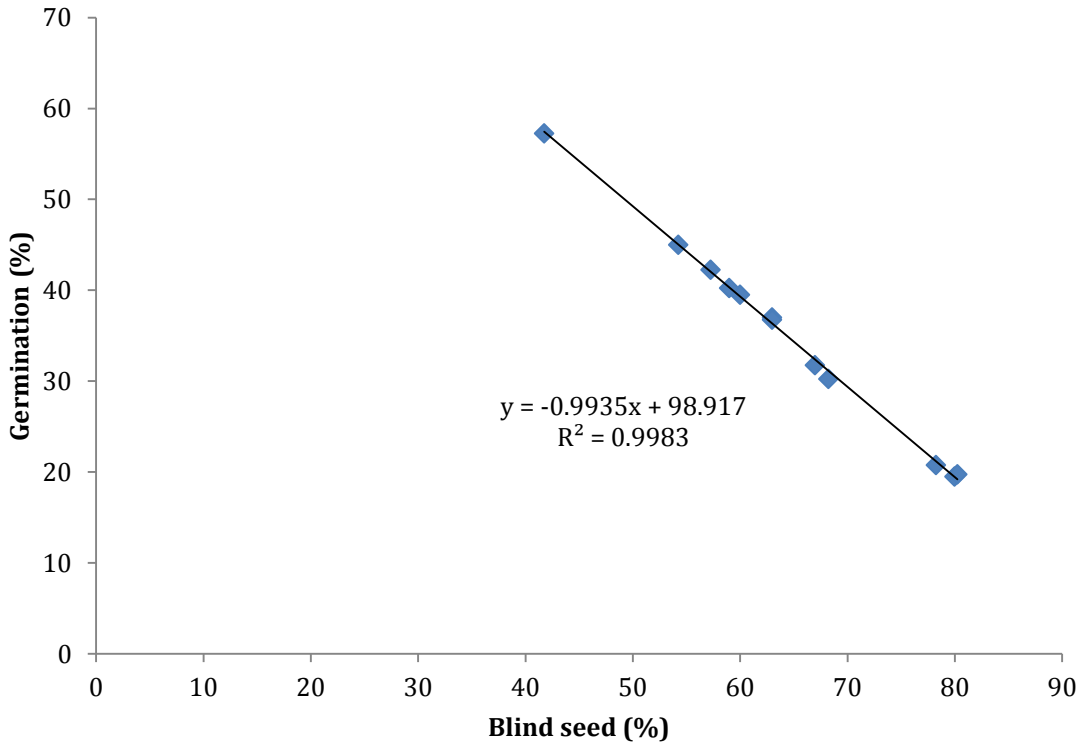


Figure 17: Scatter plot and linear regression: Means of germination (%) vs Blind Seed (%) for perennial ryegrass cv. Horizon.

4.1.6. TPIB test: Endophyte transmission Horizon

Kolmogorov-Smirnov was used to test the normal distribution of the data. This indicated that the data was normally distributed (P>0.130). According to the t-test, this cultivar had a very low percentage of endophyte-infected seed; ranging between 2 (control) and 9 % (T7).

4.2. Trial one: Samson

4.2.1. Thousand seed weight (TSW) test Samson

Data obtained from the average thousand seed weight test (TSW), was normally distributed (P>0.150) according to the Kolmogorov-Smirnov test. The Samson harvested seed weights across treatments were highly different to each other, and to the control treatment. The average seed weight ranged from 1.96 g (control) to 2.25 g (T4). Treatments that did not vary from the control were: T3, T5, T7, T9, T10, T11 and T12 (Figure 18). Treatments T2, T4, T6 and T8 had a greater seed weight than the control.

4.2.2. Purity test Samson

The data collected from the Samson purity test had a normal distribution. None of the components in the purity test were different amongst treatments.

4.2.3. Germination test Samson

The Kolmogorov-Smirnov test indicated that the germination data from trial one (Samson cultivar), was normally distributed (P >0.150). The t-test grouped the treatments in four groups (Figure 19). These data had a germination mean of 52%, with a range from 73% to 20%. The highest quantity of germinated seedlings was obtained in treatments: T4, T2, T8 and T9.

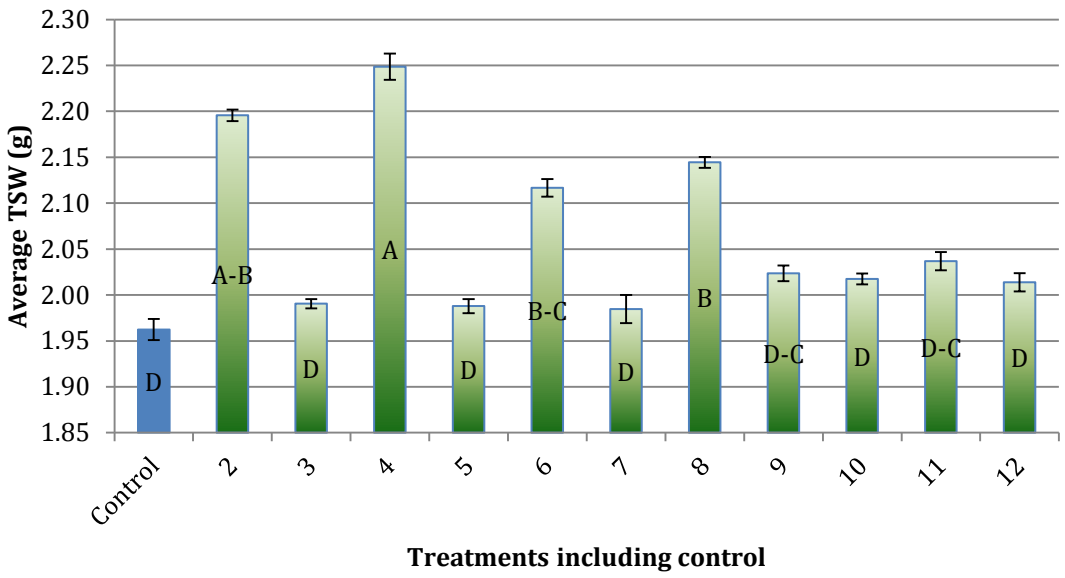


Figure 18: Average thousand seed weight test: Samson (control vs fungicide treatments at up to two applications – 13 Dec; 27 Dec). The t-test grouped the treatments in four groups. Treatment means for groups with same letter do not differ significantly.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

In this cultivar, all fungicide combinations used, except folpet, improved the germination in comparison with the control (Figure 18). The four treatments that had the highest germination (T2, T4, T8 and T9) included triazole, benzimidazole, strobilurin and pyrazole fungicides. The use of triazoles applied at mid- flowering, alone or in combination with other fungicides, gave the best germination responses. In contrast, a mid-seed fill application of prothioconazole (triazole) alone (T3) had a pronounced reduction in germination than when applied at earlier anthesis (T2). Furthermore, most treatments that included carbendazim (a benzimidazole) (T5, T6, T7, T10 and T11) gave poorer results. The four most effective treatments increased Samson germination from

20% (control) to an average of 71% germination, while treatments T3, T5, T6, T7, T10 and T11 increased germination to an average of 50%, 30% above the control.

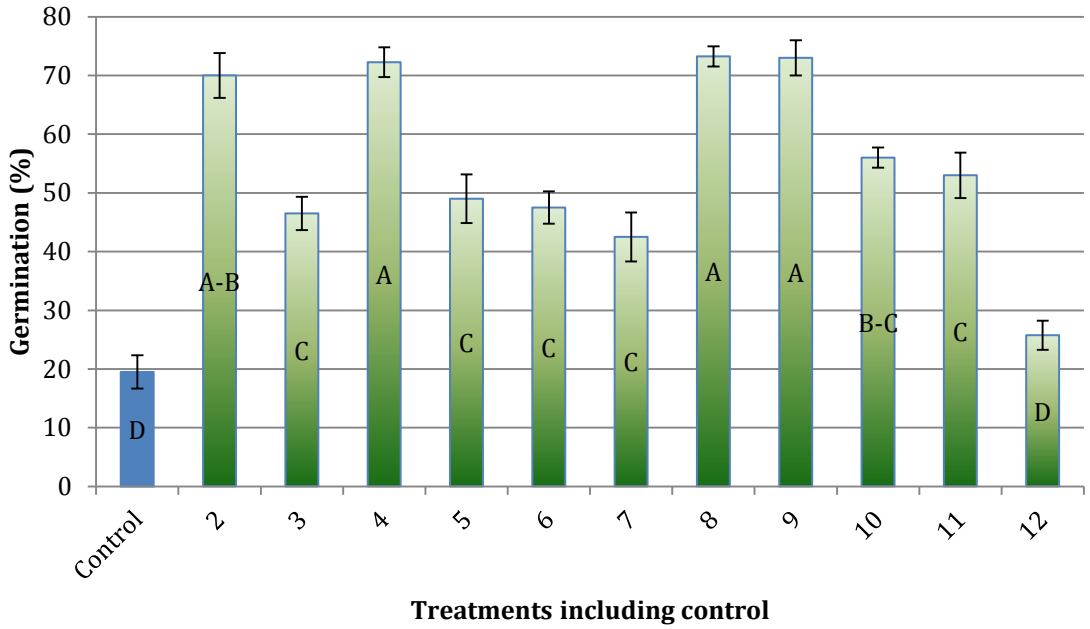


Figure 19: Percentage of germination test: in Samson (control vs fungicide treatments at up to two applications – 13 Dec; 27 Dec). T-test grouped the treatments in four groups. Groups with the same letter are not different from each other.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.2.4. Blind seed test Samson

As with Horizon, the BS data in Samson was collected from seeds remaining at the end of the germination test. Ungerminated, dead seed and abnormal seedling (rated between 0 to 6% in some seed lots) were discarded from this data, after having been evaluated for the presence of BS. The Samson BS data had a normal distribution (P >0.150). The t-test grouped the treatments in four groups. Figure 20 shows 10 out of 11 fungicide

treatments reduced the incidence of BS, in comparison to the control treatment. Treatments T2, T4, T8 and T9 had the lowest incidence of BS present in the harvested seed, while treatments T3, T5, T6, T7, T10 and T11 had a more moderate effect. T12 was not different from the control.

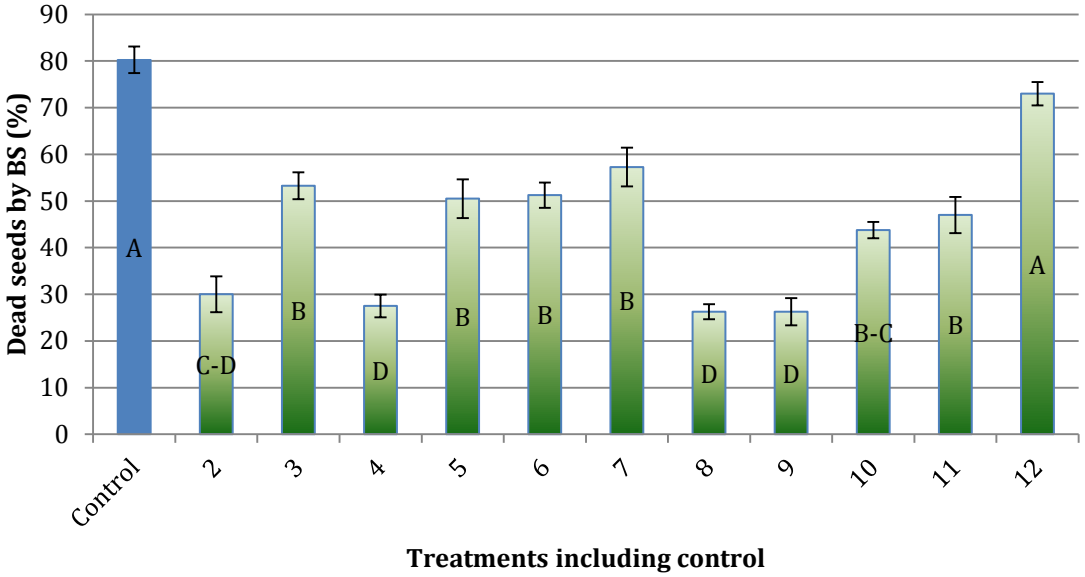


Figure 20: Mean of blind seed (%) vs treatments (control vs fungicide treatments at up to two applications – 13 Dec; 27 Dec) in Samson from four replicates. The t-test grouped the treatments in four groups from A to D. Treatment means for groups with same letter did not differ significantly.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.2.5. Relationship between germination and blind seed Horizon

The relationship between germination and BS on Samson is displayed in Figure 21. There is again a strong negative linear relationship between germination and BS, i.e. the

percentage of germination increases when the incidence of BS decreases in the same manner as for the cultivar Horizon.

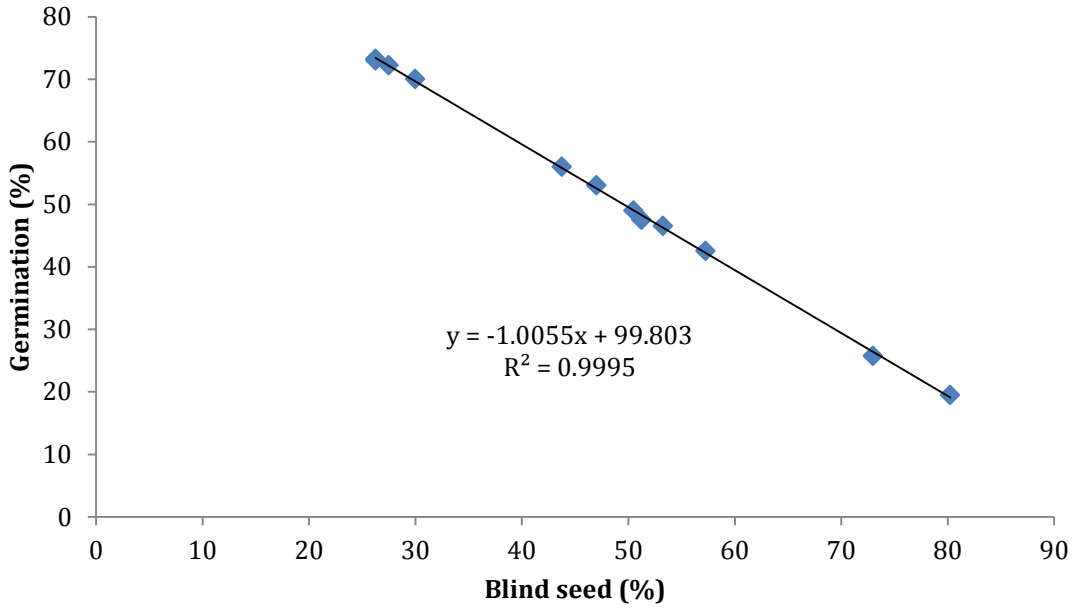


Figure 21: Scatter plot and linear regression: Means of Germination (%) vs Blind Seed (%) for perennial ryegrass cv. Samson.

4.2.6. TPIB test: Endophyte transmission Samson

Viable endophyte ranged from 58 to 78% (Figure 22). The Kolmogorov-Smirnov test determined that Samson data from the TPIB test was normally distributed (P >0.150). Analysis of the data used a t-test to identify differences amongst treatments. The endophyte levels were not significantly lower than that of the control for any treatment except (T12).

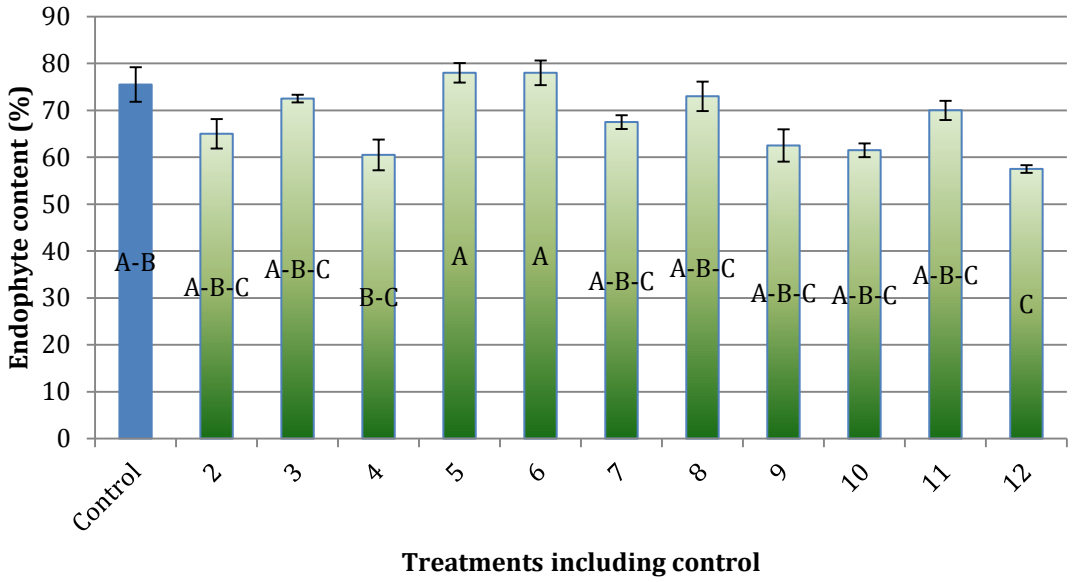


Figure 22: Mean % viable AR37 endophyte content for each seed lot treated in Samson. The t-test grouped the treatments in four groups from A to D. Treatment means for groups with same letter did not differ significantly.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.2.7. Comparison of TSW and BS between Horizon and Samson

The difference in seed weights between the control treatments of Horizon and Samson were 3.059 g vs 1.962 g, respectively. In 4n Horizon, the mean weight range was between 2.859 to 3.771 g for the treatments, whereas in 2n Samson, the mean seed weight ranged from 1.962 to 2.249 g. Overall, Samson had a lower seed weight.

After comparing the BS infection between cultivars, treatments T3, T4, T5, T8 and T9 in Horizon has a higher incidence of BS in comparison to Samson. There was no difference between the cultivars for T1 (control), T2, T6, T7, T10, T11, and T12 ($P>0.05$).

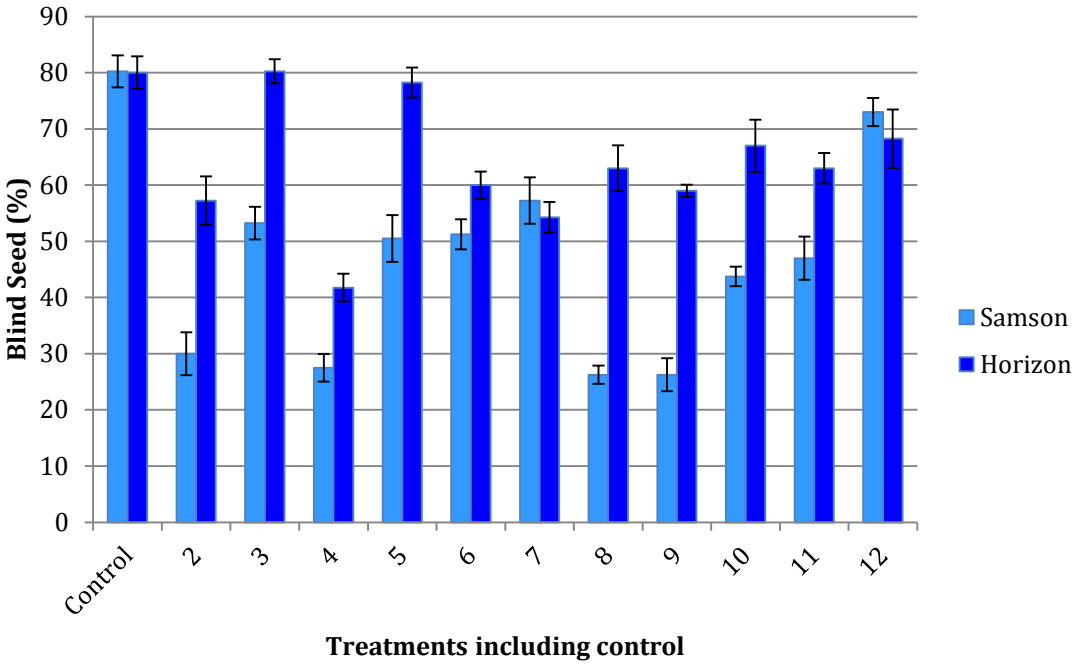


Figure 23: Comparison of blind seed incidence between ryegrass cultivars Horizon and Samson for each fungicide treatment used.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.2.8. Comparison between Horizon and Samson germination after different fungicide treatments

The t-test indicated germination differences in T3, T4, T5, T8, and T9 between Samson (2n) and Horizon (4n) (Figure 24), similar to the results obtained from BS. Samson had higher germination as a result of better control of BS.

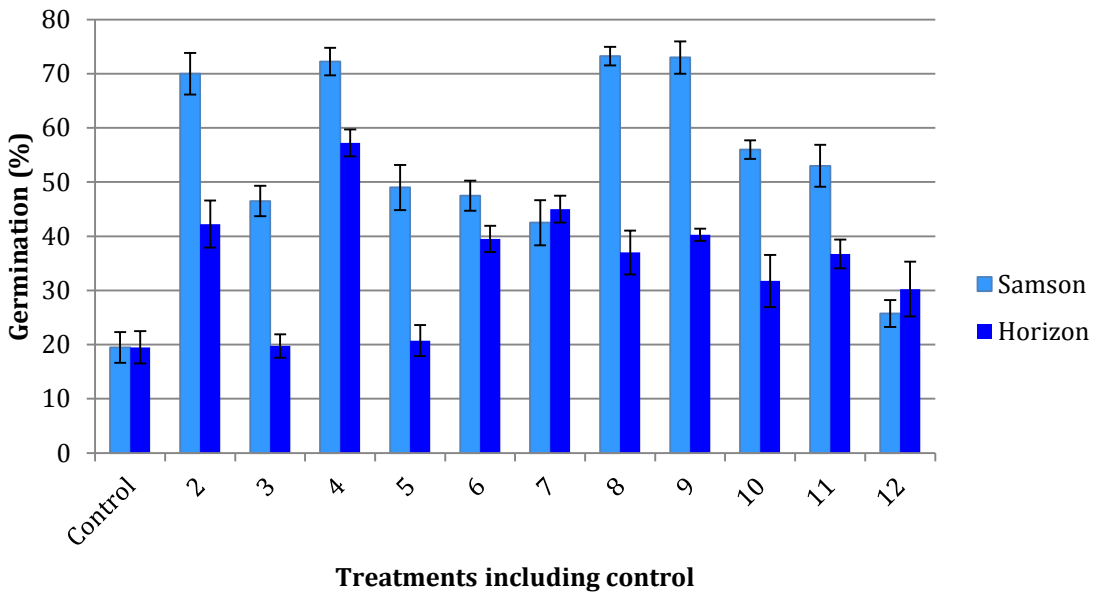


Figure 24: Comparison of germination (%) between ryegrass cultivars Horizon and Samson for each fungicide treatment used.

1= control

2= 100 g/ha prothioconazole – 1st application

3= 100 g/ha prothioconazole 2nd application

4= 100 g/ha prothioconazole + 250 g/ha carbendazim – applied twice

5= 250 g/ha carbendazim – 1st application

6= 250 g/ha carbendazim – 2nd application

7= 250 g/ha carbendazim – applied twice

8= 125 g/ha azoxystrobin + 189.2 g/ha tebuconazole – applied twice plus 250 g/ha carbendazim 2nd application

9= 100 g/ha prothioconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

10= 189.2 g/ha tebuconazole + 75 g/ha isopyrazam + 250 g/ha carbendazim – applied twice

11= 189.2 g/ha tebuconazole + 250 g/ha carbendazim – applied twice

12= 375 g/ha folpet - applied twice

4.2.9. Seed squash test

A seed squash test was performed to check the incidence of endophyte in seeds from Horizon and Samson cultivars. The results obtained from this test were not satisfactory because in many seeds AR37 and BS were both present amongst the aleurone cells (N. Grbavac, personal communication, December, 2013), and the high incidence of BS hyphae made it impossible to observe endophyte hyphae.

4.3. Trial two: PGone50

4.3.1. PGone50 thousand seed weight (TSW)

The average TSW variable of the PGone50 ryegrass cultivar had a normal distribution according to the Kolmogorov-Smirnov test (P >0.150). The t-test separated the data into five groups (Figure 25). Only T2 and T8 had a TSW significantly lower than the control.

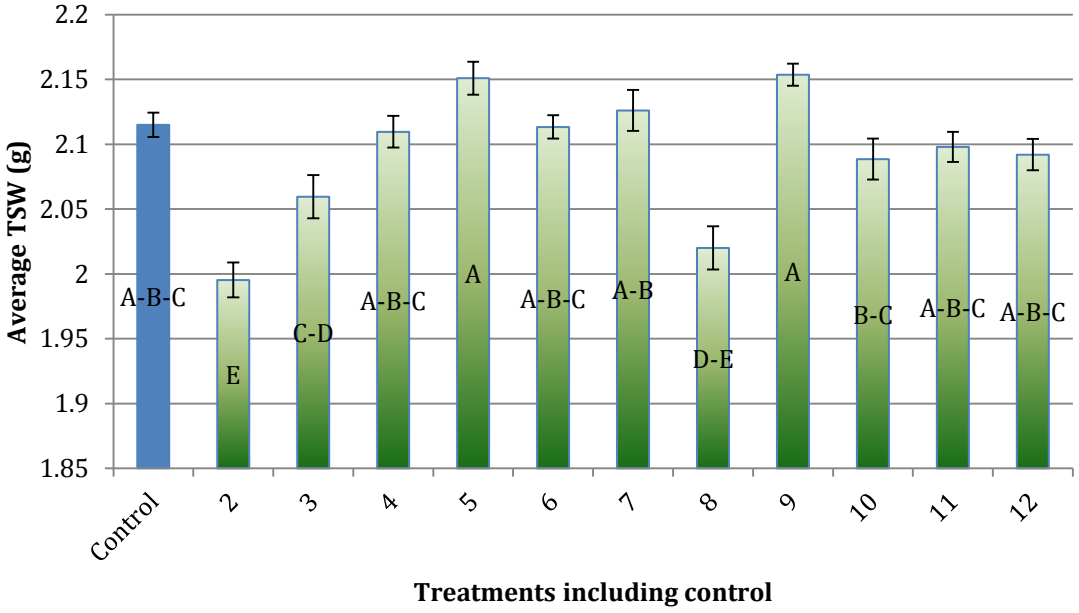


Figure 25: Average thousand seed weight test: PGone50 (control vs fungicide treatments at one application – 24 December). The t-test grouped the treatments in five groups. Treatment means for groups with same letter did not differ significantly.

1= Control

2= 75 g/ha prothioconazole

3= 75 g/ha prothioconazole + 125 g/ha pyraclostrobin

4= 75 g/ha prothioconazole + 75 g/ha isopyrazam

5= 125 g/ha pyraclostrobin + 75 g/ha isopyrazam

6= 75 g/ha isopyrazam

7= Product A

8= Product B

9= 375 g/ha folpet

10= 375 g/ha folpet + 125 g/ha pyraclostrobin

11= 189.2 g/ha tebuconazole + 125 g/ha azoxystrobin

12= 125 g/ha azoxystrobin + 75 g/ha isopyrazam

4.3.2. Purity Test

Before analysis the purity test components (pure seeds, other seeds, inert matter, ergot, empty seeds), were assessed by Kolmogorov-Smirnov, and found not to be normally

distributed. To normalise it, the data was transformed using an arc sin square root transformation and then analysed using the Kruskal-Wallis test, where P≥0.05 was considered to have a normal distribution. This indicated that there was no significant difference between the control and any fungicide treatments.

4.3.3. Germination PGone50

Germination data had a normal distribution, with a P>0.091 from the Kolmogorov-Smirnov. The t-test indicated that germination did not differ amongst treatments. Mean germination was 86% and the range was from 84 to 89% (Figure 26).

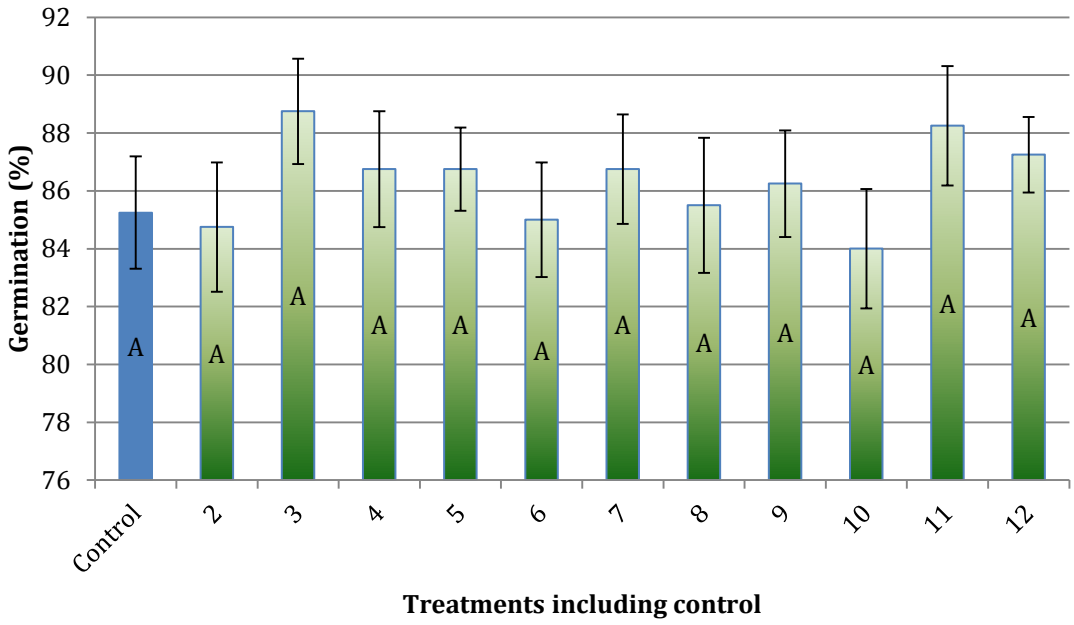


Figure 26: Percentage of germination test: In PGone50 (control vs fungicide treatments at one application – 24 December). Treatments did not different from each other.

1= Control

2= 75 g/ha prothioconazole

3= 75 g/ha prothioconazole + 125 g/ha pyraclostrobin

4= 75 g/ha prothioconazole + 75 g/ha isopyrazam

5= 125 g/ha pyraclostrobin + 75 g/ha isopyrazam

6= 75 g/ha isopyrazam

7= Product A

8= Product B

9= 375 g/ha folpet

10= 375 g/ha folpet + 125 g/ha pyraclostrobin

11= 189.2 g/ha tebuconazole + 125 g/ha azoxystrobin

12= 125 g/ha azoxystrobin + 75 g/ha isopyrazam

4.3.4. PGone50 endophyte content

These data had a normal distribution according to the Kolmogorov-Smirnov test (P>0.064). The t-test showed that the endophyte level did not differ significantly between the control and fungicide treatments. Endophyte content ranged between 70 to 77%.

4.3.5. PGone50 seed yield by treatment

Seed yield data was provided from the Lincoln trial and analysed as part of this research. Seed yield from all the treatments was analysed (without performing an arc sin transformation) with the Kolmogorov-Smirnov test (P>0.150). The t-test demonstrated that T4, T7, T10, and T11 have a higher seed yield than the control. The seed yield mean across all treatments was 2,348 kg/ha, with a range of 2.230 kg/ha to 2,413 kg/ha (Figure 27).

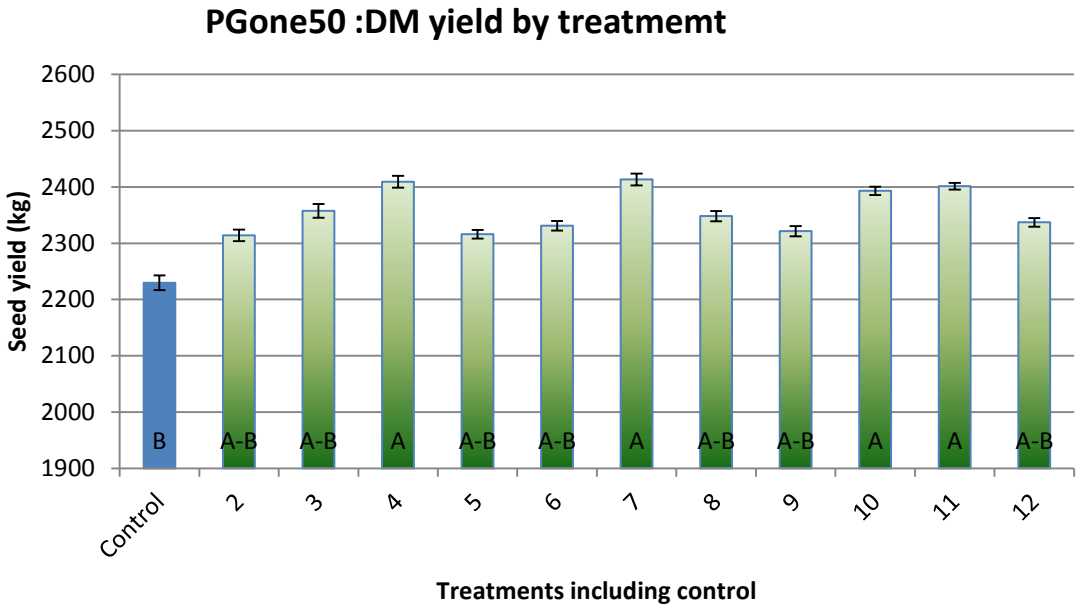


Figure 27: Seed yield (kg): PGone50 (control vs fungicide treatments at one application – 24 December). Treatments four, seven, ten and eleven were significantly different from control.

1= Control

2= 75 g/ha prothioconazole

3= 75 g/ha prothioconazole + 125 g/ha pyraclostrobin

4= 75 g/ha prothioconazole + 75 g/ha isopyrazam

5= 125 g/ha pyraclostrobin + 75 g/ha isopyrazam

6= 75 g/ha isopyrazam

7= Product A

8= Product B

9= 375 g/ha folpet

10= 375 g/ha folpet + 125 g/ha pyraclostrobin

11= 189.2 g/ha tebuconazole + 125 g/ha azoxystrobin

12= 125 g/ha azoxystrobin + 75 g/ha isopyrazam

Chapter 5. Discussion

It is usual for ryegrass seed growers to apply foliar fungicides to control *G. temulenta* (Hardison, 1970; Rolston and Falloon, 1998; FAR, 2006). However, use of fungicides may affect the vertical transmission of desirable novel endophytes from the parent plant into the developing seed in perennial and hybrid ryegrass cultivars (FAR, 2008). It has been reported by FAR (2008) that the use of triazole fungicides such as prothioconazole or tebuconazole depress AR37 endophyte viable levels in harvested seed more than the use of carbendazim.

5.1. Trial One: Blind seed Horizon

5.1.1. Thousand seed weight (TSW)

With respect to TSW, the use of prothioconazole, carbendazim and especially the combination of azoxystrobin plus tebuconazole at mid-flowering (13 Dec) and azoxystrobin, tebuconazole, and carbendazim at mid-seed fill (28 Dec), increased the seed weight in Horizon (T2, T7, T8). The use of triazoles (prothioconazole and tebuconazole) increased seed weight in resultant seed lots (PA *et al.*, 2010). The use of carbendazim also increased the seed weight in T7 when applied at mid-seed fill. Differences in weight between prothioconazole application timing (T2 and T3) demonstrated that application at mid-flowering has a greater effect in increasing the seed weight in this cultivar (Mebalds, 2010). Prothioconazole targets primary infection during anthesis, therefore reducing BS in T2 compared with T3. In T3 the higher presence of BS means that the TSW is a combination of both healthy seed and BS infected seed. The seed containing BS are lighter than uninfected seed resulting in a lower TSW (Alderman, 2001; Neill, 1939).

5.1.2. Effectiveness of fungicide treatments on germination and control of blind seed disease

The primary purpose of this trial was to determine the effectiveness of a range of fungicide combinations on the control of BS caused by the fungal pathogen *G. temulenta*. Infection and the parameters used in the trial establishment and subsequent management during the growing season were designed to promote the incidence of BS. As a result, the BS incidence was very high (control=80%) and consequently seed germination was low (control=20%). Several fungicide combinations were able to improve the germination performance by controlling BS. Despite the low performance that Horizon had in this study, the application of 100 g/ha prothioconazole with 250 g/ha carbendazim applied twice (mid-flowering and mid-seed fill) (T4) had the best results, reducing BS incidence from 80% to 42%, and also increasing germination from 20% to 58%. It is likely that this was effective due to the combined action of both fungicides: with prothioconazole[1] targeting primary infection (as seen in the results), and carbendazim targeting later secondary infections (Bayer, 2013; Zelam, 2014; FRAC, 2013). Chynoweth (2012) used a double application of prothioconazole combined with carbendazim (mid-seed fill) with Samson, similar to T4 in this study, and also found good control of BS. This result is discussed in 5.2.1.

The use of prothioconazole in T2 (mid-flowering) in this study and (Chynoweth *et al.*, 2012), gave a higher germination than T3 (mid-seed fill), again demonstrating the importance of controlling primary infection during flowering (Bayer, 2013).

The poor germination in T5 is also the result of inappropriate timing of carbendazim being applied at mid-flowering rather than at mid-seed fill where it controls secondary infections (FRAC, 2013). In T6 and T7, with a single late application or double application of carbendazim respectively, secondary infection is controlled and as a consequence, germination in T6 and T7 is significantly higher than T5. Moreover, T6 and T7 did not differ significantly in their germination indicating that the early application of carbendazim was ineffective and not required. According to recommendations from FAR (FAR, 2007b), carbendazim treatments should be repeated at seven day intervals if rain

[1] Prothioconazole stimulates important plant physiological factors, including photosynthesis (Bayer, 2013). Also has a target action on the carbon 14 demethylation in sterol biosynthesis, removing vital molecules of the fungi, whereas carbendazim is an important and potent inhibitor of ß-tubulin polymerization (FRAC, 2013).

(approximately 3 mm or more) or irrigation events occur up to and immediately before mowing. During the production of the seed used in this study, a rainfall event of greater than 3 mm (6.6 mm) occurred four days (31 Dec) after the application of carbendazim (Figure 6) which may explain the incomplete control of BS in those treatments where carbendazim was applied as a late treatment.

5.1.3. Seed endophyte levels

The percentage of Horizon plants with endophyte infection in this study was low as determined by the examination of seedlings grown from the seed obtained from the different plots of this trial. The cause of this was most likely that the trial area was sown with Horizon seed containing a low level of viable endophyte.

5.2. Trial one: Blind seed disease in Samson

5.2.1. Thousand seed weight

The single applications of prothioconazole (T2 and T4), increased significantly the seed weight of Samson in this study. This can be a direct result of the substantially reduction of BS incidence in the seed lot by the use of prothioconazole.

5.2.2. Effectiveness of fungicide treatments on germination and control of blind seed disease

Germination in Samson with all fungicide treatments was higher, and conversely BS was lower, than the control (except T12). Within fungicide treatments some (T2, T4, T8 and T9) gave better control of BS than others (T3, T5, T6, T7 and T11). As in Horizon three of the four best treatments included prothioconazole (T2, T4 and T9) as a first application at mid-flowering. The ability of prothioconazole at first application (mid-flowering) to control BS in Samson has also been shown by Chynoweth (2012) in seed produced in the 2007-2008 season, suggesting that this treatment is effective over a range of growing conditions. In Chynoweth (2012), application of 200g/ha prothioconazole at mid-flowering on 1 Dec increased germination to 82% compared with 70% in this study. This may be partly due to the much higher levels of BS inoculum in this study (80%).

The present study applied 100 g/ha prothioconazole, suggesting that the higher 200 g/ha rate may not be necessary. Regardless, neither trial achieved complete control of BS. This may be because of the extended period available for infection from the beginning of anthesis until close to cutting. In contrast to Horizon, a second application (mid-seed fill) of prothioconazole in Samson gave better control of BS than the control. This may be due to Samson having smaller seeds possibly resulting in a less tightly packed seedhead and, therefore, allowing better penetration of the fungicide into sites of secondary infection in the developing seeds.

In addition, T4, T8 and T9 included carbendazim at the later application (mid-seed fill). This suggests, as in Horizon, the key to effective control of BS disease is the early application of prothioconazole and the late application of carbendazim. In Samson, azoxystrobin combined with tebuconazole (T8) appears to be able to be substituted for prothioconazole. In contrast these two fungicides in combination, and with carbendazim as a second application, did not give any better control in Horizon compared with most other fungicide treatments. The reason for the better response in Samson is unclear but may be related to differences between ploidy cultivars. However, this was not reflected in the untreated treatments of both cultivars, where both have exactly the same amount of BS and germination rate. But it is more likely that the later flowering of Horizon (3 days later than Samson) at the moment of the fungicides application may have affected the protection of the flower given by the fungicide. This could have caused a difference in the uptake of the product by the flower. Loss of the foliar application by runoff in that time was unlikely, because the amount of rain in this period was quite low according to the Lincoln, Broadfield Ews climate data.

There was no difference in BS control between T4 and T9. These treatments were identical except for the addition of 75 g/ha isopyrazam in T9. This fungicide is known to be effective against crown and stem rusts but not BS (Syngenta, 1997). This study has demonstrated that the addition of isopyrazam is not detrimental to the control of BS, but also in the absence of rust, its inclusion is not required, although standard agronomic practice in ryegrass seed production is to include fungicides for rust control (IHSG, 2013).

No control of BS was achieved with 375 g/ha folpet (T12). This fungicide is not known to control BS, and moreover this product is not recommended by the label to be use on ryegrass (PMRA, 2008), therefore this result was not unexpected.

5.2.3. Seed endophyte levels

None of the fungicides reduced endophyte transmission compared with the control, except folpet (T12). A major concern with the use of fungicides to control crown and stem rusts, as well as BS in ryegrass is their potential to reduce endophyte transmission (FAR, 2008; Harvey and Harvey, 2009; Chynoweth *et al.*, 2012). The results of this trial have identified a range of fungicide combinations that include fungicides used to control crown and stem rusts, and BS, but those do not significantly reduce endophyte transmission. The effects of triazole fungicides, such as prothioconazole and tebuconazole, on endophyte transmission is variable and appear to depend on the endophyte strain and fungicide combination effects (Rolston *et al.*, 2002a). The identification of other fungicide families that do not have a detrimental effect on AR37 endophyte transmission but which are also able to control BS disease and are labelled for use against crown and stem rust provides an alternative to the use of triazoles. However, the use of fungicides needs to be evaluated under a range of conditions and with different endophyte strains. Rolston *et al.* (2002) investigated the use of strobilurin fungicides in AR1 and found no detrimental effect on the transmission of endophyte. Chynoweth *et al.* (2012) used the strobilurin fungicide azoxystrobin in combination with tebuconazole, prothioconazole and carbendazim and found detrimental effects on endophyte transmission in one combination but not others. None of the combinations used by Chynoweth *et al.* (2012) are the same as the one used in this trial which also did not have a detrimental effect on endophyte transmission.

There are reports in the literature that endophytes have antifungal action in grasses (Cheplick *et al.*, 1989; Christensen, 1996; Yue *et al.*, 2000; Rubini *et al.*, 2005; Popay and Bonos, 2008) but none have been reported for BS. In Samson only folpet (T12) had significantly lower endophyte content. This treatment also gave no control of BS. This is due that it has as target black rot and downy mildew (PMRA, 2008) and it is not labelled to be used in ryegrass. Also, it may suggest that the endophyte is having an anti-BS effect but this is speculative because the majority of fungicide combinations did not differ in

their endophyte level, and moreover, all other fungicide treatments controlled BS, therefore any effect of endophyte is confounded with fungicide effects.

5.2.4. Controlling blind seed without reducing endophyte transmission

Only four fungicide combinations gave the highest control of BS without adversely affecting endophyte transmission: T2, T4, T8 and T9. T2, T4 and T9 included fungicides from the triazole family, whereas T8 included both strobilurin and triazoles. Early application of prothioconazole gave effective control of BS on its own. This is in contrast to Chynoweth et al. (2012), where all prothioconazole treatments were in combination with other fungicides and involved more than one application of prothioconazole. For control of BS, multiple applications and the inclusion of other fungicides do not appear to be required, but the effect on other diseases such as crown and stem rust was not evaluated in this trial.

This study is also the first to report the effect of isopyrazam on BS and endophyte transmission. According to International Herbage Seed Group (2013) the combination of isopyrazam with strobilurin fungicides are an effective control of stem rust. In comparison to this study, the application of isopyrazam combined with prothioconazole plus carbendazim applied at mid-flowering and mid-seed fill, seems to give a better control of BS (T9=27% BS) than combined isopyrazam with tebuconazole and carbendazim (T10=44% BS). But T10 is no more effective than T4 which is the same as T10 except T10 had isopyrazam. This suggests that the addition of isopyrazam is not needed.

5.3. Trial Two: Endophyte transmission PGone50

5.3.1. Thousand seed weight

In trial two, thousand seed weight (TSW) demonstrated that most of the treatments do not differ from the control, except T2 and T8, in the reduction of seed weight. Due to the confidentiality of T8, only the reduction of seed weight relating to T2 will be discussed. In this trial, the use of prothioconazole applied at mid-flowering had the lower seed weight. In contrast, Samson with also a mid-flowering application, prothioconazole

increased the seed weight. This is because Samson had high levels of BS which were controlled by the fungicide whereas PGone50 there was no BS.

5.3.2. Effectiveness of fungicide treatments on germination

All treatments used in this trial did not differ from the control treatment. All treatments (including control) had germination between 84 and 89%. However, as explained earlier (Section 3.2.), in contrast to trial one, trial two was not established or managed to especially promote *G. temulenta* pathogen infection; in addition, most of the fungicides used in this trial are more targeted to control stem rust and crown rust. Therefore the result of low BS and high germination across all fungicide treatments is not unexpected.

5.3.3. Effectiveness of fungicide treatments on the endophyte content

The transmission of AR37 endophyte in perennial ryegrass diploid cultivar PGone50 was not affected by the fungicide treatments used. Most of the fungicides used in this trial are known to control stem rust and BS, as a fungicide management approach to control both, maintaining the noble endophyte (IHSG, 2013). The content of AR37 endophyte in the seed ranged from 71 to 77%, av. 73% (control included). It is therefore suggested that the use of any of the fungicides at the rates reported in Table 9, can be used to control rust pathogens without having a negative effect on AR37 endophyte transmission in PGone50. However, their effectiveness as a control of pathogens in this trial was not performed and therefore remains unknown.

It seems that the use of prothioconazole can maintain effective endophyte transmission , only if a lower amount (T2)of prothioconazole is applied to the seed crop (75 g/l/ha prothioconazole); this finding is in contrast to previous studies where prothioconazole decreased endophyte transmission by applying 125 g/ha prothioconazole (Rolston *et al.*, 2002a), and 200 g/ha prothioconazole (Chynoweth *et al.*, 2012).

In this trial, T4 maintained the AR37 endophyte as it appears that isopyrazam does not affect the endophyte content. However, in trial one, the use of isopyrazam did not affect the endophyte and BS. Its effects in this trial confirm that isopyrazam was not needed in these fungicide combinations. The combination in T5 of isopyrazam (that not affected

the fungus in this study) with pyraclostrobin (a strobilurin member) in this treatment provided protection against pathogens possibly present in that time, thus having seeds with high germination rates. In a different genus, (tall fescue), Walker (2008), applied 600 g/ha of pyraclostrobin, affecting the endophyte content in tall fescue. In contrast, the application of lesser quantity in perennial ryegrass (125 g/ha of pyraclostrobin) in this trial did not affect the endophyte content. The difference of response in this study in comparison to Walker (2008) is due to the high rate applied by Walker, and by the differences of genus used in these studies.

5.3.4. Seed yield

According to the data obtained from FAR in relation to seed yield, the results demonstrated that the use of 75 g/ha prothioconazole + 75 g/ha isopyrazam; 375 g/ha folpet + 125 g/ha pyraclostrobin; 189.2 g/ha tebuconazole + 125 g/ha azoxystrobin, increase the seed yield, without also affecting the AR37 endophyte. It is possible that the increase of TSW in these treatments was related to the reduction of possible diseases present in this trial such as rusts (unidentified in this study), that were better controlled by these treatments.

5.3.5. Comparison trial one and trial two: Samson and PGone50

Foliar application of prothioconazole (100g/ha) at mid-flowering (13 Dec) in trial one (T2) had a lower performance (70% germination) in comparison to T2 from trial two, where the application of 75 g/ha of prothioconazole on PGone50 (a late flowering cultivar) and also at mid-flowering (24 Dec) had greater germination. This difference was because the incidence of pathogens in trial two was absent in comparison to trial one.

The use of folpet (T12) in trial one, despite being applied at both mid-flowering and mid-seed fill, had a low effect in controlling BS as it is a fungicide that targets black rot and downy mildew (PMRA, 2008) and is not labelled to be used in ryegrass. In trial two, folpet appears to have no effect on the AR37 endophyte when it is applied once, but it does when applied twice as in trial one. Therefore, a double application of 375g/ha of folpet is not suggested.

Chapter 6. Conclusion

This study was undertaken to test different fungicides treatments designed to control BS disease (and other pathogens), and its effects on the transmission of the AR37 endophyte into the new seed generation. Two trials were part of this study, where trial one was designed to test some fungicides treatments that (applied to Samson and Horizon ryegrasses) would control the incidence of BS, assessing also any detrimental effect from these fungicides on the transmission of AR37 endophyte in seed. Trial two (PGone50) solely evaluated the effect of different fungicide treatments used to control crown rust, septoria and powdery mildew, among others, on the transmission of the AR37 endophyte.

In trial one, Horizon performed poorly in relation to germination, with a low control of BS disease. In addition, the endophyte content in this cultivar was almost nil, preventing any assessment of the effects of fungicides on endophyte transmission. There were no differences between Samson and Horizon controls regarding BS content demonstrating that ploidy level did not influence BS incidence. The differences between Horizon and Samson in BS content after fungicide treatments may be due to the fact that Samson has smaller seeds, possibly resulting in a less tightly packed seedhead and, therefore, allowing better penetration of the fungicide into sites of secondary infection in the developing seeds.

Only four fungicide combinations gave the highest control of BS without reducing the endophyte: T2, T4, T8 and T9. T2, T4 and T9 included fungicides from the triazole family, whereas T8 included both strobilurin and triazoles. The ability of prothioconazole to control BS in Samson, using a lower quantity of product than previous studies, has been demonstrated, but only when it is applied at mid-flowering. Regardless, neither trial achieved complete control of BS. This may be because of the extended period available for infection from the beginning of anthesis until close to cutting.

No control of BS was achieved with the use of folpet (T12). This fungicide is not known to control BS, and, moreover, this product is not recommended by the label to be use on ryegrass (PMRA, 2008); this result, therefore, was not unexpected.

Regarding the transmission of the endophyte, none of the fungicides reduced endophyte transmission compared with the control, except folpet (T12) where a double application seemed to reduce endophyte content.

This study is also the first to report the effect of isopyrazam on BS and endophyte transmission. The application of isopyrazam combined with prothioconazole plus carbendazim applied at mid-flowering and mid-seed fill gave a better control of BS (T9=27% BS) than combined isopyrazam with tebuconazole and carbendazim (T10=44% BS). This suggests that the combination of isopyrazam with prothioconazole and carbendazim is an effective control of BS when applied at mid-flowering and mid-seed fill. In comparison, there was no difference in BS control between T4 and T9. These treatments were identical except for the addition of 75 g/ha isopyrazam in T9. This study has demonstrated that the addition of isopyrazam did not adequately control BS disease, therefore is not needed.

In this study, it is seen that the use of prothioconazole did not greatly affect the endophyte, used solo or combined. The use of prothioconazole at mid-flowering had better outcomes in controlling *G. temulenta* than when used at mid-seed fill, as occurred with Samson and Horizon (13th December). There were no differences of AR37 endophyte content between Samson and PGone50, except by the combined use of prothioconazole and carbendazim; and the use of folpet applied to the sward in Samson. This suggests that 75g/ha of prothioconazole has better outcomes in increasing germination and maintaining the endophyte content on 2n perennial ryegrass cultivars. In addition, it is recommended to control BS using prothioconazole combined with other product such as benzimidazole, avoiding thus a possible loss of sensitivity from BS to the use of prothioconazole, as is reported happening with Septoria.

Regarding to trial two (relating to AR37 endophyte transmission), there were not visible deleterious effects on PGone50 from the treatments utilised. Therefore, all treatment used in this trial maintained the presence of AR37 in the seed lot. All treatments (including the control) have a germination level between 84 to 89%. The content of the AR37 endophyte in the seed samples ranged from 71 to 77%.

It seems that the AR37 endophyte has less viability when fungicides used to control pathogens are applied at too high concentrations (such as prothioconazole, folpet) or if an overlapping of fungicides is used. Endophyte transmission and BS disease were not eliminated totally by any of the treatments.

To increase the control of BS while maintaining endophyte content, a good fungicide programme should be designed. This should not contain excessive fungicide rates and be designed with an awareness of application frequency. This accompanied, as seen in previous studies, by adequate crop architecture; applications of nitrogen used to increase vegetative growth which creates a barrier to ascospore movement; and an appropriate application of plant growth regulator (trinexapac-ethyl). Ongoing research is required into treatments that will enhance seed production while not adversely affecting transmission of endophyte into seed or the retention of viability of endophyte during seed storage.

Chapter 7. Recommendations

Four fungicide application treatments that have provided good control of BS disease while not adversely affecting the transmission of AR37 endophyte into the seed were revealed in these studies. These fungicide regimes are: 1) the combination of 125 g/ha azoxystrobin with 189 g/ha tebuconazole applied twice (at mid-flowering and mid-seed fill) and 250 g/ha carbendazim at mid-seed fill; 2) the use of 100 g/ha prothioconazole + 250 g/ha carbendazim applied twice (at mid-flowering and mid-seed fill); 3) application of 100 g/ha prothioconazole, 75 g/ha isopyrazam and 250 g/ha carbendazim at mid-flowering; 4) and 100 g/ha prothioconazole applied at mid-flowering and mid-seed fill control BS disease and maintain the presence of the AR37 endophyte in the resulting seed lot. However, these findings were based on a single season in one location and so further trials are essential to confirm these findings.

However, the continued application of group members of benzimidazole (such as carbendazim) produces positive cross resistance. Ten mutations have also been found that confer resistance to benzimidazoles and which have been identified in the ß-tubulin gene (FRAC, 2013). It is recommended that there is no less than 10 to 14 days between applications to avoid resistance from the fungus. The use of prothioconazole is also recommended in lesser quantities and in combination with other products, due to the reduction of seed endophyte transmission. There are further problems relating to the sole reliance on triazole-based fungicides with reports of developing disease resistance.

Folpet (T12) is not recommended due to its poor control of BS. In addition, the double application affects the AR37 transmission (reduced 18% AR37 content); therefore, growers need to be cautious when applying this product to seed grasses containing this endophyte. It is recommended that application happens once at early flowering with 375 g/ha of folpet (as trial two) to maintain the endophyte transmission.

The current study performed on Horizon should be repeated with known and high endophyte content due to the possibility that endophyte survival and transmission may be detrimentally affected by a fungicide cultivar interaction. It would be interesting to study the differences in responses of different diploid level cultivars to fungicides treatments.

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Chapter 9. Appendices

All statistical analysis performed with SAS 9.3 (SAS, 2009) in this thesis are attached next. Results for each test are attached along with the section number.

9.1. Trial one: Horizon (refer Section 4.1)

9.1.1. TSW test Horizon: T- tests (LSD) (refer Section 4.1.1)

Alpha	0.05
Error Degrees of Freedom	371
Error Mean Square	0.084695
Critical Value of t	1.96638
Least Significant Difference	0.1431

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	2.95563	32	8
A			
B A	2.92381	32	2
B A			
B A	2.87756	32	7
B A			

Means with the same letter are not significantly different.

t Grouping			Mean	N	treatment
B	A	C	2.83619	32	4
B		C			
B	D	C	2.81075	32	6
	D	C			
E	D	C	2.71484	32	11
E	D				
E	D		2.68478	32	9
E	D				
E	D		2.67625	32	12
E					
E			2.66016	32	10
E					
E	F		2.59419	32	5
	F				
G	F		2.51053	32	1
G					
G			2.42469	32	3

9.1.2. TSW test Horizon: Kolmogorov-smirnov test (refer Section 4.1.1)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.04078248	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03927997	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.35202106	Pr > A-Sq	>0.250

9.1.3. Germination test horizon: T- tests (LSD) (refer Section 4.1.3)

Alpha	0.05
Error Degrees of Freedom	36
Error Mean Square	0.022648
Critical Value of t	2.02809
Least Significant Difference	0.2158

Means with the same letter are not significantly different.				
t Grouping		Mean	N	treatment
	A	0.8586	4	4
	A			
B	A	0.7351	4	7

Means with the same letter are not significantly different.

t Grouping			Mean	N	treatment
B	A				
B	A		0.7030	4	2
B	A				
B	A		0.6872	4	9
B	A				
B	A	C	0.6790	4	6
B	A	C			
B D	A	C	0.6500	4	11
B D	A	C			
B D	A	C	0.6474	4	8
B D		C			
B D		C	0.5758	4	10
B D		C			
B D		C	0.5491	4	12
D		C			
D		C	0.4674	4	5
D					

Means with the same letter are not significantly different.			
t Grouping	Mean	N	treatment
D	0.4585	4	3
D			
D	0.4498	4	1

9.1.4. Germination test horizon: Kolmogorov-smirnov test (refer Section 4.1.3)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.06572932	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.02799849	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.16736228	Pr > A-Sq	>0.250

9.1.5. Blind seed test Horizon: T-tests (LSD) (refer Section 4.1.4)

Alpha	0.05
Error Degrees of Freedom	36
Error Mean Square	0.022372
Critical Value of t	2.02809
Least Significant Difference	0.2145

Means with the same letter are not significantly different.

t Grouping			Mean	N	treatment
	A		1.1142	4	1
	A				
	A		1.1123	4	3
	A				
B	A		1.0895	4	5
B	A				
B	A	C	1.0067	4	12
B	A	C			
B	A	C	0.9774	4	10
B	A	C			
B	A	C	0.9234	4	8
B	A	C			
B	A	C	0.9182	4	11
B		C			
B	D	C	0.8866	4	6
B	D	C			
B	D	C	0.8759	4	9

Means with the same letter are not significantly different.				
t Grouping		Mean	N	treatment
D	C			
D	C	0.8622	4	2
D	C			
D	C	0.8281	4	7
D				
D		0.7020	4	4

9.1.6. Blind seed test Horizon: Kolmogorov-Smirnov test (refer Section 4.1.4)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.09173675	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03187235	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.17886945	Pr > A-Sq	>0.250

9.1.7. TPIB test: Endophyte transmission Horizon: T-tests (LSD) (refer Section 4.1.6)

Alpha	0.05

Error Degrees of Freedom	**12**
Error Mean Square	**0.011209**
Critical Value of t	**2.17881**
Least Significant Difference	**0.2307**

Means with the same letter are not significantly different.

t Grouping		Mean	N	treatment
	A	0.2859	2	7
	A			
B	A	0.2024	2	6
B	A			
B	A	0.1371	2	1
B	A			
B	A	0.1007	2	3
B	A			
B	A	0.0870	2	12
B	A			
B	A	0.0870	2	2
B	A			

Means with the same letter are not significantly different.

t Grouping		Mean	N	treatment
B	A	0.0870	2	5
B	A			
B	A	0.0709	2	8
B	A			
B	A	0.0709	2	4
B				
B		0.0000	2	9
B				
B		0.0000	2	11
B				
B		0.0000	2	10

9.1.8. TPIB test: Endophyte transmission Horizon: Kolmogorov-Smirnov test (refer Section 4.1.6)

Goodness-of-Fit Tests for Normal Distribution

Test	Statistic	p Value

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic		p Value
Kolmogorov-Smirnov	D	0.15656076	Pr > D	0.130
Cramer-von Mises	W-Sq	0.12706601	Pr > W-Sq	0.046
Anderson-Darling	A-Sq	0.84848223	Pr > A-Sq	0.025

9.2. Trial one: Samson (refer Section 4.2)

9.2.1. TSW test Samson: T-tests (LSD) (refer Section 4.2.1)

Alpha	0.05
Error Degrees of Freedom	180
Error Mean Square	0.018931
Critical Value of t	1.97323
Least Significant Difference	0.096

Means with the same letter are not significantly different.				
t Grouping		Mean	N	treatment
	A	2.24856	16	4
	A			
B	A	2.19569	16	2
B				

Means with the same letter are not significantly different.

t Grouping		Mean	N	treatment
B		2.14450	16	8
B				
B	C	2.11669	16	6
	C			
D	C	2.03681	16	11
D	C			
D	C	2.02356	16	9
D				
D		2.01744	16	10
D				
D		2.01381	16	12
D				
D		1.99056	16	3
D				
D		1.98794	16	5
D				
D		1.98463	16	7

Means with the same letter are not significantly different.			
t Grouping	Mean	N	treatment
D			
D	1.96231	16	1

9.2.2. TSW test Samson: Kolmogorov-Smirnov test (refer Section 4.2.1)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.05337168	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.07867622	Pr > W-Sq	0.221
Anderson-Darling	A-Sq	0.46343562	Pr > A-Sq	>0.250

9.2.3. Germination test Samson: T-test (LSD) (refer Section 4.2.3)

Alpha	0.05
Error Degrees of Freedom	36
Error Mean Square	0.013076
Critical Value of t	2.02809
Least Significant Difference	0.164

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	1.02834	4	9
A			
A	1.02760	4	8
A			
A	1.01791	4	4
A			
B A	1.00000	4	2
B			
B C	0.84562	4	10
C			
C	0.81621	4	11
C			
C	0.77490	4	5
C			
C	0.76014	4	6
C			
C	0.75014	4	3

Means with the same letter are not significantly different.			
t Grouping	Mean	N	treatment
C			
C	0.70615	4	7
D	0.53025	4	12
D			
D	0.45184	4	1

9.2.4. Germination test Samson: Kolmogorov-Smirnov test (refer Section 4.2.3)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic		p Value
Kolmogorov-Smirnov	D	0.08397039	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.06261867	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.48420319	Pr > A-Sq	0.226

9.2.5. Blind seed test Samson: T-tests (LSD) (refer Section 4.2.4)

Alpha	**0.05**
Error Degrees of Freedom	**36**
Error Mean Square	**0.012911**
Critical Value of t	**2.02809**
Least Significant Difference	**0.163**

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	1.11573	4	1
A			
A	1.02622	4	12
B	0.86193	4	7
B			
B	0.81813	4	3
B			
B	0.79800	4	6
B			
B	0.79064	4	5

Means with the same letter are not significantly different.

t Grouping		Mean	N	treatment
	B			
	B	0.75459	4	11
	B			
C	B	0.72266	4	10
C				
C	D	0.57079	4	2
	D			
	D	0.55026	4	4
	D			
	D	0.53757	4	8
	D			
	D	0.53401	4	9

9.2.6. Blind seed test Samson: Kolmogorov-Smirnov test (refer Section 4.2.4)

Goodness-of-Fit Tests for Normal Distribution

Test		Statistic		p Value
Kolmogorov-Smirnov	D	0.09462950	Pr > D	>0.150

Goodness-of-Fit Tests for Normal Distribution				
Test	Statistic		p Value	
Cramer-von Mises	W-Sq	0.06769797	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.51288659	Pr > A-Sq	0.194

9.2.7. TIPB test: Endophyte transmission Samson: T-tests (LSD) (refer Section 4.2.6)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.007459
Critical Value of t	2.17881
Least Significant Difference	0.1882

Means with the same letter are not significantly different.				
t Grouping		Mean	N	treatment
	A	1.08510	2	6
	A			
	A	1.08348	2	5
	A			
B	A	1.06068	2	1

Means with the same letter are not significantly different.

t Grouping			Mean	N	treatment
B	A				
B	A	C	1.02771	2	8
B	A	C			
B	A	C	1.01880	2	3
B	A	C			
B	A	C	0.99163	2	11
B	A	C			
B	A	C	0.96428	2	7
B	A	C			
B	A	C	0.93947	2	2
B	A	C			
B	A	C	0.91378	2	9
B	A	C			
B	A	C	0.90149	2	10
B		C			
B		C	0.89247	2	4
		C			

Means with the same letter are not significantly different.			
t Grouping	Mean	N	treatment
C	0.86069	2	12

9.2.8. TIPB test, endophyte transmission Samson: Kolmogorov-Smirnov test (refer Section 4.2.6)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.08970147	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.03115677	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.24675126	Pr > A-Sq	>0.250

9.3. Trial two: PGone50 (refer Section 4.3)

9.3.1. PGone50 TSW: T-test (LSD) for Seed Weight (refer Section 4.3.1)

Alpha	0.05
Error Degrees of Freedom	372
Error Mean Square	0.01575
Critical Value of t	1.96636
Least Significant Difference	0.0617

Means with the same letter are not significantly different.

t Grouping			Mean	N	Treatment
	A		2.15366	32	9
	A				
	A		2.15100	32	5
	A				
B	A		2.12606	32	7
B	A				
B	A	C	2.11494	32	1
B	A	C			
B	A	C	2.11331	32	6
B	A	C			
B	A	C	2.10959	32	4
B	A	C			
B	A	C	2.09797	32	11
B	A	C			
B	A	C	2.09197	32	12
B		C			
B		C	2.08853	32	10

Means with the same letter are not significantly different.				
t Grouping		Mean	N	Treatment
	C			
D	C	2.05953	32	3
D				
E D		2.02003	32	8
E				
E		1.99528	32	2

9.3.2. PGone50 TSW: Kolmogorov-Smirnov test (refer Section 4.3.1)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.03491223	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.07666508	Pr > W-Sq	0.234
Anderson-Darling	A-Sq	0.59445175	Pr > A-Sq	0.125

9.3.3. Germination PGone50: Kolmogorov-Smirnov test (refer Section 4.3.3)

Goodness-of-Fit Tests for Normal Distribution		
Test	Statistic	p Value

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic		p Value
Kolmogorov-Smirnov	D	0.12414390	Pr > D	0.064
Cramer-von Mises	W-Sq	0.09890738	Pr > W-Sq	0.116
Anderson-Darling	A-Sq	0.64214496	Pr > A-Sq	0.091

9.3.4. PGone50 endophyte content: T-Tests (LSD) (refer Section 4.3.4)

Alpha	0.05
Error Degrees of Freedom	36
Error Mean Square	0.00429
Critical Value of t	2.02809
Least Significant Difference	0.0939

Means with the same letter are not significantly different.			
t Grouping	Mean	N	Treatment
A	1.06912	4	11
A			
A	1.04748	4	5
A			
A	1.04741	4	4

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treatment
A			
A	1.04649	4	10
A			
A	1.03083	4	8
A			
A	1.02401	4	3
A			
A	1.02389	4	12
A			
A	1.01871	4	6
A			
A	1.01376	4	7
A			
A	1.01356	4	9
A			
A	1.00175	4	2
A			

Means with the same letter are not significantly different.			
t Grouping	Mean	N	Treatment
A	0.98720	4	1

9.3.5. PGone50 endophyte content: Kolmogorov-Smirnov test (refer Section 4.3.4)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.09300289	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.10987672	Pr > W-Sq	0.084
Anderson-Darling	A-Sq	0.70563405	Pr > A-Sq	0.064

9.3.6. PGone50 yield by treatment: T-tests (LSD) (refer Section 4.3.5)

Alpha	0.05
Error Degrees of Freedom	36
Error Mean Square	9828.326
Critical Value of t	2.02809
Least Significant Difference	142.17

Means with the same letter are not significantly different.

t Grouping		Mean	N	treatment
	A	2413.00	4	7
	A			
	A	2408.75	4	4
	A			
	A	2401.25	4	11
	A			
	A	2393.00	4	10
	A			
B	A	2357.50	4	3
B	A			
B	A	2348.00	4	8
B	A			
B	A	2337.25	4	12
B	A			
B	A	2331.25	4	6
B	A			
B	A	2321.50	4	9
B	A			

Means with the same letter are not significantly different.				
t Grouping		Mean	N	treatment
B	A	2316.00	4	5
B	A			
B	A	2314.00	4	2
B				
B		2229.75	4	1

9.3.7. PGone50 yield by treatment: Kolmogorov-Smirnov test (refer Section 4.3.5)

Goodness-of-Fit Tests for Normal Distribution				
Test		Statistic	p Value	
Kolmogorov-Smirnov	D	0.09826691	Pr > D	>0.150
Cramer-von Mises	W-Sq	0.06319614	Pr > W-Sq	>0.250
Anderson-Darling	A-Sq	0.36126783	Pr > A-Sq	>0.250

9.3.8. PGone50 yield by treatment: Kolmogorov-Smirnov test (refer Section 4.3.5). Data provided by FAR trial.

			F.D. Yield	Dress	**MD Yield**		Plot Yields MD kg/ha		
Treatments			kg/ha	Loss %	**kg/ha**	Rep 1	Rep 2	Rep 3	Rep 4
1	nil		2506	6.8	**2230**	2008	2227	2254	2430
2	300ml/ha Proline		2597	6.7	**2314**	2176	2331	2318	2431
3	300 ml/ha Proline + 500 ml/ha Comet		2646	6.7	**2357**	2174	2380	2338	2538
4	300 ml/ha Proline + 600 ml/ha S.F.		2702	6.6	**2409**	2296	2430	2353	2556
5	500 ml/ha Comet + 600 ml/ha S.F.		2595	6.5	**2316**	2245	2289	2375	2355
6	600 ml/ha S.F.		2608	6.3	**2331**	2262	2309	2436	2318
7	1.2l/ha BAS 700 01F		2707	6.6	**2413**	2341	2324	2419	2568
8	1.2l/ha BAS 701 00F		2635	6.7	**2348**	2261	2382	2441	2308
9	750 g/ha folpan		2600	6.5	**2321**	2226	2322	2313	2425
10	750 g/ha folpan + 500 ml/ha Comet		2686	6.6	**2393**	2344	2466	2376	2386
11	440 ml/ha Folicur + 500 ml/ha Az		2694	6.6	**2401**	2420	2368	2374	2443
12	500 ml/ha Az + 600 ml/ha S.F		2610	6.2	**2337**	2306	2279	2349	2415
		Mean	2632	6.6	**2348**	**2255**	**2342**	**2362**	**2431**
Application Date:		sig	*	NS	*				
24.12.12		CV%	2.8	14.8	**3.0**	Soil in sample			
		LSD	107	1.4	**101**	Missing Plot used for Yield			

End of Thesis

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