ECOLOGY OF *BACILLUS* ON WHEAT FLORETS: INSIGHTS FOR IMPROVED BIOLOGICAL CONTROL ON AERIAL PLANT SURFACES

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ECOLOGY OF *BACILLUS* ON WHEAT FLORETS: INSIGHTS FOR IMPROVED BIOLOGICAL CONTROL ON AERIAL PLANT SURFACES

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The biological control agent *Bacillus amyloliquefaciens* strain TrigoCor provides significant and consistent control of Fusarium head blight in greenhouse settings but not in field environments. To investigate potential ecological factors contributing to the poor performance of TrigoCor in field trials, we measured the levels of *Bacillus* populations and of a critical *Bacillus*-produced antifungal metabolite, iturin, on wheat spikes in the greenhouse and the field for 14 days post-TrigoCor application. We found that on wheat spikes in both environments, the levels of *Bacillus* populations remained fairly stable throughout the sampling period, but the levels of iturins declined drastically by 3 days post-application. Results from greenhouse trials and antibiosis assays indicated that the biological activity of TrigoCor-synthesized compounds was close to the minimum threshold needed for successful *Fusarium graminearum* inhibition and disease suppression. The decline observed for iturins on wheat spikes is therefore likely limiting for disease control, particularly in field environments where natural *Fusarium* infections can occur for a period of over 10 days post-anthesis.

To increase the production of iturins on wheat spikes, we sought to stimulate the germination of metabolically dormant *Bacillus* spores present on wheat surfaces. Using the terbium chloride assay and phase contrast microscopy, we screened potential nutrient germinants of TrigoCor spores at room temperature and at 37 ºC, and identified two germinant combinations that produced high levels of TrigoCor spore germination *in vitro*. One of these germinant

combinations, consisting of an equimolar mixture of L-asparagine, D-glucose, D-fructose, and potassium chloride, also produced significant *Bacillus* germination on wheat surfaces. However, neither germinant combination was able to significantly improve disease control with TrigoCor spores in greenhouse settings, in one case likely due to its stimulatory effect on *Fusarium* growth.

Finally, we used epifluorescent stereomicroscopy and confocal laser scanning microscopy to visualize the spatial distribution of TrigoCor inoculum on wheat spikes post-application, and found that the coverage of inoculum sprayed onto wheat spikes in the greenhouse was much higher than that in the field. We also observed that significant inhibition of *Fusarium* ascospores only occurred when the spores were in direct contact with TrigoCor inoculum droplets. Inadequate spray coverage of wheat spikes in field settings may therefore limit disease control in this environment by leaving large regions of the wheat spike unprotected against *Fusarium* germination and colonization.

Future optimization efforts for above-ground biological control with *Bacillus* should focus on improving *in situ* metabolite production by *Bacillus*, as well as on increasing application coverage on plant surfaces in the field. *Bacillus* biological control might be best suited for diseases with short, predictable infection periods or where multiple applications are feasible.

BIOGRAPHICAL SKETCH

Julia was born in 1983 to Patricia (Pat) and John Robert (Bob) Crane. As is true for many political families like Julia's, the Cranes lived in the District of Columbia suburbs, principally in Silver Spring, Maryland. Even as a child Julia was an academic generalist, and had as equal and strong a love for the sciences as she did for the humanities. As an illustration for this generalist mentality and for her intellectual lust (nerdiness), Julia attended a humanities and communications magnet program for middle school, and then a math, science, and computer science magnet program for high school. She also spent a significant amount of time volunteering for environmental organizations, and developed an appreciation for nature through these experiences, as well as through her explorations of the parks in her community and on trips with her parents.

Julia attended the University of Maryland in College Park from 2002 to 2006. When she started college she focused her studies on the humanities, however Julia could not ignore the siren call of the sciences, and eventually she started taking more biology classes. Julia finally decided to concentrate her studies on Anthropology and Environmental Science & Policy, and ended up graduating summa cum laude with a double degree in these subjects.

While in college, Julia interned for a summer as a horticulture intern at the Baltimore Aquarium, and realized through this job that she enjoyed working with plants. The following summer she interned in Dr. William Turechek's strawberry pathology lab at the US Department of Agriculture's Agricultural Research Service site in Beltsville, Maryland. While in this position Julia was introduced to research on bacteria, fungi, and oomycetes, and spent a good deal of time culturing microbes and performing PCRs, as well as working long hours in the fields. This was Julia's first foray into plant pathology, and she enjoyed the research and free

strawberries so much that she continued in this position for an additional year. During this time Julia also assisted a wheat breeder, Dr. Jose Costa, at the University of Maryland on his analysis of wheat genetic and agronomic data.

Julia began her studies at Cornell in Gary Bergstrom's lab in 2007. Julia has been very involved in her department's graduate student association, and served as both president and vice president for this organization. She also helped organize many of the social events for the department. Julia has met many inspirational and fun people at Cornell, and has enjoyed her integration into upstate New York life.

For my parents.

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CHAPTER 1

BACILLUS ECOLOGY AND BIOLOGICAL CONTROL ON ABOVE-GROUND PLANT PARTS: LITERATURE REVIEW AND RESEARCH OBJECTIVES

Biological control on above-ground plant parts[1]

Microbial ecology on above-ground plant surfaces

The environment on above-ground plant surfaces is fairly inhospitable for microbes. As described in this section, environmental stresses on above-ground plant parts shape the communities, spatial distributions, and interactions of microbes on plant surfaces. However, in many cases, *Bacillus* spores appear to be fairly resilient to the stresses faced by their microbial peers due to their ability to form environmentally resilient spores.

One major environmental stress on aerial plant surfaces is UV radiation, which affects microbes directly by causing DNA damage, or indirectly by altering plant physiological processes (151). Defense against UV radiation in fungi can take the form of protective pigments or hyphal thickenings, whereas in bacteria UV radiation defense is focused on repairing damaged DNA through photoreactivation (151). Avoidance of radiation through colonization of the abaxial surface of leaves (152) or internal plant regions (172) can also facilitate microbial survival on aerial plant surfaces. In *Bacillus*, spore formation enables an additional layer of defense against UV radiation. During radiation exposure, spores form photoproducts that are less harmful than the photoproducts formed in vegetative cells, and spores are efficient at repairing these photoproducts when they resume vegetative growth. The alternative

[1] Please note that several reviews have been written on the information presented in this section (83-85, 88, 95, 96, 190), and should be consulted for more detailed information on these topics.

photoproducts formed in spores are caused by the binding of protective proteins to spore DNA, and also possibly by the low water content of spore cores (142).

Desiccation stress is another major constraint faced by microbial inhabitants of above-ground plant surfaces. Monier and Lindow (102) studied the survival of *Pseudomonas* cells in response to desiccation, and found that desiccation led to the death of a large proportion of cells, but also that the formation of large aggregates facilitated greater survival of bacterial cells as compared to a solitary existence. However, in an experiment where *Bacillus* was exposed to desiccation stress on plant surfaces, it did not form aggregates or experience a rapid population decline (90). Most of the *Bacillus* cells in this experiment were found to be in the spore form, which is unable to form aggregates but is also more resistant to desiccation stress through the binding of DNA by protective proteins (108, 142).

Nutrient limitation is a third stress faced by microbial inhabitants of aerial plant surfaces. Bacterial biosensors have been used to map the availability of iron (71) and sugars (82) across leaves, and have indicated that iron is fairly available on plant surfaces but that sugars are present mainly in small "oases." As a result, bacteria such as *Pseudomonas* often form aggregates around areas of water or nutrient abundance, such as near trichomes, in grooves between veins (103), or near stomates (92). *Bacillus* cells on leaves have not been found to associate with particular structures (90), again presumably because they are typically in the spore form.

The microbial inhabitants of above-ground plant surfaces are complex (177), and vary across time and space. For instance, the abundance and diversity of bacterial inhabitants, mainly *Bacillus*, were found to decline on the soybean phyllosphere as the season progressed, and the amount of *Bacillus* on lower versus upper leaves changed over time as well (5). On wheat, the

population levels of microbial inhabitants were higher on leaves as opposed to spikes, and changed through time in a pattern specific to the particular microbial species studied (79). Additionally, the levels of microbial populations on wheat plants were approximately three times greater in the field as compared to the greenhouse (79). Bacteria, particularly proteobacteria, appear to be dominant on many phyllosphere surfaces, however yeast are also quite common, as are some filamentous fungi, commonly present as spores (84, 170). Although *Bacillus* is frequently recovered from plant surfaces, including wheat spikes (182), it is likely there only transiently, and instead its main habitat is believed to be the soil and/or insect and animal intestines (61, 153).

Fusarium head blight background and biological control

Relevant information on Fusarium head blight biology and on the utility of biological control in this system is discussed in several of the research chapters of this dissertation, as well as in several reviews (81, 95, 96, 163). Briefly, the main causal pathogen of this disease in North America is *Fusarium graminearum*, which lands on wheat spikes in the form of asexual conidia or, primarily, as sexual ascospores. *Fusarium* spores germinate on wheat surfaces then grow to more vulnerable adaxial plant regions, typically reaching them through openings in the palea and lemma (17). Fungal hyphae invade the plant through direct penetration (17) then colonize the spike by moving through the rachis. Throughout the spike colonization process, *Fusarium* produces the mycotoxin deoxynivalenol (68, 72). Deoxynivalenol-contaminated grain induces vomiting following ingestion, and is referred to as a vomitoxin. Reductions in deoxynivalenol are a major management goal for Fusarium head blight. Disease management is currently limited by the lack of highly resistant wheat cultivars (10) and by the variable or incomplete

control by commercially available fungicides (14, 96, 100, 118, 171). In addition to the commonly cited advantages for biocontrol in general, an additional benefit of biological control in this system is that there are no legal restrictions on the timing of biocontrol applications. Biocontrol agents can therefore be applied to prevent late-season deoxynivalenol-producing infections (31, 33) that cannot be prevented by fungicides due to a 30 days pre-harvest restriction on their application timing (39, 95).

Numerous biological control agents have been developed for control of Fusarium head blight, and a list of bacterial, yeast, and filamentous fungi biocontrol agents can be found in the literature (88). Several strategies have been employed for Fusarium head blight biological control. For biocontrol agents applied to wheat spikes, the Schisler group has worked to develop a *Cryptococcus* strain, and to a lesser extent strains of *Bacillus* and *Pseudomonas*, which were isolated from wheat anthers and selected based on their ability to metabolize choline (75, 136). The proposed mode of action of these biocontrol agents is through nutrient competition with *Fusarium*. Such efforts have resulted in successful disease control in greenhouse settings, and moderate/inconsistent disease control in field settings (136, 137). Competition by non-pathogenic or moderately pathogenic *Fusarium* strains has also been tested, and has been successful at reducing deoxynivalenol production *in vitro* (30) and in field settings (32), although preliminary experiments with one competitor strain led to mild head blight severity symptoms (186).

The production of antifungal metabolites to inhibit the growth or germination of *Fusarium* on wheat surfaces is another proposed mode of action for biocontrol agents, particularly for *Bacillus* species. Antibiosis has also been proposed for several of the choline-metabolizing *Bacillus* strains isolated by the Schisler group (34, 35), and was attributed to iturins and fengycins.

Several groups have reported *Fusarium* inhibition or hyphal distortions *in vitro* from application of *Bacillus*-produced fengycin (23) or more generally *Bacillus* culture filtrate (22). The role of metabolites in *Fusarium* inhibition is also illustrated by reports of reductions in Fusarium head blight symptoms following application of *Bacillus* culture filtrate to wheat spikes in the greenhouse (23), as well as a report of a culture filtrate application reducing deoxynivalenol levels in the field by suppressing Gibberella ear rot in corn (23), which is also caused by *F. graminearum*. Inhibition of *Fusarium* spore germination and growth has also been documented with a *Clonostachys* antagonist, possibly through the production of antifungal compounds, although this mode of action was not confirmed (176).

Production of lytic enzymes with antifungal activities, such as chitinases and β-1,3-glucanases has been proposed for a *Lysobacter* biocontrol agent of Fusarium head blight (70). This *Lysobacter* biocontrol agent is also believed to provide protection against *Fusarium* infection by inducing host defenses, although as opposed to induced systemic resistance, the activity of *Lysobacter* had only local effects, and thus it requires application to above-ground plant parts (70).

Reductions in saprophytic survival, perithecial development, and/or ascospore production on crop residues is another strategy that has been employed for Fusarium head blight biocontrol, using the fungi *Microsphaeropsis* (16), *Clonostachys* (87), and *Trichoderma* (64, 65). However this method would be less effective in regions with large areas of wheat or corn production, where local inoculum contributes less to Fusarium head blight infections compared to regional effects of crop residues (13).

Finally, several other novel microbial-based modes of action for Fusarium head blight and deoxynivalenol control have been explored. For instance, bacteria (63, 143, 184), fungi (53), and

mixed microbial cultures (67, 161) have been used to detoxify deoxynivalenol by transforming it to a less toxic compound. Additionally, small *Fusarium* mating pheromone peptides have been applied to wheat spikes to inhibit ascospore germination, and have shown preliminary potential in reducing spikelet infection (187).

On the whole, biological control of Fusarium head blight has produced ineffective or inconsistent disease control in field settings, particularly when compared to fungicides (50, 137, 139, 168, 175, 176, 189). Efforts to improve the biological control of Fusarium head blight have focused on altering the formulation of biological control agents. The Schisler group, for instance, has worked to optimize formulations for biocontrol agents by altering the C:N ratio of culture media (137, 138, 195). Additionally, Chan et al. (23) found a positive effect on *Bacillus* antifungal metabolite production by adding casamino acids, and Pryor (122) documented the role of several factors, particularly moisture, on the antifungal metabolite production by TrigoCor in solid state fermentation. Mixtures of biological control agents have also been investigated, with some improvement over the inhibition by each agent individually (139). Other efforts have focused on the timing of biological control application. For instance, the disease control efficacy of most biological control agents appears to be greatest when they are applied prior to *Fusarium* infections (16, 64, 70). Finally, other studies have investigated the feasibility of applying biocontrol agents later in the season to augment chemical controls (50, 188), although these trials have produced variable results.

Bacillus *above-ground biological control and ecology*

Bacillus is most commonly applied as a biological control agent in the soil environment. In the soil, *Bacillus* biocontrol agents prevent disease either directly through inhibitory compound

production, or indirectly, by inducing systemic resistance or by improving plant health. The application of *Bacillus* as a soil biocontrol agent has been covered by several reviews (97, 98) and will not be discussed here.

Although *Bacillus* biocontrol agents are commonly applied to the soil, they are also frequently applied to above-ground plant parts for protection against pathogens invading stem, leaf, or floral tissues. The range of crops, pathogens, and potential modes of action for which *Bacillus* biocontrol agents have been tested is illustrated by a sampling of recent Plant Disease Management Reports (45, 48, 49, 58, 69, 93, 94, 113, 125, 133, 196) and publications in peer-reviewed journals (2, 21, 27, 99, 106, 145, 159, 193) (more examples listed in (20)). For the most part, *Bacillus* biocontrol agents are used for protection against fungal diseases, likely due to their production of antifungal metabolites, especially the cyclic lipopeptides described in more detail in the next section. However, *Bacillus* also displays antibacterial activity through alternative modes of action, such as through interference with pathogen biofilm formation and colonization (159).

Numerous studies have been conducted on the ecology of *Bacillus* biocontrol on above-ground plant parts. For instance, a Spanish group has published several papers (128-130) on the ecology of a *Bacillus* biological control agent and its interaction with the cucurbit powdery mildew fungus, *Podosphaera fusca*. Using scanning electron microscopy, they observed the *Bacillus* biological control agent colonizing fungal hyphae on plant surfaces, and forming aggregates surrounded by an extracellular matrix. Additionally, conidial vacuolization, shrinkage, and retraction of the plasma membrane were observed following treatment of the fungal pathogen with *Bacillus*-synthesized lipopeptides. They detected fairly stable *Bacillus* population levels on leaves after 16 days, and recovered lipopeptides from leaves 5 days

following application of washed cells, potentially indicating *in situ* metabolite production. Additional work by this group identified bacillomycin to be the main inhibitory component of the culture filtrate, and found that high relative humidity (75-90 %) was associated with the most effective disease control.

Several other groups have also studied *Bacillus* colonization and survival on aboveground plant parts. Specific foci of these research projects include the degree of secondary colonization to other plants or plant regions, the population dynamics of *Bacillus* applied to different plant parts, and comparisons in the colonization abilities of different biocontrol species. In general, and with some exceptions, *Bacillus* populations do not appear to be capable of significant secondary colonization on plants or plant parts, or of thriving for long periods of time on aerial plant surfaces, particularly in relation to other biocontrol species. For instance, Yuen (185) compared the population dynamics of *Erwinia* and *B. polymyxa* biocontrol strains applied to bean leaves, and found that in contrast to *Erwinia* which survived at significant levels on leaves and also spread to blossoms, populations of the *Bacillus* biocontrol strain on leaves declined and did not colonize blossoms. Thus *Bacillus* was found to be ineffective as a biocontrol agent in field settings for white mold, caused by *Sclerotinia sclerotiorum*. Alexandrova et al. (1) observed that populations of a *Bacillus subtilis* biocontrol agent were stable on pear flowers for 4-7 days and on leaves for 2 days, then declined in both environments, especially on leaves. Researchers investigating the insect biocontrol agent *Bacillus thuringiensis* found that it showed poor phyllosphere primary and secondary colonization relative to other phyllosphere inhabitants such as *Pseudomonas*, and that unlike *Pseudomonas* it also did not form significant aggregates on plant surfaces (89, 90). On the other hand, Broggini (15) detected secondary colonization of apple blossoms at variable levels by a *Bacillus subtilis* biocontrol agent, which they attributed to

movement by honeybees. A study on *Bacillus* and *Brevibacillus* biocontrol agents on barley spikes found that although populations declined they were found to be present in significant levels for 37 days post-application. Additionally, these populations were found to be present mainly on chaff but also on awns and grain, and declined then rebounded after rain storms (174). Finally, although populations of a commercial *Bacillus* biocontrol strain declined on blueberry flowers after 3-4 days whereas a commercial *Pseudomonas* biocontrol strain increased in numbers, the *Bacillus* product (Serenade) was better able to reduce *in vivo* germination and growth of the fungal pathogen *Monilinia vaccinii-corymbosi*, which the authors attributed to the non-cellular component of its formulation (135).

The inability of *Bacillus* to significantly colonize above-ground plant parts may be due in part to its transition to the spore form following application to plant surfaces, or more generally to the fact that it is not primarily adapted for survival in this environment. Formulation additives, as well as an understanding of the biocontrol agent ecology, may facilitate improvements in the survival and spread of *Bacillus* on aerial plant surfaces. For instance, collaborators working with sugar beet cercospora leaf spot, caused by *Cercospora beticola*, have attempted to investigate and modify the ecology of a *Bacillus subtilis* biocontrol agent to optimize its disease control efficacy. They used a β-glucan formulation amendment to alter the ratio of *Bacillus* spores:vegetative cells in inoculum, and found that having a higher percentage of vegetative cells was advantageous for control, and that control was also enhanced with earlier applications (28). In a separate study by the same group, β-glucan applied with *Bacillus* inoculum was also found to increase the population of vegetative cells on leaves after 2 weeks, and to alter the spatial distribution of cells on leaves by reducing the aggregation of *Bacillus* populations, although the

authors did not find a correlation between cell density and disease reductions (29). Additional examples of formulation amendments for *Bacillus* can be found in this review (138).

Bacillus amyloliquefaciens *strain TrigoCor*

Bacillus amyloliquefaciens strain TrigoCor was isolated from the wheat rhizosphere and selected for further study based on its consistent ability to inhibit the growth of common cereal crop pathogens, including *F. graminearum*, in antibiosis assays (12). TrigoCor, previously described as *B. subtilis*, was properly identified as *B. amyloliqeufaciens* based on phylogenetic analysis using a concatenated sequence of five genes (16S ribosomal RNA gene, gyrase subunit alpha, phosphoribosylaminoimidazolecarboxamide formyltransferase, DNA polymerase III subunit alpha, and RNA polymerase subunit beta), following the method of Rooney et al. (131).

The inhibitory potential of TrigoCor has been tested in *in vitro* (123, 149), greenhouse (73), and field settings (74, 149, 167, 168). In laboratory and field settings TrigoCor consistently reduces *Fusarium* growth and disease symptoms, however in field settings the disease control efficacy of TrigoCor is inconsistent and often ineffective.

The main mode of action for TrigoCor is believed to be inhibition of *Fusarium* spore germination and/or growth from TrigoCor-synthesized antifungal compounds. A former graduate student identified the major *Fusarium*-inhibitory metabolites produced by TrigoCor and optimized the solid state fermentation of TrigoCor for increased production of these compounds (121-124).

The role of metabolites in **Bacillus** *biological control*

As mentioned previously, *Bacillus* BCAs are believed to control plant disease through numerous mechanisms, one of which being the inhibition of pathogens through production of antimicrobial compounds. Visualization of the disruption of primarily fungal (62, 160, 194) but also bacterial (36) cell membranes following application of *Bacillus*-synthesized compounds highlights the importance of such compounds on disease control, as do findings that increases in metabolite production lead to corresponding improvements in disease control (78, 101, 112). Several recent reviews have been written on the biosynthesis, structure, mode of action, and further involvement in biological control of *Bacillus*-produced secondary metabolites (37, 109, 110, 146), and much of this section draws from information found therein. This section is mainly focused on the metabolites and processes relevant to disease control by TrigoCor, and the reader is encouraged to consult these reviews for additional information on *Bacillus* secondary metabolites, including for those metabolites not covered in this chapter (146).

Bacillus, particularly *B. amyloliquefaciens*, is a super-producer of secondary metabolites. Greater than 8.5 % of the *B. amyloliquefaciens* genome is dedicated to production of antibiotics and siderophores, which is more than the average for other *Bacillus* species (4 - 5 %), as well for the well-recognized metabolite producer *Streptomyces* (6.5 % for S. *avermitilis*) (24). Many of the *Bacillus*-produced compounds involved in biological control are produced by nonribosomal peptide synthetases (NRPSs) or NRPS-polyketide synthetases (NRPS-PKS), although some are ribosomally synthesized and post-translationally modified. The nonribosomal synthesis of many *Bacillus* secondary metabolites, along with natural rearrangements of synthetic clusters and proposed exchanges of clusters between strains via horizontal gene transfer, has enabled a great

diversity in metabolic profiles across *Bacillus* strains and species (146), and indicates the potential of this genus for biological control of a range of organisms.

Three main groups of secondary metabolites– iturins, fengycins, and surfactins – are produced by TrigoCor (123). Iturins, fengycins, and surfactins are cyclic lipopeptides, due to their composition of a polypeptide ring interlinked with a fatty acid side chain. The amphiphilic nature of these compounds contributes to their activity, because it enables them to integrate into cell membranes, although for fengycin this mode of action is less well understood. These metabolites can be inhibitory on their own, or in combinations (126, 129), and sometimes synergistically (91) as has also been reported for TrigoCor (121). In addition to their direct antimicrobial activity, the metabolites surfactin and fengycin have also been implicated in stimulating an induced systemic response in plants (40), which for surfactin may be caused by small non-destructive disruptions of plant membranes (57).

Interestingly, surfactins, fengycins, and iturins are not produced by the main *Bacillus* laboratory workhorse, *B. subtilis* 168. For surfactins and fengycins, the synthetic genes are present in *B. subtilis* 168; however a frameshift mutation in a gene coding for an enzyme that activates these metabolites, *sfp*, prevents their successful production (146). Transfer of the iturin A operon (158), as well as the introduction of a functional allele for the *sfp* gene (105, 146, 157) have allowed production of these metabolites by *B. subtilis* 168, as well an evaluation of the positive influence of such introductions on biological control (112).

Surfactins

Surfactins are produced by many *Bacillus* species, including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, and *B. coagulens* (110). Surfactins are produced by NRPSs coded

for by three open reading frames, *srf-A*, *srf-B*, and *srf-C* (120), as well as a fourth which initiates surfactin biosynthesis, *srf-D* (147). The synthesis of surfactins occurs mainly in the transition from exponential to stationary growth, and its production, as well as the expression of the *ComS* gene involved in competence which is located within the surfactin operon, is regulated in part through quorum sensing (51).

Surfactins consist of a cyclic heptapeptide linked with a 13-16 carbon β-hydroxy fatty acid chain. Of the cyclic lipopeptides produced by *Bacillus*, surfactin is the most amphiphilic, and at high concentrations it can form pores in and disrupt lipid membranes, forming mixed micelles (54). Surfactins are typically anti-bacterial (11, 193) or anti-viral (162). The presence of sterols in membranes has been shown to decrease the incorporation and disruption of such membranes by surfactin (19), and this has been hypothesized as a reason why surfactins show little antifungal activity (110). Recently, however, cell wall disruptions have been observed following application of surfactin to *F. oxysporum* (160).

Although surfactins typically do not display direct antifungal activity, they are still involved in biological control of fungal pathogens through their role in the colonization and survival of *Bacillus* BCAs. For instance, surfactin facilitates movement of *Bacillus* cells by reducing the surface tension of surrounding areas (3, 77), and may also be involved in biofilm formation (11). Additionally, surfactins appear to be involved in competitive interactions with the soil inhabitant *Streptomyces*. Surfactins were found to restrict aerial structure formation and sporulation by *Streptomyces coelicolor* (150), and possibly functioned by repressing the production of a *Streptomyces*-synthesized surfactant (178).

Iturins

The iturin family of metabolites is synthesized by *B. subtilis* and *B. amyloliquefaciens*, and includes the compounds iturin A and C, bacillomycin, and mycosubtilin (110). Iturins are synthesized by a NRPS-PKS hybrid coded for by four open reading frames, *ituA-B*, *ituB*, *ituC*, and *ituD* (110). Iturins are produced in late stationary growth, and consist of a cyclic heptapeptide linked to a 14-17 carbon β-amino fatty acid chain. Unlike surfactins, iturins are mainly antifungal, and upon integrating into membranes they form pores which leak ions (4). Iturins appear to be attracted to and interact with sterols in lipid membranes, however intriguingly they show less preference for ergosterol, found in fungal membranes, than for cholesterol, which is more dominant in animal membranes (37).

Yeasts are particularly susceptible to iturins (38), and iturins have been found to cause invaginations of the yeast cytoplasmic membrane, and even to distort the nuclear membrane of yeast cells (154). Also inhibited by iturins are pathogenic and saprophytic filamentous fungi, such as *Fusarium* (52, 194), *Bipolaris* (179), *Rhizoctonia* (8, 101, 183), *Penicillium* (25) *Gloeosporium* (26), and a range of post-harvest citrus pathogens (6). Overproduction of iturin has been linked to improvements in disease control against fungi (101) and oomycetes (78). Occasionally, iturins are implicated in bacterial inhibition, such as in the inhibition of *Streptomyces* sporulation but not its mycelial growth (52), and in the disease suppression and *in vitro* plasma membrane disruption of *Pectobacterium* and *Xanthomonas* (191).

Fengycins

Fengycins are produced by *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, and *B. thuringiensis* (110). The fengycin family consists of fengycin A and B, which are also known as plipastatins,

and consist of a cyclic lipodecapeptide with a 14-18 carbon β-hydroxy fatty acid chain. The fengycin operon consists of five open reading frames, *fenA, fenB, fenC, fenD,* and *fenE,* and codes for an NRPS (110). Fengycin production occurs during the early stationary phase of culture growth.

Fengycins have strong antifungal toxicity, which is less well understood but is believed to be due to their ability at high concentrations to form large pores in lipid membranes (117) through alterations in the membrane curvature. Fengycins are commonly described as the main inhibitory compound of *Fusarium graminearum* (23, 126, 164), and were previously identified as a key antifungal metabolite produced by TrigoCor (123). Fengycins are also believed to be integral in the biological control of the fungal pathogens *Sclerotinia* (126), *Rhizoctonia* (86), *Fusarium oxysporum* (86), and *Colletotrichum* (76), among others. Additionally, cells of bacteria (*E. coli*) and insects (cabbage white butterfly larvae) observed using scanning electron microscopy were found to shrink in the presence of fengycin-type compounds, possible due to membrane permeabilization (76).

In situ *metabolite dynamics*

Not many studies have been conducted on the synthesis and/or persistence of *Bacillus* cyclic lipopeptides in the soil or above-ground environment, despite such information being useful for understanding the success or failure of biological control. Most research has been conducted on the dynamics of metabolites in the rhizosphere, however a few studies have focused on the phyllosphere.

In the rhizosphere, surfactins have been found to be fairly stable, but iturins decline. Asaka and Shoda (8) found that the levels of surfactin were stable for at least 20 days, but that iturin A

decreased quickly, by more than 50 % over 5 days. Mizumoto et al. (101) also observed a decline in iturin A in the rhizosphere, and Ongena et al (112) recovered surfactin at least 8 days post-application in the rhizosphere, although the quantity of surfactin recovered was not reported.

The levels of surfactin recovered by Asaka and Shoda (8) in the rhizosphere were only slightly higher when whole culture broth was applied rather than culture filtrate, indicating that minimal surfactin production was occurring. However, surfactin production on root surfaces was reported by Bais et al. (11) following addition of *Pseudomonas* to the soil, possibly suggesting that competition with antagonists induces the production of this metabolite.

A few attempts to measure the production of *Bacillus* metabolites have been conducted on above-ground plant parts, typically through detection of these metabolites following application of washed cells. On melon leaves, for instance, Romero et al. (129) recovered bacillomycin, iturin A, and fengycin 5 days but not 12 d after application of washed cells, perhaps suggesting production but not maintenance of these compounds in the phyllosphere. However the initial level of these metabolites was not measured in this study. Similarly, Ongena et al. (111) recovered fengycins 5 days post-application of washed *Bacillus* to apple tissues, again potentially but not definitively indicating *in situ* production of this metabolite, due to insufficient data regarding the starting levels of this compound on plants or in inoculum. Finally, Touré et al. (156) detected fengycins at 1.5 µg/g apple tissue and iturins below 70 ng/g apple tissue 120 hours post-application of *Bacillus* spores. This report provides slightly more conclusive evidence of *in situ* compound production due to the lower chance of retention of metabolites with washed spores as compared to vegetative cells. However again the initial levels of metabolites were not reported in this study.

Bacillus *spores and spore germination*

Upon conditions of nutrient depletion, *Bacillus* cells transition from a metabolically active state to a dormant spore form. *Bacillus* spores and sporulation have been studied in detail, and were reviewed recently (56, 59, 80).

Much of the process of sporulation is initiated and driven by a transcription factor, Spo0A, which activates sporulation at high levels of phosphorylation (59). The phosphorylation state of Spo0A integrates environmental and biological information, theoretically to better guarantee that conditions are inhospitable for continued vegetative growth before cells commit to the sporulation process, which is irreversible after an early stage. One tactic utilized by *Bacillus* cells to prevent sporulation under conditions of only transient nutrient depletion is for sporulating cell to produce a 'killing factor'. This killing factor lyses nearby cells, thereby releasing a short burst of nutrients and prolonging the sporulation of cells that have low levels of Spo0A phosphorylation but have not yet committed to sporulation (46).

Spore formation begins with asymmetric division via septum formation near one pole of the cell, which leads to the creation of a prespore and a mother cell. As sporulation progresses, the mother cell engulfs the prespore, forming a forespore with inner and outer membranes within the mother cell. Next, the peptidoglycan-containing spore cortex is formed around the forespore, followed by a spore coat. The forespore accumulates the compound pyridine-2,6-dicarboxylic acid, which facilitates its dehydration. Finally, further spore maturation and mother cell lysis occurs, releasing the spore to the environment. Throughout the process, the mother cell and developing spore communicate through numerous transcription factors, which regulate the

timing and progression of spore formation, as well as the production by the mother cell of spore components, and eventual mother cell lysis (59).

At the center of the spore is the spore core, containing pyridine-2, 6-dicarboxylic acid and Ca^{2+} as well as DNA bound by small acid-soluble proteins which protect the DNA and keep it in a compact state. Exterior to the core is the germ cell wall and then the spore cortex, which together are bound by inner and outer membranes. The spore cortex and germ cell wall contain peptidoglycan, and the cortex likely contributes to the heat resistance of the spore. The germ cell wall becomes the cell wall during spore germination. Outside the outer membrane is the spore coat, consisting of the two proteinaceous inner and outer coat layers, which probably enable the chemical and mechanical resistance of spores, and which provide a barrier for large molecules. Finally, a mainly protein and lipid-containing exposporium layer is on the outside of the spore.

Bacillus spores are resistant to multiple terrestrial and even extraterrestrial environmental stresses (108), and are able to survive for long periods of time in a metabolically dormant state until more conducive environmental conditions return.

As is the case for spore formation, spore germination has also been studied extensively, and is described in several reviews (116, 141). The overall process of *Bacillus* spore germination consists of activation, germination, and outgrowth phases. Spore activation typically involves a sub-lethal heat treatment that primes the spore for germination in response to added germinants. Germination is a two-step germination phase in which monovalent cations and pyridine-2, 6-dicarboxylic acid chelated with calcium (Ca-DPA) are released, and then the spore core is hydrated by core lytic enzymes, enabling loss of resistance and a regaining of metabolism. Finally, in outgrowth, macromolecules are synthesized, and vegetative growth resumes.

Whereas the activation stage is reversible, the germination phase is not, and will continue even if germinants are removed (181).

Bacillus spores monitor their environments through a series of germinant receptors located in the spore's inner membrane, which are clustered together along with other germination proteins in a 'germinosome' (47). By studying the germination properties of mutants with overexpressed, deleted, or otherwise altered receptors and/or in combination with germination inhibitors such as D-alanine, specific receptors have been linked with the nutrients they bind, and the synergism between receptors has also been determined (9, 18, 114, 115, 148, 180). In *B. subtilis* the main receptors are GerA, which binds alanine and valine and their analogs and can trigger germination on its own; and GerB and GerK, which together stimulate germination in response to the germinant combination L-asparagine (or L-alanine), D-glucose and the potassium ion. GerK binds glucose, and GerB probably binds fructose as well as amino acids although the binding of amino acids is facilitated by interactions with GerK. The potassium ion likely can interact with all receptors rather than being bound by one, and is required for interactions between GerA and GerK or GerB.

Spore germination can also be stimulated by non-nutrient agents, such as pressure (173), cationic surfactants (140), lysozyme, salts, and Ca-DPA. The mechanism of germination stimulation appears to be different with many of these inducers, and often does not involve germinant receptors, but rather functions by directly activating core lytic enzymes (for Ca-DPA) or by opening channels allowing release of Ca-DPA or small molecules (for surfactants such as dodecylamine) (42).

Germination rate and efficiency of germination in a population of spores can be determined through several factors. For instance, temperature has a documented influence on the

germination rate and interactions between receptors (18, 66, 165, 166), possibly due to conformational changes in the receptors which make them more accessible for binding. The number of germinant receptors in a spore also influences germination rate. The number of germinant receptors in a spore is likely determined stochastically, and heterogeneity in spore receptor numbers among spores in a population leads to spores that germinate at different rates, potentially ensuring that the entire population does not germinate in response to a deceptive trigger (42). Increases in germinant receptor number can be achieved either through genetic or cultural manipulations, and lead to faster germination within a population (18, 127). However, a recent study (192) suggests that germination receptor numbers do not entirely explain the heterogeneity observed in the time required for spore germination, and suggests that other proteins or processes may also be involved. Finally, the concentration of germinants also influences the time required for germination, particularly in synergistic interactions between receptors in spores that have a low total number of receptors (180).

Many *Bacillus* spore populations contain a small percentage (<15%) of spores referred to as superdormant spores. These spores respond much more slowly to nutrient germinants but typically germinate normally with nonnutrient germinants (169), although recently superdormant spores for nonnutrient germinants have been isolated as well (119). Superdormant spores have a higher optimal heat activation temperature, and are more resistant to wet heat than are normal dormant spores (42, 44). The superdormancy of some spores may come from their lower germinant receptor content and/or lower core water content as compared to normal spores. Other factors influencing the percentage of superdormant spores in a population include the type of germinant (single vs multiple) used and the use of heat in spore activation (41-44).

A few research groups have studied the dynamics of *Bacillus* sporulation and spore germination in the environment (107). For instance, the sporulation and/or germination of *Bacillus* cells following application to the soil has been tracked (55, 104, 144), and the speed of sporulation was found to be positively correlated with the level of populations surviving over time (7, 155). Germination dynamics of beneficial and harmful *Bacillus* spores have also been studied in animal and insect intestines (55, 60, 61, 134, 153), and the large-scale levels of *Bacillus* germination observed in the gastric system have led researchers to conclude that spores have evolved to respond to germination cues produced in this environment. Finally, alterations in the spore:vegetative cell ratio of foliar-applied *Bacillus* biocontrol agents have been produced through genetic manipulations (132) and culture amendments (28), with mixed results on disease control.

Research objectives

The overall goal of this research project is to identify strategies for the optimization of biological control by *Bacillus* on above-ground plant application by investigating the ecology and biological control interactions of a model *Bacillus* biocontrol agent. To address this goal, we evaluated the temporal and spatial dynamics of TrigoCor cells and metabolites on wheat spikes in greenhouse and field settings, and related this information to *Fusarium graminearum* inhibition and disease suppression. We also assessed whether altering the form of TrigoCor cells on wheat spikes by stimulating spore germination would lead to enhanced *in situ* metabolite production and improvements in disease control.

The specific objectives were:

1. To assess viable cell populations and antifungal metabolite levels over time on wheat spikes in the greenhouse and in the field, in relation to Fusarium head blight and deoxynivalenol suppression.

2. To identify potential germinants that could stimulate TrigoCor spore germination on plant surfaces, and to determine if induction of TrigoCor spore germination improves Fusarium head blight and deoxynivalenol control in controlled environments.

3. To evaluate the coverage of TrigoCor inoculum on wheat spikes in controlled and field settings, and to characterize the *in planta* spatial interactions between TrigoCor and *F. graminearum* on wheat surfaces.

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CHAPTER 2

ITURIN LEVELS ON WHEAT SPIKES LINKED TO BIOLOGICAL CONTROL OF FUSARIUM HEAD BLIGHT BY *BACILLUS AMYLOLIQUEFACIENS*

Abstract

The TrigoCor strain of *Bacillus amyloliquefaciens* provides consistent control against Fusarium head blight of wheat in controlled settings but there is a lack of disease and deoxynivalenol suppression in field settings. Since production of antifungal compounds is thought to be the main mode of action of TrigoCor control, we quantified levels of a key family of antifungal metabolites, iturins, as well as monitored *Bacillus* populations on wheat spikes over 14 days post-application in both the greenhouse and the field. We found that initial iturin levels on spikes in the greenhouse were three times greater than on spikes in the field. Mean iturin concentrations could not be subjected to statistical comparison between field and greenhouse experiments as these were distinct experiments with different experimental protocols and environments. By 3 days post-application, iturin levels were equivalent and very low in both settings. We also determined that iturins declined rapidly over a 3 day post-application period on wheat spikes in both environments, despite the presence of significant *Bacillus* populations. Greenhouse trials and antibiosis tests indicated that the lower iturin levels on wheat spikes in the field could be a major factor limiting disease control in field settings. Future efforts to improve *Bacillus* disease control on wheat spikes and in the phyllosphere of various plants should focus on maintaining higher levels of iturins over critical infection periods.

Introduction

Managing fungal diseases of plants with the gram-positive bacterium *Bacillus subtilis* or the closely related *Bacillus amyloliquefaciens*, either as an alternative or a supplement to fungicides, could be of great benefit. Biological control agents (BCAs) such as *Bacillus* are attractive for disease control due to their low environmental impact, and to their ability to help reduce growers' dependence on chemicals, thereby slowing the development of fungicide resistance in pathogen populations (22). There have been notable successes with *Bacillus* BCAs applied in the soil (40), whereas biocontrol of fungal diseases by BCAs applied to aerial plant parts has been more challenging (21). Several *Bacillus* BCAs have shown consistent suppression of aerial diseases under controlled environments, but have shown less consistent control under field conditions. However, few studies have pinpointed the reasons for failed disease control by *Bacillus* BCAs in the field.

The TrigoCor strain of *Bacillus amyloliquefaciens* is one such *Bacillus* BCA, that when applied to above-ground plant surfaces, is unable to consistently control disease in the field, despite reproducibly suppressing disease in the greenhouse (6, 12, 24, 52). Like many *Bacillus* biocontrol strains, TrigoCor shows potential as a BCA because it produces a diverse arsenal of antifungal metabolites in culture. Production of these compounds, particularly cyclic lipopeptides in the iturin and fengycin families, is believed to be the main mechanism through which TrigoCor (30, 42) and other *Bacillus* BCAs (36) inhibit fungal spore germination and growth on plant surfaces, prior to pathogen invasion of plant tissues. In addition to the production of antifungal compounds, another aspect of TrigoCor and other *Bacillus* species

which makes them promising as BCAs is that they can transition to a spore form in adverse environmental conditions, thereby potentially extending their lifespans on aerial plant surfaces.

TrigoCor has been evaluated extensively for control of Fusarium head blight (FHB) of wheat (11, 12, 18, 24, 47, 53), caused in the United States by the filamentous ascomycete *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch). Management of this disease is currently challenged by the general lack of highly resistant wheat cultivars (4), and by variable or incomplete control by currently available fungicides (8, 32, 33, 39, 54). A major obstacle to FHB management using chemical control is that *F. graminearum* infections can occur beyond the 30 day pre-harvest interval when fungicides can be legally applied (17, 31). These late-season infections contribute to accumulation of the *Fusarium*-produced mycotoxin deoxynivalenol (DON) (10, 13), and consequently, to rejection or severe dockage of grain at grain elevators or flour mills. Because there are no restrictions on application timings of BCAs, a BCA sprayed onto wheat spikes either with or following a fungicide could add significantly to an integrated management plan by protecting against late-season infections, particularly if the BCA could survive and be active for long periods of time on wheat surfaces. In addition to this potential role of BCAs in managing FHB within conventional wheat production, BCAs could also be a valuable tool for managing FHB in organic systems, for which control measures are severely limited. In addition to TrigoCor, several other BCAs have been evaluated for control of FHB, including yeasts and both gram-positive and gram-negative bacteria (7, 23, 25-27, 30, 38, 55), however many exhibit the same problems with consistency when it comes to field applications (23, 46).

Using TrigoCor as a model BCA, we sought to identify the key factors that influence the success or failure of *Bacillus*-based disease control on aerial plant surfaces. Understanding why

TrigoCor frequently fails at controlling FHB in the field despite being consistently effective in the greenhouse should allow the formulation of intelligent strategies for enhancing biocontrol effectiveness in the field.

We hypothesize that the success of disease control depends on the presence of sufficient levels of viable *Bacillus* populations and /or of *Bacillus*-produced antifungal metabolites over critical infection periods. Our objectives in this study were to assess viable cell populations and antifungal metabolite levels over time on wheat spikes in the greenhouse and in the field, and to relate these data to FHB/DON suppression.

Materials and Methods

Microbial cultures. *Bacillus amyloliquefaciens* strain TrigoCor was isolated from the wheat rhizosphere and selected for further study based on its consistent ability to inhibit the growth of common cereal crop pathogens, including *F. graminearum*, in antibiosis assays (6). TrigoCor, previously described as *B. subtilis*, was properly identified as *B.amyloliqeufaciens* based on phylogenetic analysis using a concatenated sequence of five genes (16S ribosomal RNA gene, gyrase subunit alpha, phosphoribosylaminoimidazolecarboxamide formyltransferase, DNA polymerase III subunit alpha, and RNA polymerase subunit beta), following the method of Rooney et al. (44).

For inoculum production, TrigoCor liquid starter cultures were grown for 48 h in 100 mL nutrient broth plus yeast extract (NBYE; 8 g Difco nutrient broth (Becton, Dickinson and Company, Franklin Lakes, NJ), 7 g Bacto yeast extract (Becton, Dickinson and Company, Franklin Lakes, NJ), 1 liter RO water), in 250 mL flasks. Starter cultures were transferred to

fresh media and grown to stationary phase, typically 5 to 7 days. The volume of inoculum and duration of growth varied depending on the experiment, as the larger inoculum volumes used for field trials required longer periods of growth than did the smaller inoculum volumes used for greenhouse trials. Unless otherwise noted, all cultures were grown at room temperature (22 to 26 ºC) with shaking (155 rpm).

For greenhouse assays, TrigoCor starter cultures were transferred 1:1000 to 100 mL aliquots of NBYE amended with 0.1 mM $MnSO_4 \cdot H_2O$ (NBYE.Mn) in 250 mL flasks, then were grown for 5 days. For the 2009 field season experiments, TrigoCor starter cultures were transferred 1:100 to 1 liter aliquots of NBYE amended with 0.5 mM $MnSO_4 \cdot H_2O$ (NBYE.5Mn) in 2 liter Fernbach flasks, then grown for 7 days. In order to reproduce the typical greenhouse protocol as much as possible for field trials in 2010, TrigoCor cultures were grown according to the normal greenhouse inoculum growth conditions, with the exception that inoculum for the Ithaca location was grown for 6 rather than 5 days. In the 2011 field season, inoculum was grown in 1 liter aliquots of NBYE.5Mn, however due to an earlier than expected wheat flowering and consequently, a rapid need for inoculum, the cultures were grown for 2 days at elevated temperature, 30 ºC, to reach stationary phase. For each experiment, culture aliquots were combined just prior to inoculations; samples of inoculum were set aside at 4 ºC and -80 ºC for later quantification of viable cell populations and metabolites, respectively. For the 2009 field experiments, inoculum was frozen at -80 ºC for 5 to 7 days prior to use.

F. graminearum strain Gz014 NY98 was isolated from a FHB-infected wheat spike. For *F. graminearum* growth, an approximately 3 mm x 5 mm sample from a glycerol stock was spread onto a plate of Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Franklin Lakes, NJ) and grown at room temperature with 12 h UV (40 W, 350 nm) light/day for

approximately 14 days. Conidia were transferred to fresh PDA and grown until adequate conidia were formed, typically 13 to 15 days. Cultures were flooded with sterile water then filtered through a 250 micron sieve (Dual Manufacturing Company, Chicago, IL) to remove mycelia prior to use. Conidia were adjusted with sterile deionized water to the desired final concentration prior to challenge. Conidial concentrations of 6×10^4 spores/ml were used for greenhouse experiments, and 1×10^5 spores/ml for field experiments. The increase in inoculum concentration for field trials was intended to compensate for the less conducive conditions for infection in this environment.

Standard greenhouse bioassay. Seeds of 'Norm' spring wheat were sown in soilless Cornell Mix (3 to 3.8 ft^3 compressed peat moss, 2 to 6 ft^3 vermiculite, 20 lbs dolomitic limestone, 6 lbs 11-5-11 fertilizer) in 6-in clay pots at 10 seeds/pot. Spring wheat was selected for greenhouse assays instead of winter wheat because it does not require a vernalization period and because it takes less time to mature. Norm was selected because it is highly susceptible to FHB and therefore allows greater separation between treatments and more reliable disease symptom development. Plants were grown for roughly 8 weeks in the greenhouse at 25 to 35 ºC under 12 to 14 h light/day, and sprayed for powdery mildew and insects as needed. No fungicide sprays were applied after spike emergence. Prior to TrigoCor inoculations, tillers within each pot were trimmed so that each pot contained 10 spikes of roughly equivalent growth stage. Tillers were held in an upright standing position by a large loop of twist tie attached to a bamboo pole. For TrigoCor inoculations, approximately 30 ml TrigoCor inoculum was sprayed onto spikes in a single pot using a pressurized Preval Spray Gun (Preval, Coal City, IL). Unless otherwise noted, control spikes were treated with deionized water. During TrigoCor inoculations, pots were rotated 45 degrees so that each was sprayed in eight positions, with four passes of the sprayer in

each position. Wheat plants remained in the greenhouse for 24 h post-TrigoCor spray, then were sprayed with roughly 30 ml *F. graminearum* conidia (6 x 10^4 spores/ml) using a household handheld sprayer (Consolidated Plastics, Stow, OH). During *F. graminearum* inoculations pots were rotated 45 degrees so that each was sprayed in eight positions, with four sprays in each position. Following *F. graminearum* application, pots were placed into a mist chamber for 48 h, then into a controlled climate chamber set at 25.5 ºC, 14 h light: 9 h dark cycle where they remained until harvest. Appearance of disease symptoms, characterized by spikelet bleaching and bending of awns, was monitored daily until symptoms stabilized at approximately 12 days post- *F. graminearum* application, when FHB severity (percent of symptomatic spikelets per spike) and incidence (percent of symptomatic spikes per pot) were recorded.

Population dynamics of *Bacillus* on wheat spikes in the greenhouse. Three greenhouse experiments to investigate *Bacillus* temporal population dynamics on wheat spikes were conducted using the standard greenhouse protocol, and results from a single representative experiment are shown. In the representative experiment presented, inoculum *Bacillus* cell concentration was 1.1 x 10^9 CFU/ml, and spikes were sprayed at anthesis (Feekes growth stage 10.51). In other experiments spikes were slightly older (Feekes growth stage 10.54, 11.2). Control pots were nontreated. Plants were not challenged with *F. graminearum* and were therefore not rated for FHB index. There were seven replicates per treatment and two pots per replicate. For population quantification at each time point, two spikes from each pot in a given replicate were collected and combined for processing (four spikes total per replicate).

Population dynamics of *Bacillus* on wheat spikes in the field. Field trials were conducted all years at the Ithaca field site (IFS) in Cornell's Fusarium head blight nursery in Ithaca, NY, and in 2009 and 2010 at the Aurora field site (AFS) in Cornell's Musgrave Research Farm in

Aurora, NY. At the IFS, wheat was irrigated for 12 h/day at a rate of 30 s on, 4.5 min off. Wheat in the AFS field site was not irrigated. At the IFS, rows were planted with 'Caledonia' winter wheat in all years and were roughly 1 m long with approximately 150 spikes/row. At the AFS field site, rows of 'Jensen' (2009) and 'Pioneer 25R47' (2010) winter wheat were marked off within larger fields, and each row was on average 1.3 m long with 100 wheat spikes/row. Cultivars were selected based on availability of excess rows from other experiments at field sites. The average surface areas of Norm and either Caledonia or Pioneer 25R47 spikes are not significantly different (data not shown), suggesting that population levels on spikes can be compared between greenhouse and field environments without a complicating factor of spike size.

In the 2009 trials, spikes were either nontreated or were treated with TrigoCor ("TrigoCor low"). In 2010, two application rates (Table 1) of the same TrigoCor inoculum were applied to wheat spikes as a "high" volume and a "low" volume TrigoCor treatment, and control spikes were nontreated.

Table 1. Inoculum delivery to wheat spikes in the greenhouse, and in the field at three application rates.

Application method and rate	**Approx. volume delivered (ml/spike)[z]**
Hand spray greenhouse (30 ml/pot [y])	0.532 ± 0.014
Hand spray field 'high' (250 ml/row [x])	0.230 ± 0.008
Hand spray field 'low'(65 ml/row [w])	0.037 ± 0.002
Commercial spray (187 liters/hectare)	0.007 ± 0.001

[w] Sprayed by hand; approximately 0.5 ml applied per wheat spike
[x] Sprayed by hand; approximately 2.3 ml applied per wheat spike
[y] Sprayed by hand; approximately 3.0 ml applied per wheat spike
[z] Calculated based on linear fit equation from standard curve (Appendix Figure 1). Numbers represent means from 50 spikes ± standard error.

Spikes were sprayed with TrigoCor at anthesis (Feekes growth stage 10.52 in 2009 and 10.51 in 2010). The following day, *Fusarium* conidia ($1x10^5$ conidia/ml) were sprayed onto wheat spikes using an R & D Sprayer (CO_2), Model GS set at a pressure of approximately 30 psi, with 4 fan nozzles at 19" (48cm). In 2010 at the AFS field site, *Fusarium* was sprayed onto spikes 3 to 4 h post-TrigoCor application. All rows, including rows between treatments and negative controls, were sprayed with conidia.

Each treatment consisted of 13 replicates in 2009 and 10 replicates in 2010. In 2009 there were two rows per replicate, however due to time constraints population data were only processed from the first row within each replicate. FHB index and DON data are presented for the same rows as for population data. In 2010, each replicate was one row. Treatments in 2009 were alternating and in 2010 were arranged in a randomized block design, with replicate as the blocking factor. In 2009 there was one nontreated row separating treatments, and in 2010 there were five nontreated rows separating treatments. At the AFS, there was more variation in spike density among rows, so that the most suitable row was selected for use, if spike density was too inconsistent within a row.

Disease data was estimated once incidence levels had stabilized. High levels of FHB were expected in 2009 at the IFS field site, so to obtain an unbiased representative sample of disease symptoms, incidence data were collected from 50 spikes tagged with colored time tape (VWR, Batavia, IL) prior to TrigoCor spray. Lower levels of FHB were expected at the AFS in 2009 and at both field sites in 2010, so incidence ratings were taken from entire rows. FHB severity at all

field sites was recorded based on 10 representative symptomatic spikes per row, and was quantified using a FHB severity scale (15).

***Bacillus* population quantification.** At 0, 1, 3, 7, and 14 days post-TrigoCor application, wheat spikes were collected from either the greenhouse or the field and stored in a -80 ºC freezer until processing. The 0 day samples were collected after wheat spikes had dried following *Bacillus* spray, usually 4 to 5 h post-spray. In the greenhouse, the 1 day sample was collected prior to pots being transferred to the mist chamber, and the 3 day sample was collected after pots were removed from the mist chamber and spikes had fully dried. In the 2009 field trials, spikes were marked for collection prior to TrigoCor inoculations. In the 2010 field trials, representative spikes were collected randomly from various positions within each row.

Bacterial quantification was conducted as described in (23), with the modification that the phosphate buffer was amended with 0.1% Triton X-100. Colonies were counted based on similarity to typical TrigoCor morphology.

Background *Bacillus* level on spikes in the greenhouse and field. Wheat spikes were collected prior to application of any treatments to assess the starting background level of *Bacillus* on wheat surfaces. From the greenhouse, a total of four samples from two separate experiments were collected and processed using the standard protocol. For the 2010 field sites, two samples were processed from IFS and one sample was processed from AFS. Samples were processed to provide a rough estimate of background population level on wheat spikes, and were not subjected to statistical analysis.

Estimation of *Bacillus* population present as spores. The percentage of *Bacillus* cells present in the spore form was evaluated for three replicates per treatment per time point from a greenhouse experiment and from both field locations in 2010. Samples were removed from the

dilution series made to measure population dynamics, then were heated at 85 ºC for 10 m to kill vegetative cells, and plated. Colony numbers were compared to the corresponding counts from the non-heat treated dilution series to calculate percent spores.

Estimation of inoculum delivery to wheat spikes. To estimate the amount of Pyranine dye delivered to wheat spikes at different application rates and in different environments, a solution of Pyranine 10G dye (Keystone. Aniline Inc., Chicago, IL) (0.5 g/l water) was sprayed onto spikes in the greenhouse using the typical protocol; onto 1 m rows of Caledonia at IFS by hand at 65 ml/row or 250 ml/row; and onto a plot of Pioneer 25R47 at AFS with a backpack sprayer at 187 liters/hectare. All field applications were conducted in 2012. The application rates used on Caledonia are equivalent to the rates used for the low and high TrigoCor applications in the 2010 field season. For each environment and application rate, 50 treated spikes were collected. Ten nontreated spikes per cultivar were also collected and processed as negative controls. Stalks were removed, then spikes were dipped 20 times in 20 ml water in 13 mm x 100 mm glass tubes to remove dye, and the absorbance of the resulting solution was measured at 400 nm on a Spectronic 20 spectrophotometer. The amount of dye delivered to spikes was calculated using the linear fit equation from the standard curve (Appendix Figure 1).

Time course of metabolite dynamics. For studies on metabolite dynamics in the field at the AFS in 2010, blocks within a field of 'AC Superb' spring wheat were used. Spring wheat was used instead of winter wheat because there were larger numbers of spring wheat spikes available, and therefore multiple replicates of each treatment could be analyzed. A high and a low application volume of TrigoCor inoculum were applied, and the control block was nontreated. Plots were not inoculated with *F. graminearum*. There was one block per treatment and one to three block lengths between treatments. At 0, 1, and 3 days post-TrigoCor application, three sets

of 100 spikes were collected from each treatment block for quantification of metabolites, and two sets of four spikes were collected for quantification of populations. The 0 day sample was taken 2 to 3 h after TrigoCor application, after wheat spikes had dried. FHB incidence and severity were recorded from 10 spike samples selected from the corners and center of each plot.

Due to the large sample size needed for quantification of metabolites and the limited space available in the greenhouse, it was difficult to perform a single experiment in which spikes were collected for metabolite quantification with multiple replicates and/or with more than two data collection time points. Instead, data from three greenhouse experiments were combined to determine the fate of iturin metabolites on wheat spikes in the greenhouse. In each experiment, pots of wheat at anthesis (Feekes growth stage 10.53) were sprayed with TrigoCor and *F. graminearum* using the standard greenhouse protocol. Inoculum cell and metabolite concentrations for the three experiments were 7.2×10^8 CFU/ml and 0.64 mg/ml; 6.2×10^8 CFU/ml and 0.39 mg/ml; and 8.8×10^8 CFU/ml and 0.22 mg/ml. All other conditions remained the same across experiments.

During the course of each greenhouse trial, sets of 100 TrigoCor-treated wheat spikes were collected from 10 pots at 0 (all trials), 1 (trials 1 and 3), and 3 (trials 2 and 3) days post-TrigoCor application. Control spikes were treated with deionized water and collected at the 0 day time point. The 0 day sample was taken 4 to 5 h after TrigoCor application, after wheat spikes had dried. In the third trial, *Bacillus* population dynamics were estimated from one 4- spike replicate per treatment at each time point.

***Bacillus* metabolite quantification.** Wheat spikes for metabolite analysis were placed into individual freezer bags and stored at -80 ºC prior to extraction. Bulk samples consisting of 100 wheat spikes were placed upside down into a beaker containing approximately 500 ml methanol

to cover the spike surfaces and then sonicated for 10 min. The mixture was filtered using a Buchner funnel, and the filtrate evaporated to dryness on a rotoevaporator under minimal heat. The residue was then reconstituted into 1 ml methanol, of which 100 µl was filtered through a 0.4 µm PTFE syringe filter for HPLC analysis using a modification of the method as described (41). HPLC employed a Polaris RP C18-A column (Varian, 5 µm 250 mm x 4.6 mm) column, using a gradient of solvent A- acetonitrile containing 0.1 % trifluoroacetic acid and solvent B- hplc-grade water with 0.1 % trifluoroacetic acid, flow rate of 1 ml/min. Gradient conditions were as follows: 30 % A:70 % B for 2 min, linear gradient to 65 % A:35 % B over 35 min; linear gradient to 90 % A:10 % B over 10 min, then held for 10 min prior to return to starting conditions at 55 min and held for 10 min prior to the next injection. Primary detection was by UV at 220 nm (extracted from a 190 to 350 nm scan on a Waters 996 diode array detector). Iturins were estimated using an external standard containing primarily iturin A as well as two minor iturins with longer fatty acid tails (Sigma Chemical Co., St. Louis, MO) using a 5 point calibration curve and integrated areas for the peaks in extract chromatograms that matched the retention times and spectral scans of the authentic standard (Sigma Chemical Co., St. Louis, MO).

For quantification of iturins on wheat spikes, two peaks in the HPLC chromatogram that showed good separation from the background trace of control plants and that had the same retention time and spectral identity as those in an iturin A standard were chosen (Appendix Figure 2). Their identity as iturins had also been confirmed in earlier studies of TrigoCor by MS analysis (41). These two peaks were also easily visible in HPLC traces from extracted broth samples and therefore, were useful as specific biomarkers to follow the time course of metabolites on the wheat spike surface. To further confirm identities of these peaks, electrospray

(ESI) mass spectra of standard mixtures of iturin (Sigma Chemical Co., St. Louis, MO), fermentation extracts, and wheat spike extracts were analyzed by direct infusion of methanolic solutions into the mass detector at a flow rate of 1 µl/min using a syringe pump (Model 55-1199; Harvard Apparatus, Holliston, MA). Detection in the positive ion mode gave major quasimolecular ions [M+Na] of iturins at 1065 and 1079 using a cone voltage of 80V, and a capillary voltage of 4.0 kV, source block temperature held at 100° C, and desolvation gas (N_2) heated to 150° C.

Concentration effects of TrigoCor inoculum. Three greenhouse experiments were conducted according to the standard protocol using full strength TrigoCor inoculum as well as inoculum diluted 10- and 100- fold dilutions in deionized water. There were seven replicates per treatment for measuring FHB symptoms and one replicate per treatment for examining *Bacillus* population levels at 0 days and 14 days post-application where each replicate was one pot. Spikes were sprayed with TrigoCor and *F. graminearum* at anthesis (Feekes growth stage 10.53). Wheat spikes were collected following application on day 0, and after 14 days, and processed for *Bacillus* populations. Iturin levels on wheat spikes were calculated based on the iturin concentration in the inoculum and on the average volume delivered to wheat spikes in greenhouse experiments (Table 1).

Agar well diffusion assay. 500 µL *F. graminearum* conidial suspension (1 x 10^5 conidia/ml) was uniformly spread on the surface of 100 x 15 mm plates containing 15 ml PDA. After plates had dried (approximately 60 min), a 4.72 mm diameter cork borer was used to cut holes in the agar. Holes were filled with 29 µL of a freshly made serial dilution of an iturin A standard (Sigma Chemical Co., St. Louis, MO) in 9 % methanol, and with a 5-day TrigoCor culture filtered through a 0.1 µm filter. Prosaro (prothioconazole 1.76 + tebuconazole 1.76 lb ai/gal)

(0.3 % v/v) and 9 % methanol were used as positive and negative controls, respectively. Prosaro was diluted to a concentration similar to that recommended for use in the field (6.5 fl oz/20 gal). There were three replicates/sample and each replicate was on a separate plate, although a single plate contained up to five different samples. Plates were incubated at room temperature under 12 h UV light/day for 6 days. At 6 days, inhibition zone measurements were taken using a digital caliper along two perpendicular lines drawn to intersect in the center of the sample well. Inhibition zone lengths were measured from the edge of the well to the edge of the zone of inhibition. Minimum inhibitory concentration was defined as the smallest iturin concentration that produced a zone of inhibition. The assay was repeated three times.

Effect of repeated *Bacillus* applications in the field. To examine the effect on disease control of repeated TrigoCor applications, wheat was grown and irrigated as above for a field trial (IFS). The experiment was performed in 2011. There were four treatments: nontreated; TrigoCor applied once (day 0); TrigoCor applied three times (day 0, 2, 4); and TrigoCor applied once (day 0) with noninoculated NBYE.Mn broth applied twice (day 2, 4) to control for the effect of nutrient addition to wheat surfaces in the triple TrigoCor treatment. There were two replicates per treatment, where each replicate consisted of seven 1-m long rows, and there were four rows between treatments. Treatments were arranged in a randomized block design, with replicate as the blocking factor. At the day 0 spray time, wheat was at the anthesis stage (Feekes 10.52). *F. graminearum* conidia were applied the day following the first TrigoCor application as described for field trials above.

To calculate FHB incidence, all symptomatic spikes were counted per replicate per treatment, and this number was divided by total number of spikes. Spikes at the very front and back of

rows were excluded. FHB severity ratings were taken from 20 representative spikes from each treatment replicate.

DON analysis. Wheat spikes in greenhouse and field experiments were harvested by hand. Grain was cleaned using a belt thresher for field experiments and by hand for greenhouse experiments, and was ground using a coffee grinder (Cuisinart, Stamford, CT). DON quantification using gas chromatography was conducted by US Wheat and Barley Scab Initiative-supported DON Testing Labs at Virginia Tech and University of Minnesota.

Statistical analysis. FHB index was calculated by multiplying FHB incidence by FHB severity. *Bacillus* population levels, FHB index, and DON (ppm) were $\log_{10}$, arcsine, and natural log transformed respectively prior to statistical analysis. Data was analyzed using the general linear model within the JMP statistical program version 9.0.2 (SAS Institute Inc., Cary, NC). The response variable was population level, FHB index, DON, or inhibition zone radius, and the independent variables were treatment and replicate. Means were separated by t-test or by Tukey HSD (P = 0.05), depending on the number of treatments. Where applicable, trends in means separation are shown for transformed means. Reported means and standard error were calculated using nontransformed data.

Results

Population dynamics of *Bacillus* on wheat spikes in the greenhouse. The level of *Bacillus* populations recovered from wheat spikes was stable throughout the 14 day sampling period at 10^8 *Bacillus* CFU/spike (Fig. 1), while the quantity of *Bacillus* on nontreated spikes ranged over time from 10^4 to 10^6 *Bacillus* CFU/spike. At each time point, the *Bacillus* populations on

TrigoCor-treated wheat spikes were significantly higher than on nontreated spikes (P = 0.05). FHB index data was not recorded, however TrigoCor applications at the rate used in these trials have consistently resulted in significant FHB/DON control (48).

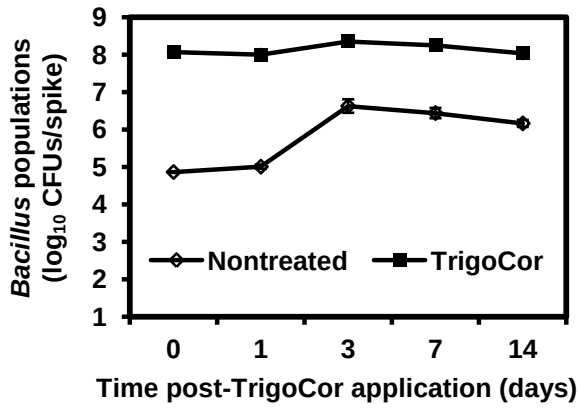

Fig. 1. *Bacillus* population dynamics on wheat spikes in the greenhouse following application of the biocontrol agent *Bacillus amyloliquefaciens* strain TrigoCor. *Bacillus* population levels were quantified using dilution plating. The wheat spikes were in a mist chamber in the period from 1 to 3 days post-TrigoCor application. Experiment was repeated three times with comparable results. Data shown is from a representative experiment. Each data point represents the mean of seven replicates, using population levels from four spikes per replicate. Vertical lines represent the standard error of the mean for each treatment at each time point.

Population dynamics of *Bacillus* on wheat spikes in the field. In field trials at two locations over two years, *Bacillus* populations on TrigoCor-treated wheat spikes declined by one order of magnitude over a 14 day sampling period post-application (Fig. 2). This trend was consistent despite differing patterns in temperature and rainfall among field locations and years. Although *Bacillus* populations decreased throughout the 14 day period, *Bacillus* was still recovered from treated wheat spikes in each experiment at significant levels at harvest.

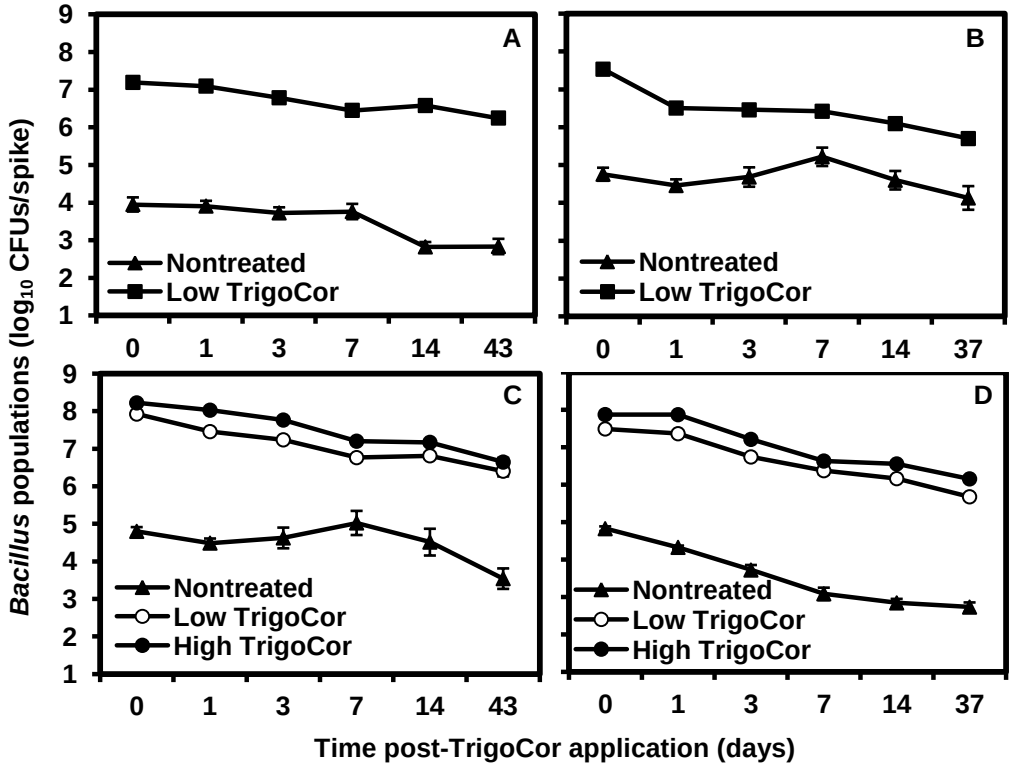

Fig. 2. *Bacillus* population dynamics on wheat spikes in the field following application of the biocontrol agent *Bacillus amyloliquefaciens* strain TrigoCor. *Bacillus* population levels were quantified using dilution plating from wheat spikes collected from two winter wheat field locations in **A and B,** 2009 and **C and D,** 2010. The **A and C,** Ithaca, NY field site was irrigated whereas the **B and D,** Aurora, NY field site was not. In 2010 spikes were treated with a low or a high volume of TrigoCor inoculum. Corresponding inoculum concentrations, application volumes, and disease data for all years and field sites are presented in Table 2. The last time point in each series is the day of harvest. Data points represent the means of 13 (2009) and 10 (2010) individual wheat rows, using population levels from four spikes per row. Vertical lines represent the standard error of the mean for each treatment at each time point.

In the 2009 field trials (Figs. 2A and B), the *Bacillus* population level on TrigoCor-treated wheat spikes was generally one to two orders of magnitude lower than the level observed at corresponding times in the greenhouse (Fig. 1) at each time point, although no direct statistical comparison was performed between environments. The level of *Bacillus* populations on TrigoCor-sprayed spikes was still significantly higher than on nontreated wheat spikes at all time

points (P = 0.05). Treatment with TrigoCor did not produce any significant reductions in FHB/DON in 2009 at either field site (Table 2).

In the 2010 field trials, the high TrigoCor treatment resulted in *Bacillus* population levels that on days 0 and 1 were similar (IFS, Fig. 2C) or slightly lower (AFS, Fig. 2D) than the 10^8 CFU/spike observed in the greenhouse, although again no statistical comparison was performed between environments. At both field sites, the population level on spikes in the low TrigoCor treatment generally approximated the level observed on TrigoCor-treated spikes in 2009. Although the *Bacillus* population level on spikes remained numerically greater in the high TrigoCor treatment compared to the low TrigoCor treatment at all time points in both locations, this difference was only statistically significant (P = 0.05) in IFS from 0 to 3 days and at AFS on all days except day 7. At all time points at both field sites the *Bacillus* population levels on spikes treated with the two TrigoCor treatments were significantly greater than the level on nontreated wheat spikes (P = 0.05).

TABLE 2. Inoculum concentrations and application rates, and ineffective disease control in field experiments using the biocontrol agent *Bacillus amyloliquefaciens* strain TrigoCor.

			TrigoCor inoculum characteristics			FHB disease parameters	
Year	**Location**	**Treatment**	**Cells (CFUs/ml)[u]**	**Iturins (mg/ml)[v]**	**Volume applied (ml/ spike)[w]**	**FHB Index[x]**	**DON (ppm)[y]**
2009	Aurora	TrigoCor low	$4.4x10^8$	0.12	0.4	16 ± 2	19.90 ± 5.77
		Nontreated	--	--	--	15 ± 2	12.43 ± 3.86
2009	Ithaca	TrigoCor low	$3.8x10^8$	0.13	0.8	21 ± 3	20.10 ± 1.74
		Nontreated	--	--	--	24 ± 3	17.20 ± 1.75
2010	Aurora	TrigoCor low	$8.6x10^8$	0.08	0.3	9 ± 2	3.28 ± 0.25 a
		TrigoCor high	$8.6x10^8$	0.08	1.0	4 ± 1	1.96 ± 0.29 b
		Nontreated	--	--	--	10 ± 4	3.25 ± 0.67 ab
2010	Ithaca	TrigoCor low	$7.7x10^8$	0.03	0.5	8 ± 1	4.41 ± 0.15 y
		TrigoCor high	$7.7x10^8$	0.03	2.1	6 ± 1	4.04 ± 0.28 y
		Nontreated	--	--	--	7 ± 1	2.78 ± 0.20 x
2010	Aurora[z]	TrigoCor low	$7.0x10^8$	0.05	0.5	20 ± 5	0.81
		TrigoCor high	$7.0x10^8$	0.05	1.1	14 ± 3	0.72
		Nontreated	--	--	--	18 ± 7	1.20

[u] Inoculum *Bacillus* cell concentrations, quantified by dilution plating. Corresponding population levels on wheat spikes are shown in Figs 2 and 3.

[v] Inoculum iturin concentrations. Data represent the means of two HPLC peak areas quantified based on comparisons with an iturin standard. Corresponding iturin levels on wheat spikes for the 2010 spring wheat experiment are shown in Fig. 3.

[w] Per row, the total volume of inoculum applied was divided by the number of wheat spikes, then these values were averaged for each treatment. In 2010, these amounts correspond to the 'high' and 'low' designations in the treatment names.

[x] FHB index = a combined measure of Fusarium head blight severity and incidence. Data represent the mean ± standard error. Means were arcsine transformed prior to separation by t-test in 2009 and ANOVA followed by Tukey HSD in 2010. There were no statistically significant differences between treatments in 2009 ($P = 0.6409$ for Ithaca and $P = 0.8300$ for Aurora), 2010 population dynamics trials ($P = 0.3938$ for Ithaca and $P = 0.1695$ for Aurora), or 2010 metabolite dynamics trial ($P = 0.7729$).

[y] DON = deoxynivalenol. Data represent the mean ± standard error. Means were natural log transformed prior to separation by t-test in 2009 and ANOVA followed by Tukey HSD in 2010. Means followed by the same letter were statistically different in 2010 population dynamics trials ($P < 0.0001$ for Ithaca and $P = 0.0118$ for Aurora). There were no statistically significant differences between treatments in 2009 ($P = 0.2282$ for Ithaca and $P = 0.2927$ for Aurora).

[z] Spring wheat field experiment for measuring metabolite dynamics. Population and iturin levels on wheat spikes are shown in Fig. 3. All other experiments listed were winter wheat trials for measuring population dynamics.

There was a numerically small but statistically significant difference in DON levels at IFS and AFS in 2010, however in neither location did TrigoCor treatment significantly reduce DON from the nontreated control. At AFS the high TrigoCor treatment resulted in significantly less DON accumulation than the low TrigoCor treatment. There were no statistically significant differences in FHB index among treatments in either location, however at AFS the high TrigoCor treatment resulted in a numerically lower FHB index than did the other two treatments.

Background *Bacillus* level on spikes in the greenhouse and field. In the greenhouse and in both field locations in 2010, the *Bacillus* level on wheat spikes collected prior to TrigoCor application was estimated at 10^2 CFU/spike. The two or more orders of magnitude discrepancy between this pre-TrigoCor application *Bacillus* level and the post-TrigoCor application level observed on nontreated spikes in the greenhouse (Fig. 1) and field (Fig. 2) suggests that a significant portion of the *Bacillus* recovered from nontreated spikes in our experiments resulted from the TrigoCor spray, perhaps through drift of inoculum in the air or through physical spreading of cells by insects or humans.

Estimation of *Bacillus* population present as spores. The percentage of spores from TrigoCor-treated spikes fluctuated in both the greenhouse and the field, but was typically 60 to 90 %. The percentage of spores from nontreated spikes was 60 to 90 % in the field, and 40 to 100 % in the greenhouse. Typical TrigoCor inoculum contains 50 to 80 % spores.

Estimation of inoculum delivery to wheat spikes. Pyranine dye applied at the normal greenhouse rate resulted in a volume delivered to wheat spikes that was approximately two times higher than the volume delivered to wheat spikes for the 'high TrigoCor' Ithaca field site rate, one order of magnitude higher than the volume delivered to wheat spikes at the 'low TrigoCor'

Ithaca field site rate, and two orders of magnitude higher than dye delivered to wheat spikes at the rate typical for commercial spray equipment (Table 1).

Time course of metabolite dynamics. In both the greenhouse and in the field samples, iturin biomarker levels on wheat spikes decreased over a 3 day period post-TrigoCor application (Fig. 3). Initially, the level of iturin biomarkers was approximately three times greater on greenhouse spikes than on field spikes using a high TrigoCor application rate, however by 3 days post-TrigoCor application, iturin biomarker levels on spikes from the two environments were similar (Fig. 3). No statistical comparison was performed due to the fact that trials were performed in separate environments with different experimental protocols. Iturin biomarker levels on field wheat spikes treated with a low TrigoCor application volume were detectable but low.

In both the greenhouse and in the field, iturin biomarker levels decreased over the sampling period despite the presence of *Bacillus* populations in significant quantities (Fig. 3). The levels of *Bacillus* populations recovered from spikes in both environments were in keeping with the range of values observed in population dynamics trials (Figs. 1,2).

There were no statistically significant differences in FHB/DON among the three treatments in the field experiment (Table 2). Due to the large number of wheat spikes needed for detection of iturins, there was insufficient space available to simultaneously measure FHB/DON control in the greenhouse; however TrigoCor applications at the rate used in these trials have consistently resulted in significant FHB/DON control (48).

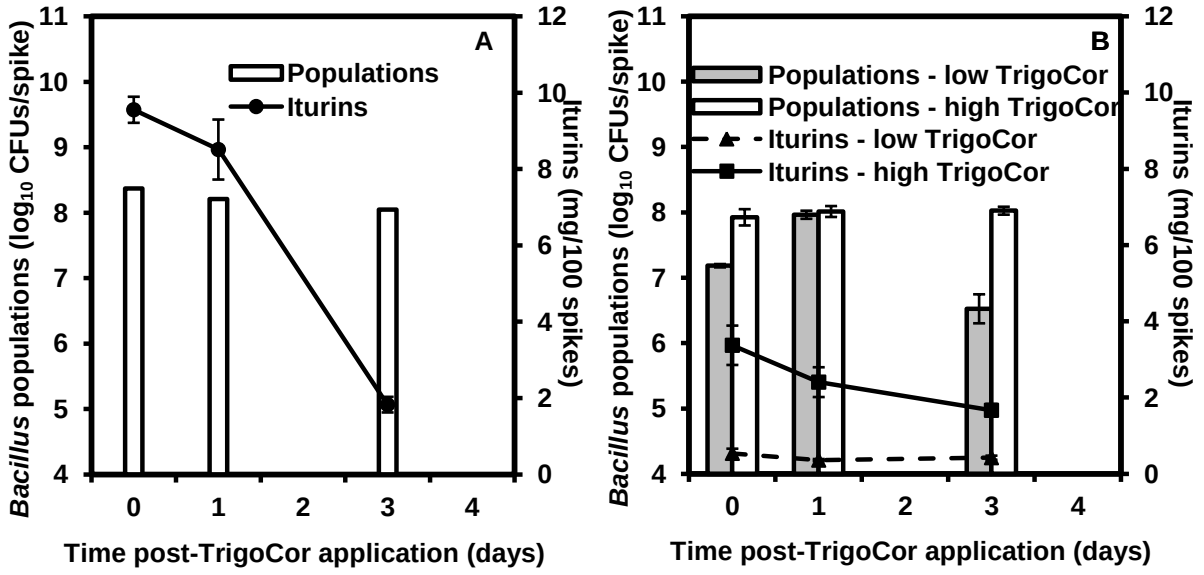

Fig. 3. Recovery of *Bacillus* populations and *Bacillus*-synthesized iturins from wheat spikes following TrigoCor application in **A,** the greenhouse and **B,** a field of spring wheat in 2010. In **B**, TrigoCor inoculum was applied in high and low application volumes. Inoculum concentrations, application volumes, and disease data for **B** is presented in Table 2. Iturin levels were calculated by averaging the quantities estimated from two peaks in the HPLC profile, and *Bacillus* population levels were quantified using dilution plating. In **A**, iturin quantities shown are averaged from three experiments, and population levels shown are from the third experiment. In **B**, iturin quantities shown for each treatment are averaged from three replicates within the same experiment, where each replicate was a 100-spike sample collected within the same field plot. Population means for each treatment are based on two samples of four bulked spikes collected at each time point within the same field plot. Vertical error bars represent the standard error of the mean.

Concentration effects of TrigoCor inoculum. Treatment with full strength and diluted inoculum produced *Bacillus* populations that approximated the range of levels observed on wheat spikes in the greenhouse and in the field, respectively. The calculated level of day 0 iturins on wheat spikes for each treatment indicated that the 10- fold dilution produced iturin levels on wheat spikes that were similar to levels in the 'high TrigoCor' treatment in the field, and that the full-strength inoculum resulted in iturin levels roughly equivalent to those recorded previously in the greenhouse (Fig. 3, Table 3).

TABLE 3. Comparison of disease control efficacy in the greenhouse with 10- and 100- fold diluted TrigoCor inoculum. [z]

TrigoCor inoculum characteristics			***Bacillus* population levels on wheat spikes (CFUs/spike)** [x]		**Iturins on wheat spikes, calculated (mg/spike)**[y]	**FHB disease parameters**	
Cells (CFUs/ml)[t]	**Iturins (mg/ml)**[u]	**Volume applied (ml/spike)**	**Day 0**	**Day 14**	**Day 0**	**FHB Index** [v]	**DON (ppm)**[w]
5.8×10^8	0.25	3.0	1.8×10^8	1.1×10^8	0.13	6 ± 3 a	6.17 ± 2.16 a
9.6×10^7	0.06	3.2	3.6×10^7	1.4×10^7	0.03	27 ± 4 b	28.87 ± 5.10 b
9.3×10^6	N.m.[s]	3.0	1.2×10^6	1.1×10^6	--	30 ± 4 b	25.93 ± 2.14 b
--	--	--	5.2×10^4	1.1×10^4	--	33 ± 4 b	31.96 ± 3.50 b

[s] N.m.= not measurable
[t] Inoculum *Bacillus* cell concentrations, quantified by dilution plating.
[u] Inoculum iturin concentrations. Data represent the means of two HPLC peak areas quantified based on comparisons with an iturin standard.

[v] FHB index = a combined measure of Fusarium head blight severity and incidence. Means were arcsine transformed prior to statistical analysis. Means followed by the same letter were not statistically different ($P < 0.0001$) according to the Tukey HSD test within the general linear model procedure. Numbers represent the mean ± the standard error using nontransformed data.
[w] DON = deoxynivalenol. Means were natural log transformed prior to separation using the Tukey HSD test within the general linear model procedure. Means followed by the same letter were not statistically different ($P < 0.0001$). Numbers represent the mean ± the standard error using nontransformed data.
[x] Quantified using dilution plating from a bulked sample of four spikes per treatment.
[y] Calculated based on inoculum concentration and an inoculum delivery rate of 0.5 ml/spike.
[z] TrigoCor inoculum was diluted to produce *Bacillus* population levels on wheat spikes that were similar to levels observed in the field. All dilutions were made with sterile water, and water alone was applied as a negative control. Experiment was conducted three times with reproducible results. Data shown is from a representative experiment.

Treatment with full-strength inoculum provided the only statistically significant reduction in FHB and DON from the water-treated control. There were no significant differences in FHB index or DON between any of the other treatments.

Agar well diffusion assay. With each two-fold reduction in iturin concentration in an agar well diffusion assay, there was a statistically significant decrease in *F. graminearum* growth inhibition (Table 4). A minimum inhibitory concentration of 0.16 mg iturin/ml was identified, based on this concentration being the lowest to produce *F. graminearum* inhibition. A TrigoCor culture filtrate sample run alongside the iturin dilutions produced a zone of inhibition that was statistically equivalent to the zone produced by this minimum inhibitory concentration. This assay was repeated two additional times with comparable results (data not shown).

TABLE 4. Relationship between iturin concentration and *Fusarium* growth inhibition in well diffusion assays.[z]

Sample	Inhibition zone radius (mm)[y]
Prosaro (0.3 % v/v)[x]	18.3 ± 0.2 a
Iturin (5.00 mg/ml)	11.8 ± 0.1 b
Iturin (2.50 mg/ml)	10.6 ± 0.1 c
Iturin (1.25 mg/ml)	9.2 ± 0.1 d
Iturin (0.63 mg/ml)	7.3 ± 0.2 e
Iturin (0.31 mg/ml)	5.2 ± 0.2 f
TrigoCor culture filtrate	3.2 ± 0.2 g
Iturin (0.16 mg/ml)	2.9 ± 0.2 g
Iturin (0.08 mg/ml)	0.0 ± 0.0 h
Iturin (0.04 mg/ml)	0.0 ± 0.0 h
Neg control (9 % MeOH)	0.0 ± 0.0 h

[x] Prosaro 421 SC (19 % prothioconazole, 19 % tebuconazole) is a fungicide registered for use against Fusarium head blight. Rate is based on concentration applied to acre of wheat according to label (6.5 fl oz/20 gal).

[y] Radius defined as the distance from the edge of the agar well to the edge of the inhibition zone. Data shown are means ± standard error. Data represent the mean inhibition from three plates, with four radius measurements recorded and averaged per sample per plate. Means followed by a different letter are significantly different ($P < 0.0001$) according to the Tukey HSD test within the general linear model procedure.

[z] Assay was repeated three times with comparable results. Results shown are from a representative assay.

Effect of repeated *Bacillus* applications in the field. Triple applications of TrigoCor in the field resulted in significant reductions in FHB index and DON compared to a nontreated control and a single TrigoCor treatment (Table 5). The triple TrigoCor application treatment had significantly lower DON, and numerically but not statistically significantly lower FHB index than did the single application of TrigoCor followed by two broth applications, indicating that nutrient addition by the two later inoculations may have contributed to the reduction in disease symptoms observed. The single TrigoCor application treatment resulted in FHB index and DON values that were similar to the nontreated control, which has been a common trend in previous field trials (Table 2).

TABLE 5. Reduced contamination of grain with deoxynivalenol following multiple applications of the biocontrol agent *Bacillus amyloliquefaciens* strain TrigoCor in the field.

Treatment [x]	FHB index [y]	DON (ppm) [z]
TrigoCor applied day 0, 2, 4	6 ± 0 a	2.45 ± 0.15 a
TrigoCor applied day 0; noninoculated broth applied day 2, 4	13 ± 1 ab	6.80 ± 0.70 b
TrigoCor applied day 0; no treatment day 2, 4	20 ± 0 b	9.25 ± 0.15 b
Nontreated	20 ± 4 b	9.70 ± 1.80 b

[x] TrigoCor inoculum cell (CFU/ml) and iturin (mg/ml) concentrations, respectively, by application day were 1.0×10^8 and 0.05 (day 0); 9.8×10^7 and 0.08 (day 2); and 4.0×10^7 and 0.04 (day 4). On all days approximately 1.2 ml inoculum was applied per spike.
[y] FHB index = a combined measure of Fusarium head blight severity and incidence. Means were arcsine transformed prior to statistical analysis. Means not followed by the same letter were statistically different (P = 0.0286) according to the Tukey HSD test within the general linear model procedure. Numbers represent the mean ± the standard error using nontransformed data.
[z] DON = deoxynivalenol. Means were natural log transformed prior to separation using the Tukey HSD test within the general linear model procedure. Means not followed by the same letter were statistically different (P = 0.0078). Numbers represent the mean ± the standard error using nontransformed data.

Discussion

In this study we found that there were large differences in the levels of both *Bacillus* cells and *Bacillus*-synthesized iturins on wheat spikes in the greenhouse versus the field over time, although the overall trends were similar in both settings. *Bacillus* populations recovered from spikes in the greenhouse (Fig. 1) were typically one or more orders of magnitude higher than in the field (Fig. 2), and in both environments populations were fairly stable although in the field there was a single order of magnitude decline over 14 days. Populations in the field survived at significant levels through harvest. Despite the presence of significant populations, iturin levels declined rapidly on wheat spikes throughout 3 days post-application in both the greenhouse and in the field, but the initial iturin amount recovered from spikes was at least three times higher on spikes in the greenhouse as compared to the field (Fig 4). Statistical analysis was not performed comparing population or metabolite values recovered from spikes in the greenhouse and field due to the distinct nature of these environments and because different experimental protocols were used in these settings.

Our observation that *Bacillus* population levels are relatively constant on wheat surfaces is similar to trends reported for *Bacillus* populations in the soil (2, 28, 29, 34, 50), which often stabilize as spores following an initial dying off of non-sporulating or slowly sporulating vegetative cells. On foliar surfaces, *Bacillus* populations have typically been found to decrease, often by two orders of magnitude or more, over a period of 14 to 15 days (1, 9, 45, 49, 57), which is a larger decline than that observed in this study. Bacterial colonization in the phyllosphere is controlled by many factors (35), so it is difficult to predict why the population loss we observed was less extreme than that from other phyllosphere studies.

Our result that iturins decrease quickly on wheat spikes despite the presence of significant populations is consistent with Mizumoto (34) and Asaka and Shoda (3), who described a similar trend following a *Bacillus* soil application. Little information is available on *Bacillus* metabolite persistence in the phyllosphere, although other studies (37, 43, 51) have reported the recovery of antifungal metabolites 5 to 6 days following application of washed *Bacillus* cells to detached melon leaves or apples. Possible mechanisms for the iturin loss from wheat surfaces we observed include microbial, chemical, or photo-degradation, or runoff from rainfall or irrigation. However, the lack of significant rainfall (1.3 mm) in the 3 day period when we observed decreasing iturin levels on wheat spikes in the field suggests that washing off is likely not the main cause of iturin loss from wheat spikes in this environment, although it is still possible that a large rain event might exacerbate iturin decline.

Fungal antagonism by iturins is the main mode of action for *Bacillus* biocontrol of FHB (14, 42), so information on iturin dynamics is particularly informative for understanding and enhancing disease control. We believe the role of *Bacillus* cells *in situ* to be relatively minor in FHB control, as indicated by replicated greenhouse trials in which treatment with culture filtrate provided more FHB/DON suppression than did treatment with washed cells (Kawamoto, Vaughan, Gibson, Bergstrom, and Crane, unpublished data). Furthermore, our finding that there was not a significant improvement in FHB/DON control when *Bacillus* populations on wheat spikes in the 2010 field trials were increased to a level initially matching that in the greenhouse (Table 2; Figs. 1, 2C&D), suggests that cell levels do not by themselves determine the success or lack of disease control.

Most *Bacillus* cells are present post-application as metabolically-dormant spores, which is probably why cells do not provide a major contribution to disease control. The insufficient

metabolite production of cells following application to wheat spikes is indicated by our result that there was a drastic decline in iturins despite the presence of significant *Bacillus* cell concentrations (Fig. 3). Other groups (37, 43, 51), have reported recovery of iturins and/or fengycins following application of washed *Bacillus* cells to post-harvest apples or to detached melon leaves, indicating that *Bacillus* cells may be able to produce metabolites on plant surfaces, however it is also possible that cells can produce a small amount of metabolites *in situ* but that the rate of production is not large enough to compensate for the quantity that is lost.

While information on *Bacillus* population dynamics does not directly explain disease control, it is still useful for understanding the ecology of this BCA and for identifying strategies to improve metabolite production. For instance, we found that *Bacillus* cells can survive on wheat spikes in the field for at least 40 days post-application (Fig. 2), indicating that if they can be modified genetically or through formulation to produce more antifungal compounds post-application, then they could potentially prevent infections throughout grain ripening.

Disease control in this system was dose-dependent (Table 3,3), and the dose of iturins currently being applied in greenhouse experiments was already fairly close to the minimum amount needed for control, as evidenced by the fact that even a 10-fold dilution in inoculum compromised FHB/DON suppression in the greenhouse (Table 3), and that a TrigoCor culture filtrate was not statistically more effective than the minimum iturin concentration needed for *Fusarium* inhibition in antibiosis assays (Table 4). Thus, any major reduction from the iturin level observed in the greenhouse, such as that observed on wheat spikes in the field, will decrease these essential *Bacillus*-synthesized components past the point of effectiveness. One potentially major limiting factor for FHB control by *Bacillus* in the field is inadequate delivery of inoculum, which by itself will reduce iturin levels below the threshold for successful control.

For instance, the amount of inoculum reaching spikes in the field at a high commercial-scale rate (187 liters/hectare) is two orders of magnitude lower than the volume delivered in the greenhouse (Table 1), and an even greater reduction can be expected with application rates typical in commercial-scale agriculture (47 liters/hectare for aerial application and 97 liters/hectare for ground application (19, 20)).

One option for enhancing BCA performance under field conditions may be increasing the concentration of metabolites in inoculum. Increased iturin concentrations in inoculum enhanced control of southern corn leaf blight, caused by *Bipolaris maydis* (56), and tomato damping-off, caused by *Rhizoctonia solani* (34). In our system, based on the minimum inhibitory concentration for *Fusarium* inhibition in antibiosis assays (Table 4), and on the rate of inoculum delivered to wheat spikes in the greenhouse and in field at a high commercially relevant rate (Table 1), iturin concentrations in inoculum would need to be increased greatly, 100 to 300-fold, to ensure that the resulting iturin levels on wheat spikes in the field would match the minimum effective level in the greenhouse (calculated at 8 mg iturins/100 spikes). For inoculum applied at the recommended rates for commercial-scale application (47 liters/hectare for aerial application and 97 liters/hectare for ground application (19, 20)), iturin concentrations would need to be increased even further. In antibiosis assays, even the most concentrated iturin sample only provided a little over half the *Fusarium* inhibition as did a registered FHB fungicide (Table 4), showing that very high inoculum concentrations of iturins will be essential for BCAs to compete with chemical controls. Despite the importance of antifungal metabolites in control of foliar diseases, however, commercial BCA products list *Bacillus* CFUs as the descriptor of product activity.

Due to the rapid decline of iturins we observed on wheat spikes (Fig. 3), we expect that increasing inoculum metabolite concentrations would provide the greatest advantage for disease control in the days immediately following application. Thus, alterations in *Bacillus* inoculum quality would be most beneficial when controlling diseases with predictable, narrow infection periods. For diseases such as FHB where DON-contributing *Fusarium* infections can occur over a period of 10 or more days post-anthesis (10, 13), such short-term enhancements in disease control would be less advantageous. Instead, having a high level of iturins that are maintained on plant surfaces over a longer period of time may be a more reliable means of improving biocontrol efficacy. Part of the success of TrigoCor in greenhouse trials may come from the fact that *Fusarium* is applied to spikes 1 day after TrigoCor treatment and then the wheat is placed in a mist chamber which facilitates rapid disease development while iturin levels are high. In contrast, in field experiments although we apply *Fusarium* at the same time post-TrigoCor treatment, natural infections are likely occurring at later times when iturin levels are too low or almost non-existent to be effective. When *Fusarium* infections in the greenhouse are delayed to 3 days post-TrigoCor application the magnitude of FHB control resulting from TrigoCor pre-treatment decreases (Kawamoto and Bergstrom, unpublished data), supporting the idea that longevity of antifungal activity is key to biocontrol success in this system.

In the 2011 field trial, we showed that a triple TrigoCor application provided significant FHB/DON suppression, whereas a single application did not (Table 5). Our assumption is that the triple application maintained high iturin levels over much of the critical FHB infection period following anthesis, however due to space and time constraints iturin levels were not measured. The effect of repeated applications of *Bacillus* BCAs has also been investigated in other disease systems with mixed results (5, 16), indicating that the advantage of increasing metabolite

longevity might be environment- or disease-dependent. Although the enhancement in biocontrol we observed from repeating TrigoCor applications is useful for experimental purposes, it is likely not economical in a wheat commercial setting. However, multiple applications of *Bacillus* should be considered for crops where this method might be more economically viable, such as for high value horticultural crops, crops grown on a small scale, or for organic production where options for disease control are more limited. We are currently exploring other avenues for improving metabolite longevity on wheat surfaces, such as by activating metabolite production from *Bacillus* cells post-application, or by adding stabilizers to our inoculum.

In summary, we propose that successful control of FHB/DON by *Bacillus* relies on adequate levels of a key antifungal compound, iturin, and that insufficient levels of this metabolite on wheat spikes in the field are responsible for the lack of disease control in this environment. Furthermore, our finding that iturins decline rapidly on wheat spikes in the field and greenhouse despite the presence of significant *Bacillus* populations indicates that *Bacillus* cells are not adequately replenishing lost iturins on wheat surfaces. Our results suggest that future research on enhancing *Bacillus* control of foliar diseases should focus on increasing the level and longevity of iturins in the phyllosphere, particularly when combating diseases with long infection periods.

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CHAPTER 3

NUTRIENT-INDUCED SPORE GERMINATION OF A *BACILLUS* AMYLOLIQUEFACIENS BIOCONTROL AGENT ON WHEAT SPIKES

Abstract

One of the challenges of applying *Bacillus* biological control agents to aboveground plant parts is that *Bacillus* transitions to a metabolically dormant spore state on plant surfaces, and therefore is unable to produce sufficient antimicrobial compounds for effective control. In this study we investigated the feasibility of applying nutrient germinants to wheat surfaces to stimulate the germination and metabolic functioning of the plant disease biocontrol agent *B. amyloliquefaciens* strain TrigoCor. Using the terbium chloride assay and phase contrast microscopy, we screened potential germinants of TrigoCor spores, and found that a combination of D-glucose, D-fructose, and potassium chloride (GFK), in addition to either L-asparagine (Asn-GFK) or L-alanine (Ala-GFK), induced maximal levels of TrigoCor spore germination *in vitro*. However neither Asn-GFK nor Ala-GFK was able to improve control of the wheat disease Fusarium head blight in controlled settings. Despite not having the desired effect on disease control, the germinant mixture Asn-GFK was able to significantly stimulate TrigoCor spore germination on plant surfaces. The successful *in vivo* stimulation of TrigoCor germination suggests that nutrient-induced germination of *Bacillus* spores on plant surfaces is a feasible strategy for improving *Bacillus* biocontrol, and should be pursued further.

Introduction

Under certain conditions of nutrient depletion, bacteria in the Firmicutes classes *Bacilli* and *Clostridia* can transition from their normal vegetative state to that of a spore, a metabolically dormant resting structure capable of surviving for thousands to possibly millions of years (20). Although dormant, bacterial spores can sense their environments, and when favorable conditions return these spores lose their resistance capabilities in exchange for a regaining of metabolic functions and vegetative growth through the processes of germination and outgrowth (31, 32).

Germination of *Bacillus* spores can be triggered by both nutrient and non-nutrient germinants, and occurs in a species- and strain- specific manner. Nutrient germination is receptor-mediated, and in *B. subtilis* is driven by three types of receptors: GerA, which binds L-valine and L-alanine and its derivatives, and GerB and GerK, which together recognize a germinant composed of a combination of L-asparagine, D-glucose, D-fructose, and the potassium ion. Other *Bacillus* species respond to a range of different nutrient germinants and combinations of germinants (27). For instance, *B. cereus* responds to purine ribonucleosides and several amino acids either individually or in mixtures (16), and *B. anthracis* only responds independently to L-alanine, and for all other germinants must have either alanine or purine ribonucleosides as cogerminants (17). Non-nutrient germination in *Bacillus* is not receptor-based, and can occur following exposure to salts, cationic surfactants, lysozyme, high pressures, or a chelate of Ca^{2+} and pyridine-2, 6-dicarboxylic acid (DPA), a compound released from germinating spores (40).

Significant research effort has been devoted to understanding the process of spore germination, particularly in the model gram-positive organism *B. subtilis*, and great strides have been made in understanding the physical and mechanistic properties of germinant receptors and

other proteins involved in the germination process (12, 23, 32, 41, 42, 52). Most of these studies are conducted *in vitro* using lab strains or mutants derived from such strains, and while these experiments are useful in elucidating the intricacies of spore germination, less is known about the feasibility of inducing spore germination of environmental and/or commercially relevant *Bacillus* strains *in vivo* to obtain a commercially-desirable outcome.

In this study, we examined the *in vitro* and *in vivo* germination of spores from the TrigoCor strain of *B. amyloliquefaciens*, a species closely related to but distinct from *B. subtilis* (34). TrigoCor is being developed as a biological control agent for the wheat and barley disease Fusarium head blight (FHB), caused in North America by the filamentous fungus *Fusarium graminearum*. FHB causes significant economic damage resulting in large part from the production by *Fusarium* of the grain-contaminating mycotoxin deoxynivalenol (DON) (25). Control of FHB is currently hindered by the lack of completely resistant wheat cultivars (3) and by inconsistent reductions in FHB and DON from fungicides (5, 26, 33, 49). Biological control is an attractive management option in this system because unlike fungicides, biocontrol agents can be used by organic farmers, are more socially acceptable due to their lower human health and environmental impact, and also can be legally applied later in the growing season, thereby potentially protecting against later-season DON-producing *Fusarium* infections that fungicides cannot prevent due to restrictions on their application timings (25).

The impetus for this study came from an earlier observation (10) that a TrigoCor-produced antifungal lipopeptide, iturin, declined drastically on wheat surfaces by 3 days post-TrigoCor application. Iturin is critical for *Fusarium* inhibition by TrigoCor (24, 35), and its decline was believed to contribute to the inconsistent and often insufficient control of FHB and DON in the field, where DON-producing *Fusarium* infections can occur over a period of 10 or more days

during wheat development (9, 11). Iturin is delivered in the TrigoCor inoculum along with a mixture of TrigoCor vegetative cells and spores, and the production of additional iturins on wheat surfaces is detectable but small compared to the amount that is delivered initially. The drastic decline in iturins was observed despite the presence of significant *Bacillus* populations on wheat spikes for 14 or more days post-application, likely due to the fact that the cells were primarily in the metabolically dormant spore form and therefore unable to replenish iturins as they were lost (10). Thus, it was hypothesized that stimulating *Bacillus* spore germination on wheat surfaces could activate *Bacillus* cells to produce additional iturins, therefore prolonging the antifungal activity of this biological control agent and improving its efficacy.

The objectives of this study were therefore to identify potential germinant(s) that could stimulate TrigoCor spore germination *in vitro*, to determine if these germinant(s) stimulated *Bacillus* spore germination on plant surfaces, and then to assess if application of these germinant(s) to wheat plants pre-treated with TrigoCor spores improved FHB/DON control in controlled environments.

Materials and Methods

Microbial cultures and *Bacillus* spore purification. All germination assays were conducted using *Bacillus amyloliquefaciens* strain TrigoCor (4, 10). Additional trials were performed using *Bacillus subtilis* var. *amyloliquefaciens* NRRL B-50349 (Taegro®, Novozymes BioAg), as noted. *Fusarium graminearum* sensu stricto strain Gz014 NY98 (10) was grown as described previously (10), with exceptions noted for individual experiments.

Bacillus starter cultures were grown in nutrient broth plus yeast extract (10), then were transferred 1:1000 to a modified Schaeffer's sporulation medium (30) containing per liter 2.012 g KCl, 0.492 g $MgSO_4 \cdot 7H_20$, no NaOH, and 0.0197 g $MnCl_2 \cdot 4H_20$, and grown for 72 h. All cultures were grown in baffled flasks at 37 ºC with 225 RPM shaking. Spores for greenhouse assays were grown in 500 mL aliquots in 1 l flasks, and for all other assays were grown in 50 mL aliquots in 100 mL flasks.

For *Bacillus* spore purification, liquid cultures were spun down at 4 ºC for 10 min at 10,000 RCF, washed 10 times in cold deionized water, and resuspended in cold deionized water. To remove vegetative cells, cultures were treated with lysozyme (final concentration 50 µg/mL) for 10 min on ice, followed by six rounds of 15 sec sonication on ice, then were washed twice with cold deionized water. Spore preparations were stored in deionized water at 4 ºC, and prior to use were washed once with cold deionzied water, then resuspended in 25 mM HEPES (Sigma-Aldrich, St. Louis, MO) pH 7.4 to produce a final concentration of 10^8 CFUs/mL, unless otherwise noted. Purified spore cultures contained over 98 % phase-bright spores and minimal cell debris, as verified through phase contrast microscopy.

Terbium chloride and germinant preparations. Unless otherwise noted, terbium chloride (1 mM) (Sigma-Aldrich, St. Louis, MO) and all germinant solutions were prepared in 25 mM HEPES pH 7.4. Germinants tested were selected from the literature (6, 31) and included D-glucose (glucose), D-fructose (fructose), KBr, KCl, L-alanine (alanine), L-asparagine (asparagine), L-proline (proline), L-valine (valine), calcium-DPA (Ca-DPA, mixture of $CaCl_2$ and DPA), and the germinant combinations alanine-glucose (equimolar solution of alanine and glucose), Asn-GFK (equimolar solution of asparagine, glucose, fructose, and KCl), and Ala-GFK (equimolar solution of alanine, glucose, fructose, and KCl). Unless otherwise noted, all

germinants were prepared to produce a final concentration of 10 mM following mixture with *Bacillus* spores.

Terbium chloride assay. For the initial screen of potential germinants, 90 µL of TrigoCor spores was mixed with 10 µL of each germinant, or HEPES, in the wells of a 96-well plate (Thermo Scientific, Waltham, MA) and incubated at 37 ºC for 1, 10, 30, 60, or 120 min. Samples were prepared so that all incubations finished simultaneously and were analyzed together. For each germinant tested, two spore-germinant mixtures were loaded per incubation time. Following addition of 100 µL terbium chloride to all samples, fluorescence was measured immediately as explained below. Assay was repeated three times, and typically with separate spore preparations, with comparable results.

In all other terbium chloride assays, 450 µL TrigoCor spores were mixed with 50 µL germinant or buffer, and incubated at 37 °C. Two *Bacillus*-germinant samples were prepared for each germinant and concentration being tested. At each sampling time, 100 µL was transferred from each incubation tube to a 96-well plate. For an initial measurement of spore germination, two 100 µL samples of spores not mixed with germinants were analyzed either alongside the 10 minute post-germinant addition sample, or separately prior to the start of the assay. Immediately after samples were loaded into the plate, 100 µL terbium chloride was added to each well and fluorescence was measured as described below. Assays were repeated twice, with separate spore preparations, and with comparable results.

Fluorescence was measured at 545 nm emission and 273 nm excitation according to Yi and Setlow (53) using a Synergy 4 plate reader (Biotek Instruments Inc., Winooski, VT). At each plate reader measurement time, fluorescence was also measured from wells containing two 90 µL samples of TrigoCor spores autoclaved for 60 min mixed with 10 µL HEPES, or a sample

containing a mixture of all germinants and HEPES, mixed with 100 µL terbium chloride. A final sample of pure deionized water was also analyzed alongside all other samples in the plate. The samples containing the germinant mixture and deionized water served as negative controls, and did not produce any significant fluorescence in any of the trials. Percent spore germination for each sample at each time point was calculated by dividing its relative fluorescence units (RFUs) by the corresponding average RFUs of the autoclaved cells, which have released all their dipicolinic acid (DPA) due to lysis (50). Spore germination results were routinely confirmed using phase contrast microscopy (data not shown).

***Bacillus* spore germination at room temperature.** For analysis of spore germination with alanine-glucose and Asn-GFK, TrigoCor and Taegro germination were performed in a 96-well plate (USA Scientific, Orlando, FL) by mixing 160 µL spore suspension with 40 µL of germinant or HEPES. Spores and germinants were incubated at room temperature (24 - 26 ºC) with slow continuous shaking, and the optical density at 600 nm (OD600) was recorded periodically. For analysis of spore germination with alanine substituted for asparagine, TrigoCor samples were incubated in tubes which were vortexed periodically but were otherwise motionless, and OD600 was not measured.

Spores and germinants were incubated for 6 h, which was previously identified as the time required for the majority of spore germination to occur (data not shown). Three replicates per treatment were analyzed and assays were performed twice, with separate spore preparations, and with comparable results. Percent spore germination was examined using the phase contrast setting of a compound microscope (Carl Zeiss, Oberkochen, Germany) and was calculated by dividing the number of cells that were phase dark spores or vegetative cells by the total number of cells (phase bright and dark spores, and vegetative cells). At least five fields of view

representing a minimum total of 200 spores per replicate were analyzed. The results of spore germination as monitored through OD600 readings matched the trends observed with microscopy (data not shown).

Germination was measured using microscopy instead of the terbium chloride assay due to the extended incubation times necessary for germination to occur at room temperature; for the terbium chloride assay a decline in fluorescence was occasionally noticed at 120 and 180 minutes post-germinant application, possibly because of DPA degradation or re-uptake by cells.

TrigoCor germination on plant surfaces. The highly susceptible to Fusarium head blight spring wheat cultivar 'Norm,' which is the standard cultivar used for greenhouse experiments in our lab, was grown in the greenhouse and trimmed for experiments as described previously (10). Wheat spikes were sprayed with a hand-held atomizing sprayer according to our typical greenhouse protocol (10), with pre-treatments of either TrigoCor spore suspensions in deionized water or deionized water alone, such that two pots with 10 spikes each were sprayed per pre-treatment. Wheat spikes were at late anthesis (Feekes wheat developmental scale 10.5.3) at time of spraying. After drying for 4.5 h, 4 (trial 1) or 5 (trial 2) spikes per pot were removed for quantification and characterization of *Bacillus* spore populations as described below. HEPES or Asn-GFK (100 mM in HEPES) were sprayed onto spikes from one pot of each of the pre-treatments, according to the same protocol as used for the TrigoCor sprays. After drying for 3 h, spikes were sprayed with a fine mist of deionized water using a household sprayer (Consolidated Plastics, Stow, OH) and covered with a plastic bag (AEP Industries Inc., Peabody, MA) to produce a humid environment conducive for spore germination. Plastic bags were removed 24 h later and 5 spikes per pot were removed for quantification and characterization of *Bacillus* spore

populations. Although the wheat was grown in the greenhouse, all applications were conducted at room temperature (24 - 26 ºC) in the laboratory.

Bacillus spore populations from wheat spikes were quantified as described in Jochum et al. (19), except that spikes were processed individually using, per spike, 1 mL potassium phosphate buffer amended with 0.1 % Triton X-100. Colonies were identified as *Bacillus* based on visual appearance, such as white and non-shiny. To measure the percentage of *Bacillus* cells present as dormant spores, samples were removed from the dilution series made to measure total population size, then were heated at 85 ºC for 10 min to kill germinated spores, and dilution-plated. Colony numbers were compared to the corresponding counts from the non-heat treated dilution series plates to calculate percent dormant spores.

***Fusarium* growth assay.** *F. graminearum* starter cultures grown on potato dextrose agar as described previously (10) for 7 - 9 days were flooded with sterile deionized water, filtered through 8 layers of sterile cheesecloth, and adjusted to a final concentration of 1×10^4 conidia/mL. Conidia (200/plate) were plated onto 3 plates of 1 % water agar amended with each germinant being tested, or HEPES.

Plates were incubated at 25 °C in the dark for the initial 24 h, then were grown at room temperature with 12 h UV (40 W, 350 nm) light/day. Photos of mycelial growth were taken after 3 days growth. Trials were repeated twice with comparable results.

Greenhouse experiments. Greenhouse trials were performed as described in previously (10) with the following exceptions.

For the trial with Asn-GFK, TrigoCor spores (4×10^8 CFU/mL) in deionized water or deionized water alone were sprayed onto wheat spikes at the milk stage (Feekes wheat developmental scale 11.1). TrigoCor spores were grown and purified as described for *in vitro*

assays, except that spores were grown in larger volumes. Pure TrigoCor spores were applied rather than a mixture of vegetative cells and spores, to more effectively test the ability of spores to germinate and produce biologically relevant levels of antifungal compounds. TrigoCor spores were kept at 4 °C until time of spray. There was no measurable level of antifungal compounds from TrigoCor spore suspensions, as determined by high-performance liquid chromatography analysis (data not shown). After wheat spikes had dried for approx 4 h, spikes from 7 pots of each treatment were sprayed with either HEPES or Asn-GFK (100 mM in HEPES) according to the same protocol as used for the TrigoCor sprays. After drying for 45 min, wheat was put into a mist chamber for 24 h. The mist chamber was set at 72 °F and adjusted to produce a fine mist on wheat surfaces, 78 % RH for 17 h followed by 40% for 8 h. Following removal from the mist chamber and drying for approximately 45 min, wheat spikes were sprayed with *Fusarium* conidia from a culture grown for 9 days on full-strength potato dextrose agar.

For the trial with Ala-GFK, the germinant treatments tested were deionized water or Ala-GFK (10 mM) in deionized water; the mist chamber was set to 65 % RH for the initial 24 h period post-germinant application but pre-*Fusarium* application; and the *Fusarium* conidia were harvested from an 11 day old culture which had been transferred from an 8 day old starter culture. Additionally, each treatment had an additional pot of wheat from which spikes were collected and assayed for spore germination. Five wheat spikes were collected from each pot prior to application of germinant, as well as after removal of wheat from the mist chamber following germinant application but before *Fusarium* application. *Bacillus* populations from each spike were quantified and the percent spores were estimated by heat treatment as described above.

At the end of the experiments, wheat spikes were harvested and cleaned by hand. Grain was ground for 1 min/sample using a coffee grinder (Capresso, Closter, NJ), and DON quantification was performed in the laboratory of Dr. Yanhong Dong at the University of Minnesota.

Statistical analysis. FHB index was calculated by multiplying FHB incidence by FHB severity. FHB index and percent germination were arcsine transformed prior to statistical analysis. Data was analyzed using the PROC GLM function of the SAS statistical program version 9.3 (SAS Institute Inc., Cary, NC), and means were separated by Tukey HSD (P = 0.1). Where applicable, trends in means separation are shown for transformed means, and reported means and standard error were calculated using nontransformed data.

Results

Identification of potential *Bacillus* spore germinants using the terbium chloride assay. Because *Bacillus* germination is strain- and species- specific, we conducted several screens using the terbium chloride assay to determine which germinant(s) most stimulated TrigoCor germination. Of the nine candidate germinants selected from the literature (6, 31) only Asn-GFK, glucose, and alanine produced significant spore germination in the initial TrigoCor spore germination screen (Fig. 4). The percent spore germination produced by each treatment varied slightly among the trials, however the trends remained consistent. Asn-GFK produced the highest level of germination, which was consistently 80 % or higher by 120 min post-germinant addition. In all trials the extent of germination produced by alanine and glucose was significantly lower than that produced by Asn-GFK, and in the majority of trials, alanine and glucose produced similar levels of germination by 120 min post germinant-addition. Alanine is a

germinant for most *Bacillus* species, and Asn-GFK is a well-established germinant for *B. subtilis*, however in *B. subtilis* glucose is typically considered to be a co-germinant which enhances the germination of other germinants (2, 18, 51) but does not activate germination on its own (48), although it has been characterized as an independent germinant for other *Bacillus* species (36).

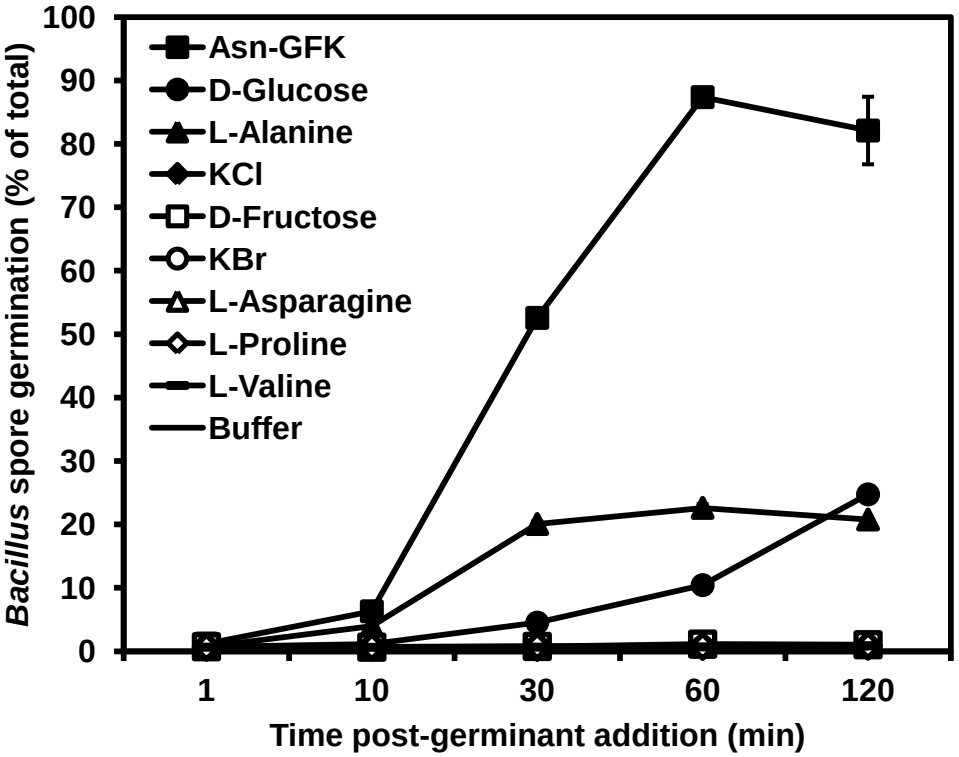

Fig. 4. TrigoCor spore germination at 37 °C following addition of candidate nutrient germinants (10 mM final concentration). Asn-GFK is an equimolar solution of L-asparagine, D-glucose, D-fructose, and KCl. Data points represent the mean percent spore germination of 2 replicates per sample, and vertical lines represent the standard error of the mean for each treatment.

Because the delivery rates of inoculum to wheat spikes sprayed in the field are variable, and because we wanted to ensure that by testing germinants at 10 mM we were not missing a larger-scale stimulation at higher concentrations, we tested each of the top germinants from our initial screen at a higher concentration. We also combined the two lower-performing candidate

germinants, L-alanine and D-glucose, in a combination germinant, alanine-glucose. We found that Asn-GFK and alanine-glucose produced a relatively high level of TrigoCor spore germination at both 100 mM and 10 mM by 120 min post-addition (Fig. 5). The amount of spore germination stimulated by 100 and 10 mM Asn-GFK was greater than that stimulated by alanine-glucose at comparable concentrations at both 60 and 120 min post-germinant application, and there appeared to be a slight effect of concentration for both germinant mixtures at 30 and 60 min post-application, but only for alanine-glucose by 120 min post-application. Although alanine at 100 mM stimulated spore germination to an extent similar to Asn-GFK and alanine-glucose, at 10 mM alanine the level of spore germination produced was significantly lower. Glucose produced a low level of spore germination at both concentrations tested, and again the level was similar to that of alanine 10 mM. There was no detectable TrigoCor spore germination in the absence of germinant in any of the trials.

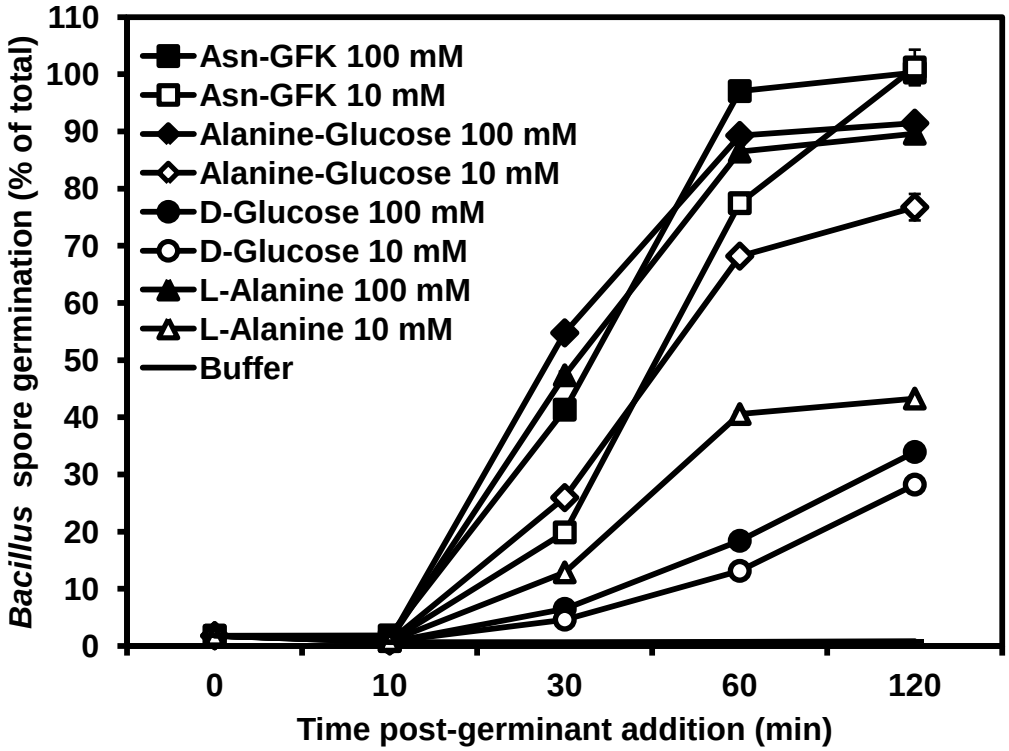

Fig. 5. Stimulation of TrigoCor spore germination at 37 °C following addition of nutrient germinants to a final concentration of 100 mM (closed symbol) or 10 mM (open symbol). Asn-GFK is an equimolar solution of L-asparagine, D-glucose, D-fructose, and KCl, and Alanine-Glucose is an equimolar solution of L-alanine and D-glucose. Data points represent the mean percent spore germination of 2 replicates per sample, and vertical lines represent the standard error of the mean for each treatment.

***Bacillus* germination at room temperature is most stimulated by Asn-GFK.**

Temperatures in New York field locations during the time of most FHB infections, i.e., early June (avg. daily max in 2008-2010 25 ºC) are typically closer to room temperature (24 - 26 ºC) than they are to 37 ºC. Assays at 37 ºC were useful due to the shortened time required for completion, however to better predict field performance of each germinant we also felt it was important to measure spore germination at room temperature, particularly because spore germination has previously been found to be temperature-dependent (18, 47, 48).

At room temperature, Asn-GFK stimulated nearly 100 % of TrigoCor spores to germinate (Fig. 6). Alanine-glucose produced a significantly lower level of TrigoCor spore germination

stimulation than did Asn-GFK, and cells mixed with buffer had an almost undetectable level of spore germination. Most germination was in the form of phase-dark spores, and only a minimal number of vegetative cells were observed. Compared to germination at 37 ºC (Fig. 5), at room temperature (Fig. 6) Asn-GFK produced a roughly equivalent amount of germination and alanine-glucose produced less germination. However it is possible that the alternative method for measuring germination contributed to this difference.

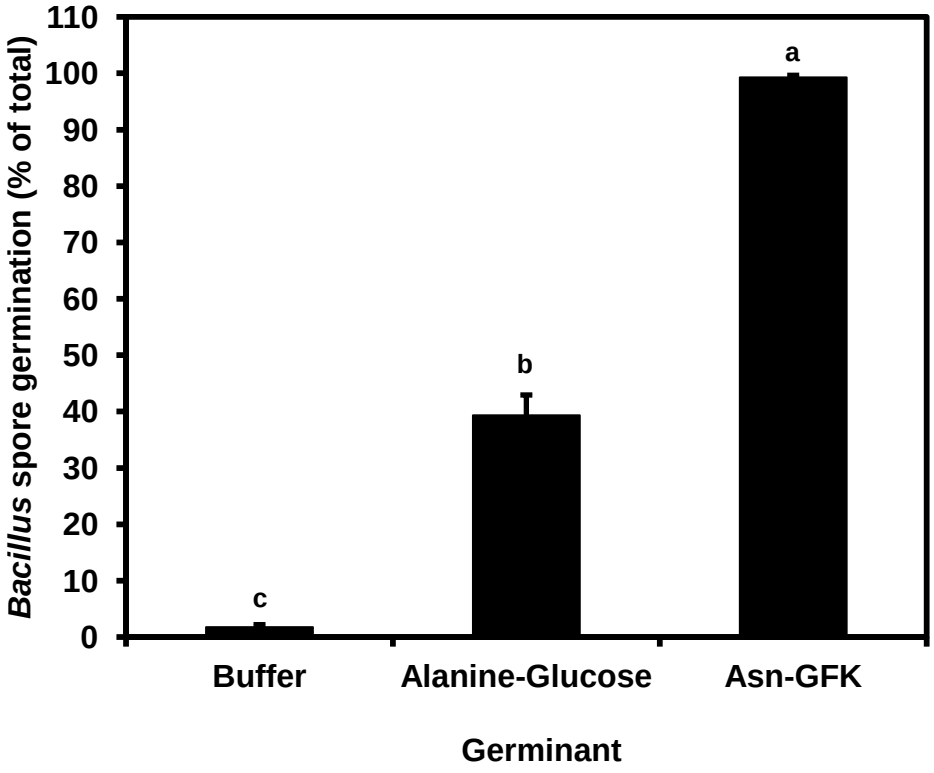

Fig. 6. Enhanced TrigoCor spore germination at room temperature following addition of nutrient germinants (10 mM final concentration). TrigoCor spore germination was measured 6 h after germinant addition. Asn-GFK is an equimolar solution of L-asparagine, D-glucose, D-fructose, and KCl, and Alanine-Glucose is an equimolar solution of L-alanine and D-glucose. Data points represent the mean percent spore germination of 3 replicates per sample, and vertical lines represent the standard error of the mean for each treatment. Means not followed by the same letter were statistically different ($p<0.0001$).

Only 1 and 21 % of Taegro spores germinated following alanine-glucose and Asn-GFK addition, respectively (data not shown), supporting the strain-specific concept of spore germination.

An additional germinant (Ca-DPA, 60 mM final concentration) was also tested in initial assays at room temperature but did not produce any significant TrigoCor germination (data not shown).

Asn-GFK stimulates *Bacillus* germination on plant surfaces. In both trials, *Bacillus* spores were able to germinate significantly on TrigoCor-treated wheat spikes with and without Asn-GFK application (Fig. 7). However, Asn-GFK stimulated a greater level of spore germination on wheat spikes (nearly 0 % dormant spores or 100 % germination) than did buffer (approximately 40 % dormant spores) by 24 h post-application, despite similar starting levels for these two treatments (Fig. 7). The level of *Bacillus* spore germination following Asn-GFK treatment was similar to that seen in *in vitro* trials (Fig. 4-6), and for buffer treatment it was greater, presumably because of the availability of additional germinants on the plant surface.

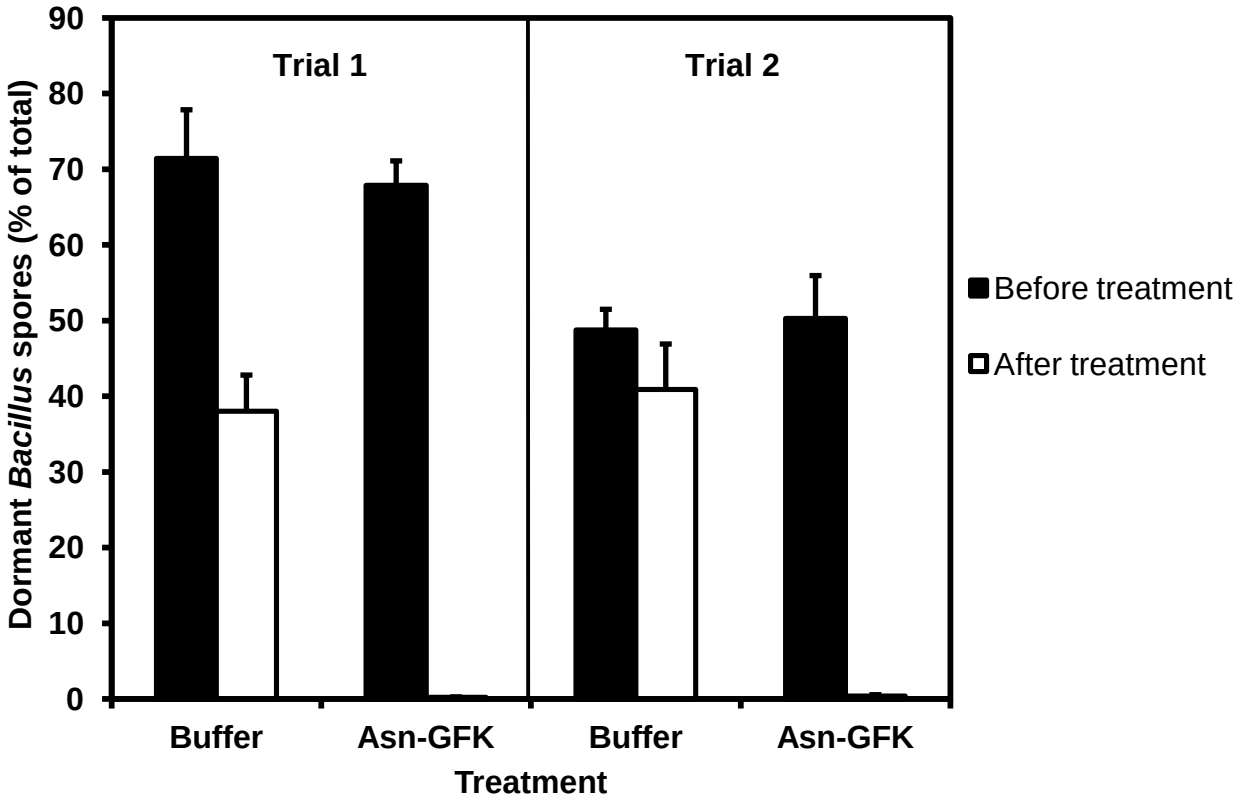

Fig. 7. Enhanced germination of *Bacillus* spores on wheat surfaces following treatment with an equimolar solution of L-asparagine, D-glucose, D-fructose, and KCl (Asn-GFK). Wheat spikes pre-treated with TrigoCor spores were sprayed with Asn-GFK (100 mM) in HEPES buffer, or with buffer alone, and incubated in a mist chamber for 24 h. The percentage of *Bacillus* cells in the dormant spore phase was estimated before germinant application and after misting, in two trials. Data represent mean percent dormant spores as estimated through heat treatment from 5 wheat spikes at each collection time (4 wheat spikes for trial 1 'Before Treatment' data), and vertical error bars represent standard error of the mean.

The total *Bacillus* population level on TrigoCor treated spikes was typically 10^7 CFUs/spike (data not shown). There was an increase in *Bacillus* population size following both buffer and Asn-GFK treatment, however the degree of increase was only great enough in one of the trials (trial 1, 3 – 4 scale increase for both treatments) to suggest any significant cell multiplication may have occurred (data not shown). On wheat spikes not treated with TrigoCor, buffer and Asn-GFK application followed by misting resulted in larger-scale *Bacillus* population increases,

particularly on spikes treated with Asn-GFK, however the populations were still one or more orders of magnitude lower at all time points than on TrigoCor-treated spikes (data not shown).

Greenhouse experiment with Asn-GFK. Treatment of wheat spikes with Asn-GFK as compared to buffer produced an approximately two-fold increase in FHB index and DON accumulation when the wheat spikes were pre-treated with water (Table 6).

Table 6. Increase in Fusarium head blight (FHB) symptoms and deoxynivalenol (DON) contamination of grain following treatment with an equimolar solution of L-asparagine, D-glucose, D-fructose, and KCl (Asn-GFK).

Treatment, Germinant [x]	**FHB Index** [y]	**DON (ppm)** [z]
Water, Buffer	24 ± 7 b	37.14 ± 13.15 b
Bacillus spores, Buffer	13 ± 4 b	22.33 ± 4.32 b
Water, Asn-GFK	50 ± 10 a	80.84 ± 17.65 a
Bacillus spores, Asn-GFK	18 ± 4 b	25.31 ± 5.57 b

[x] Water or TrigoCor spores were sprayed onto wheat spikes, followed by either HEPES buffer or Asn-GFK (100 mM of each component, in buffer). After 24 h in a mist chamber, all spikes were treated with *F. graminearum*.
[y] FHB index = a combined measure of Fusarium head blight severity and incidence. Means not followed by the same letter were statistically different (P = 0.0086). Numbers represent the mean ± the standard error using nontransformed data.
[z] DON = deoxynivalenol. Means not followed by the same letter were statistically different (P = 0.0055). Numbers represent the mean ± the standard error.

Pre-treatment of wheat spikes with TrigoCor spores instead of water produced a statistically significant reduction in FHB index and DON accumulation only when it was followed by an Asn-GFK application (Table 6). However, Asn-GFK application to spikes pre-treated with spores did not significantly improve disease control compared to spikes pre-treated with water or

spores followed by buffer. Without Asn-GFK, TrigoCor spores reduced FHB symptoms and toxin accumulation compared to water, but the difference was not statistically significant.

TrigoCor germination with Asn-GFK component mixtures and with alanine substituted for asparagine. The asparagine-glucose combination was present in the top four treatments to stimulate TrigoCor spore germination (Fig. 8). TrigoCor germination with mixtures containing asparagine and glucose was synergistic with each additional component. While neither KCl nor fructose produced any significant germination on their own (Fig. 4), both added to the germination stimulation provided by the asparagine-glucose combination, particularly KCl (Fig. 8), and all four components of Asn-GFK were necessary for maximal TrigoCor germination. These results differ from those of Wax and Freese (47) who found that the removal of any of the four A-GFK components would completely eliminate *B. subtilis* germination, and from Atluri et al. (2) who suggested a larger role for fructose than for KCl in *B. subtilis* spore germination.

When alanine was substituted for asparagine, the relative effectiveness of each 2-, 3-, and 4-component treatment was conserved (Fig. 9). Each germinant combination containing asparagine was more effective at stimulating germination than was the corresponding combination containing alanine, despite the fact that alanine but not asparagine could produce germination on its own (Fig. 4). These results differ from those of Wax and Freese (47) who found Ala-GFK to be more stimulatory than Asn-GFK in *B. subtilis*, however these differences could be due to species or strain specificities of spore germination.

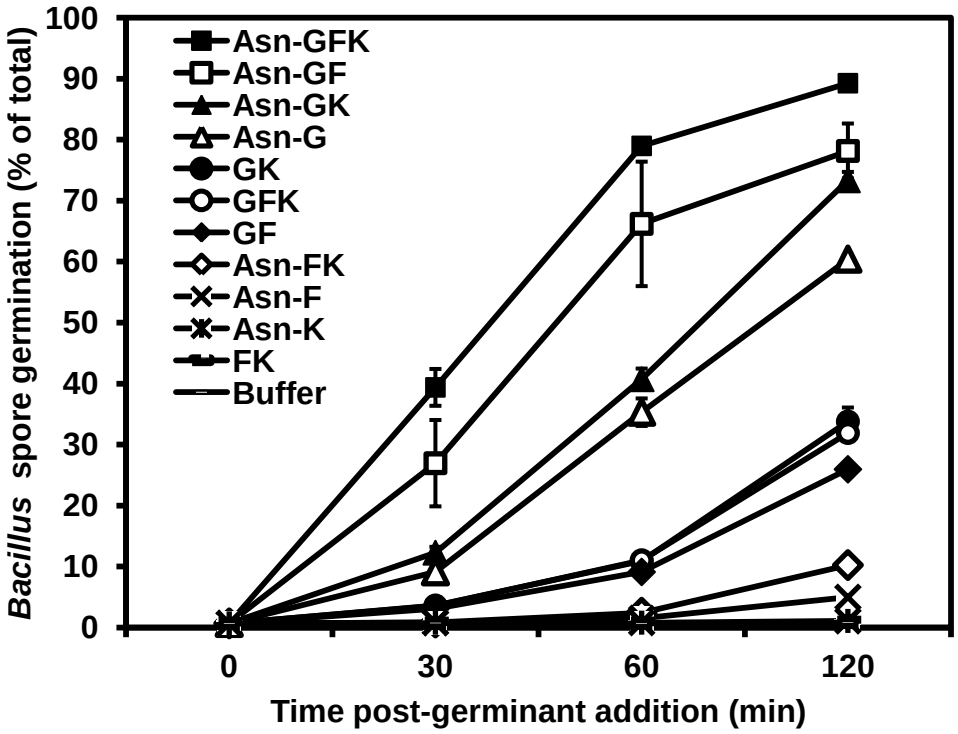

Fig. 8. Relative activation of TrigoCor spore germination at 37 °C by Asn-GFK component mixtures. TrigoCor spore germination was measured with all possible 2-, 3-, and 4- germinant combinations from an equimolar solution of L-asparagine (Asn), D-glucose (G), D-fructose (F), and KCl (K) (Asn-GFK) (10 mM final concentration of each component). Data points represent the mean percent spore germination of 2 replicates per sample, and vertical lines represent the standard error of the mean for each germinant.

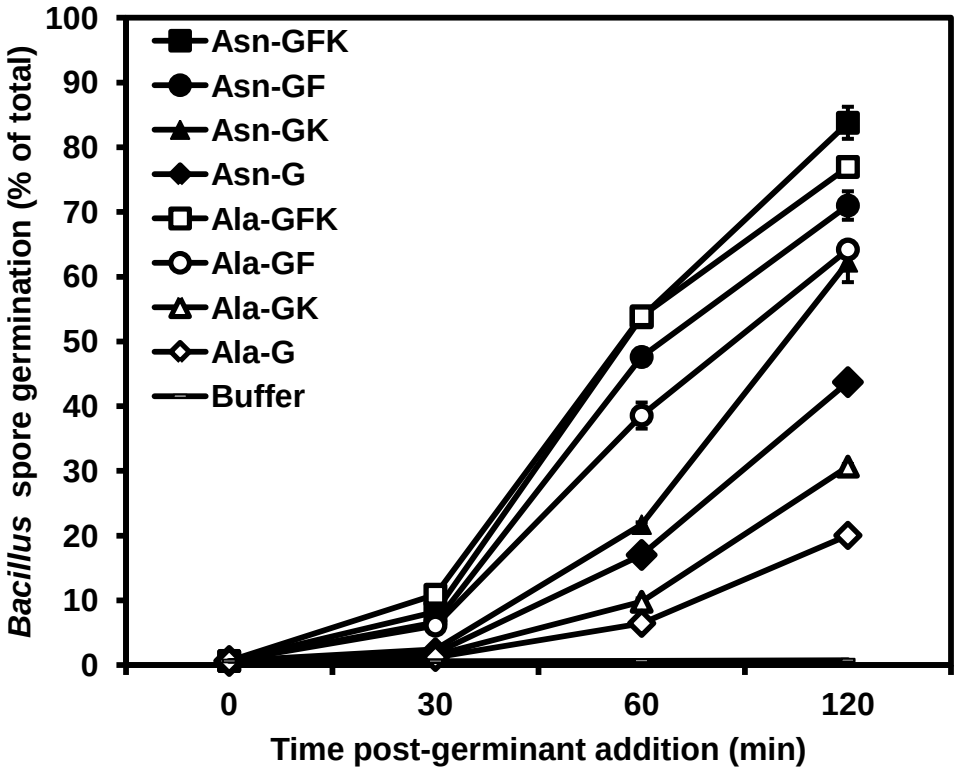

Fig. 9. Similarity in *Bacillus* spore germination at 37 °C following addition of germinant mixtures containing either L-asparagine or L-alanine. TrigoCor spore germination was measured following addition of the most stimulatory combinations of components from the germinant mixture L-asparagine (Asn), D-glucose (G), D-fructose (F), and KCl (K) (Asn-GFK), that contained either L-asparagine (closed symbol) or L-alanine (Ala) (open symbol) (10 mM final concentration). Data points represent the mean percent spore germination of 2 replicates per sample, and vertical lines represent the standard error of the mean for each germinant.

TrigoCor germination at room temperature was comparable with Asn-GFK and Ala-GFK. Asn-GFK, Asp-GF, and Ala-GFK all produced a very high level (90 – 100 %) of TrigoCor spore germination at room temperature (Fig. 10). The mean degree of germination stimulation by Ala-GF was lower than for the other treatments, and was significantly different from all other treatments except for Ala-GFK. In general, the levels of stimulation at room temperature were slightly greater than those observed at 37 ºC (Fig. 9). As before (Fig. 6), there was a very low level of germination with cells mixed with buffer (Fig. 10).

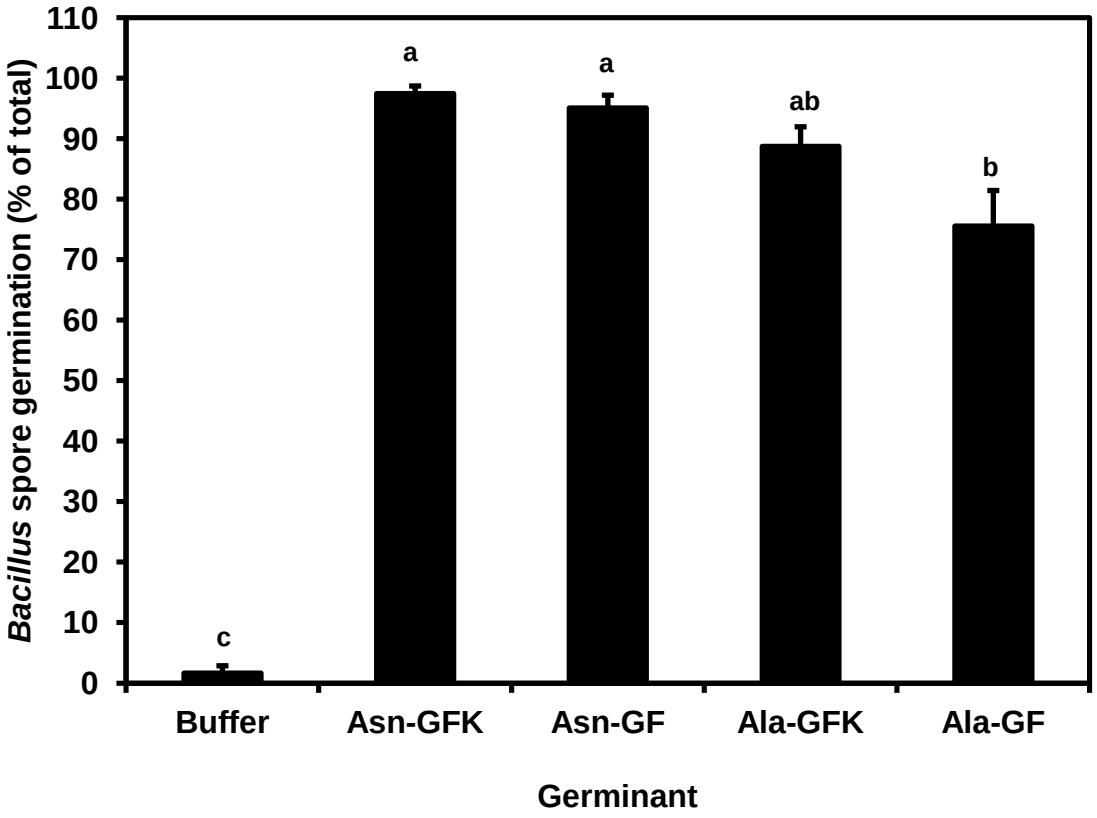

Fig. 10. Stimulation of TrigoCor spore germination at room temperature 6 h after addition of mixtures of L-asparagine (Asn) or L-alanine (Ala) with D-glucose (G), D-fructose (F), and KCl (K) (10 mM of each component). Data points represent the mean percent spore germination of 3 replicates per sample, and vertical lines represent the standard error of the mean for each treatment. Means not followed by the same letter were statistically different ($p<0.0001$).

***Fusarium* growth with individual germinants, Asn-GFK, and Ala-GFK.** *Fusarium* mycelial growth was stimulated on plates amended with asparagine, slightly reduced on plates amended with glucose, and unaffected on plates amended with fructose and KCl, as compared to plates amended with buffer (Fig. 11A). Alanine did not stimulate *Fusarium* growth compared to the buffer control, and produced much less growth than did asparagine.

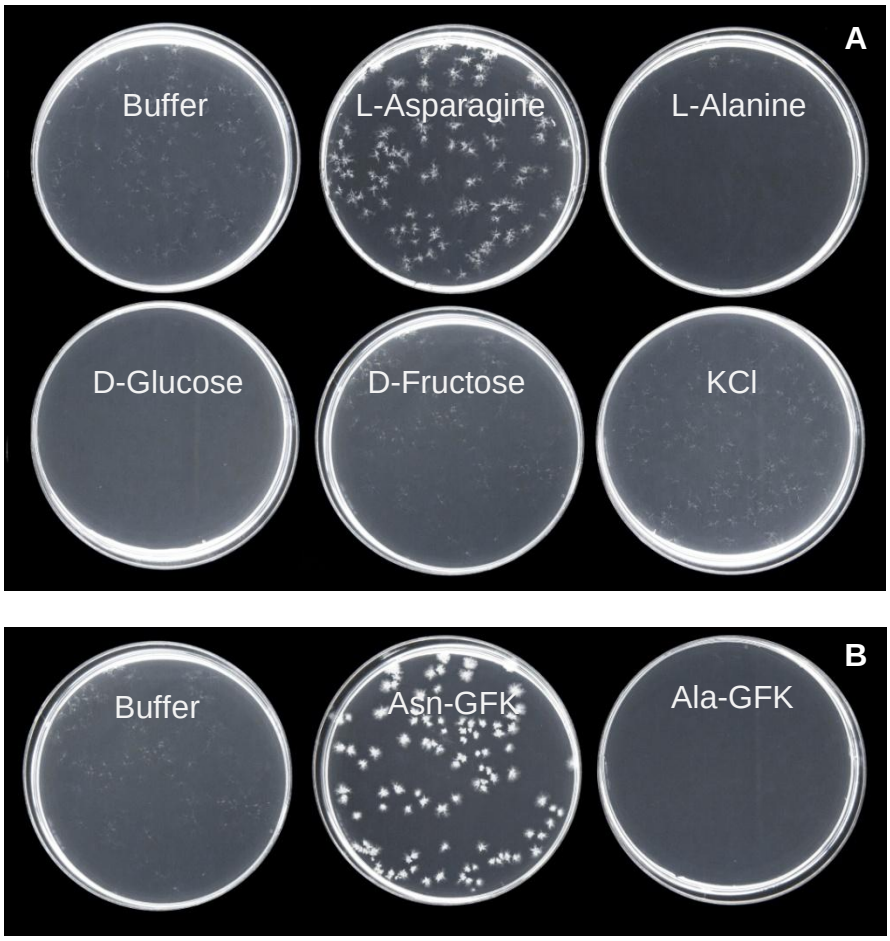

Fig. 11. Asparagine and Asn-GFK stimulate *Fusarium* growth. *Fusarium* conidia were germinated and grown on water agar plates **(A)** amended with alanine and the individual components of the germinant mixture L-asparagine, D-glucose, D-fructose, and KCl (Asn-GFK), or **(B)** on plates amended with Asn-GFK or with the germinant mixture L-alanine, D-glucose, D-fructose, and KCl (Ala-GFK). All germinants (10 mM of each individually and in mixtures) were prepared in HEPES buffer and plates amended with buffer were used as a control. Mycelial growth from germinated conidia on representative plates was photographed after 3 days.

Asn-GFK increased *Fusarium* mycelial growth relative to the buffer control, but Ala-GFK did not (Fig. 11B). The *Fusarium* colonies produced on Asn-GFK plates were consistently denser than those produced on plates with just asparagine. In separate trials (data not shown), Asn-GFK did not consistently effect conidial germination rate as compared to a buffer control.

Greenhouse experiment with Ala-GFK. When Ala-GFK (10 mM) rather than water was applied to wheat spikes pre-treated with either TrigoCor spores or water, it caused a slight increase in FHB index and DON, however in neither case was the increase agriculturally or statistically significant (Table 7). There was a lower FHB index and level of toxin accumulation when wheat spikes were treated with TrigoCor spores as opposed to water, however the difference was only statistically significant when the spores were followed by water.

Table 7. Fusarium head blight (FHB) symptoms and deoxynivalenol (DON) contamination of grain not changed following treatment with an equimolar solution of L-alanine, D-glucose, D-fructose, and KCl (Ala-GFK).

Treatment, Germinant [x]	**FHB Index** [y]	**DON (ppm)** [z]
Water, Water	95 ± 3 a	31 ± 8
Bacillus spores, Water	82 ± 5 b	25 ± 6
Water, Ala-GFK	98 ± 1 a	33 ± 6
Bacillus spores, Ala-GFK	88 ± 4 ab	27 ± 7

[x] Water or TrigoCor spores were sprayed onto wheat spikes, followed by either water or Ala-GFK (10 mM of each component, in water). After 24 h in a mist chamber, all spikes were treated with *F. graminearum*.
[y] FHB index = a combined measure of Fusarium head blight severity and incidence. Means not followed by the same letter were statistically different (P = 0.0054). Numbers represent the mean ± the standard error using nontransformed data.
[z] DON = deoxynivalenol. Means not significantly different (P=0.8980). Numbers represent the mean ± the standard error.

Analysis of wheat spikes from each treatment collected before application of germinant and after the initial 24 h period in the mist chamber indicated that Ala-GFK was not able to stimulate *Bacillus* germination on plant surfaces in this trial (data not shown).

Discussion

The main purpose of this study was to develop a treatment that could be applied to wheat spikes to stimulate TrigoCor spore germination, thereby potentially activating the metabolic functioning and antibiotic production of this potential biological control agent. To this end, we found that Asn-GFK was a highly effective TrigoCor spore germinant, which stimulated significant TrigoCor germination *in vitro* (Figs. 4-6,8-10) and on plant surfaces (Fig. 7). However in a greenhouse assay Asn-GFK increased the amount of FHB/DON on wheat spikes not pre-treated with TrigoCor (Table 6), presumably because *Fusarium* growth was stimulated by the asparagine in this treatment (Fig. 11). In a secondary round of spore germination trials we determined that Ala-GFK also produced significant TrigoCor germination *in vitro* (Figs. 9-10). Although this treatment did not lead to drastic increases in FHB in the greenhouse, it was also not able to improve disease control (Table 7), likely due to insufficient TrigoCor spore germination. Thus, although we were successful in characterizing the dynamics of TrigoCor germination and in identifying two promising candidate treatments for greenhouse applications, neither of these treatments was able to produce the desired effect on FHB/DON control.

The *in vitro* germination of TrigoCor in response to specific nutrient germinants was overall similar to that of characterized lab strains of *B. subtilis*, despite the fact that TrigoCor is an environmental strain of a different species. In addition to the similarity between TrigoCor and *B. subtilis* strains in germinating with Asn-GFK and Ala-GFK, TrigoCor also germinated, albeit to a lesser extent, with the most common *Bacillus* germinant, L-alanine (Figs. 4,5). TrigoCor germination with L-alanine was enhanced by the addition of glucose (Fig. 5), particularly at lower concentrations of each germinant, which mirrors the well-characterized role of glucose as

an important co-germinant for *B. subtilis* germination (2, 18, 51). Furthermore, it corroborates a previous finding (52) suggesting that synergistic interactions between germinants occur to a greater extent at lower germinant concentrations. Germinant specificity is influenced by the specificity of receptors that recognize particular germinants. Although *B. subtilis* and *B. amyloliquefaciens* are separate species they are closely related, and the genome of *B. amyloliquefaciens* contains orthologues of all the main germination proteins in *B. subtilis* (32). Consequently, it is perhaps not surprising that there are similarities among the patterns in germination between strains from these two groups.

Some differences existed between the germination of TrigoCor and lab strains of *B. subtilis*. For instance, TrigoCor did not germinate with the *B. subtilis* germinants L-valine or Ca-DPA, although it is possible that germination could have occurred with higher concentrations of each germinant. Additionally, unlike in *B. subtilis*, where glucose is considered to be incapable of stimulating spore germination in the absence of other germinants (48), for TrigoCor glucose was able to consistently stimulate germination (Fig. 4,5). Although the level of germination with glucose was low, it was similar to that of alanine at 10 mM and consistently higher than the level of germination with buffer. These differences in germination dynamics may be due to differences in receptor specificities among the species or strains, or in experimental design. For instance, our assays were frequently carried out for longer periods of time and used spores that had not received sublethal heat activation, and the concentrations of nutrients tested were on the high end of the range used in other studies.

In general, the presence of glucose appeared to be a key factor in stimulating TrigoCor germination. Aside from its role in stimulating germination on its own and in enhancing spore germination with alanine (Fig. 5), glucose also stimulated germination with asparagine (compare

Figs. 4,8). The asparagine-glucose combination also appeared to be a necessary component for significant germination with any of the possible 2- 3- or 4- component combination of Asn-GFK (Fig. 8). Knowledge of the positive role of glucose in enhancing TrigoCor germination might be useful in modifying treatments for spore germination on plant surfaces, which is fortunate because glucose appeared to retard *Fusarium* growth on agar plates (Fig. 11), and probably also contributed to a statistically significant reduction in *Fusarium* conidial germination on plates containing alanine-glucose plates (data not shown).

Our finding that Asn-GFK could stimulate TrigoCor germination on wheat spikes is to our knowledge the first description of a nutrient-induced alteration of *Bacillus* germination on plant surfaces. For the most part studies on *Bacillus* sporulation and germination *in vivo* have focused on observing natural processes (13, 14, 29, 44, 46). A few attempts however have also been made to alter *Bacillus* germination, sporulation, or cell type, *in vivo*. For instance, researchers have induced *Bacillus* germination (28) or sporulation (45) in the soil; increased the amount of vegetative cells in a biocontrol agent inoculum (7); applied an amendment with a *Bacillus* biological control agent to maintain cells in a vegetative form (8, 22); or introduced asporogenic *Bacillus* mutants into the environment (1, 39).

Although neither Asn-GFK nor Ala-GFK application were successful in producing the desired effects on disease control in the greenhouse, we believe that modifications of these or other germinant treatments might still produce a viable commercial treatment for on-plant biological control agent spore germination. Both Asn-GFK and Ala-GFK produced a subset of promising results; while Asn-GFK was able to germinate *Bacillus* spores on plant surfaces, it was overly stimulatory to *Fusarium*, and the opposite was true for Ala-GFK. Perhaps it would be helpful to modify the Ala-GFK treatment so that it is more stimulatory to *Bacillus in vivo*, such as through

increasing the concentration of one or more of the components so that it is closer to that used in the Asn-GFK treatment (100 mM vs 10 mM for Ala-GFK). Such increases might be particularly helpful because the volume delivered to wheat spikes is an order of magnitude or more lower than the volume sprayed (10), although at some point raising germinant concentrations too high would make such treatments financially unfeasible for application in large-scale agriculture. Another option is to screen for a new germinant to test, such as a different amino acid to combine with GFK, like L-valine which in *B. subtilis* binds to the same receptor as L-alanine, or a non-nutrient germinator whose activity might be more specific to *Bacillus* germination and whose application might therefore be less stimulatory of *Fusarium* growth.

Modifications to facilitate faster germination in response to nutrients or to lower concentrations of nutrients might also be helpful in improving the success of biological control agent germination. Faster or less specific germination could be especially useful on a plant surface, where competition for such nutrients post-application is fierce. Fast germination also would be particularly beneficial for a biological control agent such as TrigoCor, where the effectiveness of such germination would be dependent on the biological control agent's ability to quickly produce adequate levels of antifungal compounds prior to pathogen invasion. One option for improving germination rate would be to alter the spore culture conditions (15, 37, 38), which is believed to alter germination rate by changing the number of germinant receptors in the spores (37). Another possibility would be to activate the *Bacillus* biological control agent spores immediately prior to application; such activation would prime the spores to germinate faster when conditions become favorable. Although heat activation is the main method used in *Bacillus* labs this would not be feasible for environmental application, however reducing agents or lowered pH have also been shown to activate *Bacillus* spores (21). Finally, screening for fast-

germinating strains or for strains that germinate upon contact with lower concentrations of germinants or a broader range of germinants could also achieve this goal. However, an unintended consequence of using spores that respond to lower germinant concentrations or a broader range of germinants might be a steady and low-level number of *Bacillus* cells germinating within the population over time. This pattern of germination might be useful against a pathogen with a constant influx of inoculum, but less powerful against one with a narrower inoculation or infection period, for which a more desirable germination response would be one that affects the entire biological control agent population for a more confined period of time. For FHB, *Fusarium* infections are favored by periods of warm (25-30 ºC), wet weather (43), so our hypothesis would be that a stronger *Bacillus* response directed at these critical periods would be most effective in this system.

Several additional factors should be considered for future trials on induction of *Bacillus* germination for biocontrol. As evidenced by the increase in *Fusarium* growth with Asn-GFK, future germinant treatments should be tested against the pathogen target, and the potential effect on the background microbial community should be considered as well. An additional consideration is that in an actual application scenario, *Bacillus* cells presumably have transitioned to the spore form because there were inadequate nutrients on the wheat surface, and by inducing them to germinate via a nutrient treatment we are assuming that this treatment will provide enough for *Bacillus* to proliferate. However we do not know if this is the case. Additionally, the strain-specific nature of spore germination, as evidenced by the lower extent of germination by the commercial biological control agent strain Taegro, suggests that treatments should be optimized based on the needs of the particular biocontrol agent, and that this strategy might not be appropriate for all biocontrol agents.

Finally, although TrigoCor was able to germinate on wheat surfaces by 24 h post-Asn-GFK application, we did not directly investigate the extent to which these cells were able to undergo outgrowth and vegetative cell proliferation on the wheat surface, nor did we measure if they were able to produce antifungal compounds. There was a slight increase in *Bacillus* populations 24 h post-Asn-GFK application suggesting that some multiplication may have occurred, but we cannot be certain. In liquid culture, production of a key TrigoCor compound, iturin, begins after 2 days of culture growth and stabilizes around 3-4 days of growth, however it is difficult to predict how these dynamics compare to metabolite production on wheat surfaces. Due to practical constraints on wheat producers, we believe a germinant treatment must activate germination and compound production within 24 h to be considered feasible. Measuring iturin production would have been difficult in these experiments due to a large space requirement needed to achieve adequate plant material for compound detection, however future efforts might benefit from estimating the time scale of *in situ* inhibitory compound production. Similarly, although we did not measure production of *Bacillus* antifungal compounds on the wheat surface, the reduction in FHB/DON in the greenhouse when TrigoCor spores were followed by Asn-GFK (Table 6) suggests that a low-level production of inhibitory levels of antifungal compounds may be possible within 24 h. However the extent of this metabolite production is still unknown.

In summary, we propose that stimulation of *Bacillus* biological control agent germination on aboveground plant surfaces through the application of nutrient germinants is possible, and that it is a promising option for improving disease control. We found *in vitro* assays to be useful in identifying treatments capable of activating *Bacillus* germination on plant surfaces, however these *in vitro* trials were not able to fully predict the *in vivo* disease control outcome. Additional work is needed to further explore the possibility of stimulating *Bacillus* biocontrol agent

germination in the environment, taking into consideration the specific needs and complexities of the system at hand.

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CHAPTER 4

SPATIAL DISTRIBUTION AND ANTIFUNGAL INTERACTIONS OF A *BACILLUS* BIOLOGICAL CONTROL AGENT ON WHEAT SURFACES

Abstract

The proximity of a biological control agent and its associated anti-microbial metabolites on wheat surfaces to plant pathogens and to sites of plant invasion can determine the outcome of disease control. In this study we investigated whether differences in the extent and/or localization of inoculum coverage on wheat spikes sprayed in controlled and field environments could explain the inability of the biological control agent *Bacillus amyloliquefaciens* strain TrigoCor to consistently control Fusarium head blight in the field, despite producing effective and consistent disease control in greenhouse settings. Using epifluorescent stereomicroscopy and confocal laser scanning microscopy, we visualized the coverage of wheat spike surfaces by TrigoCor inoculum post-application in greenhouse and field environments, and determined that there are large deficiencies in the coverage of wheat spikes sprayed with commercial-scale field equipment, as compared to typical greenhouse applications. Additionally, we found that in conditions of low relative humidity, antifungal compounds produced by TrigoCor were not able to diffuse across wheat surfaces in biologically relevant amounts, further suggesting that the low coverage of wheat surfaces by TrigoCor inoculum could be directly limiting disease control. TrigoCor cells were easily rinsed off of wheat surfaces within 8 hours of application, indicating that rainfastness might be an additional limitation for biological control in field settings. Finally, we observed *in vivo* inhibition of *Fusarium graminearum* spore germination by TrigoCor

inoculum on wheat surfaces, confirming this as a mode of action for TrigoCor biocontrol. Future optimization efforts for biological control agents applied to above-ground plant parts should focus on enhancing the rainfastness, quantity, and spatial coverage of inoculum on plant surfaces.

Introduction

Strategies to optimize biocontrol of plant diseases are often guided by knowledge of the ecology of the biological control agent (BCA) and the BCA-pathogen interaction. To this end, considerable efforts have been made to describe the persistence of BCAs on plants or in the soil following application (2, 4, 28, 64, 72), as well as the mode of action of pathogen inhibition (32, 44, 59). Significantly less is known about the distribution and spatial interactions of BCAs, particularly *Bacillus*-based BCAs, on aerial plant surfaces, despite the fact that this information is relevant for disease control. Through visualization of the interactions and colonization of plant surfaces by the more commonly studied rhizosphere-applied BCAs, researchers have identified novel BCA modes of action, and have determined some of the processes underlying variations in disease control efficacy (11, 12, 36, 47, 49). Parallel insights would undoubtedly be useful for optimizing biological control on aerial plant surfaces, where the variable and inhospitable environment has a documented effect on the distribution of naturally-occurring epiphytic and pathogenic microbial communities (31, 35, 42, 62). However with a few exceptions (17, 21, 53) these visual or spatial avenues of aerial BCA ecology have remained largely unexplored.

In this study we visualized the spatial coverage of above-ground plant surfaces post-application by the BCA *Bacillus amyloliquefaciens* strain TrigoCor, as well as the *in vivo* interactions between TrigoCor and its target fungal pathogen, *Fusarium graminearum*. *F. graminearum* is the main causal agent in North America of the economically devastating (38-40) wheat and barley disease Fusarium head blight. Successful management of this disease is currently hindered by the lack of completely resistant wheat cultivars (5) and by the inconsistent control provided by fungicides (14, 41, 46, 70). Biological control could be useful as an enhancing supplement to fungicides in conventional cereal production and as an alternative to chemical controls for organic wheat production.

F. graminearum spores are deposited onto wheat spikes by air currents, in overhead raindrops, or by upward rain splash from lower in the crop canopy. Spores that reach the spikes are primarily airborne ascospores (sexual) but rain-splashed conidia (asexual) may also inoculate the spikes. The majority of spores land on the exterior surface of the wheat spike which contains thick-walled and lignified epidermal and/or hypodermal cells resistant to penetration (15). After germinating, most of these spores must grow across the plant surface as hyphae or a mycelial mat to reach more vulnerable interior entry points on the plant, via openings between the palea and lemma (15, 26) or possibly through stomates (26, 50). As with several other BCAs being developed for above-ground application to manage Fusarium head blight (37, 39), TrigoCor is believed to suppress disease by inhibiting the initial germination and/or growth of *F. graminearum* on exterior plant surfaces. Thus adequate coverage of the wheat surface and/or localization near potential sites of *Fusarium* invasion could be critical to control of this pathogen.

Unfortunately, like many of the other *Bacillus* BCAs (25, 55) applied for control of above-ground diseases, TrigoCor is unable to suppress disease consistently in field environments, despite being able to do so in controlled settings (27, 68, 69). An ongoing goal of our research program is to investigate the ecology of TrigoCor on wheat surfaces in the greenhouse and the field in order to enhance disease control efficacy of *Bacillus*-based BCAs in field environments. The antifungal activity of TrigoCor, like many other *Bacillus* BCAs (45), is believed to be derived from its production of antifungal compounds, which for TrigoCor inhibit fungal spore germination and growth *in vitro* (18, 51). Because the majority of the TrigoCor-synthesized antifungal compounds are delivered in the inoculum rather than being produced on the plant (18), the distribution of this inoculum on the plant surface could potentially delimit TrigoCor's disease control efficacy. Previously (18), we compared levels of *Bacillus* populations and of a critical *Bacillus*-produced antifungal compound on wheat spikes in the greenhouse and field; however these measurements did not take into account the spatial coverage of TrigoCor inoculum or its proximity to *F. graminearum* spores or points of entry on the wheat surfaces.

In the present study we aimed to determine if there were differences in the spatial coverage of wheat spikes sprayed with TrigoCor in controlled versus field settings that could explain the disparity in disease control between these environments. Our main objectives were to compare the coverage of wheat surfaces by TrigoCor inoculum between typical greenhouse and field applications; to quantify the coverage by TrigoCor inoculum following application in controlled environments; and to characterize the interaction between TrigoCor and *F. graminearum* on wheat surfaces, including any spatial requirements for successful inhibition to occur.

Materials and Methods

***Bacillus* strains, transformations, and culture.** *Bacillus amyloliquefaciens* strain TrigoCor was originally isolated from the wheat rhizosphere, and has been developed as a biological control agent based on its *in vitro* antibiosis of *Fusarium* and on its ability to consistently reduce disease in the greenhouse (10, 18).

The GFP-TrigoCor strain was a gift of Novozymes BioAg Limited. Transformation of TrigoCor to produce GFP-Trigocor was performed using a two-step process involving transformation of a competent *Bacillus* donor strain followed by conjugation between the donor strain and TrigoCor. The transformation vector contained the GFP gene Gfpmut3a and constitutive uracil phosphoribosyltransferase promoter P_{uup} from the *E. coli-Bacillus* shuttle vector pAD43-25 (*Bacillus* Genetic Stock Center), as well as the *neo* gene for neomycin resistance (23). The plasmid conferred neomycin resistance and constitutive expression of GFP, which was highly visible in vegetative cells but only slightly visible in spores. On average, over 70 % of vegetative cells were highly fluorescent, and those which were not fluorescent were usually within aggregates formed during liquid growth.

For visualization of wild-type TrigoCor inoculum on wheat spikes sprayed in the greenhouse, TrigoCor was cultured at room temperature as described previously for greenhouse experiments (18). For all other experiments, TrigoCor was grown under comparable culturing conditions except that cultures were grown at 30 ºC, and the starter and inoculum cultures were grown for one and four days, respectively. All GFP-TrigoCor culture media was amended with 6 µL/mL neomycin sulfate.

***Fusarium* strains, transformations, and ascospore production.** The Ph-1 strain of *Fusarium graminearum* (65) is readily transformable and a highly pathogenic causal agent of Fusarium head blight.

Transformation of Ph-1 was performed using the pIGRedPAPA plasmid (B.G. Turgeon, pers. comm.) which confers production of the DsRed protein under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase gene promoter, as well as resistance to Hygromycin B (100µg/mL). For protoplast transformation, Ph-1 cultures were grown for 9-13 days on quarter-strength Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Franklin Lakes, NJ), then transferred to new agar and grown for 13-14 days. Conidial suspensions were filtered through sterile cheesecloth, inoculated (1×10^7 total) into 100 mL quarter-strength Difco potato dextrose broth (Becton, Dickinson and Company, Franklin Lakes, NJ), then grown, germinated, and protoplasted as described in (7), using 14.5 h for germination and using lysing enzyme (Sigma Chemical Co., St. Louis, MO) rather than mureinase for protoplast preparation. Protoplasts were resuspended, washed, and transformed as described in (66), with the exceptions that only 2.6×10^6 protoplasts were used per transformation and that plates were incubated at 27 ^{o}C.

For ascospore production, DsRed-Ph1 mutants were grown on full-strength PDA containing 100 µg/mL Hygromycin B for 10-12 days, then were transferred and grown as described previously (16), except that it took 4 – 5 weeks after initial carrot agar plating for cultures to produce ascospores. Carrot agar plates (Earthbound Farm Organic, San Juan Bautista, CA) were either amended with 100 µg/mL Hygromycin B or nonamended; amendment produced ascospores with brighter fluorescence but lower numbers, and cultures with amendment were slower to mature. In some trials, rather than initiating the *Fusarium* culture in the center of each

carrot agar plate using a plug of mycelia, a lawn of conidia was spread over the plate, to ensure more even perithecial maturation. Cultures were grown at room temperature (24 - 26 ºC) with 12 h warm white (34 W) light/day.

Visualization of TrigoCor inoculum spray deposition on wheat spikelets in the greenhouse and field. Wild type TrigoCor cultures (5×10^8 - 1×10^9 CFU/mL) were mixed with Pyranine 10G dye (Keystone Aniline Inc., Chicago, IL) (0.5 g/l) and sprayed onto wheat spikes. In the greenhouse, wheat was grown and sprayed with a hand held atomizer as described previously (18). For field applications, wheat spikes at Musgrave Research Farm in Aurora, NY were sprayed using an R & D Sprayer (CO_2), Model GS set at a pressure of approximately 30 psi, with 4 fan nozzles spaced 48 cm apart at 19". Field experiments were conducted in 2013. Wheat spikes in both locations were either awned ('Norm' in the greenhouse, 'Pioneer Brand P25R46' in the field) or awnless ('AC Barrie' in the greenhouse, 'Otsego' in the field), and were sprayed at kernel watery ripe to early milk stage (Feekes wheat developmental scale 10.54). Wheat cultivars were spring wheat in the greenhouse, and winter wheat in the field.

After spikes had dried, individual spikelets were removed and visualized with an Olympus SZX12 stereomicroscope (Olympus, Japan) equipped with a GFP filter cube (excitation 470/40, emission LP 500). Photographs were taken using a Magnafire (Optronics Engineering, East Muskogee, OK) camera for greenhouse spikelets, or a ProgRes C14 (Jenoptik Group, Germany) camera for field spikelets, using a 0.5 x parfocal objective and a 16 x zoom. For fluorescence photos, images were taken at 282 and 200 ms exposures for greenhouse and field spikelets, respectively. All fluorescent illumination photos were modified in Photoshop (Adobe Systems, Inc., San Jose, CA) by adjusting the maximum and midpoint levels of the green channel to facilitate visualization of dye, and equivalent adjustments were used on images of treated and

nontreated spikelets from each environment. Between 9 and 11 spikelets per cultivar per location were visualized, and representative photographs are shown.

Visualization of TrigoCor cellular distribution on wheat glumes following spray application in controlled environments. GFP-TrigoCor (2 x 10^8 CFU/mL) was sprayed onto 'Norm' wheat spikes at kernel watery ripe to milk stage (Feekes wheat developmental scale 10.54 to 11.1) using the greenhouse protocol previously described (18), except that application was performed in the laboratory with air conditioning (approx. 21 ºC). Following application, spikes were allowed to dry for approximately 4 h, then thin sections of glume (approximately 0.5 cm^2) were excised using a razor blade and mounted onto a glass microscope slide atop a smear of molten 1 % water agar. Care was taken to minimize contact with the surface of the glume segments.

After the agar dried for approximately 10 min, glume sections were visualized using the 10 x EC Plan-Neofluar dry objective of a Zeiss LSM710 (Zeiss, New York, NY) confocal laser scanning microscope and a 488 nm laser. Images were collected using Zen 2010B SP1 software package (Zeiss, New York, NY). Due to the size and three-dimensional nature of the glume segments, photographs were taken as z-series using an automated tiling function, where consecutive sections of the glume with similar depths were photographed and tiled together, and then the maximum intensity projections of these pieces were stitched together manually or using the "Image Stitching" plugin (48) for the ImageJ software package (57) to reconstruct the larger glume section. Prior to visualization, slides were kept at room temperature in a plastic container with a moist paper towel to ensure minimal drying and shrinkage. Six glume sections from four separate inoculations were imaged and analyzed.

A preliminary experiment was conducted in which glume sections were imaged using a compound microscope (Carl Zeiss, Oberkochen, Germany) 7.5 hrs after initial application. A household sprayer was then used to spray a fine mist of approximately 75-100 µL sterile deionized water onto each of the slides holding the glume samples. After drying, the glumes were re-imaged using the identical camera and microscope settings as were used for the initial set of photographs. Imaging, misting, and re-imaging were repeated the following day (approximately 30 h post-initial application) with different glume sections taken from the same pot, and images were taken as described above using the Zeiss confocal laser scanning microscope.

The percent surface area covered by GFP-TrigoCor cells was estimated using ImageJ, by dividing the area covered by GFP-TrigoCor cells by the total glume area. To measure total glume area, glume sections were freehand outlined and total areas were measured using the Analyze Particles tool. The area covered by GFP-TrigoCor cells was selected in several ways. In most cases, the threshold was adjusted to select all green pixels, corresponding to regions covered with autofluorescent TrigoCor culture medium and most of the GFP-tagged cells, then the combined area covered by GFP-TrigoCor was measured using the Analyze Particles tool. Adjustments were also made, such as by applying filters, to select GFP-TrigoCor cells more or less conservatively, and these alterations resulted in only minor changes to the calculated GFP-TrigoCor area. Occasionally, some images contained concentrated areas of GFP-TrigoCor cells, often within a defined region of autofluorescent culture media, and/or with the cells positioned at the border of these regions to form a well-defined droplet outline. In these situations, the outline of the droplet was freehand traced and filled green (Appendix Figure 3), then the area covered by these segments was analyzed alongside the individual cells not contained within such droplets

using the Analyze Particles Tool. This method estimated not only cell coverage but coverage by culture supernatant as well, which is relevant because the supernatant contains the antifungal compounds critical to disease control.

Characterization of the *Bacillus*-*Fusarium* interaction on wheat leaves. 'Norm' wheat leaves were taped to the inside of petri dish tops, such that inside of the petri dish lid and the upper side of the leaf were facing upwards. The petri dish lids were placed on boxes of appropriate height next to the wheat plant to minimize strain on the wheat stalks and leaves. Wheat leaves were chosen primarily based on a relative absence of powdery mildew, but were typically the 2nd leaf down from the flag leaf, on spikes at watery ripe stage (Feekes wheat developmental scale 10.54). At one end of each leaf a maker (Sharpie, Oak Brook, IL) was used to draw small dots in a line perpendicular to the length of the leaf. Approximately 24 h prior to the time of visualization, 1 uL droplets of either full strength GFP-TrigoCor (2 x 10^8 CFU/mL), 1/10 GFP-TrigoCor diluted in deionized water, or 1/100 GFP-TrigoCor diluted in deionized water were applied with a pipet to each leaf starting at each Sharpie dot and extending along the length of the leaf. 2-3 treatments were applied per leaf and 2-3 leaves were taped per petri dish lid. The Sharpie dots allowed faster localization of each droplet line later, and the placement of multiple droplets of each treatment helped ensure that some of these droplets would be in the correct location for adequate *Fusarium* deposition.

At 1-1.5 h post-TrigoCor application, a plate of DsRed-Ph1 actively producing and dispensing ascospores (10^4-10^5 ascospores/cm^2) was placed above the leaves, facing down, such that it fit inside the petri dish lid to which the leaves were taped. Leaves and plates were left for 16 h under ambient lighting. At 7 h prior to the time of visualization, leaves were mounted to glass microscope slides on 1 % water agar, such that the agar covered the bottom and ends of the leaf

section but not the top. Slides were put on a piece of Whatman No. 1 filter paper cut to fit inside a 150 x 15 mm petri dish lightly wetted with 2 mL deionized water, then placed under white lights (32 W) until the time of visualization. Filter paper was rewetted as necessary. The assay was conducted in the laboratory with air conditioning (approx. 21 ºC).

Leaves were imaged as described above, except that a 561 nm laser was also used. Photographs were taken of sections of or entire TrigoCor droplets, and surrounding areas (up to approx 750 nm outside of droplet borders). As needed, a tiling feature was used to image automated stitched-together images of large sections of droplets or entire droplets, with up to 9 tiles per image.

The numbers of germinated and non-germinated *Fusarium* ascospores inside and outside TrigoCor droplets were counted using the "Cell Counter" plugin within ImageJ. Germinated ascospores were defined as those which contained germ tubes approximately 1/3 the length of the ascospore or longer. If it was unclear whether an ascospore had germinated, it was not counted. TrigoCor droplet borders were fairly easily defined based on the limits of the TrigoCor culture supernatant, which was highly autofluorescent particularly in the full-strength TrigoCor droplets, and based on the presence of a high density of fluorescent TrigoCor cells. For 1/10-diluted samples the TrigoCor culture supernatant was less fluorescent but still usually visible, and there was also still an easily recognizable border to the TrigoCor cells. For 1/100-diluted samples it was more difficult to define the borders of TrigoCor droplets so photos and measurements were taken in what appeared to be the center of droplets, where there was a fairly even and low-level coverage of TrigoCor cells. In previous trials we found minimal rearrangement of TrigoCor cells and supernatant on leaf surfaces between the time of application

and imaging (data not shown), so the placement of TrigoCor droplets in our images should match the general placement of these droplets when *Fusarium* initially landed on the leaf surface.

For non-dilute TrigoCor inoculum, six images representing 1356 ascospores (620 inside droplets and 736 in surrounding areas) were counted, and for 1/10- diluted TrigoCor inoculum 12 images representing 1357 ascospores (637 inside droplets and 720 in surrounding areas) were counted. For 1/100- diluted inoculum 208 ascospores inside droplets were counted.

For each TrigoCor dilution treatment, the ascospore germination rate was calculated for areas inside and outside TrigoCor droplets, and the % inhibition by TrigoCor was calculated by comparing the mean germination inside versus outside these droplets. For the full-strength and 1/10-diluted TrigoCor treatments, the mean ascospore germination rates inside and outside of TrigoCor droplets were arcsine transformed, then means were separated using the PROC TTEST function (P=0.05) of the SAS statistical program version 9.3 (SAS Institute Inc., Cary, NC). Because the variances for both treatments were equal, the Pooled t-test p-value was reported. Means and standard errors reported are for nontransformed values.

Results

Visualization of TrigoCor inoculum spray deposition on wheat spikelets in the greenhouse and field. All wheat spikelets from spikes that were sprayed with TrigoCor mixed with fluorescent dye at a commercial-scale agriculture rate had detectable droplet(s) of TrigoCor inoculum on the spikelet surface (Fig. 12). However the coverage was very low, and typically consisted of a single droplet localized to a small section of the spikelet.

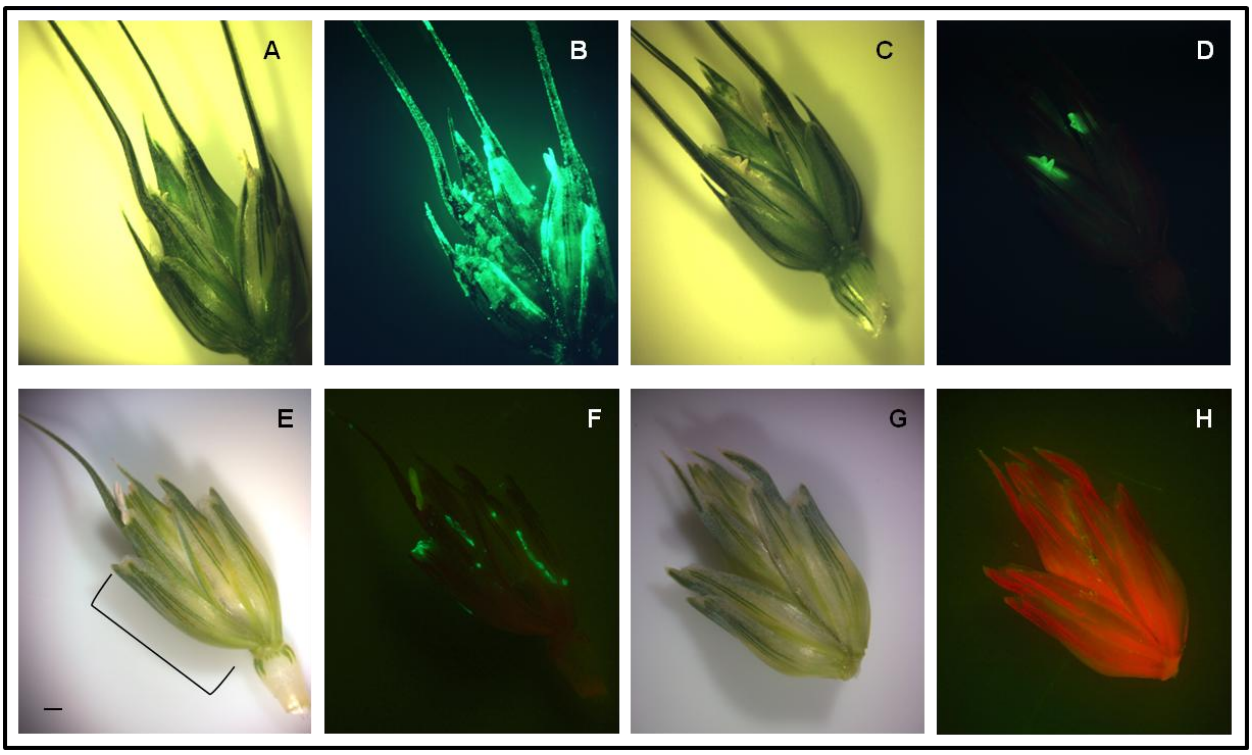

Fig. 12. TrigoCor inoculum coverage on wheat spikelets following application in the greenhouse and field. TrigoCor inoculum containing cells and culture supernatant was mixed with Pyranine dye and sprayed onto wheat spikes in **(A-B)** the greenhouse with a hand-held atomizer, or in **(E-F)** the field using a sprayer applicable for commercial-scale agriculture set at a pressure of 30 psi. Nontreated wheat spikelets from **(C-D)** the greenhouse and **(G-H)** the field were also collected, and all spikelets were visualized with a stereoscope in bright field **(A,C,E,G)** or fluorescence **(B,D,F,H)** modes. In **(E)**, the bracket marks one of the two glumes on the spikelet. All images are shown at the same magnification and scale bar represents 1 mm.

In the greenhouse, the degree of coverage on wheat spikelets from spikes sprayed with a hand-held atomizer was qualitatively greater than the coverage observed from spikelets sprayed with a commercial sprayer under field conditions (Fig. 12), although no statistical comparison was conducted. A patchy and fairly random distribution of inoculum was commonly observed on spikelet surfaces, and TrigoCor inoculum was frequently observed along the ridge formed where the glume edge contacted the floret beneath it.

In the field, no difference was seen between inoculum coverage in awned and awnless wheat cultivars, although the overall magnitude of inoculum delivered to spikes was so low that

potential differences could have been missed (data not shown). However, we also did not observe any major differences in the pattern or level of coverage between awned and awnless cultivars in the greenhouse (data not shown) where overall coverage was high, indicating that the presence of awns likely does not impact greatly the deposition pattern or coverage of inoculum delivered to wheat surfaces.

GFP TrigoCor properties. Compared to wild type TrigoCor, GFP-TrigoCor produces longer vegetative cells, and lower CFUs/mL and lower concentrations of antifungal compounds in liquid culture after 4 days of growth. GFP-TrigoCor inhibits *F. graminearum* to a slightly lesser extent in antibiosis assays than does wild type TrigoCor (data not shown).

Visualization of TrigoCor cellular distribution on wheat glumes following spray application in controlled environments. GFP-TrigoCor cells on wheat glumes were commonly observed in fairly well-defined and dense clusters associated with areas of highly fluorescent culture supernatant, but also occasionally as a less dense and more dispersed covering on glume surfaces (Fig. 13). The distribution for the most part was random, except that inoculum appeared to accumulate in spaces between veins, and less so on the surfaces of chlorenchyma (Fig. 13C). *Fusarium* colonization of chlorenchyma has been observed, and the stomates within these tissues have been described as a point of entry to the plant for *Fusarium* (15).

All glume sections visualized had less than 10 % of their total area covered by GFP-TrigoCor cells, and in 5 out of 6 sections the area covered by GFP-TrigoCor cells was 5 % or less of the total area. When inoculum droplets were freehand traced based on clearly-defined borders of cells or autofluorescent inoculum, the area covered by GFP-TrigoCor inoculum was still estimated at less than 20 % of the total glume area visualized.

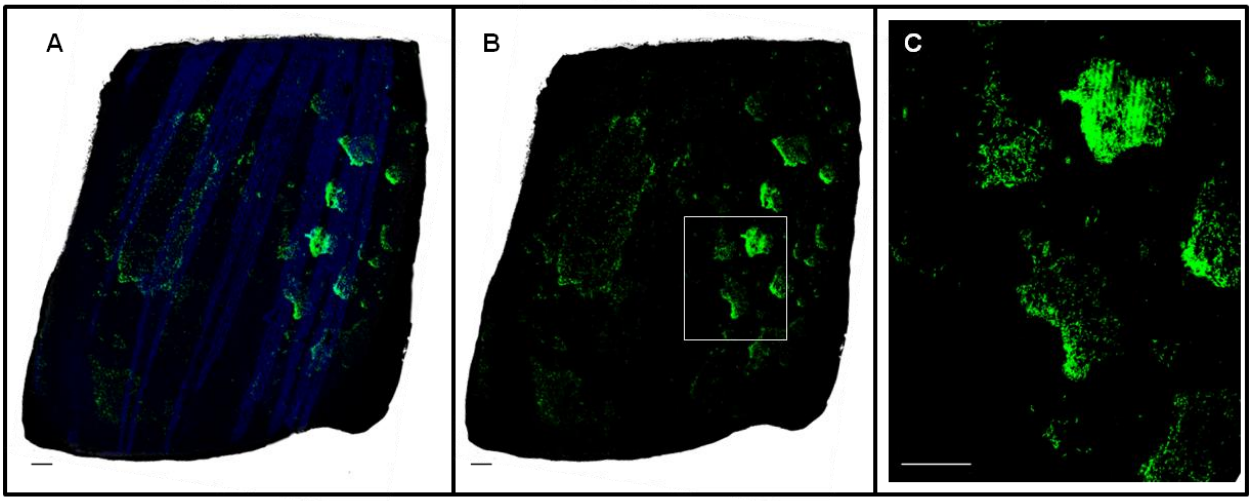

Fig. 13. Distribution of GFP-TrigoCor inoculum on wheat glumes following spray application in the laboratory. GFP-TrigoCor cells and culture supernatant were sprayed onto wheat spikes in the laboratory with a hand-held atomizer according to the standard greenhouse protocol. Glume sections were excised and their surfaces visualized using a confocal microscope. Green areas are GFP-TrigoCor cells and/or autofluorescent culture supernatant. In (A), a filter on the microscope was used to detect chlorophyll fluorescence, in blue, and these blue regions are chlorophyllous tissue. Excised glume sections are shown in their entirety in (A) and (B), and the image in (C) is a magnification of the boxed area in (B). For clarity, the background around each glume in A and B was removed. Scale bars represent 200 µm.

In a preliminary experiment, we found that when TrigoCor-treated glume sections were sprayed with a fine mist of water 7.5 h post-TrigoCor application, the majority of TrigoCor cells were washed off (Appendix Figure 4 A-B). When TrigoCor-treated glume sections were allowed to dry for one day before being sprayed with water, a greater extent of TrigoCor cells were retained following the water application (Appendix Figure 4 C-F).

Characterization of the *Bacillus-Fusarium* interaction on wheat leaves. *Fusarium* ascospore germination on wheat leaves was significantly inhibited when the ascospores were located within droplets of TrigoCor inoculum (Table 8, Fig. 14). A 10-fold dilution in TrigoCor inoculum did not appear to significantly lessen the degree of *Fusarium* inhibition. *Fusarium* ascospores that landed on leaf areas covered with 1/100 diluted TrigoCor inoculum germinated

at rates (57 %) comparable if not higher than the observed rates on areas non-treated with TrigoCor, indicating that at this dilution TrigoCor was no longer inhibitory.

Table 8. Inhibition of *Fusarium* ascospore germination on wheat leaves by GFP-TrigoCor inoculum droplets.

	% *Fusarium* germination [z]		
TrigoCor Treatment	Outside TrigoCor droplet	Inside TrigoCor droplet	% Inhibition by TrigoCor [y]
Full strength [x]	45 ± 5	18 ± 3	61
1/10 strength [w]	45 ± 4	15 ± 4	67

[w] GFP-TrigoCor cells and supernatant were diluted in deionized water. n=12 images representing 1357 ascospores (720 outside droplets and 637 inside). T-test p-value for reduced germination inside droplet: p=0.0009.
[x] n = 6 images representing 1356 ascospores (736 outside droplets and 620 inside). T-test p-value for reduced germination inside droplet: p <.0001.
[y] Determined using the equation % Inhibition = (% germination outside droplet - % germination inside droplet) / (% germination outside droplet).
[z] Mean germination rate ± standard error of the mean

Fusarium ascospores that germinated outside TrigoCor droplets often produced qualitatively longer germ tubes as compared to ascospores germinated inside droplets (Fig. 14). Extensive germination was frequently observed for ascospores in close proximity to TrigoCor droplets, suggesting that there was minimal diffusion of antifungal compounds on the plant surface. For instance, ascospores germinated at approximately 100 µm of the border of the TrigoCor inoculum droplet (Fig. 14 E,F). Additionally, TrigoCor cells were not frequently observed outside the boundaries of the inoculum droplets.

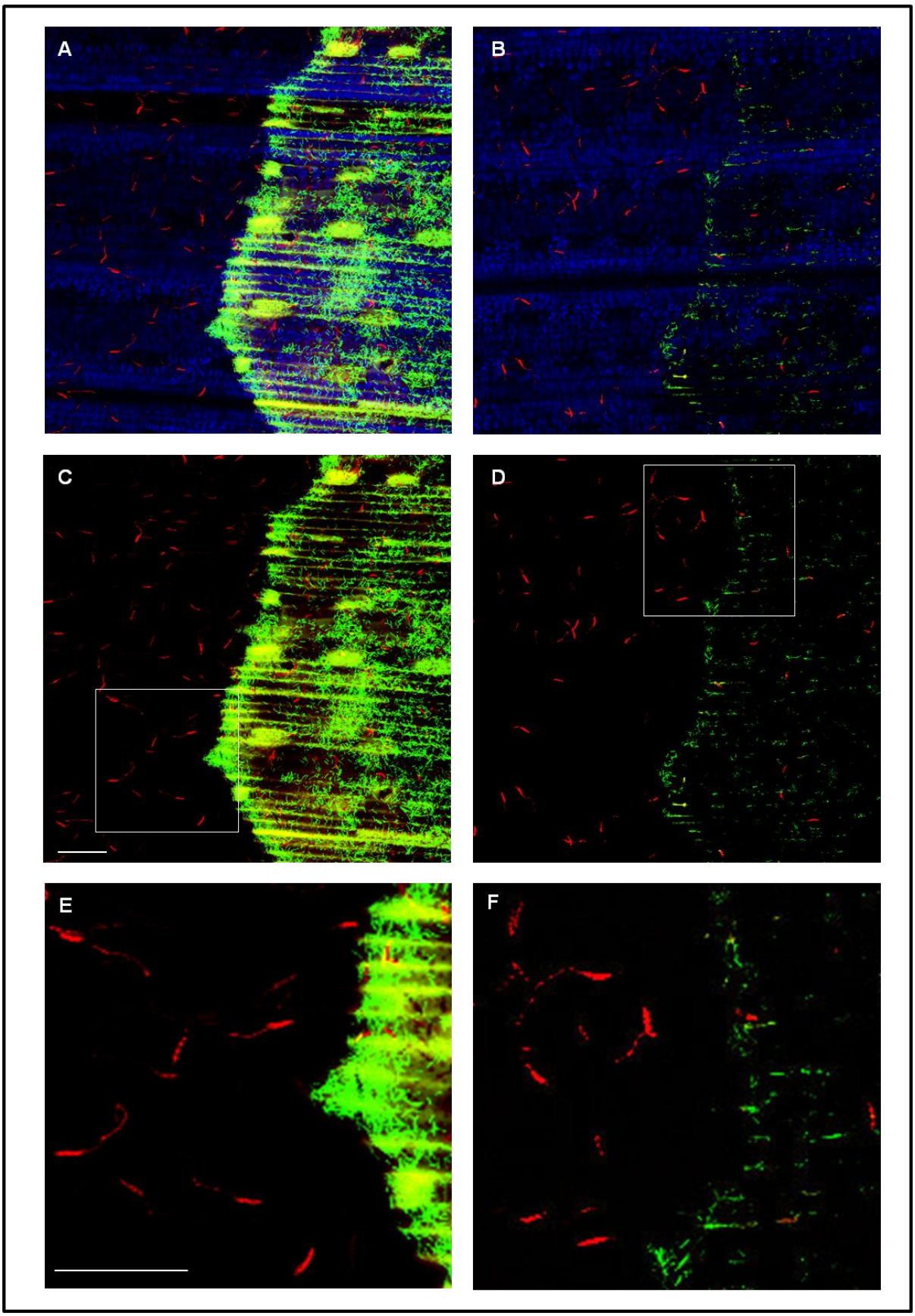
A
B
C
D
E
F

Fig. 14. Inhibition of *Fusarium* ascospore germination within TrigoCor droplets on wheat leaves. Wheat leaves were treated with 1 µL droplets of **(A,C,E)** full strength or **(B,D,F)** 1/10-diluted GFP-TrigoCor inoculum, then were exposed to dsRed-*Fusarium* cultures that were actively discharging ascospores. TrigoCor inoculum contained GFP-labeled cells, in green, as well as autofluorescent culture supernatant. Leaf sections were visualized with confocal microscopy, and in (A,B) a filter for chlorophyll fluorescence was used on the microscope which indicates the presence of chlorophyllous tissue, chlorenchyma, in blue. The images in E and F are a magnification of the boxed areas in C and D, respectively. A-D and E-F are shown at the same magnification and scale bars for all represent 100 µm.

Discussion

For BCAs that rely on competition, parasitism, or antibiosis as their mode of action against plant pathogens, proximity to the target pathogen is crucial, and inadequate coverage of plant surfaces or localization of the BCA to plant regions uninhabited by the targeted pathogen or critical sites of plant invasion will lead to corresponding decreases in biocontrol efficacy. The biological control activity of TrigoCor, like many other *Bacillus* BCAs applied to aerial plant parts, likely comes from its production of antifungal compounds primarily delivered in the inoculum (18). Therefore, we investigated the coverage of TrigoCor inoculum on wheat spikes following application in the greenhouse and field to determine if deficiencies in inoculum formulation or application methods should be targets of future optimization efforts.

We first mixed a fluorescent dye with TrigoCor inoculum to track the deposition of droplets following a typical greenhouse application and a field application using a commercial sprayer and typical application rate. The inoculum, which included not only cells but also the antifungal compounds present in the culture supernatant, had visually more complete coverage on wheat spikelets sprayed in the greenhouse as compared to the field (Fig. 12). Although we did not quantify the difference in coverage between greenhouse and field applications, a previous investigation revealed that the total volume of inoculum delivered to individual wheat spikes was

on average two orders of magnitude higher in the greenhouse as compared to the field (18), so it is probable that the difference in coverage might be along similar lines. As wheat spikes are highly complex vertical structures, the degree of spray coverage on their surfaces can vary greatly depending on the application technology (spray rate, nozzle type and angle, etc.) used. The coverage of field-sprayed spikes reported by other groups using alternative application methods (22) was greater than the coverage observed in our experiments. However, none of these other application technologies were able to deliver inoculum to spikes with a degree of coverage equivalent to what we observed following application with a hand-held atomizer in the greenhouse. Inadequate coverage therefore may be a pervasive problem for BCA delivery in the field, as it is for application of fungicides to wheat spikes (39). Some fungicides such as the strobilurins are able to redistribute across plant parts post-application through translaminar movement or by moving across the plant surface as a vapor within the boundary layer (67), and such redistribution compensates for some deficiencies in coverage. To our knowledge, no similar mechanisms have been observed for redistribution of BCA-produced antifungal compounds, although movement of bacterial cells and presumably compounds through surface wetting has been observed (8, 42, 52).

We did not observe a significant influence of awns on the degree of coverage or distribution of TrigoCor inoculum on wheat spikes in the greenhouse or the field. Other groups have observed greater water retention on awned wheat varieties (29, 30). Such differences may result from the angle or volume of liquid applied in the various experiments, or from differences in the spreading or drying properties between water and TrigoCor.

Although TrigoCor inoculum coverage on spikelets sprayed with a hand-held atomizer in the greenhouse was more extensive than that on spikelets sprayed with commercial equipment in the

field, the coverage observed on spikelets sprayed in the greenhouse was still incomplete. An even more dramatic demonstration of poor coverage was seen on the cellular level following application in the laboratory of GFP- labeled TrigoCor (Fig. 14) where we found less than 20 % of the wheat glume surface to be covered by dried culture supernatant and GFP-*Bacillus* cells, and less than 10 % to be covered by cells. The low coverage of wheat surfaces by TrigoCor cells and particularly culture supernatant under ideal controlled conditions indicates that in the field, where overall coverage by TrigoCor inoculum was already relatively low (Fig. 12), the coverage at the cellular level would be almost negligible.

It is possible that our estimates for the percent coverage by cells and/or supernatant underrepresented the coverage by *Bacillus*-produced antifungal metabolites, which were not visible in this experiment but were suspended in the culture supernatant. However, in most cases, TrigoCor cells and autofluorescent culture supernatant were co-distributed in easily recognizable droplets on the wheat surface (Fig. 13C), so it is unlikely that by measuring the coverage by these inoculum components we were missing large regions covered with inoculum but not visibly so.

Based on the lack of *Fusarium* inhibition when spores were approximately 100 µm from TrigoCor inoculum droplets (Fig. 14 E,F), it appears as though the diffusion of antifungal compounds across plant surfaces is minimal. The low coverage of wheat spikes by TrigoCor inoculum could therefore limit disease control greatly. Increases in inoculum coverage can clearly be improved in field settings. Dramatic increases from the coverage observed in controlled settings, however, may be limited by the water repellent nature of the plant surface, which is especially high for wheat and related cereal crops (8, 71). Our estimates of percent cell coverage were similar to those documented on bean leaves that were completely submerged in a

bacterial cell suspension (42), suggesting that achieving higher coverage might be difficult due to the intrinsic hydrophobic nature of plant surfaces. Consequently, either focusing on altering these physical properties or formulating biological control inoculum accordingly may be required for greater improvements in disease control.

The hydrophobicity of the wheat surface likely also contributed to the limited movement of antifungal compounds (Fig. 14 E,F) and TrigoCor cells (Fig. 14) outside the areas of initial inoculum deposition, even after 24 h. This inability of TrigoCor cells to relocate post-application is true even though TrigoCor, like many *Bacillus* strains, produces the lipopeptide surfactin, which has previously been implicated in facilitating mobility of *Bacillus* across surfaces by reducing surface tension (1, 3, 33). Although TrigoCor-produced sufactins were apparently not effective enough to enable movement of TrigoCor post-application, preliminary findings suggest that wheat spikes sprayed with TrigoCor culture filtrate, which contains surfactins, were covered more uniformly than were spikes treated with noninoculated culture broth. This indicates that surfactins may influence the initial distribution of TrigoCor inoculum over the wheat surface (data not shown), which was perhaps aided by the force of the spray delivery. Future studies should continue to investigate the role of surfactins in *Bacillus* BCA deposition or movement on plant surfaces, perhaps on plants with different degrees of hydrophobicity.

The lack of unassisted movement of TrigoCor cells and antifungal compounds outside the initial boundaries where they land indicates that the spatial distribution of inoculum deposition on wheat surfaces can potentially have a large impact on the extent of disease control, especially when *Fusarium* is delivered to wheat spikes as ascospores. Fungal spores contain adhesives which allow them to attach to hydrophobic plant surfaces (20). Because they are often deposited

through air currents in dry inoculation events, their distribution on plant surfaces would likely be different than that of TrigoCor, which is applied as a liquid and thus produces a distribution dependent on the hydrophobicty and topography of the plant surface. For instance, wind-driven *Fusarium* ascospores would be expected to land on the upper-facing surfaces of wheat spikes, and in a fairly random distribution. In contrast, TrigoCor inoculum was commonly localized along the crevasse formed where the outer edge of the glume met the underlying palea and lemma (Fig. 12), or between veins in the glume surface (Fig. 14), likely because these were locations where the liquid inoculum could pool. Similar localizations have been observed for other bacteria applied to plant surfaces (8, 42, 52), and may in one sense be advantageous in that these areas may provide protection from desiccation or UV radiation. However, such differences in distribution between TrigoCor and *Fusarium* could potentially allow germinating *Fusarium* ascospores to escape inhibition by TrigoCor. The experiments in this study did not investigate if actively growing *Fusarium* hyphae are also inhibited by TrigoCor antifungal compounds, but if they are, successful *Fusarium* inhibition might still occur if TrigoCor can block access to potential sites of plant invasion. This is particularly true for *Fusarium* mycelia that grow across large areas of the wheat surface. For instance, the large pool of TrigoCor inoculum that frequently collects along the glume edge (Fig. 12B) could inhibit *Fusarium* as it grows from the glume to the openings between the palea and lemma. On the other hand, *Fusarium* is also suspected to occasionally invade plants through stomates (26, 50), which are abundant particularly on glume surfaces and which are only protected when TrigoCor droplets are randomly deposited nearby (Fig. 13).

Although TrigoCor cells and compounds are not able to relocate on plant surfaces on their own, or under the minimal amount of humidity utilized in our ascospore germination assay, they

do appear to be redistributed upon direct surface wetting (Appendix Figure 4). The role of water in facilitating movement and colonization of other bacteria on plant surfaces is well established (24, 34, 42, 52, 54). In a preliminary experiment we observed that even a light misting of glume segments 7.5 h post-TrigoCor application removed most TrigoCor cells from the surface (Appendix Figure 4 A,B), although thus far it is unclear whether on wheat spikes the inoculum would be re-distributed, particularly in large depressions on the spikes, or whether it would simply wash off, as has been reported for other bacteria on plant surfaces (34). The fate of antifungal compounds following water application is also unclear. Although disease control could potentially be enhanced if surface wetting spreads compounds across the plant surface, it also could be detrimental if it dilutes the compounds to non-inhibitory levels. Such might be the case for inoculum applied to wheat spikes in the field, which have a low total quantity of compounds on wheat spikes to begin with due to deficiencies in initial inoculum deposition.

At one day post-TrigoCor application, we observed much less rinsing off of TrigoCor cells following misting of glume surfaces (Appendix Figure 4 C-F), suggesting that the impact of surface wetting on BCA distribution and potentially disease control performance is likely greatest in the hours immediately following BCA application. As before, however, washing off of antifungal metabolites one day post-TrigoCor application is less certain. The lessened rinsing off of TrigoCor cells one day post-application suggests that this BCA may require a rain-free period up to one day after initial application. Fungicides also require rainfast intervals although they are typically much shorter, although it is possible that with formulation alterations the rainfastness of *Bacillus* BCAs can be improved and the drying interval shortened. Our finding that TrigoCor cells are retained on wheat surfaces after one day corroborates an earlier finding in

which we did not observe significant reductions in *Bacillus* population levels on wheat spikes following a 48 h period in a mist chamber that began one day post-TrigoCor application (18).

Changes over time in the extent of bacterial adherence to plant surfaces has been documented by other groups as well (9). Bacterial adherence to aerial plant surfaces has been attributed to the formation of biofilms, which are common for phyllosphere-inhabiting bacteria (19, 43), and particularly for gram-positive bacteria (13). Biofilm formation was not explored in this work due to the short exposure of TrigoCor to plants prior to imaging, however extensive biofilm formation by TrigoCor has been observed *in vitro* (data not shown). Biofilm formation has been implicated in the biological control capabilities of a rhizosphere-inhabiting *Bacillus* BCA (6), however the environment and pathogen target, a bacterium, of that study were quite different. Depending on the role of cellular and compound re-distribution on plant surfaces in disease control of Fusarium head blight, enhanced formation of TrigoCor biofilms could either be a detriment or an asset in this system, and merits future study.

The redistribution of TrigoCor cells and antifungal metabolites with surface wetting would be relevant particularly for control of Fusarium head blight, because moisture enhances *Fusarium* spore production, dispersal, and germination, and because extended periods of wetness are correlated with large-scale epidemics of this disease (58, 60). The impact of surface wetting on disease control would be particularly significant for infections by *Fusarium* conidia, which are almost exclusively delivered to wheat spikes in water droplets (63) splashed from nearby infested debris or plants.

Our observation that *Fusarium* ascospores germinated to a lesser extent when they were in contact with TrigoCor inoculum (Table 8, Fig. 14) confirms our hypothesis that inhibition of spore germination is a mode of action of TrigoCor. Our qualitative observation that *Fusarium*

ascospores which germinated within TrigoCor inoculum droplets seemed to have shorter germ tubes indicates that inhibition of growth may be an additional mode of action of this BCA, although further studies would be needed to confirm this hypothesis. Investigation of *Fusarium* growth over a longer period of time would determine more definitively if hyphal growth inhibition is a mode of action of TrigoCor and would be relevant particularly for spores that germinated outside TrigoCor inoculum droplets.

In summary, this study provides evidence that inadequate coverage of wheat spikes by TrigoCor inoculum could limit the efficacy of TrigoCor in field settings. Insufficient coverage by TrigoCor is likely detrimental because it produces large unprotected areas where *F. graminearum* ascospore germination can occur. Coverage deficiencies may be partially overcome by redistribution of cells and antifungal compounds by surface wetting, however it is likely that the timing and nature of this wetting would dictate the effectiveness of such reorganization. Although improvements in application technologies would likely produce the greatest gains in disease control, such improvements may be slow to achieve, both technologically and in terms of grower adoption. In the short term, however, significant advancements in disease control may be derived from improving BCA coverage of plant surfaces, such as through formulation additives (17, 56, 61) or by selecting or modifying BCAs to have enhanced capabilities for initial plant retention and/or post-application surface spreading and colonization.

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CHAPTER 5

FUTURE DIRECTIONS FOR *BACILLUS* ABOVE-GROUND BIOLOGICAL CONTROL RESEARCH

Each chapter of this dissertation provides a unique insight into the ecology of TrigoCor and the TrigoCor-*Fusarium* interaction on wheat surfaces, and will hopefully advance efforts to develop consistently effective *Bacillus* biocontrol agents for above-ground plant diseases in field settings. However, although progress has been made, many questions large and small still remain unanswered.

On the smaller-scale, there are many experiments that could be performed to test hypotheses made in this dissertation, or to add depth to our findings by tying up loose ends. For instance, in chapter 2, our main conclusion is that a rapid decline in iturins is likely responsible for the inconsistent and often ineffective disease control in the field. Although I attempted to test this hypothesis by applying TrigoCor multiple times in a field trial, the broad implications of these findings may require confirmation of our hypothesis in a more controlled experiment, such as by testing the long-term disease control efficacy of a TrigoCor strain genetically manipulated to constitutively produce iturins. Similarly, it would be useful to investigate the dynamics of fengycins on wheat surfaces. TrigoCor-produced fengycins were previously found to be more inhibitory against *Fusarium oxysporum* than were iturins (1), so knowing if the trends for fengycins are equivalent to those for iturins should help direct future optimization efforts.

In chapter 3, I was intrigued by my findings that glucose represses *Fusarium* growth but that asparagine stimulates it. Possible follow-ups on this work include applying glucose to wheat by

itself or as an additive to biocontrol formulations, or, as Gary and I have discussed, screening potential biocontrol agents for their asparagine-metabolizing abilities.

Chapter 4 leaves a number of questions unanswered for me, particularly regarding the role of surface wetting in biocontrol. It is still unclear if surface wetting is beneficial, by spreading compounds and cells across the plant, or if it is detrimental, by completely washing metabolites away or by moving them to less advantageous locations on the wheat spike. It would be helpful to evaluate if inoculum is redistributed, retained, or entirely lost from plant surfaces, perhaps by simply visualizing its redistribution, or by testing the (in)ability of plant parts that have had TrigoCor 'washed-off' to inhibit *Fusarium* germination and growth. Similarly, it would be interesting to assess if stimulating biofilm formation might help prevent washing off of cells and antifungal compounds, or if instead it would limit their potentially beneficial redistribution, or hold the compounds in a matrix that makes them inaccessible for pathogen contact. A related experiment would be to determine if the extent of inoculum wash-off changes with increasing time post-application.

Finally, an earlier observation that has always perplexed me is that the distribution and retention of *Fusarium* conidia sprayed onto wheat spikes appears to be different when the spikes were pre-treated with TrigoCor, such that more of the conidial inoculum (green in the figure below) seems to stick to the plant surface when it is pre-treated with TrigoCor (see figures below). Similarly, I have observed different degrees of running off by *Fusarium* inoculum with TrigoCor pre-treatment in the greenhouse. It is possible that TrigoCor pre-inoculation alters the distribution or retention of secondary microbial inoculations, perhaps by altering the surface properties of the wheat spike such as through the involvement of surfactins or components of the culture media, which are 'sticky'.

In these experiments, *Fusarium* conidial suspensions were mixed with a green dye and applied to wheat spikes either pre-treated or not with TrigoCor, then visualized with a epifluorescent stereomicroscope (left, middle) or under UV light with a normal camera (right).

On a larger scale, a key overall conclusion of this work is that deficiencies in spray deposition and coverage appear to be extremely limiting for disease control in this system. Optimization efforts focused on improving spray technologies or on enhancing the spreading and/or sticking abilities of *Bacillus* biocontrol agents will likely yield the greatest results. Although I believe that work should continue on improving metabolite persistence, such as by stimulating *Bacillus* spore germination, such efforts might be useless if the quantity of inoculum applied to wheat plants is too low or the distribution across spike surfaces too sparse. Simultaneously pursuing both application-driven, and ecology-enhancing, tactics for improving biocontrol might be advantageous. Similarly, *Bacillus* biocontrol agents might best be suited for crops where application deficiencies are more easily overcome, such as those with more conducive plant architectures, or where multiple or more thorough applications are financially feasible.

Finally, in general this work suggests that the most effective biological control agents are likely those that are well-adapted to the environment in which they are applied. In situations where a microbe is introduced to an environment in which it is not capable of thriving on its own, then the greatest success may come from identifying the ecological shortcomings of that organism in its new environment, and allowing formulation to stand in to fill those needs.

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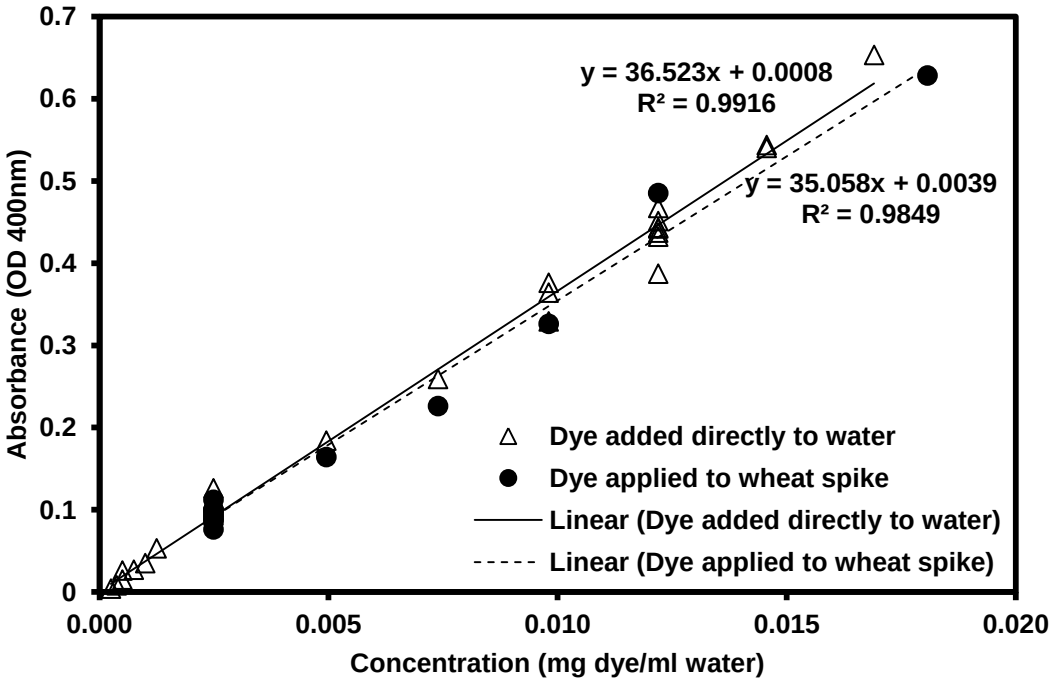
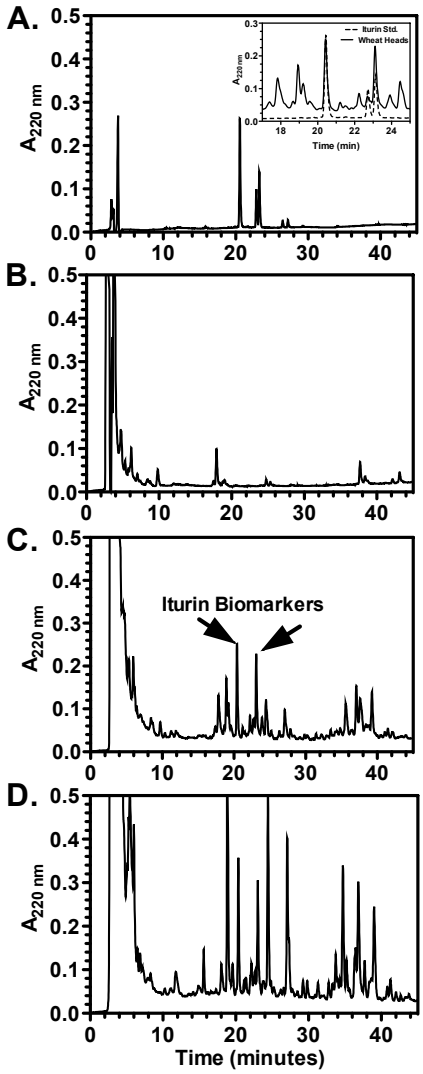
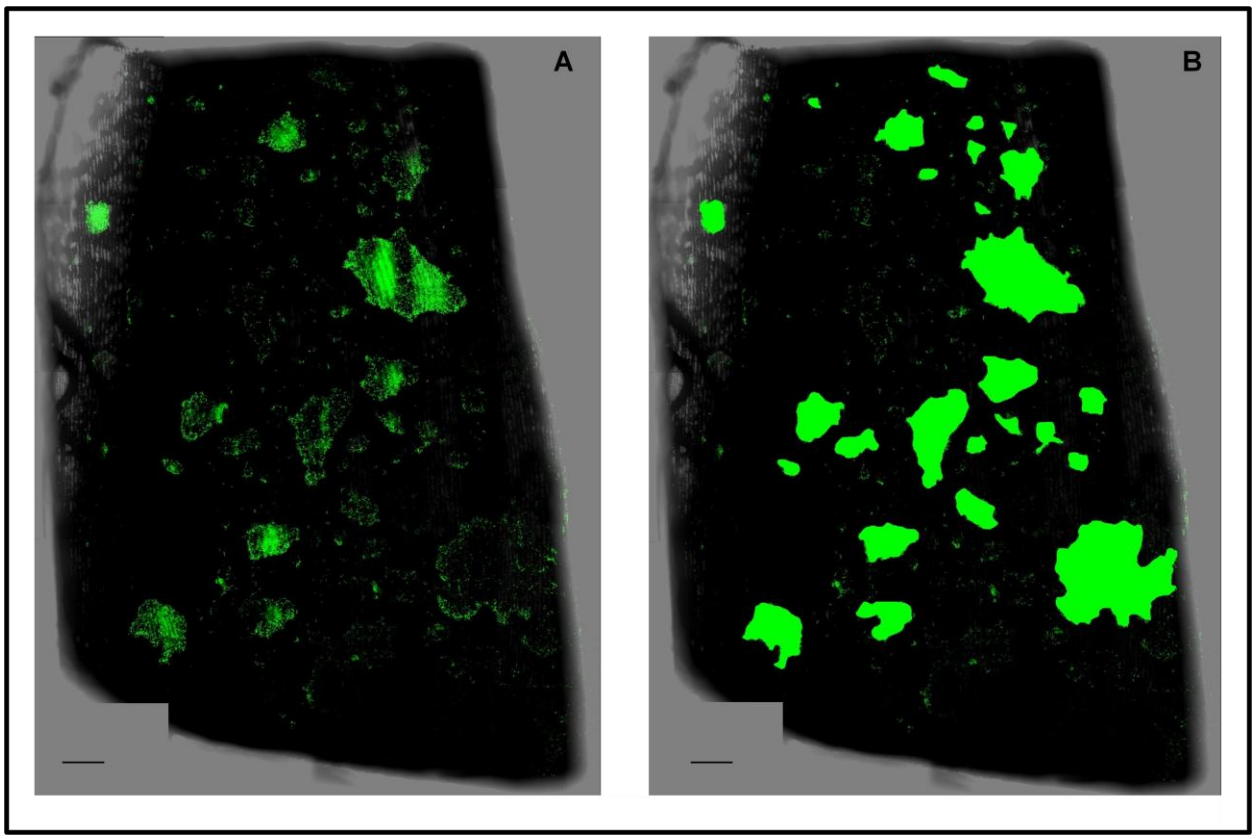
APPENDIX



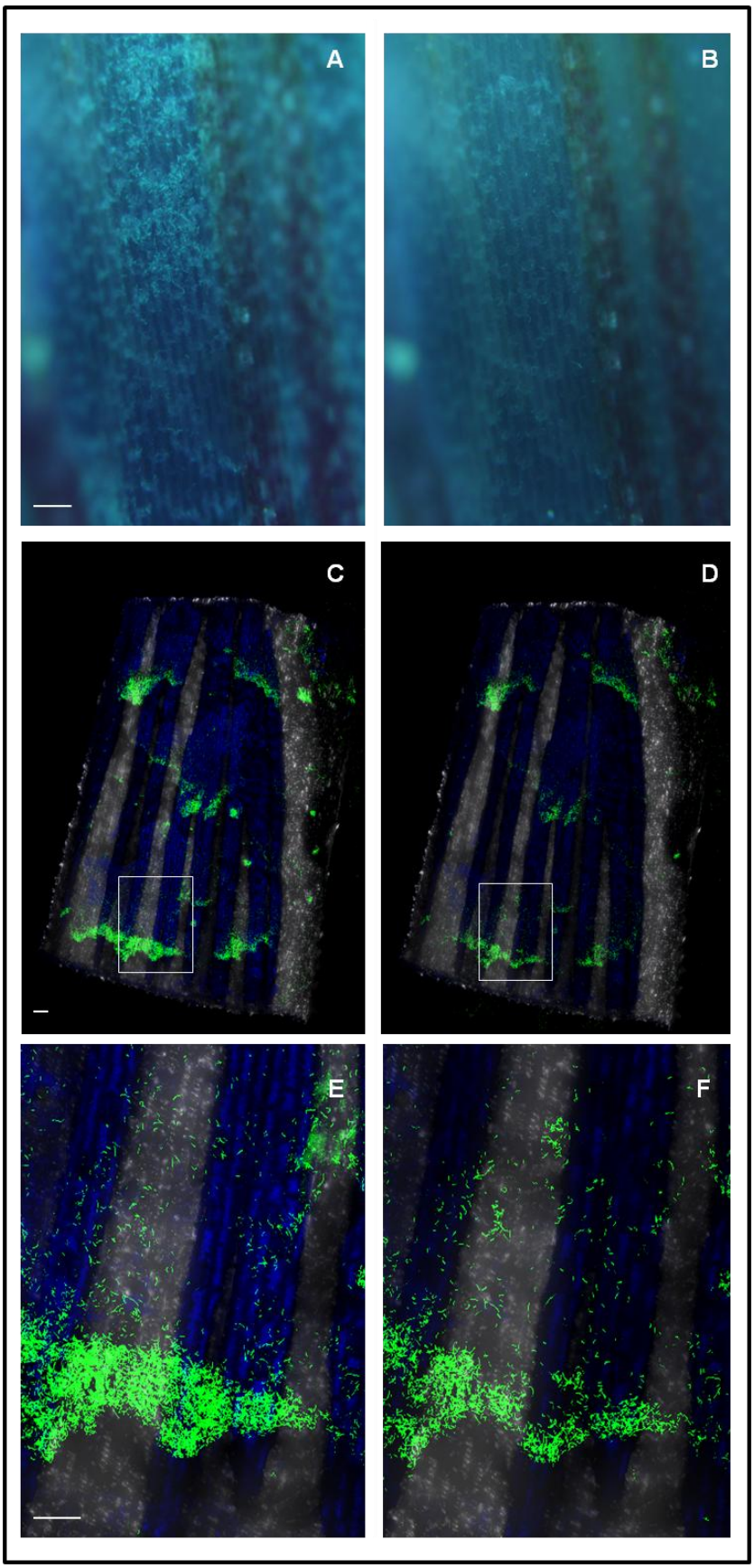
Appendix Figure 1. Linear relationship between Pyranine dye concentration and absorbance in a spectrophotometer. A solution of Pyranine 10G dye (0.5 mg/ml) was added directly to water, or pipetted onto 'Norm' wheat spikes which were allowed to dry then dipped 20 times in 20 ml water to remove dye. The absorbance of the resulting solution was measured at 400 nm on a Spectronic 20 spectrophotometer. A linear trendline was fit to the data sets from washed spikes and from dye added directly to water. The linear fit equations from the two data sets were nearly identical, so the equation for dye added directly to water was chosen for calculating inoculum delivery to spikes.



Appendix Figure 2. HPLC profiles (220 nm) of extracts of untreated and TrigoCor-treated wheat spikes. An iturin A standard (Sigma Chemical Co., St. Louis, MO) at 0.1 mg on column and TrigoCor broth used for wheat spike spray (0.5 ml broth equivalent on column) are included for comparison. **A** 0.1 mg iturin A standard; **B** untreated control wheat spike extract, representing 2 wheat spike equivalents on column, **C** high dosage wheat spike extract treated with TrigoCor, representing 2 wheat spike equivalents, and **D** 0.5 ml TrigoCor broth equivalent used for wheat spike treatment; The two peaks marked in **C** represent the two major iturins (also present in the standard) produced by TrigoCor in broth culture. The two iturin peaks (marked by arrows on C), matched by spectral identity and MS confirmation (M+Na = 1065 and M+Na= 1079) in the external standard, are also detectable in the wheat head extract sprayed with TrigoCor as seen in the insert in A, and subsequently were used as biomarkers to analyze time course of TrigoCor treatments in greenhouse and field trials.



Appendix Figure 3. Manual modification of images to assist in measuring the coverage of wheat surfaces by GFP-TrigoCor inoculum. GFP-TrigoCor cells and culture supernatant were sprayed onto wheat spikes in the laboratory according to the standard greenhouse protocol, then glume sections were excised and their surfaces visualized using a confocal microscope. In (A) green areas are GFP-TrigoCor cells and/or autofluorescent culture supernatant. In **(B)**, areas of wheat glumes covered by droplets of GFP-TrigoCor were manually outlined and filled green in the software program ImageJ, then were measured alongside non-outlined TrigoCor cells and supernatant to calculate the percent coverage by TrigoCor inoculum. Scale bars represent 200 µm.


A
B
C
D
E
F

Appendix Figure 4. Washing off of GFP-TrigoCor from wheat surfaces. Glumes pre-treated with GFP-TrigoCor inoculum were visualized before (A,C,E) and after (B,D,F) deionized water was sprayed onto wheat surfaces. Wheat surfaces were sprayed and visualized 7.5 h (A,B) and 1 day (C-F) post-TrigoCor application. The images in E and F are a magnification of the boxed areas in C and D, respectively. A,B; C,D; and E-F are shown at the same magnification, and scale bars for all represent 100 µm.