UNIVERSITY OF KWAZULU-NATAL

INYUVESI YAKWAZULU-NATALI

INTEGRATED CONTROL OF FUSARIUM HEAD BLIGHT OF WHEAT

By

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A dissertation submitted in fulfilment of the requirements for the degree of

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DISSERTATION ABSTRACT

Fusarium head blight (FHB) is a destructive fungal disease of wheat (*Triticum aestivum* L) world-wide. There are no resistant cultivars currently available, nor are there any effective fungicides against FHB of wheat. The use of biological control agents (BCAs) has been reported to be effective, if combined with other control measures. Therefore, this study was aimed at isolating and screening for antagonistic BCAs against *F. graminearum* Strain F.32 (F.32) in *in vitro* and *in vivo* bioassays. Subsequently, single and combined applications of potassium silicate (KSi) and BCAs treatments were tested for their ability to reduce FHB of wheat under greenhouse and field conditions.

In vitro bioassays showed that eight *Bacillus* isolates and two *Trichoderma* strains inhibited F.32 on potato dextrose agar (PDA) plates. *Bacillus* Isolates B13, B14 and B15 inhibited mycelial growth of F.32 by between 41 - 49%, whereas B1, B7, B9 and B16 inhibited mycelial growth of F.32 by between 20 - 28%. Two commercial *Trichoderma harzianum* strains, Strains T.kd and T.77 inhibited mycelial growth of *F. graminearum* F.32 by 30% and 24%, respectively. None of the 16 yeast isolates tested inhibited the germination of F.32 macroconidia within 7 h when screened on yeast dextrose chloramphenicol agar, ¼ strength PDA and water agar plates.

Eight *Bacillus* isolates, two commercial *T. harzianum* strains and four yeast isolates were tested against FHB under greenhouse conditions. *Bacillus* Isolate B7 significantly ($p = 0.05$) reduced FHB severity by 16% compared to the pathogen-inoculated Control treatment. None of the four tested yeast isolates significantly reduced FHB compared to the pathogen-inoculated Control treatment ($p = 0.90$). Single applications of *T. harzianum* Strains T.kd and T.77 did not significantly reduce FHB compared to the pathogen-inoculated Control treatment ($p = 0.22$). Repeated applications of *T. harzianum* Strains T.kd and T.77 did not significantly improve the reduction of FHB compared to the pathogen-inoculated Control treatment ($p = 0.76$).

Granulated KSi fertilization at 3.0 and 4.5 g reduced FHB of wheat under greenhouse conditions by 49.0 and 54.0%, respectively, on Day 12 post-pathogen inoculation (ppi) compared to the pathogen-inoculated Control treatment in the two KSi trials. Liquid KSi

fertilization at 600 mg L^{-1} was not effective in reducing FHB of wheat under greenhouse conditions in the first 12 days after pathogen inoculation. There was no significant difference in the severity of FHB of wheat heads after treatments with KSi alone, and KSi combined with the *T. harzianum* Strains T.kd or T.77.

None of the single and combined treatments with *Bacillus* Isolate B7, *T. harzianum* Strain T.kd and KSi treatments significantly reduced FHB severity and incidence compared to the pathogen-inoculated Control treatment on Day 20, Day 30 and Day 40 ppi under field conditions. None of the treatments tested under field conditions reduced deoxynivalenol levels in the wheat grains below 2 mg kg^{-1}, which is the legally recommended limit.

Biological control agents that demonstrated antagonistic potential against F.32 on agar plates were ineffective in reducing the severity of FHB by F.32 on wheat under field conditions. Potassium silicate demonstrated a limited capacity to reduce the levels of FHB under greenhouse conditions. The search for effective BCAs against FHB of wheat grown under South African conditions will need to continue.

DECLARATION

I, Zandile Nothile Consolate Mngadi declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research
2. This dissertation has not been submitted for any degree examination at any other university
3. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted. Then:
    a. Their words have been re-written but the general information attributed to them has been referenced;
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Prof M.D. Laing (Co-supervisor)

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DEDICATION

To my late parents Bayi Peterson and Nokuthula Betty Mngadi and sister Buyisiwe Pearl Mngadi for their thirst for knowledge

DISSERTATION INTRODUCTION

In South Africa, Fusarium head blight (FHB) was first noticed on wheat under irrigated areas in 1980's (Boshoff et al., 1999; Kriel and Pretorius, 2008). It affects wheat production across South Africa (Boshoff et al., 1999). FHB has become the most important disease of wheat worldwide because it reduces yield and quality of wheat (Legzdina and Buerstmayr, 2004), and releases dangerous mycotoxins into the wheat grain. Given that most human beings consume bread and other wheat products, this result in most humans being exposed to Fusarium toxins on a regular basis. Currently, there are no resistant cultivars in South Africa, nor are there any effective fungicides against FHB (Giraud et al., 2011; Willyerd et al., 2012). Levels of FHB can be reduced by the application of two or more control strategies (Palazzini et al., 2009). These include partially resistant cultivars, crop sanitation, biocontrol agents and the use of crop fertilizers that enhance plant resistance (Yuen and Schoneweis, 2007).

The main aim of this research was to investigate the application of single and combined applications of potential biological control agents and potassium silicate in order to reduce levels of FHB, under greenhouse and field conditions. The objectives of this study included:

1. Review literature on FHB: the causal microorganisms, their life cycle, climatic conditions required for infection, symptoms, economic importance, mycotoxins and control strategies;

2. Isolate and screen microorganisms for antagonism against *Fusarium graminearum* Strain F.32 *in vitro;*

3. Screen for the best antagonists against *F. graminearum* Strain F.32 on wheat under greenhouse conditions;

4. Evaluate single and combined applications of potassium silicate (KSi) and commercially available *Trichoderma* biocontrol agents against FHB on wheat under greenhouse conditions

5. Attempt to manage FHB of wheat with biological control agents (BCAs) and KSi under field conditions.

The dissertation has been written in the form of five chapters, each chapter covering a specific aspect of the research conducted on the integrated control of FHB of wheat, specifically the integration of BCAs and KSi. With the exception of the literature review, each of the chapters has been written as an independent study, and prepared in the format of a scientific paper. This creates some redundancy in the introductory information, and the references. However, it is the standard dissertation model that has been adopted by University of KwaZulu-Natal.

This research was undertaken in the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Dr K.S. Yobo and Prof. M.D. Laing.

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“Fusarium head blight is not a simple disease with simple solutions, so progress has been slower than anyone would like. With sustained and collaborative efforts, however, little steps of progress will lead to realistic and implementable solutions”

Marcia McMullen, 2003

TABLE OF CONTENTS

CHAPTER ONE

LITERATURE REVIEW

1.1. Introduction

Wheat (*Triticum aestivum* L.) is an important food crop grown throughout the world (Beyer et al., 2005; Pei et al., 2010; Yao et al., 2010). It is a staple food crop to many South Africans, and is grown in all provinces of South Africa, but its levels of production differ from one province to another (Figure 1) (Anonymous, 2011). Wheat grown in South Africa is exported to neighboring countries, particularly Angola, Democratic Republic of Congo, Malawi, Mozambique, Zambia and Zimbabwe (Anonymous, 2011).

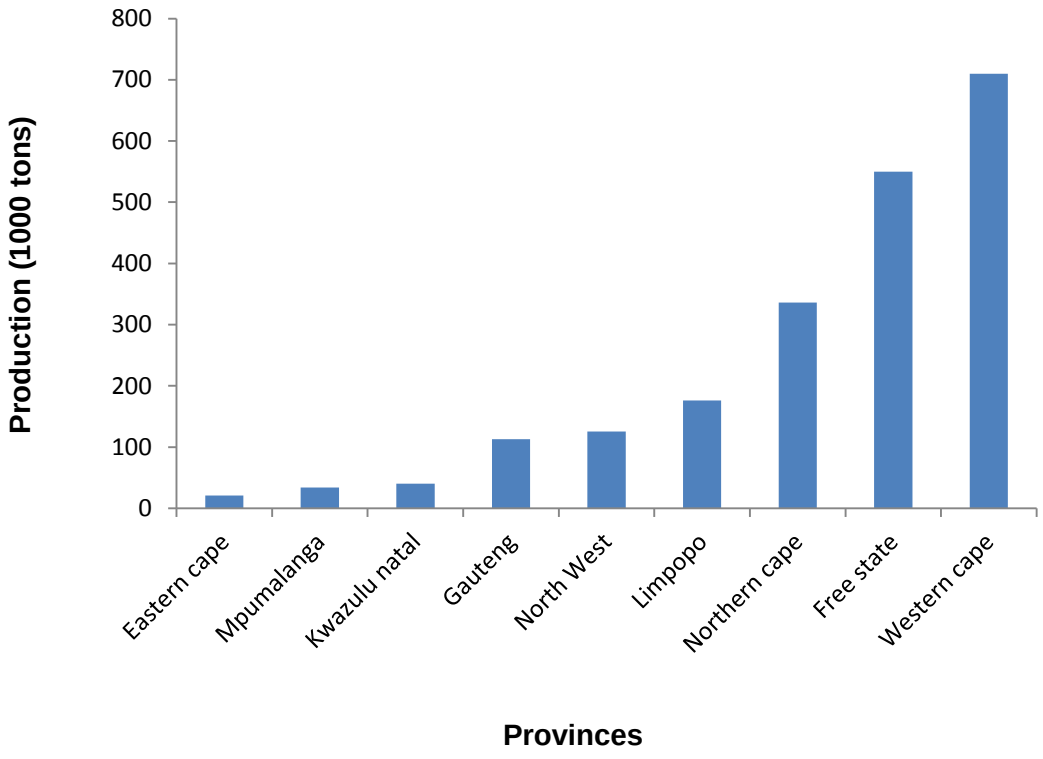

Figure 1**:** Wheat production in provinces of South Africa (Anonymous, 2011)

Wheat crops are susceptible to attacks by *Fusarium* and *Microdochium* species (Muthomi et al., 2007) and the resulting disease is called Fusarium head blight (FHB). Fusarium head blight disease has been identified as a major factor limiting wheat production in many parts of the world (Goswami and Kistler, 2004). There have been several FHB outbreaks in South Africa and the most extensive case was recorded in KwaZulu-Natal where the disease incidence was reported to have exceeded 70% (Boshoff et al., 1999). Mesterházy et al. (2003) reported that FHB incidence in some fields can reach 100%. Incidences and severity of FHB have increased over years due to adoption of simplified crop rotations, particularly with regards to wheat and maize (*Zea mays* L) production in many countries (Kriel and Pretorius, 2008; Giraud et al., 2011). Wet weather is also implicated for increased severity of FHB in many countries (Dill-Macky and Jones, 2000).

FHB pathogens produce some of the most important mycotoxins during their development within wheat heads, namely the trichothecenes deoxynivalenol (DON), zearalenone, T-2 toxin and T-2-like toxins (Miller, 1995; Wagacha and Muthomi, 2008). Trichothecene occurrences have been reported in Asia, Africa, South America, Europe and North America (Beyer at al., 2005; Zain, 2010). Deoxynivalenol is produced in higher concentrations in infected grains and hence its production is considered a major food safety issue (Legzdina and Buerstmayr, 2004), because it poses health problems to both animals and humans (Miller, 1995). Laboratory animals exposed to low concentrations of DON have become more susceptible to bacterial, viral and fungal diseases (Miller, 1995).

All known wheat cultivars are vulnerable to FHB infection (Schisler et al., 2002; Willyerd et al., 2012). Resistant cultivars are not available and fungicides are relatively ineffective in controlling the disease (Dill-Macky and Jones, 2000; Chongo et al., 2001; Aldred and Magan, 2004; Kriel and Pretorius, 2008; Giraud et al., 2011). Integrated management combining the use of fungicides, cultural practices and partially or tolerant cultivars serves as a popular strategy in managing FHB (Diamond and Cooke, 2003).

1.2. The disease: Fusarium head blight (FHB)

FHB is a fungal disease and was first described in 1884 in England (Parry et al., 1995; Stack, 2003; Goswami and Kistler, 2004; Legzdina and Buerstmayr, 2004; Trail et al., 2005). In South Africa, FHB was first noticed in 1980 on wheat fields under irrigation (Boshoff et al., 1999; Kriel and Pretorius, 2008). This disease is also known as scab or Fusarium ear blight (Bai, 2000; Stack, 2003; Bai and Shaner, 2004; Osborne and Stein, 2007). FHB is monocyclic because the secondary inoculum only becomes available when the host is less susceptible to infection, and therefore there are few host plants available to the spread inoculum (Bai and Shaner, 2004; Luongo et al., 2005). Epidemics of FHB have become more prevalent as a result of intensive agriculture (Heier et al., 2005).

1.2.1. Causal microorganisms

FHB is a complex disease because of the number of fungal species associated with it (Rossi and Giosué, 2002; Aldred and Magan, 2004). There are 17 *Fusarium* species considered to be associated with FHB (Parry et al., 1995; Muthomi et al., 2007, Audenaert et al., 2013). One genera associated with FHB is *Microdochium*, in particular *Microdochium nivale* (Fr) Samuels & I.C. Hallet (Muthomi et al., 2007; Osborne and Stein, 2007). However, the five species commonly associated with this disease in wheat fields include *Fusarium* spp., namely *F. avenaceum* (Corda: Fr) Sacc, *F. culmorum* (W.G. Smith) Sacc., *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch) and *F. poae* (Peck) Wollenw and *Microdochium nivale* (Muthomi et al., 2007; Osborne and Stein, 2007). These fungi occur as a complex and rarely exist in isolation (Muthomi et al., 2007). *F. graminearum* is a major causal microorganism of FHB in many countries (Miller, 1995; Bai and Shanner, 2004; Monds et al., 2005; Osborne and Stein, 2007; Seong et al., 2008; Palazzini et al., 2009). Climatic conditions, particularly temperature and moisture, pre-determine the distribution of different FHB pathogen species (Xu, 2003; Champeil et al., 2004). In cooler regions of the world, *F. culmorum*, *F. poae* and *M. nivale* predominate, whereas *F. graminearum* is more important in hotter regions (Xu, 2003; Muthomi et al., 2007).

1.2.2. Colonization and infection of wheat

During winter, *F. graminearum* may exist on non-decomposed residues as chlamydospores, and mycelium depending on the species involved (Figure 2) (Champeil et al., 2004; Osborne and Stein, 2007; Yuen and Schoneweis, 2007). When temperatures increase with the presence of moisture, the fungus then produces either asexual spores (macroconidia) or sexual spores (ascospores) to infect wheat plants (Figure 2) (Dufault et al., 2006; Seong et al., 2008). The sticky ascospores are forcibly dispersed into the air from perithecia formed on the host plant residues (Beyer et al., 2004; Yuen and Schoneweis, 2007). Spores can be carried by wind or rain onto a host plant (Goswami and Kistler, 2004; Jouany, 2007; Seong et al., 2008), and can also be transported by insect vectors such as wheat midges onto wheat heads (Figure 2) (Beyer et al., 2004).

Wheat is most susceptible to FHB infection at anthesis, which last for about 10 days, but can be susceptible to infection 2-3 weeks after anthesis (Figure 2) (Miller, 1995; Clear and Patrick, 2000; Walker et al., 2001; Champeil, et al., 2004; Luongo, et al., 2005; Pritsch et al., 2001). Susceptibility of the wheat flower heads declines once anthesis has been completed (McCallum and Tekauz, 2002). However, for infection to occur, climatic conditions, particularly temperature and moisture, have to be optimal for the pathogen (Boshoff et al., 1999; Clear and Patrick, 2000; Xu, 2003).

The FHB infection cycle is initiated by germinating spores that were previously deposited on, or inside floral tissue (Bai and Shaner, 2004; Yuen and Schoneweis, 2007; Seong et al., 2008). Germinated spores develop hyphae that spread out over the exterior surfaces of florets and glumes toward stomata and other entry sites on the wheat head (Bai and Shaner, 2004; Seong et al., 2008; Brown et al., 2010). Anthers, stigmas and lodicules are easily colonized by the pathogen once it has entered the floret (Pritsch et al., 2001; Goswami and Kistler, 2004). During the flowering stage wheat anthers are an important floral part that makes wheat highly susceptible to FHB infection (Yoshida et al., 2007). This is because more infections are initiated on extruded anthers than on other parts of the plant (Yoshida et al., 2007). Choline and

betaine compounds in the anthers are thought to stimulate the germination of fungal spores (Paulitz, 1999; Osborne and Stein, 2007)

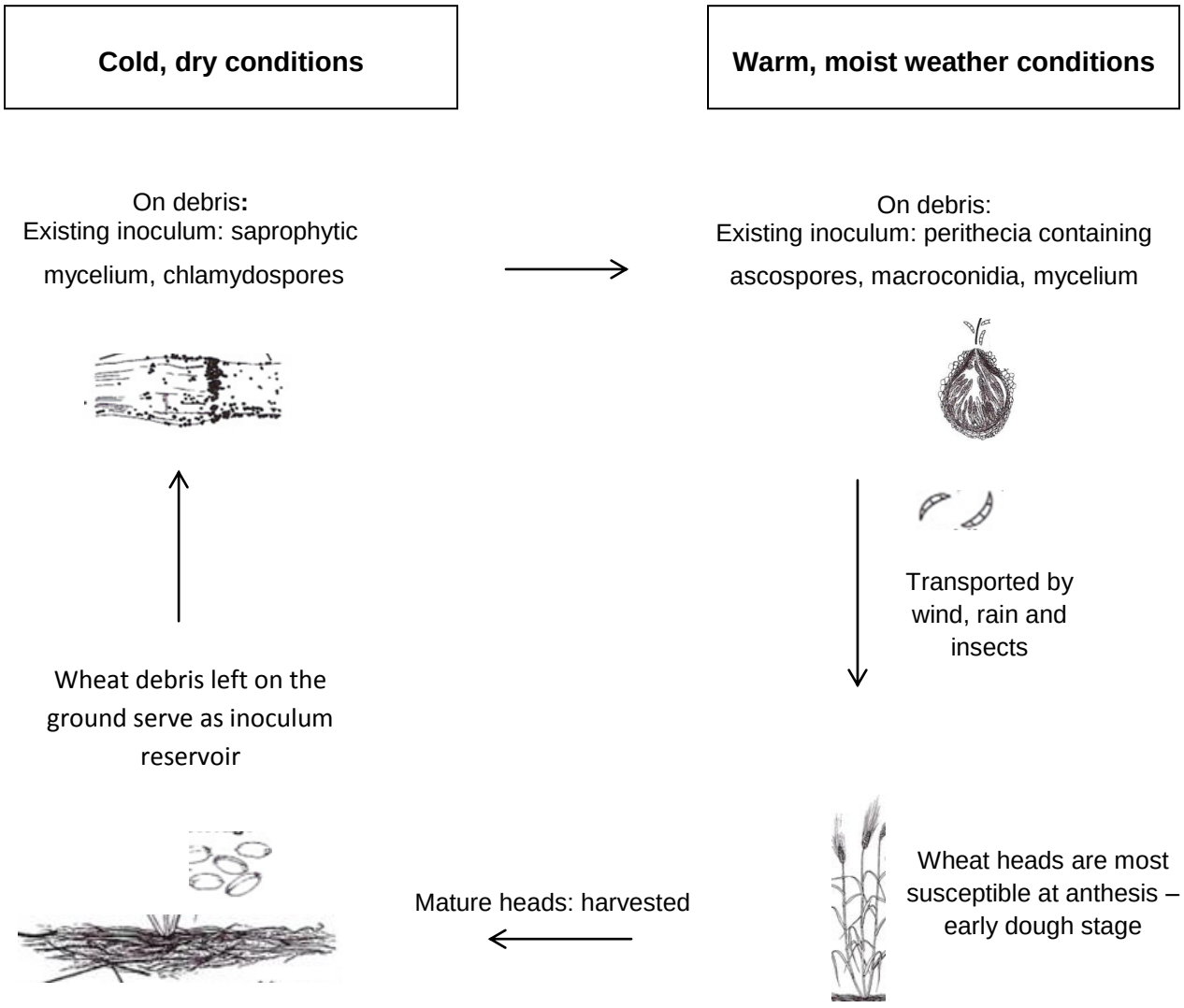

Figure 2: Schematic diagram showing the life cycle of *Fusarium graminearum* (Sutton, 1982).

However, according to Parry et al. (1995) and Gilbert and Tekauz (2000), these compounds stimulate the growth of the fungus rather than stimulate the germination of fungal spores. These compounds can also be found in paleas, lemmas, glumes, rachis and grains but at much lower concentrations than in the anthers (Parry et al., 1995).

During anthesis, it has been observed that the choline content is twice as high in susceptible cultivars than in resistant cultivars (Bai and Shaner, 2004).

The relationship between the fungus and the host plant starts by being biotrophic, and later becomes necrotrophic (Goswami and Kistler, 2004). The pathogen secretes cell wall degrading enzymes that aid the fungus to spread further within host tissues during colonization and infection (Wanjiru et al., 2002; Jackowiak et al., 2005; Brown, 2010). Cellulase, xylanase and pectinase are some of the enzymes produced by *F. graminearum* during penetration and colonization of wheat spike tissue (Wanjiru et al., 2002). Infection by the pathogen involves two steps, namely, initial infection and spread of pathogen within the spike (Bai, 2000).

The pathogen spreads from infected floret to health floret inside the spikelet and from one spikelet to another through the vascular bundles in the rachis and rachilla (Bai and Shanner, 2004; Goswami and Kistler, 2004). Eventually vascular bundles clog, leading to dysfunctional xylem and phloem tissues in the rachis, which leads to premature death of the spikelet (Bai and Shanner 2004; Goswami and Kistler, 2004).

1.2.3. Symptoms

Wheat plants may not show symptoms for a few days after being infected with FHB pathogens (Trail et al., 2005) but under favorable conditions, lesions can start showing within 3-4 days (Pritsch et al., 2001). Lesions appear as small, water-soaked brownish spots at the base or middle glumes, or on the rachis (Parry et al., 1995). A salmon pink to red FHB fungus develops as a dense mycelium along the edge of the glumes or at the base of the spikelet (Parry et al., 1995). Infected spikelets appear tan, and as more spikelets get infected with the fungus, the whole head become bleached in appearance (Figure 3A) (Chongo et al., 2001; Bai and Shaner, 2004; Osborne and Stein, 2007). Peducle tissues may also turn brown or tan (Parry et al., 1995; Osborne and Stein, 2007).

Purple black perithecia may be seen on heads, especially on glumes, giving a scabbed appearance (Parry et al., 1995; Osborne and Stein, 2007). Infected grains may appear small, shrunken or shriveled and sometimes chalky (Champeil et al., 2004; Osborne

and Stein, 2007). These shriveled grains are a result of a shortage of nutrients and water within the infected wheat head (Bai and Shaner, 2004; Argyris et al., 2005).

Figure 3: Diseased and healthy wheat heads: A. bleached wheat head infected with *F. graminearum*. B. healthy wheat head uninfected with the fungus (Photograph by Zandile Mngadi, 2013).

Empty blighted heads with no grains may also be seen in fields affected by FHB (Bai and Shaner, 2004). Grains fail to develop entirely if heads were infected by the pathogen at a very early wheat growth stages (Bai and Shaner, 2004). At a cellular level, susceptible cultivars infected with FHB show blocked, destroyed phloem and no defensive reaction, whereas infected partially resistant cultivars show thick parenchyma walls and blocked but intact phloem (Argyris et al., 2005).

1.2.4. Climatic conditions for disease occurrence

The production and dispersal of inoculum, produced, either sexually and/or asexually, is affected by environmental conditions, most importantly temperature and rainfall or wet conditions (Clear and Patrick, 2000; Doohan et al., 2003; Ramirez et al., 2006). The range of temperatures affecting the production of spores is shown in Table 1. A more detailed table on environmental factors affecting FHB fungal development can be found

in Osborne and Stein (2007). *F. graminearum* adapts to a wider range of environmental conditions than other FHB pathogens, and hence is more widespread throughout the world than other species (Osborne and Stein, 2007).

Table 1: Optimum temperatures for inoculum production and infection by FHB pathogens (Brennan et al., 2003).

Fusarium species	Inoculum	Optimum temperature
F. graminearum	Perithecial production	29^{o}C
F. graminearum	Ascospore production	25-28^{o}C
F .graminearum	Ascospore dispersal	16^{o}C
F. graminearum	Wheat ears infection	25^{o}C
F. culmorum,	Wheat ears infection	25^{o}C
F. avenaceum	Wheat ears infection	25^{o}C
F. poae	Wheat ears infection	25^{o}C
M. nivale	Wheat ears infection	15^{o}C

The production of ascospores, macroconidia, mesoconidia, microconidia and chlamydospores may switch depending on both nutritional and environmental factors (Doohan et al., 2003). Ascospores are thought to be the main source of inoculum as more of them are trapped in spore traps than macroconidia (Beyer et al., 2005). The optimum temperatures for production of ascospores are between 7-20^{o}C (Xu, 2003). However these temperatures differ from the temperature range reported by Brennan et al. (2003). Rainfall is essential for the development and maturation of ascospores, but it is not important for the release of ascospores (Xu, 2003). Ascospore release is triggered by a drop in air temperature and the rising of relative humidity (RH) but it is negatively affected by very high RH (Francl et al., 1999; Doohan et al., 2003). Dry

conditions also contribute to the release of ascospores from perithecia (Doohan et al., 2003).

Macroconidia are rain splashed over shorter distances from a few centimeters to meters during heavy rains (Champeil et al., 2004; Luongo et al., 2005; Osborne and Stein, 2007). They are rain splashed because they do not have a discharge mechanism as with ascospores (Beyer et al., 2005). Macroconidia are too heavy to be transported by wind (Champeil et al., 2004), and are therefore spread during rainfall whereas ascospores are released after the rainfall (Beyer et al., 2005). A very high RH delays macroconidia production and decreases conidia numbers (Doohan et al., 2003).

FHB seems to be severe when rainfall is more frequent, humidity is high and when persistent dew occurs at the flowering stage (Rossi et al., 2002) as a result of prolific production of macroconidia and ascospores during these times. Most disease epidemics that have been reported are associated with exacerbated wet periods during anthesis (Aldred and Magan, 2004; Lori et al., 2009). Occurrences of FHB incidence that has been reported in drier years are usually very low (5-6%) (Mesterházy et al., 2003). The microclimate in fields under irrigation is likely to be influenced by irrigation, which may lead to disease development (Parry et al., 1995; Champeil et al., 2004). Aeration and light are other climatic factors that affect the production of *Fusarium* inoculum (Doohan et al., 2003). Perithecial initiation require low intensity ultraviolet light (<390nm) (Doohan et al., 2003).

1.2.5. Source of inoculum

Inoculum production can be in the form of ascospores, macroconidia, chlamydospores and hyphal fragments (Bai and Shaner, 2004). Host plants such as grasses including oats (*Avena sativa* L), barley (*Hordeum vulgare* L), maize (*Zea mays* L), sorghum (*Sorghum bicolor* L. Moench), soybean (*Glycine max* L) and some broad-leaved weeds may provide an important alternative inoculum source (Parry et al., 1995; Paulitz, 1999; Xu, 2003; Champeil et al., 2004; Lori et al., 2009; Osborne and Stein, 2007; Brown et al., 2010). Seedling blight as a result of infected seeds may also serve as a source of inoculum (Bai, 2000).

Microdochium nivale is able to form perithecia and sporodochia (Rossi et al., 2002); hence, both macroconidia and ascospores are produced by this fungus. *F. avenaceum* produces asexual inoculum, but perithecia may also be formed (Rossi et al., 2002). Inoculum produced by *F. culmorum* is entirely through asexual reproduction (Rossi et al., 2002), whereas *F. graminearum* produces inoculum both sexually and asexually (Doohan et al., 2003; Beyer et al., 2005).

Ascospores travel great distances by air (Osborne and Stein, 2007), but locally available inoculum from previous crop residues are a major inoculum source of infection of new crops (Champeil et al., 2004; Trail et al., 2005; Osborne and Stein, 2007). Insects may carry spores onto floral parts of susceptible wheat and spores of *F. poae* have been seen on mites (*Tetranychidae* Donnadieu) (Gilbert and Tekauz, 1995). *Fusarium avenaceum*, *F. culmorum* and *F. poae* have been isolated from insects such as common housefly (*Musca domestica* L.), clover leaf weevil (*Hypera zoilus* Scopoli), grasshoppers (*Caelifera* Ander sp.) and picnic beetles (*Glischrochilus* Reitter sp.) (Gilbert and Tekauz, 1995).

1.3. Economic losses

FHB is unique because it has the ability to influence every aspect of the grain industry from harvesting to selling of wheat grains (Gilbert and Tekauz, 2000).

Infection of wheat heads in the field varies from a trace to virtually 100% (Muthomi et al., 2007). *F. graminearum* causes crop yield and quality losses (Lutz et al., 2003; Legzdina and Buerstmayr, 2004; Luongo et al., 2005; Juchum et al., 2006; Palazzini et al., 2007; Riungu et al., 2007; Yoshida et al., 2007; Seong et al., 2008; Willyerd et al., 2012). In one case, there was an estimated loss of $1 billion in one region of the United States and Canada (States of Minnesota, North Dakota, South Dakota and the Canadian province of Manitoba) due to yield reduction and mycotoxins contamination as a result of FHB (Dufault et al., 2006). In the US, the disease has caused over 3 billion dollars in losses in the 1990s (Paulitz, 1999; Schisler et al., 2002; Bai and Shaner, 2004).

Grains formed during FHB infection are discoloured, withered, shriveled and weigh less than normal grains (Boshoff et al., 1999; Bai, 2000; Goswami and Kistler, 2004; Ramirez et al., 2004; Snijders, 2004; Prange et al., 2005). This grain causes difficulties in grain marketing, exporting and processing (Goswami and Kistler, 2004). Germination of infected grains is poor, thereby affecting the number of healthy plants in the field (Gilbert and Tekauz, 1995). Cleaning of infected grains by removing chalky and light weight grains and/or treating seeds with fungicide has not been effective in decreasing the affected grain levels satisfactorily (Gilbert and Tekauz, 1995).

Baking qualities are affected by the presence of fungi in the grain (Clear and Patrick, 2000, Gilbert and Takauz, 2000; Jackowiak et al., 2005; Parry et al., 1995). The FHB fungus, particularly *F. graminearum,* produces proteases that digest storage proteins and starch within the wheat endosperm (Bechtel et al., 1985; Clear and Patrick, 2000; Champeil et al., 2004; Snijders, 2004; Heier et al., 2005; Jackowiak et al., 2005; Brown et al., 2010). Damaged storage proteins in the grains lead to lower loaf volume (Champeil et al., 2004; Jackowiak et al., 2005).

Grains contaminated with mycotoxins may be rejected at the grain elevator if the levels are higher than the established contamination standards (Dyer et al., 2005). It is forbidden to mix batches of contaminated and uncontaminated grain (Heier et al., 2005). The United States Food and Drug Administration (FDA) have set the level for deoxynivalenol (DON) to 1000µgkg^{-1} for finished products (Khan et al., 2004, Palazzini et al., 2007), and European Union has set the level for DON for processed wheat to be 1750µgkg^{-1} (Palazzini et al., 2007). DON produced by *F. graminearum*, *F. culmorum* and other *Fusarium* species during their vegetative stage in the grains, is stable and unaffected by storage or heat (Champeil et al., 2004; Prange et al., 2005). Therefore, DON levels can still be detected after wheat processing.

1.4. Mycotoxins

The presence of FHB fungi in grains in the field results in grains contaminated with mycotoxins (Doohan et al., 2003; Khan et al., 2004; Palazzini et al., 2007; Yuen and Schoneweis, 2007; Seong et al., 2008). Weather has a great influence on the quantity

and type of mycotoxins produced before and after harvest (Lacey et al., 1999; Brennan et al., 2003; Wagacha and Muthomi, 2008; Kokkenen et al., 2010). Besides weather, other factors that influence mycotoxin production under field conditions include agricultural practices, the host plant, and the complex of microorganisms present in the wheat heads (Kokkonen et al., 2010; Willyerd et al., 2012).

Fusarium culmorum and *F. graminearum* produce mycotoxins Type B trichothecenes, which include deoxynivalenol (DON), 3 acetyldeoxynivalenol (3-ACDON), 15 acetyldeoxynivalenol (15-ACDON) and nivalenol (NIV); whereas *F. poae* produces Type A trichothecenes, which include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol (DAS) (Moss, 1996; Doohan et al., 2003; Marin et al., 2004; Trail et al., 2005; Kokkenen et al., 2010). *Fusarium avenaceum* produces diacetoxyscirpenol, T-2 toxin and zearalenone, and *M. nivale* produces diacetylnivalenol, nivalenol, zearalenone and T-2 toxin (Liddel, 2003). Mycotoxins produced by FHB pathogens pose a threat to human and livestock health (Parry et al., 1995; Gilbert et al., 2002; Aldred and Megan, 2004; Beyer et al., 2004; Riungu et al., 2007; Seong et al., 2008; Wagacha and Muthomi, 2008; Kokkenen et al., 2010; Willyerd, et al., 2012). DON is produced more frequently and in higher concentrations in cereals than other trichothecenes (Goswami and Kistler, 2004; Legzdina and Buerstmayr, 2004; Beyer et al., 2005; Prange et al., 2005). DON is a sesquiterpenoid that inhibits eukaryotic protein synthesis by binding on a 60 S ribosome. It is believed that DON limits wheats' defensive response (Moss, 1996; Di et al., 2010; Goswami and Kistler, 2004; Khan et al.; 2004; Legzdina and Buerstmayr, 2004; Dyer et al., 2005; Brown et al., 2010). However, DON is less toxic than its isomers, 3-ACDON and 15-ACDON which are twice as toxic to humans and animals (Lacey et al., 1999). Nivalenol is ten times more toxic than DON (Lacey et al., 1999). Countries in Eastern Europe and Asia have reported several human disease outbreaks related to consumption of contaminated grains (Bai et al., 2002).

Adverse effects in animals that consume grains contaminated with mycotoxins include food refusal, diarrhea, emesis, alimentary haemorrhaging and contact dermatitis (Goswami and Kistler, 2004; Marín et al., 2004). Pigs are less tolerant to feed

contaminated with DON than cattle and poultry (Miller, 1995; Legzdina and Buerstmayr, 2004; Zain, 2010). Humans are sensitive to DON and experience illnesses characterized by nausea, vomiting, anorexia and convulsions and alimentary toxic aleukia (Miller, 1995). A maximum daily intake of contaminated grains with DON is estimated at 3ugkg^{-1} for humans as recommended by Swedish regulators (Lacey et al., 1999). Canadian Agriculture has recommended grain feed to animals should have less than 1 ugg^{-1} of DON (Lacey et al., 1999).

1.5. Control strategies

FHB cannot be controlled by only one control strategy but rather by two or more strategies (Bai and Shaner, 2004; Palazzini et al., 2009). These strategies include host resistance, chemical fungicides, biological control and cultural practices (Osborne and Stein, 2007; Yuen and Schoneweis, 2007; Willyerd et al., 2012).

1.5.1. Cultural practices

Rotation of soybean with wheat may be used to reduce inoculum levels in the field (Champeil et al., 2004). Soybean is attacked by *F. sporotrichoides*, which is less pathogenic than *F. graminearum* (Champeil et al., 2004). Secondly, soybean leaves less residue than maize under tillage practices (Champeil et al., 2004). Wheat has a greater risk to be severely affected by FHB if it is cultivated after a maize crop (Stack, 2003; Champeil et al., 2004; Luongo et al., 2005; Jouany, 2007). Maize is susceptible to the aggressive *F. graminearum* and provides the best substrate for ascospore production (Champeil et al., 2004; Blandino et al., 2006). The risk is lower if wheat is planted after FHB non-host crops such as clover (*Trifolium* L.), sugarbeet (*Beta vulgaris* L), flax (*Linum usitatissimum* L.) and alfafa (*Medicago sativa* L.) (Paulitz, 1999; Dill-Macky and Jones, 2000; Stack, 2003; Champeil et al., 2004). Alfalfa and flax can be used as a clean-up crop as it has been shown that disease severity after cultivation of these crops can become significantly low (Champeil et al., 2004).

Removing crop debris from soil before planting a new crop is an alternative strategy in reducing FHB inoculum (Parry et al., 1995; Luongo et al., 2005; Willyerd et al., 2012). However, removal of debris leaves the soil bare and as a result suffers from erosion,

direct sunlight and dryness (Champeil et al., 2004; Lori et al., 2009). Removing debris does not always lead to reduction of FHB because when environmental conditions are favourable during anthesis even though inoculum may be low, then the disease may still become severe (Lori et al., 2009). Debris can be buried. However this practice negates the benefits of low and no-till practice (Champeil et al., 2004; Luongo et al., 2005; Blandino et al., 2006)

1.5.2. Fertilizers

Fertilizers alter the rate of residue decomposition, rate of plant growth, soil structure and soil microbial activity (Jouany, 2007). A high nitrogen content in maize residues may lead to greater colonization of residues by fungi (Champeil et al., 2004). Using urea instead of ammonium nitrate decreases FHB severity (Parry et al., 1995; Champeil et al., 2004; Jouany, 2007; Lori et al., 2009). Urea may decrease severity of FHB in two ways: (i) it blocks reproduction of *Fusarium* fungi and inhibits the formation of chlamydospores (Champeil et al., 2004); (ii) it increases the number of actinomycetes in the soil, which are antagonists of *F. graminearum* (Champeil et al., 2004). Incidence of FHB is decreased by 31-59% when nitrolime is used instead of calcium ammonium nitrate but this had no effect on DON contamination levels (Jouany, 2007). Residues with a high silicon content may be resistant to colonization by soil saprophytes, including FHB pathogens, through the protective role it plays against pathogen infection (Champeil et al., 2004).

Silicon fertilizers have been used by several researchers to assist crops fight fungal diseases (Kanto, et al, 2007; de Melo et al., 2010; and Xavier Filha et al., 2011). The effect of silicon on fungal diseases has been carried out on powdery mildew (*Blumeria graminis* DC. Speer) and rice blast (*Magnaporthe grisea* (Hebert) Barr. (teleomorph: *Pyricularia oryzae* Cavara) (Guével et al., 2007). Plants take up silicon from the soil solution, which is deposited throughout various organs in the form of silica (Bocharnikova et al., 2010; de Melo et al., 2010). Deposited silica have been found in cell walls, cell lumens, tricombs, intracellular spaces, roots, leaves, and reproductive organs (de Melo et al., 2010). Silica deposited in these sites has been demonstrated to minimize fungal penetration (de Melo et al., 2010). In addition, silicon stimulates natural

defense reactions in plants via increases in the activity of chitinases, peroxidases and polyphenoloxydases and accumulation of phenolic compounds that affect fungal pathogens (Fauteux et al., 2005). Wheat powdery mildew, leaf blotch (*Mycosphaerella graminicola* (Fuckel) Schröter (anamorph *Septoria tritici* Rob. ex Desm.), foot rot (*Fusarium* spp) and rice blast have been controlled by application of silicon fertilizers (Rémus-Borel et al., 2005; Kanto et al., 2007; Sun et al., 2010; Xavier Filha et al., 2011). Silicon fertilizers can be used with environmentally friendly strategies for crop production (Xavier Filha et al., 2011).

1.5.3. Planting date, weed control and cultivars

It has been shown that planting wheat earlier than usual in the season saves plants being severely affected by FHB (Champeil et al., 2004; Jouany, 2007). However, there has been conflicting results on the link between resistance to head blight and maturity factors of wheat cultivars (Champeil et al., 2004). The poor management of weeds may increase FHB infection in wheat (Champeil et al., 2004; Jouany, 2007). There has been a good correlation between application of herbicides and a decrease in the incidence of FHB (Champeil et al., 2004). It has been shown that awned wheats are 80% more susceptible to FHB than awnless cultivars because awned cultivars develop a high level of humidity between awns after rain or as a result of free water (Parry et al., 1995). Free water between awns of awned wheats is not lost as quickly as on awnless cultivars (Parry et al., 1995).

1.5.4. Resistance

There are two forms of genetic resistances to FHB that exist in wheat cultivars, Type I and Type II (Bai, 2000; Argyris et al., 2005; Yuen and Schonewis, 2007). Type I is resistance to initial infection, whereas Type II is resistance to pathogen spread to nearby florets via the rachis. In general, Type II is stable and less affected by the environment (Bai, 2000). The challenge of developing better cultivars is to breed for resistance to FHB, while maintaining desired agronomic, baking qualities and resistance to other diseases (Bai and Shaner, 2004; Jouany, 2007; Yuen and Schonewis, 2007).

Sumai 3 is a widely used cultivar in breeding programs for FHB resistance (Stack, 2003; Bai and Shaner 2004; Osborne and Stein 2007). The cultivar was developed in China in 1974 and has been used since the 1980's throughout the world (Stack, 2003). The interest in Sumai 3 is that its resistance prevents the spread of the fungus beyond the site of initial infection (Gilbert and Tekauz, 2000; Pritsch et al., 2001). Other resistant lines to FHB that have been used in breeding programs are Ning from the Brazillian line Fontana, NoboekaBozu from Japan, and Praag 8 and Novukrumba from Europe (Gilbert and Tekauz, 2000). Resistant cultivars are considered to be the most effective strategy to control FHB (Bai, 2000, Gilbert and Tekauz, 2000; Schisler et al., 2006; Jouany, 2007, Riungu et al., 2007). There is a growing need for wheat varieties with greater resistance (Clear and Patrick, 2000) as it is believed that resistant cultivars are economically and environmentally feasible method of managing FHB (Paulitz, 1999; Aldred and Magan, 2004; Lemmens et al., 2004; Mesterházy et al., 2011). It has been shown that cultivars resistant to the most aggressive fungal strains can inhibit disease progression and toxin production (Aldred and Magan, 2004).

1.5.5. Fungicides

In areas where FHB is more prevalent and wheat lacks high levels of resistance, the use of fungicides is an important option to control FHB (Mesterházy et al., 2011). However, there are a few limiting factors on the use of fungicides including (i) the high cost of fungicide applications; (ii) available fungicides have not been consistently effective in controlling FHB and subsequently DON has been produced in the grains (Mesterházy et al., 2003; Bai and Shanner, 2004; Khan et al., 2004; Yuen and Schoneweis, 2007; Di et al., 2010). The inconsistent effectiveness of fungicides may be due to non-uniform spray application patterns, or narrow window for effective application (Boshoff et al., 1999; Heier et al., 2005; Yuen and Schoneweis, 2007; Mesterházy et al., 2011). The use of fungicides may stimulate the production of mycotoxins in the grains if they are not completely lethal to the targeted *Fusarium* spp. (Miller, 1995; Mesterházy et al., 2003; Aldred and Magan, 2004; Ramirez et al., 2004; Jouany, 2007). In the field, FHB pathogens coexist as a complex and if one species is controlled by a fungicide there may be a greater development of another species (Champeil et al., 2004). This

explains the increase of mycotoxins in the grains from plants treated with fungicides. There are instances where fungicides have increased yields but failed to decrease mycotoxin contamination (Boshoff et al., 1999; Bai and Shaner, 2004). Application of fungicides during the post-heading stages of wheat growth is a concern because of the residues they leave on and in the kernels (Boshoff et al., 1999, Schisler et al., 2002; Khan et al., 2004)

Fungicides that are used for controlling FHB contain either triazole or strobilurin active ingredients (Table 2). The mode of action of fungicides with a triazole ingredient is that they interfere with the metabolism of fungal pathogens by inhibiting the ergosterol synthesis (Ramirez et al., 2004; Blandino et al., 2009). The affected fungi are deformed as a result of these fungicides (Ramirez et al., 2004). Fungicides with strobilurins block electron transport in the mitochondrial respiratory chain in fungi (Ramirez et al., 2004). Consequently, fungal growth is inhibited due to reduced energy production (Ramirez et al., 2004). Fungicides containing triazoles appear to be more effective than those with strobilurins in reducing DON in the infected grains (Mesterházy et al., 2003; Riungu et al., 2007; Blandino et al., 2009). In the 1980's, carbendazim was most effective against FHB in the field in China but the FHB pathogen population have developed resistance to the fungicide (Parry et al., 1995; Mesterházy et al., 2003; Li et al., 2008).

1.5.6. Biological control agents

The interest in biological control of FHB is probably because all alternative and affordable methods of controlling FHB are only partially effective (Snidjers, 2004). Additionally, the window period for infection to take place is narrow (5-10 days), and therefore, this can be a perfect opportunity to use microorganisms at this stage (Aldred and Magan, 2004; Snidjers, 2004). The use of biological control agents has shown promise in managing FHB (Table 3) (Khan et al., 2004). According to Riungu et al. (2007), there would be a reduction in contaminated grains, environmental pollution and potential hazards to human health if biological control agents were used rather than fungicides.

Table 2: List of fungicides and their effect on FHB and DON contamination in wheat

Fungicides	Effect	Source
Azoxystrobin	Reduced FHB but sometimes increased DON levels	Gilbert and Tekauz, 2000; Mesterházy et al., 2003; Aldred and Megan, 2004; Blandino et al., 2006; Blandino et al., 2009; Jouany, 2007
Azoxystrobin+tebuconazole	Reduced FHB severity and DON levels	Jouany, 2007
Bromoconazole+metconazole	Moderately effective against FHB	Mesterházy et al., 2003
Carbendazim	Partially effective against FHB	Boshoff et al., 1999;
Difenoconazole	Stimulated 3-ACDON production	Aldred and Megan, 2004
Metconazole	Effectively reduced DON levels	Mesterházy et al., 2011
Metconazole+tebuconazole	Reduced FHB and DON levels	Aldred and Megan, 2004; Jouany, 2007
Propiconazole+triadimefon	Reduced FHB severity and DON levels	Boshoff et al., 1999; Gilbert and Tekauz, 2000; Aldred and Megan, 2004
Prothioconazole	Reduced DON levels	Mesterházy et al., 2003
Tebuconazole	Partially effective against FHB but stimulated 3-ACDON	Gilbert and Tekauz, 2000; Mesterházy et al., 2003; Aldred and Megan, 2004;
Tebuconazole+propiconazole	Inconsistently reduced DON levels	Gilbert and Tekauz, 2000
Tebuconazole+thiabendazole	Partially effective against FHB	Yu et al., 2011
Thiabendazole	Little effect on FHB but reduced DON levels	Gilbert and Tekauz, 2000

Biological agents can be applied on crop residues and subsequently interact with the saprophytic microorganisms, resulting in a reduced hindrance colonization of saprophytic microorganisms (Schisler et al., 2002; Luongo et al., 2005; Yuen and Schoneweis, 2007). Reduced colonization of saprophytic microorganisms such as *Fusarium* spp can result in a decrease of ascospore production (Paulitz, 1999). There are two other ways microorganisms can be applied in the biocontrol strategy of FHB: (i) coating seeds with a bioprotectant to prevent seedling infection, and (ii) applying microorganisms onto wheat heads at the flowering stage (Paultz, 1999; Champeil et al., 2004; Snijders, 2004). The mode of action of microorganisms used in biocontrol strategies involves mycoparasitism, competition for nutrients and space (Diamond and Cooke, 2003; Palazzini et al., 2007). Induced resistance may result from inoculation with some microorganisms (Juchum et al., 2006).

In the field, microorganisms used as biological control agents are most effective to controlling FHB in wheat crops within a narrow band of environmental conditions (Diamond and Cooke, 2003). It has been difficult to achieve consistent performance of microorganisms used as biocontrol agents in the field over time and across locations (Juchum et al., 2006).

1.5.7. Integrated control measures

The most effective approach to control FHB is one that integrates agronomic techniques (crop rotation, tillage), chemical control and resistant cultivars (Lemmens et al., 2004). Several factors such as epidemiological characteristics of pathogens, levels of genetic resistance in the host and environmental conditions have an influence on FHB development (Ramirez et al., 2004). Therefore, it is important to implement several integrated measures in order to ensure that the damage by the disease is decreased (Ramirez et al., 2004). If fungicides are used as a strategy to control FHB, other management practices should also be included, such as tillage, crop rotation and using resistant cultivars because fungicides may not provide sufficient control (Mesterházy et al., 2011).

Table 3: List of microbial genera and their site of application to control FHB

Genera	Site of application	References
Alternaria	Wheat heads; anthesis	Champeil et al., 2004
Bacillus	Wheat heads; anthesis	Schisler et al., 2002; Juchum et al., 2006; Palazzini et al., 2007; Palazzini et al., 2009
Botrytis	Wheat heads; anthesis	Champeil et al., 2004
Cladosporium	Wheat heads; anthesis	Champeil et al., 2004
Cryptococcus	Wheat heads; anthesis	Khan et al., 2004; Juchum et al., 2006
Lyzobacter	Wheat heads; anthesis	Juchum et al., 2006
Microsphaeropsis	Wheat straw; debris	Champeil et al., 2004; Khan et al., 2004; Yuen and Schoneweis, 2007
Phoma	Wheat heads; anthesis	Diamond and Cooke, 2003; Champeil et al., 2004
Pseudomonas	Wheat heads; anthesis	Juchum et al., 2006; Schisler et al., 2006; Palazzini et al., 2007; Palazzini et al., 2009
Rhodotorula	Wheat heads; anthesis	Palazzini et al., 2007; Palazzini et al., 2009
Sporobolomyces	Wheat heads; anthesis	Palazzini et al., 2007; Palazzini et al., 2009
Trichoderma	Wheat straw; debris, Anthesis	Gilbert and Tekauz, 2000; Khan et al., 2004; Luongo et al., 2005; Palazzini et al., 2007; Riungu et al., 2007; Yuen and Schoneweis, 2007

It appears that biological control is a useful tool to manage FHB if it is integrated with fungicide applications and host resistance (Khan et al., 2004; Juchum et al., 2006; Yuen and Schoneweis, 2007). The notion that biological control can be integrated with other control measures makes it more attractive to manage FHB (Schisler et al., 2002).

1.6. Conclusion

FHB affects yield and quality of wheat grains in many parts of the world. Since there are no available resistant cultivars and/or effective fungicides registered against FHB of wheat in South Africa, there is an urgent need for economically and environmental-friendly alternative control measures. Several environmental friendly control measures such as crop tillage practices, tolerant cultivars, biological control and the use of fertilizers can be combined in an attempt to reduce FHB.

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CHAPTER TWO

Isolation and *in vitro* screening of microbial antagonists against *Fusarium graminearum* Strain F.32

Abstract

Eight *Bacillus* and 16 yeasts isolates obtained from the phyllosphere of wheat and maize plants were screened *in vitro* against *Fusarium graminearum* Strain F.32 (F.32). Two commercial *Trichoderma* formulations, *T. harzianum* Strain kd (T.kd) and *T. harzianum* Strain 77 (T.77) were also tested *in vitro* against F.32. *In vitro* bioassays showed that the eight *Bacillus* isolates and the two *Trichoderma* products inhibited F.32 on potato dextrose agar (PDA) plates. *Bacillus* Isolates B13, B14 and B15 significantly (P = 0.01) inhibited F.32 compared to *Bacillus* Isolates B1, B7, B9 and B16 on Day 20. *Bacillus* Isolates B13, B14 and B15 inhibited F.32 by between 41 - 49%, whereas B1, B7, B9 and B16 inhibited F.32 by between 20 - 28%. T.77 and T.kd inhibited F.32 mycelial growth by 24.0% and 30.0%, respectively. None of the 16 yeast isolates inhibited the germination of F.32 macroconidia on yeast dextrose chloramphenicol agar (YDCA), ¼ strength PDA or water agar plates within 7 h. However, the presence of yeast isolates prevented F.32 from growing profusely on YDCA compared to Control plates over 10 days.

Keywords*: Fusarium graminearum*, *Trichoderma* spp., *Bacillus* spp., yeast spp.

2.1. Introduction

Fusarium head blight (FHB) is a devastating disease of wheat (*Triticum aestivum* L.) caused by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch (Muthomi et al., 2007; Giraud et al., 2011; Mesterházy et al., 2011). There are no resistant cultivars, nor are there any effective fungicides against FHB (Chongo et al., 2000; Dill-Macky and Jones, 2000; Aldred and Magan, 2004; Giraud et al., 2011). However, the window of vulnerability of wheat to FHB infection is narrow, and for this reason, biological control agents (BCAs) have potential for use during this period (Corio da Luz et al., 2003). Biological control of FHB has been investigated by several researchers (Schisler et al., 2002; Khan et al. 2004; Juchum et al., 2006, Nourozian et al., 2006, Palazzini et al., 2007; Riungu et al., 2007).

Potential BCAs are best isolated from the target areas of the host plant where they are to be applied (Yoshida et al., 2012). For example, the most suitable microbes for use as BCAs against FHB of wheat would be those that inhabit wheat plants (Yoshida et al., 2012). Some potential BCAs that have been effective at reducing FHB severity on wheat heads under greenhouse and field conditions have been isolated from healthy wheat anthers, intact kernels and whole wheat heads (Diamond and Cooke 2003; Palazzini et al., 2007; Riungu et al., 2007; Yoshida et al., 2012).

In vitro screening is a rapid pre-screening technique that allows large numbers of microbial strains to be tested (Dal Bello et al., 2002; Palazzini et al., 2007). There have been both positive and negative feedbacks on this technique in selecting for effective BCAs (Dal Bello et al., 2002). Some effective BCAs have been selected through this technique, but some potential BCAs selected through this technique have proven to be ineffective on plants (Dal Bello et al., 2002). *In vitro* screening remains a useful method for preliminary screening of large numbers of potential BCAs (Dal Bello et al., 2002; Palazzini et al., 2007).

Two bacterial genera that have been well studied for their ability to control FHB are *Bacillus* and *Pseudomonas* (Corio da Luz et al., 2003; Juchum et al., 2006). *Trichoderma* is a genus that has been widely studied for its potential as a biological

control agent (Punja, 1997; Scala et al., 2007; Nawrocka and Malolepsza, 2013). *Trichoderma* spp. have been tested against *F. graminearum* on crop debris (Luongo et al., 2005) and on wheat heads (Riungu et al., 2007). Another group of fungi that have been studied for biocontrol activity is the yeasts. Some yeasts have been shown to control FHB both under greenhouse and field conditions (Schisler et al., 2002; Khan et al., 2004; Schisler et al., 2006; Palazzini et al., 2007).

The mechanisms of action exhibited by BCAs include the production of antibiotics and toxins against pathogen growth, competition for infection sites or nutrients, and induction of resistance mechanisms in the plant (Punja, 1997; Scala et al., 2007).

The aim of this study was to isolate potential BCAs from the phyllosphere of wheat and maize plants and to screen them for activity against *F. graminearum* Strain F.32 *in vitro*.

2.2. Materials and Methods

2.2.1. Isolation, storage and identification of FHB pathogens

Fungi were isolated from 20 bleached heads of wheat plants growing in pots under greenhouse conditions at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. Isolated mycelium from a single head was treated as an individual isolate and the pathogen isolates were transferred separately into individual Eppendorf tubes. Fragments of mycelium were transferred onto peptone pentacloronitrobenzene agar (PPCNBA) plates [peptone 15 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.25 g, pentacloronitrobenzene (PCNB) 750 mg, agar 20 g in 1 L distilled water. Streptomycin (20 ml) and neomycin (12 ml) were also added]. The plates were incubated for seven days at 28^{o}C in the dark. *Fusarium* spp. isolates were transferred twice on PPCNBA plates before they were grown on Spezieller Nährstoffarmer Agar (SNA) plates. Macroconida were produced by transferring agar plugs carrying growing mycelia cut from PPCNBA plates onto SNA (KH_2PO_4 0.5 g, KNO_3 0.5 g, $MgSO_4$ 0.25 g, KCl 0.25 g, sucrose 0.1 g, glucose 0.1 g and agar 10 g in 500 ml distilled water) plates. The SNA plates were incubated at ambient temperature under continuous infrared light (350 nm) and sporulation was checked within 14 days. Macroconidia were harvested in a 15% sterile glycerol solution. With the aid of an L-bent metal rod, conidia were dislodged

from the agar surface. Aliquots (1 ml) of 15% glycerol solution with conidia were transferred in the 2 ml cryovials and stored in a -80^{o}C freezer.

Three *Fusarium* isolates (F.13, F.29 and F.32) were selected and sent to the Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI), Pretoria, South Africa for identification. All three isolates were confirmed to be *Fusarium graminearum*.

2.2.2 Isolation of microbial antagonists

a*. Bacillus* spp.

Potential bacterial BCAs were isolated from wheat and maize parts collected from three locations. One batch consisted of kernels (20) at the hard dough stage [11.2 Feeke's scale] (Zadoks et al., 1974) removed from wheat heads (10) collected from a wheat field at the Ukulinga Research Station, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The second batch consisted of wheat kernels (20) at the soft dough stage (11.2 Feeke's' scale, Zadoks et al., 1974) removed from wheat heads (10), collected from a wheat field in Winterton, KwaZulu-Natal, South Africa. The third source consisted of five leaves removed from maize plants cultivated at the Ukulinga Research Station, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The kernels were ground into fine particles using a coffee grinder before being submerged in 200 ml distilled water. Maize leaves were cut into smaller pieces (10 x 10 mm^{2}) before being submerged in 500 ml distilled water. Milled kernels and leaf pieces in flasks were heated in a water bath at 80^{o}C for 30 min for isolation of *Bacillus* spp. Serial dilutions from heated solutions were made after they had cooled. Aliquots (200 µl) from 10^{6} dilutions were spread plated on tryptic soy agar (TSA, Merck) and the plates were incubated at 28^{o}C in the dark for 48 h. Bacterial cultures were stored in 15% sterile glycerol in cryovials in a -80^{o}C freezer. A Gram stain was performed on all pure bacterial cultures.

Pre-screening of bacteria

Bacillus colonies isolated from specific wheat and maize parts appeared similar (by observation of colony size, surface texture and colour) on respective agar plates. Hence two *Bacillus* isolates were selected from 8 isolates obtained from the UKZN batch, one from 3 isolates obtained from the Winterton batch and all five *Bacillus* isolates obtained from maize leaves were selected for screening.

b. Yeast spp

Yeasts were isolated from maize leaves (2), spikelets (50) and anthers (50), wheat kernels (20), and glumes (50) collected from maize and wheat field plots at Ukulinga Research Station, University of KwaZulu-Natal, Pietermaritzburg. Maize leaves were cut into pieces before they were submerged in 200 ml of distilled water and all other cereal parts were separately submerged whole in 200 ml distilled water. Cereal parts in flasks were shaken for 2 h at 20^{o}C in a water bath in the dark. Serial dilutions were made from suspensions, and 200 µl aliquots from the 10^{7} dilutions were plated onto yeast dextrose chloramphenicol agar (YDCA) (yeast extract 5 g, dextrose 20 g, Chloramphenicol 0.1 g, agar 15 g in 1 L distilled water) plates. The plates were incubated in the dark at 28^{o}C for five days. Single colonies (by observation of the colony size, surface texture and pigmentation) were picked, re-streaked and grown on YDCA plates before they were stored. Pure cultures (two days old) were suspended in 15% sterile glycerol solution and 1 ml aliquots were stored at a -80^{o}C freezer in Eppendorf tubes.

c. *Trichoderma* spp

Maize grains obtained from Agricultural Research Council, Potchefstroom, North West, South Africa were surface sterilized and placed on *Trichoderma* selective media (TSM) [$MgSO_4$ 0.2 g, K_2HPO4 0.9 g, KCl 0.15 g, NH_4NO_3 1 g, glucose 1 g, rose Bengal 0.15 g, agar 20 g, with fungicides) (Askew and Laing, 1993).The plates were incubated in the dark at 28^{o}C and the appearance of *Trichoderma* colonies was checked regularly for 14 days. In addition, aliquots (200 µl) of the 10^{6} dilutions were transferred from solutions of maize leaves, maize anthers, wheat kernels, and wheat glumes (Section 2.2.2b) submerged in distilled water onto TSM plates. Plates were incubated for 14 days at

28^{o}C in the dark and checked regularly for the appearance of typical *Trichoderma* colonies.

2.2.3. *In vitro* dual culture bioassays

a. *Bacillus* spp

Agar plugs carrying mycelium of *Fusarium graminearum* Strain F.32 (F.32) were transferred aseptically from McCartney bottles onto fresh PPCNBA plates. After five days incubation in the dark at 28^{o}C, mycelial plugs were transferred from PPCNBA onto PDA plates, which were then incubated at 28^{o}C for four days. One plug (4 x 4 mm^{2}) carrying F.32 mycelia was placed in the center of a fresh PDA plate (90 mm diameter) and incubated at 28^{o}C for 48 h in the dark. *Bacillus* spp. cultures (B1, B7, B9, B12, B13, B14, B15 and B16) taken from -80^{o}C storage were cultured twice on TSA plates to obtain pure colonies. A day old pure bacterial culture was introduced by streaking (10 mm) in a circular mode at four spots, 30 mm away from a 48 h old growing F.32. Plates were stored in the dark in a 28^{o}C incubator for five days. The diameters of the zones of inhibition were measured on Days 5, 10 and 20. The percentage inhibition was calculated using the following formula (Dal Bello et al., 2002).

PI=100(R1 - R2)/R1

Where:
PI = percentage inhibition
R1 = radius of the colony of *F. graminearum* in the direction with no bacterial growth
R2 = radius of the *F. graminearum* in the direction of the bacterial growth

b. Yeast spp

Yeast cultures (Y50, Y51, Y52, Y53, Y54,Y55, Y56, Y57 Y58, Y59, Y60, Y61, Y62, Y63, Y64, Y65) taken from -80^{o}C storage were cultured on YDCA plates at 28^{o}C for 48 h in the dark. Single colonies were picked and streaked onto fresh YDCA plates and re-incubated at 28^{o}C. After 24 h, yeast cells were suspended in sterile distilled water and adjusted to 1x10^{4} cells/ml using a haemocytometer. A 300 µl suspension of each yeast

isolate and a 600µl suspension of $1x10^4$ macroconidia/ml of F.32 produced from SNA were mixed and spread onto YDCA, ¼ strength PDA and water agar plates. The bioassay was replicated two times and plates were incubated in the dark for 7 h at 28^{o}C. Macroconidia germinations were stopped by flooding the agar plates with 1-2 ml of lactophenol bromide dye.

In a second bioassay test, 300 µl aliquots of yeast suspensions containing $1x10^4$ cells/ml were spread onto YDCA plates and incubated at 28^{o}C in the dark for 24 h. One milliliter (1 ml) of F.32 suspensions containing $1x10^4$ macroconidia/ml were introduced and mixed with already established yeast cultures on YDCA plates. Plates were incubated at 28^{o}C for 10 days and the growth of F.32 was assessed and compared to the Control. Control plates were inoculated with F.32.

c. *Trichoderma* spp

Two formulation of *Trichoderma harzianum,* Strain kd (T.kd) and Strain 77 (T.77) were obtained from Plant Health Products (Pty) Ltd, Nottingham Road, KwaZulu-Natal, South Africa. Three loop-fulls of each *Trichoderma* powder formulation were inoculated onto TSM plates. The plates were incubated for 5 days at 28^{o}C in the dark. Mycelial plugs with green conidia of the two *Trichoderma* strains were aseptically transferred separately onto PDA plates.

An agar plug of F.32 was introduced two days early on one end of fresh PDA plate (90 mm diameter) before a plug with a *Trichoderma* strain was introduced at the opposite end of the plate. The bioassay was replicated two times and the plates were incubated in the dark at 28^{o}C. Controls for each *Trichoderma* strain, as well as the test pathogen, were also cultured on PDA plates and incubated with the test plates. After five days of incubation, each dual-culture bioassay plate was assessed for inhibition of F.32. The percentage inhibition was calculated using the following formula by Musyimi et al. 2012:

$$\text{Inhibition \%} = \frac{\text{colony diameter} - (\text{colony diameter of pathogen + antagonist}) \times 100}{\text{Colony diameter of pathogen alone}}$$

Mycoparasitism studies under phase contrast microscope

Water agar slabs (5 cm x 2 cm) were cut from 2% bacteriological agar plates and transferred onto a microscope slide. *Fusarium graminearum* Strain F.32 plugs (2 x 2 mm^2) from a PDA plate was introduced at one end of the agar slab. The slides were incubated in a sterile petri dish (90 mm diameter) for 24 h at 28^oC in the dark. Plugs of T.kd and T.77 (2 x 2 mm^2) from PDA plates were singly transferred onto a slide at the opposite end to the pathogen. Each bioassay slide was replicated three times and slides were re-incubated at 28^oC in the dark. Two days after introduction of the *Trichoderma* isolates, sections where *Trichoderma* had met with F.32 were viewed under a phase contrast microscope to look for evidence of mycoparasitism.

Mycoparasitism studies using Environmental Scanning Electron microscope (ESEM).

Mycelial plugs (4 x 4 mm^2) of a *Trichoderma* strain and the pathogen were cut from the actively growing edge of a 4-day old mycelial mat on PDA, and were placed opposite each other on PDA plates. Each bioassay was replicated two times and incubated for 5 days at 28^oC in the dark. Mycelial plugs from regions of *Trichoderma*-pathogen interaction were cut out of the agar 5 days post-inoculation, and were fixed in 3% (v/v) cacodylate buffer (0.1M; PH 7.0). After 3 h of refrigeration at 4^oC, the specimens were dehydrated in a graded ethanol series [10, 20, 50, 70% (v/v)]. The cultures were stored in 70% ethanol at 4^oC. Specimens were further dehydrated in 90% ethanol and three times in 100% (v/v) the following day. Dehydrated samples were further dried in a critical point dryer (Model HCP-2 Hitachi) and mounted on copper stubs with double-sided sticky tape. Samples were sputter coated with gold-palladium and then kept in a desiccator until examination with Philips XL30 Environmental Scanning Electron Microscopy (ESEM) (http://www.innovationservices.philips.com) on high vacuum at 10Kv. Three samples per *Trichoderma* – F.32 interactions were examined.

2.2.4 Data analysis

Data collected was subjected to analysis of variance (ANOVA) using Genstat (VSN International Ltd, Version 14.1 Edition). Treatment means were separated using Duncan's multiple range test at a 5% probability level.

2.3. Results

2.3.1. Isolation of microbial antagonists

There were 11 *Bacillus* isolates that were initially isolated from wheat kernels collected from University of KwaZulu-Natal (8 isolates) and Winterton (3 isolates). Five more *Bacillus* spp. isolates were isolated from maize leaves. The *Bacillus* colonies isolated from wheat heads were visually distinct from the *Bacillus* strains isolated from maize leaves. Both *Bacillus* groups obtained from wheat heads and maize leaves stained Gram positive. Sixteen yeast spp. were isolated in this study: 1,2,3,5 and 5 isolates were obtained from wheat kernels, maize spikelets, maize anthers, wheat glumes and maize leaves, respectively. No *Trichoderma* strains were isolated from wheat kernels, maize leaves and maize kernels.

2.3.2. Dual culture bioassays

Interaction of *Bacillus* isolates against F.32 on agar plates

All 8 *Bacillus* isolates inhibited F.32 on PDA. Inhibition was shown by the zone of inhibition around *Bacillus* colonies (Figure 1). *Bacillus* Isolates B1 and B7 were the only two isolates that had percentage inhibition significantly different from Isolates B14 and B15 on Day 5 (Table 1). On Day 10, percentage inhibition by Isolate B16 was significantly lower than all other isolates except Isolates B7 and B9 (Table 1). On Day 20, percentage inhibition demonstrated by Isolates B13, B14 and B15 were significantly different from inhibition demonstrated by Isolates B7, B9 and B16 (Table 1). Overall, percent inhibition demonstrated by *Bacillus* Isolates B1, B7, B9 and B16 decreased sharply compared to *Bacillus* Isolates B12, B13, B14 and B15, which decreased slightly between Day 5 to Day 20 (Table 1).

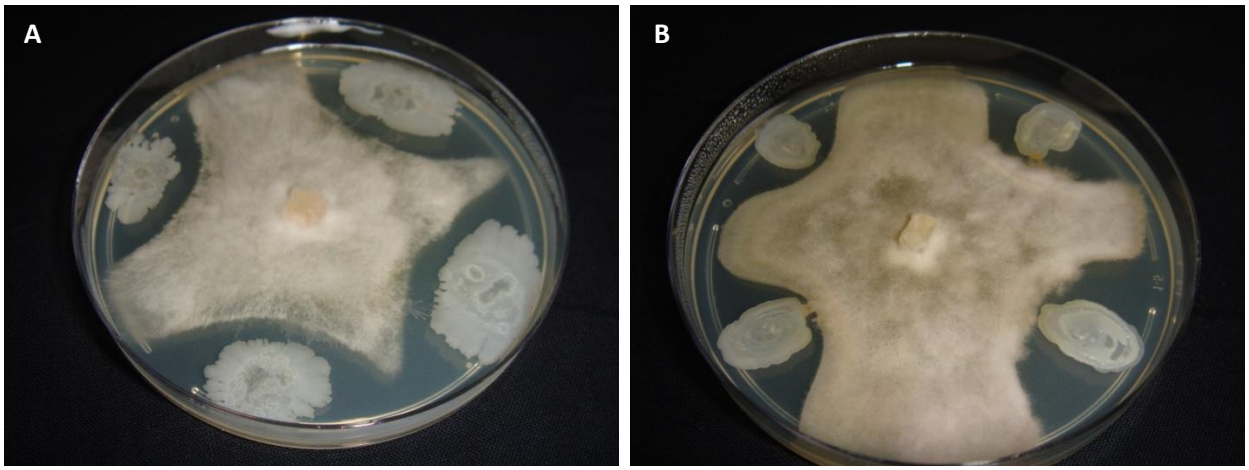

Figure 1: Zones of inhibition created by two *Bacillus* isolates B14 (A) and B9 (B) against *F. graminearum* Strain F.32 on PDA plates on Day 20.

Table 1: Inhibition of *F. graminearum* Strain F.32 by *Bacillus* isolates on PDA plates (%), over a period of 15 days of incubation of the bioassay plates at 28^{o}C

Bacillus spp.	Day 5	Day 10	Day 20
B1	38.75b	38.0cd	27.5bc
B7	42.0b	30.5bc	20.5b
B9	47.0bc	36.75bcd	25.5b
B12	46.25bc	43.25de	38.25cd
B13	47.25bc	46.0de	41.25d
B14	53.0c	49.0e	49.25d
B15	52.75c	47.5e	47.25d
B16	44.25bc	28.25b	28b.0c
F.32	0a	0a	0a
F-ratio	28.17	25.47	17.21
P-value	0.01	0.01	0.01
CV%	14.7	17	24.0

Means followed by the same letters in the same column are not significantly different from each other using Duncan multiple Range Test at 5% Probability level.

Effect of yeast isolates against F.32 on agar plates

None of the 16 yeast isolates were able to inhibit germination of F.32 macroconidia on YDCA, ¼ PDA or water agar plates within 7 h of incubation at 28^{o}C. Macroconidia germinated well (97-100%) in the presence of the tested yeasts. However, the yeasts appeared to suppress the mycelial growth of F.32. Only sparse growth of F.32 was seen on YDCA plates with yeast isolates compared to the Control plates within 10 days incubation, whereas F.32 grew well on YDCA plates in the absence of yeasts. Newly produced F.32 macroconidia were observed under the microscope on YDCA plates in the presence of yeast isolates.

Interaction of two *Trichoderma* strains against F.32 on agar plates

T.kd and T.77 inhibited the growth of F.32 on agar plates by 30% and 24%, respectively. Yellow–green conidia of T.kd and T.77 were seen forming on F.32 mycelia on PDA plates, which were incubated for 10 days at 28^{o}C. However, no mycoparasitism was exhibited by T.kd and T.77 against F.32 under phase contrast microscope. This was confirmed with the ESEM studies.

2.4. Discussion

Biological control agents offer a promising control strategy against FHB (Corio da Luz et al., 2003). In this study, eight *Bacillus* and 16 yeast isolates were tested against F.32 *in vitro*. The putative biocontrol agents were obtained from the phyllospheres of maize and wheat plants. Strains of *Bacillus* and yeast have previously been isolated from parts of wheat plants, and have demonstrated the ability to to reduce FHB severity (Khan et al., 2001; Schisler et al., 2002; Schisler et al., 2006). No isolates of *Trichoderma* were found in the maize and wheat phyllosphere in this study. In other studies on the endophytes of wheat, *Trichoderma* spp. were also not found (Larran et al., 2002; Larran et al., 2007). *Trichoderma* spp. are common inhabitants of the rhizosphere (Rachniyom and Jaenaksorn, 2008), but they can also be found in foliar environments (Reino et al., 2008).

Isolates of *Bacillus* spp. have been demonstrated to reduce FHB severity (Corio da Luz et al., 2003, Nourozian et al., 2006, Riungu et al., 2007, Chan et al., 2009; Blandino et

al., 2012.). The advantage of *Bacillus* spp. as a BCA is that it produces endospores, which make it resistant to harsh conditions such as desiccation and ultraviolet radiation (Corio da Luz., et al., 2003). In this study, the number of *Bacillus* isolates obtained from wheat heads was low (3 - 8 colonies). The number of *Bacillus* isolates recovered from wheat heads and leaves by Larran et al. (2002 and 2007) and Yoshida et al. (2012) were also low. For example, the proportion of *Bacillus* spp. isolated by Yoshida et al. (2012) was 4.1% out of the total bacterial populations isolated from one spikelet. However, *Bacillus* spp. were isolated from 3 out of 10 wheat spikelets (Yoshida et al., 2012). According to Larran et al. (2002), *Bacillus* spp. are only found occasionally on wheat plants. The dominant bacterial genera isolated by Yoshida et al. (2012) from wheat heads were *Pseudomonas* spp., *Sphingomonas* spp., *Arthrobacter* spp. and *Curtobacterium* spp. There are other several bacterial and fungal taxa commonly found on wheat plants (Larran et al., 2007), which compete with *Bacillus*. Five *Bacillus* spp. were isolated from maize leaves and appeared to be similar on TSA plates. There is limited literature on the species identification of *Bacillus* spp. isolated from maize leaves. Those that have been isolated and identified from maize leaf surfaces include *Bacillus thuringiensis* (Jara et al., 2006) and *Bacillus pumilus* (Ersayin Yasinok et al., 2008).

All eight *Bacillus* isolates tested inhibited F.32 on agar plates. Zones of inhibitions were seen around *Bacillus* isolates, but after Day 5 incubation of bioassay plates at 28^{o}C, the zones diminished in size. Zones of inhibitions by *Bacillus* spp. against *F. graminearum* were reported by Riungu et al. (2007) and Nourozian et al. (2006) in their *in vitro* studies The zones of inhibitions around antagonists are indicators that antibiotics were secreted by the antagonists (Nourozian et al., 2006, Riungu et al., 2007; Wachowska et al., 2013). Iturin and fengycin produced by *Bacillus* spp. strains are classes of antibiotics that have demonstrated antifungal activity (Nourazian et al., 2006; Bladdino et al., 2012).

The *Bacillus* Isolates B1 and B7 were obtained from the same wheat kernel sample. These two *Bacillus* isolates caused levels of inhibition that were not significantly different. In contrast, *Bacillus* isolates obtained from leaves appeared similar on agar

plates, and caused zones of inhibition that were significantly different. Nourazian et al. (2006) compared two *Bacillus* isolates obtained from wheat heads against *F. graminearum*. They found that *B. subtilis* Strains 71 and 53 inhibited *F. graminearum* by 15 mm and 5 mm on agar plates, respectively.

None of the 16 yeast isolates were able to inhibit F.32 macroconidia from germinating on agar plates. Macroconidia germination was between 97-100 % in the presence of the yeast isolates, irrespective of the type of media. These results agree with the study by Khan et al. (2001) who found that isolated yeasts did not antagonize *Gibberella zeae* on the petri dish. However, the yeast isolate *Cryptococcus albidus* var. albidus (CBS 2991) has been shown to inhibit mycelial growth of *F. graminearum* (Wachowska et al., 2012). In this study, it was also found that F.32 produced macroconidia in the presence of yeast isolates. However, F.32 did not grow normally in the presence of the yeasts. Mechanisms of control demonstrated by biocontrol yeasts include antibiotic production and competition for food and space (Corio da Luz et al., 2003).

The two *Trichoderma* strains, T.kd and T.77 inhibited the mycelial growth of *F. graminearum* F.32 by 24-30%. However, this inhibition was less than that provided by *Trichoderma* isolates tested against *F. graminearum* by Musyimi et al. (2012) and Riungu et al. (2007). The two *Trichoderma* isolates inhibited *F. graminearum* on agar by 53 and 64%, respectively. Three isolates (*T. gamsii* 6085, 6317 and *T. veluntinum*) out of ten *Trichoderma* isolates studied by Matarese et al. (2012) inhibited *F. graminearum in vitro*. Mycoparasitism is a key biocontrol mechanism for most, if not all, *Trichoderma* strains (Lutz et al., 2003). There was no coiling by T.kd nor T.77 around F.32, as observed under phase contrast microscope, and ESEM. Hajieghrari et al. (2008) found *Trichoderma harzianum* Isolate T. 969 significantly inhibited *F. graminearum* by secreting diffusible no-volatile inhibitory substance. However, this strain did not coil around the hyphae of *F. graminearum*. Neither T.kd nor T.77 caused a zone of inhibition.

Three *Bacillus* Isolates, B13, B14 and B15, and the two commercial *Trichoderma* strains demonstrated antagonistic potential against F.32. Greenhouse and field studies would

be needed to test the effectiveness of these putative biocontrol agents against *F. graminearum* F.32.

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CHAPTER THREE

In vivo screening of microbial antagonists against *F. graminearum* Strain F.32 on wheat

Abstract

Eight *Bacillus* and four yeast isolates previously selected from *in vitro* studies were tested against *Fusarium graminearum* Strain F.32 under greenhouse conditions on wheat. Two *Trichoderma* formulations, *T. harzianum* Strain kd (T.kd) and *T. harzianum* Strain 77 (T.77) were also tested. *Bacillus* Isolate B7 significantly ($p = 0.05$) reduced Fusarium head blight (FHB) severity by 16% 22 days after inoculation. None of the four tested yeast isolates significantly ($p = 0.90$) reduced FHB. Neither T.kd nor T.77 reduced FHB under greenhouse conditions, irrespective of the number of applications

Keywords: *Bacillus, Fusarium graminearum*, Fusarium head blight, *Trichoderma harzianum*, yeast

3.1. Introduction

Fusarium head blight (FHB) is the most important disease affecting wheat (*Triticum aestivum* L.) worldwide (Boshoff et al., 1999; Juchum et al., 2006). In South Africa, FHB on wheat is especially severe under irrigated areas (Boshoff et al., 1999; Kriel and Pretorius, 2008). *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch is the major causal microorganism of FHB of wheat in five of the nine provinces of South Africa (Boshoff et al., 1999). *Fusarium graminearum* has been reported to be more aggressive than other *Fusarium* spp. causing head blight (Osborne and Stein, 2007). Mycotoxins produced by *F. graminearum* during its development within wheat heads (Muthomi et al., 2007; Yuen and Schoneweis, 2007) are a food safety issue (Legzdina and Buerstmayr, 2004). Mycotoxins reduce the quality of wheat grains and as a result, grains contaminated with mycotoxins may be rejected at the grain elevators if the levels are higher than the established maximum contamination standards (Dyer et al., 2005). Developed grains in wheat heads infected with FHB are discoloured, shriveled and weigh less than normal, thereby resulting in yield losses (Snijders, 2004; Prange et al., 2005; Ramirez et al., 2006).

Moisture and temperature play a significant role in the intensity and severity of FHB under field conditions (Rossi et al., 2002; Champeil et al., 2004), but moisture is considered much more important (Yuen and Schoneweis, 2007). FHB infection occurs when relative humidity is relatively high (100%). Usually infection takes place within 24 h – 60 h on wheat heads (Champeil et al., 2004).

Fungicides and resistant cultivars are the obvious measures to use to control FHB (Schisler et al., 2002; Schisler et al., 2006). However, there are no currently available wheat cultivars resistant to FHB (Giraud et al., 2011). Strobilurin fungicides such as azoxystrobin can actually stimulate the production of DON within wheat heads (Aldred and Magan, 2004). Triazole fungicides such as tebuconazole are sometimes effective in reducing FHB severity (Mesterházy et al., 2003; Mesterházy et al., 2011), but inconsistent reduction of FHB by triazole fungicides has been reported (Mesterházy et al., 2011). Carbendazim is a benzimidazole fungicide that was effective against FHB in

the 1980's but was rendered ineffective due to the resistance of the FHB pathogens to the fungicide (Li et al., 2008).

Biocontrol of FHB offers a promising alternative strategy to control FHB (Juchum et al., 2006; Palazzini et al, 2007; Wachowska et al., 2013). The use of biological control agents (BCAs) to control FHB disease will help reduce and eliminate the need to use of fungicides to control this disease (Riungu et al., 2007). Eliminating the use of fungicides may lead to a reduction of environmental pollution and potential hazards to human beings (Riungu et al., 2007). Promising BCAs can be incorporated in an integrated management programme for FHB (Corio da Luz et al., 2003; Palazzini et al., 2007).

There are three ways BCAs can be applied to manage FHB disease: (i) Seeds can be coated with BCAs; (ii) BCAs can be applied onto wheat heads between the anthesis and the dough growth stages; and (iii) BCAs can be applied onto crop debris to reduce inoculum that will affect subsequent crops (Paulitz, 1999; Khan et al., 2004; Luongo et al., 2005). The most popular biocontrol technique used to control FHB under greenhouse and field conditions is the application of BCAs on wheat heads between the anthesis and dough growth stages (Diamond and Cooke, 2003; Riungu et al., 2007; Yoshida et al., 2012). Bacterial, yeast and fungal BCAs have been shown to control FHB disease (Pirgozliev et al., 2003; Juchum et al., 2006). For example, application of the fungus *Phoma betae* A.B. Frank, the bacterium *Bacillus* spp. Strain AS 43.4 and the yeast *Cryptococcus* spp. Strain OH 182.9 reduced FHB severity under greenhouse conditions by 60%, 67-95%, and 48-95%, respectively (Pirgozliev et al., 2003).

In general, *In vivo* screening tests are essential in confirming the potential BCAs selected from *in vitro* tests (Elad, 2000; Dal Bello et al., 2002). *In vivo* screening assays are essential when screening putative BCAs for their ability to reduce FHB (Nourozian et al., 2006). Situations where potential BCAs have been selected from *in vitro* screening studies reduced FHB on plants have been reported (Schisler et al., 2002; Nourozian et al., 2006; Palazzini et al., 2007). On the other hand, there have been situations where BCAs did not work *in vitro* but worked *in vivo* (Khan et al., 2001).

In this chapter, fourteen antagonists selected from *in vitro* screening for activity against *F. graminearum* Strain F.32 (F.32) were studied on wheat plants under greenhouse conditions for ability to reduce FHB development.

3.2. Materials and Methods

3.2.1. Production of *Fusarium graminearum* Strain F.32

Agar plugs carrying the mycelium of F.32 stored in double sterilized distilled water was grown on plates of peptone pentacloronitrobenzene agar (PPCNBA) (Chapter Two) to select for the growth of F.32. Plates were incubated for seven days at 28^{o}C in the dark. Agar plugs carrying mycelium from PPCNBA were then transferred onto plates of potato dextrose agar (PDA; Merck) and incubated for four days at 28^{o}C in the dark. Conidial suspensions were made by transferring plugs carrying mycelia from PDA plate onto plates of Spezieller Nährstoffarmer Agar (SNA) (Chapter Two) plates, which were then incubated for 14 days at ambient temperature under continuous black light (350 nm). Macroconidia were harvested in sterile distilled water by dislodging them off the agar plates with an L-bent metal rod and adjusted to the required concentration using a haemocytometer.

3.2.2. Greenhouse conditions on *in vivo* bioassays

All *in vivo* bioassays under greenhouse conditions were treated with the same conditions as described in this section.

Five wheat seeds (PAN 3494) obtained from Pannar Seeds (Pty) Ltd, Greytown, South Africa, were planted in 24-cm-diameter pots containing composed pine bark (CPB) potting mix media. Plants were watered twice from the first week of germination until maturity. After the first week of germination, plants were fertilized once weekly with a nutrient solution (per liter) containing NPK Easy Grow Starter fertilizer 2:1:2 (43) (1g) (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa) and Easy Grow Calcium 10:0:1 (1g) (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa). Temperature in the greenhouse was maintained at 18^{o}C at night and 30^{o}C during the day. Humidity was maintained between 75-90%.

3.2.3. Pathogenicity of *Fusarium graminearum* F.32

Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. Conidial suspensions of F.32, at concentrations of $1x10^2$, $1x10^3$, $1x10^4$ and $1x10^5$ conidia.ml^{-1} were each sprayed separately using a 1 L hand sprayer onto seven wheat heads per pot at anthesis [10.51 Feeke's scale] (Zadoks et al., 1974). Wheat heads were allowed to air-dry before covering them with transparent plastic bags for 24 h. Each treatment was replicated four times. The trial was arranged in a randomized complete blocks design.

3.2.4. Effect of wheat head wetness period on the establishment of FHB

Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. An optimum wheat head wetness period for the development of FHB on wheat plants was investigated. Using a 1 L hand sprayer, F.32 inoculum at $1x10^3$ conidia.ml^{-1} was sprayed onto seven wheat heads per pot at anthesis. Wheat heads were allowed to air-dry before covering them with transparent plastic bags. Four periods of head covering were tested: 0, 6, 12, and 36 h. Each treatment was replicated three times. The trial was arranged in a randomized complete blocks design.

3.2.5. Biocontrol efficacy under greenhouse conditions

a. *Bacillus* isolates

Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. Eight *Bacillus* isolates, B1, B7, B9, B12, B13, B14, B15 and B16, were aseptically grown on tryptic soy agar (TSA; Merck) plates and incubated in the dark at 28^{o}C for two days. Pure single colonies were re-streaked onto freshly prepared TSA plates. Inoculum was prepared by growing the pure bacteria culture of each putative BCA on 15 TSA plates (90 mm diameter) in the dark at 28^{o}C for 48 h. Each bacterial isolate was separately suspended in sterile distilled water and adjusted to $1x10^7$ cells.ml^{-1} using a haemocytometer. At anthesis, using a 1 L hand sprayer, the bacterial suspensions were sprayed onto five wheat heads per pot until run off. Control wheat heads were sprayed with distilled water only. The wheat heads were allowed to air-dry before covering them

with transparent plastic bags for 24 h. Conidial suspensions ($1x10^3$ conidia.ml^{-1}) of F.32 were sprayed onto bacterial and water-treated wheat heads, which were allowed to air-dry before covering them again for another 24 h. Each treatment was replicated seven times. The trial was arranged in a randomized complete blocks design.

b. Yeasts isolates

Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. Four yeast cultures, Y52, Y53, Y57 and Y63, were grown overnight on yeast dextrose chloramphenicol agar (YDCA) (Chapter Two) plates to select for pure colonies. Single colonies were sub-cultured onto freshly prepared YDCA plates and incubated at 28^oC in the dark. Loop-fulls of each yeast isolate were re-streaked onto 10 YDCA plates (90 mm diameter) and incubated for 24 h at 28^oC in the dark. Each yeast isolate was separately suspended in sterile distilled water and adjusted to $1x10^8$ cells/ml using a haemocytometer. At anthesis each potential BCA was sprayed onto seven wheat heads per pot until run off, using a 1 L hand sprayer. Control wheat heads were sprayed with distilled water only. Wheat heads were allowed to air-dry before covering them with transparent plastic bags for 24 h. Conidial suspensions ($1x10^3$ conidia/ml) of F.32 were sprayed onto yeast and water-treated wheat heads, which were then allowed to air-dry before covering them again for another 24 h. Each treatment was replicated four times. The trial was arranged in a randomized complete blocks design.

c. *Trichoderma harzianum* strains

(i). Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. Samples of two commercial *Trichoderma* biocontrol agents, *Trichoderma harzianum* Strain kd (T.kd) and *Trichoderma harzianum* Strain 77 (T.77) were obtained from Plant Health Products (Pty) Ltd, Nottingham Road, South Africa. Inoculum was prepared by suspending 1 g of each formulation in 2 L tap water, as recommended by the manufacturer. Each suspension contained $1x10^9$ conidia/ml. Using a 1 L hand sprayer, each BCA was separately sprayed onto five wheat heads per pot until run off, when the plants were at anthesis. Control plants were sprayed with tap water only. Wheat heads were allowed to air-dry before covering them with transparent plastic bags for 24 h. Conidial suspensions ($1x10^3$ conidia/ml) of F.32 was sprayed onto the treated wheat

heads, which were allowed to air-dry before covering them again for another 24 h. Each treatment was replicated seven times. The trial was arranged in a randomized complete blocks design.

(ii). Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. A second trial was done in the same way as (i) above except that *Trichoderma* treatments were applied twice onto the wheat heads. Eight wheat heads were sprayed using 1 L hand sprayer with the *Trichoderma* inocula. The first application was done 24 h before inoculation with F.32, and the second 4 h after F. 32 application. There were four replicates per treatment. The trial was arranged in a randomized complete blocks design.

3.2.6. Survival of *Trichoderma* on wheat heads

(i). Wheat seeds were planted, watered and fertilized as described in Section 3.2.2. One gram of each T.kd and T.77 were separately suspended in 2 L of water to make up concentrations of $1x10^9$ conidia/ml. Each *Trichoderma* formulation was sprayed onto 60 wheat heads in eight pots until run-off, using a 1 L hand sprayer. The heads were at different growth stages, namely: before anthesis (GS58-59, Zadoks et al., 1974); at anthesis (GS 64-65, Zadoks et al., 1974); and after anthesis (GS 68-69, Zadoks et al., 1974). The wheat heads were allowed to air-dry before covering them with transparent plastic bags. Three wheat heads were sampled at each growth stage on Day 1, 3, 7 and 16, post inoculation. The sampled heads were surface sterilized by dipping them in 70% ethanol for 2 min, followed by 2% sodium hypochlorite (Jik®) for 3 min and rinsed three times in sterile distilled water. Excess water on wheat heads was blotted dry with paper towel. Sections of glumes, ovary/soft kernels and palea were removed with a pair of laboratory tweezers from nine wheat heads. Fifty glumes, 50 palea and 25 ovaries were plated on *Trichoderma* selective media (TSM; Chapter Two). The first 16 days, groups of glumes, palea and ovaries to be plated were selected from combined wheat heads at all growth stage to only assess the *Trichoderma* strains survival. An additional five wheat heads for each growth stage were sampled on Day 25. However, these wheat heads were not surface sterilized before plating them on agar plates, in order to compare them with the surface sterilized wheat heads. One hundred glumes and

kernels per treatment were placed onto TSM plates. On Day 25, groups of glumes and kernels to be plated were selected from wheat heads separated into different growth stages in order to evaluate both the survival of *Trichoderma* strains on wheat as well as the effect of different growth stages on the survival of *Trichoderma* strains. Plates were incubated at 25^{o}C. The emergence of *Trichoderma* spp. on wheat glumes, palea, ovaries and kernels was checked within 15 days of incubation. White colonies that emerged from wheat parts were transferred onto PDA plates and incubated under black light to confirm whether they were *Trichoderma* colonies. Cultures that produced typical green and greenish-yellow conidia on PDA plates were counted as recovered *Trichoderma*.

(ii) Two sets of ten wheat glumes picked from wheat heads growing under greenhouse were separately suspended for 5 min in 10 ml of T.kd and T.77 inoculum (1x10^{9} conidia ml^{-1}). Subsequently, glumes were removed from the suspension and placed on sterile Petri dish plates. The glumes were air-dried under laminar flow for 1 h. Dried glumes were incubated in closed sterile dishes on a laboratory bench at ambient temperature. Mycelial growth of *Trichoderma* on glumes was checked for under phase contrast microscopy for three consecutive days.

The rate of mycelial growth of *Trichoderma* on glumes was checked against the rate of F.32 mycelial growth on glumes. Ten wheat glumes were suspended for 5 min in 10 ml of F.32 inoculum (1x10^{4} conidia ml^{-1}). Treated glumes with F.32 were air- dried under laminar flow for 1 h. Dried glumes were incubated in closed sterile Petri dish on a laboratory bench at ambient temperature. Untreated glumes which served as controls were also incubated separately in a sterile Petri dish plate.

3.2.7. Disease scoring

Head blight was evaluated as severity on Day 12 and Day 22 as the number of bleached spikelets in a spike using the following formular:

$$\text{Disease severity} = \frac{\text{number of bleached spikelets} \times 100}{\text{total number of spikelets (bleached and unbleached)}}$$

3.2.8. Data analysis

Disease severity data was transformed for all treatments, using the Common Log (base 10) Transformation, except the pathogenicity data. Data was subjected to analysis of variance (ANOVA) using GenStat (VSN International Ltd, Version 14.1 Edition). Treatment means were separated using Duncan's multiple range test at the 5% probability level.

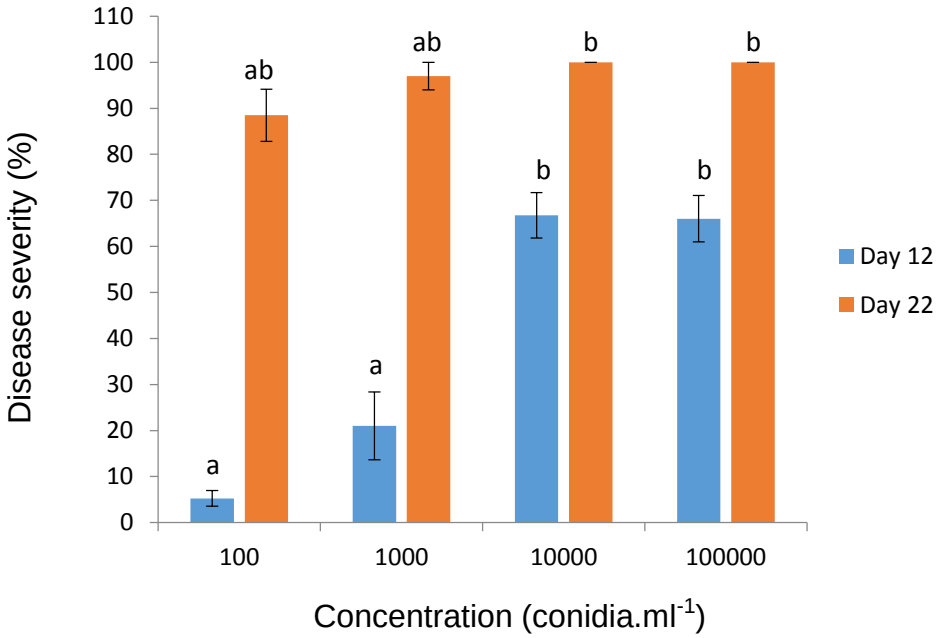
3.3. Results

3.3.1. Effect of *F. graminearum* Strain F. 32 concentrations on FHB severity.

The use of different F.32 concentrations caused different levels of FHB severity by Day 12 ($p = 0.001$) (Figure 1). Disease severities caused by lower doses ($1x10^2$ and $1x10^3$ conidia ml^{-1}) were less than or equal to 21%, whereas higher doses ($1x10^4$ and $1x10^5$ conidia ml^{-1}) caused 67% FHB severity (Figure 1). On Day 22, there was no significant difference in disease severity between concentrations (Figure 1).

3.3.2. Effect of wetness period of wheat heads on FHB severity

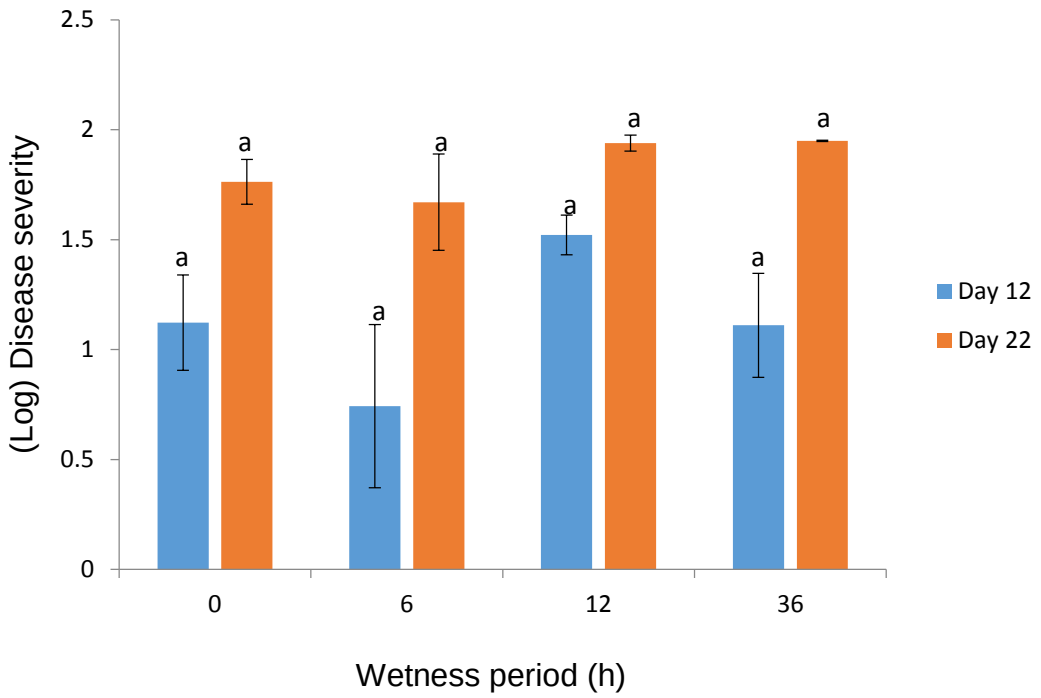
The duration of wheat head wetness had no significant effect on the development of FHB of wheat under greenhouse conditions. Disease severities on wheat heads that were covered for 6, 12 and 36 h on Day 12 ($p = 0.26$) and Day 22 ($p = 0.35$) were not significantly different (Figure 2). Disease severity after the 12 h treatment was the highest but it was not significantly higher than the other treatments on Day 12. The Control treatments were not significantly different compared to 6, 12 and 36 h treatments on Day 12 and Day 22 (Figure 2).



Day 12: P-value = 0.001; CV% = 26.1; S.E.D = 7.33

Day 22: P-value = 0.08; CV% = 6.7, S.E.D = 4.54

Figure 1: Effect of different concentrations of *F. graminearum* Strain F.32 on Fusarium head blight of wheat under greenhouse conditions



Day 12: P-value = 0.26; CV% = 38.4; S.E.D = 0.35

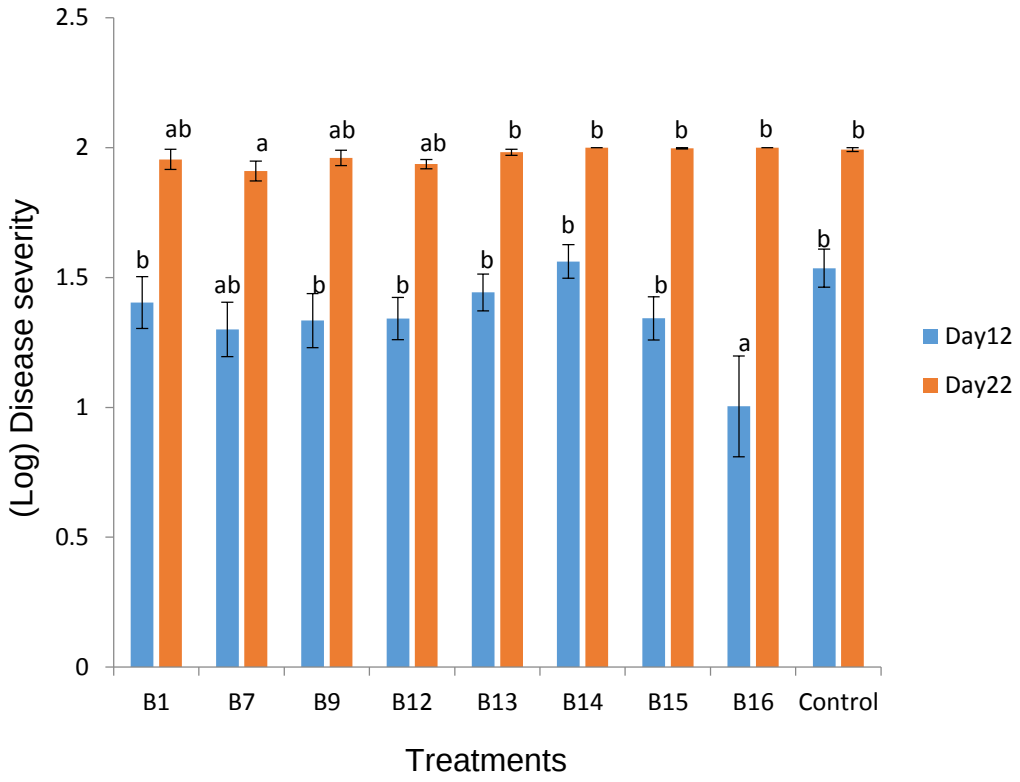
Day 22: P-value = 0.35; CV% = 11.6; S.E.D = 0.17

Figure 2: Effect of wheat head wetness period on Fusarium head blight of wheat under greenhouse conditions.

3.3.3. Efficacy of antagonists in reducing FHB severity

a. *Bacillus* isolates

Under greenhouse conditions some *Bacillus* isolates caused a reduction in disease severity. Isolate B16 reduced disease severity by 58% on Day 12 (Figure 3). The rest of the isolates reduced disease severity between 0 - 34%. Over time, disease severity increased, despite all treatments. On Day 22, Isolate B7 reduced disease severity by 16%. There was no significant difference between the FHB severity on the Control plants and those treated with the seven *Bacillus* isolates by Day 22 (Figure 3).



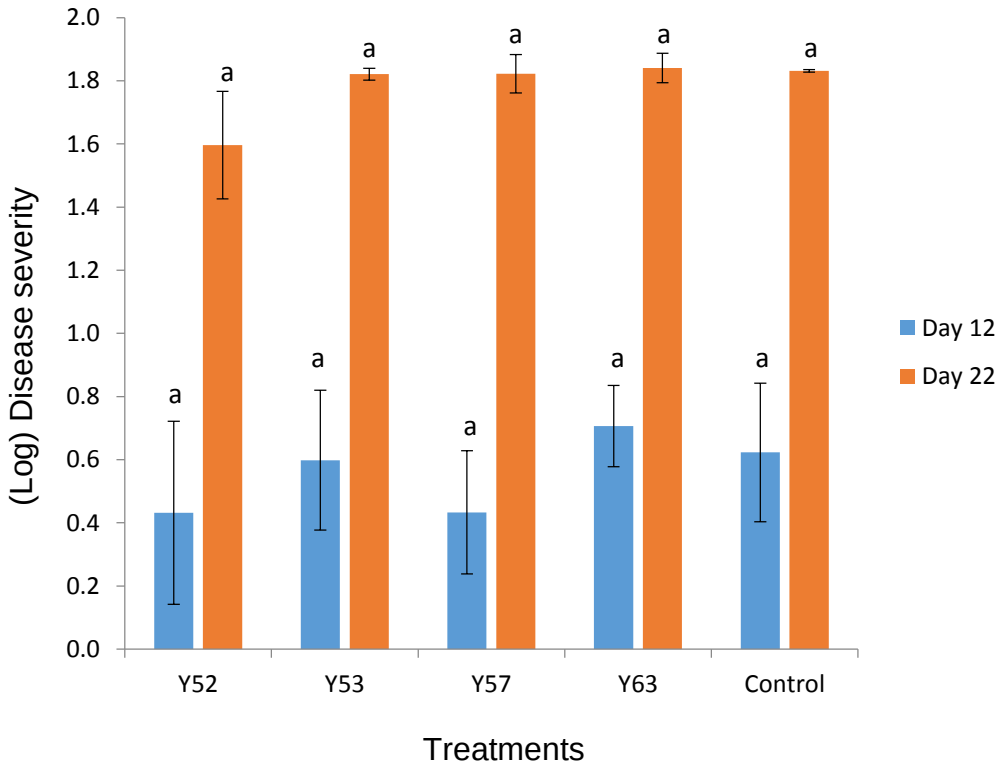
Day 12: P-value = 0.03; CV% = 18.7; S.E.D = 0.15

Day 22: P-value = 0.05; CV% = 2.7; S.E.D = 0.03

Figure 3: Levels of Fusarium head blight of wheat after treatment with eight *Bacillus* isolates under greenhouse conditions

b. Yeast isolates

There was no significant difference in reduction of disease severity between yeast isolates on both rating days (Figure 4). On Day 12, Isolates Y52, Y53 and Y.57 reduced disease severity, but not significantly, by 23%, 2% and 36%, respectively. ($p = 0.78$). However, by Day 22, Isolates Y52 and Y53 reduced disease severity by only 3% and 2%, respectively. Yeast Isolate Y57 did not cause any reduction in disease severity by Day 22.



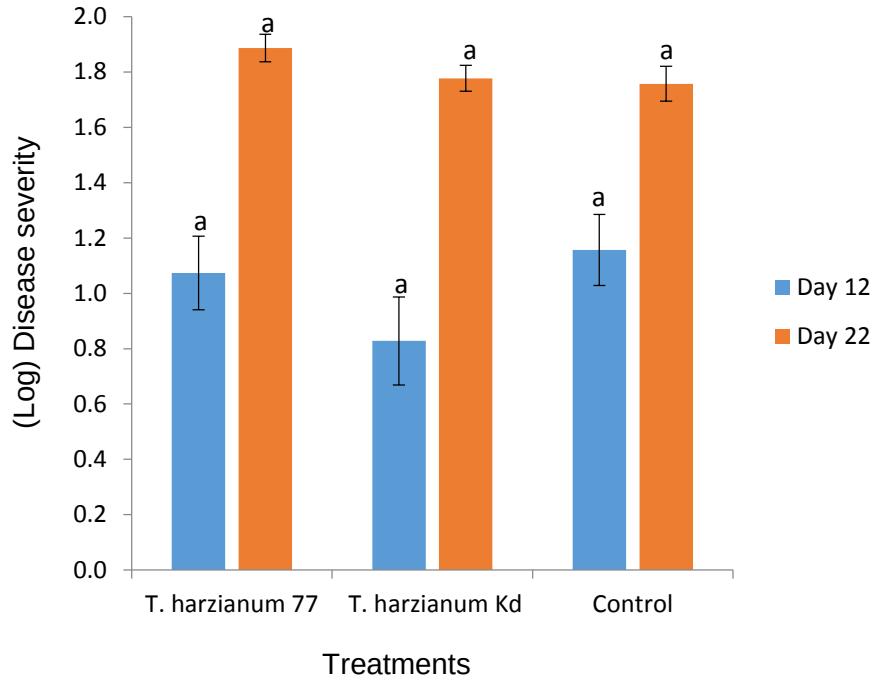
Day 12: P-value = 0.78; CV% = 62.7; S.E.D = 0.35

Day 22: P-value = 0.90; CV% = 11.1; S.E.D =0.20

Figure 4: The effect of yeast isolates on Fusarium head blight of wheat under greenhouse conditions

C. *Trichoderma harzianum* strains

One application of the two *Trichoderma* formulations, T.kd or T.77, did not significantly reduce disease severity under greenhouse conditions on both rating days (p = 0.26; p = 0.22) (Figure 5). Although, T.kd reduced disease severity slightly by Day 12, disease severity after treatment with T.kd increased to almost to the same level as the Control plants on Day 22 (Figure 5). Repeated applications of the two *Trichoderma* formulations did not reduce disease severity under greenhouse conditions, on both rating days (p = 0.56; p = 0.76) (Figure 6). A slight reduction of disease severity was seen on Day 22 on wheat heads that received repeated applications of T.kd or T.77. However, this was not the case on Day 12 (Figure 6).



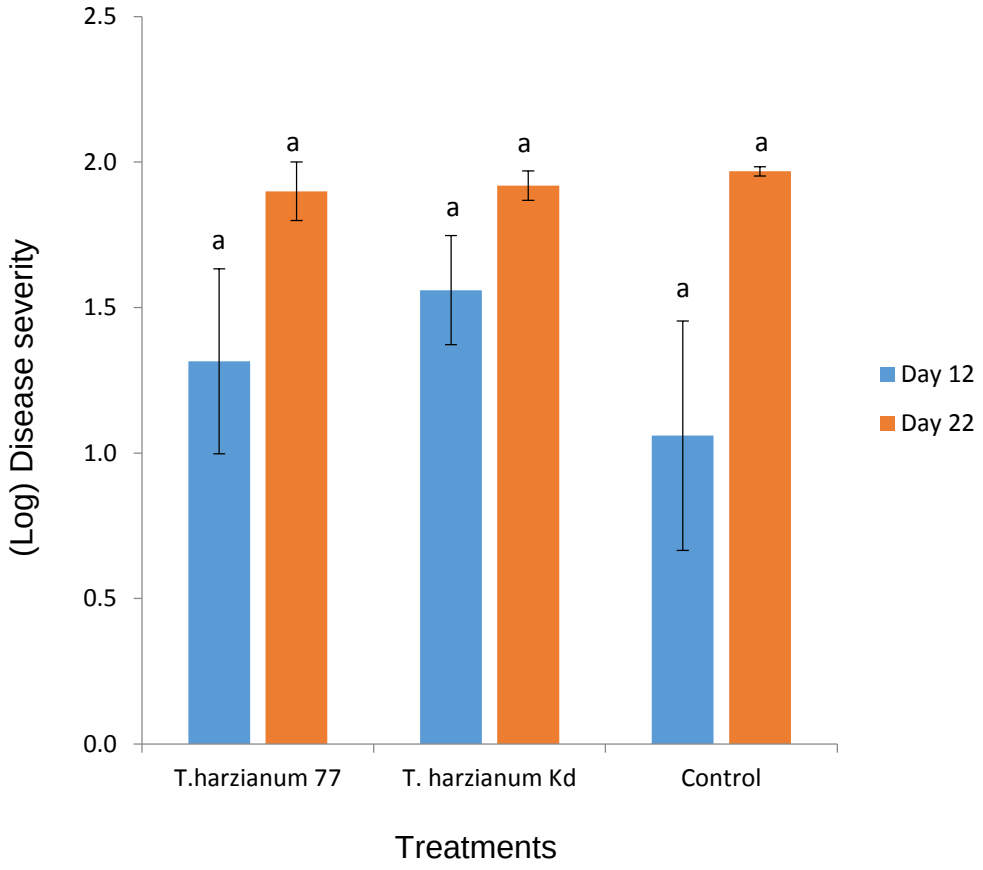
Day 12: P-value = 0.26; CV% = 36.5; S.E.D = 0.20

Day 22: P-value = 0.22; CV% = 7.9; S.E.D = 0.08

Figure 5: The effect of treating wheat heads with one application of *T. harzianum* Strain T.kd and *T. harzianum* Strain T.77 on Fusarium head blight under greenhouse conditions

3.3.4. Colonization of wheat heads by two *Trichoderma* strains

The two *Trichoderma* formulations, T.kd and T.77, demonstrated the ability to survive on glumes, palea and kernels of wheat grown under greenhouse conditions (Table 1, Figure 7 and 8). T.kd and T.77 survived for up to 25 days on wheat heads under greenhouse conditions. There was a high recovery percentage of the two *Trichoderma* strains from wheat glumes compared to kernels removed from the same unsterilized wheat heads (Figure 7 and 8). The levels of recovery of the two *Trichoderma* strains from the sterilized glumes were lower than the recovery from unsterilized glumes (Table 1 and Figure 7 and 8).



Day 12: P-value = 0.56; CV% = 41; S.E.D = 0.44

Day 22: P-value = 0.76; CV% = 5.9; S.E.D = 0.09

Figure 6: The effect of treating wheat heads with repeated applications of *T. harzianum* Strain T.kd and *T. harzianum* Strain 77 on Fusarium head blight under greenhouse conditions.

No hyphae of T.kd or T.77 were observed under the phase contrast microscope on wheat glumes (Figure 9). There were no visual differences between *Trichoderma*-treated and Control wheat glumes on all observation days. A network of hyphae of F.32 was observed on wheat glumes as early as Day 1 post pathogen inoculation (Figure 9).

Table 1: Recovery of *T. harzianum Strain* kd , *T. harzianum Strain* 77 and unidentified contaminant fungal spp. on glumes, palea and ovaries removed from sterilized *Trichoderma* treated wheat heads collected on Day 1, 3, 7 and 16 post *Trichoderma* inoculation at different wheat growth stages.

Treatment	Day	growth stage	floral part	% contaminant fungal spp.	*% Trichoderma* spp.
T. harzianum kd	1	GS58 -69	glumes	0	0
T. harzianum kd	1	GS58 -69	palea	0	17
T. harzianum 77	1	GS58 -69	glumes	0	0
T. harzianum 77	1	GS58 -69	palea,	0	0
T. harzianum kd	3	GS58 -69	glumes	0	0
T. harzianum kd	3	GS58 -69	ovaries	0	0
T. harzianum kd	3	GS58 -69	palea	35	6
T. harzianum 77	3	GS58 -69	glumes	0	0
T. harzianum 77	3	GS58 -69	palea	25	0
T. harzianum 77	3	GS58 -69	ovaries	0	0
T. harzianum kd	7	GS58 -69	glumes	7	2
T. harzianum kd	7	GS58 -69	ovaries	0	32
T. harzianum kd	7	GS58 -69	palea,	19	6
T. harzianum 77	7	GS58 -69	glumes	3	4
T. harzianum 77	7	GS58 -69	palea	21	0
T. harzianum 77	7	GS58 -69	ovaries	5	4
T. harzianum kd	16	GS58-69	glumes	27	0
T. harzianum kd	16	GS58-69	palea	43	
T. harzianum 77	16	GS58-69	glumes	25	
T. harzianum 77	16	GS58-69	palea	38	4
Un-inoculated	16	GS58-69	glumes	2	0

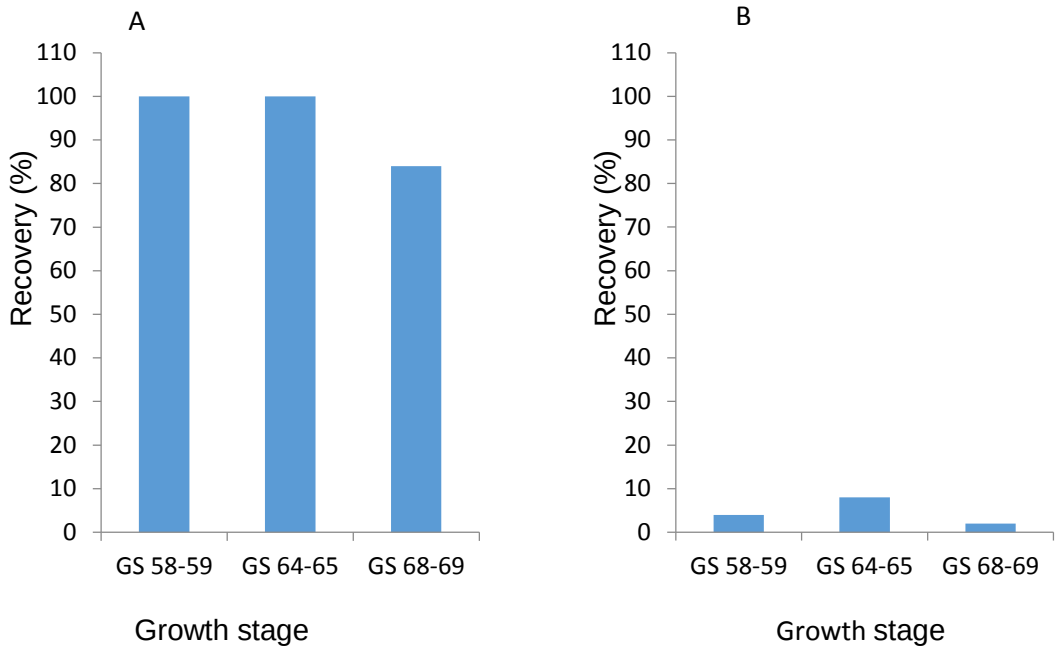


Figure 7: Percentage recovery of *T. harzianum Strain* 77 on glumes (A) and kernels (B) on Day 25 post inoculation at different wheat growth stages

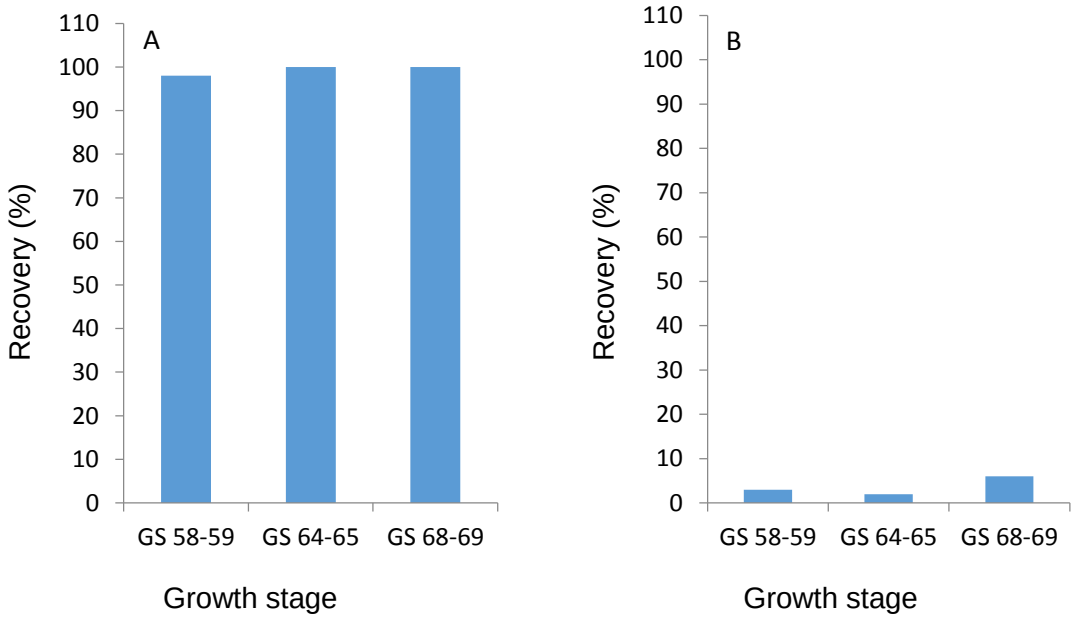


Figure 8: Levels of re-isolation of *T. harzianum Strain* kd on glumes (A) and kernels (B) on Day 25 after inoculation, when applied at different wheat growth stages

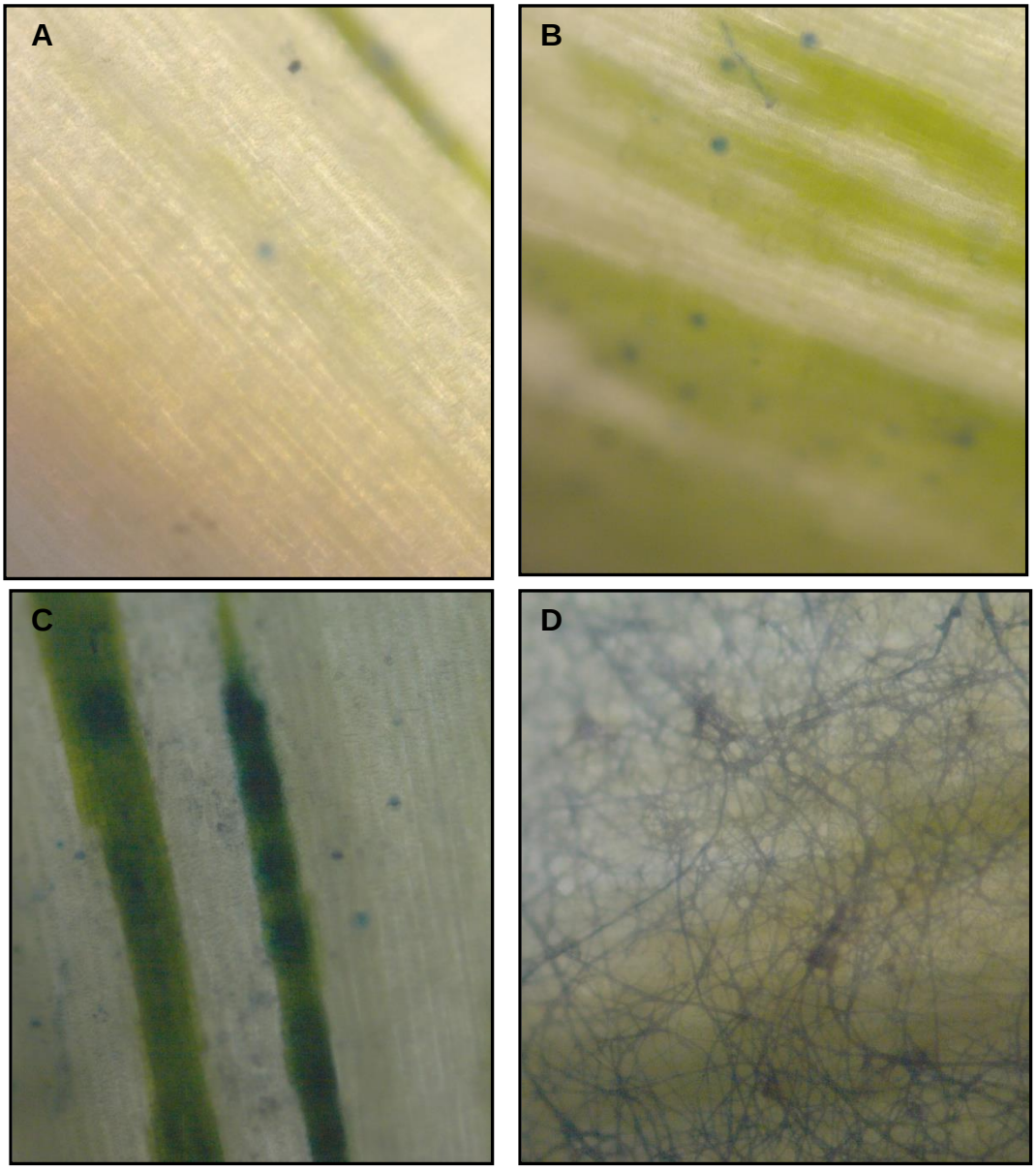


Figure 9: Colonization of wheat glumes by *T .harzianum* Strain kd and *T. harzianum* Strain *77* and *F. graminearum* Strain F.32 on Day 3 post inoculation. Wheat glumes were viewed under a phase contrast microscope (10x): **A**. Control. **B**. *T. harzianum* Strain 77. **C**. *T. harzianum* Strain kd. **D**. *Fusarium graminearum* Strain F.32.

3.4. Discussion

In this study, the pathogenicity of F.32 was confirmed under greenhouse conditions on wheat. A concentration of $1x10^2$ conidia ml^{-1} of F.32 was sufficient to cause infection and subsequently disease severity of 88% by Day 22 post pathogen inoculation (ppi) on wheat under greenhouse conditions (Figure 1). According to Lori et al. (2009), a minimal inoculum is sufficient to cause FHB infection provided that environmental conditions especially temperature and moisture are favaurable. Disease severity levels resulting from different concentrations of F.32 were not different on Day 22. However, a concentration of $1x10^3$ condia ml^{-1} was used in all subsequent greenhouse trials because this concentration caused a level of disease severity that was neither too low nor too high at the early disease stages.

The duration of wheat head wetness had no effect on FHB severity under greenhouse conditions. The disease severities on previously covered wheat heads (6, 12; 36 h) were not significantly different from the disease severity of uncovered wheat heads (0 h) by the final disease rating. Moreover, FHB severity on wheat heads that were covered for a short period (6 and 12 h) was not significantly different from the level of FHB severity after covered for a longer period (36 h) (Figure 2). The results from our study differ from a study that found a higher infection level of *F. graminearum* on wheat heads than that were covered for 24 h as compared to 17 h (Lemmens et al., 2004). Moreover, wheat heads that were bagged post-inoculation developed more disease heads that were not covered (Lemmens et al., 2004). In our study, significant differences in FHB severity between covered and uncovered wheat heads were not observed because the percentage RH in the greenhouse was high and favourable (70-90%) for F.32 infection. Hence covering wheat heads did not cause any difference in disease level compared to uncovered heads. Although there were no significant differences in disease severity between covered and uncovered wheat heads; in subsequent greenhouse trials, the wheat heads were covered for 24 h post-inoculation to ensure that all wheat heads received the same amount of moisture after inoculation

Bacillus is one of the well-studied bacterial genera for its biological control activity against FHB. Several *Bacillus* spp. have proven to be good antagonists against *F.*

graminearum (Schisler et al., 2002; Nourozian et al., 2006; Palazzini et al., 2007). However, the *Bacillus* strains tested by Nourozian et al. (2006) under greenhouse conditions did not reduce FHB severity, possibly because: i. Different fungal isolates respond to different *Bacillus* strains; ii. Fungi may have ability to detoxify metabolites; and iii. Antibiotics may become inactive because of the environment. In this study, *Bacillus* Isolate B7 reduced disease severity by 16% by Day 22 ppi.

Another issue is that the *Bacillus* isolates tested in this study were not grown in liquid culture media but rather on agar plates. *Bacillus* spp. tested under greenhouse conditions by Riungu et al. (2007) and Nourozian et al. (2006) were grown on agar plates and did not reduce disease severity. In research by Schisler et al. (2002), *Bacillus* Strain AS 43.3 was grown in liquid media. This product reduced FHB severity and incidence by 96% and 78% under greenhouse conditions. Antibiotics were produced during cells growth, and excreted into the culture liquid, and they were retained on wheat heads, where they were presumed to have acted against *F. graminearum*. Juchum et al. (2006) reported suppression of FHB severity when a broth of bacterial cells and their secondary metabolites were sprayed on wheat heads. Therefore, it is possible that the efficacy of *Bacillus* strains tested to reduce FHB severity on wheat heads in this study was affected by the inoculum production method used in the laboratory.

Bacillus Isolate B16 reduced disease severity more than the other seven *Bacillus* isolates by Day 12 ppi. This *in vivo* result was not expected because Isolate B16 performed poorly in the prior *in vitro* bioassay (Chapter Two). In contrast, Isolate B14 was one of the best isolates in the *in vitro* study (Chapter Two), but it failed to reduce FHB severity on all disease rating days. Therefore, this study confirms that *in vitro* results do not correlate well with *in vivo* studies (Dal Bello et al., 2002).

Yeast isolates, in particular *Cryptococcus* strains have been shown to reduce FHB severity and incidence by 50-60% and 57-60%, respectively, under greenhouse and field conditions (Schisler et al., 2002; Khan et al., 2004; Schisler et al., 2013). In this study, yeast Isolates Y52 and Y57 reduced disease severity by between 23 - 36%, but not significantly, by Day 12 ppi. Thereafter, there was no further improvement in control

of FHB by any of the yeast isolates. The mechanisms of control of yeasts for the control of FHB have not been elucidated, but postulated mechanisms include competition by yeasts for nutrients and space in the infection court of wheat flowers, and production of antibiotics (Corio da Luz et al., 2003). Yeast strains that have reduced FHB severity under greenhouse and field conditions were grown in liquid media (Schisler et al. 2002, Schisler et al. 2013). As with bacterial biocontrol agents, yeast produces antibiotics in the liquid media that act against *Fusarium* spp. after being sprayed onto wheat heads. In this study, yeast were grown on solid media and as a result, they were as not effective as yeast isolates used in other studies by Khan et al. (2001), Khan et al. (2004) and Schisler et al. (2013) who all grew their yeast biocontrol agents in liquid media. Khan et al. (2001) reported that yeast isolates that did not inhibit *F. graminearum* on agar plates successfully reduced FHB levels under greenhouse conditions. In this study, the four tested yeast isolates did not inhibit the germination of F.32 macroconidia on agar plates (Chapter Two), and they did not reduce FHB severity under greenhouse conditions.

Fungal physiology and the production of secondary metabolites are affected by nutrient availability (Matarese et al., 2012). *Trichoderma gamsii* Strain 6085, which has demonstrated mycoparasitism and subsequently inhibited *F. graminearum in vitro,* was not able to inhibit *F. graminearum in vivo* on wheat haulms (Matarese et al., 2012). Nutrient levels on wheat haulms are poor, hence *T. gamsii* Strain 6085 developed poorly on wheat haulms, and the growth of *Fusarium* species were unaffected by the presence of the antagonist (Matarese et al., 2012). In this study, it is possible that the conidia of the two *Trichoderma harzianum* strains were not able to germinate on wheat glumes because of the poor nutrient levels on the surface of the glumes. On the other hand, *F. graminearum* does not need nutrients to germinate, simply moisture (Bushnell et al., 2003). Hence, a vigorous hyphal network of F.32 developed on the glumes, compared to the lack of growth of the two *T. harzianum* strains, which did not appear to germinate or grow. However, the conidia of both strains survived for 25 days on wheat under greenhouse conditions. *Trichoderma harzianum* Strain T39 has been reported to survive on wheat leaves for 1 year (Perazolli et al., 2008).

In this study, T.kd and T.77 reduced disease severity by less than 10% by Day 22 ppi, irrespective of the number of applications on wheat heads. Riungu et al. (2007) studied the efficacy of *Trichoderma* spp. against *F. graminearum* and observed a reduction of disease severity of 25% under greenhouse conditions. The strains of *Trichoderma* they tested were more effective than *Bacillus* spp., *Epicoccum* spp. and *Alternaria* spp. (Riungu et al., 2007). The species of *Trichoderma* tested in the study of Riungu et al. (2007) was not mentioned. Different *Trichoderma* species may respond differently to a particular *F. graminearum* strain (Matarese et al., 2012). At least three *Trichoderma* species, *T. gamsii* 6085*, T. gamsii 6317, T. velutinum 4837,* were able to parasitize and inhibit *F. graminearum* on agar plates (Matarese, 2012). Strains of other *Trichoderma* species (*T. citrinoviride* 8218, 8244; *T. harzianum* 8243; *T. viride* 7634, 7636, 7646) were not active *in vitro* against the same *F. graminearum* (Matarese et al., 2012). The two *Trichoderma* strains were unable to parasitize F.32 on agar plates (Chapter Two). They were also unable to establish well on wheat glumes. The combination would explain why these two strains of *T*. *harzianum* were unable to reduce the severity of FHB under greenhouse conditions.

Overall, *Bacillus* Isolate B7 reduced FHB severity on wheat heads slightly better than the other tested *Bacillus* isolates. None of the tested yeast isolates significantly reduced disease severity under greenhouse conditions, nor did the two strains of *T. harzianum*, irrespective of the number of applications.

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CHAPTER FOUR

Efficacy of single and combined applications of potassium silicate and *Trichoderma* strains on Fusarium head blight of wheat

Abstract

Three rates (1.5, 3 and 4.5 g) of a granulated formulation and three concentrations (200, 300 and 600 mg L^{-1}) of a liquid formulation of potassium silicate (KSi) were tested against Fusarium head blight (FHB) of wheat under greenhouse conditions. Granulated potassium silicate (KSi) gave slightly better control of FHB than the liquid KSi on wheat under greenhouse conditions in two trials. Granulated KSi (3 g) reduced FHB by 49% compared to the KSi untreated Control Control treatment on Day 12 during the first KSi trial ($p = 0.004$). In the second trial, granulated KSi (4.5 g) reduced FHB by 54% compared to the untreated Control treatment on Day 12. Control of FHB severity did not improve after Day 12 as a result of either the granulated or liquid treatments during the two KSi trials. Treatment with granulated KSi at 3 and 4.5 g had lower disease severities than the 1.5 g granular KSi treatment on Day 12 during the two KSi trials. However, the liquid formulation of KSi 200 and 300 mg L^{-1}gave better protection than the application at 600 mg L^{-1}. There was no significant difference in the severity of FHB of wheat heads, or the number of infected grains, after treatments with KSi alone, or KSi combined with the two *Trichoderma* formulations, *T. harzianum* Strain kd and/ or *T. harzianum* Strain 77.

Keywords: Biocontrol, *Fusarium graminearum*, Fusarium head blight, potassium silicate, *Trichoderma harzianum*.

4.1. Introduction

The best way to control Fusarium head blight (FHB) of wheat is through cultivation of resistant cultivars. However there are no highly resistant cultivars currently available (Mesterházy et al., 2011). Fungicides are an option in regions prone to the disease (Lacey et al., 1999). However, the application of fungicides such as azoxystrobin has been correlated with the accumulation of deoxynivalenol (DON) within wheat (*Triticum aestivum* L.) grains (Lacey et al., 1999; Khan et al., 2004; Giraud et al., 2011). Other problems associated with the use of fungicides include: the high cost (Lacey et al., 1999); poor levels of control of 50% or lower (Mesterházy et al., 2011); and residues in the wheat grains (Bai et al., 2003; Corio da Luz et al., 2003; Khan et al., 2004). Therefore, there is a need for safer, more effective strategies for FHB control. Biocontrol of FHB offers a promising strategy and has been demonstrated to work in some studies (Schisler et al., 2002; Diamond and Cooke, 2003; Juchum et al., 2006; Schisler et al., 2006, Riungu et al., 2007; Palazzini et al., 2007; Palazzini et al., 2009). Biological control of FHB is effective if it is incorporated into an integrated management program for FHB (Corio da Luz et al., 2003; Palazzini et al., 2007). The genus *Trichoderma* is among the most effective biopesticides used against fungal diseases (Scala et al., 2007). Strains of *Trichoderma* spp. have been applied onto wheat heads (Riungu et al., 2007) and crop residues (Lutz et al., 2003), to reduce FHB on wheat.

Silicon is the second most abundant element after oxygen (Hull, 2004; Ma and Yamaji, 2006, Van Bockhaven et al., 2013) but soluble Si that is readily available for uptake by plant roots in soil solution is low (Hull, 2004). Silicon assists plants in alleviating biotic and abiotic stress (Ma and Yamaji, 2006; Smith, 2011; Dallagnol et al., 2012, Van Bockhaven et al., 2013). The biotic stresses include pest and microorganisms, and abiotic stresses include water logging, metal toxicities, salinity and temperature extremes (Ma and Yamaji, 2006; Rains et al., 2006; Epstein, 2009). Important diseases of wheat, barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), cucumber (*Cucumis sativus* L.), grapes (*Vitis vinifera* L.), rice (*Oryza sativa* L), rye (*Secale cereale* L.), soybean (*Glycine max* (L.) Merr, strawberry (*Fragaria x ananassa* Duchesne) and melon (*Cucumis melo* L.) have been efficiently controlled by supplying soluble Si to crops

(Xavier Filha et al., 2011; Dallagnol et al., 2012). Silicon plays two roles in plant's defense against diseases: (1). it acts as a physical barrier beneath the cuticle hindering pathogens from entering the plant through the epidermis and; (2). it acts as a priming agent of primary the plant defense response (i.e. enhancing the speed and strength of response in the production of antifungal compounds) once pathogens induce the resistance reaction by passing through the epidermis (Fauteux et al., 2005; Ma and Yamaji, 2006; Van Bockhaven et al., 2013).

Wheat is classified as a Si accumulator (Maryland et al., 1991). Silicon present in bracts of inflorescences (lemma, palea and glumes) is heavily deposited in the trichomes, papillae and macrohairs (Hodson and Sangster, 1988; Hodson and Sangster, 1989).

The aim of this chapter was to investigate the effect of Si application in two formulations of potassium silicate on FHB on wheat. The effect of combining the granulated formulation of potassium silicate with two strains of *Trichoderma harzianum* Strain kd (T.kd) and Strain 77 (T.77) was also investigated.

4.2. Materials and Methods

4.2.1. Production of *F. graminearum* Strain F.32 inoculum

An agar plug containing mycelium of *F. graminearum* Strain F.32 (F.32), stored in sterile distilled water in the laboratory at ambient temperature, was grown on peptone pentacloronitrobenzene agar (PPCNBA) (Chapter Two) plates for selection of F.32. Plates were incubated for seven days at 28^{o}C in the dark. Agar plugs containing mycelium on PPCNBA were transferred onto potato dextrose agar (PDA; Merck) plates and incubated for four days at 28^{o}C in the dark. Conidial suspensions were made by transferring plugs containing mycelia from PDA plates onto 15 Spezieller Nährstoffarmer Agar (SNA) (Chapter Two) plates. These plates were incubated for 14 days at 20-22^{o}C under continuous black light (350 nm). Macroconidia were harvested in sterile distilled water by dislodging them off the agar plates with an L-bent metal rod. The conidial concentration was adjusted to 1x10^{3} conidia ml^{-1} using a haemocytometer.

4.2.2 Greenhouse conditions for *in vivo* bioassays

All *in vivo* bioassays under greenhouse conditions were treated with the same conditions as described in this section.

Seven wheat seeds (PAN 3494) (Pannar Seeds (Pty) Ltd, Greytown, South Africa) were planted in 24 cm diameter pots containing a composted pine bark (CPB) seedling mix media. Two weeks after germination, plants were thinned to five seedlings per pot and plants were watered until maturity. Plants were fertilized once a week with a nutrient solution (per liter) containing NPK Easy Grow Starter fertilizer 2:1:2 (43) (1 g) (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa) and Easy Grow Calcium 10:0:1 (1 g) (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa). Temperature in the greenhouse was maintained at 18^{o}C at night and 30^{o}C during the day. Humidity was maintained at 75-90% during the day and at night.

4.2.3. Effect of potassium silicate on FHB under greenhouse conditions.

Wheat seeds were planted, watered and fertilized as described in Section 4.2.2 unless otherwise specified.

Potassium silicate (KSi) in the form of granulated and liquid AgriSil K50 (PQ Corporation, South Africa) were used as the sources of soluble Si. Granulated KSi at three rates (1.5, 3 and 4.5 g) was dispersed evenly into the media at planting. A light layer of CPB medium was used to cover the seeds in each pot, and then the seeds were watered. Liquid KSi was first applied at the three leaf stage (Growth Stage 13, Zadoks et al., 1974) at concentrations of 200, 300 and 600 mg L^{-1}. The plants were drenched (250 ml per pot) separately with each KSi concentration once a week until plants were completely blighted by FHB. The Control treatment plants were watered and fertilized as described in Section 4.2.2. At anthesis (10.51 Feeke's scale, Zadoks et al., 1974), inoculum of F.32 was sprayed onto five wheat heads per pot at 1x10^{3} conidia ml^{-1}, using a 1 L hand sprayer. Wheat heads were allowed to air-dry before covering them with transparent plastic bags for 24 h, to enhance humidity. Treatments were replicated seven times and arranged in a randomized complete block design. The experiment was repeated twice.

4.2.4. The effect of combining potassium silicate with *Trichoderma* spp. on FHB.

Wheat seeds were planted, watered and fertilized as described in Section 4.2.2 unless otherwise specified.

Granulated KSi (1.5 g) was weighed and uniformly spread onto the surface of CPB media in a pot at planting of seeds. Seeds were introduced after KSi application, covered and watered. Wheat plants were grown until the stage of anthesis, when *Trichoderma* formulations were sprayed on. Suspensions of two *Trichoderma* strains, T.kd and T.77 (Plant Health Products (Pty) Ltd, South Africa) were prepared by mixing one gram of the biocontrol agent in 2 L of tap water to make up $1x10^9$ spores ml^{-1}, as recommended by the manufacturer. The *Trichoderma* inoculum was sprayed onto wheat heads until run off using a 1 L hand sprayer. Subsequently the plants were allowed to air-dry. After 24 h, inoculum of F.32 at $1x10^3$ conidia ml^{-1} was sprayed onto the wheat heads using a hand sprayer. Treatments consisted of **1***.* T.kd (applied once) + KSi, **2**. T.kd (applied twice) + KSi (2T.kd+KSi), **3**. T.77 (applied once) + KSi (T.77+KSi), 4. T.77 applied twice + KSi (2T.77+KSi), 5. KSi alone and 6. Control. Wheat heads that were inoculated twice with a *Trichoderma* strain were sprayed with *Trichoderma* 24 h before and 24 h after *F. graminearum* F.32 inoculations. Control heads were sprayed with water only. Treatments were replicated five times and the pots were arranged in a randomized complete block design.

Grains were harvested from all mature wheat plants. Subsequently, seeds were threshed by hand; surface sterilized by dipping them in 70% ethanol for 2 min, followed by dipping in 2% sodium hypochlorite (Jik®) for 3 min and rinsing three times in sterile distilled water. Excess water on the wheat seeds was blotted off with sterile paper towels. Sterilized seeds were plated on PPCNBA and incubated at 28^oC in the dark. The seeds were examined after 14 days to quantify the emergence from F.32.

4.2.5. Disease scoring

Head blight severity was recorded as the number of bleached spikelets in a spike using the following formula:

$$\text{Disease severity} = \frac{\text{number of bleached spikelets} \times 100}{\text{total number of spikelets (bleached and unbleached)}}$$

Disease scoring for KSi trials was done on Day 12, 22 and 28, while disease scoring for the interaction experiment was recorded on Day 18 and 28 due to delayed disease development.

4.2.6. Data analysis

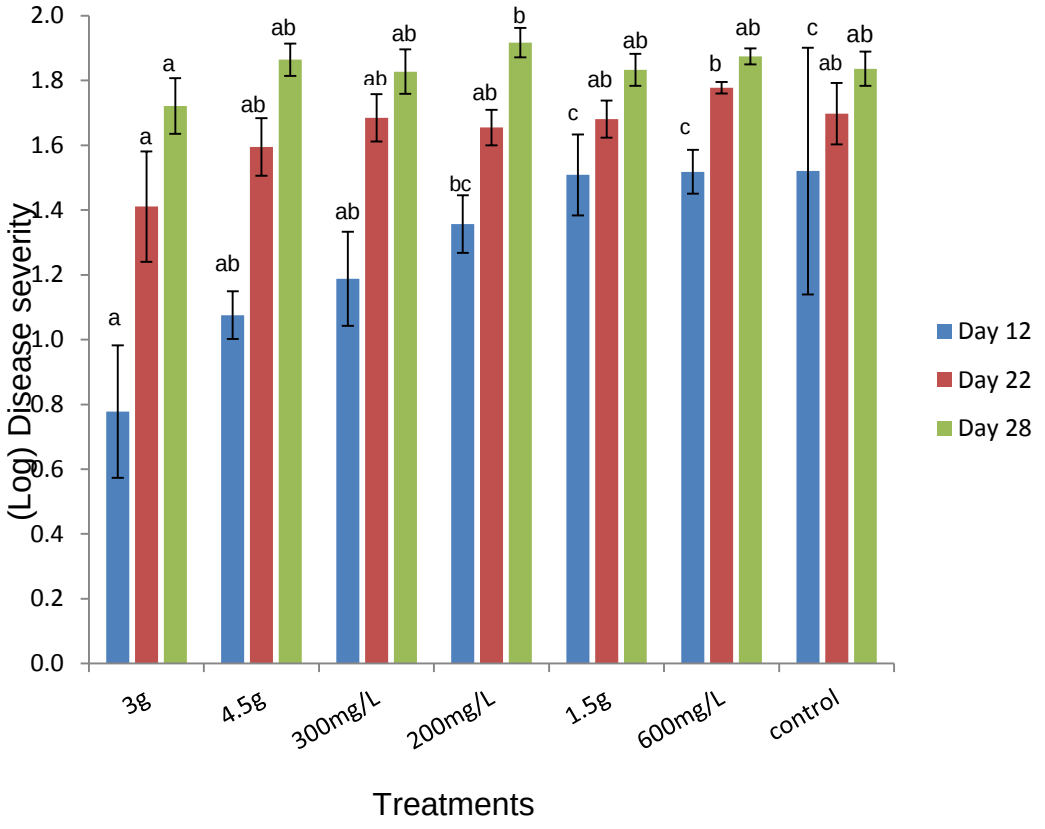
Disease severity data was transformed to Log (base 10) before subjected to analysis of variance (ANOVA) using Genstat (VSN International Ltd, Version 14.1 Edition). Treatment means were separated using Duncan's multiple range test at the 5% probability level.

4.3. Results

4.3.1. Efficacy of potassium silicate on Fusarium head blight

1st potassium silicate trial

Significant disease severity differences among granular KSi treatments were seen on Day 12 ($p = 0.004$) but not on Day 22 and Day 28, post-pathogen inoculation (Figure 1). Wheat plants treated with 3 g granular KSi had lower disease severity on all three rating days (Day 12, Day 22 and Day 28), although these were not significant on Day 22 and Day 28 (Figure 1). Disease severity on wheat plants treated with 3 g granular KSi was reduced by 49%, 17% and 6% on Day 12, Day 22 and Day 28, respectively, compared to the KSi untreated Control treatment. Increasing granulated KSi concentrations reduced disease severity but only on Day 12 (Figure 1).



Day 12: P = 0.004; %CV = 17.5; S.E.D = 0.15

Day 22: P = 0.18; %CV = 12.4 ; S.E.D = 0.13

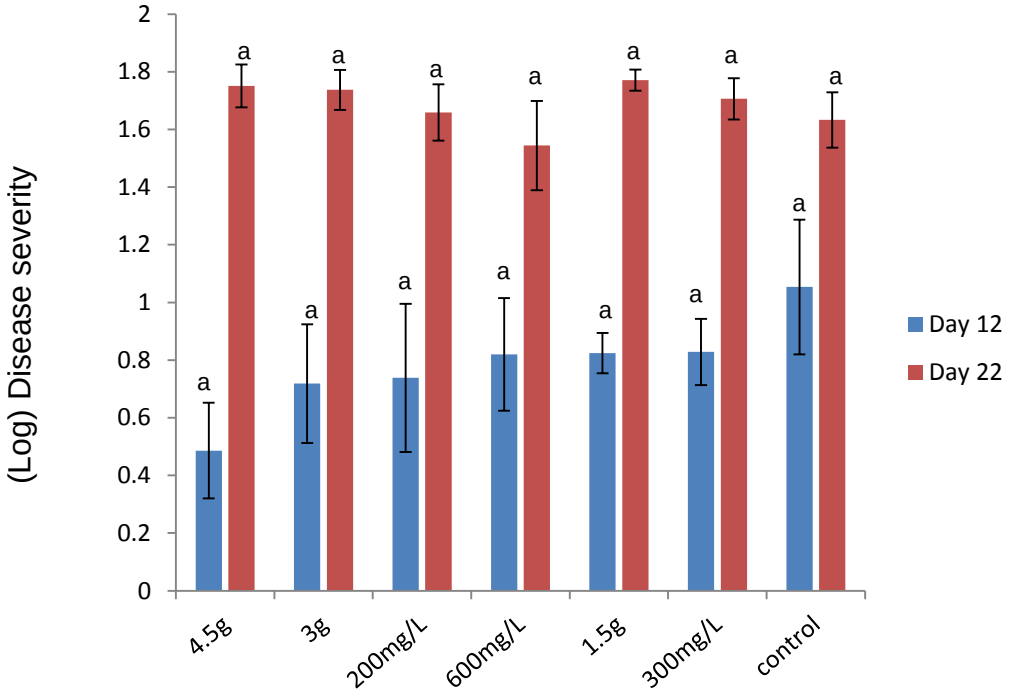
Day 28: P = 0.37; %CV = 6.9 ; S.E.D = 0.08

Figure 1: First trial on the efficacy of granulated and liquid potassium silicate (KSi) fertilization at three concentrations each against Fusarium head blight of wheat under greenhouse conditions

Liquid (300 mg L^{-1}) KSi treatments significantly reduced disease severity compared to 600 mg L^{-1} liquid KSi on Day 12 (Figure 1). There was no significant difference in the effect of 200 mg L^{-1} and 300 mg L^{-1} liquid KSi treatments on disease severity on Day 12 (Figure 1). Disease severity increased after Day 12 on all wheat plants treated with both granulated and liquid KSi products. Significant differences were not observed between granulated and liquid KSi treatments on Day 22 and 28.

2nd potassium silicate trial

Neither the granulated nor liquid KSi treatments significantly reduced FHB severity compared to the pathogen-inoculated Control treatment on Day 12 in the repeat KSi trial (Figure 2). Application of granulated KSi at 4.5 g resulted in the lowest level of disease severity, reducing it by 54% compared to the Control treatment. Wheat plants treated with granulated KSi at 3 and 4.5g developed lower levels of disease, although not significantly lower than those treated with 1.5 g granulated KSi, when evaluated on Day 12 (Figure 2). Application of the liquid KSi at 200 mg L^{-1}resulted in less FHB, although not significantly less than plants treated with 300 and 600 mg L^{-1}, when evaluated on Day 12 (Figure 2). Unlike in the first KSi trial, all wheat plants developed completely bleached heads by Day 28 in the second KSi trial.



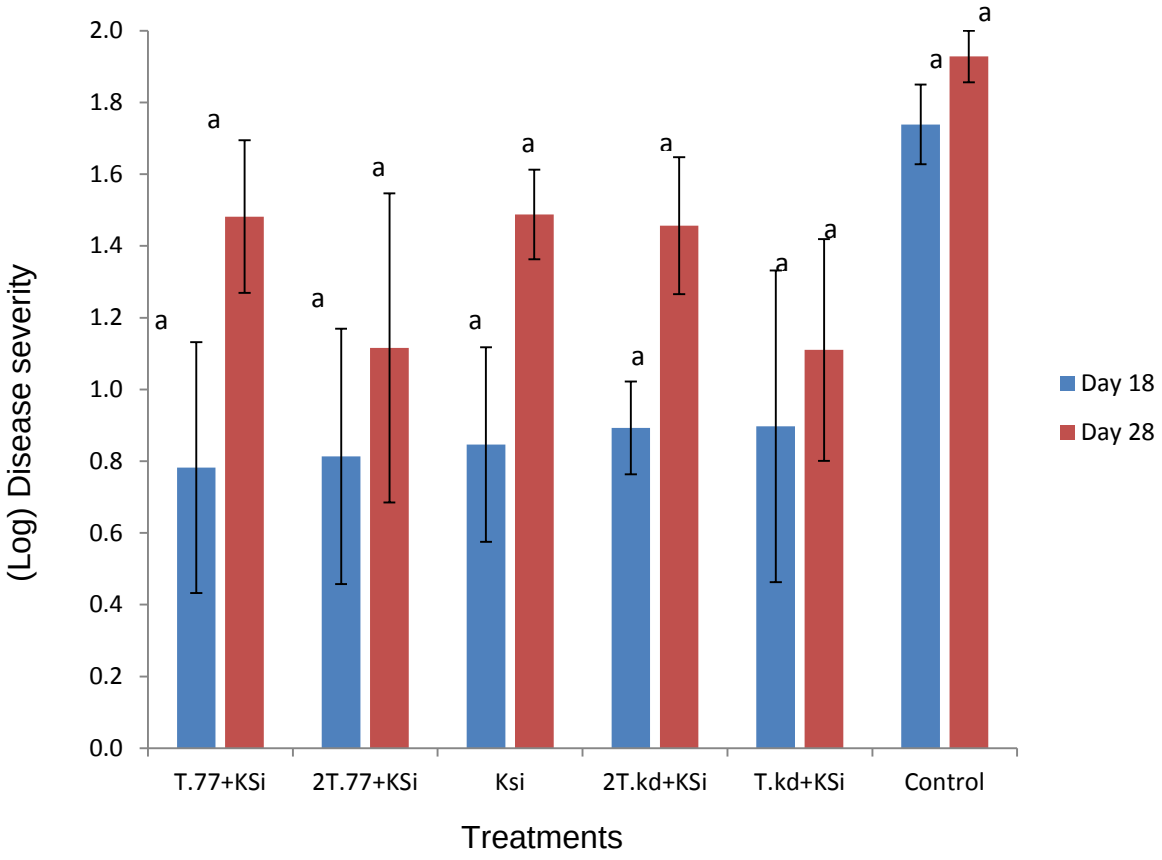
Day 12: P = 0.59; %CV = 49; S.E.D = 0.25

Day 22: P = 0.61 %CV = 12.2; S.E.D =0.13

Figure 2: Second trial on the efficacy of granulated and liquid potassium silicate (KSi) fertilizerd against Fusarium head blight of wheat under greenhouse conditions.

4.3.2. The effect of combining potassium silicate fertilization with two *Trichoderma* strains on the development of FHB

No significant differences were observed as a result of the treatments on Day 18 (p = 0.38) and Day 28 (p = 0.25) (Figure 3). Increasing the number of applications of T.77 and T.kd did not significantly reduce FHB severity compared to the KSi untreated Control treatment on both Days 18 and 28. Combining either of the *Trichoderma* treatments with KSi did not significantly reduce FHB severity compared to the treatment with KSi alone.

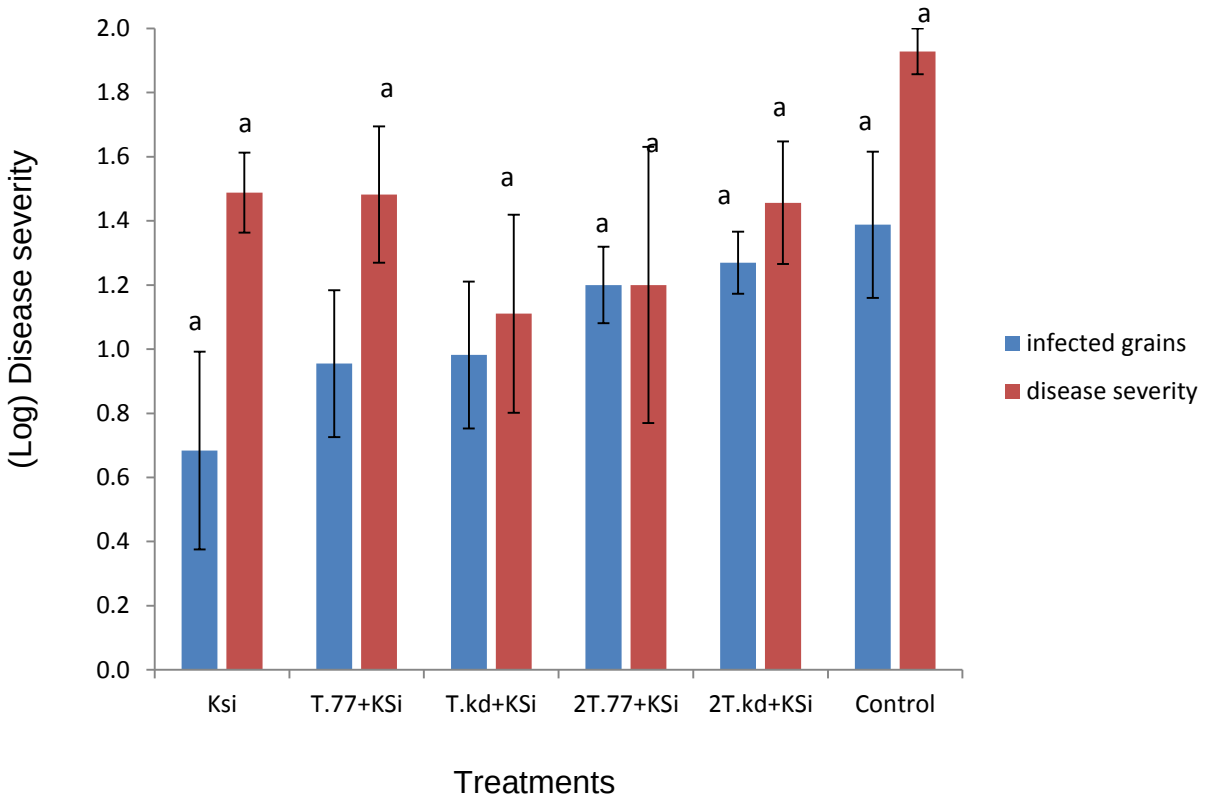


Day 18: P = 0.38, %CV=59.1; S.E.D = 0.55

Day 28: P = 0.25; %CV=39.5; S.E.D. = 0.36

Figure 3: The efficacy of combining potassium silicate (KSi) fertilization and two *Trichoderma* strains, Strain T.kd (T.kd) and Strain 77 (T.77) against Fusarium head blight on wheat.

There was no significant differences in the frequency of emergence of F.32 from infected seeds as a result of the various treatments (p = 0.53) (Figure 4). The number of infected seeds with F.32 infections was constantly lower than the number of infected heads as a result of all the treatments (with the exception of the 2T.77+KSi treatment) (Figure 4).



Infected grains: P = 0.53; %CV=35; S.E.D = 0.27

Disease severity: P = 0.25; %CV=39.5; S.E.D = 0.36

Figure 4: Comparison between disease incidence (% infected grains) and disease severity on wheat heads (% blighted heads) treated with *T. harzianum* strains, StrainT.kd (T.kd), Strain T.77 (T.77) and potassium silicate (KSi).

4.4. Discussion

Silicon fertilization has been shown to control grass diseases such as blast (*Magnaporthe grisea* (Hebert) Barr) in rice, sheath blight (*Thanatephorus cucumeris* (A.B. Frank) Donk.), brown spot (*Cochliobolus miyabeanus* (S. Into & Kurib.) Drechsler

ex Dastur) in rice (Dordas, 2008) and powdery mildew (*Blumeria graminis* (DC.) E.O Speer *f.sp. tritici* Em. Marchal) (Belanger et al., 2003) and spot blotch (*Bipolaris sorokiniana* (Sacc.) Shoemaker) in wheat (Domiciano et al., 2010).

However, neither the granulated nor the liquid KSi fertilizers were effective in reducing FHB caused by *F. graminearum* Isolate F.32 on wheat plants under greenhouse conditions on Days 22 and 28 in the KSi trials. Application of granulated KSi treatments resulted in a greater reduction in FHB levels than the application of liquid KSi treatments, when assessed on Day 12 during the two KSi trials, although the results were not significantly different in the second trial.

To the best of our knowledge, there is no literature on the use of KSi against FHB of wheat and therefore these results cannot be compared to other studies with the similar content. However, wheat diseases such as powdery mildew (Bélanger et al., 2003; Guével et al., 2007; Côté-Beaulieu et al., 2009) and spot blotch (Domiciano et al., 2010) were successfully reduced by Si fertilizer applications. The successful reduction of these diseases was due to the fact that these studies focused on wheat leaf tissues. Approximately 90% of absorbed silicon by the plant is deposited in the leaves (ZanãoJúnior et al., 2010). The effect of Si deposition in leaves might have been stronger because silicon played two roles. Firstly, silicon acted as a physical barrier that reduced the number of germ tubes entering the epidermis. Secondly, germ tubes which successfully entered the epidermis were controlled by the release of plant antifungal compounds, enhanced by the presence of soluble Si in the cytoplasm (Van Bockhaven et al., 2013).

The postulated mechanism by KSi against FHB in this study is through priming the plants for enhanced production of antifungal compounds. This hypothesis is based on (i) *Fusarium graminearum* enters wheat floret by forming a mycelial network over wheat glumes that extends to stomata, the crevices between palea and lemma, and anthers (Bushnell et al., 2003, Seong et al., 2008); and (ii) it is unlikely that *F. graminearum* can penetrate the outer surfaces of the floret because they have highly thickened lignified walls (Bushnell et al., 2003).

A reduction of FHB severity in the presence of KSi compared to the Control treatment demonstrated that KSi did have an effect on FHB. However, the reduction of FHB severity by KSi was not significant, possibly because *F. graminearum* produces mycotoxins during its development within wheat heads, which inhibit protein synthesis in heads (Paulitz, 1999, Beyer et al., 2004). Mycotoxins produced by *F. graminearum* are assumed to suppress or delay plant defense responses against fungal attack because they impair protein synthesis in plants (Harris et al., 1999). Therefore, in both KSi trials F.32 would have produced mycotoxins within the wheat heads that would have suppressed the capacity of the wheat plants to resist ongoing infection of the heads by F.32 after Day 12.

The two higher doses of granulated KSi (3 and 4.5 g) resulted in a reduction of disease severity compared to that of the lower dose (1.5 g), on Day 12 on both KSi trials. At 200 and 300 mg L^{-1} the liquid KSi treatments were moderately effective on Day 12. At 600 mg L^{-1} the KSi was entirely ineffective, probably because the level of soluble Si inside the plant exceeded the critical concentration at which silicic acid polymerizes (Van de Ent et al., 2009). The polymerized form of Si is no longer physiologically active, hence these plants performed similarly to the Control plants. A reduction in wheat spot blotch severity by increasing the amount of silicon application has been reported (Domiciano et al., 2010; ZanãoJúnior et al., 2010).

Control of FHB may be enhanced by the use of two or more strategies (Palazzini et al., 2007). Thw use of a single control strategy is usually inadequate against FHB (Bai et al., 2003). Two *Trichoderma* strains, T.kd and T.77 have been demonstrated to reduce diseases on green bean (*Phaseolus vulgaris* L.), potato (*Solanum tuberosum* L.), maize (*Zea mays* L.), cucumber (*Cucumis sativus* L.), tomato (*Solanum lycopersicum* L.) and grapevine (*Vitis vinifera* L.) (www.plant-health.co.za.). However, there was no significant reduction in FHB severity on wheat plants treated with KSi alone, and KSi and the two *Trichoderma* strains, compared to the Control treatment. The number of harvested infected grains from wheat heads treated with KSi and the two *Trichoderma* strains were not significantly different. The incidence of infected grains among treatments ranged between 11% and 34%, whereas the levels of disease severity on wheat heads

ranged between 31% and 89%. It was expected that the levels of infected grains with F.32 would closely match the levels of FHB severity. Bleaching of heads by *F. graminearum* may be a result of mycotoxins production, or the clogging of phloem and xylem by the pathogen (Bai et al., 2003; Bushnell et al., 2003). Deoxynivalenol (DON) is phytotoxic to plants and is responsible for the loss of chloroplast pigment and browning of the wheat head (Bushnell et al., 2003). Moreover, DON is diffusible, i.e., it moves into wheat tissues that are not colonized by *F. graminearum* (Jackowiak et al., 2005). The clogging of phloem and xylem vessels disrupts the movement of nutrient and water to un-colonized tissues, resulting in premature death of spikelets. Therefore, in this study, the low level of infected grains from bleached heads infected with F.32 may be explained as being the result of poor colonization of grains, production of DON and/or the clogging of xylem and phloem vessels by F.32.

High percentage coefficient of variation (CV%) i.e (greater than 30%) were observed from some of the data analyses of KSi experiments under greenhouse conditions. The high CV values could have been a result of the following reasons: i. the number of replications was few and ii. biocontrol trials are very sensitive to the environment and block effects (tritrophic interaction) increases variations in the greenhouse.

Granulated KSi has a limited potential to reduce FHB caused by *F. graminearum* Strain F.32 under greenhouse conditions for the first 12 days after pathogen inoculation. Field trials are necessary to confirm the efficacy of granulated KSi in reducing Fusarium head blight on wheat in practice.

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CHAPTER FIVE

Management of Fusarium Head Blight of wheat with biological control agents and potassium silicate under field conditions

Abstract

Bacillus Isolate B7 (B7), *Trichoderma harzianum* Strain T.kd (T.kd) and potassium silicate (KSi), and their combination treatments were tested against Fusarium head blight (FHB) on wheat cultivar 'Gariep' under field conditions. None of the single or combined treatments significantly reduced FHB severity and incidence compared to the untreated Control treatment on all disease rating days. Neither the single nor multiple applications of B7, T.kd, KSi nor their combination treatments were significantly different ($P > 0.05$) from the untreated Control treatment for the final evaluation of disease severity (Day 40 post-pathogen inoculation) ($P = 0.90$ and 0.88, respectively) and incidence ($P = 0.94$ and 0.78, respectively). The treatment with T.kd resulted in an increase of 27% in grains per head. The thousand grain weights were not increased by any of the treatments used. None of the treatments significantly ($P = 0.95$ and $P = 0.96$) reduced deoxynivalenol levels in the wheat grains compared to the pathogen-inoculated control treatment.

Keywords: Biological control agents, potassium silicate and Fusarium head blight.

5.1. Introduction

Fusarium head blight (FHB) is sometimes considered to be the most destructive disease of wheat (*Triticum aestivum* L.) in the world (Bai and Shaner, 1996). The International Maize and Wheat Improvement Center (CIMMYT) has identified this disease as a major limiting factor of wheat production in many parts of the world (Goswami and Kistler, 2004). The threat posed by this disease is multifaceted (Goswami and Kistler, 2004). It bleaches wheat heads (Bai and Shaner, 1996; Bai, 2000), and affected wheat heads can become sterile (Goswami and Kistler, 2004). If grains are formed, they can appear withered and discoloured (Bai and Shaner, 1996; Goswami and Kistler, 2004). Affected grains usually weigh much less than unaffected grains (Palazzini et al., 2007). Most importantly, the FHB pathogens produce mycotoxins within the grain (Goswami and Kistler, 2004). Mycotoxins pose a health threat to both humans and livestock (Paulitz, 1999; Beyer et al., 2004; Kokkenen et al., 2010; Willyerd et al., 2012). The mycotoxin deoxynivalenol (DON) is the most prevalent mycotoxin produced by *Fusarium* spp. within wheat grains (Legzdina and Buerstmayr, 2004; Kokkenen et al., 2010) and as a result FHB has become a food safety issue (Legzdina and Buerstmayr, 2004). DON is immunosuppressive (Moss, 1996), inhibiting eukaryotic protein synthesis via binding to a 60S ribosome (Bushnell et al., 2003; Goswami and Kistler, 2004). Therefore, the levels of DON in grain fit for human consumption has been regulated to less than 0.5 – 2 mg kg^{-1} in unprocessed grains (Harris et al., 1999; Lacey et al., 1999; Heier et al., 2005; Mesterházy et al., 2011).

Control measures that have been suggested to reduce the intensity of FHB include crop rotation, tillage, fungicides, resistant cultivars and biological control agents (Paulitz, 1999; Lemmens et al., 2004; Yuen and Schoneweis, 2007). The use of biological control agents (BCAs) to reduce FHB has become the most attractive control method following the use of resistant cultivars (Khan et al., 2004; Juchum et al., 2006, Riungu et al., 2007). Resistant cultivars are still considered a more reliable and a safe control measure of FHB than other FHB control measures (Paulitz, 1999; Lemmens et al., 2004; Mesterházy et al., 2011) but there are no highly resistant wheat cultivars available (Mesterházy et al., 2011).

Bacterial BCA's that have been studied to control FHB belong to the genera of *Bacillus*, *Pseudomonas* and *Lyzobacter*, (Yuen and Schoneweis, 2007). *Trichoderma* spp. (Lutz et al., 2003, Luongo et al., 2005), *Phoma spp.* (Diamond and Cooke, 2003), *Clonostachys* spp. (Luongo et al., 2005), *Epicoccum spp.* (Riungu et al., 2007) and *Microsphaeropsis spp.* (Yuen and Schoneweis, 2007) are some of the fungi that have been tested for control of FHB. Yeasts belonging to the genera *Rhodotorula*, *Sporobolomyces* and *Cryptococcus* have also been tested for control of FHB (Palazzini et al., 2007). The antagonistic mechanisms involved in BCAs include the production of antibiotics and toxins against pathogen growth, competition for infection sites or nutrients, and induction of resistance mechanisms in the plant (Punja, 1997; Scala et al., 2007). So far, the application of BCAs has proven to be ineffective when used alone to control FHB (Bai et al., 2003). However, the efficacy of BCAs has been enhanced when combined with reduced fungicide levels (Schisler et al., 2002) and moderate resistant cultivars (Lacey et al., 1999; Khan et al., 2004).

Application of silicon (Si) fertilizers is a safe environmental practice to reduce the levels of disease severity and incidence of several crop diseases. Important diseases of wheat, barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), cucumber (*Cucumis sativus* L.), grape (*Vitis vinifera* L.), rice (*Oryza sativa* L.), rye (*Secale cereale* L.), soybean (*Glycine max* (L.) Merr, strawberry (*Fragaria x ananassa* Duchesne) and melon (*Cucumis melo* L) have been efficiently controlled by supplying Si fertilizers to these crops (Xavier Filha et al., 2011, Dallagnol et al., 2012). Silicon plays two important roles in the plant's defense against diseases: (i) it acts as a barrier beneath the cuticle hindering pathogens from entering the plant through the epidermis; and (ii) it primes the plants for enhanced resistance after the pathogens have passed through the epidermis and induce the resistance reactions (Ma and Yamaji, 2006). The use of Si fertilizer applications is an attractive approach to manage crop diseases because it can be integrated with other control measures (Rezende et al., 2009; Dallagnol et al., 2012).

Combining BCAs can be an effective method to enhance the efficacy and reliability of biocontrol of FHB (Palazzini et al., 2007; Palazzini et al. 2009). The BCA combinations treatments that have been studied to reduce FHB include, *Brevibacillus* + *Streptomycin*

(Palazzini et al., 2007) and *Pseudomonas* + *Cryptococcus* (Schisler et al., 2006). Mixing biological control agents from different species ensures that the treatments have a lasting effect and provide a wide array of biological mechanisms and/functions under a broader range of environmental conditions (Hervás et al., 1998; Yobo, Laing and Hunter., 2011).

Therefore, the aim of this chapter was to investigate whether *Trichoderma harzianum* Strain T.kd (T.kd), *Bacillus* Isolate B7 (B7) and potassium silicate (KSi) could reduce FHB of wheat, when applied alone or in a combination, under field conditions.

5.2 Materials and methods

5.2.1 Inoculum production for field trials

a. *Bacillus* Isolate B7

Bacillus Isolate B7 initially isolated from wheat heads was aseptically cultured on tryptic soy agar (TSA; Merck) plates for 48 h in the dark at 28^{o}C. Fifty (50) TSA plates were sub-cultured with B7 from a pure culture and plates were incubated in the dark for 48 h at 28^{o}C. Bacterial inoculum for field trial was prepared by suspending the 48 h old B7 culture in sterile distilled water and adjusted to 1x10^{7} cells ml^{-1} using a haemocytometer.

b*. Trichoderma harzianum* Strain T.kd

Trichoderma harzianum T.kd (T.kd) is a formulated biocontrol agent that is manufactured by Plant Health Products (Pty) Ltd, Nottingham road, South Africa. The T.kd treatment was prepared by suspending 3g of the product in six litres of tap water, as recommended by the manufacturer, making up a suspension containing 1x10^{9} conidia ml^{-1}. The T.kd inoculum was prepared in the field just before spraying onto wheat plants.

c. FHB pathogen

Mycelial plugs (4 mm x 4 mm) of *Fusarium graminearum* Strain F.32 (F.32) stored in sterile distilled water at ambient temperature were transferred onto peptone pentacloronitrobenzene agar (PPCNBA) (Section 2.2.1) and incubated for seven days at 28^{o}C in the dark. A growing mycelium on PPCNBA plug was transferred onto potato dextrose agar (PDA; Merck) plates and incubated for four days at 28^{o}C in the dark. Conidial suspensions were made by transferring plugs with mycelia from PDA plate onto 70 Spezieller Nährstoffarmer Agar (SNA) (Section 2.2.1) plates, which were incubated for 14 days at 20 – 22^{o}C under continuous black light (350 nm). Macroconidia were harvested in sterile distilled water by dislodging them off the agar plates with an L-bent metal rod and adjusted to 1 x 10^{3} conidia ml^{-1} using a haemocytometer. *Fusarium graminearum* F.32 inoculum was carried in an ice box to the trial site in the field.

5.2.2. Efficacy of biological control agents and potassium silicate under field conditions

The field site (Ukulinga Research Station, University of KwaZulu-Natal, Pietermaritzburg, South Africa) was pre-treated with Kleenup® and Karate® (3 ℓ/ha) to remove weeds and cutworms, followed by removing all vegetation. The site was divided into three blocks which were separated by 1 m spacing. Each block (30 m x 10.5 m) represented one replicate and was further divided into 14 mini plots representing 14 treatments. The mini plots were separated by 0.5m spacing. Each mini plot (2 m x 1.8 m) had six rows which were separated by 10 cm spacing. Treatments were arranged in a randomized complete block design. The treatments were as follows: **1**. Control (untreated), **2**. KSi alone, **3**. T.kd applied once, **4**. T.kd applied twice (2T.kd), **5**. B7 applied once (B7), **6**. B7 applied twice (2B7), **7.** T.kd + KSi, **8**. 2T.kd + KSi, **9**. B7 + KSi, **10**. 2B7 + KSi, **11**. T.kd + B7, **12**. 2T.kd +2B7, **13**. T.kd + B7 + KSi and **14**. 2T.kd + 2B7 + KSi. Twenty winter wheat (Cultivar ‘Gariep’) seeds, obtained from Pannar Seeds (Pty) Ltd, Greytown, South Africa, were planted in each row by hand. KSi treatments received 4 g AgriK50 (manufactured by PQ Corporation; South Africa) in the form of potassium silicate (KSi) as a source of Si at planting. Potassium silicate (4 g) was uniformly distributed in all six rows in KSi subplot treatments. At early anthesis (10.51 Feeke’s scale, Zadoks et al., 1974), wheat plants that received T.kd and B7 inoculum were

sprayed separately with 1 x 10^9 conidia ml^{-1} of T.kd and 1 x 10^7 cells ml^{-1} of B7 inoculum using a Ryobi pressure sprayer GS-600 (0.15-0.3 Mpa) (Stevens & Co Pty (Ltd). On Day 3 post biological control agents inoculation, inoculum of F.32 containing 1 x 10^3 conidia ml^{-1} was sprayed onto wheat heads of all treatments using a clean Ryobi pressure sprayer. After 48 h post pathogen inoculation, T. kd and B7 were sprayed again accordingly on the following treatments: 2T.kd, 2B7, 2T.kd + KSi, 2B7 + KSi, 2T.Kd +2B7, 2T.kd + 2B7 + KSi treatments.

5.2.3. Disease scoring

FHB incidence and severity was recorded on Day 20, 30 and 40. Six spots in each plot were randomly selected and 30 heads at each spot were scored for FHB severity and incidence. Therefore, 180 heads in total per plot were scored for disease incidence and severity. Disease incidence was recorded as the proportion of bleached spikelets in a sample using the following formulae:

$$\text{Disease incidence} = \frac{\text{proportion of bleached spikes} \times 100}{\text{total number of spikes sampled}}$$

Disease severity was recorded as the number of bleached spikelets in a spike using the following formulae:

$$\text{Disease severity} = \frac{\text{number of bleached spikelets} \times 100}{\text{total number of spikelets (bleached and unbleached)}}$$

All disease values were analysed using AUDPC as described by Shaner and Finney (1977) before statistical analysis.

5.2.4. Wheat grains per head and thousand grains weight

Matured and dry wheat heads were harvested five months after planting using secateurs. Two hundred and fifty wheat heads were randomly selected, harvested from each subplot and oven dried at 60^{o}C for 48 h. Dried heads were threshed using sorghum threshing machine and grains were weighed.

Grains per head were calculated using the proportion formula:

$$\frac{a}{b} = \frac{c}{d}$$

One thousand grains were randomly selected from all treatments threshed grains and weighed.

5.2.5. Mycotoxin detection from wheat grains

Deoxynivalenol (DON) was quantified in all treatments from threshed grains. Twenty grains were randomly selected from each treatment and milled. A sample (2g) was used to quantify DON using MaxSignal® DON Enzyme-linked immunosorbent assay (ELISA) test kit (Inqaba Biotechnical Industries Pty (Ltd), South Africa) as per manufacture's instruction with DON levels of 0.25 -5 ppm. The ELISA plate was read with FLUOStar Optima microplate Reader (Optima scientific Pty (Ltd), South Africa) at 405nm.

5.2.6. Data analysis

A general linear model was used to run a factorial analysis on the results using the computer statistical Package, Statistical Analysis System 2002 (SAS Institute Inc, USA)

5.3. Results

5.3.1. Efficacy of biological control agents and potassium silicate on FHB incidence and severity in the field

There was no significant difference between treatments in FHB severity (Table 1 and 2) on all disease rating record days. However, significant differences between treatments in FHB incidence were observed only on Day 20 but not on Day 30 and 40 (Table 3 and 4). The Control treatment was not significantly different from BCA inoculated treatments in terms of FHB severity and incidence (Table 1, 2, 3 and 4).

Table 1: Efficacy of one application of *Bacillus* Isolate B7 (B7), *T. harzianum* Strain T.kd (T.kd) and potassium silicate (KSi) and their combination treatment on FHB severity under field conditions.

Treatment	*Bacillus*	*Trichoderma*	Silicon	Day 20	Day 30	Day 40	AUDPC
B7	Yes	No	No	4.6a	6.5a	12.8a	123.50a
T.kd	No	Yes	No	1.4a	6.7a	8.8a	77.50a
KSi	No	No	Yes	1.4a	6.0a	10.3a	111.50a
B7+T.kd	Yes	Yes	No	1.8a	5.4a	6.2a	59.50a
B7+KSi	Yes	No	Yes	3.4a	10.5a	15.1	164.75a
T.kd+KSi	No	Yes	Yes	0.9a	4.7a	9.9a	75.75a
B7+T.kd+KSi	Yes	Yes	Yes	1.6a	4.4a	15.4a	131.25a
Control	No	No	No	1.7a	5.9a	12.5a	89.0a
Treatment effects				P value	P value	P value	P value
B7				0.0995	0.6433	0.6034	0.3460
T.kd				0.1351	0.3186	0.5020	0.2773
KSi				0.5428	0.8839	0.5020	0.3161
B7+T.kd				0.2926	0.3843	0.8803	0.7001
B7+KSi				0.8634	0.5070	0.4227	0.4779
T.kd+KSi				0.7891	0.3482	0.5140	0.9581
B7+T.kd+KSi				0.7167	0.7074	0.8138	0.6748
				%CV =100	%CV =72	%CV = 65	%CV = 60
				MSE =2.1	MSE =4.5	MSE =7.4	MSE = 62

Means with the same letter in the same column are not significantly different (P =0.05) according to Duncan's multiple range tests.

None of the combination treatments of B7 + T.kd + KSi significantly decreased FHB severity and incidence compared to B7, T.kd and KSi treatments when used alone (Table 1 and 3). Increasing the number of applications of B7 and T.kd did not significantly reduce FHB incidence and severity compared to the Control treatment (Table 2 and 4). There was also no significant difference in area under disease progress (AUDPC) values between treatments (Table 1 and 2).

Table 2: Efficacy of repeated application of *Bacillus* Isolate B7 (B7), *T. harzianum* Strain T.kd (T.kd) and potassium silicate (KSi) and their combination treatments on FHB severity under field conditions.

Treatment	*Bacillus*	*Trichoderma*	Silicon	Day 20	Day 30	Day 40	AUDPC
2B7	Yes	No	No	1.2a	6.4a	11.4a	120.50a
2T.kd	No	Yes	No	2.4a	8.1a	7.8a	88.25a
KSi	No	No	Yes	1.4a	6.0a	10.3a	111.50a
2B7+2T.kd	Yes	Yes	No	2.0a	4.9a	15.6a	122.50a
2B7+KSi	Yes	No	Yes	1.0a	5.1a	11.7a	107.75a
2T.kd+KSi	No	Yes	Yes	1.2a	5.4a	13.4a	122.25a
2B7+2T.kd+KSi	Yes	Yes	Yes	0.87a	6.0a	9.2a	86.00a
Control	No	No	No	1.7a	5.9a	12.5a	
Treatment effects				P value	P value	P value	P value
2B7				0.4686	0.9318	0.8733	0.8646
2T.kd				0.5195	0.6066	0.5603	0.7526
KSi				0.1874	0.4578	0.7418	0.9656
2B7+2T.kd				0.9402	0.3939	0.2963	0.9605
2B7+KSi				0.9601	0.3740	0.1677	0.2997
2T.kd+KSi				0.5026	0.5673	0.4230	0.7185
*2B7+2T.kd+KSi				.	.	.	
				%CV = 78	%CV = 63	%CV = 48	%CV= 42
				MSE =1.3	MSE =3.7	MSE =5.5	MSE = 45

Potassium silicate (KSi) was constant and applied once at planting
Means with the same letter in the same column are not significantly different (P =0.05) according to Duncan's multiple range tests.
* Missing P values were as a result of insufficient degrees of freedom

5.3.2. Efficacy of biological control agents and potassium silicate on grains yield per head, thousand grain weights and deoxynivalenol content.

Treatment T.kd was the only treatment that resulted in an increase of grains per head compared to the pathogen-inoculated Control treatment increasing grains per head by 27% (Table 5). Treatment with T.kd alone significantly increased grains per head compared to yields after treatments with B7 + T.kd, and B7 + T.kd + KSi combination

treatments. However, T.kd treatment did not significantly increase grains per head compared to the T.kd + KSi combination treatment (Table 5). Treatment with

Table 3: Efficacy of one application of *Bacillus* Isolate B7 (B7), *T. harzianum* Strain T.kd (T.kd) and potassium silicate (KSi) and their combination treatment on FHB incidence under field conditions.

Treatment	*Bacillus*	*Trichoderma*	Silicon	Day 20	Day 30	Day 40
B7	Yes	No	No	12.97ab	42.60a	70.85a
T.kd	No	Yes	No	10.93ab	33.87a	65.25a
KSi	No	No	Yes	10.53ab	45.17a	59.15a
B7+T.kd	Yes	Yes	No	8.80b	32.30a	51.15a
B7+KSi	Yes	No	Yes	21.67a	39.83a	67.25a
T.kd+KSi	No	Yes	Yes	7.033b	30.93a	60.55a
B7+T.kd+KSi	Yes	Yes	Yes	12.40ab	38.73a	70.00a
Control	No	No	No	13.30ab	36.10a	63.85a
Treatment effects				P value	P value	P value
B7				0.1644	0.5892	0.4377
T.kd				0.0844	0.6273	0.8516
KSi				0.6449	0.9566	0.6825
B7+T.kd				0.4128	0.5187	0.9559
B7+KSi				0.1140	0.5604	0.9641
T.kd+KSi				0.8708	0.5550	1.000
*B7+T.kd+KSi				.	.	.
				%CV = 49	%CV = 46	%CV = 26
				MSE = 5.4	MSE = 17	MSE = 16

Means with the same letter in the same column are not significantly different (P =0.05) according to Duncan's multiple range tests.
* Missing P values were as a result of insufficient degrees of freedom

B7 alone significantly increased grains per head compared to the B7+ T.kd combination treatment (Table 5). None of the BCAs + KSi combinations significantly increased grains per head compared to the treatment with KSi alone (Table 5). Increasing the number of applications of either B7 or T.kd did not significantly increase grains per head compared to the Control treatment (Table 6). The combination treatments of repeated applications of the two biocontrol agents, T.kd and B7, with or without KSi did not result in

significantly increased grains per head compared to repeated applications of T.kd and B7 (Table 6).

Table 4: Efficacy of repeated application of *Bacillus* Isolate B7 (B7), *T. harzianum* Strain kd (T.kd) and potassium silicate (KSi) and their combination treatments on FHB incidence under field conditions.

Treatment	*Bacillus*	*Trichoderma*	Silicon	Day 20	Day 30	Day 40
2B7	Yes	No	No	9.833a	45.20a	64.15a
2T.kd	No	Yes	No	12.033a	36.10a	55.00a
KSi	No	No	Yes	10.533a	45.17a	59.15a
2B7+2T.kd	Yes	Yes	No	8.800a	32.80a	51.15a
2B7+KSi	Yes	No	Yes	9.067a	41.67a	64.15a
2T.kd+KSi	No	Yes	Yes	8.133a	45.73a	76.65a
2B7+2T.kd+KSi	Yes	Yes	Yes	10.933a	46.83a	63.60a
Control	No	No	No	13.30ab	36.10a	63.85a
Treatment effects				P value	P value	P value
2B7				0.7055	0.7266	0.5236
2T.kd				0.9890	0.9662	0.4906
KSi				0.5527	0.5926	0.2409
2B7+2T.kd				0.9673	0.5916	0.8465
2B7+KSi				0.2970	0.8484	0.6692
2T.kd+KSi				0.6114	0.4493	0.5651
*2B7+2T.kd+KSi				.	.	.
				%CV =78	%CV =63	%CV = 48
				MSE =1.3	MSE =3.7	MSE = 5.6

Potassium silicate (KSi) was constant and applied once at planting
Means with the same letter in the same column are not significantly different (P =0.05) according to Duncan's multiple range tests.
* Missing P values were as a result of insufficient degrees of freedom

There were no significant differences in the thousand grain weights (TGW) as a result of the different treatments (Table 5 and 6). None of the treatments significantly increased TGW compared to the Control treatment. None of the B7, T.kd and KSi combination treatments significantly increased TGW compared to B7, T.kd and KSi treatments when used alone (Table 5 and 6).

Deoxynivalenol (DON) levels found in wheat grains were not significantly different as a result of the various treatments (Figure 5 and 6). Grain from all plots had more than 2 mg kg^{-1} DON levels within the grains, i.e., more than the legally recommended limit (Table 5 and 6).

5.4. Discussion

Treating the wheat cultivar 'Gariep' with two biological control agents (BCAs) and potassium silicate (KSi) had no significant effect on the levels of Fusarium head blight (FHB) disease severity and incidence compared to the Control treatment under field conditions. There were no significant differences in AUDPC values between treatments. Moreover, combining BCAs and KSi did not reduce FHB severity and incidence compared to the Control treatment. Increasing the number of BCA applications did not reduce FHB severity or incidence. Various factors such as ultraviolet light, arrival of pathogen inoculum on wheat head over an extended period of head susceptibility and fluctuating environmental conditions such as temperature, moisture and available nutrients on the phylloplane could have affected the efficacy of BCAs under field conditions (Schisler et al., 2002).

In similar research, *Bacillus* strains were found to be less effective under field conditions than in the greenhouse when used against *Fusarium graminearum* (Schisler et al., 2002; Palazzini et al., 2009). In this study, B7 tested under field conditions did not significantly reduce FHB, whereas it had been effective under greenhouse conditions (Chapter Three). Rain might have negatively affected the efficacy of B7 under field conditions since there were showers of rain shortly after the inoculations of the BCAs and the pathogen. According to Schisler et al. (2002), bioactive compounds produced by *Bacillus* strains that suppress FHB on wheat heads are prone to wash-off during rain events, or with supplemental mist irrigation in the field. The B7 inoculum used in the field was grown on a solid medium. *Bacillus* strains tested in other studies that successfully reduced FHB incidence and severity under field conditions were produced in liquid culture (Schisler et al., 2002; Khan et al., 2004; Juchum et al., 2006; Palazzini

et al., 2009). Bioactive compounds produced in liquid media were retained on wheat heads after spraying the *Bacillus* broth onto the wheat heads, and acted against FHB. Therefore, the efficacy of B7 against FHB might have been additionally affected by the inoculum production method

Table 5: Efficacy of one application of *Bacillus* Isolate B7 (B7), *T. harzianum* Strain T.kd (T.kd) and potassium silicate (KSi) and their combination treatments on grains per head (G/H), thousand grain weights (TGW) and deoxynivalenol (DON) under field conditions

Treatment	*Bacillus*	*Trichoderma*	Silicon	G/H (g)	TGW (g)	DON ($mg.kg^{-1}$)
B7	Yes	No	No	0.9750ab	29.835a	4.650a
T.kd	No	Yes	No	1.0200a	31.137a	5.200a
KSi	No	No	Yes	0.7933bc	29.737a	4.333a
B7+T.kd	Yes	Yes	No	0.7100c	31.153a	4.200a
B7+KSi	Yes	No	Yes	0.7700bc	29.567a	5.133a
T.kd+KSi	No	Yes	Yes	0.8400abc	31.363a	3.767a
B7+T.kd+KSi	Yes	Yes	Yes	0.7700bc	30.467a	4.600a
Control	No	No	No	0.80333bc	28.810a	5.067a
Treatment effects				P value	P value	P value
B7				0.1408	0.9943	0.9309
T.kd				0.9575	0.0953	0.6579
KSi				0.1365	0.9551	0.9203
B7+T.kd				0.0067	0.6241	0.8709
B7+KSi				0.6057	0.5522	0.3223
T.kd+KSi				0.4339	0.7515	0.8117
B7+T.kd+KSi				0.0182	0.9363	0.8285
				%CV =12	%CV = 6.8	%CV = 36
				MSE= 0.1	MSE = 2.0	MSE = 1.6

Means with the same letter are not significantly different (P =0.05) according to Duncan's multiple range test

Table 6: Efficacy of repeated application of *Bacillus* Isolate B7 (B7), *T. harzianum* Strain T.kd (T.kd) and potassium silicate (KSi) and their combination treatments on grains per head (G/H), thousand grain weights (KGW) and deoxynivalenol (DON) under field conditions

Treatment	*Bacillus*	*Trichoderma*	Silicon	G/H (g)	KGW (g)	DON ($mg.kg^{-1}$)
2B7	Yes	No	No	0.8300a	29.807a	5.176a
2T.kd	No	Yes	No	0.7633a	29.170a	4.233a
KSi	No	No	Yes	0.7933a	29.737a	4.333a
2B7+2T.kd	Yes	Yes	No	0.6933a	29.670a	4.633a
2B7+KSi	Yes	No	Yes	0.8500a	29.737a	4.500a
2T.kd+KSi	No	Yes	Yes	0.7433a	29.993a	5.067a
2B7+2T.kd+KSi	Yes	Yes	Yes	0.7367a	29.490a	3.600a
Control	No	No	No	0.8033a	28.810a	5.067a
Treatment effects				P value	P value	P value
2B7				0.8983	0.8267	0.5910
2T.kd				0.0729	0.6725	0.3839
KSi				0.8535	0.8593	0.8729
2B7+2T.kd				0.3877	0.9984	0.8766
2B7+KSi				0.5688	0.5141	0.3694
2T.kd+KSi				0.8330	0.7163	0.8652
2B7+2T.kd+KSi				.	.	.
				%CV = 12	%CV = 8.6	%CV = 35.
				MSE = 0.1	MSE = 2.5	MSE = 1.6

Potassium silicate (KSi) was constant and applied once at planting
Means with the same letter are not significantly different (P =0.05) according to Duncan's multiple range tests.
* Missing P values were as a result of insufficient degrees of freedom

When applied alone T.kd was ineffective against FHB under field conditions. Under greenhouse conditions T.kd was also not effective at reducing FHB (Chapter Three). Although the number of applications of T.kd was increased, this did not reduce FHB severity in the field. Therefore, it is probable that the particular strain of *T. harzianum* used, T.kd, does not have the potential to compete in the phylloplane of wheat, and therefore to reduce FHB severity. To the best of our knowledge there is no information on studies done on the use of *T. harzianum* against FHB on wheat heads under field

conditions. However, strains of *Trichoderma* spp. have been shown to reduce FHB severity under greenhouse conditions (Riungu et al., 2007).

Application of KSi had no significant effect on FHB severity under field conditions. This confirmed the results obtained under greenhouse conditions (Chapter Four) that treating wheat plants with KSi was ineffective against FHB disease. According to Epstein (1994) the effect of applying silicon on plants becomes more noticeably in the field than in the greenhouse, but it did not work in this case.

Combining biocontrol agents can be an effective method to enhance the efficacy and reliability of biocontrol of FHB (Palazzini et al., 2009). In this study, combining T.kd and B7 did not increase their activity against FHB compared to either of the BCAs when applied alone. Increasing the number of applications of T.kd and B7 in a combined treatment did not reduce FHB severity and incidence compared to one applications of the two BCAs in a combined treatment. Enhanced biocontrol of *Fusarium* spp. by combining *Trichoderma* + *Bacillus* has been reported (Morsy et al., 2009). However, *Trichoderma* and *Bacillus* combination treatments do not always give better disease control (Hervás et al., 1998). Combining T.kd, B7 and KSi did not reduce the levels of FHB (severity and incidence), despite the prior hypothesis that this would be the case.

High percentage coefficient of variation (%CV) values (46-100) were observed for analysed FHB severity and incidence on all record days, with an exception on Day 20 for FHB incidence. These high % CV values could have been as a result of the following reasons: i. few replications were used (3 replications were used under field conditions) and ii. biocontrol trials are very sensitive to the environment and block effects (tritrophic interaction) increases variations in the field trials.

Although T.kd was ineffective in reducing FHB severity, this treatmet resulted in increased grains per wheat head. In prior research, T.kd has been shown to be effective against sublethal pathogens and to stimulate the production of plant roots (data not shown here). A single application was enough to establish T.kd on the roots of wheat plants, and a second spray did not enhance this effect.

There were no significant differences in thousand grain weights (TGW) between treatments in this study, which reflects the ineffectiveness of the different treatments, both biocontrol and silicon. Diamond et al. (2003) commented that, “it would appear that heavy FEB infection at early anthesis is capable of reducing grains per ear by preventing grain formation at some grain sites already established prior to anthesis, and that compensation can then occur in grain size at remaining healthy sites leading to TGW values which do not differ significantly from control values”. Other studies have reported an increased in TGW from wheat plants that have been treated with effective antagonists compared to the Control treatment under field conditions (Schisler et al., 2002; Schisler et al., 2006; Khan et al., 2004).

The application of BCAs and KSi to wheat plants failed to reduce the deoxynivalenol (DON) levels significantly within grains. However, treating wheat plants with T.kd +B7 twice + KSi resulted in lower DON levels in the grains than the rest of the treatments. This treatment should have had lower DON levels, if the products worked against *F. graminearum* F.32. Khan et al. (2004) found that DON levels were rarely reduced by BCAs, despite significantly reducing FHB severity and incidence.

None of the treatments or their combinations reduced Fusarium head blight under field conditions, nor did they lower DON levels. There is still a need to search for more effective biocontrol agents against *F. graminearum* F.32.

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DISSERTATION OVERVIEW

Fusarium head blight (FHB) is a destructive fungal disease of wheat (*Triticum aestivum* L) world-wide (Bai and Shaner, 2004). The disease was first noticed in South Africa in 1980's on wheat under irrigation areas (Boshoff et al., 1999; Kriel and Pretorius, 2008). *Fusarium graminearum* is the major causal agent of FHB globally (Osborne and Stein, 2007). During the growth of *F. graminearum* within wheat grains, mycotoxins are produced, which reduces the quality of wheat grains (Legzdina and Buerstmayr, 2004).

There are no available resistant cultivars nor are there effective fungicides against FHB (Giraud et al., 2011; Willyerd et al., 2011). Biocontrol of FHB is a promising alternative control measure. Combined control measures are more effective than single strategies in reducing the severity of FHB (Palazzini et al., 2009). The application of silicon fertilization has been reported to control various crop diseases including those of wheat (Xavier Filha et al., 2011). However, it has never been reported to have been used against FHB.

Therefore, this study was aimed at isolating potential BCAs and screening them *in vitro* and *in vivo* against South African *F. graminearum* Strain F.32 (F.32), to evaluate single and combined applications of potassium silicate (KSi) and *Trichoderma* strains on FHB of wheat *in vivo,* and lastly, to investigate the effect of single and combined biological control agents (BCAs) and KSi treatments on FHB of wheat under field conditions.

In this study, it was established that:

- *Bacillus* isolates obtained from phyllosphere of wheat and maize inhibited F.32 growth on potato dextrose agar (PDA) plates.
- Two commercial strains of *Trichoderma harzianum,* Strain T.kd (T.kd) and Strain T.77 (T.77), inhibited F.32 on PDA.
- None of the 16 yeast isolates obtained from the phyllosphere of wheat and maize inhibited the germination of F.32 macroconidia within 7 h on agar plates. However, the presence of yeast in the dual culture bioassay inhibited F.32 from

growing profusely on YDCA plates compared to the pathogen-inoculated Control treatment.

- *Bacillus* Isolate B7 (B7) significantly reduced FHB compared to a pathogen-inoculated Control treatment of wheat, under greenhouse conditions.
- *Trichoderma harzianum* Strains T.kd and T.77 were not effective in reducing the severity of FHB under greenhouse conditions, irrespective of the number of applications.
- Four tested yeast isolates were not effective in reducing the severity of FHB of wheat under greenhouse conditions.
- Granular KSi fertilization at two doses moderately reduced FHB of wheat under greenhouse conditions on Day 12, post-pathogen inoculation.
- None of the single and combined treatments of B7, T.kd and KSi significantly reduced FHB severity and incidence under field conditions.

***In vitro* bioassays**

In this study, *Bacillus* isolates from the phyllosphere of wheat and maize demonstrated significant inhibitions against F.32 on agar plates. Moreover, this study demonstrated that *Bacillus* isolates isolated from the same environment can inhibit the growth of F.32 differentially. Laboratory screening of antagonistic microbes against a pathogen is time consuming (Khan et al., 2001). However, this study has proven that it is important to test all bacterial isolates of interests because the capabilities of antagonistic microbes of the same group against the pathogen may not be the same.

The presence of yeast isolates in dual culture plates slowed mycelial growth of F.32 but failed to prevent F.32 from producing macroconidia. This indicated that the yeasts isolated here were not good biocontrol agents against F.32 *in vitro*.

The two commercial *Trichoderma* strains, T.kd and T.77, inhibited F.32 on agar plates but failed to mycoparasitise the pathogen. Effective *Trichodema* strains against *F. graminearum*, mycoparasitise and create zone of inhibitions against the pathogen (Matarese at al., 2012). Therefore, *Trichoderma* strains to be tested against F.32 *in vivo*

should demonstrate zone of inhibitions and the ability to mycoparasitise the pathogen in agar plate dual culture assays.

Greenhouse trials

The correlation between *in vitro* antibiosis and biological control in plants is frequently inconsistent (Palazzini et al., 2007). This was also true in this study, because some *Bacillus* strains selected for their good *in vitro* activity performed poorly *in vivo* on wheat plants against F.32 under greenhouse conditions (e.g., B14). Conversely, some strains that performed poorly *in vitro* performed better under greenhouse conditions (e.g., B7).

Wheat heads do not reach anthesis at the same time and as a result, this created variability in disease severity during assessment between the replicates. The symptoms of bleached spikelets varied greatly between replicates in the first 12 days after the inoculation of the pathogen.

It was difficult to rate FHB severity during bioassays under greenhouse conditions. The symptoms would not show for several days after inoculation, and then suddenly mycelia would develop on wheat heads. The bleaching of heads would occur subsequently. After this, the head would prematurely senesce. Hence, disease severity was best assessed from Day 12 to Day 22 post-inoculation.

Field trial

Bacillus Isolate B7 reduced FHB significantly compared to the pathogen-inoculated Control treatment under greenhouse conditions but was not effective in reducing FHB under field conditions. The efficacy of BCAs may be affected by environmental conditions under field conditions (Schisler et al., 2002). It rained during the inoculations of BCAs and pathogen, and the rain is presumed to have affected the efficacy of B7 under field conditions.

Trichoderma harzianum Strain T.kd reduced FHB slightly better than T.77 under greenhouse conditions in the first 12 days after pathogen inoculation. However, under field conditions, T.kd was not effective in reducing FHB of wheat. Although T.kd showed a potential under greenhouse conditions, T.77 should have been used under the field

conditions because it is a phyllosphere strain. Possibly T.77 would have adapted better on wheat heads and might have reduced FHB. Strains of *Trichoderma* isolated from the heads of wheat plants would be better adapted to this unique environment, and should be more competitive.

Overall conclusion

This study was the first in South Africa to investigate the effect of applying BCAs and KSi to reduce FHB of wheat. It was demonstrated that there is a potential in the application of BCAs and KSi against FHB of wheat. However, there is a need to search for more effective BCAs against *F. graminearum* F.32.

Proposed future research priorities

- To isolate and screen numerous virulent bacterial and fungal BCAs from the phyllosphere of wheat across South Africa against *F. graminearum* Strain F.32.
- To screen *Trichoderma* strains with mycoparasitic activity against *F. graminearum* F.32 and other isolates causing FHB.
- To evaluate inoculum production method of BCA inoculum used in the laboratory on FHB of wheat.
- To screen wheat cultivars for partial resistance to FHB and use only moderately resistant cultivars during bioassay studies.
- To test the effect of potential BCAs against FHB on the accumulation of deoxynivalenol content in the wheat grains under greenhouse conditions.

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