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Dual Biological Control: Characterization of Fungi and Bacteria to Control Granary Weevil and Fungal Pathogens of Stored Grain

Gülçin Ercan
University of Nebraska - Lincoln

DUAL BIOLOGICAL CONTROL: CHARACTERIZATION OF FUNGI AND BACTERIA TO CONTROL GRANARY WEEVIL AND FUNGAL PATHOGENS OF STORED GRAIN

by

Gülçin Ercan

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DUAL BIOLOGICAL CONTROL: CHARACTERIZATION OF FUNGI AND BACTERIA TO CONTROL GRANARY WEEVIL AND FUNGAL PATHOGENS OF STORED GRAIN

Gülçin Ercan, M.S.

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Advisors: Julie A. Peterson & Sydney E. Everhart

Cereals are main food sources for humans and animals. However, during storage, cereal grains can be infested by insects and fungi. One of the most important insect storage pests is *Sitophilus granarius* (L., Coleoptera: Curculionidae). Adults and larvae can cause serious grain losses. In addition to insect pests, fungal pathogens may also invade the grain and cause economic loss, including contamination with mycotoxins, which threaten mammal health by causing serious disease. The most common mycotoxigenic grain fungi are species that belong to the genera *Fusarium*, *Aspergillus* and *Penicillium*. Currently, the most commonly used management strategies for insect and fungal storage pests are based on conventional pesticides and cultural methods. However, there is a need for alternatives to conventional pesticides due to their side effects, insecticide resistance, and consumer demand for uncontaminated food. Cultural methods may not be cost-effective or practical in all storage facilities. Moreover, both insect and fungal pests share the same niche and have possible interactions with each other that increase economic losses. Therefore, the aim of this study was to find potential biocontrol agents that showed dual biocontrol effect against granary weevil and three common fungal pests in stored grain. We determined that two fungal isolates

(*Trichoderma gamsii* E1032 and E1064) and one bacterial isolate (*B. amyloliquefaciens* C415) achieved dual control against both the insect pest and the fungal pathogens by causing mortality of *S. granarius* and suppression of three grain fungal pathogens. In addition, a specific aim of this study was the examination of lethal and sublethal effects on *S. granarius. Metarhizium anisopliae* E213 showed strong sublethal effect by reducing ovipositio rate and grain infestation additionally, *Cladosporium halotolerans* E126 minimally reduced oviposition rate yet was significantly different from negative control. Also, all tested bacterial treatments had significantly lower survival than the negative control. And, *Bacillus thuringiensis* C423 showed strong sublethal effect by reducing feeding damage and oviposition rate. Additionally, candidate biocontrol agents were tested against grain fungal pathogens with two different method, antibiosis and wheat seed bioassays, to confirm performance of the effective isolate on the real stored grain. *Bacillus amyloliquefaciens*, *Lysobacter enzymogenes*, and *Burkholderia ambifaria* demonstrated the highest antifungal activity. This study demonstrates dual biocontrol against insect and fungal pests, which has potential as a component of Integrated Pest Management strategies for stored grain.

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DEDICATION

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CHAPTER 1: LITERATURE REVIEW

Cereal grains contain high amounts of proteins, carbohydrates and fiber and are the main food sources for humans and many animals (Neethirajan et al. 2007). Wheat has the leading position among cereal crops in terms of cultivation and production (Piasecka-Kwiatkowska et al. 2014). The worldwide production of wheat was 750 million metric tons between 2016 and 2017 and it is estimated that demand for wheat will reach 1,300 million metric tons by 2050 (Bolanos-Carriel 2018). However, cereal grains can be infested by fungi and insects (Bryden 2012). Storage insect pests are an important problem worldwide. These pests can lead to significant economic loss, which can reach 9–20% in developed countries and more in developing countries. One of the most important storage pests is *Sitophilus granarius* (L., Coleoptera: Curculionidae), especially in temperate climates. Adults and larvae can cause serious grain losses. Losses result from feeding damage, and weevils may also contaminate the grain with their byproducts (such as frass) and body parts. According to the United States Department of Agriculture, grain is graded as infested when two live insects are present in 1,000 grams of wheat, rye, or triticale, which may be a significant economic loss to the producer (USDA 2019). In addition, the metabolic activity of granary weevils can increase heat and moisture in stored grain (Hagstrum et al. 2012).

In addition to insect pests, fungal pathogens may also invade the grain and cause economic loss. Cereal contaminated by fungi and toxic secondary metabolites cause loss of dry matter, nutrition, and grain quality (Magan and Aldred 2007). This contamination can occur in the field and during storage (Bullerman and Bianchini 2007). The most

common mycotoxigenic grain fungi are species that belong to the genera *Fusarium*, *Aspergillus* and *Penicillium* (Bothast 1978). Globally, nearly 25% of crops are affected by mycotoxins each year (Whitlow 2010). In addition to economic loss, mycotoxins threaten mammal health by causing serious disease (Fleurat-Lessard 2017).

Currently, the most commonly used management strategies for insect and fungal storage pests are based on conventional pesticides and cultural methods. However, there is a need for alternatives to conventional pesticides due to their side effects, insecticide resistance, and consumer demand for food that is free of insect pests, grain fungi and insecticide residues. Cultural methods, such as drying of grain, controlling heat and moisture, and modifying the atmosphere, may not be cost-effective or practical in all storage facilities.

Biological control is an alternative management strategy to the use of chemical pesticides and is compatible with many cultural controls due to its unique features, including safety for mammalian health and low non-target and environmental effects. Biological control, which is defined as suppression of the pest population or reduction of the influence of the pest by using living organisms (Eilenberg et al. 2001). Biological control agents can be different depending on the discipline; for example, for the control of invertebrate pests, predators, parasitoids and pathogens are used as biological control agents. For weed control, herbivores and pathogens are used as biological control agents and for plant pathogens, antagonistic microorganisms and induced plant resistance are used (Eilenberg et al. 2001). Some desirable characteristics of biocontrol agents are cost effectiveness, suitability for mass production, and host specificity. These characteristics make them a desirable option and important component of Integrated Pest Management

strategies. As mentioned previously, there is need for biological control strategies for insect and fungal pests of stored grains. The present research was conducted to determine the potential of using individual biocontrol agent strains to suppress both types of the pests. Therefore, topics presented in this literature review include:

- The biology of the targeted insect and fungal storage pests;
- The potential and limitations of current management strategies for these pests;
- Biological control research relevant to management of the these pests;
- The stored grain environment in which the pests are problematic and in which biocontrol agents must function.

Granary weevils (*Sitophilus granarius*)

The granary weevil (*Sitophilus granarius* L., Coleoptera: Curculionidae) is one of the most destructive insect pests of stored grain (Gaino and Fava 1995, Kljajić and Perić 2006, Piasecka-Kwiatkowska et al. 2014). It is distributed worldwide, but is especially problematic in temperate zones (Campbell et al. 2004). They are known as primary pests, due to being internal feeders that lay eggs directly inside of the grain kernel, where the larvae bore into the kernel to complete their development into the adult stage.

Identification

Adult weevils are 2−3 mm, shiny reddish-brown, and flightless. They have some specific and distinctive features that make them different from other beetles, such as, a prolonged head or snout, elongated pits on the thorax, absence of flight wings, and four light-colored markings on the wing covers. Larvae are creamy white, legless and immobile; size of larvae is dependent upon grain size (Rees 2004).

Life Cycle

The adult females bore into the grain kernel using their rostrum and oviposit inside of the kernel; after that, the egg cavity is closed with a mucous plug (Gaino and Fava 1995). The female may lay 50−250 eggs, but on average oviposits 200 eggs, with oviposition rate varying according to food availability. Thus, oviposition rate can reach the maximum level in storage facilities with unlimited grain. Usually, the female lays one or two eggs into the endosperm or germ of one grain kernel. Although the female may oviposit more than one egg per grain, because of larval cannibalism, only one larva will grow and emerge from a single grain kernel (Bothast 1978). Larvae excavate a tunnel inside the kernel, where they complete their juvenile life stage. They have four instars and at the end of the fourth instar, the larva combines frass and larval secretions to close their feeding tunnel to form a pupal cell (Stephensons 1983). Newly emerged adults usually stay inside of the grain kernel until sclerotization (the cuticle hardens) and they may continue to feed there for up to one week. Adults can live seven to eight months. The life cycle from egg to adult can be completed within four to six weeks depending on humidity and grain temperature. The life cycle of the weevil will be shorter when humidity and temperature increases (Mason and McDonough 2012). The shortest development period for the life cycle is 25 days, which is accomplished at 30°C and 70% relative humidity (Rees 2004).

Occurrence

The granary weevil is a cosmopolitan pest and it can be found all over the world. However, it mostly prefers temperate climates (Mason and McDonough 2012) and leads to economic loss in the Mediterranean, central Europe, Asia, North America and

Australia (Plarre 2010). *Sitophilus granarius* are more tolerant of low temperatures than other *Sitophilus* species, surviving down to 5°C (Ku 2007). Conditions for reproduction are between 11−34°C with relative humidity more than 40% (Rees 2004, Table 1.1). However, *S. granarius* develops best between 25−30°C and 65−70% relative humidity (Hansen and Steenberg 2007, Athanassiou et al. 2017).

Granary weevils are able to feed on both unbroken and broken grains, including wheat, rice, barley, buckwheat, corn, oats, and rye (Campbell et al. 2004). Additionally, they can live on manufactured or refined cereal material, such as pasta, bread, and cereal. There are reports that they have also been found in bird seed, sunflower seed, and chestnuts (Mason and McDonough 2012).

Damage

Granary weevils are considered a significant pest that can cause qualitative and quantitative losses to stored grain kernels. Damage results from both adults and larvae, although most damage is caused by larvae. The larva can destroy greater than 60% of a wheat kernel (Hurlock 1965). Temperature, humidity and food type affect the larval feeding rate. There is a positive relationship between temperature and grain damage by *Sitophilus* spp., with higher damage observed at 26°C compared to 18°C (Pramono et al. 2018). Previous research on *Sitophilus oryzae* showed that endosperm thickness increased feeding damage on sorghum seeds (Russell 1962). In addition to feeding damage, female weevils damage the grain by excavating a hole to lay eggs. Female weevils prefer seeds that are relatively large for that seed type for oviposition, due to the benefit of larger sized seeds for larval survival (Campbell 2002). In addition, grain hardness causes a decrease in oviposition (Russell 1962). According to the standards of

the United States Department of Agriculture's Federal Grain Inspection Service, grain is determined as infested when two or more live grain pest insects have been found in a 1,000 gram grain sample (Mason and Obermeyer 2010, USDA 2019).

Management Strategies for Granary Weevil

Sanitation

Application of sanitation practices plays a key role in controlling storage pests (Phillips and Throne 2010). It is essential that freshly harvested grain is stored in clean storage facilities. The other important point is that older products may host pests and should therefore be stored apart from newly harvested grain. Harvesting equipment, transportation containers, loading region, and storage silos should also be kept clean, as much as possible. If sanitation practices are applied correctly, prevention of pest contamination is an advantage; however, famers might be limited in following sanitation practices because of cost and practical considerations, such as the need to store more than one year's harvest in the same area.

Irradiation

An irradiation technique is used in many countries as a method to control storage pests (Phillips and Throne 2010). Irradiation is used to sterilize insects by damaging the chromosomes of eggs and sperm (Bakri et al. 2005). A dose of 0.5 kilogray is needed to prevent reproduction of storage pests, and a much higher dose is necessary for acute mortality. Although the insect pest is alive after irradiation at 0.5 kilogray, their damages would be reduced due to decreased feeding. However, use of irradiation to control insect grain pests in bulk grain storage facilities is not practical, due to the high cost of facility construction and potential adverse effects on human health (Hallman 2013).

Modified Atmospheres

A modified atmosphere creates an unsuitable condition for storage pests. A concentration of 3% or less of oxygen and/or 60% or more of carbon dioxide shows a toxic impact on insect pests in storage facilities. Application of either low oxygen or high carbon dioxide levels to an infested product can provide effective control in the storage area. This method is especially effective on active stages of the pest (larvae and adults; Phillips and Throne 2010). Modified atmospheres are considered a safe and environmentally friendly method compared to the use of conventional chemical insecticides (Navarro 2006). Despite these benefits, modifying the stored grain atmosphere is often not cost-effective due to the expense of gas and need for special facilities (Phillips and Throne 2010).

Humidity Control & Desiccants

A reduction in moisture in stored grain is another method to control storage pests. Since most insect pests that arise in stored grain grow well when the grain moisture content is 12 to 15%, thus reduction of the humidity level of the grain by using desiccants should help to reduce pests (Phillips and Throne 2010). Desiccant insecticides include diatomaceous earth, inert dust, and kaolin. Diatomaceous earth is composed of fossilized skeletons made of silicon dioxide from aquatic algae, called diatoms. Diatomaceous earth has a sharp structure and creates an insecticidal effect via mechanical abrasions and absorption of the hydrocarbons from the insect cuticle, which leads to loss of water from the insect body (Phillips and Throne 2010; Hosseini et al. 2014). Similarly, inert dust provides pest control by removal of the epicuticle lipid layers of the insect cuticle, which leads to extreme water losses and eventual insect death (Storm et al. 2016). Kaolin is

hydrated aluminum silicate, which has demonstrated high mortality against *Sitophilus* spp. under laboratory conditions (El-Sayed et al. 2010). The structure of kaolin is softer than diatomaceous earth, therefore the effective dose which is used to control stored grain pests is higher for kaolin than for diatomaceous earth (Storm et al. 2016).

Desiccants have many advantages, which include being safe for mammals, having a long-lasting effect, and leaving no residue on the grains (Wakil et al. 2010). Moreover, desiccants can be combined with biocontrol agents. There are many studies that show that use of diatomaceous earth with biocontrol agents has a synergistic effect with higher mortality than when used alone (Sabbour et al. 2012, Wakil et al. 2015). Synergistic effects of kaolin have also been shown with a strain of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin against *S. granarius* (Storm et al. 2016). However, application on the grain of the effective rate for controlling weevils can lead to loss in bulk mass due to desiccation of the grain, which results in lower quality, less dense grain. Moreover, drying of the grain kernel may lead to cracks, which cause the grain to be more vulnerable to pests (Phillips and Throne 2010). The other adverse effect of diatomaceous earth is that personnel can be irritated because of high dust levels. Even though there are some challenges with diatomaceous earth, it is still accepted as one of the most successful and safest (non-toxic) nonchemical methods that can be used for controlling insect pests (Phillips and Throne 2010).

Chemical Control

Organophosphate, pyrethroid, and carbamate insecticides are used as grain protectants (Arthur 1996). Some of the recommended insecticides used as residual surface treatments and registered by the United States Environmental Protection Agency

are: cyfluthrin (a pyrethroid; Zettler and Arthur 2000), chlorpyrifos-methyl (an organophosphate; Fang et al. 2002), and deltamethrin (a pyrethroid), which can be combined with chlorpyrifos-methyl (Mason and Obermeyer 2010). However, application of these insecticides has many drawbacks, such as toxicity to mammals, leaving residue on the product, and insecticide resistance (Arthur 1996).

Magnesium phosphide and aluminum phosphide are solid granules that release phosphine gas, which is a common fumigant, and has been used as a decontaminant worldwide for control of common storage pests, including *Sitophilus* spp. Due to its frequent use worldwide, there are many reports that have shown resistance to phosphine by *Sitophilus* spp. (e.g., Monro et al. 1972, Alam et al. 1999). In addition to resistance problems, it can be difficult to maintain optimum concentrations of phosphine gas and a special license is needed for application, so that this management strategy is not always effective or possible.

Biologically Based Controls

Pheromones and other semiochemicals. Pheromone traps are a highly sensitive monitoring tool (Subramanyam and Hagstrum 2000). Many pheromones are commercially available for around 20 species of post-harvest pests, including *Sitophilus oryza*e and *S. zeamais*. However, there is no pheromone available for *S. granarius*. Although pheromone traps are an important monitoring tool for making decisions, they are not a direct alternative to chemical control (Subramanyam and Hagstrum 2000).

Insect growth regulators. Insect juvenile hormone analogs, which include methoprene, hydroprene, and pyriproxyfen, are used in the United States. These insect growth regulators disrupt natural development of insects by imitating the effect of the

insect juvenile hormone (Arthur et al. 2009). Methoprene is accepted as nontoxic and can be applied to stored grain at 1 ppm to maintain insecticidal activity for more than one year (Phillips and Throne 2010). Research on the effect of hydroprene on *Sitophilus oryzae* shows that it can prevent the production of a new generation by causing abnormalities in the ovaries of the weevils (Mkhizel and Gupta 1982, Eisa and Ammar 1992). Activity of juvenile hormone compounds are selective to insects, so they have a low toxicity to non-targets. Juvenile hormone compounds do not kill adults; however, these compounds can inhibit or decrease production of progeny by affecting development of immature stages. These are considered to be safe products for use in stored grain, especially if there is insecticide resistance. Although insect growth regulators have low-level toxicity to mammals and pose a low risk in food safety, they are not broadly adopted to control stored grain pests when compared to commonly used contact insecticides and fumigants (Phillips and Throne 2010).

Plant-derived materials. There are many *in vitro* studies on plant volatile oils, which show insecticidal effectiveness against *Sitophilus* spp. (Shaaya et al. 1991, Abdelgaleil 2006, Follett et al. 2014, Rajashekar et al. 2010, Ebadollah and Mahboubi 2011). For example, volatile oils from thuja, eucalyptus and peppermint were effective against *S. granarius* as a fumigant, indicating that these volatile oils can be used for control of this pest (Hamza et al. 2016).

Vegetable oils, including cottonseed, soybean, maize, and peanut oil, can have repellent and insecticidal effects against the granary weevil (Qp and Burkholder 1981). A toxic and repellent effect of eugenol, which is the main compound of the plant *Ocimum suave* (Lamiaceae), was found against *S. granarius* and *S. oryzae* (Obeng-Ofori and

Reichmuth 1997). Azadirachtin, which is obtained from the neem tree (*Azadirachta indica*) has been tested for control of many storage pests (Adarkwah and Obeng-Ofori 2010, Lale and Mustapha 2000, Athanassiou et al. 2005). Azadirachtin can act as an anti-feedant and an insect growth regulator, causing mortality of *Sitophilus* spp. (Athanassiou et al. 2005). Azadirachtin caused high mortality against *S. oryzae* at 55% relative humidity, however this effect did not persist when the relative humidity reached 75% (Kavallieratos et al. 2007). Although there are many commercial formulations available (Liang et al. 2003), there is not enough information about the effectiveness of azadirachtin compounds on post-harvest insect pests (Kavallieratos et al. 2007). Furthermore, botanical insecticides have some disadvantages, such as lack of stability, safety concerns, and sometimes odor problems (Phillips and Throne 2010).

Bacteria-derived material. Spinosad is a commercial insecticide, which is produced by the soil actinomycete bacterium *Saccharopolyspora spinosa* Mertz & Yao as a fermentation product (Subramanyam et al. 2007). It is a commercial reduced-risk insecticide due to its low toxicity to mammalians (Subramanyam et al. 2012). Spinosad affects the insect nervous system (Hertlein et al. 2011) and has been used to control insect pests (Fang et al. 2002). The performance of spinosad and deltamethrin was evaluated against both *S. granarius* and *S. oryzae* (Vélez et al. 2017). Although spinosad takes longer to show effectiveness compared to deltamethrin, it was able to kill both *S. granarius* and *S. oryzae* 12 days after exposure. (Vélez et al. 2017).

Biological Control

Biological control is described in entomology as the use of living predators, parasitoids, or entomopathogenic microorganism to suppress a pest population (Pal and

Gardener 2006). Biological control is defined with three approaches, which include classical, augmentative, and conservation biological control:

1) **Classical biological control** is defined as the introduction of natural enemies to a new place where they are not native to control a pest that is also not native. In this approach, an economically important pest is targeted and the main purpose is to modify the natural balance in the introduced range to inhibit outbreak of the pest (Hajek et al. 2016).
2) **Augmentative biological control** is releasing of the biocontrol agents for control of the pest. The strategies include two methods, which are inoculative and inundative control. Inundative control is releases of large number of biocontrol agents, with the goal of immediate mortality of a high level of the pest population or diminishing damage occurred by the pest. In this approach, the goal is related to releasing biocontrol agents with enough population to suppress the pest. Progeny of the biocontrol agent is not expected (Eilenberg et al. 2001) and permanent establishment is not the aim (Hajek et al. 2016). Inoculative control methods include releasing of the biocontrol agents with the expectation that they will provide pest control after propagation. Success of this type of application is highly dependent on the population of the biocontrol agent adapting and reproducing where it has been released (Eilenberg et al. 2001). For application of the most appropriate biocontrol method, the goal of the control method should be considered. Such as, if the aim of releasing natural enemies is for persistence and proliferation in the area, classical biological control and inoculation biological control could be effective approaches. In contrast, if the

purpose is decreasing pest population significantly in the short term, inundation biological control may be a suitable approach (Eilenberg et al. 2001).

3) **Conservation biological control** is adjustment of the environment or existing practices to protect and enhance specific natural enemies or other organisms to diminish the influence of pests. The most important point in this approach is that natural enemies are not released, which distinguished this from the other biological control approaches (Eilenberg et al. 2001). When this approach includes a combination of protecting biocontrol agents and providing resources for them, it can be more effective (Eilenberg et al. 2001).

Parasitoids. Use of natural enemies against weevils in stored grain has shown potential for adoption in Europe (Hansen and Steenberg 2007). There are many studies that claim larval parasitoids, *Lariophagus distinguendus* (Förster) and *Anisopteromalus calandrae* (Howard; Hymenoptera: Pteromalidae), have promise for control of *Sitophilus* spp. (Smith 1992). *Anisopteromalus calandrae*, which is a parasitic wasp, can suppress *S. oryzae* by 99.4% (Press and Mullen 1992). Another study showed that *S. granarius* were attacked by *A. calandrae* and *L. distinguendus* (Schmid et al. 2012). The authors of this study recommended that the larval parasitoid *L. distinguendus* can be used for control of *S. granarius* in storage bins (Steidle and Schöller 2002). The effectiveness of these two parasitoid species were tested alone and together with the entomopathogenic fungus *Beauveria bassiana* (Hansen and Steenberg 2007). Results showed highest suppression (99.9%) with parasitoids only, while the treatment that included both parasitoids and *B. bassiana* had a moderately low level of suppression. Although *B. bassiana* also had a negative effect on the two parasitoids, the population of *S. granarius* was suppressed by

83-98% (Hansen and Steenberg 2007). Moreover, the researchers have claimed that *L. distinguendus* is a candidate for biological control of *S. granarius* (Hansen and Steenberg 2007). However, there is some challenge about releasing parasitoids because timing needs to be careful planned. The parasitoids should be released early enough that they can reach sufficient population to control the pests. If released later, a much higher population of parasitoids is needed, which may not be feasible or affordable (Mason and McDonough 2012). In addition, parasitoids must be reared and obtained from a high quality source that requires special expertise that is not common.

Entomopathogenic bacteria. Bacteria are unicellular prokaryotic microorganisms that have become popular as microbial biopesticides because of some favorable features, such as cost-effective mass production, specificity, and environmental friendliness. Commercialized entomopathogenic bacteria possess an obligate or facultative relationship with their host or they create toxins, which can be used for insect control. Entomopathogenic bacteria first move into the host body through the hemocoel and then propagate inside of the insect body. They cause disease by producing virulence factors, such as crystalline proteins, and eventually kill the host (Glare et al. 2017). Most bacterial entomopathogens that perform well commercially, such as species from the genera *Bacillus*, *Lysinibacillius*, and *Paeanibacillus*, are gram-negative.

In the United States, the entomopathogenic bacterium *Bacillus thuringiensis* has been recognized as a grain protectant (Ramos-Rodríguez et al. 2006). *Bacillus thuringiensis* is more successful against insect pests that belong to the Orders Lepidoptera and Diptera (Phillips and Throne 2010). Commercial production of *B. thuringiensis* has been used for control of Indian meal moth larvae. However, pest resistance to *B.*

thuringiensis has been reported (McGaughey and Beeman 1988). *Bacillus thuringiensis* subsp. *tenebrionis* has promise for the control of Coleopteran insect pests of stored wheat, such as *S. oryzae*, under *in vitro* conditions (Mummigatti et al.1994). Other research with *B. thuringiensis* against *Sitophilus* spp. demonstrated that an isolate of *B. thuringiensis* can be used to manage *S. oryzae* larvae (Silva et al. 2010).

Entomopathogenic fungi. Entomopathogenic fungi are present in nature, broadly, and they can live in a wide range of environmental conditions, including arid to tropical and terrestrial to aquatic areas, where they can infect a wide range of insects (Skinner et al. 2014). The infection process of entomopathogenic fungi starts with penetration of the insect cuticle by using appressoria, which use enzymatic and physical pressure to penetration the host cuticle (Kaya and Vega 2012). In addition, entomopathogenic fungi can enter via openings of the insect body, such as spiracles, sensory pores, and wounds (Skinner et al. 2014). When entomopathogenic fungi reach the inside of the insect body, they proliferate and feed on the host interior content. During this time, the fungus may impact the host by changing host behavior and feeding, reducing body weight and fertility, and causing other abnormalities (Zimmermann 2007). Eventually, the host is killed by disrupting key biological functions, which lead to nutritional deficiency and tissue devastation (Skinner et al. 2014). When the host dies, the entomopathogenic fungus begins its saprophytic phase and breaks out of the host body to produce conidia on the surface of the cadaver (Zimmermann 2007).

Entomopathogenic fungi have numerous features that make them excellent tools for use in Integrated Pest Management. For example, entomopathogenic fungi are relatively host-specific and moderately innocuous to beneficial insects and so have

minimum influence on the natural biodiversity. In addition, most entomopathogenic fungi are facultative saprophytes, which makes them suitable for mass production on artificial media. Moreover, spores from species of entomopathogenic fungi can be produced by dehydration and, if stored under suitable conditions, can remain viable. Many species of entomopathogenic fungi can show activity under a wide range of environmental conditions (Skinner et al. 2014, Table 1.1). Thus there are several studies with entomopathogenic fungi against *Sitophilus* species.

Isolates of *B. bassiana* have been screened against three important storage pests: *S. oryzae*, *S. zeamais*, and *Rhyzopertha dominica* (Fabricius; Coleoptera: Bostrichidae) (Moino et al. 1998). Although *R. dominica* was more vulnerable in general than *Sitophilus* spp., the results showed that two isolates of *B. bassiana* caused highest mortality to *Sitophilus* spp. Strains of *B. bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* were screened against *S. oryzae*, with the highest mortality being obtained when fungal spores were sprayed on the pest (Kavallieratos et al. 2014). In addition, isolates of *B. bassiana*, *M. anisopliae*, *Purpureocillium lilacinum* (Thom) Luangsa-ard, Hou-braken, Hywel-Jones & Samson and *Lecanicillium lecanii* Zare & Gams have been analyzed in terms of insecticidal effect against *S. zeamai*s (Ahmed 2010). Results of this study showed that *M. anisopliae* and *V. lecanii* have an effect against *S. zeamais*; however, the authors claimed that this result was dependent on conidial concentration (Ahmed 2010). Although *Fusarium* spp. are generally accepted as plant pathogenic fungi, direct application of *F. avenaceum* to wheat has shown a high mortality rate against *S. oryzae* (94.86%; Batta 2012).

Grain fungi (*Fusarium*, *Penicillium*, and *Aspergillus*)

Fungi are ubiquitous in nature (Pettigrew et al. 2010) and are well adjusted to the environments where grain is grown pre-harvest and stored post-harvest. Fungal infection is considered one of the most significant risks to stored grain (Fleurat-Lessard 2017). Fungal infection can cause loss of dry matter, nutritional value and seed germination. Fungi have also been linked with serious plant diseases and can lead to low grain quality and quantity. In addition to these adverse effects, the presence of fungal infections in grain threaten human and animal health due to mycotoxins, which are produced by fungi as secondary metabolites (Fleurat-Lessard 2017).

Among the species of fungi that are known to cause contamination of stored grain, these can be divided into two groups, which are field fungi and storage fungi (Atanda et al. 2011). This classification is not a taxonomic division, but is based on the moisture requirements of these fungi. Field fungi infect grain in the field with at least 20% moisture content or relative humidity of 90−100%. The main species of field fungi are *Alternaria, Cladosporium, Fusarium,* and *Helminthosporium*. After harvest, storage fungi infect stored grain with 13−20% moisture content or 70−90% relative humidity (Bothast 1978, Herceg et al. 2015). Storage fungi include species of *Aspergillus* and *Penicillium.* Although *Fusarium* is considered to be a field fungus, it can continue to develop on grain in the storage area when humidity is adequate (Magan and Aldred 2007). *Aspergillus*, *Fusarium,* and *Penicillium* are the most important fungal species of interest for stored grain globally (Atanda 2011, Bryden 2012).

Fusarium graminearum

Fusarium graminearum (Schwabe; teleomorph = *Gibberella zeae* (Schwein)), is the primary causative agent of Fusarium head blight disease, which is one of the major economically destructive diseases of small grains including wheat, barley, maize, oats, and wild rice (Jochum et al. 2006, Wegulo et al. 2015). The optimum temperature for *F. graminearum* growth is 25 to 28 ºC (Miller 2008; Table 1.1), with 0.90 water activity (Cheli et al. 2013). Fusarium head blight needs high humidity (>90%) and warm temperatures (15−30 ºC) for infection (Schmale and Bergstrom 2003). Fusarium head blight is a source of mycotoxin contamination and causes over a billion dollars of damage worldwide. (Wegulo et al. 2015). *Fusarium graminearum* produces secondary metabolites, which include zearalenone and deoxynivalenol. Deoxynivalenol is a vomitoxin that can threaten animal and human health by disrupting cell function and protein synthesis, affecting the digestive system of animals. According to recommendations from the United States Department of Agriculture, safe consumption levels of deoxynivalenol in human food should be less than 1 ppm. However, deoxynivalenol levels can reach more than 20 ppm when wheat is contaminated with Fusarium head blight (Schmale and Bergstrom 2003).

Penicillium chrysogenum

Penicillium chrysogenum can produce multiple different important mycotoxins which can be found on a variety of grains, but citrinin is the most common with mycotoxin levels up to 0.2–0.4 μg/g) (Krejci et al. 1996). The optimum temperature for growth of *P. chrysogenum* and production of citrinin is 25−30 ºC (Reiss 1977, Table 1.1). The mycotoxin citrinin causes severe problems in countries with hot climates. Citrinin

has adverse effects on human and animal health by creating kidney, liver and gastro-intestinal problems (Ammar 2000).

In addition to citrinin, many *Penicillium* spp. can cause additional problems for infected food and grains by the production of other mycotoxins, including ochratoxin, sterigmatocysin, rubratoxins, and patulin (Williams and McDonald 1983). Ochratoxin is another important mycotoxin in stored grain, which is secreted by species of *Aspergillus* and *Penicillium*. Specifically, *A. ochraceus* and *P. verrucosum* are associated with ochratoxin (Richard 2007). The optimum conditions for growth of *P. verrucosum* are 20 ºC, with pH from 6−7, and 0.80 water activity. The mycotoxin ochratoxin is produced at a temperature of 4−20 ºC with 0.86 water activity (Cheli et al. 2013). Ochratoxin is produced during the storage period (Luo et al. 2018), when it can contaminate a variety of foods, but it has been reported most often in cereal grains and grape products. Ochratoxin has strong carcinogenic potential, especially for liver cancer (Clark and Snedeker 2006). According to the European Union, the maximum allowable concentration in raw cereals should be 5 mg/kg and processed cereals should be 3mg/kg (Luo et al. 2018).

Aspergillus parasiticus

Aspergillus flavus and *A. parasiticus* produce aflatoxins as secondary metabolites, which are known as highly toxic, mutagenic, teratogenic, and carcinogenic compounds (Bhat et al. 2010). *Aspergillus parasiticus* will grow at a wide range temperatures (from 10−43 ºC) with an optimum temperature for the production of aflatoxins around 28−30 ºC with 0.87 water activity (Cheli et al. 2017, Table 1.1). *Aspergillus* spp. can produce 18 altered forms of aflatoxins, with the most important being B_1, B_2, G_1, G_2, M_1, and M_2.

(Luo et al. 2018). Aflatoxins are considered mutagenic, teratogenic, and carcinogenic compounds. They cause serious health problems, such as acute or chronic liver necrosis and tumors (Fleurat-Lessard 2017). Aflatoxins are designated as Class 1 carcinogens with a maximum allowable concentration of 2 mg/kg in all cereals (Ostry et al. 2017).

Factors Affecting Fungal Growth in Grain

Many factors affect fungal growth and spoilage in grain, including temperature, atmosphere, pH, grain type, relative humidity, grain moisture content, and water activity. Grain moisture content is the percentage of moisture in the material. Moisture content is calculated as: wet weight – dry weight / wet weight (Hellevang 1995). Water activity is the availability of the free water in the food (Sancho-Madriz 2003). Water activity is measured between 0 (no moisture) to 1.0 (pure water) (Tiefenbacher 2019). There is a relationship between water activity (a_w) and relative humidity (RH), as shown in this formula: RH = a_w x 100% (Segers et al. 2016).

In addition, existence of insect, mites, and rodents can affect fungal infection, which will be further addressed in a later section. The presence of broken grains and the harvest process can also impact infection with fungal pathogens (Bothast 1978, Neme and Mohammed 2017). Nevertheless, temperature and moisture content are the main dynamics that influence fungal growth in stored grains (Jayas and White 2003). Relative humidity is the rate of the partial pressure of water vapor to the equilibrium vapor pressure of water at a given temperature. Relative humidity is an important factor for development of fungal pathogens. Storage fungal pests need greater than 0.7 a_w for growth (Bothast 1978). Research shows that *Aspergillus* species are not able to infest grain when the humidity level is less than 70% (Kabak et al. 2006).

The pH of the environment is another critical factor. Grain fungal pathogens are able to grow at a wide range of pH (2−8.5; Bothast 1978). *Aspergillus parasiticus* can grow between 2.1−11.2 pH, but the optimum pH for its growth is 3.5−8.0. Usually, *Fusarium* species need a pH between 2.4−3.0, but this range will also depend on temperature (Cheli et al. 2013). Atmosphere (the relative amount of carbon dioxide and oxygen) is another important factor for fungi. Fungi are aerobic microorganisms and need oxygen. Therefore, low oxygen levels or high carbon dioxide concentrations can negatively affect their activity. For example, decreasing the oxygen level from 5 to 1% significantly inhibits growth of *A. flavus* and the production of aflatoxins (Cheli et al. 2013). In addition, grain type may affect fungal infection. Such as, softer types of wheat respire more rapidly than harder types, affecting temperature and moisture levels (Bothast 1978).

Although grain respires slowly when it is stored dry, if water activity increases to 0.75-0.85 a_w (15−19% moisture content), respiration activity significantly increases, which causes release of energy. Consequently, this process causes increasing temperatures in the storage area (Magan and Aldred 2007).

Management Strategies for Grain Fungi

Pre-harvest Control Methods

Pre-harvest control methods play an important role in the management of fungal growth and mycotoxin development in stored grain (Cheli et al. 2017). For example, one of the most important wheat diseases is Fusarium head blight, which originates from infection in the field. Moreover, planting time is also another effective pre-harvest method that affects later mycotoxin contamination. For example, it has been reported that

late planting cause four times higher fumonisin contamination (Magan and Aldred 2007). Therefore, pre-harvest control has crucial importance to diminish pre-harvest contamination. Pre-harvest control methods include agronomic practices (such as tillage, crop rotation, and irrigation), planting of resistant varieties, and sanitation.

Tillage is an important agronomic practice that helps to mitigate Fusarium head blight. Higher contamination of the harvested grain with *Fusarium* and deoxynivalenol has been determined with minimum tillage or no-till practices in wheat (Dill-Macky and Jones 2000). The practice of suitable crop rotation is another technique that helps to mitigate infection with *Fusarium* spp. Maize is highly vulnerable to *Fusarium* spp., and contamination of maize in the field in the preceding year is a contributing factor to deoxynivalenol infection of wheat (Cheli et al. 2017, Schaafsma et al. 2005). A soybean-wheat rotation can lower deoxynivalenol concentration compared to a wheat-wheat or corn-wheat rotation (Dill-Macky and Jones 2000, Schaafsma et al. 2005). Irrigation plays an important role in reduction of pre-harvest contamination. Both water stress and over-irrigation can create favorable condition for infection by *Fusarium*. Water stress should be avoided during the period of development and maturation of the seed. In addition, over-irrigation during the flowering and early grain fill period can provide suitable conditions for the development of *Fusarium* disease (Cheli et al. 2017).

Planting resistant crops is the most cost effective strategy to reduce fungal problems. Numerous studies have been conducted since 1990 to assess the resistance of crop cultivars against Fusarium head blight (Dill-Macky and Jones 2000). Although some cultivars have been discovered to have some resistance to Fusarium head blight, complete

resistance has not been confirmed in wheat (Li et al. 2010). Currently, ‘Sumai 3’ which is a partially resistant wheat cultivar is used extensively worldwide (Niwa et al. 2014).

Application of sanitation practices during both pre-harvest and post-harvest periods is the first step to lower the risk of fungal growth during grain storage (Fleurat-Lessard 2017). Grain cleaning is an important technique to reduce contamination of fungal growth. Most of the post-harvest fungi that produce mycotoxins are localized on the surface of the grain. Thus, brushing or removing part of the grain which has fungal growth can help to mitigate fungal contamination and mycotoxin production in the storage area (Fleurat-Lessard 2017). However, this technique is not common for removing fungal pathogens.

Post-harvest Control Methods

Drying. After harvesting, reducing the moisture content of the grain is particularly important to protect the grains against fungal development and mycotoxin production (Neme and Mohammed 2017). Insufficient drying can allow colonization by *Penicillium* spp. in storage facilities (Magan and Aldred 2007). Thus, it is suggested that the harvested crop should be dried to safe moisture content levels. Recommended moisture content for safe storage of wheat grain is 14.0−14.5% (0.70 a_w), although the wheat should be kept at 1−3% less than this value if the grain will be stored for more than one year (Jayas and White 2003). Drying grain is one of the most effective control methods for both insect pests and fungi. However, grain drying with high temperatures may cause reduction of grain quality because of protein denaturation (Mohapatra et al. 2017).

Irradiation. Irradiation is another recommended technique for inhibition of fungal growth. Application of 6 kilogray (kGy) of gamma radiation can eliminate deoxynivalenol and zearalenone, mycotoxins produced by *Fusarium*, in flour and wheat (Aziz et al. 1997). However, application of ionizing irradiation in storage areas is not common because of public demand for food free of radioactivity (Phillips and Throne 2010).

Modified atmospheres. Changing the gas concentration in the storage facility has the potential to inhibit fungal growth. Reducing oxygen (<0.14%) and increasing carbon dioxide (>50%) is recommended to control fungal growth and inhibit mycotoxin activity (Magan 2006). Although controlling and modifying atmospheres is an applicable method for management of both insect and fungal pests in stored grain, adequate control of these pests may not be achieved if relative humidity is high. Moreover, application of carbon dioxide for reduction of mycotoxin accumulation in maize may not be cost-effective, as the concentration needed for control is considerably high (Chulze 2010).

Plant-derived materials. Many studies have investigated the efficacy of plant extracts against grain fungal pathogens. Clove oil, *Syzgium aromticum* (L.) Merrill & Perry, has been found to have antifungal effects against *Aspergillus* spp. and *Fusarium graminearum* (Cardiet et al. 2012). Moreover, clove oil also had insecticidal activity against *Sitophilus oryzae.* These results show that clove oil has promise as a protectant for stored grain (Cardiet et al. 2012). Extracts from *Azadirachta indica* have inhibitory effects against biosynthesis of aflatoxins (groups B and G) which are produced by *Aspergillus* spp. (Bhatnagar and McCormick 1988). Also, five essential oils, including oregano, cinnamon, lemongrass, clove and palmarose, have reduced zearalenone and

deoxynivalenol, mycotoxins produced by *F. graminearum* (Velluti et al. 2004). Essential oils from garlic and wild oregano have also been found to have an antifungal effect against *Penicillium* spp. (Ozcakmak et al. 2017). Although these studies have shown success with natural plant materials against storage pests, these tests were conducted under *in vitro* conditions, and large-scale studies are needed. Thus, to determine the real effectiveness of plant-derived compounds, these products should be tested in grain storage areas (Mannaa and Kim 2017).

Chemical control. Phenolic antioxidants, such as butylated hydroxyanisole, have antifungal activity against species of *Aspergillus*. However, it has been reported that butylated hydroxyanisole has limited effect against species of *Fusarium* and *Penicillium* (Thompson 1996). A combination of butylated hydroxyanisole and propyl paraben can be used as an effective fungitoxicant to control *Aspergillus flavus* and *A. parasiticus* (Nesci et al. 2003). Application of fungicides is one of the effective ways to control fungal pests. Fungicides, including propiconazole, prothioconazole, tebuconazole, and metconazole, have been used against Fusarium head blight (Paul et al. 2010). Particularly, treatment of wheat with tebuconazole can reduce deoxynivalenol accumulation (Wegulo et al. 2011). However, synthetic fungicides provide only partial control against Fusarium head blight in the field, due to problems with application technique and timing (Schmale and Bergstrom 2003, Wegulo et al. 2015). Additionally, although, these fungicides could help to reduce fungal pathogens, they are not registered for application to harvested grain.

Biological control. Biological control for plant pathogens is described as the use of microorganisms which diminish the activity of disease causative agents or survival of the pathogens (Ownley et al. 2010). Different biological control mechanisms are

identified, such as antibiosis, competition, parasitism, induced systemic resistance, increased growth response, and endophytic colonization in the plant. Antibiosis is when the biocontrol agent overcomes the pathogen by secretion of secondary metabolites (antibiotics, volatile organic compounds and several lytic enzymes; Ownley et al. 2010). Competition is another biocontrol mechanism, which is competition among the microorganisms for nutrients and space (Verma et al. 2007). Parasitism is when one organism is getting a benefit from another organism where they coexist for a period of life (Pal and McSpadden Gardener 2006). Induced systemic resistance is another mechanism, which is when control is provided by non-pathogenic fungi and bacteria. Induced resistance occurs when the plant reacts to the existence of a pathogen by defense related genes (Ownley et al. 2010). Endophytic colonization by the biocontrol agent is another mechanism, which is described as the presence of the biocontrol agent on the plant tissue, providing suppression of the plant disease (Ownley et al. 2010).

Biological control approaches have the potential to manage pathogenic fungi and production of mycotoxins. Application of biocontrol agents can be a viable option for the protection of organic products, where synthetic fungicides are not allowed (Wegulo et al. 2015).

The most commonly tested bacterial biocontrol agents against *Fusarium graminearum* are species from the genera *Bacillus*, *Lysobacter*, and *Pseudomonas* (Jochum et al. 2006). *Streptomyces albidoflavus* Waksman & Henrici and *Bacillus velezensis* Ruiz-García can suppress fungal growth and mycotoxin accumulation by *F. graminearum* (Palazzini et al. 2018). Spraying *Streptomyces* sp. spores on wheat during flowering can provide protection of the wheat (Jung et al. 2013). Strains of *Lactobacillus*

and *Propionibacterium* are able to reduce deoxynivalenol by 55% and zearalenone by 88% (Niderkorn et al. 2006). Under storage conditions, strains of *Bacillus megaterium*, *Microbacterium testaceum*, and *Pseudomonas protegens* have shown antifungal activity against *Aspergillus* spp. and *Penicillium* spp. in stored rice (Mannaa and Kim 2018). The *B. megaterium* strain was the most effective isolate among those tested (Mannaa and Kim 2018).

In addition to bacteria, many fungal isolates have been examined for antagonistic activity against grain fungal pathogens. *Trichoderma* spp. are the most important promising biocontrol agents due to their fast growth ability, which allows them to be strong competitors capable of suppressing *Fusarium* spp. Although *Trichoderma* spp. had an antagonistic effect against *Fusarium graminearum* and *F. culmorum* on rice, this effect could not be confirmed on wheat haulms (Matarese et al. 2012). *Clonostachys rosea*, *Cryptococcus flavescens* and *C. aureus* can reduce Fusarium head blight severity on wheat (Schisler et al. 2011, Xue et al. 2014). *Bacillus amyloliquefaciens* has also shown antagonistic activity against *F. graminearum* under *in vitro* conditions (Shi et al. 2014). *Pichia anomala*, *P. guilliermondii*, and *Saccharomyces cerevisiae* have shown potential to inhibit the growth of *Penicillium roqueforti* and *Aspergillus candidus* (Petersson and Rer 1995). *Streptomyces* sp. was able to inhibit growth of *Aspergillus parasiticus* on peanut under *in vitro* conditions (Zucchi et al. 2008). Another study has shown that *S. cerevisiae* can control *Aspergillus* spp. in postharvest coffee bean (Velmourougane et al. 2011). Under simulation of realistic wheat grain storage conditions in a pilot scale silo, the biocontrol yeast *Pichia anomala* inhibited growth of *Penicillium roqueforti*, which is known as one of the most serious spoilage fungi of

stored grain (Druvefors et al. 2002). Although there are many studies and promising results with potential biocontrol agents for control of Fusarium head blight, there are no registered biocontrol agents against this pest (Yuen and Schoneweis 2007). Performance of biological control agents in the field is affected by abiotic conditions, application technique and timing, and persistence of biocontrol agents (Yuen and Schoneweis 2007, Wegulo et al. 2015). The same challenges can occur for the use of biological control agents for post-harvest pest management, due to difficulties in application and persistence. Although several studies have shown effectiveness of biocontrol agents (Druvefors et al. 2002, Velmourougane et al. 2011), their use is limited in storage facilities (Mannaa and Kim 2017) since their effectiveness is highly dependent on the target host, and usually, they are acting slowly (Copping and Menn 2000). Temperature is another factor that affects the activity of biocontrol agents; generally, entomopathogenic fungi can tolerate temperatures between 0 to 40 °C. However, 20 to 30 °C is optimal for germination, growth and sporulation (Goettel et al. 2000). Moreover, humidity is another crucial factor that affects their activity; low humidity may cause failures of the biocontrol agents (Skinner et al. 2014). However, selection of the biocontrol agents according to the storage environment could help to reduce disadvantages. The potential antagonist should have the capability to rapidly colonize (Janisiewicz and Korsten 2002) and keep their persistence and survive under unfavorable conditions (Wilson and Wisniewski 1989).

Integrated Pest Management

Integrated pest management uses as many control strategies as possible, combined, to reduce pest populations or to suppress pest activity below an economic

threshold with minimal injury to humans and the environment (Elzinga 2004). Integrated Pest Management for stored products includes establishing action levels that take into account information about the stored product, the pests arising in the product, abiotic dynamics of the system, and tolerance levels for damage and contamination. In addition to using a variety of complementary (or even synergistic) management strategies, pest monitoring and the use of economic thresholds to help make decisions and manage risk are important components of Integrated Pest Management.

Pest monitoring via observation and sampling is important for estimating pest populations. However, monitoring is done at a minimum level by many producers; a survey conducted in Indiana and Illinois showed that monitoring often consisted of just visual and odor testing when the storage bin door was open (Yigezu et al. 2008). Grain should be checked every 21 days with a deep-bin probe trap or digital x-ray equipment if grain temperature is above 15°C (Phillips and Throne 2010, Mason and McDonough 2012). Monitoring for signs of fungal activity, such as gas production and moldy scent using an electronic nose, is another important step. Detection of granary weevil infestation is difficult due to the internal feeding of larvae, making infestation hidden and difficult to detect (Piasecka-Kwiatkowska et al. 2014). There is equipment for detection of internally-feeding pests, such as digital x-ray, but this is not practical for use on-farm, as the equipment is relatively costly (Phillips and Throne 2010).

To help avoid economic loss, there are some computer assisted tools that help determine risk and aid decision-making for stored grain (Phillips and Throne 2010). The Stored Grain Advisor program from the United States Department of Agriculture may be

used to make decisions about stored grain based upon information on the temperature and moisture content of the grain and the level of pest infestation as determined by sampling.

Interactions between Insects and Fungi in Stored Grain

It is important to consider the presence of both insect and fungal pests in stored grains. In addition to direct feeding damage on the stored grain, many insect pests, such as *Sitophilus* spp., *Rhyzopertha dominica,* and *Tribolium castaneum*, promote the development of fungal pests in stored grains as well. For example, *Sitophilus granarius* promotes infestation of wheat grains by *Aspergillus restrictus* (Agrawal et al. 1957). Moreover, *S. zeamais* may serve as a vector of many species of *Aspergillus*, *Penicillium*, and *Fusarium* (Mason and McDonough 2012). Insect activity in grain can cause increased temperature and moisture accumulation, which promotes favorable conditions for fungal growth and mycotoxin production (Chulze 2010, Mohapatra et al. 2017). Therefore, control of insect pests with appropriate management techniques can also be advantageous for the control of fungal invasion.

Management strategies to control insect pests in storage areas are considerably similar to those used for management of storage pathogens (Fleurat-Lessard 2017). Determination of storage conditions that are not suitable for insect and fungal growth is the first step for this strategy. Maintaining grain moisture content and humidity below the lower limit of fungal and insect growth is essential to reduce economic losses. Poor storage conditions lead to the presence of both insect pests and fungal pathogens in stored grains, and thus the application of sanitation rules are necessary to obtain pest-free and cost effective food (Skinner et al. 2014).

Therefore, dual biological control against these two group of the pest is important. There are several studies that test candidate biocontrol agent isolates against insect and fungal pests. *Metarhizium brunneum* and *Clonostachys rosea* have shown dual effect against *Fusarium culmorium* and *Tenebrio molitor* (Keyser et al. 2016). *Leaniciliium* spp. (formerly *Verticillium lecanii*) are effective entomopathogens against cotton aphid (*Aphis gossypii*) and white fly (*Trialeurodes vaporariorum*) insect pests (Kim et al. 2001), as well as root-knot nematode (*Meloidogyne incognita*; Gan et al. 2007). *Verticillium lecanii* was tested against potato aphid and cucumber powdery mildew, with effectiveness of *V. lecanii* against both aphids and fungi = reported under laboratory conditions (Askary et al. 1998). Additionally, this fungus has activity against plant pathogens such as green mold (*Penicillium digitatum*) (Benhamou and Brodeur 2000). Moreover, *Beauveria bassiana*, which has more than 700 species of insect hosts, has also shown antifungal ability. For example, *B. bassiana* strain 11-98 was able to inhibit *Rhizoctonia solani*, which is a soilborne plant disease (Ownley et al. 2010).

Situations that Favor the Use of Biological Control

As described in the previous sections, there are many different management strategies that can be applied against granary weevils and stored grain fungal pathogens; however, there are many reasons why specific management strategies may not be applicable, possible, and/or effective in all situations. First, it is not always possible to harvest grain at the desired moisture content. Field conditions are dynamic due to weather, equipment, timing, and other practical considerations. In other words, temperatures required for grain drying do not always occur in the field. If grain is harvested with high moisture content, there is a need for artificial drying to prevent

spoilage. However, grain drying expenses may be too high and drying equipment and facilities may not be available. Therefore, there are practical situations where grain enters storage with high moisture content, potentially already infected with *Fusarium* from the field, leading to a high-risk scenario for insect and fungal pest infestation.

Once the grain has been stored, farmers may not be able to maintain humidity and temperature levels during the long storage period. For example, farmers do not aerate the grain during the winter or summer (Yigezu et al. 2008). Furthermore, if the weevils have already entered the grain, the best option to eliminate the pest is fumigation. Nevertheless, fumigation can be applied only by personnel with specific certification (Holscher 2000) and some populations of *Sitophilus* spp. have developed resistance to phosphine gas. Another chemical option is residual surface treatments with insecticides; however, this cannot provide immediate control against weevil larvae, which are the most destructive stage, due to their internal feeding (Holscher 2000).

Considering this situation, microbial biocontrol agents are a promising management tool. Many studies have shown that biocontrol agents are important tools in Integrated Pest Management; they provide many advantages with their unique features. However, several abiotic factors can negatively influence effectiveness; for example, exposure to UV radiation can cause inactivation or delayed germination of conidia, optimal temperature is needed for germination, and low humidity can limit activity.

Adaptation of the biocontrol agents to the target environment is one of the main features needed to achieve successful biocontrol (Mannaa and Kim 2017). Biocontrol agents, which will be tested in this research, have a similar abiotic niche as granary

weevils and grain fungal pathogens (Table 1.1). The pests and biocontrol agents found in the same environment should increase the chance of effectiveness of biological control.

Rationale & Objectives

Cereals play a crucial role in human and livestock diets. In particular, wheat provides approximately 19% of global dietary energy consumption (Piasecka-Kwiatkowska et al. 2014). However, due to the need to store this grain for long periods, it can be invaded by insect and fungal pests. The granary weevil, *Sitophilus granarius*, is considered a major pest of stored grains worldwide (Kljajić and Perić 2007). Adult and larval stages of the pest cause detrimental economic loss to stored grain, specifically wheat. In addition, fungi have been ranked as the second most important pest in stored grain after insects (Yigezu et al. 2008). *Fusarium graminearum, Aspergillus parasiticus*, and *Penicillium chrysogenum* are accepted among the most important grain fungal pathogens globally (Bryden 2012). These species lead to economic damage by affecting grain quality and quantity. Moreover, their secondary metabolites cause serious health problems for humans and livestock. Thus, there is a need for management strategies to minimize economic loss and side effects on mammalian health. Although many preventative and reactionary actions have been taken against both granary weevils and grain fungal pathogens, still the most common and effective management strategy is chemical control. However, broad use of chemicals for decades has resulted in ecological and human health problems, as well as pesticide resistance (El-Bakry et al. 2015). Because of global public concern, the need to find alternative strategies to chemical management has been promoted. Microbial biocontrol agents are one of the most promising control tools due to their many advantages, including safety for humans,

animals, and the environment, relative host-specificity, and suitability for mass production.

There are several studies which have tested biocontrol agents against insect and fungal pests (e.g., Moino et al. 1998, Zucchi et al. 2008, Kavallieratos et al. 2014, Shi et al.2014, Palazzini et al. 2018, Mannaa and Kim 2018). However, these studies have tested the individual effectiveness of the biocontrol agents against insect or fungal pests. Testing the dual effect of candidate biocontrol agents against these two groups has additional importance because they share the same ecological niche and they both cause damage in stored grain. Additionally, *Sitophilus* spp. can promote the development of fungal pests in stored grains as well. For example, *S. granarius* promotes infestation of wheat grains by *Aspergillus restrictus* (Agrawal et al. 1957). Another example is that *S. zeamais* may serve as a vector of many species of *Aspergillus*, *Penicillium*, and *Fusarium* (Mason and McDonough 2012). Moreover, insect activity in grain can cause increased temperature and moisture accumulation, which promotes favorable conditions for fungal growth and mycotoxin production (Chulze 2010, Mohapatra et al. 2017).

There are no effective chemical pesticides that are registered against both granary weevil and fungal grain pathogen pests. Therefore, control of both insect pests and fungal pathogens with the same biocontrol agents is significantly important in terms of providing effective control and economic benefit by reducing number of applications of grain protectants. Although there are several reports of a dual effect of a biocontrol agent against insect and fungal pests (e.g. Kim et al. 2001, Keyser et al. 2016), there are no studies looking specifically at one of the most important grain insect pests, *Sitophilus granarius*, and three common grain fungal pathogens, *Fusarium graminearum*,

Aspergillus parasiticus, and *Penicillium chrysogenum*, in the same study, which makes this research unique.

The primary aim of the research described in this thesis is to test the hypothesis that individual microorganism strains that have the ability to inhibit both granary weevil and grain fungal pathogens can be found. Towards this end, a selection of fungi and bacterial strains, from genera with demonstrated activity against insect and/or fungal pests, were investigated. Therefore, the specific objectives of this study are to:

1. Determine the lethal (mortality) and sublethal (grain damage and oviposition) effects of strains of bacteria and fungi as entomopathogens of granary weevil, compared to commercial biological and chemical treatments.
2. Determine the ability of strains of bacteria and fungi to inhibit growth of three common grain fungal pathogens under *in vitro* and wheat seed conditions.

This research is the first stage or "proof of concept" in evaluating whether these microorganisms have an effect on insect and fungal pests. If these biocontrol agents are effective on the targeted pests, future studies should be done to determine the mode of action of biocontrol agents and examine results under realistic storage facility conditions.

Table 1.1. Environmental conditions for pests and biocontrol agents.

Category	Organism	Preferred Abiotic Conditions			References
		Temperature (°C)	Relative Humidity (%)	Water Activity (A_w)	
Insect Pest	*Sitophilus granarius*	25–30	65–70	[a]	Hansen and Steenberg 2007, Athanassiou et al. 2017
Grain Fungal Pathogens	*Fusarium graminearum*	25–28	>90	0.90	Miller 2008, Cheli et al. 2013
	Aspergillus parasiticus	28–30	70–90	0.84–0.87	Bothast 1978, Herceg et al. 2015, Cheli et al. 2017
	Penicillium chrysogenum	25–30	70–90	0.80	Reiss 1977, Bothast 1978, Herceg et al. 2015
Biocontrol Fungi	*Beauveria bassiana*	23–28	60–90	0.90	Zimmerman 2007
	Metarhizium anisopliae	25–30	86–100	0.97–0.99	Zimmerman 2007
	Cladosporium sp.	24–25	>88	0.80–0.91	Aihara et al.2002, Mason and Strait 2012
	Trichoderma sp.	25–30	[c]	[c]	Mason and Strait 2012
Biocontrol Bacteria	*Bacillus thuringiensis*	10–45[b]	[c]	[c]	Logan and De Vos 2015
	Lysinibacillus sphaericus	10–45[b]	[c]	[c]	Logan and De Vos 2015
	Burkholderia sp.	30–35 [b]	[c]	[c]	Govan et al. 1996
	Lysobacter enzymogenes	[c]	[c]	[c]	-
	Bacillus amyloliquefaciens	30-40[b]	[c]	[c]	Logan and De Vos 2015

[a]Water activity is not commonly measured for insects.
[b]General range for survival of this species; preferred conditions were not found in the literature.
[c]Conditions were not found in the literature.

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CHAPTER 2: EVALUATION OF POTENTIAL BIOCONTROL AGENTS TO DETERMINE LETHAL AND SUBLETHAL EFFECTS AGAINST GRANARY WEEVIL

Introduction

The granary weevil (*Sitophilus granarius* L., Coleoptera: Curculionidae) is one of the most important pests of stored grain (Gaino and Fava 1995, Kljajić and Perić 2006, Piasecka-Kwiatkowska et al. 2014). The granary weevil is a cosmopolitan pest, but particularly, it causes economic damage in the temperate zone (Campbell et al. 2004). Granary weevils are able to feed on both unbroken and broken grains, including wheat, rice, barley, buckwheat, corn, oats, and rye (Campbell et al. 2004). They are a primary pest and they complete their early life stage in the grain. The weevils cause economic damage by causing reduction of the grain quality and quantitative losses on the stored grain product. Damage is caused by both the adult and larval stages of the insect, but larvae are the most destructive. Each larva can devastate more than 60% of a wheat kernel (Hurlock 1965). Larvae excavate a tunnel inside of the grain kernel and complete their early life stage in the same grain (Stephensons 1983).

In addition to feeding damage, adult females contaminate the grain by laying eggs. The female bores a hole in the grain and deposit eggs inside of the grain before closing the hole with a gelatinous substance, which is called the egg plug (Szewczuk et al. 2010). Females may oviposit 50 to 250 eggs, but average oviposition is 200 eggs (Mason and McDonough 2012). Thus, the female causes infestation of the grain with larvae by oviposition. In addition, granary weevils have a relationship with important fungal grain pests. For example, *S. granarius* promotes infestation of the wheat grains by *Aspergillus restrictus* (Agrawal et al. 1957). Furthermore, *Sitophilus zeamais*, another

important *Sitophilus* species, can serve as a vector of many species of *Aspergillus*, *Penicillium*, and *Fusarium* (Mason and McDonough 2012).

Currently, the most commonly used management strategies are based on conventional pesticides and cultural methods. Cultural methods, such as drying of grain, controlling heat and moisture, and modifying atmosphere are considered safe and environmentally friendly methods compared to the use of conventional chemical insecticides (Navarro 2006). Such cultural methods, unfortunately, may not be cost-effective or practical in all storage facilities (Phillips and Throne 2010). For chemical control, organophosphate, pyrethroid, and carbamate insecticides are used as grain protectants (Arthur 1996). Some of the recommended insecticides used as residual surface treatments and registered by the United States Environmental Protection Agency are: cyfluthrin (a pyrethroid; Zettler and Arthur 2000), chlorpyrifos-methyl (an organophosphate; Fang et al. 2002), and deltamethrin (a pyrethroid), which can be combined with chlorpyrifos-methyl (Mason and Obermeyer 2010). However, application of these insecticides has many drawbacks, such as toxicity to mammals, leaving residue on the product, and insecticide resistance (Arthur 1996). Using phosphine gas is another common method against pests of stored products. However, due to frequent use worldwide, there are many reports of phosphine resistance in *Sitophilus* spp. (e.g., Monro et al. 1972, Alam et al. 1999). Consequently, there is a need for alternatives to conventional pesticides due to their side effects, insecticide resistance, and consumer demand for food, which must be free of insect pests and insecticide residues. Biological control agents are strong candidates for alternative management strategies to chemical pesticides and are compatible with many cultural controls, due to their unique

features, including safety for mammalian health and the environment, cost effectiveness, suitability for mass production, and low non-target effects. These characteristics make them a desirable option and important component of Integrated Pest Management strategies. Currently, there is no specifically registered biocontrol agent against *Sitophilus* spp. However, some commercial bioinsecticides, which are produced from entomopathogenic fungus, can be used against weevils. For example, Met 52 (*Metarhizium anisopliae*) and Botanigard (*Beauveria bassiana*) are registered against weevils that cause damage to ornamental plants, including vine weevil, strawberry root weevil, rose curculio and black vine weevils. However, these commercial products are not specifically registered against granary weevils. There are also commercial products derived from entomopathogenic bacteria that can be used against pests that cause foliar plant damage. For example, Dipel (*Bacillus thuringiensis* subsp. *kurstaki*) can be used against lepidopteran larvae and Novodor (*Bacillus thuringiensis* subsp. *tenebrionis*) is effective against foliar coleopteran pests, but neither of these are registered against granary weevils.

There are many studies conducted investigating the potential efficacy of biocontrol agents against stored product pests (e.g. Mummigatti et al.1994, Hansen and Steenberg 2007, Silva et al. 2010, Kavallieratos et al. 2014). However, while these studies assessed the effect of the agents on viability of the target pests, none of these studies have reported sublethal effects (such as reduced feeding and oviposition) of biocontrol agents on stored product pests. Most of these studies are also conducted only with fungal isolates (e.g., Hansen and Steenberg 2007, Kavallieratos et al. 2014). Therefore, the aim of this study was to characterize candidate bacterial and fungal strains

for potential as biocontrol agents by determining the lethal and sublethal effects against *Sitophilus granarius*.

Materials and Methods

Sources and Preparation of Organisms

Grain

Untreated, freshly-harvested grain of winter wheat (WestBred, St. Louis, MO) grown at the University of Nebraska-Lincoln (UNL) West Central Research & Extension Center's Dryland Farm in North Platte, NE (GPS: 41.058331°, -100.752677°) was used for this study. Before starting to experiment, the moisture content was measured by using a bench grain moisture tester (GAC® 2100 Agri, Dickey-John, Auburn, IL). Distilled water was added to reach 13.5% moisture content, the optimum for *S. granarius*. Grains were placed in a sealed plastic container (77 mm × 77 mm × 97 mm, Magenta Corp, Lockport, IL) and stored at 4 °C to prevent loss of grain moisture.

Insects

A colony of *Sitophilus granarius* originating from the USDA-ARS Center for Grain and Animal Health Research (Manhattan, KS) was reared on whole winter wheat (WestBred, St. Louis, MO) in an incubator at 25 ± 2 °C and 62 ± 3% RH with 16:8 (L:D) photoperiod. Colony rearing protocols followed Toews et al. (2006). Adult *S. granarius* (within 14 days from emergence) from this colony were used in the bioassays

Candidate Bacteria

Five bacterial strains belonging to four genera were chosen for testing based on past reports of entomopathogenic or antifungal efficacy (Parikh et al. 2018), ability to grow under laboratory conditions, and representation of taxonomic diversity (Table 2.1).

Burkholderia ambifaria strain C628, *Bacillus thuringiensis* strain C423, and *Bacillus amyloliquefaciens* strain C415 were collected from the roots of wheat plants from commercial wheat fields in Lincoln County (Nebraska, USA); *Lysinibacillus sphaericus* strain W341 was collected under the same conditions in Keith County (Nebraska, USA; Parikh et al. 2018). These strains were isolated and maintained in the laboratory of Dr. Tony Adesemoye, West Central Research & Extension Center, University of Nebraska-Lincoln, North Platte, NE. *Lysobacter enzymogenes* strain C3R5 was obtained from the foliage of Kentucky bluegrass grown in Nebraska (Giesler and Yuen 1998). Bacterial strains were grown on tryptic soy agar (TSA, (Difco Laboratories Inc., Franklin Lakes, NJ). Cells of bacterial strains from a 48 hr old TSA plate were used to inoculate 25 ml tryptic soy broth (TSB; 30 g/liter) contained in a 50 ml centrifuge tube and incubated for 48 hr on a rotary shaker at 150 RPM and 28±1°C to obtain broth cultures. Bacterial suspensions were prepared by centrifuging at 3500 RPM for 15 min, and suspending the pellets in sterile water. Cell concentrations were adjusted to $1x10^8$ CFU/ml using a spectrophotometer.

Candidate Fungi

Ten fungal strains belonging to four genera were chosen for testing based on past reports of entomopathogenic or antifungal efficacy (Oliveira Hofman 2018), ability to grow under laboratory conditions, and representation of taxonomic diversity tested (Table 2.2). All fungi were collected from commercial, continuous cornfields in Keith and Perkins County (Nebraska, USA; Oliveira Hofman 2018). *Metarhizium* spp. and *Beauveria bassiana* were cultured on Sabouraud dextrose agar which provide repeat cultured, and the isolates of *Cladosporium* spp. and *Trichoderma gamsii* were grown on

potato dextrose agar at 39 g/liter (Difco Laboratories Inc., Franklin Lakes, NJ) amended with 0.01% tetracycline (Fisher Bioreagents, Leicestershire, UK) . Fungal spores from 14-day-old cultures were harvested by scraping the surface of Petri dishes with a sterile scalpel into 10 ml sterilized water containing 0.1% Tween 80 (Sigma-Aldrich®, St. Louis, MO). The conidial suspension was mixed using a benchtop homogenizer (Vortex, Bohemia, New York) and filtered through a rayon-polyester filtration cloth (22–25 µl pore size, Miracloth, MilliporeSigma, Burlington, MA). A hemocytometer was used to determine the concentration of conidia and the spore concentration was adjusted to $1x10^6$ cells/ml.

Commercial Products

Although there are no commercial biocontrol agents specifically registered for *S. granarius*, we chose two commonly used commercial bioinsecticides as a comparison. Commercial bioinsecticides were prepared with distilled water according to their labelled dose. BotaniGard® including *Beauveria bassiana* strain GHA (Arbico Organics, Oro Valley, AZ) was prepared at a concentration of 2.4mg/mL. Met52® containing *Metarhizium anisopliae* strain F52 (Novozymes Biologicals Inc, Catawba, VA) was prepared at a concentration of 6.22 µl/mL. DiPel® containing *Bacillus thuringiensis* subspecies *kurstaki* (Valent BioSciences Corporation, Libertyville, IL) was prepared at a concentration of 62.5mg/mL. The chemical insecticide Delta Gold® (active ingredient deltamethrin; Winfield United, Arden Hills, MN) was prepared at a concentration of 2.4 µl/mL and used to provide a comparison with a commercial insecticide.

Weevil Bioassays

Two separate experiments were conducted to assess the effects of 1) the five potential biocontrol bacterial strains, commercial bacterial bioinsecticide, insecticide positive control, and water-only negative control; and 2) the ten potential biocontrol fungal strains, commercial fungal bioinsecticides, commercial synthetic insecticide positive control, and water-only negative control on *S. granarius* survival, feeding damage, and oviposition rate (Table 2.1).

All treatments were applied to grain using plastic fingertip spray bottles (59 mL) that had been sterilized with 10% bleach solution and rinsed with tap water and then distilled water. Each experimental unit consisted of a 100 x 150 mm Petri dish containing 10 g moisturized winter wheat grain, replicated three times for each treatment. Wheat was sprayed with 1 mL application suspension and was dried under a laminar flow hood. Moisture level of the wheat started at 13.5% prior to application and may have reached as high as 23.5% due application of 1 mL of treatment solution. After drying, 10 adults of *S. granarius* were placed with the treated wheat in each Petri dish and covered with Parafilm to prevent escape of weevils. Petri dishes were kept in an incubator with photoperiod of 16:8 (L:D) at 25 ± 2 °C and 62 ± 3% relative humidity for the experimental period. Each of the two experiments was repeated three times between September 2018 and January 2019. In each experiment, weevil survival, feeding damage and oviposition rate were assessed as follows:

Survival

Survival was evaluated daily for the first 7 days, and then every other day until 28 days after inoculation. At each observation, weevils were touched using forceps and if the insect did not move, it was recorded as dead. To assess the growth of fungal mycelium on

the insects, which would indicate insect mortality caused by the biocontrol agents, all dead granary weevils were removed from the Petri dishes and placed in new Petri dishes with filter paper moistened with distilled water, incubated at 25 °C, and evaluated daily for up to 14 days under a stereomicroscope to observe fungal growth on the insect cadaver.

Feeding Damage

Feeding damage was assessed 7, 14, 21, and 28 days after inoculation. The evaluation was conducted using a 0-6 point rating scale based on the percentage of wheat grains that were damaged by weevil feeding: 0 (0%), 1 (1-10%), 2 (11-25%), 3 (26-50%), 4 (51-75%), 5 (76-94%), and 6 (95-100%).

Oviposition

At the conclusion of the experiment (28 days after entomopathogen application), 25 grains of wheat were randomly selected from each Petri dish to quantify oviposition. The presence of egg plugs was determined using an acid fuchsin stain (Frankenfeld 1948) with methods modified from Sharifi (1972). The grains were soaked in warm tap water (approximately 25 to 30 °C) for 30 seconds, immersed in acid fuchsin solution for 60 seconds, rinsed with tap water for 30 seconds to remove the acid fuchsin solution on the grain, and dried at room temperature on a paper towel. Each wheat grain was examined under a dissecting microscope to quantify egg plugs, which appeared as bright or cherry red (Figure 2.10).

Data Analysis

The data from bacterial and fungal biocontrol agent experiments were analyzed separately. All analyses were conducted using SAS version 9.4 (SAS Institute, Cary,

NC). Survival data were analyzed using the PROC PHREG procedure to fit the data to a Cox proportional hazards model with treatment and trial as fixed effects. This analysis method is designed for survival data that is not normally distributed, and is therefore preferred over least squares means regression or nonparametric methods. Means were considered significantly different at $\alpha = 0.05$ if the 95% confidence interval of their hazard ratio does not include 1.0.

Feeding damage data were analyzed using a linear mixed model (PROC GLIMMIX) with a Beta distribution (appropriate for discrete proportion data) with treatment and trial as fixed effects. Least Squares Means analysis was used for means comparisons and determined to be significantly different if the Tukey adjusted p-value was less than 0.05.

The mean number of egg plugs per wheat grain were analyzed using a linear mixed model (PROC GLIMMIX) assuming a normal distribution with trial as a random effect and treatment as a fixed effect. The proportion of grain infested with any eggs was analyzed using a generalized linear mixed model (PROC GLIMMIX) assuming a binomial distribution (appropriate for continuous proportion data), the logit link function, and Laplace's method for estimation (to minimize the –log likelihood function) with trial as a random effect and treatment as a fixed effect. For these analyses, means comparisons were conducted by Least Squares Means and determined to be significantly different if the Tukey adjusted p-value was less than 0.05.

Results

Weevil Survival: Bacteria Experiment

The survival analysis indicated that there were significant simple effects of trial ($df = 2$, $p = 0.0007$) and treatment ($df = 5$, $p < 0.0001$) with no significant interaction between trial and treatment ($df = 10$, $p = 0.8596$). Overall survival was higher in Trial 1 compared to Trials 2 and 3, with no significant difference between Trial 2 and Trial 3 (Figure 2.1). Among the treatments tested, only Delta Gold (commercial insecticide positive control) caused all weevils to die (0% survival) one day after inoculation (Figure 2.2). Survival of *S. granarius* adults was high in all other treatments for the first 14 days after inoculation. All treatments had significantly reduced survival compared to the negative control. For the entomopathogens tested, the lowest survival was observed for *L. sphaericus* W341. This isolate and *B. amyloliquefaciens* C415 were the only bacterial agents to cause significantly lower survival than Dipel, the commercial biocontrol comparison (Figure 2.2).

Feeding Damage: Bacteria Experiment

At 7 and 14 days after inoculation, feeding damage was consistently very low (rating of 0.5) across all treatments; therefore, statistical analyses were conducted on results for 21 and 28 days after inoculation only.

The 21 days after inoculation analysis indicated that there were significant main effects of treatment ($df = 6$, $p < 0.0001$) and trial ($df = 2$, $p < 0.0001$), as well as a significant effect of the interaction between trial and treatment ($df = 12$, $p < 0.0001$). Overall, Trial 1 had less damage than Trials 2 and 3, with no significant differences between Trials 2 and 3. Two treatments showed a difference in performance based on

Trial: *L. enzymogenes* C3R5 had less feeding damage in Trial 1 compared to Trials 2 and 3 ($df = 42$, $p = 0.0003$) and Dipel had less feeding damage in Trial 1 compared to Trials 2 and 3 ($df = 42$, $p = 0.0001$). In Trial 1, deltamethrin, *L. enzymogenes* C3R5, and Dipel all reduced feeding damage significantly lower than the negative control; however, only deltamethrin did so in Trials 2 and 3 (Table 2.3).

The 28 days after inoculation analysis indicated that there were significant simple effects of treatment ($df = 6$, $p < 0.0001$) and trial ($df = 2$, $p < 0.0025$), as well as a significant effect of the interaction between trial and treatment ($df = 12$, $p < 0.0001$). In Trials 1 and 2, the negative control had significantly higher feeding damage than all other treatments, but this was not true in Trial 3. Deltamethrin had significantly lower feeding damage than all other treatments in all trials. The only candidate biocontrol agents with lower feeding damage than the commercial biocontrol comparison (Dipel) were *L. sphaericus* W341 and *L. enzymogenes* C3R5 in Trial 2 (Table 2.3).

Oviposition: Bacteria Experiment

The analysis of mean number of egg plugs per wheat grain showed a significant effect of treatment only ($df = 6$, $p < 0.001$). All tested bacterial isolates were significantly different from the negative control, except for *B. ambifaria* C628 (Figure 2.5). The candidate bacterial biocontrol agents *B. amyloliquefaciens* C415, *L. sphaericus* W341, and *B. thuringiensis* C423 reduced oviposition as well as the commercial bioinsecticide Dipel (*Bacillus thuringiensis* sub. *kurstaki*). None were as effective as the chemical insecticide Delta Gold (deltamethrin), which reduced the oviposition rate to zero (Figure 2.5).

The analysis of the proportion of wheat grains with one or more egg plugs showed a significant effect of treatment only ($df = 6$, $p < 0.001$). The chemical insecticide Delta Gold (deltamethrin) reduced the proportion infested to zero (Figure 2.6). Four treatments reduced the proportion of infested grains significantly below the negative control: *B. amyloliquefaciens* C415, *L. sphaericus* W341, and *B. thuringiensis* C423, as well as the commercial bioinsecticide Dipel (Figure 2.6).

Weevil Survival: Fungi Experiment

The survival analysis indicated that there was a significant simple effect of treatment ($df = 11$, $p < 0.0001$) and significant interaction between trial and treatment ($df = 22$, $p = 0.0006$), but no significant simple effect of trial ($df = 2$, $p = 0.2303$). There was a significant difference in survival based on trial for the treatments Botanigard, *B. bassiana* E1040, *Cladosporium* sp. E1060, and *M. robertsii* E652 (Figure 2.3). In all trials, deltamethrin achieved 0% survival one day after inoculation, whereas survival of adults was high on day one is all other treatments. The commercial comparison Met 52 achieved 0% survival within 7-8 days in all trials and was significantly different from all other treatments. All other treatments had high survival until approximately14 days after inoculation. The candidate biocontrol agents with the lowest survival were *Metarhizium* sp. E369, *M. robertsii* E652, *T. gamsii* E1064, and *B. bassiana* E1040. Two candidate BCAs were not significantly different from the negative control: *M. anisopliae* E213 and *C. halotolerans* E126 (Figure 2.3).

Growth of fungal mycelia was confirmed on the cadavers of dead weevils from the fungal isolates *Beauveria bassiana* (E1040 and E1041), *Trichoderma gamsii* (E1032

and 1064), *Metarhizium robertsii* (E1056 and E652), *Metarhizium anisopliae* E213, *Metarhizium* sp. E369 and *Cladosporium* sp. 1060 (Figure 2.4).

Feeding Damage: Fungi Experiment

At 7 and 14 days after inoculation, feeding damage was consistently very low across all treatments (rating of 0.50 or lower); therefore, statistical analyses were conducted on results for 21 and 28 days after inoculation only.

The 21 days after inoculation analysis indicated that there were significant simple effects of treatment ($df = 12$, $p < 0.0001$) and the interaction between trial and treatment ($df = 24$, $p < 0.0001$), but the simple effect of trial was not significant ($df = 2$, $p < 0.8049$). Five treatments showed a difference in performance based on Trial: Botanigard, *B. bassiana* E1041, *C. halotolerans* E126, *M. anisopliae* E213, and *M. robertsii* E652. In each of these cases, Trial 2 was significantly different than Trials 1 and 3, but whether feeding damage was higher or lower in Trial 2 was not consistent. Deltamethrin had the lowest feeding damage in all trials and was significantly different from all other treatments. Additional treatments that were significantly lower than the negative control in all trials were the commercial biocontrol products Botanigard and Met52, as well as candidate biocontrol agents *M. robertsii* E652, *Metarhizium* sp. E369, and *T. gamsii* E1064.

The 28 days after inoculation analysis indicated that there was a significant simple effect of treatment ($df = 12$, $p < 0.0001$) and the interaction between trial and treatment ($df = 24$, $p = 0.0041$), but the simple effect of trial was not significant ($df = 2$, $p = 0.7068$). Two treatments showed a difference in performance based on Trial: *Metarhizium* sp. E369 had higher feeding damage in Trial 2 compared to Trials 1 and 3

and *M. robertsii* E652 had higher feeding damage in Trial 3 compared to Trials 1 and 2 (Table 2.4). Deltamethrin had the lowest feeding damage in all trials and was significantly different from all other treatments. The only other treatments that were significantly lower than the negative control in all trials were the commercial biocontrol product Met52 and the candidate biocontrol agent *T. gamsii* E1064.

Oviposition: Fungi Experiment

The analysis of mean number of egg plugs per wheat grain showed a significant effect of treatment only ($df = 12$, $p < 0.0001$). All tested fungal isolates had significantly lower mean egg plugs per grain compared to the negative control (Figure 2.7). The most effective isolate was *Metarhizium* sp. E369, which reduced the oviposition rate better than Botanigard. The chemical insecticide Delta Gold (deltamethrin) reduced the oviposition rate to zero (Figure 2.7).

The analysis of the proportion of wheat grains with one or more egg plugs showed a significant effect of treatment only ($df = 12$, $p < 0.0001$). All tested fungal isolates had significantly lower mean proportion infested grains compared to the negative control (Figure 2.8). The most effective isolate was *Metarhizium* sp. E369, which reduced the proportion infested grains to less than the Botanigard treatment, and not different from the Met52 treatment.

Discussion

All of the microorganisms tested in this study affected granary weevil viability. The number of insects surviving was high for a two week period after treatment. The application method of the biocontrol agents plays a key role in the speed at which

mortality of the pest occurs, as well as the efficacy against the targeted pest (Kavallieratos et al. 2014). Direct application of biocontrol agents onto the pest is more effective than application on the grain in laboratory studies (Batta 2012, Kavallieratos et al. 2014). Although direct application proved more effective than application on the grain, it is not always possible to apply products directly to storage bin pests. The granary weevil is an internal feeder, which completes part of its life cycle inside of the seed and is therefore protected from direct application. Moreover, grain is generally stored for a long period of time, thus, application of biocontrol agents on the grain as a grain protectant should be a more reliable and applicable method in storage facilities. Additionally, the aim of this study is to find potential biocontrol agents to replace chemical insecticides used as grain protectants, which are commonly applied to grain rather than applied directly to the pest.

The mode of action of the biocontrol agents may also have played a role in the delayed mortality observed in this study. Entomopathogenic fungi must contact the host to be infective. The infection process of entomopathogenic fungi starts with penetration of the insect cuticle by using appressorium, which uses enzymatic and physical pressure to penetrate the host cuticle (Kaya and Vega 2012). When entomopathogenic fungi reach the inside of the insect body, they proliferate and feed on the host interior content. During this time, the fungus may impact the host by changing host behavior and feeding, reducing body weight and fertility, and causing other abnormalities (Zimmermann 2007). Eventually, the host is killed by disrupting key biological functions, which lead to nutritional deficiency and tissue devastation (Skinner et al. 2014). When the host dies, the entomopathogenic fungus begins its saprophytic phase and breaks out of the host body to

produce conidia on the surface of the cadaver (Zimmermann 2007). This infection process is highly dependent on interactions between the host and pathogen, although there is still much that is not known about the interaction among the pathogen and host immune system (Chandler 2016).

The modes of action of entomopathogenic bacteria also might contribute to a delay in insecticidal activity. For some entomopathogenic bacteria the mode of penetration is oral. The infection process is affected by factors such as pathogenicity of the biocontrol agent and host immune defense system. Entomopathogenic bacteria first move into the host body through the hemocoel and then propagate inside of the insect body. They cause disease by producing virulence factors, such as crystalline proteins, and eventually kill the host (Glare et al. 2017). Other entomopathogenic bacteria, referred to as 'antagonists', do not require ingestion by the target insect. Antagonists can affect insects by the excreting insecticidal enzymes or secondary metabolites into the environment. One bacterial species tested in this study, *L. enzymogenes* C3, was reported to produce chitinases and an antibiotic that have activity against nematodes (Chen et al., 2006; Yuen et al., 2018), and it is possible these mechanisms may affect insects as well.

Additionally, insect cuticle structure is another important factor that affects speed of infection. The insect cuticle is the first point of contact and barrier between pathogens and their host (Ortiz-Urquiza et al. 2013). The cuticle arises from a thin deposition that consists of cement and waxy layers, which consist of lipids and other compounds. Cuticular lipids and waxes show significant variations between different insect species and life stages. Moreover, cuticular lipids are able to stimulate or inhibit fungal attachment to the insect cuticle. For example, cuticle lipids and aldehydes of the southern

stink bug (*Nezara viridula* L.) show fungistattic impact against *M. anisopliae* (Sosa-Gomez et al. 1997) and cuticular extracts of the corn earworm *Helicoverpa zea* demonstrate toxicity to *B. bassiana* (Smith and Grula 1982). However, there are no studies on the specific interactions between granary weevil cuticle structure and entomopathogen biological control agents.

Another factor that could have affected the mortality results is the life stage of the insect pest. The most susceptible stage of *S. granarius* to entomopathogenic bacteria and fungi is not determined. However, the larval stage was determined to be the most susceptible stage to phosphine (Howe 1973). The same result could be true for entomopathogens as well, due to the lack of protective cuticle in the larval stage. However, *Sitophilus* species larvae are typically protected within the grain kernel, therefore the larva is not the life stage that is targeted for control.

In addition to the mortality data, it is important to determine the sublethal effect of biocontrol agents on the granary weevil as well. In this study, sublethal effects, including reduction of feeding damage and oviposition rate was determined. Results of this study indicated that biocontrol agents are not able to perform as quickly as the chemical insecticide tested. Infection and death of the pest by a biocontrol agent is a relatively long process when compared to the quick knockdown effect of chemical insecticides (Skinner et al. 2014). Moreover, during this period, the pest may continue to feed and damage the stored grain. Therefore, determining the sublethal effect of the tested biocontrol agents has crucial importance to provide high-level control of the pest. In this present study, high mortality was achieved after 20 day and according to the pest biology this term is highly destructive in terms of insect biology. Moreover, *S. granarius*

damage results from both adult and larval stage and larva live inside of the grain. Also, it is important because weevils not only cause quantitative yield losses but also can cause qualitative yield losses. Adult females bore into the grain for feeding or for oviposition, which can damage the germ (embryo) part of the grain, affecting grain germination. Larvae feed mostly on the endosperm and fill the inside of the grain with frass. Therefore, ability of the biological control agent to suppress feeding damage and oviposition were tested.

This study showed that feeding damage was low for the first 15 days after application. Differences in the results were not observed until 21 days after inoculation, after this time damage caused by the pest increased. This result was similar to a previous study, where differences in damage was not observed until 20–30 days after infestation (Piasecka-Kwiatkowska et al. 2014).

Total feeding damage end of the experiment was reduced by *Lysinibacillus sphaericus* W341, *Lysobacter enzymogenes* C3R5 and *Bacillus thuringiensis* C423. Especially, *Lysinibacillus sphaericus* W341, *Lysobacter enzymogenes* C3R5 and Dipel showed more antifeedant effect. For the fungal treatments, *Trichoderma gamsii* E1064, *Metarhizium* sp. E369, *Beauveria bassiana* E1041, *Metarhizium robertsii* E652 and Botanigard can reduce feeding damage. During the 28 day evaluation period of this study, granary weevil feeding damage was not high. Thus, isolate showing promise of effectiveness from this study can be tested for a longer period to evaluate the potential antifeedant effect of the biocontrol agents over a longer time, such as the typical storage period of grains.

Many of the candidate biocontrol agents tested achieved reduction of the oviposition rate of *S. granarius.* Another important result from this study was that some isolates were also capable of reducing the proportion of grains infested. Granary weevils complete their life cycle inside of the grain, thus detection of their hidden infestation in grain is difficult (Piasecka-Kwiatkowska et al. 2014). Therefore, preventing oviposition plays an important role in management of this internal feeder.

In this present study, among the tested fungal isolate, group of *Metarhizium* strains and *Beauveria bassiana* strains have shown significantly preventive effect on oviposition rate against granary weevils. Especially, *Metarhizium* sp. E369 and *M. robertsii* E652 performed the same as Met52, which is a commercial bioinsecticide. Moreover, *M. anisopliae* E213 showed the same suppression as Met52. Furthermore, isolates of *B. bassiana* (E1040 and E1041) had same inhibition result on the oviposition rate as Botanigard, also a commercial bioinsecticide produced from *Beauveria bassiana.*

Also, tested *Bacillus* strains were capable reduction of the oviposition rate. The isolate most effective at reducing oviposition was *B. thuringiensis* C423. Although this isolate showed low insecticidal effect against *S. granarius*, it was able to reduce the oviposition rate, showing a sublethal effect of the isolate against *S. granarius* during the 28 days after inoculation. This result is important for pest management, since *S. granarius* reaches its highest oviposition rate between 10–30 days old (Howe and Hole 1967).

Overall, *Metarhizium anisopliae* E213 showed strong sublethal effect by reducing oviposition rate and grain infestation. Additionally, *Cladosporium halotolerans* E126 minimally reduced oviposition rate yet was significantly different from negative control.

Moreover, all tested bacterial treatments had significantly lower survival than the negative control.

Although fungal and bacterial isolates were not tested in the same bioassays, fungal isolates showed more promise than bacterial isolates. Even the commercial bacterial comparison Dipel (*Bacillus thuringiensis* subsp. *kurstaki*) did not achieve high mortality on the targeted pest. The subspecies of the *Bacillus* strain is important in determining their efficacy against a given pest. For example, *B. thuringiensis* subsp. *kurstaki* is most effective on lepidopteran larvae but is not very effective on coleopteran pests (Lacey et al. 2015). However, *B. thuringiensis* subsp. *tenebrionis* has promise for the control of coleopteran insect pests of stored wheat, such as *S. oryzae*, under *in vitro* conditions (Mummigatti et al.1994). Also, there is a report of resistance to *B. thuringiensis* by Indian meal moth larvae (McGaughey and Beeman 1988).

This bioassay was conducted under environmental conditions (temperature and relative humidity) based on *S. granarius* optimum life conditions. However, there are many abiotic factors (temperature, humidity, water content of grain) that can affect granary weevil. For instance, when the temperature increases, *S. granarius* completes their life cycle faster and consequently, oviposition rate increases (Eastham and McCully 1943). There are also many biotic factors (structure of grain, female age, availability of grain, population density) affecting oviposition rate (Niewiada et al. 2005). Such as, there is a positive correlation between grain availability and both female fecundity and grain infestation rate (Fava and Burlando1995). Also, feeding damage is highly dependent on the initial population of the pest (Campbell and Sinha 1976). In this study, *S. granarius* populations were established at a concentration of ten adult insects per ten grams of

grain, and this grain availability may affect the feeding damage and oviposition rate observed.

Furthermore, the pest strain is another important factor affecting the results, as research has shown that different strains of *S. granarius* have different oviposition rates (Longstaff 1981). In this bioassay, *S. granarius* strain and environmental conditions were kept the same for all treatments, so that the effect of environmental factors on the oviposition rate and feeding damage was equal across treatments. However, in a storage facility conditions are dynamic. Particularly if a grain mass has become significantly large, maintaining temperature and humidity levels is difficult in such a large storage area. These conditions may favor *S. granarius*, which promotes their feeding damage and oviposition ability.

Although there were promising results from tested biocontrol agents, using beneficial bacteria and fungi in stored grain has some limitations. One of the most important limitations is their slow effect when compared to chemical insecticides. In the present study, high mortality was not achieved until after 20 days, which is related to the biological interactions between the host and biocontrol agent. A combination of a grain desiccant with entomopathogenic fungus has shown to be more effective than application of biocontrol agents only (Athanassiou and Steenberg 2007). Therefore, these less effective biocontrol agents can be combined with kaolin or diatomaceous earth to increase their efficacy and kill the weevils faster.

Sitophilus spp. can coexist in the same commodity, especially, *S. granarius* and *S. oryzae* (Athanassiou et al. 2001). Therefore, application of biocontrol agents as grain protectants may not only help to control *S. granarius*, but also they may be helpful

against other *Sitophilus* species. Moreover, when both *S. granarius* and *S. oryzae* coexist, it leads to a higher oviposition rate. Since *S. oryzae* completes its life cycle in a shorter time period than *S. granarius* (Mason and McDonough 2012), it can reach the highest oviposition rate earliest. However, *S. granarius* continues to lay eggs for a longer period than *S. oryzae* (Longstaff, 1981). Therefore, coexistence of both *Sitophilus* species causes a higher pest population and consequently their damage will be greater.

Grain may be stored for a long time period. Residual effect of the biocontrol agent is significantly important to safely protect stored grains. Therefore, residual ability of the effective isolate should be tested on the grain as a grain protectant over a long-term period. Contrary to other *Sitophilus* species, *S. granarius* does not have wings and therefore must walk on the grain. An effective isolate could be formulated with oil to enhance its contact effect. Additionally, the efficacy of the biocontrol agents against granary weevil under different temperatures, humidity, and grain types, which play a key role in pest biology and consequently the performance of biocontrol agents.

Table 2.1. List of treatments for granary weevil bioassays with candidate bacteria.

Treatment Category	Description
Negative Control	Distilled water only
Positive Control	Delta Gold (deltamethrin) insecticide
Commercial Biological Insecticide	Dipel (*Bacillus thuringiensis*)
Candidate Biocontrol Agent	*Lysinibacillus sphaericus* W341
Candidate Biocontrol Agent	*Burkholderia ambifaria* C628
Candidate Biocontrol Agent	*Lysobacter enzymogenes* C3R5
Candidate Biocontrol Agent	*Bacillus thuringiensis* C423
Candidate Biocontrol Agent	*Bacillus amyloliquefaciens* C415

Table 2.2. List of treatments for granary weevil bioassays with candidate fungi.

Treatment Category	Description
Negative Control	Distilled water only
Positive Control	Delta Gold (deltamethrin) insecticide
Commercial Biological Insecticide	Met52 (*Metarhizium anisopliae*)
Commercial Biological Insecticide	Botanigard (*Beauveria bassiana*)
Candidate Biocontrol Agent	*Beauveria bassiana* E1040
Candidate Biocontrol Agent	*Beauveria bassiana* E1041
Candidate Biocontrol Agent	*Cladosporium halotolerans* E126
Candidate Biocontrol Agent	*Cladosporium* sp. E1060
Candidate Biocontrol Agent	*Metarhizium anisopliae* E213
Candidate Biocontrol Agent	*Metarhizium robertsii* E652
Candidate Biocontrol Agent	*Metarhizium* sp. E369
Candidate Biocontrol Agent	*Metarhizium robertsii* E1056
Candidate Biocontrol Agent	*Trichoderma gamsii* E1032
Candidate Biocontrol Agent	*Trichoderma gamsii* E1064

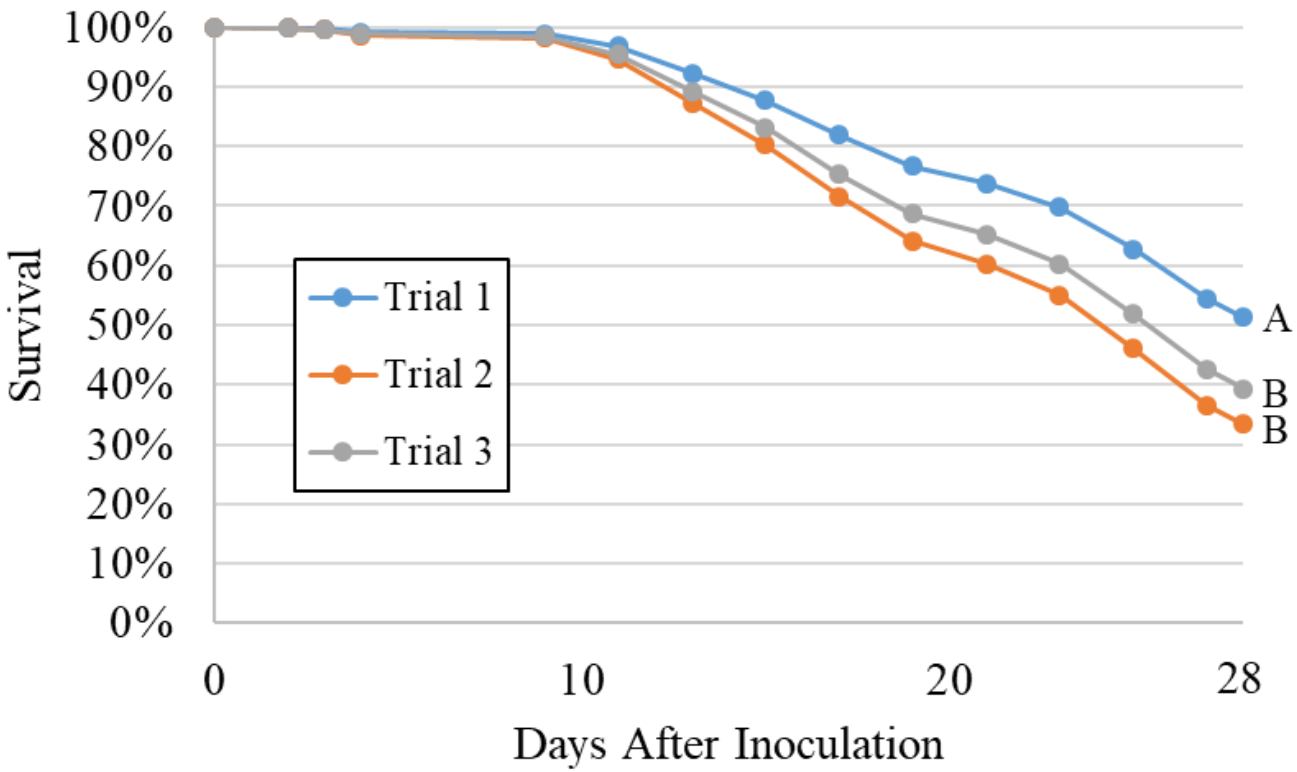

Figure 2.1. Survival of granary weevils over time for each bacterial entomopathogen trial. Letters indicate significant differences at $p < 0.05$.

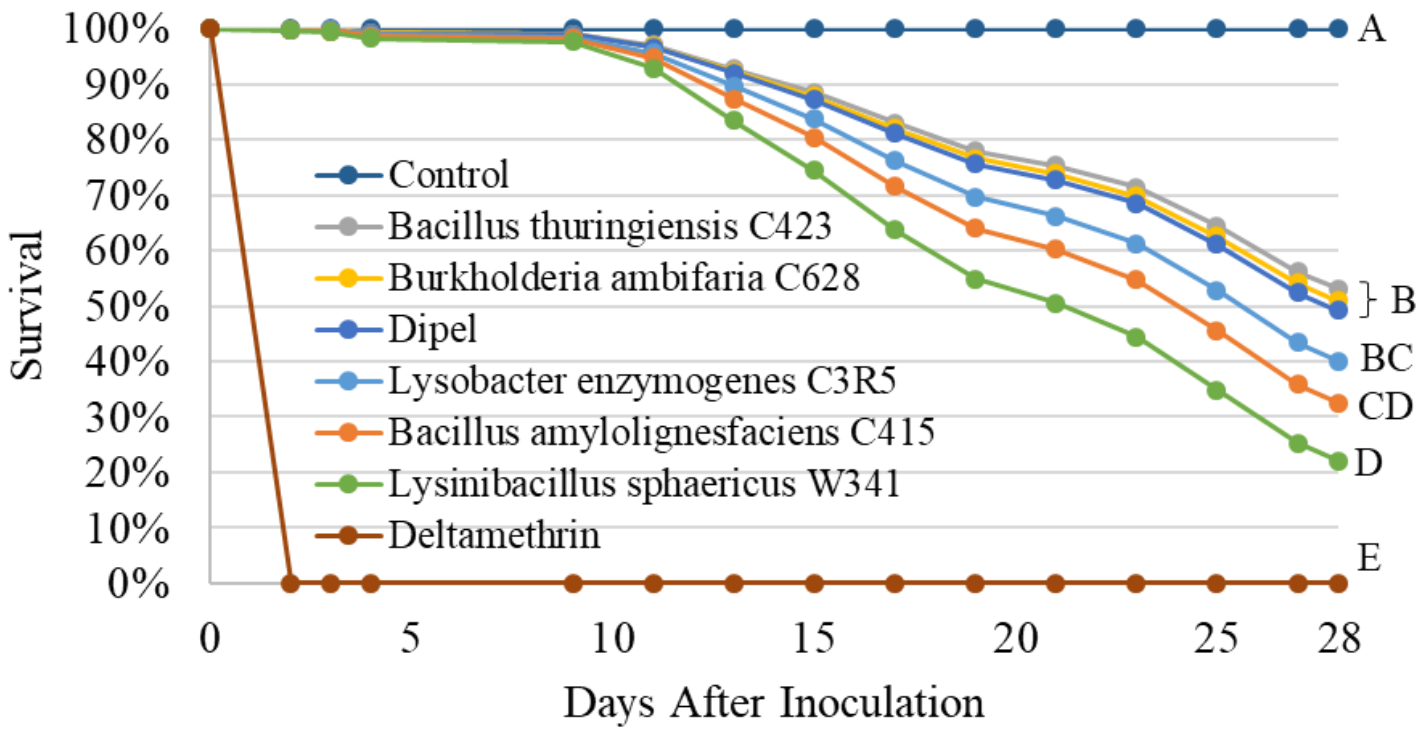

Figure 2.2. Survival of granary weevils over time for each bacterial entomopathogen treatment. Letters indicate significant differences at $p < 0.05$.

Table 2.3. Mean feeding damage of granary weevil (*Sitophilus granarius*) on wheat grains following treatment of entomopathogenic bacterial strains, commercial bioinsecticide and chemical insecticide. Means within a column with the same letter are not significantly different at $p \leq 0.05$.

	Mean (±SEM) feeding damage (0-6 scale)					
Days after Inoculation:	21			28		
Trial:	1	2	3	1	2	3
Treatment						
Negative Control	1.00 (±0) A	1.00 (±0) A	1.00 (±0) A	2.00 (±0) A	2.00 (±0) A	1.67 (±0.33) A
Bacillus amyloliquefaciens C415	1.00 (±0) A	1.00 (±0) A	1.00 (±0) A	1.50 (±0) B	1.50 (±0) B	1.33 (±0.17) ABC
Burkholderia ambifaria C628	1.00 (±0) A	1.00 (±0) A	1.00 (±0) A	1.00 (±0) C	1.50 (±0) B	1.50 (±0) AB
Bacillus thuringiensis C423	1.00 (±0) A	1.00 (±0) A	1.00 (±0) A	1.50 (±0) B	1.33 (±0.17) BC	1.17 (±0.17) BC
Dipel (*Bacillus thuringiensis*)	0.50 (±0) C	1.00 (±0) A	1.00 (±0) A	1.00 (±0) C	1.50 (±0) B	1.00 (±0) C
Lysinibacillus sphaericus W341	1.00 (±0) A	1.00 (±0) A	1.00 (±0) A	1.00 (±0) C	1.00 (±0) C	1.00 (±0) C
Lysobacter enzymogenes C3R5	0.83 (±0.17) B	1.00 (±0) A	1.00 (±0) A	1.17 (±0.17) BC	1.00 (±0) C	1.00 (±0) C
Delta Gold (deltamethrin)	0 (±0) D	0 (±0) B	0 (±0) B	0 (±0) D	0 (±0) D	0 (±0) D

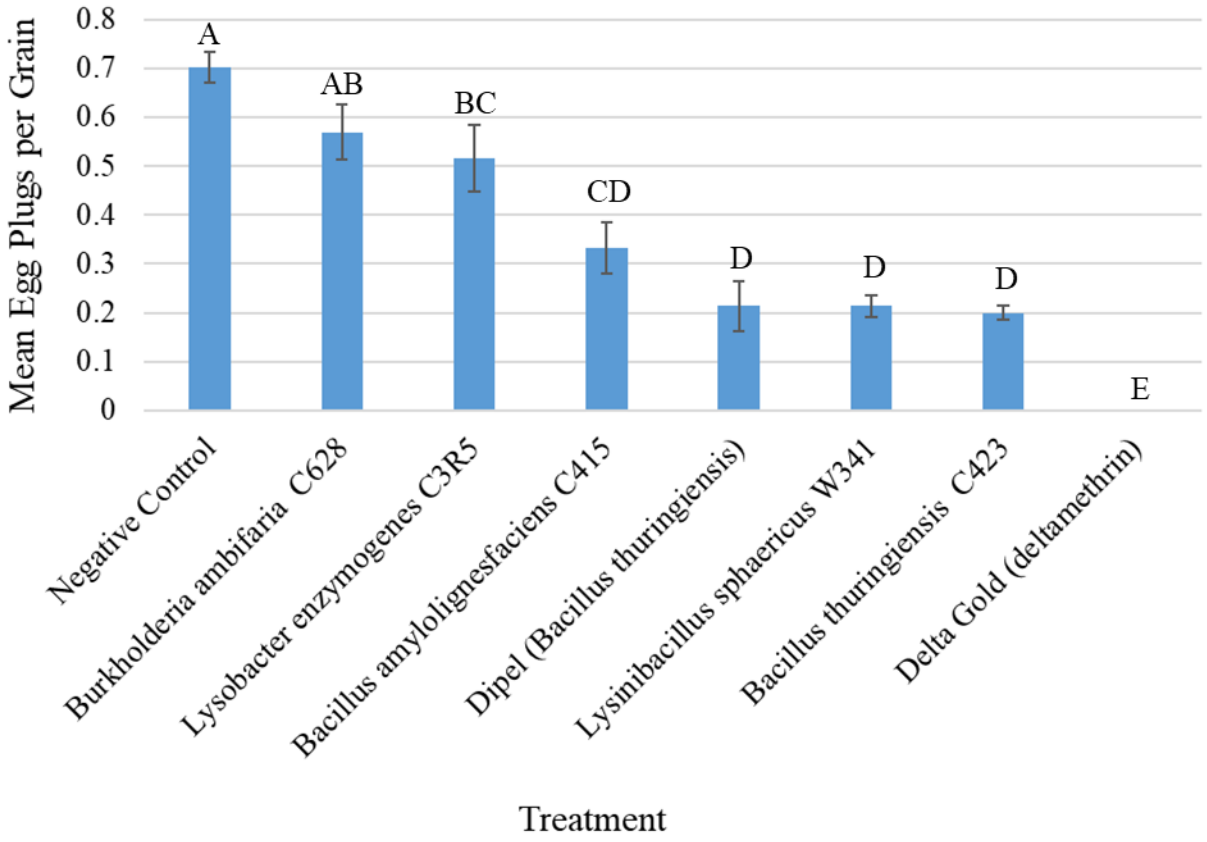

Figure 2.4. Mean oviposition of S. granarius adults for negative control, bacterial biocontrol agents, and commercial bioinsecticides, 28 days after inoculation. Letters indicate significant differences at $p < 0.05$. Errors bars show +/- standard error of the mean (SEM).

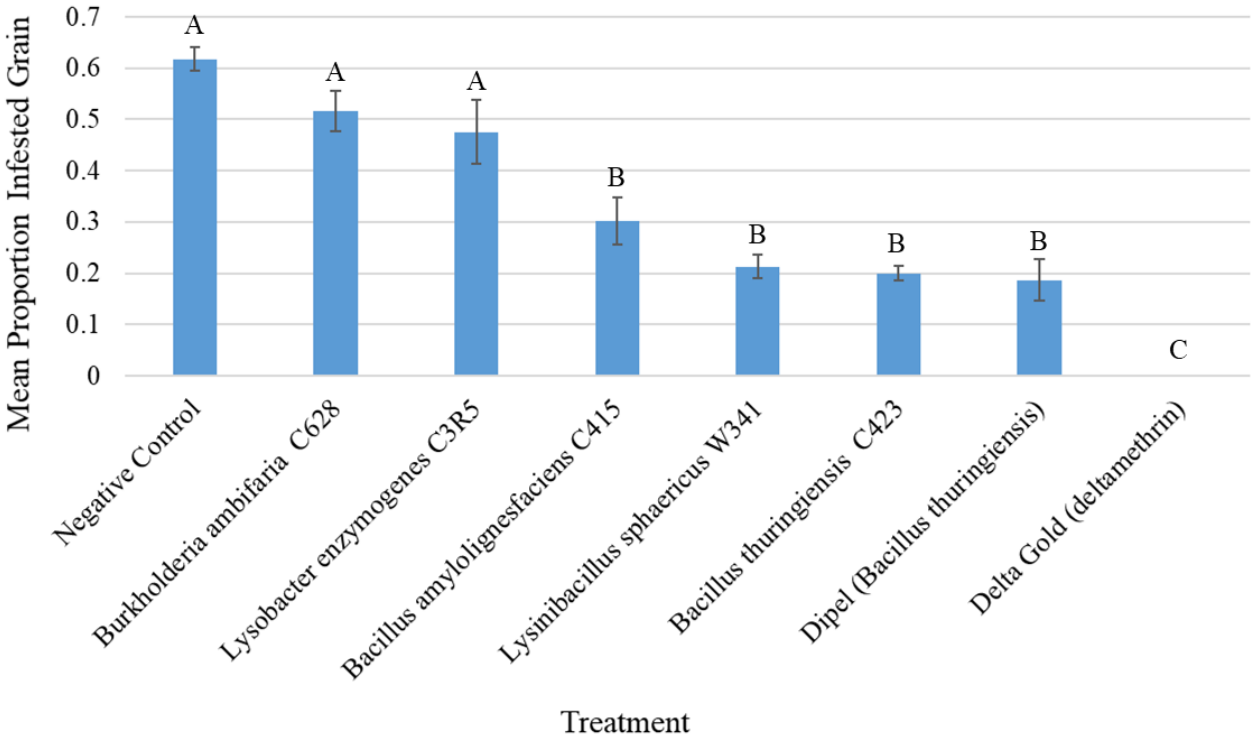

Figure 2.5. Mean proportion of grains infested with at least one egg plug for negative control, bacterial biocontrol agents, and commercial bioinsecticides, 28 days after inoculation. Letters indicate significant differences at $p < 0.05$. Errors bars show +/- standard error of the mean (SEM).

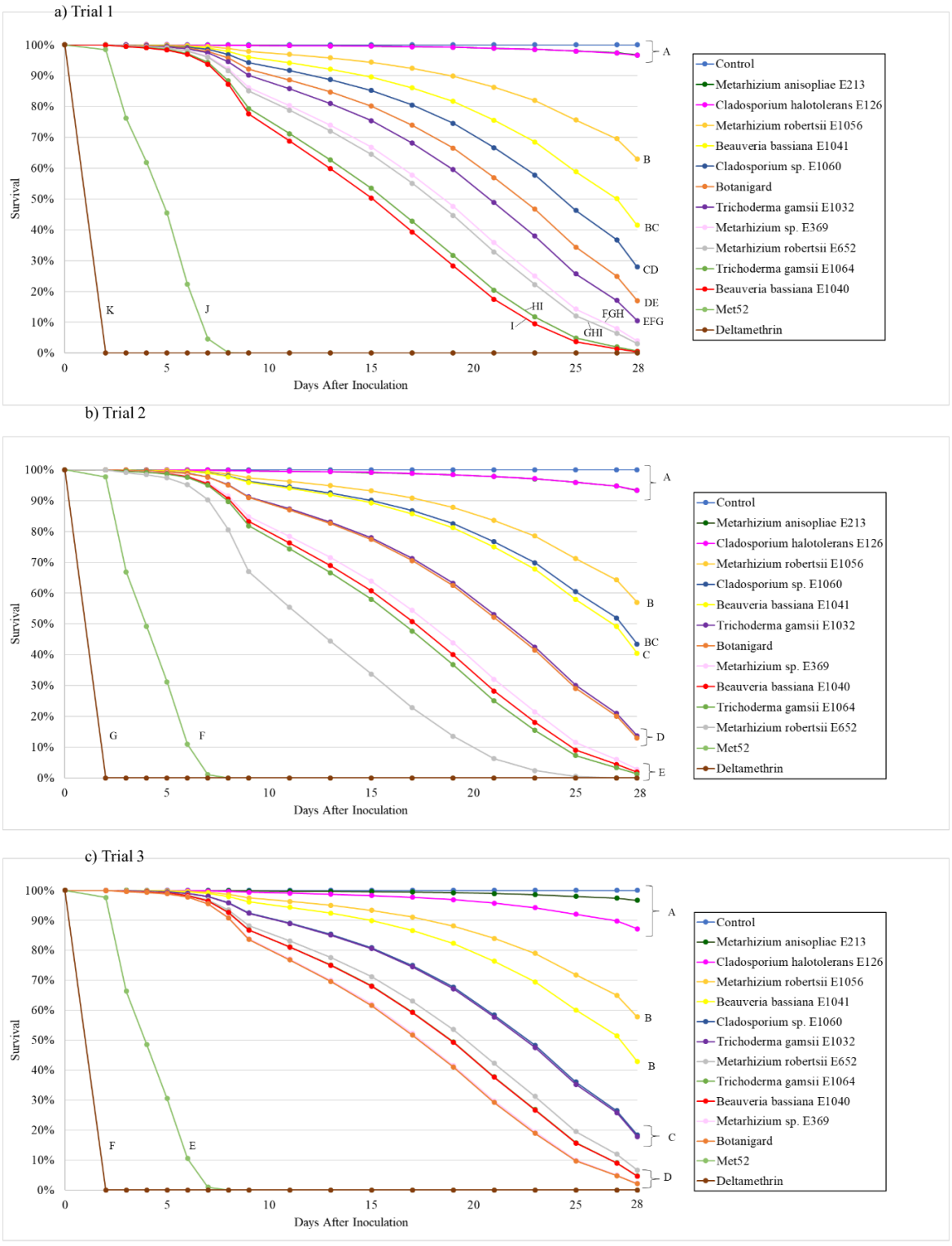

Figure 2.6. Survival of granary weevils over time for each fungal entomopathogen treatment and each trial. Letters indicate significant differences at $p < 0.05$.

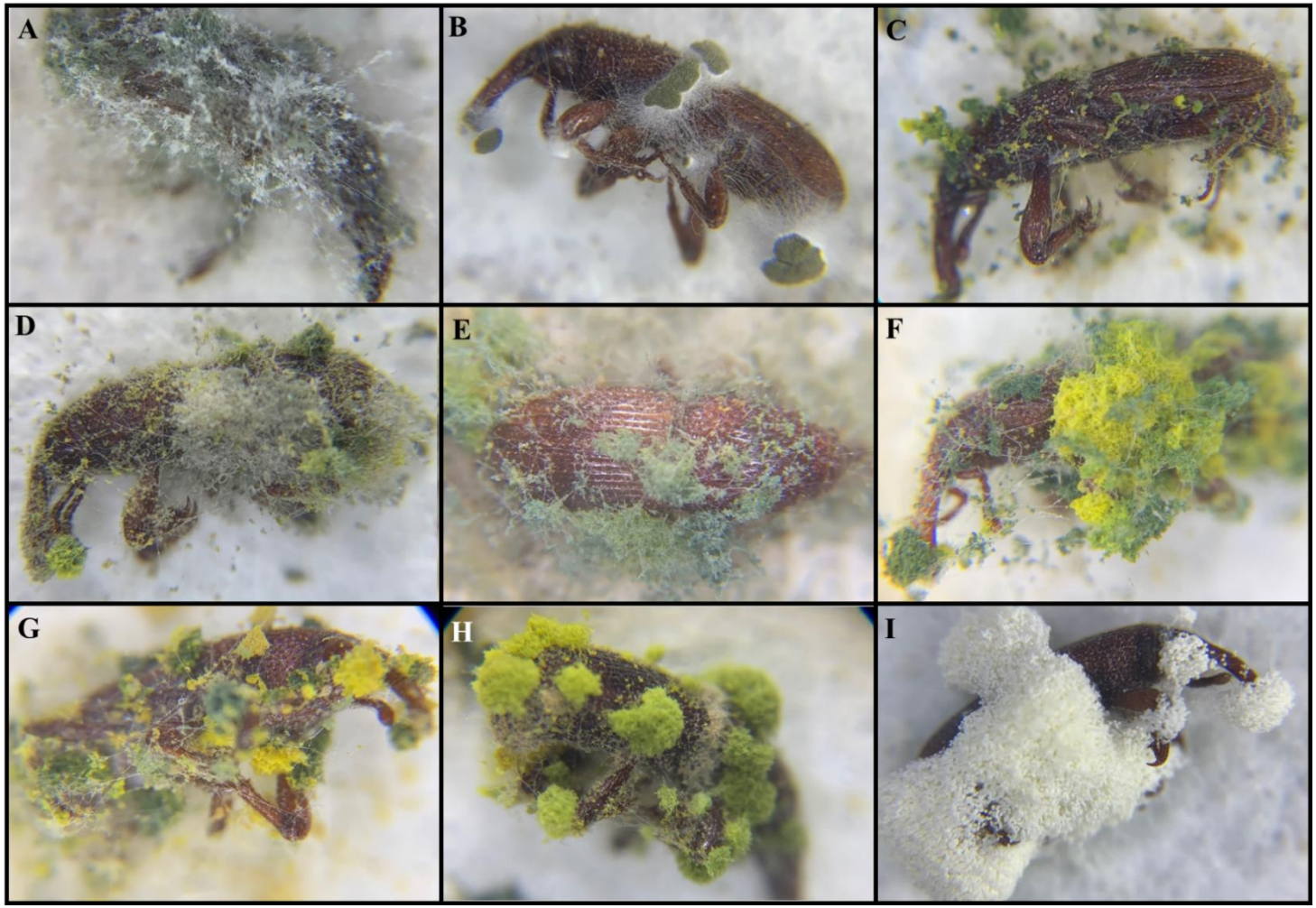

Figure 2.7. Fungal mycelia growing on granary weevil cadavers from *Metarhizium anisopliae* E213 (A-B), *Metarhizium robertsii* E1056 (C-D), *Metarhizium* sp. E369 (E), *Trichoderma gamsii* E1032 (F-G), *T. gamsii* E1064 (H), and *Beauveria bassiana* E1041 (I).

Table 2.4. Mean feeding damage of granary weevil (*Sitophilus granarius*) on wheat grains following treatment of entomopathogenic fungal strains, commercial bioinsecticides and chemical insecticide. Means within a column with the same letter are not significantly different at $p \leq 0.05$.

	Mean (±SEM) feeding damage (0-6 scale)					
Days after Inoculation:	21			28		
Trial:	1	2	3	1	2	3
Treatment						
Negative Control	1.17 (±0.17) A	1.00 (±0) A	1.17 (±0.17) A	1.83 (±0.17) A	1.83 (±0.17) A	1.83 (±0.17) AB
Cladosporium halotolerans E126	1.33 (±0.17) A	1.00 (±0) A	1.33 (±0.17) A	2.00 (±0) A	1.50 (±0) AB	1.83 (±0.17) AB
Metarhizium robertsii E1056	1.00 (±0) AB	1.00 (±0) A	1.00 (±0) AB	1.50 (±0) AB	1.00 (±0) AB	1.50 (±0) ABC
Cladosporium sp. E1060	1.00 (±0) AB	1.00 (±0) A	1.00 (±0) AB	1.33 (±0.17) AB	1.00 (±0) AB	1.33 (±0.17) ABC
Metarhizium anisopliae E213	0.50 (±0) C	1.00 (±0) A	0.50 (±0) C	1.50 (±0) AB	1.50 (±0) AB	1.50 (±0) ABC
Trichoderma gamsii E1032	1.00 (±0) AB	1.00 (±0) A	1.00 (±0) AB	1.00 (±0) AB	1.00 (±0) AB	1.00 (±0) ABC
Beauveria bassiana E1040	1.00 (±0) AB	1.00 (±0) A	1.00 (±0) AB	1.00 (±0) AB	1.00 (±0) AB	1.00 (±0) ABC
Metarhizium robertsii E652	0.83 (±0.17) B	0.50 (±0) B	0.83 (±0.17) B	0.83 (±0.17) AB	0.50 (±0) B	2.33 (±1.33) A
Botanigard (*Beauveria bassiana*)	0.83 (±0.17) B	0.50 (±0) B	0.83 (±0.17) B	0.83 (±0.17) AB	1.17 (±0.17) AB	0.83 (±0.17) BC
Metarhizium sp. E369	0.50 (±0) C	0.67 (±0.17) B	0.50 (±0) C	0.50 (±0) B	2.17 (±1.42) A	0.50 (±0) C
Beauveria bassiana E1041	0.50 (±0) C	1.00 (±0) A	0.50 (±0) C	0.50 (±0) B	1.00 (±0) AB	0.50 (±0) C
Trichoderma gamsii E1064	0.50 (±0) C	0.50 (±0) B	0.50 (±0) C	0.50 (±0) B	0.50 (±0) B	0.50 (±0) C
Met52 (*Metarhizium anisopliae*)	0.50 (±0) C	0.50 (±0) B	0.50 (±0) C	0.50 (±0) B	0.50 (±0) B	0.50 (±0) C
Delta Gold (deltamethrin)	0 (±0) D	0 (±0) C	0 (±0) D	0 (±0) C	0 (±0) C	0 (±0) D

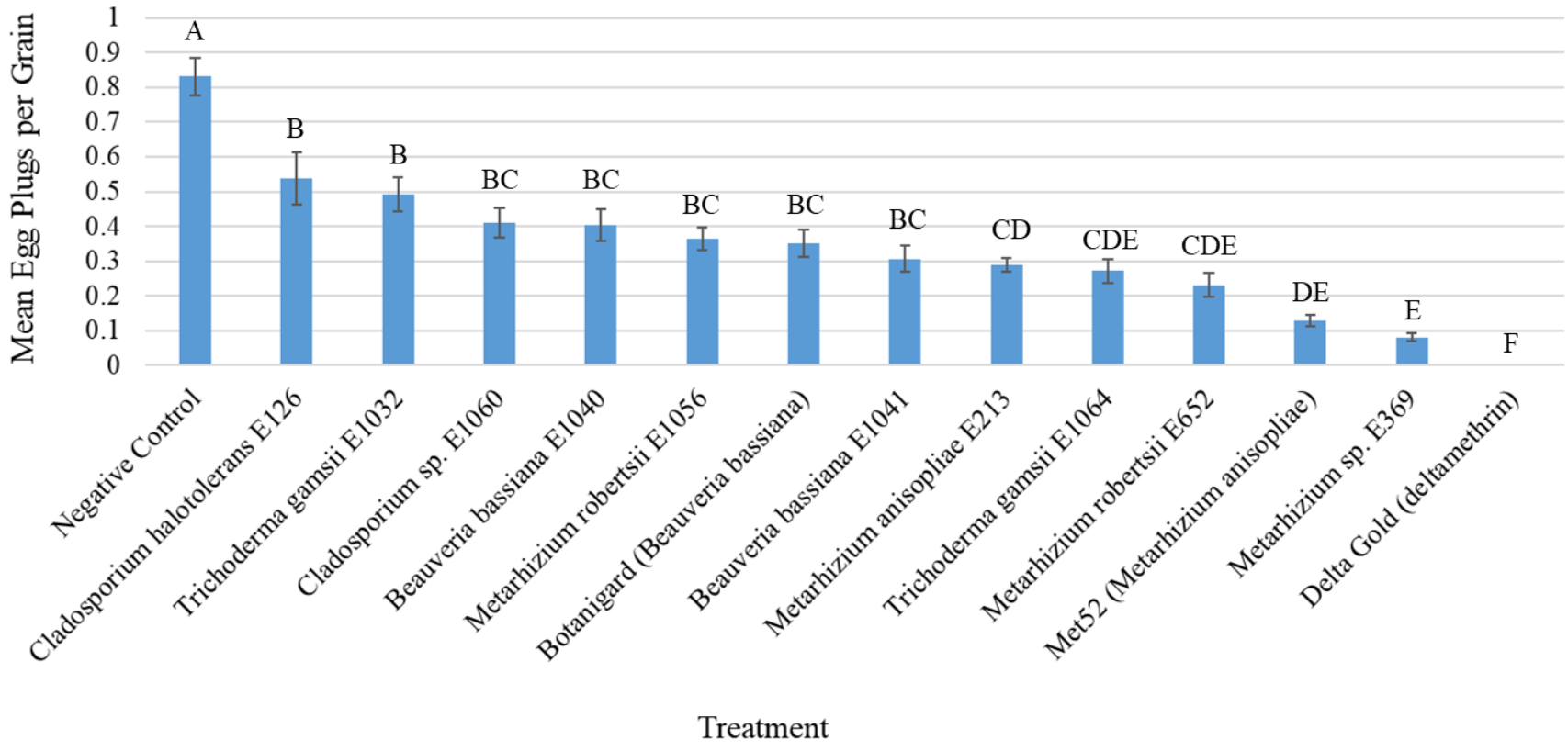

Figure 2.8. Mean oviposition of *S. granarius* adults for negative control, fungal biocontrol agents, and a commercial bioinsecticide, 28 days after inoculation. Letters indicate significant differences at $p < 0.05$. Errors bars show +/- standard error of the mean (SEM).

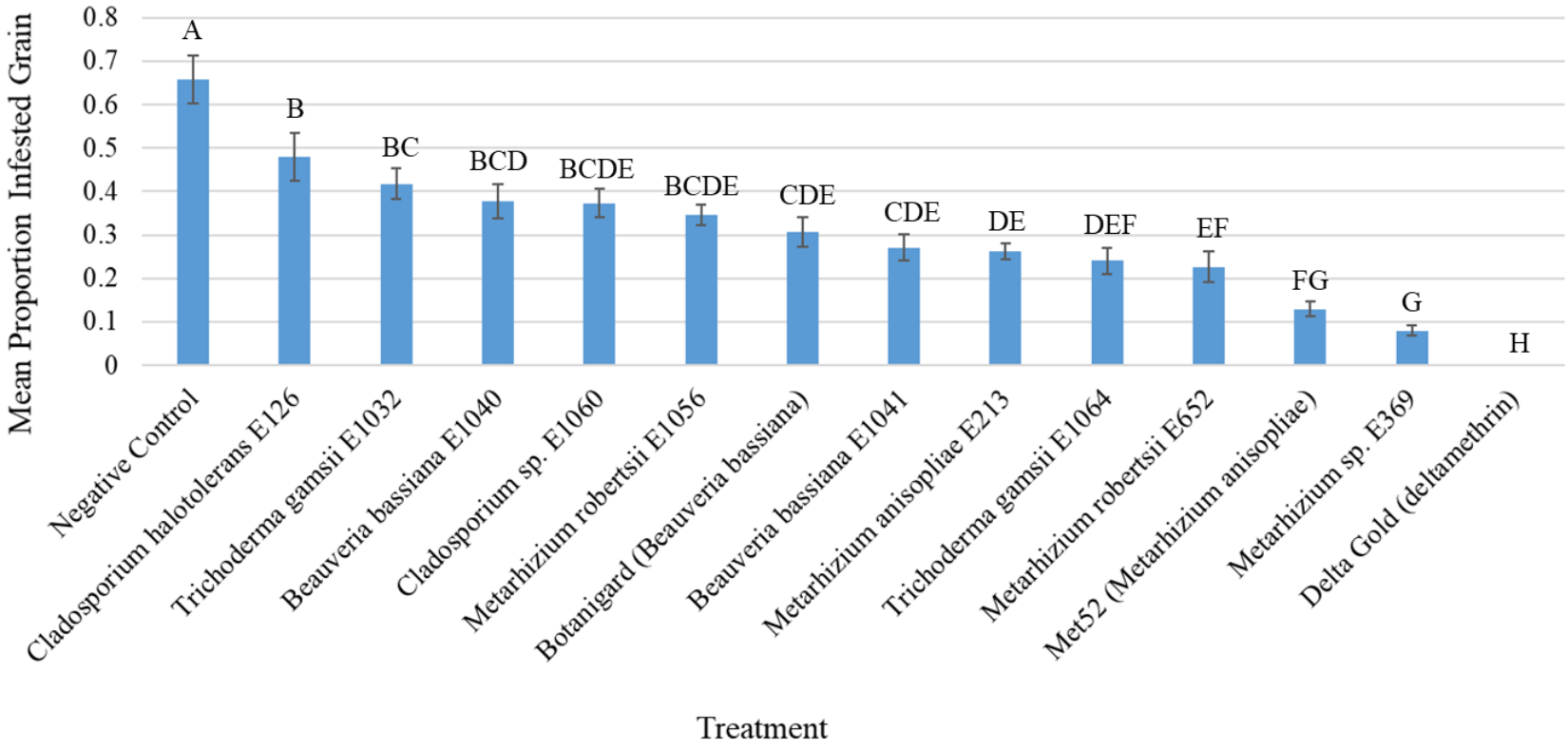

Figure 2.9. Mean proportion of grains infested with at least one egg plug for negative control, fungal biocontrol agents, and commercial bioinsecticides, 28 days after inoculation. Letters indicate significant differences at $p < 0.05$. Errors bars show +/- standard error of the mean (SEM).

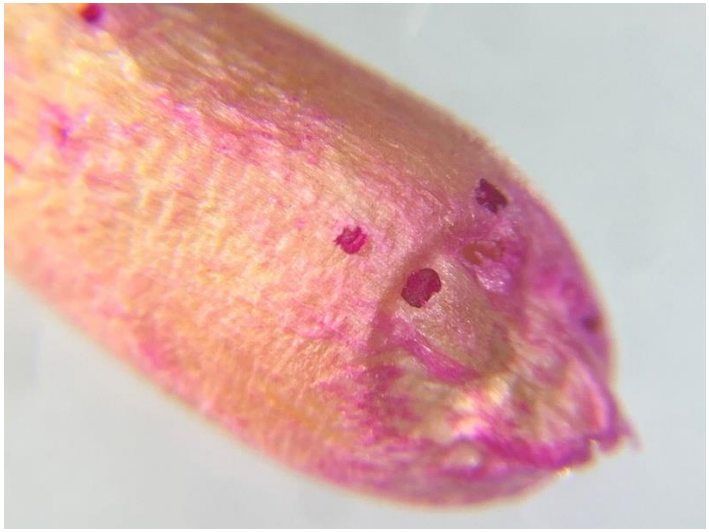

Figure 2.10. The view of egg plug under the microscope after treated with acid fuchsin solution.

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CHAPTER 3: EVALUATION OF POTENTIAL BIOCONTROL AGENTS TO DETERMINE ANTIFUNGAL ACTIVITY AGAINST THREE COMMON FUNGAL PATHOGENS OF STORED GRAIN

Introduction

Cereal grains contain high levels of proteins, carbohydrates and fiber and are the main food source for humans and many other animals (Neethirajan et al. 2007). Of all cereal crops worldwide, wheat is produced on the largest number of hectares (Piasecka-Kwiatkowska et al. 2014). However, cereal grains can be infested by a range of pests, including insects and fungi (Bryden 2012). Cereal contaminated by fungi and their toxic secondary metabolites (mycotoxins) have lower dry matter, nutrients, and grain quality (Magan and Aldred 2007). Contamination can occur in the field and during storage (Bullerman and Bianchini 2007). The most common mycotoxigenic grain fungi are species that belong to the genera *Fusarium*, *Aspergillus* and *Penicillium* (Bothast 1978). Globally, nearly 25% of crops are affected by mycotoxins each year (Whitlow 2010). In addition to economic loss, mycotoxins threaten mammal health by causing serious disease (Fleurat-Lessard 2017).

Current management strategies are based on physical, cultural, and chemical techniques, such as drying the grain after harvesting by reducing the moisture content of the grain for safe storage (Neme and Mohammed 2017), application of gamma irradiation for inhibition of fungal growth (Aziz et al. 1997), and modifying atmospheres in the storage facility (Magan 2006). Phenolic antioxidants, such as butylated hydroxyanisole, have antifungal activity against species of *Aspergillus* (Thompson 1996, Nesci et al. 2003). Fumigation with ozone is another practice, used in some European countries such as France, Italy, Spain, and the United Kingdom. However, ozone treatment can cause

adverse effects on the viability of the seed (Fleurat-Lessard 2017). Application of phosphine can be used as a control practice to inhibit fungal growth and mycotoxins for short-term storage periods. However, during long-term storage periods phosphine loses its effectiveness (Hocking and Banks 1991). Although, there are management strategies against fungal pathogens on grain, they have limited efficacy and potential negative side - effects against mammals and the environment.

Increasing public concern over insecticide residues on grains and potential health problems this may cause has promoted exploration of the feasibility of controlling grain pathogens using biological control. There are also certain situations in which a zero tolerance for pesticides is employed, such as products destined for baby food. As an alternative to chemical pesticides, there are studies that show antagonistic activity of bacterial and fungal biocontrol agents (BCA) against mycotoxigenic fungi under *in vitro*, greenhouse and field conditions (e.g., Niderkorn et al. 2006, Matarese et al. 2012, Shi et al. 2014, Mannaa and Kim 2018, Palazzini et al. 2018). However, there are few studies under stored grain conditions (Druvefors et al. 2002, Velmourougane et al. 2011). Most studies on the mechanisms of how bacteria inhibit growth of fungi come from the soil rhizosphere. In this system, these biological control agents prevent growth or metabolic activity of fungi by secreting enzymes and extracellular metabolites, and competing for space and nutrients (Raaijmakers et al. 2002). For example, lactic acid producing bacteria, such as *Lactobacillus* spp., have shown antifungal activity against fungal pathogens via production of antimicrobial compounds that cause inhibition of fungal growth (Lipińska et al. 2016). *Bacillus amyloliquefaciens* has also shown antagonistic activity against *F. graminearum* under *in vitro* conditions (Shi et al. 2014). *Streptomyces*

sp. was able to inhibit growth of *Aspergillus parasiticus* on peanut grains under *in vitro* conditions (Zucchi et al. 2008). The most commonly tested bacterial biocontrol agents against *Fusarium graminearum* are species within the following genera: *Bacillus*, *Lysobacter,* and *Pseudomonas* (Jochum et al. 2006). In addition to bacterial biocontrol agents, some species of fungi can also behave as fungal antagonists. For example, *Trichoderma* spp. are known as important biocontrol agents that can be used for control of many pathogens (Verma et al. 2007, Gehlot and Singh 2018). *Trichoderma* spp. have unique characteristics, such as rapid growth and production of anti-microbial metabolites, which make them excellent control tools. Currently, 50% of commercial fungal biocontrol products are derived from or include *Trichoderma* spp. (Verma et al. 2007). Although there are some commercial biological agents against these fungal pathogens, they are not registered for use on stored grain. These products include Kodiak, which is produced by *Bacillus subtilis* GBO3 (Bayer CropScience, North Carolina), which is registered for root disease caused by *Aspergillus* spp. and *Fusarium* spp. for peanut and wheat. In addition, Alfa guard, which is produced by *Aspergillus flavus* NRRL 21882 as active ingredient (Syngenta Crop Protection, North Carolina) is recommended for field corn. However, none of them is registered for control of post-harvest grain disease.

Given the need to find effective control strategies as alternatives to chemical fungicides, the aim of this study is to test potential bacterial and fungal biocontrol agents for the control of three common fungal pathogens, which include *Fusarium graminearum, Aspergillus parasiticus,* and *Penicillium chrysogenum,* on wheat seeds. This will be achieved using two different methods, to determine the efficacy of biocontrol

agents against the fungal pathogens *in vitro* on artificial growth media and wheat seed on wheat seeds.

Materials and Methods

Sources and Preparation of Organisms

Fungal Pathogens

Isolates of grain fungal pathogens, *Fusarium graminearum* G2649, *Aspergillus parasiticus* G2650, and *Penicillium chrysogenum* G2651, were incubated at 24 ± 1 °C on potato dextrose agar (39 g/liter, Difco Laboratories Inc., Franklin Lakes, NJ) amended with 0.01% tetracycline (PDAt). For the antibiosis assays, plugs were taken from the edge of an actively growing colony from the PDAt plate with a 4 mm diameter cork-borer after 5 days of incubation. For the wheat seed assays, spores were harvested after 14 days by adding 10 ml of water containing 0.1% Tween 80 detergent to the fungal plate and scraping with a sterile scalpel. Fungal spores were harvested from mycelia by straining spore suspension through autoclaved sterile Miracloth (pore size: 22-25 µm) into a beaker. The spore concentration was estimated with a hemocytometer and adjusted to approximately 10^6 spores/ml. This concentration was chosen based on preliminary tests used to determine the most appropriate concentration for disease development.

Candidate Bacteria & Fungi

Methods for preparing suspensions of candidate bacteria and fungi are described in Chapter 2.

Antibiosis Assays

Bacteria were tested for activity against the three grain fungal pathogens using the impregnated filter paper and spread plate techniques. Fungi were also evaluated against

the three grain fungal pathogens using an agar plug technique. All microorganisms used in the assays were prepared as described above.

Impregnated Filter Paper Assay with Bacterial Agents

The filter paper method was set up by inoculating the bacteria and the fungal pathogens onto the filter paper so that there was no initial contact between the two organisms. Filter paper (Whatman 40) discs were cut into 2 mm diameter circles using a paper punch and the discs were autoclaved. The discs were impregnated with bacterial by soaking in a bacterial suspension in the laminar flow hood. Filter paper discs were taken out of the suspension with sterile forceps and excess solution was removed by touching the disc against a sterile Petri dish. Two impregnated filter paper discs were placed at the two opposite edges of a potato dextrose agar (PDA) 90 mm Petri plate (Figure 3.1). The negative control consisted of filter paper discs dipped in sterile distilled water. After two days of incubation, a 4 mm diameter agar plug of a grain pathogens was placed onto the center of PDA plate previously inoculated with bacteria. Three replicate plates were prepared for each bacteria–grain pathogen combination. The plates were incubated for two weeks. At 7 and 14 days after inoculation (DAI), zones of inhibition were measured from the margin of the grain fungi colony to the margin of the bacterial colony (Figure 3.1). The entire experiment was repeated three times.

Spread Plate Assay with Bacterial Agents

The spread plate technique was used as a second method to assess antibiosis activity in bacteria. It was designed to allow for contact between the bacterium and the grain fungi from the time of inoculation. A 100 µl suspension of bacteria was applied onto a PDA Petri plate and spread with a sterile glass rod while the plate was rotated on

an inoculating turntable to obtain a uniform lawn of culture. The negative control used the same technique of inoculation of the PDA Petri plate with sterile distilled water spread using a sterile glass rod. Immediately after bacterial inoculation, a 4 mm-diameter agar plug was obtained from the actively growing edge of the grain fungal mycelium, flipped over, and inoculated onto the center of each prepared PDA plate. Three replicate plates were prepared for each treatment. The plates were incubated for a total of two weeks and growth measured twice, at 7 and 14 DAI. Growth of the grain fungi was regular in shape and evaluated by measuring colony diameter as total growth, minus the size of the agar plug (4 mm; Figure 3.1). The entire experiment was repeated three times.

Agar Plug Assays with Fungal Agents

The agar plug method was set up so that the potential biocontrol fungus and the grain fungal pathogen had no initial contact during incubation. Two 4 mm diameter agar plugs of a potential fungal biocontrol agent cultured on PDAt were placed at opposite edges of the PDA Petri plate (Figure 3.1). A 4 mm diameter agar plug of a grain fungal pathogen cultured on PDAt was transferred to the center of the same PDA plate. For the negative control treatment, the grain fungal pathogen was inoculated onto the center of a PDA plate. Three replicate plates were prepared for each treatment. Cultures were incubated for two weeks. At 7 and 14 DAI, zones of inhibition were measured from the margin of the grain mold colony to the margin of the potential biocontrol fungal colony (Figure 3.1). The entire experiment was repeated three times.

Wheat seed Assays

Freshly harvested winter wheat (WestBred, St. Louis, MO) was autoclaved at 121 °C for 30 min to eliminate contamination with unwanted microorganisms, and allowed to

dry under a laminar flow hood. Distilled water was added to obtain 21% grain moisture (0.90 aw), which is the most suitable moisture content for growth of fungal pathogens (Table 1.1), as determined using a Dickey John GAC 2100 Agri Bench Grain Moisture Tester. Wheat was stored for 24 h at 25 °C with frequent mixing before placing 10 g of wheat grains into Petri dishes (90 mm diameter).

Potential biocontrol bacteria and fungi were prepared as described above and, for each, a 1 mL suspension of inoculum was transferred into a plastic fingertip spray bottle (59 mL) that had been pre-sterilized with 10% bleach solution and rinsed with tap water, followed by distilled water. 10 g of wheat seed were placed per Petri dish. The negative control treatment was an application of sterile distilled water containing 0.1% Tween 80. After inoculation, grains were agitated to mix and evenly distribute seeds in the Petri dish. Subsequently, plates were placed under a laminar flow hood, without petri lid, to dry prior to wrapping with Parafilm. Plates were incubated for 2 days in the growth chamber set to 25 ºC and 75% relative humidity.

After two days of incubation with the potential biocontrol agent, a 1 ml of suspension of spores from a grain fungus that was at a concentration of 10^6 spores/ml was inoculated onto the wheat grains. After application of treatments, Petri dishes were placed under the hood, allowed to dry and were sealed with Parafilm. Petri dishes were placed in the growth chamber set to 25 °C and 75% RH and incubated for 14 days. Four replicate plates were prepared for each biocontrol agent-fungal pathogen combination. The entire experiment was repeated two times.

Evaluation of disease severity and damage to wheat grains was assessed at 7 and 14 days after inoculation using a 0–4 scale. The scale was created according to disease

growth (Figure 3.2). For *Fusarium graminearum* disease development on the wheat seed: A rating of 0 % is no disease with all seeds appearing healthy; 1 is nearly all seeds healthy with 1-10% seed appearing symptomatic (signs of the pathogen, such as white fungal mycelial growth and conidia); 2 is between 11 -25% seeds symptomatic; 3 is between 26 -50% seeds symptomatic; 4 is more than 50% of seeds appearing symptomatic. For *Aspergillus parasiticus* disease development on the wheat seed: A rating of 0 % is no disease with all seeds appearing healthy; 1 is nearly all seeds healthy with 1-10% seed appearing symptomatic (signs of the pathogen, such as green fungal mycelial growth and conidia); 2 is between 11 -25% seeds symptomatic; 3 is between 26 -50% seeds symptomatic; 4 is more than 50% of seeds appearing symptomatic. For *Penicillium chrysogenum* disease development on the wheat seed: A rating of 0 % is no disease with all seeds appearing healthy; 1 is nearly all seeds healthy with 1-10% seed appearing symptomatic (signs of the pathogen, such as green or greenish-blue fungal mycelial growth and conidia); 2 is between 11 -25% seeds symptomatic; 3 is between 26 -50% seeds symptomatic; 4 is more than 50% of seeds appearing symptomatic

Data Analysis

Bacterial and fungal biocontrol agent experiments were analyzed separately. All analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC). For the filter paper and spread plate assays, results were bimodal and therefore could not be modelled using a normal distribution. As such, we analyzed two groups (inhibition zones close to zero: control, W341 and C423; and large inhibition zones: C628, C3R5, and C415) separately with a generalized linear mixed model (PROC GLIMMIX) with pathogen and treatment as fixed effects and trial as a random effect. For the agar plug assays, all

treatments were analyzed using PROC GLIMMIX with pathogen and treatment as fixed effects and trial as a random effect. For all assays, data collected at 7 DAI and 14 DAI were analyzed separately. Post hoc means were determined using Tukey's test.

For the wheat seed assays, data were analyzed separately for 7 and 14 DAI, as well as for each fungal grain pathogen, using generalized linear mixed models (PROC GLIMMIX) with a multinomial distribution and a cumulative logit link function (appropriate for discrete, ordinal data such as the disease scale used). Treatment was a fixed effect, with trial as a random effect.

Results

Antibiosis Assays

Candidate Bacteria

For the impregnated filter paper assay at 7 DAI, there was a significant effect of pathogen ($F = 70.38$, $df = 2$, $p < 0.0001$), treatment ($F=78.47$, $df = 4$, $p < 0.0001$), and the interaction between pathogen and treatment ($F = 14.18$, $df = 8$, $p < 0.0001$). Three bacterial strains (*Bacillus amyloliquefaciens* C415, *Lysobacter enzymogenes* C3R5, and *Burkholderia ambifaria* C628) inhibited growth of all three common grain fungal pathogens tested (Figure 3.3). *Bacillus amyloliquefaciens* C415 was the most effective isolate against all fungal pathogens with mean zones of inhibition values of 14.0 mm for *F. graminearum*, 13.1 mm for *A. parasiticus*, and 15.2 mm for *P. chrysogenum*. Two bacterial strains (*Lysinibacillus sphaericus* W341 and *Bacillus thuringiensis* C423) exhibited less inhibition activity than *B. amyloliquefaciens* C415, *L. enzymogenes* C3R5, and *B. ambifaria* C628. For *F. graminearum*, the zone of inhibition was still significantly

larger (2.6 mm for *L. sphaericus* W341 and 3.9 mm for *B. thuringiensis* C423) than the untreated negative control (0.0 mm; Figure 3.3). However, there were no significant differences between these two bacteria and the negative control for *A. parasiticus* and *P. chrysogenum*.

For the impregnated filter paper assay at 14 DAI, there was a significant effect of pathogen ($F = 6.81$, $df = 2$, $p = 0.0020$), treatment ($F=12.40$, $df = 4$, $p < 0.0001$), and the interaction between pathogen and treatment ($F = 2.61$, $df = 8$, $p = 0.0429$). The same three top effective strains from 7 DAI (*B. amyloliquefaciens* C415, *L. enzymogenes* C3R5, and *B. ambifaria* C628) maintained their activity up until 14 days (Figure 3.4). However, the effect of the two weaker strains (*L. sphaericus* W341 and *B. thuringiensis* C423) did not last more than one week of incubation (Figure 3.4)

For the spread plate assay at 7 DAI, there was a significant effect of pathogen ($F = 4.27$, $df = 2$, $p = 0.0179$), treatment ($F=4.30$, $df = 4$, $p = 0.0173$), and the interaction between pathogen and treatment ($F = 3.16$, $df = 8$, $p = 0.0191$). Three bacterial strains (*B. amyloliquefaciens* C415, *L. enzymogenes* C3R5, and *B. ambifaria* C628) inhibited growth of *F. graminearum* (with mean fungal growth diameters of 1.8, 0.9, and 0.2 mm, respectively), *A. parasiticus* (with mean fungal growth diameters of 1.7, 0.4, and 1.9 mm, respectively), and *P. chrysogenum* (with mean fungal growth diameters of 0.7, 0.3, and 0.6 mm, respectively). Two bacterial strains (*L. sphaericus* W341 and *B. thuringiensis* C423) exhibited less inhibition activity. For *F. graminearum*, *L. sphaericus* W341 (23.3 mm) and *B. thuringiensis* C423 (32.9 mm) had significantly less fungal growth than the negative control (77.0 mm). For *A. parasiticus*, *L. sphaericus* W341 (62.1 mm) had significantly less fungal growth than the negative control (80.7 mm), but *B. thuringiensis*

C423 (78.4 mm) was not different. For *P. chrysogenum*, *L. sphaericus* W341 (52.6 mm) had significantly less fungal growth than the negative control (80.9 mm), but *B. thuringiensis* C423 (75.3 mm) was not different (Figure 3.5).

For the spread plate assay at 14 DAI, there was a significant effect of pathogen ($F = 10.71$, $df = 2$, $p < 0.0001$), treatment ($F=7.75$, $df = 4$, $p = 0.0009$), and the interaction between pathogen and treatment ($F = 5.73$, $df = 8$, $p = 0.0005$). Three bacterial strains (*B. amyloliquefaciens* C415, *L. enzymogenes* C3R5, and *B. ambifaria* C628) inhibited growth of *F. graminearum* (with mean fungal growth diameters of 3.6, 1.6, and 1.3 mm, respectively), *A. parasiticus* (with mean fungal growth diameters of 4.3, 1.2, and 6.2 mm, respectively), and *P. chrysogenum* (with mean fungal growth diameters of 2.0, 1.3, and 1.4 mm, respectively). Two bacterial strains (*L. sphaericus* W341 and *B. thuringiensis* C423) exhibited less inhibition activity. For *F. graminearum*, *B. thuringiensis* C423 (51.0 mm) had significantly less fungal growth than the negative control (82.0 mm), but *L. sphaericus* W341 (73.6 mm) was not different. For *A. parasiticus*, there were no differences between *B. thuringiensis* C423 (81.1 mm), *L. sphaericus* W341 (80.3 mm), and the negative control (82.0 mm). For *P. chrysogenum*, there were no differences between *B. thuringiensis* C423 (79.6 mm), *L. sphaericus* W341 (79.1 mm), and the negative control (82.0 mm; Figure 3.6). Furthermore, the three effective strains (*B. amyloliquefaciens* C415, *B. ambifaria* C628, and *L. enzymogenes* C3R5) showed longevity of their activity in that they retained their efficacy at both 7 and 14 DAI (Figures 3.5 and 3.6). Abnormal discolored mycelium growth of the pathogen was observed for *P. chrysogenum* in assays with *Lysinibacillus sphaericus* W341 (Figure 3.7).

Candidate Fungi

For the agar plug assay at 7 DAI, there was a significant effect of pathogen ($F=499.97$, $df = 2$, $p < 0.0001$), treatment ($F=482.93$, $df = 10$, $p < 0.0001$), and the interaction between pathogen and treatment ($F=25.12$, $df = 20$, $p < 0.0001$). All ten candidate fungal strains were able to inhibit growth of *F. graminearum* and *P. chrysogenum* compared to the negative control (Figure 3.8). However, three of the ten strains (*Metarhizium* sp. E1056, *Cladosporium* sp. E1060, and *M. anisopliae* E213) were not significantly different from the negative control against *A. parasiticus* (Figure 3.8). Two candidate biocontrol agents (*Trichoderma gamsii* strains E1064 and E1032) had significantly larger zones of inhibition than all other treatments: respectively, 21.7 and 21.7 mm for *F. graminearum*, 20.6 and 21.2 mm for *A. parasiticus*, and 21.6 and 21.3 mm for *P. chrysogenum* (Figure 3.8).

For the agar plug assay at 14 DAI, there was a significant effect of pathogen ($F=651.70$, $df = 2$, $p < 0.0001$), treatment ($F=806.80$, $df = 10$, $p < 0.0001$), and the interaction between pathogen and treatment ($F=30.48$, $df = 20$, $p < 0.0001$). All ten candidate fungal strains were able to inhibit growth of all three pathogens compared to the negative control (Figure 3.9). The same two candidate biocontrol agents (*Trichoderma gamsii* strains E1064 and E1032) had significantly larger zones of inhibition than all other treatments: respectively, 20.6 and 20.4 mm for *F. graminearum*, 20.8 and 20.7 mm for *A. parasiticus*, and 20.6 and 20.6 mm for *P. chrysogenum* (Figure 3.9).

Wheat Seed Assays

Fusarium graminearum

Results for *F. graminearum* indicated that all candidate bacteria (Figure 3.10) and candidate fungi (Figure 3.11) suppressed the growth of the pathogen below the negative control at both 7 and 14 DAI. However, growth was poor in the negative controls.

Aspergillus parasiticus

Results from the wheat seed bioassays against *A. parasiticus* showed that all candidate biocontrol bacteria and fungi suppressed fungal pathogen growth at both 7 and 14 DAI (Figures 3.12 and 3.13). For bacteria, *Lysinibacillus sphaericus* W341 had the strongest effect against *A. parasiticus* at 7 DAI (mean 0.0 fungal disease score). For fungi, *Trichoderma gamsii* isolates 1032 and 1064 and *Cladosporium halotolerans* E126 showed the most effectiveness against *A. parasiticus* (Figure 3.13).

Penicillium chrysogenum

All tested isolates against this grain pathogen had significantly lower disease ratings than the negative control (Figures 3.13 and 3.14). For bacteria, *Burkholderia ambifaria* C628 performed best against *P. chrysogenum* (0.0 mean disease score). *Lysinibacillus sphaericus* W341 was one of the best performers at 7 DAI; however, at 14 DAI had lost relative efficacy. For fungi, *Trichoderma gamsii* isolates 1032 and 1064 and *Cladosporium halotolerans* E126 showed the most effectiveness against *P. chrysogenum*.

Discussion

This study indicated that bacterial strains characterized in this study showed the ability to suppress growth of grain fungal pathogens under *in vitro* conditions. The results of the two antibiosis techniques supported each other and followed a similar trend. Based

on the results of zone of inhibition and growth of fungal pathogen, three bacterial isolates (*Bacillus amyloliquefaciens* C415, *Lysobacter enzymogenes* C3R5, and *Burkholderia ambifaria* C628) showed effectiveness against all fungal pathogens tested with two different antagonism bioassays, whereas two bacterial isolates did not (*Bacillus thuringiensis* C423 and *Lysinibacillus sphaericus* W341). Among the three effective strains, *B. amyloliquefaciens* C415 was the most effective against all three fungal pathogens.

In this study, bacterial isolates were tested with two different antibiosis methods. When the mechanism that the microorganisms could use is unknown, multiple screening methods (e.g., using both spread plate and filter paper impregnation) will help increase the chance of detecting potential biocontrol agents. This explains why both spread plate and filter paper impregnation methods were used in the current study. It is important to use both methods in the future to screen microorganisms with unknown mechanisms.

There have been several studies demonstrating that strains of *Bacillus* spp. have antifungal properties. Our results have also demonstrated that *B. amyloliquefaciens* is capable of inhibiting the growth of three species of grain fungal pathogens. In this study, two different strains of *Bacillus* sp. were tested for antifungal activity against grain fungal pathogens and *B. amyloliquefaciens* C415 showed highest *in vitro* fungal suppression, which supports findings in previous studies with strains of this species (e.g., Moyne et al. 2001, Arrebola et al. 2010,Yuan et al. 2012). On the other hand, although there are many reports of efficacy of *B. thuringiensis* against fungal pathogens under *in vitro* conditions (e.g., Reyes-Ramírez et al. 2004, Öztopuz et al. 2018), the *B. thuringiensis* strain in this study did not inhibit fungal growth under the conditions evaluated.

Another important point in our study is that *Lysinibacillus sphaericus* W341 was not able to prevent the growth of the grain fungal fungi evaluated. However, this bacterial strain did cause abnormal mycelia growth of *Penicillium chrysogenum*, which caused the mycelium to appear differently colored. *Penicillium chrysogenum* grown on potato dextrose agar produces green mycelium; however, after treatment with *L. sphaericus* W341, yellow coloration was observed (Figure 3.7). This result may have been caused by enzymes or volatile compounds produced by the bacterial strain. In this study, the mechanisms of antibiosis were not evaluated, but several studies have reported that *Bacillus* spp. can produce volatile compounds and fungal cell wall degrading enzymes (Fiddaman and Rossall 1993, Chowdhury et al. 2015). Research shows that *Bacillus subtilis* showed antifungal activity against *Rhizoctania soloni* and *Pythium ultimum* by production of the volatile compound in PDA (Fiddaman and Rossall 1993). Additionally, temperature is an important factor that affects volatile production, with the highest inhibition by antifungal volatile activity occurring at 30 ºC (Fiddaman and Rossall 1993). It is reasonable to conclude that such mechanisms may be the underlying reason for the similar results observed in the present study.

Two of the strains that exhibited low antifungal activity at 7 DAI (*Lysinibacillus sphaericus* W341 and *Bacillus thuringiensis* C423) appeared to have lost all activity at 14 DAI. Thus, the small amount of observed activity lacked longevity. Therefore, if these strains are to be considered in further studies, they may need to be combined with other microorganisms or other management approaches to achieve sustained effect on grain fungal pathogens.

All candidate fungal strains evaluated in the present study showed antifungal activity against all three fungal pathogens that was significantly greater than the negative control. The most effective strains in the antibiosis assay were *Trichoderma gamsii* strains E1032 and E1064. This result is similar to previous studies (Matarese et al. 2012, Schöneberg et al. 2015). This result may be due to unique features of the biocontrol agents that play an important role in interactions with pathogens. Fungal biocontrol agents and plant pathogens are in competition for niche, carbon, nitrogen and various microelements (Ownley et al. 2010). Accordingly, *Trichoderma* spp. have unique characteristics, such as rapid growth and production of anti-microbial metabolites, which makes them excellent management tools (Verma et al. 2007). Moreover, inhibition of grain pathogens by *T. gamsii* strains E1064 and E1032 persisted at both 7 and 14 DAI (Figures 3.8 and 3.9), which is important when considering long term storage periods.

Strains of *Metarhizium* spp. have been more frequently used as biocontrol agents against insect pests (Kavallieratos et al. 2014); however, there are reports that *Metarhizium* spp. can also suppress plant disease (Keyser et al. 2016). For example, *M. brunneum* was able to inhibit growth of the pathogen *Fusarium culmorium* (Keyser et al. 2016). Moreover, recent research has shown that *M. robertsii* can also serve as a plant promoter (Sasan and Bidochka 2012). In the present study, the *Metarhizium* sp. strains tested were able to suppress grain pathogens. However, none of the strains of *Metarhizium* sp. tested in the present study were among the top effective strains. These results could be affected by the aggressiveness of this strain or colonization on the seed may not be enough to suppress pathogens completely.

Another important well-known entomopathogenic fungus is *Beauveria bassiana*, which is highly virulent and is easily cultured. These features make it a good candidate for use in pest control (Li et al. 2001). Although this species is known primarily as an entomopathogenic fungus, there are reports that it also can suppress several plant pathogens under *in vitro* conditions through production of secondary metabolites (e.g. beauvericin, beauvrolides, bassianlides, oosporein and oxalic acid; Renwick et al. 1991, Culebro-Ricaldi et al. 2017). However, the two *B. bassiana* strains tested against fungal pathogens in the present study did not show strong suppression. This may be a result of features of the strains tested, since the effectiveness of *B. bassiana* is known to be highly variable among strains or even within sub-cultures of the same isolate (Li et al. 2001). Additionally, *Beauveria* spp. can be weak competitors for organic resources (Hajek 1997, Ownley et al. 2010). These could both be reasons that the *B. bassiana* strains tested in this study did not show high antifungal activity against the tested pathogens.

One of the most effective fungal strains evaluated in the present study was *Cladosporium halotolerans* E126 (second only to the *T. gamsii* strains). However, another tested *Cladosporium* sp. strain (E1060) did not show antifungal activity.

Most studies use *in vitro* testing to determine a subset of effective isolates to subsequently test under more realistic *in vivo* conditions (Verma et al. 2007, Etcheverry et al. 2009, Matarese et al. 2012). In this study, all isolates were tested under both *in vitro* and *in vivo* conditions. Research has shown that microbial colony growth is affected by the source of carbon and nitrogen (Calistru et al.1997). For example, *B. bassiana* is able to grow well in the potato dextrose agar and Sabouraud dextrose agar artificial growth media, while it grows poorly in malt extract agar and czepeck dox agar media (Dale and

Shinde 2017). Therefore, the differences in results from the antibiosis and wheat seed assays may be due to the differences in resources available under *in vitro* and *in vivo* conditions.

The wheat seed bioassay showed that there were several bacterial isolates with promising activity against fungal pathogens of grain. In particular, *B. amyloliquefaciens* C415, *L. enzymogenes* C3R5, and *B. ambifaria* C628 inhibited the growth of fungal grain pathogens. In contrast to the antibiosis assay, all isolates were significantly different from the negative control. In addition, *L. sphaericus* W341 and *B. thuringiensis* C423 inhibited fungal growth during the wheat seed study, but did not in the *in vitro* study. Bacteria can inhibit fungi through antibioisis (production of toxic enzymes or antibiotics) or through nutrient competition. All of these mechanisms are dependent on the nutrient composition and concentration available to the bacteria and the fungi. The nutrient composition and concentration in the agar was very different from that of the autoclaved seed. The nutrient environment in the agar did not support the production of toxic compound by the bacteria or allow nutrient competition to take place, while the nutrient environment in the seed allowed the mechanisms to be expressed.

Similarly, all fungal biocontrol agents were able to inhibit pathogen growth during the wheat seed study. *Trichoderma gamsii* strains 1032 and 1064 and *C. halotolerans* E126 showed the most effectiveness against fungal growth. Overall, *F. graminearum* did not grow well compared to *P. chrysogenum* and *A. parasiticus*. It is possible that this was due to the biocontrol agents being more effective against *F. graminearum*. However, this might have occurred due to the nature of growth of the fungi; for instance, *A. parasiticus* produces many spores, which are easily dispersible,

allowing them to spread more quickly on the Petri plate than either of the other grain fungal pathogens. Moreover, fungal growth of *F. graminearum* was slow even in the negative control. This result may have occurred because the strain has reduced capacity to colonize autoclaved wheat kernels or might have occurred due to the relative humidity requirement of *F. graminearum*. *Fusarium graminearum* usually needs high relative humidity to grow compared to the other fungal grain pathogens, *P. chrysogenum* and *A. parasiticus* (Table 1.1), although the relative humidity inside the Parafilm-sealed Petri dish was unknown, this evidence suggests that the moisture conditions were not suitable for growth of *F. graminearum*. Due to this, evaluation of the ability of biocontrol agents to inhibit *F. graminearum* was not as robust in this study, as it is not known whether suppression of the pathogen was a result of the efficacy of biocontrol agents or due to low pathogenicity of *F. graminearum*.

Although some of the tested organisms have potential to be considered in further studies as biocontrol agents against an insect pest and fungal pathogens in both *in vitro* and *in vivo* assays, there is the need to evaluate survival, reproduction, and antibiosis activity under real storage bin conditions in order to determine whether these strains should be considered as biocontrol agents. Additionally, the potential synergistic effects of applying multiple biocontrol agents together should be tested. Finally, the inhibitory activity and potential secondary metabolites of effective isolates should be determined.

Table 3.1. List of treatments used in antibiosis and wheat seed assays for candidate bacteria.

Treatment Category	Description
Negative Control	Sterile, distilled water
Candidate Biocontrol Agent	*Lysinibacillus sphaericus* W341
Candidate Biocontrol Agent	*Burkholderia ambifaria* C628
Candidate Biocontrol Agent	*Lysobacter enzymogenes* C3R5
Candidate Biocontrol Agent	*Bacillus thuringiensis* C423
Candidate Biocontrol Agent	*Bacillus amyloliquefaciens* C415

Table 3.2. List of treatments used in antibiosis and wheat seed assays for candidate fungi.

Treatment Category	Description
Negative Control	Sterile, distilled water
Candidate Biocontrol Agent	*Beauveria bassiana* E1040
Candidate Biocontrol Agent	*Beauveria bassiana* E1041
Candidate Biocontrol Agent	*Cladosporium halotolerans* E126
Candidate Biocontrol Agent	*Cladosporium* sp. E1060
Candidate Biocontrol Agent	*Metarhizium anisopliae* E213
Candidate Biocontrol Agent	*Metarhizium robertsii* E652
Candidate Biocontrol Agent	*Metarhizium* sp. E369
Candidate Biocontrol Agent	*Metarhizium robertsii* E1056
Candidate Biocontrol Agent	*Trichoderma gamsii* E1032
Candidate Biocontrol Agent	*Trichoderma gamsii* E1064

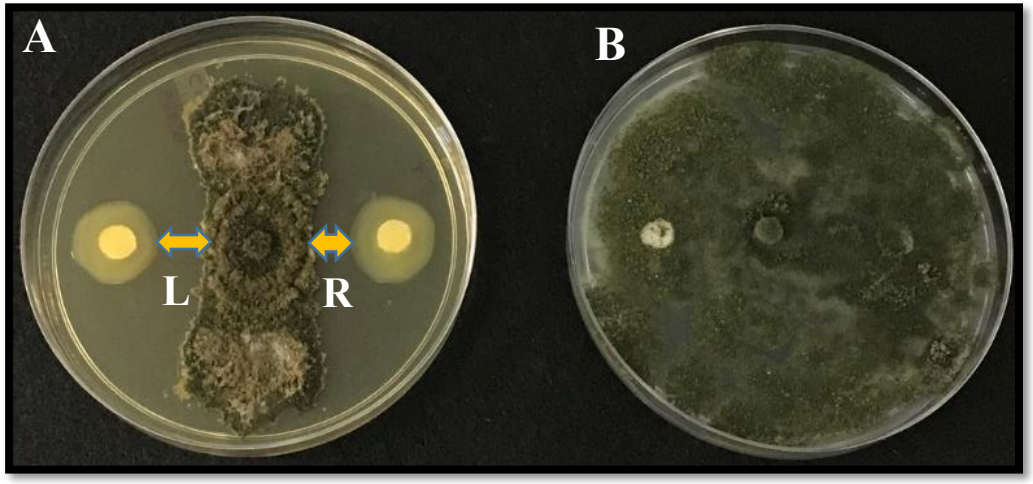

Figure 3.1. A) Zone of inhibition was measured from border of the fungal pathogen growth ring to the border of the bacterial biocontrol agent growth ring and the inhibition zone was measured on the left and right side and the average was used for analysis. B) Negative control.

Figure 3.2. Evaluation Scale for development fungal disease for: A) *Fusarium graminearum*, B) *Aspergillus parasiticus*, and C) *Penicillium chrysogenum*. A rating of 0 is no disease with all seeds appearing healthy; 1 is 1–10% of seeds appearing symptomatic (signs of the pathogen, fungal mycelial growth and conidia); 2 is 11–25% of seeds symptomatic; 3 is 26–50% of seeds symptomatic; 4 is >50% of seeds appearing symptomatic.

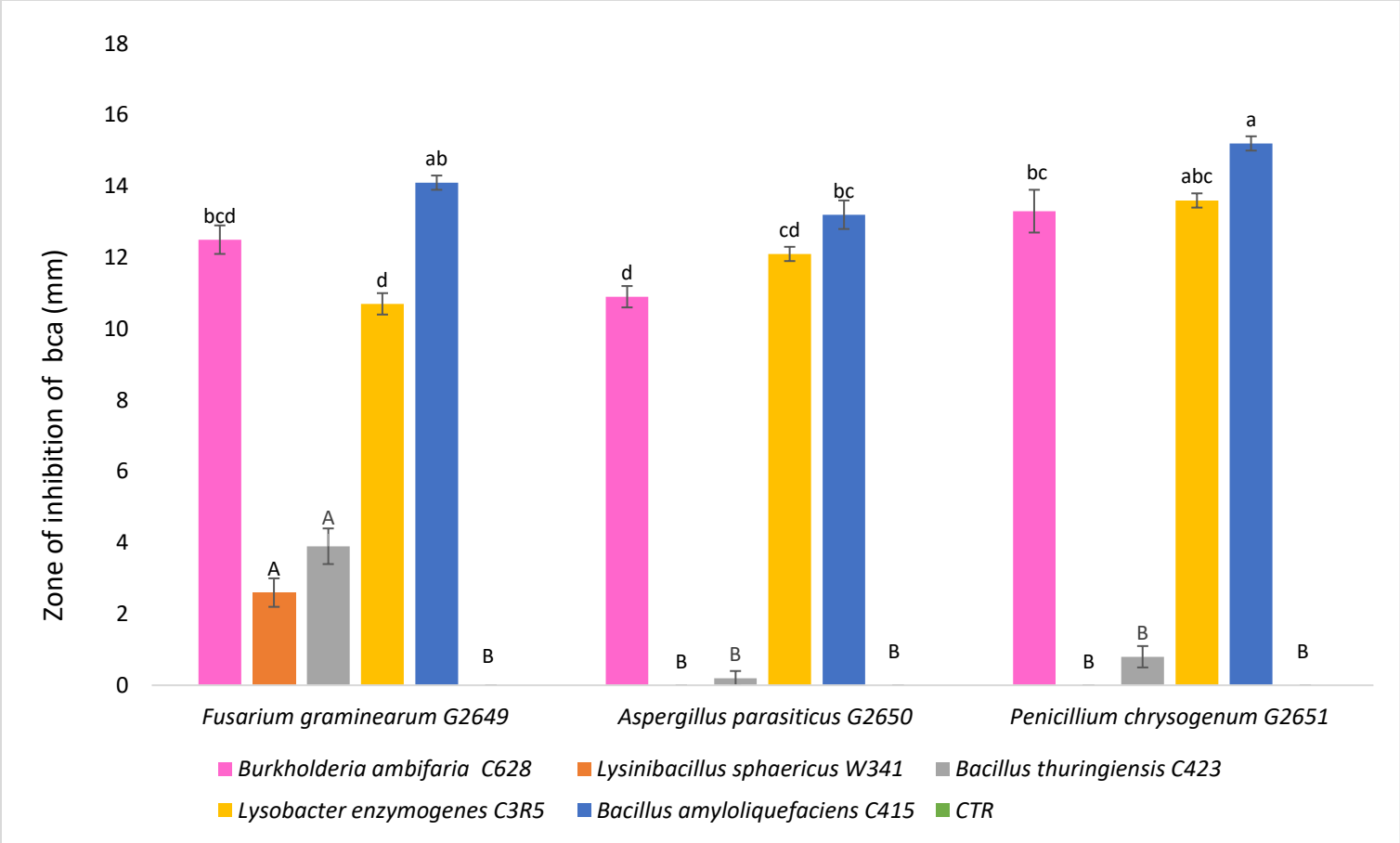

Figure 3.3. Zone of inhibition of five biocontrol agents against three fungal pathogens after 7 days after inoculation (DAI) using filter paper technique. Biocontrol treatments include five bacteria strains listed in Table 3.1, C628, W341, C423, C3R5, C415, and water control (CTR). Bars that share a lowercase letter are not significantly different from one another at $\alpha = 5\%$; similarly, bars that share an uppercase letter are not significantly different from one another at $\alpha = 5\%$.

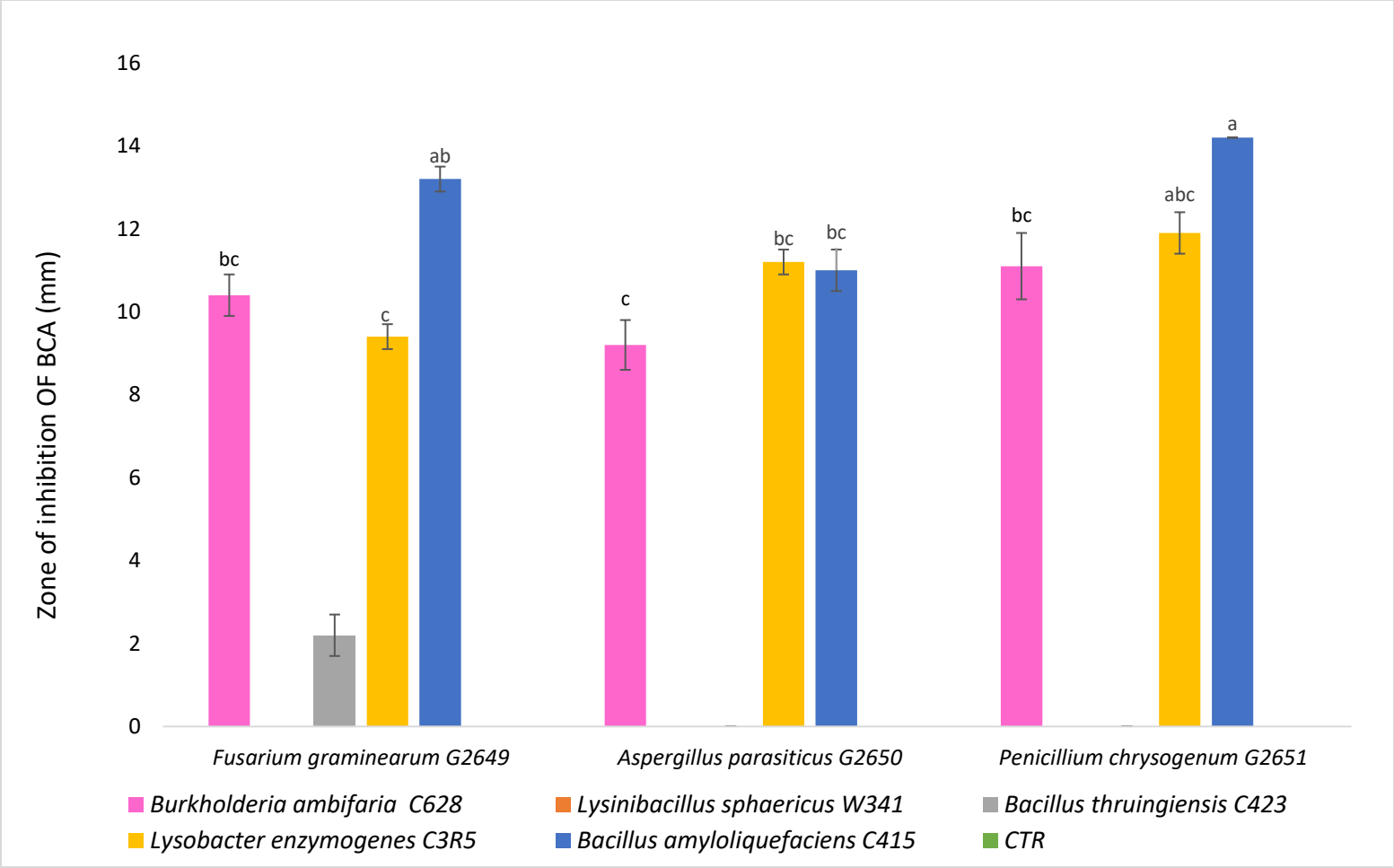

Figure 3.4. Zone of inhibition of five biocontrol agents against three fungal pathogens after 14 days after inoculation (DAI) using filter paper technique. Biocontrol treatments include five bacteria strains listed in Table 3.1, C628, W341, C423, C3R5, C415, and water control (CTR). Means that share a lowercase letter are not significantly different from one another at $\alpha = 5\%$.

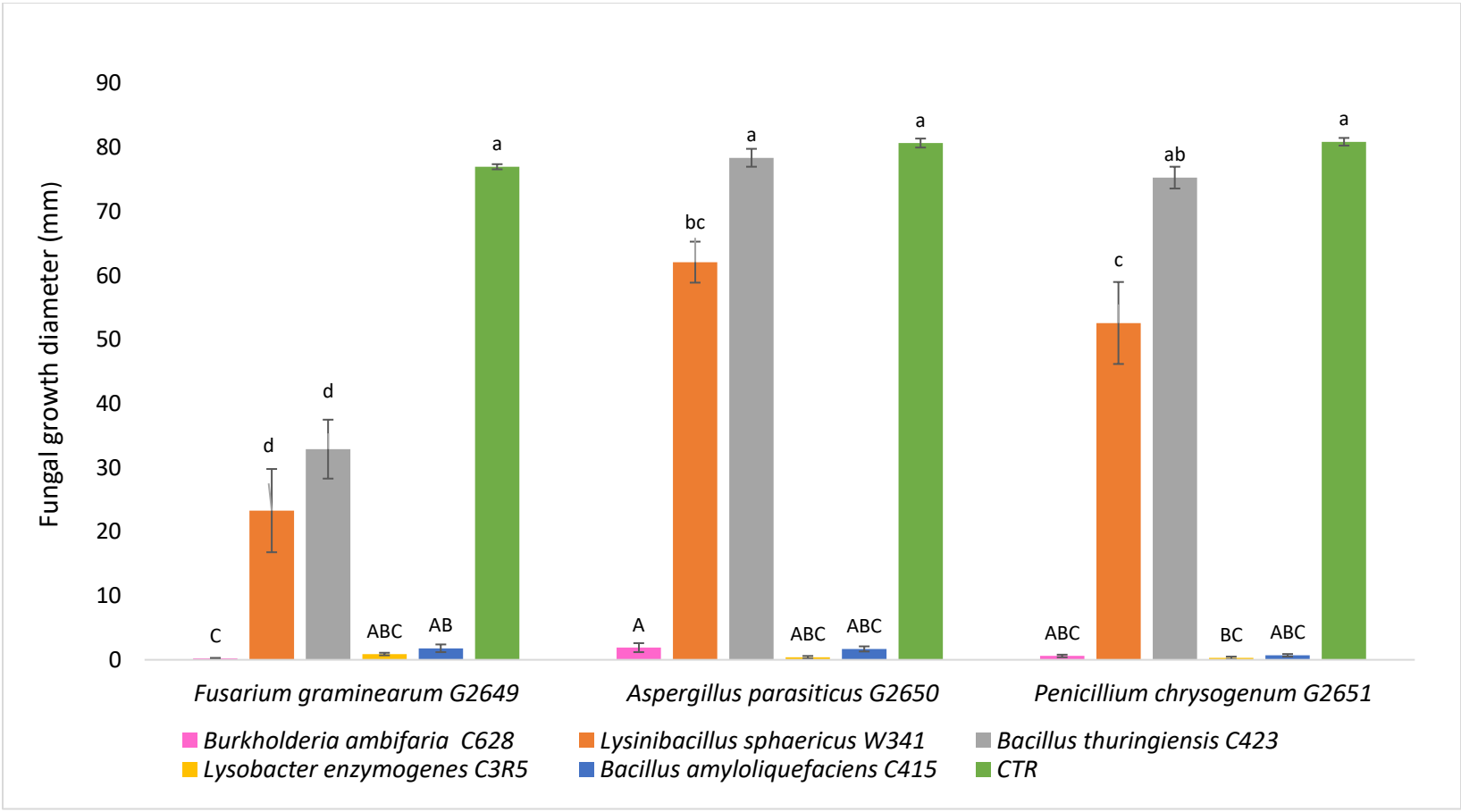

Figure 3.5. Growth of three pathogens – G2649, G2650, and G2651 against five biocontrol agents 7 days after inoculation (DAI) using spread plate technique. Biocontrol treatments include five bacteria strains listed in Table 3.1, C628, W341, C423, C3R5, C415, and water control (CTR). Bars that share a lowercase letter or that share an uppercase letter are not significantly different from one another at $\alpha = 5\%$.

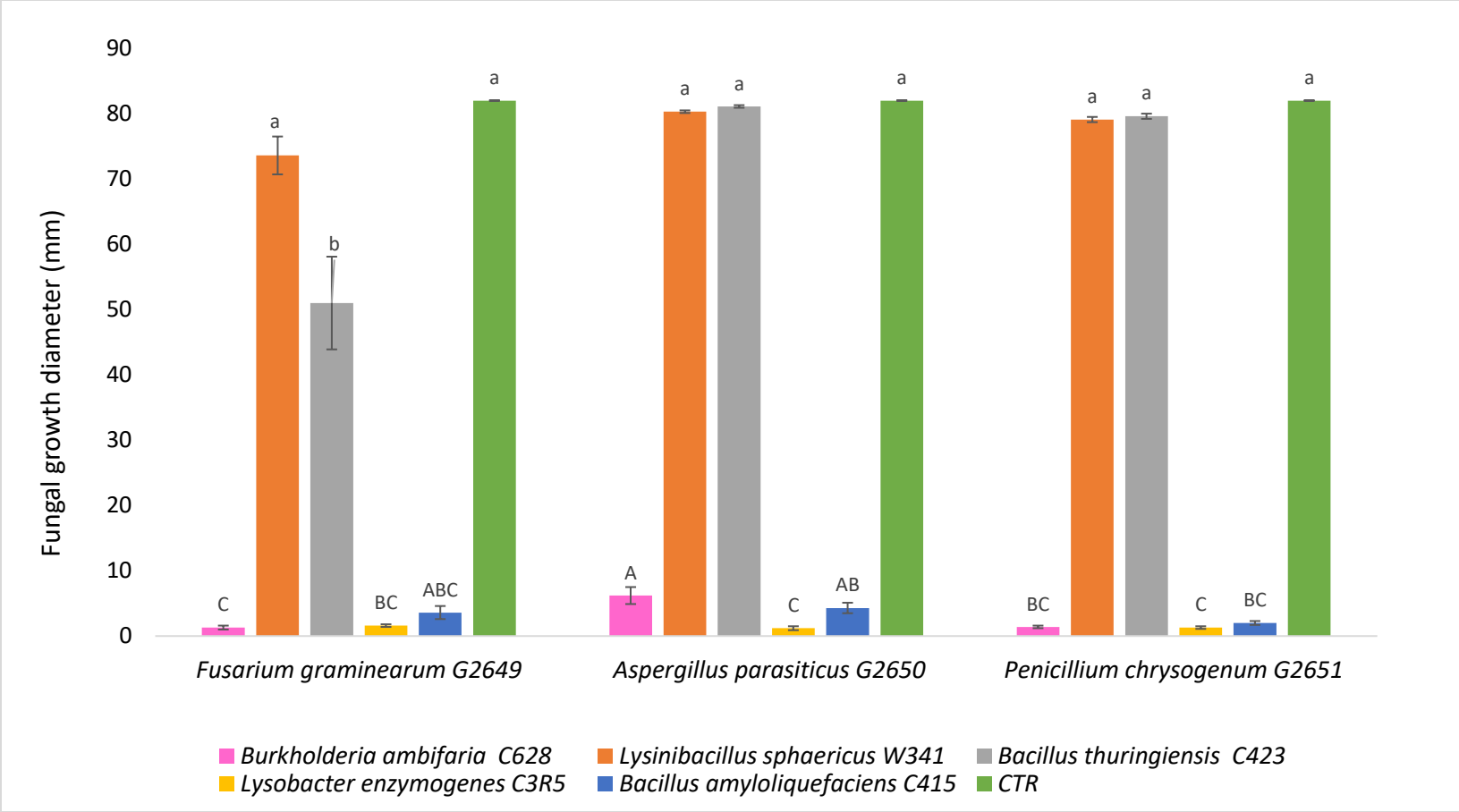

Figure 3.6. Growth of three pathogens – G2649, G2650, G2651 – against five biocontrol agents 14 days after inoculation (DAI) using spread plate technique. Biocontrol treatments include five bacteria strains listed in Table 3.1, C628, W341, C423, C3R5, C415, and water control (CTR). Bars that share a lowercase letter or that share an uppercase letter are not significantly different from one another at $\alpha = 5\%$.

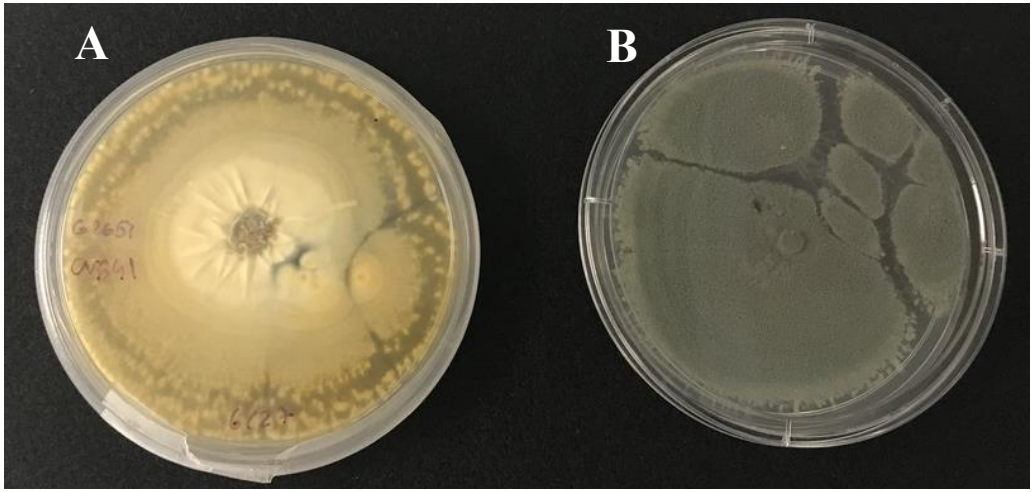

Figure 3.7. A) Spread plate antibiosis assay with *Lysinibacillus sphaericus* W341 against fungal pathogen *P. chrysogenum* showing abnormal growth of *P. chrysogenum.* B) Control petri of the *Penicillium chrysogenum*

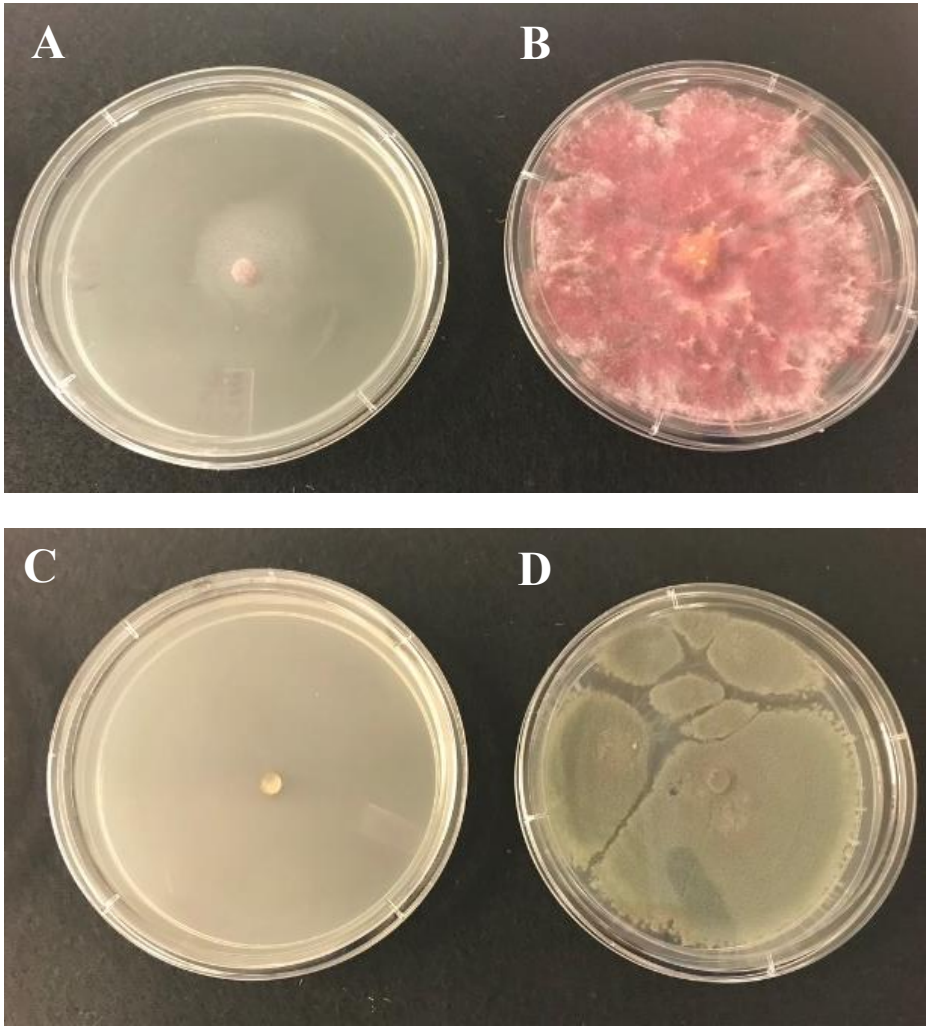

Figure 3.8. (A) *Burkholderia ambifaria* C628 co-inoculated with *Fusarium graminearum* B) growth of only *F. graminearum* as a comparison to plate (A), (C) *Lysobacter enzymogenes* C3R5 co-inoculated with *Penicillium chrysogenum* (D) growth of only *Penicillium chrysogenum* as a comparison to plate.

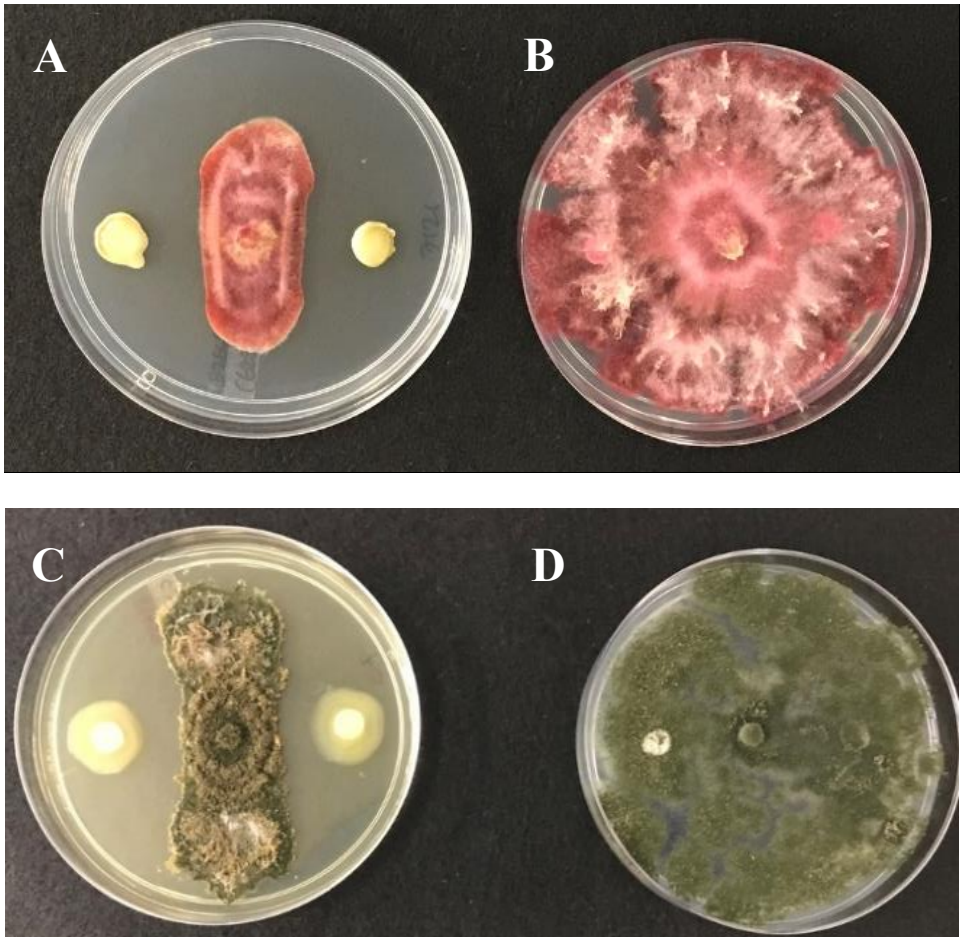

Figure 3.9. Antibiosis assay using filter paper method. (A) *Burkholderia ambifaria* C628 co-inoculated with *Fusarium graminearum*, (B) growth of only *F. graminearum* as a comparison to plate (A), (C) *Lysobacter enzymogenes* C3R5 co-inoculated with *Aspergillus parasiticus,* and (D) growth of only *A. parasiticus* G2650 as a comparison to plate (C).

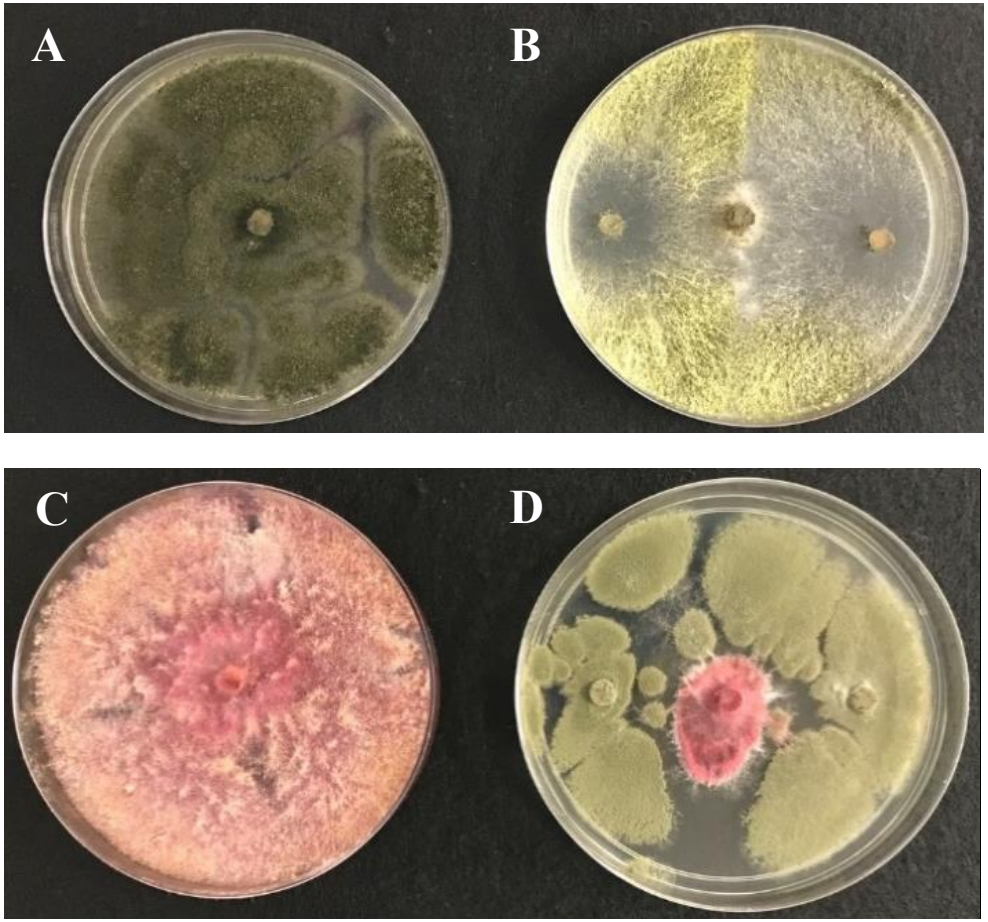

Figure 3.10. Effect of two biological control fungi on two different fungal pathogens. (A) PDA plate with *Aspergillus parasiticus*, (B) *Trichoderma gamsii* E1032 co-inoculated with *A. parasiticus* (C) PDA plate with *F. graminearum*, (D) *Cladosporium* sp. E126 co-inoculated with *F. graminearum*

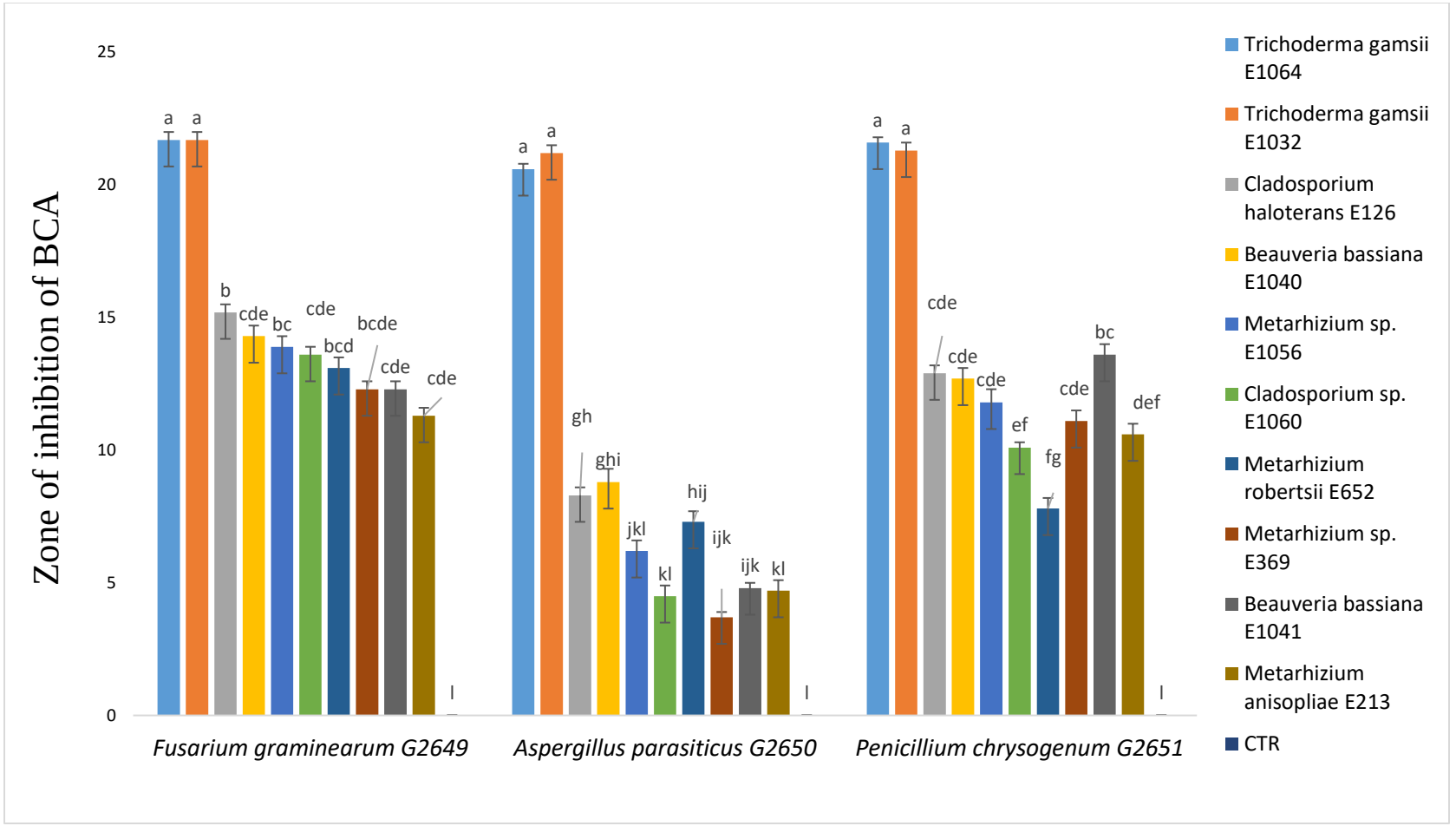

Figure 3.8. Zone of inhibition for agar plug assays with ten fungal biocontrol agents against three pathogens at 7 days after inoculation (DAI). Means with the same letter are not significantly different at p=0.05.

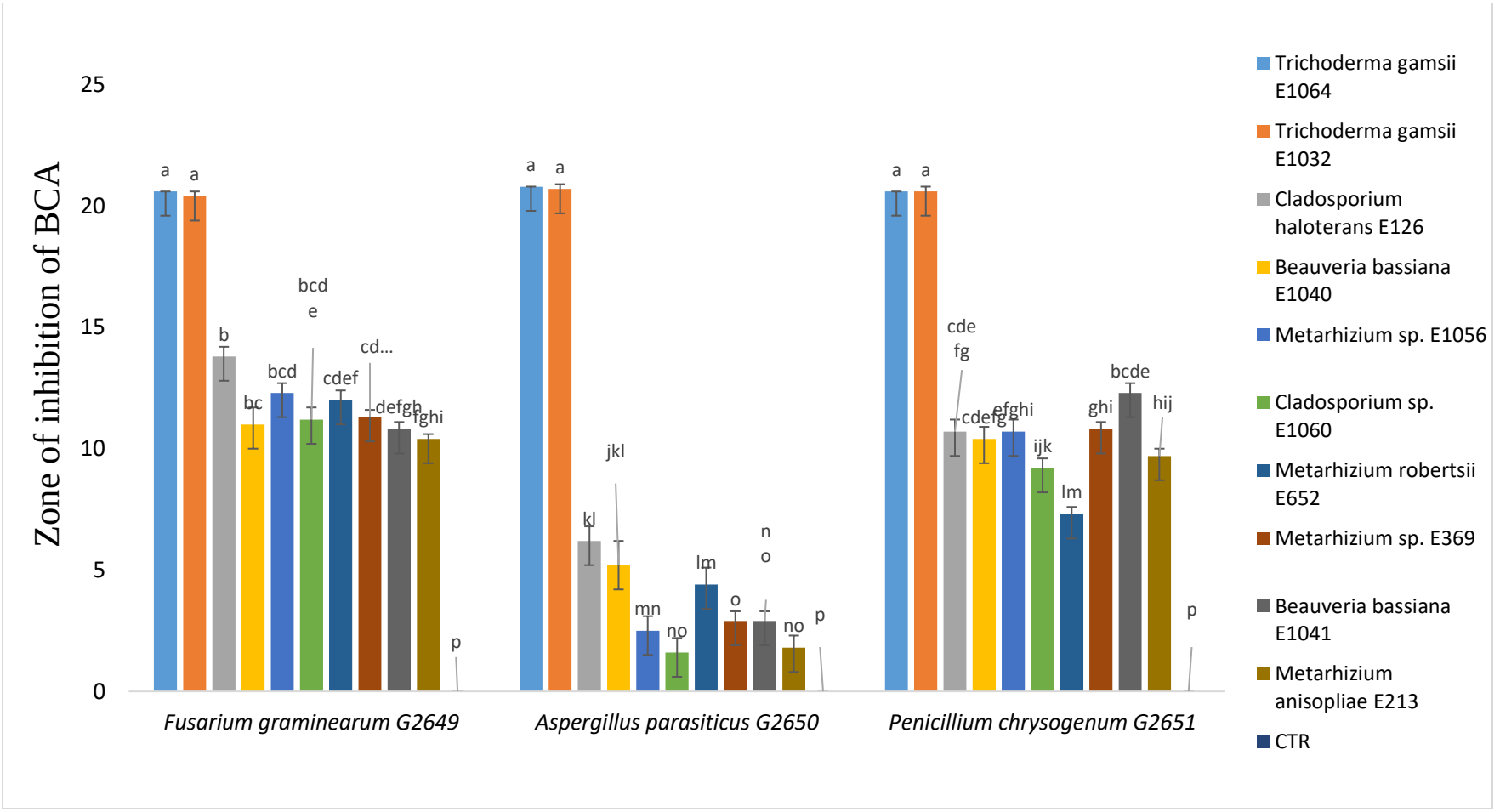

Figure 3.9. Zone of inhibition for agar plug assays with ten fungal biocontrol agents against three pathogens at 14 days after inoculation (DAI). Means with the same letter are not significantly different at p=0.05.

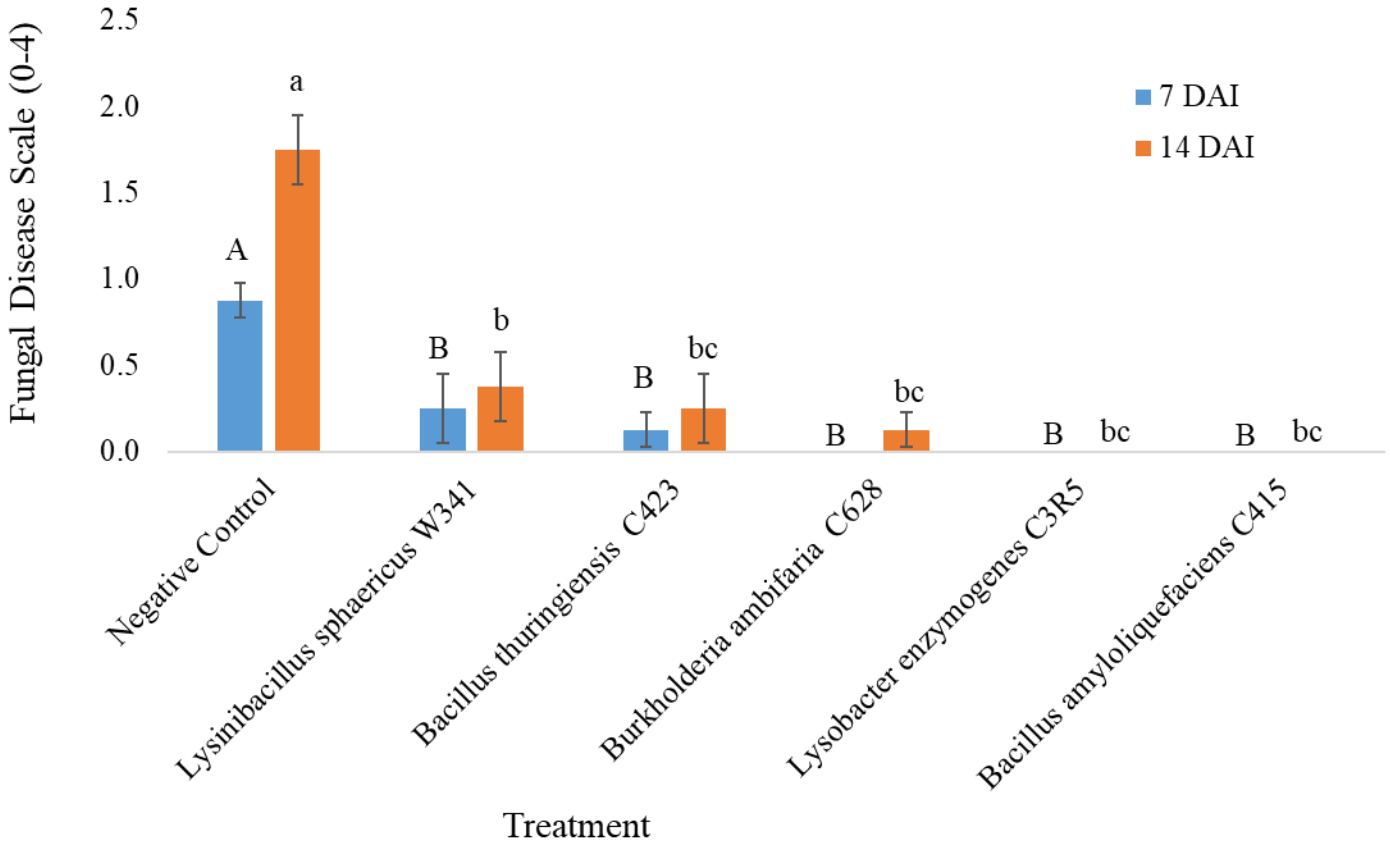

Figure 3.10. For wheat seed assays, growth of *Fusarium graminearum* against five candidate biocontrol bacteria, 7 (blue) and 14 (orange) days after inoculation (DAI). Means with the same letter are not significantly different at p = 0.05; upper case letters indicate comparisons at 7 DAI and lower case letters indicate comparisons at 14 DAI.

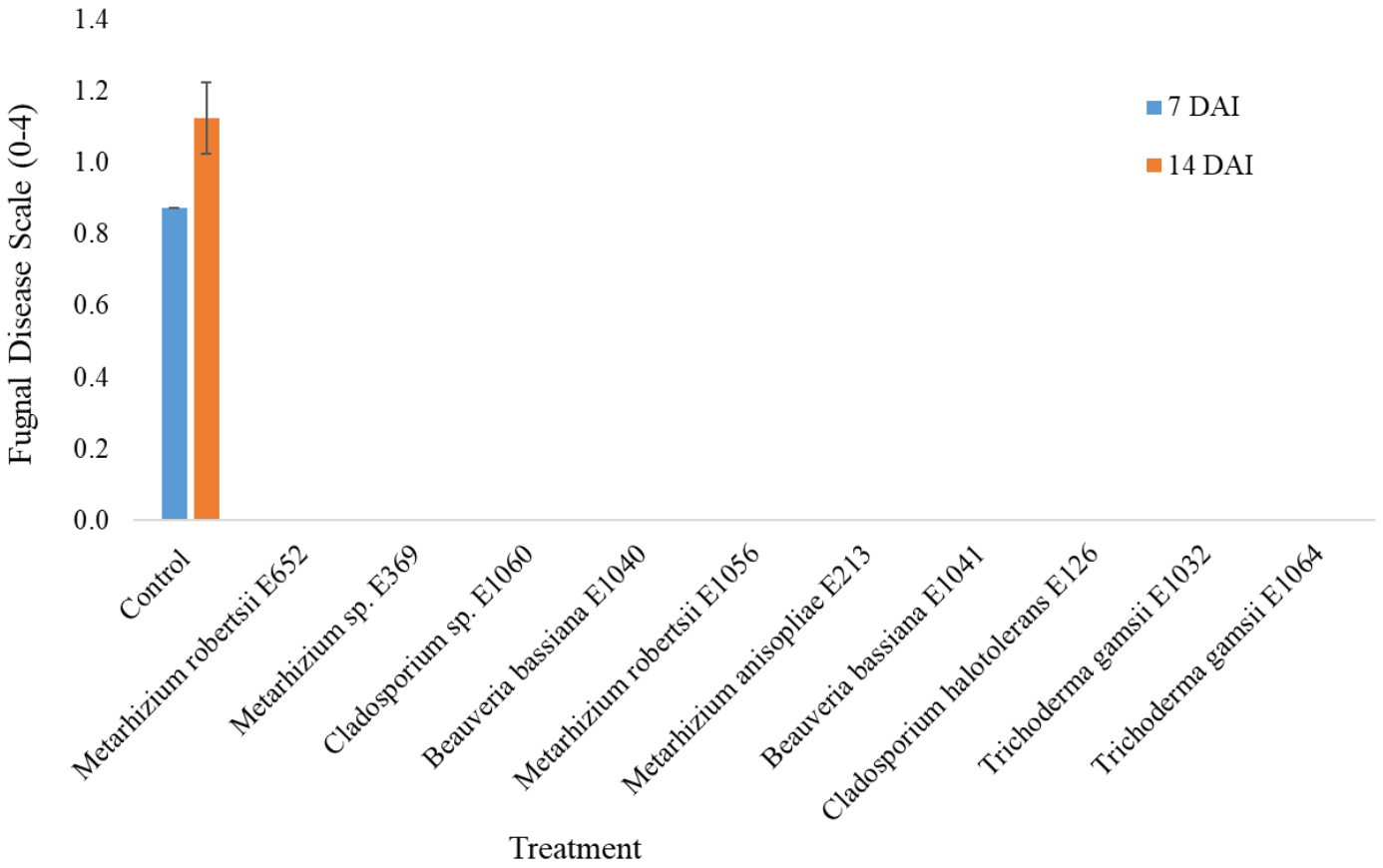

Figure 3.11. For wheat seed assays, growth of *Fusarium graminearum* against ten candidate biocontrol fungi, 7 (blue) and 14 (orange) days after inoculation (DAI). Means with the same letter are not significantly different at p = 0.05; upper case letters indicate comparisons at 7 DAI and lower case letters indicate comparisons at 14 DAI.

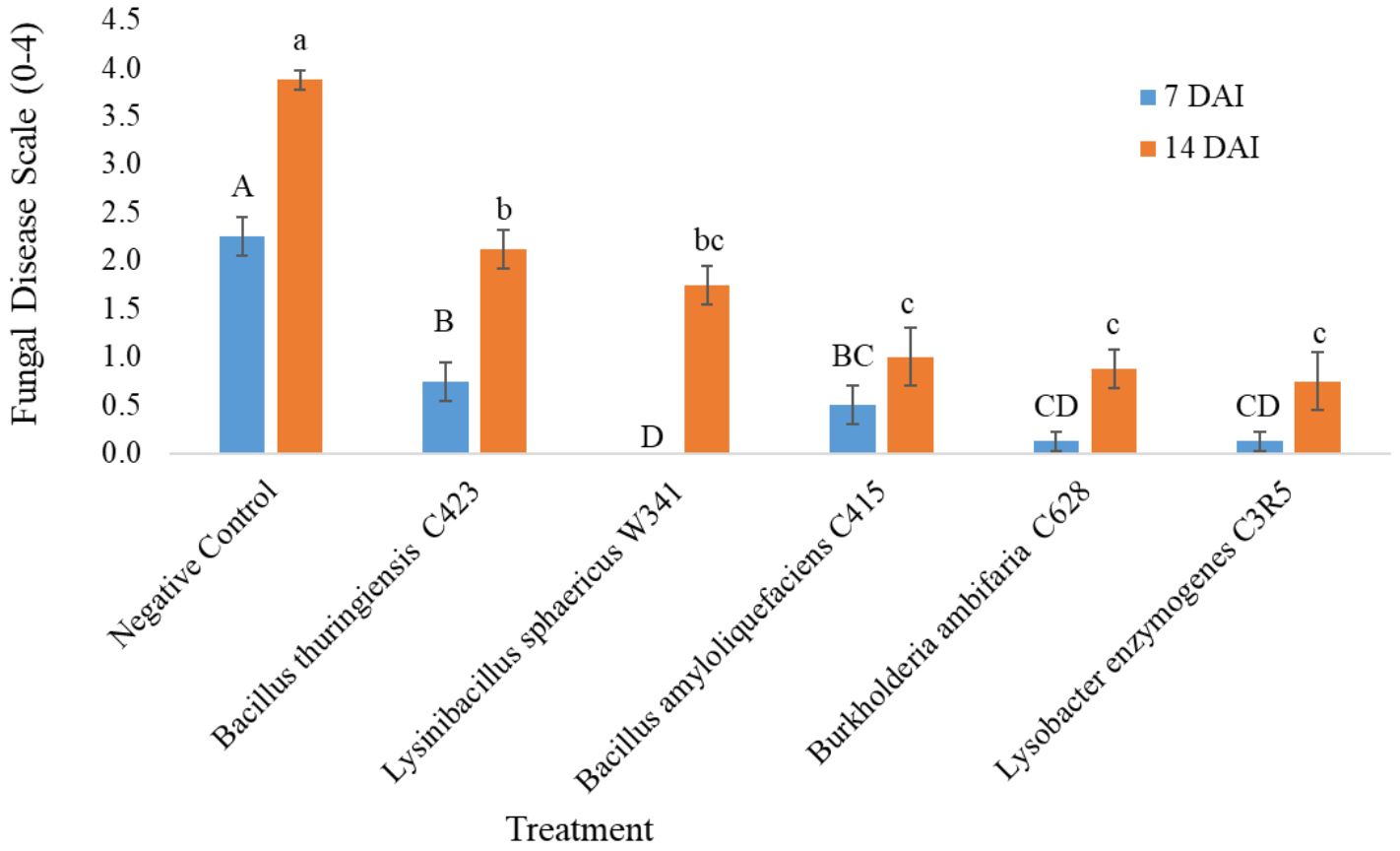

Figure 3.12. For wheat seed assays, growth of *Aspergillus parasiticus* against five candidate biocontrol bacteria, 7 (blue) and 14 (orange) days after inoculation (DAI). Means with the same letter are not significantly different at p = 0.05; upper case letters indicate comparisons at 7 DAI and lower case letters indicate comparisons at 14 DAI.

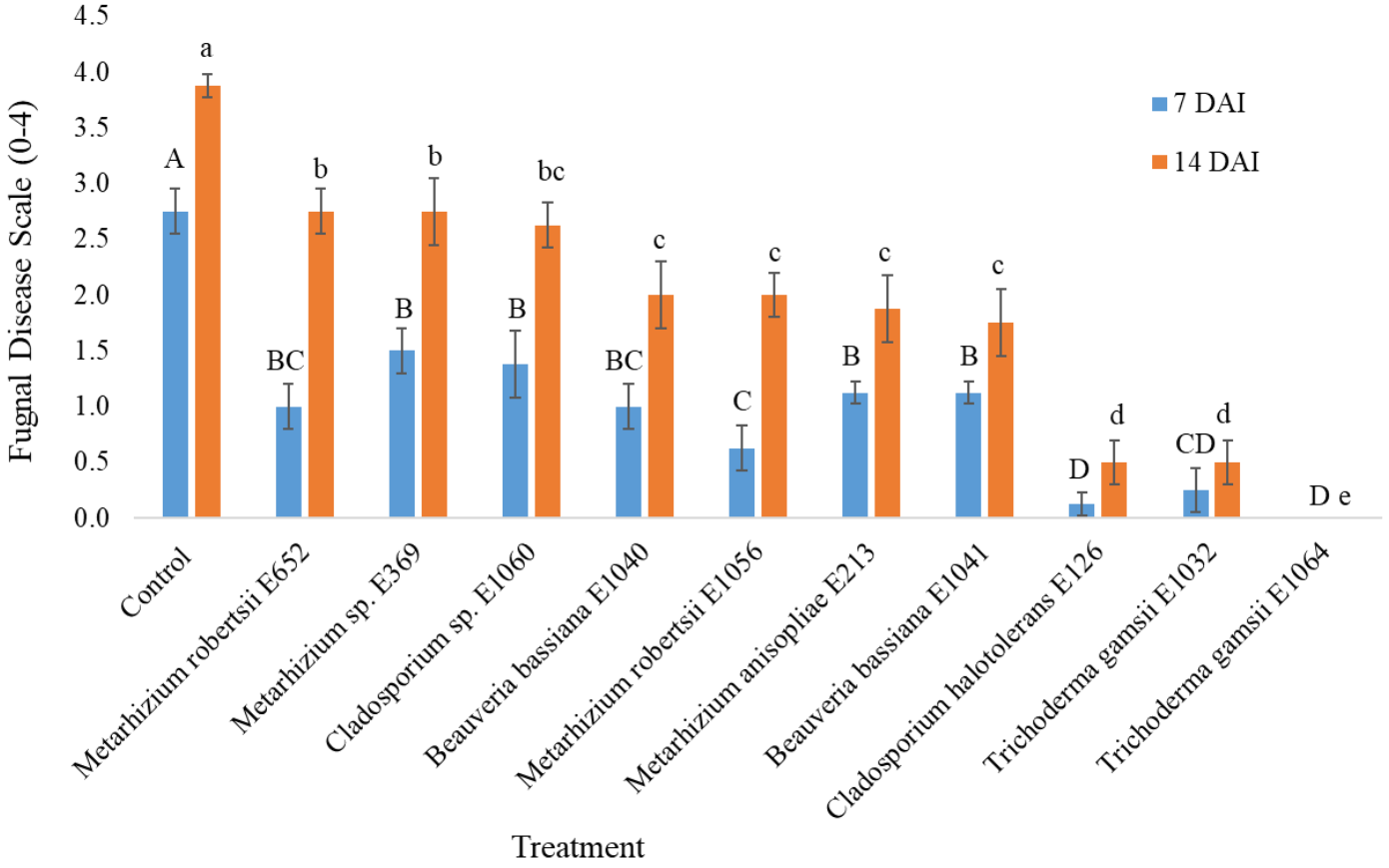

Figure 3.13. For wheat seed assays, growth of *Aspergillus parasiticus* against ten candidate biocontrol fungi, 7 (blue) and 14 (orange) days after inoculation (DAI). Means with the same letter are not significantly different at p = 0.05; upper case letters indicate comparisons at 7 DAI and lower case letters indicate comparisons at 14 DAI.

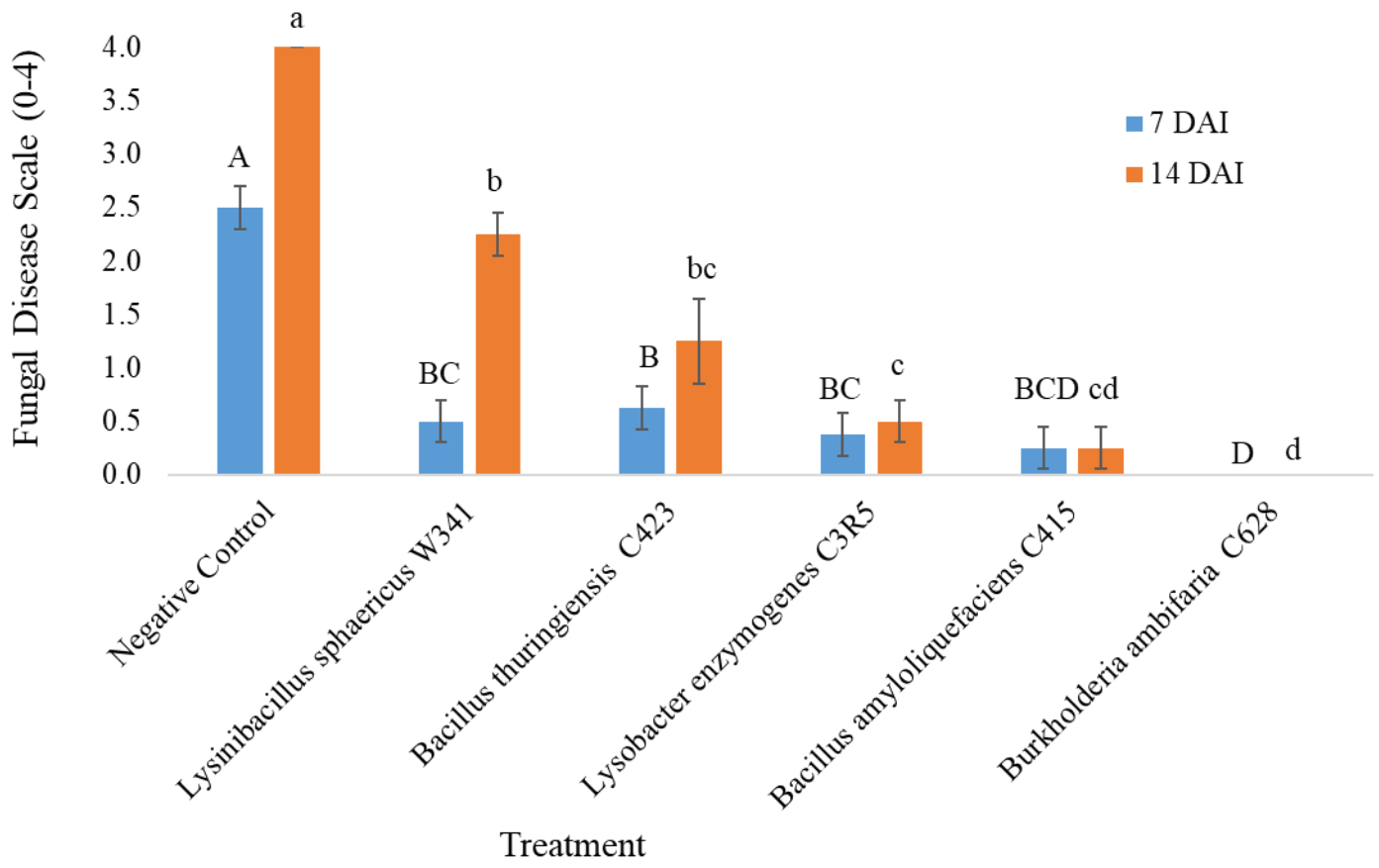

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Figure 3.14. For wheat seed assays, growth of *Penicillium chrysogenum* against five candidate biocontrol bacteria, 7 (blue) and 14 (orange) days after inoculation (DAI). Means with the same letter are not significantly different at p = 0.05; upper case letters indicate comparisons at 7 DAI and lower case letters indicate comparisons at 14 DAI.

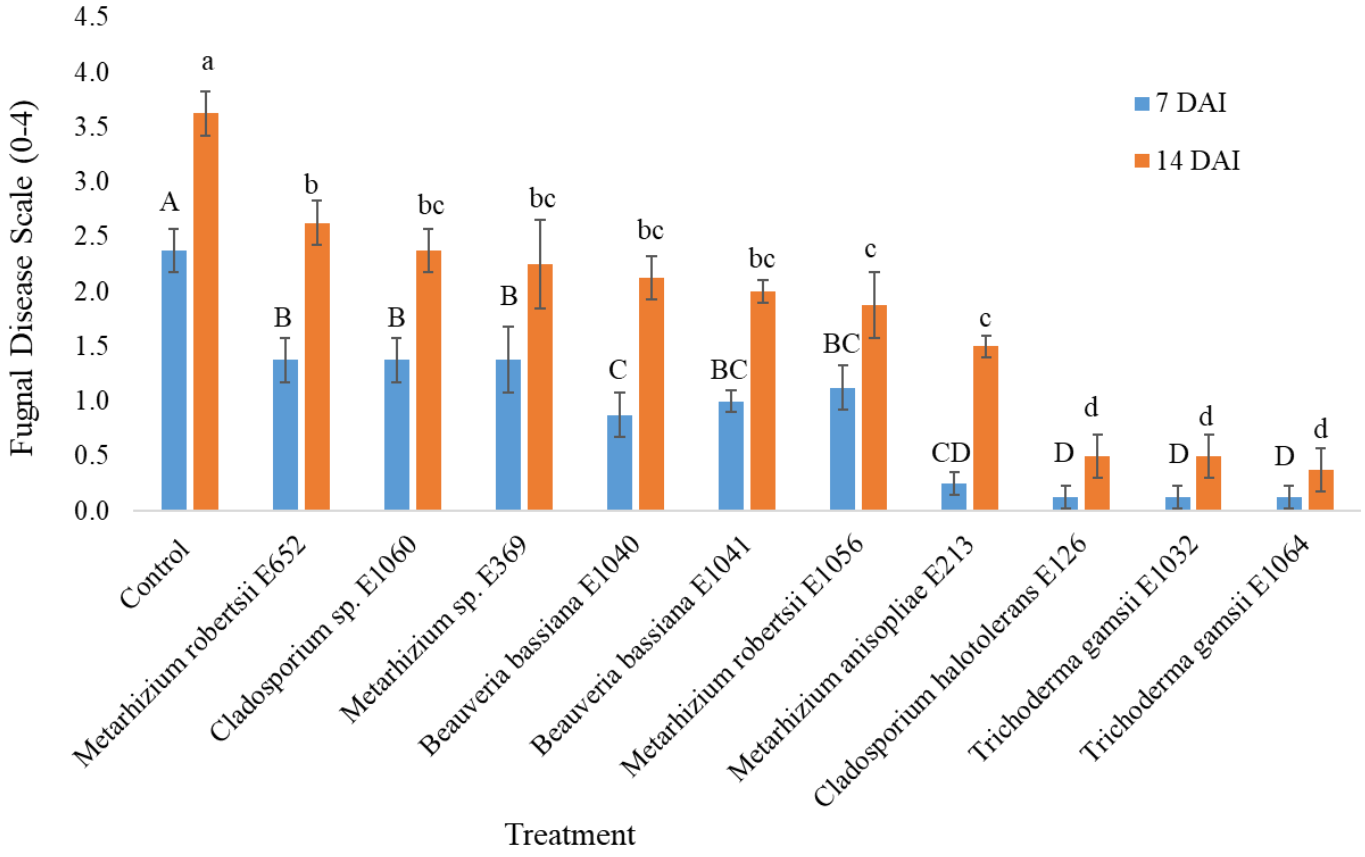

Figure 3.14. For wheat seed assays, growth of *Penicillium chrysogenum* against ten candidate biocontrol fungi, 7 (blue) and 14 (orange) days after inoculation (DAI). Means with the same letter are not significantly different at p = 0.05; upper case letters indicate comparisons at 7 DAI and lower case letters indicate comparisons at 14 DAI.

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CHAPTER 4: CONCLUSIONS

The primary aim of the research described in this thesis was to test the hypothesis that individual microorganism strains that have the ability to inhibit both granary weevil and grain fungal pathogens can be found. Therefore, the specific objectives were created to investigate fungal and bacterial strains to determine activity of potential biocontrol agents against *S. granarius* in terms of their lethal and sublethal effect as well as comparison of their performance with commercial biological and chemical treatments (Chapter 2). The second specific objective was to determine the ability of strains of bacteria and fungi to inhibit growth of three common grain fungal pathogens under *in vitro* and wheat seed assays (Chapter 3).

The research on insect pests (Chapter 2) indicated that all tested fungal isolates showed lethal effects, except two fungal agents that were not significantly different from the negative control (*Metarhizium anisopliae* E213 and *Cladosporium halotolerans* E126). However, *Metarhizium anisopliae* E213 showed strong sublethal effect by reducing ovipositio rate and grain infestation additionally, *Cladosporium halotolerans* E126 minimally reduced oviposition rate yet was significantly different from negative control. Additionally, all tested bacterial treatments had significantly lower survival than the negative control. However, *Bacillus thuringiensis* C423 demonsrated low lethal effect on *S. granaries*. In contrast, *the Bacillus* strain showed strong sublethal effect by reducing feeding damage and oviposition rate.

The study on fungal pathogens (Chapter 3) demonstrated that three out of the five bacteria (*Bacillus amyloliquefaciens* C415, *Lysobacter enzymogenes* C3R5, and *Burkholderia ambifaria* C628) and all ten tested fungi were able to inhibit the three grain

fungal pathogens in antibiosis assays. Result were the same between *in vitro* antibiosis assays and the wheat seed assay, except for one strain. *Lysinibacillus sphaericus* W341 did not suppress the fungal diseases in the antibiosis assays, but was effective in the weed seed assay.

Two fungal isolates (*Trichoderma gamsii* E1032 and E1064) and one bacterial isolate (*B. amyloliquefaciens* C415) achieved dual control against both the insect pest and the fungal pathogens. *Trichoderma* species have strong potential to be biocontrol agents. They are able to produce bioactive metabolites, which help them to complete a mycoparasitic or entomopathogenic life cycle and induce resistance in the host, such as production of hydrolytic enzymes including chitinase and glucanase (Ownley et al. 2010). Due to their potential, *Trichoderma* spp. have been widely tested against fungal pathogens that cause plant disease, particularly soilborne pathogens (e.g. Elad et al.1980, Elad et al. 1981, Chen et al. 2016). In addition, studies with insect pests showed that *Trichoderma hamatum* can cause mortality of cotton aphid, *Aphis gossypii*, and yellow mealworm beetle, *Tenebrio molitor* (Shakeri and Foster 2007, Khaleil et al. 2016). *Bacillus amyloliquefaciens* is another promising biocontrol agent which can produce antibacterial and antifungal metabolites (Ji et al. 2013). However, the effectiveness of *T. gamsii* and *B. amyloliquefaciens* on *S. granarius* has not been previously reported. This study is the first to report mortality to *S. granarius* caused by these species, indicating that they may be potential entomopathogens. Moreover, this is the first report that *T. gamsii* and *B. amyloliquefaciens* have dual effect in artificial conditions on *S. granarius* and three grain fungal pathogens, *F. graminearum, A. parasiticus,* and *P. chrysogenum.*

Another important point of the study was characterizing bacterial biocontrol agents using two different antibiosis methods. Several different screening methods can be used to screen and detect microorganisms for use as biocontrol agents and the methods to use depends on the potential mechanisms that the microorganisms might use (Fravel 2005) or the combination of methods that appear to be the most logical. For example, while the spread plate technique might be used to screen for a biocontrol agent that uses the mechanism of predation and/or competition, the method of impregnation of filter papers with the potential biocontrol agent will be better to detect organisms that use the mechanism of antibiotic production (Pal and McSpadden Gardener 2006, Parikh et al., 2018). When the mechanisms that the microorganisms could use is unknown, multiple screening methods (e.g., using both spread plate and filter paper impregnation) will help increase the chance of detecting potential biocontrol agents. This explains why both spread plate and filter paper impregnation methods were used in the current study. It is important to use both methods in the future to screen microorganisms with unknown mechanisms.

In this study, all bacterial and fungal isolates were tested against three fungal grain pathogens with both *in vitro* antibiosis assays and wheat seed assays. Results obtained from *in vitro* studies may not show parallel results with studies conducted under *in planta* or more realistic conditions. Thus, wheat seed assays were important to make conclusions about the effectiveness of the tested isolates under more realistic conditions. However, testing isolates using both *in vitro* and wheat seed assays is not always practical in terms of cost and time, especially when the number of tested isolates is high. Therefore, under these circumstances, *in vitro* Petri plate assays should be conducted to

select the most effective isolates, which are then tested via *in planta* assays. So that, the more effective isolates can be tested under conditions which are closer to the real scenario.

In the wheat seed assay, the seed was autoclaved to decontaminate any kind of microorganisms that were present on the wheat prior to the start of the experiment. Although the autoclaved grain was able to germinate within 7 to 10 days in this study, the process of high heat and pressure during autoclaving can affect seed viability. Therefore, the conditions of the wheat seed assays may not have exactly represented conditions of stored grain in a real storage area. In future studies, soaking the seeds in a dilute bleach solution could be used as an alternative method to autoclaving the grain for surface disinfection and keep the seed viable.

To increase the success of biological control, candidate bacterial/fungal agents were chosen based on several characteristics, including past record of entomopathogenic or antifungal properties, ability to grow in the laboratory, and taxonomic diversity. For example, *Trichoderma* spp. was used due to their unique characteristics of rapid growth and production of anti-microbial metabolites, which makes them excellent management tools (Verma et al. 2007). *Metarhizium* spp. have been frequently used as biocontrol agents against insect pests (Kavallieratos et al. 2014) and plant diseases (Keyser et al. 2016). For example, *M. brunneum* was able to inhibit growth of the pathogen *Fusarium culmorium* (Keyser et al. 2016). *Metarhizium robertsii* can also serve as a plant promoter (Sasan and Bidochka 2012). Moreover, tested fungal biocontrol agents were screened previously against the insect pest western corn rootworm (*Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae), with *M. anisopliae* E213, *M robertsii* E1056, and

Cladosporium sp. E1060 showing high mortality (Oliveira Hofman 2018). This demonstrated ability of these entomopathogenic fungi against western corn rootworm was an important reference for choosing these isolates to test against the granary weevil and three fungal grain pathogens. In addition, *Burkholderia ambifaria* C628 was tested previously against *Fusarium graminearum* and it was able to suppress fungal growth under *in vivo* conditions (Parikh et al. 2018). Other important consideration in choosing the isolates for this study was their environmental requirements, such that the niche of the insect pest and fungal pathogens overlapped with the candidate biocontrol agents (Table 1.1). Additionally, we tested bacteria and fungi, from multiple genera so that we were able to evaluate organisms representing a broad range of diversity. Although species may be morphologically indistinguishable, they have different genetic biochemical, and physiological features. For example, one of the most effective fungal strains evaluated in the present study was *Cladosporium halotolerans* E126, yet *Cladosporium* sp. E1060 did not show antifungal activity. In addition, *Bacillus thuringiensis* C423 was not effective in causing granary weevil mortality and disease suppression; however, *Bacillus amyloliquefaciens* C415 achieved dual control against both insect and fungal pathogens.

The modes of action of entomopathogenic bacteria might also be a contributing factor to the observed delay in insecticidal activity. For some entomopathogenic bacteria, the mode of requires oral consumption. Thus, the infection and colonization processes are affected by factors such as agressiveness of the biocontrol agent and host immune defense. For example, entomopathogenic bacteria first move into the host body through the hemocoel and then propagate inside of the insect body. They cause disease by producing virulence factors, such as crystalline proteins, and eventually kill the host

(Glare et al. 2017). Other entomopathogenic bacteria, referred to as ‘antagonists’, do not require ingestion by the target insect. Antagonists can affect insects indirectly via excretion of insecticidal enzymes or secondary metabolites into the environment. One bacterial species tested in this study, *L. enzymogenes* C3, was reported to produce chitinases and an antibiotic that have activity against nematodes (Chen et al., 2006; Yuen et al., 2018), and it is possible these mechanisms may affect insects as well.

This study is important and unique in several ways. First, there are no effective chemical pesticides that are registered against both granary weevil and fungal grain pathogens. Therefore, finding isolates that showed effectiveness on these two pests is significant. Moreover, although there are several reports of a dual effect of a biocontrol agent against insect and fungal pests, there are no studies looking specifically at one of the most important grain insect pests, *Sitophilus granarius*, and three common grain fungal pathogens, *Fusarium graminearum, Aspergillus parasiticus,* and *Penicillium chrysogenum,* in the same study, which makes this research unique. Many studies have investigated the efficacy of biocontrol agents against stored product pests, however, none reported sublethal effects of biocontrol agents on stored product pests. Most previous studies were only conducted with fungal isolate so, our results for bacteria represent a novel contribution of information to the literature. Additionally, *Sitophilus granarius* may coexist with other *Sitophilus* species, therefore, isolates found to be effective have may hold promis to control to control other *Sitophilus* species.

Collectively, the results of the current study suggest that there is potential to find biological control agents with dual function to affect the granary weevil and grain fungal pathogens. However, there are several limitations to the current study and need

for additional research. This includes how the fungi were inoculated onto the grain. Although the technique used for fungal incoluations used a suspension of spores in water, this is not the usual way for these fungal pathogens to disperse and infect the host plant. For example, *Aspergillus parasiticus* and *Penicillium chrysogenum* both use wind as a mechanism for dispersal, not water. Thus, future studies will need to create or implement inoculation methods that are more realistic. Another limitation of the current study was that the conditions under which this study was conducted were not favorable to colonization of the wheat seed by *Fusarium graminearum*. So future studies should conduct these types of studies under higher temperature and humidity conditions that are more similar to the conditions that F. graminearum requires for growth. In the insect studies, a limitation was the homogeneous nature of the inoculation of insects in the artificial conditions of this study, which means that in large storage bins, this could be a limitation to the effectiveness of these strains. One possible outcome is that such biocontrol agents would be more appropriate for smaller seed lots, such as in silo bags, where a more even distribution of the inoculum could be achieved.

Ultimately, the novelty of this research is as a proof-of-concept study in evaluating whether these microorganisms have a dual effect on insect and fungal pests. Although the aim of finding isolates with the ability to control both an insect pest and grain fungal pathogens was achieved, there is the need to evaluate survival, feeding damage and reproduction, and antibiosis activity under real storage bin conditions in order to determine whether these strains should be considered as biocontrol agents. Additionally, to determine potential synergistic effects of applying multiple biocontrol agents together or with other control strategies should be tested. Such as wheather

effective biocontrol bacteria fungi can be and combinated with diatomaceous earth, which causes mortality of *S. granarius* and increase effectiveness and/or speed of mortality.

Grain is usually, stored for a long period, so that residual effect of the biocontrol agent plays a significant role in safely protecting stored grains. Therefore, survival and /or residual activity of the effective isolates on the grain should be tested over a long period. Contrary to other *Sitophilus* species, *S. granarius* does not have wings and therefore must walk on the grain. An effective isolate could be formulated with oil or other adjustment to enhance its contact effect. Additionally, these isolates should be tested under different temperatures, humidity, and grain types, which play a key role in pest biology and also the performance of biocontrol agents. The effective isolates should be tested at a higher temperature and relative humidity to see their effectiveness against *F. graminearum.* Moreover, isolates that showed effectiveness in this study should be tested to determine their risk for toxicity towards mammals prior to use in stored grains.

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Table 4.1. Summary of results from all experiments conducted with potential biocontrol agents against granary weevils and grain fungal pathogens.

Bacterial/Fungal Agents:	Granary Weevil			*Fusarium graminearum*				*Aspergillus parasiticus*				*Penicillium chrysogenum*			
	Mortality	Feeding Damage	Oviposition	Filter Paper	Spread Plate	Agar Plug	Wheat Seed	Filter Paper	Spread Plate	Agar Plug	Wheat Seed	Filter Paper	Spread Plate	Agar Plug	Wheat Seed
Bacillus amyloliquefaciens C415	+	-	+	+	+		*	+	+		+	+	+		+
Burkholderia ambifaria C628	+	-	-	+	+		*	+	+		+	+	+		+
Bacillus thuringiensis C423	+	-	+	-	-		*	-	-		+	-	-		+
Lysinibacillus sphaericus W341	+	+	+	-	-		*	-	-		+	-	-		+
Lysobacter enzymogenes C3R5	+	+	+	+	+		*	+	+		+	+	+		+
Beauveria bassiana E1040	+	-	+			+	*			+	+			+	+
Beauveria bassiana E1041	+	+	+			+	*			+	+			+	+
Cladosporium halotolerans E126	-	-	+			+	*			+	+			+	+
Cladosporium sp. E1060	+	-	+			+	*			+	+			+	+
Metarhizium anisopliae E213	-	-	+			+	*			+	+			+	+
Metarhizium robertsii E1056	+	-	+			+	*			+	+			+	+
Metarhizium robertsii E652	+	+	+			+	*			+	+			+	+
Metarhizium sp. E369	+	+	+			+	*			+	+			+	+
Trichoderma gamsii E1032	+	-	+			+	*			+	+			+	+
Trichoderma gamsii E1064	+	+	+			+	*			+	+			+	+

(+) the result is different from negative control (in all trials)

(-) the result is same as negative control (in at least one trial)

(*) no reliable result