Influence of Propiconazole and Metconazole Formulations on Bacillus subtilis Vegetative Cell Growth and Disease Control of Fruit Crops

Article *in* Phytopathology · March 2024

DOI: 10.1094/PHYTO-01-24-0029-R

CITATIONS
2

READS
154

2 authors, including:

Johanna Wesche
Clemson University
13 PUBLICATIONS **11** CITATIONS

SEE PROFILE

Phytopathology® • XXXX • XX:X-X • https://doi.org/10.1094/PHYTO-01-24-0029-R

Disease Control and Integrated Management

Influence of Propiconazole and Metconazole Formulations on *Bacillus subtilis* Vegetative Cell Growth and Disease Control of Fruit Crops

Johanna Wesche and Guido Schnabel†

Department of Plant and Environmental Science, Clemson University, 105 Collings Street Clemson, SC 29634
Accepted for publication 13 March 2024.

Abstract

Biological control agent *Bacillus subtilis* formulated as Theia is registered for control of fungal and bacterial diseases of fruit crops. Combinations of Theia and strategic concentrations of two demethylation inhibitor (DMI) fungicides were investigated to explore potential synergisms. Bacteria were cultured in nutrient broth and combined with technical grades and two formulations of propiconazole (emulsifiable concentrate [EC] and wettable powder) and metconazole (EC and water-dispersible granule) at 0, 10, 50, 100, and 150 μg/ml of active ingredient. After cocultivation, the optical density (OD_{600}) and colony forming units (CFU/ml) were evaluated. In contrast to EC formulations, the wettable powder or water-dispersible granule formulations at 10 or 50 μg/ml of both DMIs did not affect vegetative cell growth. The mixture of Theia and each formulated DMI at 50 μg/ml of active ingredient resulted in a significant reduction of *Monilinia fructicola* lesion development on apple, *Colletotrichum siamense* lesion development on cherry, and *Botrytis cinerea* lesion development on cherry. The combination of Theia with EC formulations showed weaker disease reduction due to antagonism. Only Theia plus non-EC formulated propiconazole and metconazole significantly reduced brown rot disease incidence of apple compared with the respective solo treatments and anthracnose disease incidence of cherry compared with the untreated control. Our results indicated that at least some DMI fungicides possess bactericidal effects depending on the formulation and concentration. The combination of Theia with a lower-than-label-rate concentration (50 μg/ml) of the DMI fungicides propiconazole and metconazole showed potential for synergistic effects, especially when non-EC formulations were used.

Keywords: chemical control, disease control and pest management, biological control

Synthetic fungicides used in integrated pest management (IPM) systems have been successfully used to control plant diseases since the 1950s (Beckerman et al. 2023). However, regulatory authorities in the United States are implementing plans to drastically reduce their use. For example, California released a roadmap to eliminate high-risk pesticides by 2050 (California Department of Pesticide Regulation 2023). These initiatives will increase the pressure on growers to apply more sustainable IPM systems for high-quality food production with fewer synthetic fungicides.

One possible solution could be to replace at least some conventional fungicide sprays in rotation programs with biological control agents (BCAs), which have become increasingly popular in recent decades (Bonterra et al. 2022; Ongena and Jacques 2008). The most used bacteria for biological control are *Bacillus* species (Bonterra et al. 2022; Ongena and Jacques 2008). Their ability to form resilient spores that withstand unfavorable environmental conditions can be used to produce formulations with a relatively long shelf life (Bonterra et al. 2022). The fungicide Theia contains endospores of *Bacillus subtilis* ASF032321 and antimicrobial metabolites from the fermentation process (AgBiome 2023). *B. subtilis* has different modes of action. When the spores germinate on the plant surface and the bacteria multiply, they compete with plant pathogens for nutrients and space or parasitize (Chen et al. 2020). In addition, *B. subtilis* produces volatile organic compounds, bacteriocins (ribosomally synthesized peptides), and cyclic lipopeptides (non-ribosomally synthesized peptides) that can interfere with the synthesis of the cell wall and form pores in the membrane of the pathogen (Bonterra et al. 2022; Raaijmakers et al. 2010). Hydrolitic enzymes such as chitinases also efficiently hydrolyze the main components of fungal cell walls (Bonterra et al. 2022). In addition, *Bacillus* spp. can trigger induced systemic resistance in the plant and thus reduce susceptibility to infections (Bonterra et al. 2022; Chen et al. 2020). However, BCAs, such as Theia, are not routinely used in current IPM systems, as their performance under field conditions often lagged behind the label rate of synthetic fungicides. For example, the control efficacy of Theia was inferior against gray mold (*Botrytis cinerea*) and mummy berry (*Monilinia vaccinii-corymbosi*) of blueberries (Cline 2022), bacterial spot of peach (Breeden et al. 2021), and Phytophthora crown rot in strawberries (Marin et al. 2022) compared with the label rate of synthetic fungicides.

BCAs have been used in alternations with synthetic fungicides in IPM programs. Another possibility for integrating BCAs in production systems is to use them in mixtures with synthetic fungicides (Brannen and Kenney 1997; Kondoh et al. 2001; Ons et al. 2020; Peng et al. 2014). Such mixtures should be economical to increase the likelihood of implementation, and therefore, mixtures with reduced rates leading to synergistic interactions are of particular interest. Few studies have been conducted to examine such mixtures. Brannen and Kenney (1997) observed a significant reduction in the incidence of cotton blight caused by *Rhizoctonia solani* when they combined *B. subtilis* with seed treatment chemicals as opposed to the chemical alone. Peng et al. (2014) reported a synergistic effect in the control of eyespot in wheat when *B. subtilis* was mixed with full or half concentration of formulated difenoconazole or flutolanil. Other results showed a high tolerance of vegetative cell growth of *B. amyloliquefaciens* to 200 μg/ml of difenoconazole, and combinations of the fungicide at a dosage of 100 or 120 g/ha improved control of Fusarium wilt (*Fusarium oxysporum*) in tomato in field

†Corresponding author: G. Schnabel; schnabe@clemson.edu

Technical Contribution number 7222, Clemson University Experiment Station.

Funding: Support was provided by AgBiome Inc. and the U.S. Department of Agriculture-National Institute of Food and Agriculture (project number SC-1700594).

The author(s) declare no conflict of interest.

trials (Xu et al. 2022). Ji et al. (2019) showed synergistic effects of *B. methylotrophicus* in combination with the benzamide fungicide fluopimomide at 50 and 100 g/ha against gray mold on tomato. The mixture of the high label rate of the quinone outside inhibitor azoxystrobin with *B. subtilis* also showed better efficacy in controlling powdery mildew on zucchini than either product for itself (Gilardi et al. 2008). However, mixtures can also lead to antagonism. Liu et al. (2023) showed that tebuconazole strongly inhibits vegetative cell growth of *B. subtilis* at higher concentrations. The latter study showed a synergistic effect for rice false smut (*Ustilaginoidea virens*) control of rice only at a lower concentration of 45 μg/ml of tebuconazole. Specifically, for the DMI fungicide propiconazole, two studies evaluated its impact on *B. subtilis*. Yadav et al. (2022) appeared to show that 0.1 and 1% propiconazole inhibited the bacteria on nutrient agar. In contrast, Vijay et al. (2011) reported compatibility between 1,000 μg/ml of propiconazole and *B. subtilis* strain MBI 600. The latter may have been a result of potential differences between *B. subtilis* strain susceptibility. We found no information on the influence of metconazole on *B. subtilis* cell growth.

Both, propiconazole and metconazole are important fungicides in fruit production in the Southeastern United States and are marketed mainly in the form of emulsifiable concentrate (EC), water-dispersible granule (WDG), or wettable powder (WP) formulated products (Knowles 2008). Formulations can also be biologically active and influence interactions (Backman 1978; Oliver and Beckerman 2022), and thus, most studies have used technical-grade DMIs to investigate effects on bacterial growth (Ji et al. 2019; Liu et al. 2023; Peng et al. 2014; Xu et al. 2022). To the best of our knowledge, there is no information publicly available on the influence of DMI fungicides and their different formulations on the vegetative cell growth of BCAs.

The objective of this study was to evaluate the influence of propiconazole EC and WP formulations and metconazole EC and WDG formulations on the vegetative cell growth of *B. subtilis* and to examine the efficacy of their strategic mixtures with Theia against economically important fruit diseases caused by three fungal pathogens.

Materials and Methods

BCAs, fungicides, and growth media

Theia (AgBiome, Durham, NC), propiconazole (98.0%, Sigma Aldrich, Saint Louis, MO; PropiStar EC 41.8%, Agri-Star, Ankeny, IA; and Mentor WP 45%, Syngenta Crop Protection, Greensboro, NC), and metconazole (98.0%, Sigma Aldrich; Caramba EC 8.6%, BASF, RTP, Durham, NC; and Quash WDG 50%, Valent, San Ramon, CA) were used for all the experiments. Theia contained endospores of *B. subtilis* strain ASF032321 together with its metabolites from the fermentation process. Technical grades of propiconazole and metconazole were dissolved in methanol and adjusted to stock concentrations of 20 g/liter, and the final concentration of methanol in the experiment for all concentrations was less than 1.0%. For the commercial propiconazole and metconazole fungicide formulations, a 10 g/ml stock solution was prepared. Nutrient standard broth (NB; Sigma Aldrich) and nutrient standard agar (Sigma Aldrich) were used for cultivation of *B. subtilis*. Fungal isolates were cultured on potato dextrose agar (Hardy Diagnosis, Santa Maria, CA). We used the commercial Theia product rather than the purified bacteria for our studies because biological activity may be determined by the biologically active metabolites, the dividing bacteria, or any other inert ingredients in the formulated product. Thus, when referring to the activity of Theia against diseases, we refer to the mixture of the bacteria together with the activity of fermentation residue against diseases.

Fungal isolates and inoculum preparation

Monilinia fructicola isolate TF005 (Gura et al. 2024), *Colletotrichum siamense* isolate EY12-6 (Hu et al. 2015), and *Botrytis cinerea* isolate NC4 (Fernández-Ortuño et al. 2015) (Table 1) were revived from filter paper (stored at −20°C) and cultured on potato dextrose agar at 25°C. For the detached fruit studies on apple, an *M. fructicola* resistant isolate to FRAC 3 was used to assess synergistic interactions because DMI-sensitive isolates were completely inhibited, even at the low concentration of 50 μg/ml used in this study (data not shown).

Fresh conidia from 14-day-old cultures were harvested in sterile deionized water by scraping the colony surface with a sterile glass slide and filtering the spore suspension through sterile cheesecloth. *M. fructicola* isolate TF005 was not producing spores in the culture; therefore, apples were infected with a mycelial plug (0.5-mm diameter) applied to a wound. Spores of TF005 were then harvested directly from apple fruit infected for 1 week in sterile deionized water and filtered through sterile cheesecloth. The suspension was stored at 4°C prior to inoculation but no longer than 6 h. To confirm the germination ability of the conidia, 500 μl of the suspension was plated on potato dextrose agar and incubated for 24 h. The germination rate was always above 90% for all conidia suspensions and all experiments.

Influence of fungicide formulations on vegetative cell growth

Fungicides at concentrations of 0, 10, 50, 100, and 150 μg/ml of the active ingredient were added to the autoclaved NB that had been cooled to room temperature. Three sterile falcon tube replicates, each filled with 30 ml of NB, were used for each concentration and each DMI treatment. Each experiment was repeated twice for a total of nine technical replicates. A stock solution of not less than 1×10^8 CFU/ml was prepared for the formulated BCA based on the product information. To each falcon tube, 300 μl (dilution of 1:100) of the stock solution was pipetted to achieve a *B. subtilis* endospore concentration of not less than 1×10^6/tube at 0 h of the experiment. The bacteria were incubated at 30°C and shaken in a rotary shaker at 180 rpm. After 0, 24, and 48 h, the optical density (OD_{600}) relative to uninoculated NB for each chemical suspension was measured using a photometer (GeneQuant pro; Biochrom Ltd., Cambridge, England). In addition, a subsample was taken from each replicate and treatment after 24 and 48 h, and a dilution series from 10^{-1} to 10^{-4} was created to determine the CFU/ml values. A total of 15 μl of each dilution was pipetted onto the nutrient standard agar in the upper middle of Petri dishes and allowed to run down the agar by turning the dishes on their sides until the drop disappeared. Petri dishes were incubated for 2 days at 30°C. The colonies of 10^{-4} dilution per streak were counted with a digital counter pen (Thermo Fisher Scientific, Florence, SC). Experiments were conducted independently three times. In an additional experiment, the potential biological activity of the 0.76% methanol used to dissolve the technical grade of DMIs to 150 μg/ml was investigated by measuring the OD_{600} values after 0, 24, and 48 h. To determine more detailed differences between the EC and WP formulations on the vegetative cell growth of *B. subtilis*, an additional experiment was created to measure OD_{600} values at 0, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h when exposed to 50 μg/ml of propiconazole. The experimental design was the same as previously described.

Detached fruit assays

Ten organic apple fruits (cultivar 'Gala') and 12 conventional dark sweet cherries of unknown cultivar per treatment were used in detached fruit experiments. Each experiment was independently repeated three times. All fruit was purchased at local grocery stores; no organic cherries were available. Apples were rinsed with warm tap water and then disinfected by wiping the fruit surface with a paper towel soaked in 70% ethanol (wt/vol). Cherries were immersed in disinfectant solution (32 ml of 0.5% sodium hypochlorite, 32 ml of 95% ethanol, and 0.01 ml of Tween 20) for 5 min, rinsed with sterile deionized water, and dried under the running laminar flow hood for 30 min. Apples and cherries were gently wounded with a sterile toothpick (1-mm diameter, 1-mm depth), sprayed with fungicide

suspensions using a hand mister until they were completely wetted, and allowed to dry overnight. The experimental treatments are shown in Table 2. Theia and DMI fungicides were diluted in sterile tap water and shaken at 180 rpm at room temperature for 24 h before application. Fungicides were sprayed and had a further 24 h to establish and dry on the fruit surface before inoculation.

For inoculation, the conidial suspensions were adjusted to 2.5×10^5 conidia/ml, and 20 µl was applied per wound for a total of 5,000 conidia/wound. Fruits were fully randomized and incubated in lidded 30-cm length by 20-cm width by 16.5-cm depth plastic boxes with water-soaked paper towels on the bottom to keep humidity high. For the inoculated control, fruits were wounded, sprayed with sterile tap water, and inoculated with the spore suspension. For the uninoculated control, fruits were wounded, sprayed with sterile tap water, but not inoculated.

The smallest and largest radius of each fruit lesion were measured with a digital caliper 5 days postinoculation (dpi), and values were averaged. Disease incidence (%) was calculated by dividing the number of diseased fruits by the total number of fruits and multiplying the results by 100.

Data analysis

All statistical analyses were performed using SPSS 29.0 software (IBM, Armonk, NY).

To test for differences between the CFU/ml values after 24 and 48 h, an analysis of variance ($P < 0.05$) was performed, followed by Tukey's test. The homogeneity of variance was checked before analysis using the Levene test. If variances did not fulfill homogeneity, all individual values of the experiment were transformed with the Log_{10} before the analysis of variance was performed. In addition, a regression analysis ($P < 0.05$) was performed, and the Pearson correlation coefficient ($r > 0.7$, strong correlation; $r < 0.3$, weak correlation) was calculated to show the relationship between OD_{600} and CFU/ml values. The data for lesion radius 5 dpi, the disease incidence, and the observed control in the detached fruit assays were analyzed by analysis of variance ($P < 0.05$) followed by Tukey's test to determine statistical differences between treatments. If the data did not meet variance homogeneity, all data in that experiment were log_{10} transformed before analysis. The observed control was calculated as follows: (lesion radius 5 dpi of the inoculated control – lesion radius 5 dpi of the treated group)/lesion radius 5 dpi of the inoculated control × 100%. To compare synergistic or antagonistic interactions between the fungicides, the expected control of the fungicide mixtures based on the observed control of the single fungicides was calculated as described previously (Colby 1967; Liu et al. 2023; Peng et al. 2014): $E = I_f + I_N - I_f \times I_N/100$. I_f is the observed percentage of the control efficacy at the concentration C_f. I_N is the observed percentage of the control provided by Theia at the concentration C_n. E is the expected percentage of control by Theia plus the respective DMI formulation at a concentration of C_f and C_n. A positive value indicated synergy; a negative value indicated antagonism. A zero value would have indicated a neutral interaction.

Results

Effect of methanol on vegetative cell growth

The highest concentration of methanol 0.76% (vol/vol) used in the experiments to dissolve technical-grade DMI fungicides did not affect vegetative cell growth (Fig. 1). The optical density (OD_{600}) of *B. subtilis* cultivated only in NB was 0.404 after 24 h. The *B. subtilis* cultivated in NB plus 0.76% (vol/vol) methanol showed an OD_{600} value of 0.378. Only after 48 h was the optical density detected for the untreated control slightly higher compared with *B. subtilis* cultivated in NB plus 0.76% (vol/vol) methanol.

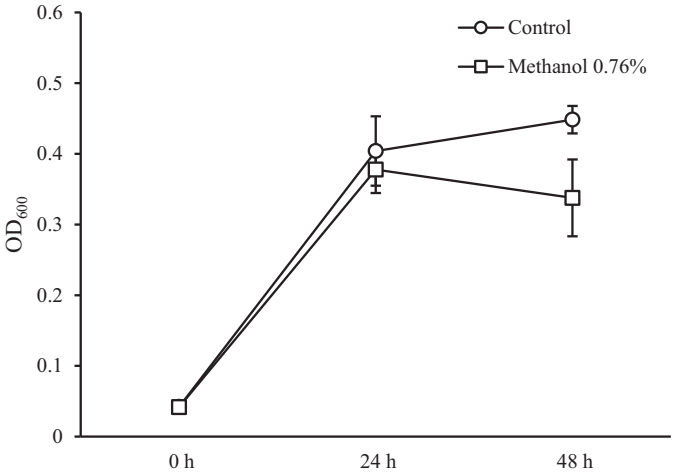

Fig. 1. Effect of 0.76% methanol in nutrient broth on the vegetative cell growth of *Bacillus subtilis*. OD_{600}, optical density at 600 nm.

TABLE 1. Fungal isolates used in the detached fruit assays and their characteristics

Fungal pathogen	Isolate	Known phenotype	Host	Reference
Botrytis cinerea	NC4	Sensitive to FRAC 1, 2, 7, 9, 11, 12, 17	Strawberry	Fernández-Ortuño et al. 2015
Colletotrichum siamense	EY12-6	Sensitive to FRAC 1, 11	Peach	Hu et al. 2015
Monilinia fructicola	TF005	Resistant to FRAC 3	Peach	Gura et al. 2024

TABLE 2. Treatments used to control brown rot of apple caused by *Monilinia fructicola*, anthracnose of cherry caused by *Colletotrichum siamense*, and gray mold of cherry caused by *Botrytis cinerea* in detached fruit assays

Treatment[z]	Active ingredient (concentration)	Product concentration	Apple (*M. fructicola*)	Cherry (*B. cinerea*, *C. siamense*)
Uninoculated control	N/A	–	x	x
Inoculated control	N/A	–	x	x
Theia	*Bacillus subtilis*	4.4 g/liter	x	x
Mentor (WP)	Propiconazole (50 µg/ml)	0.116 g/liter	x	–
PropiStar (EC)	Propiconazole (50 µg/ml)	0.119 ml/liter	x	–
Quash (WDG)	Metconazole (50 µg/ml)	0.111 g/liter	–	x
Caramba (EC)	Metconazole (50 µg/ml)	0.581 ml/liter	–	x
Theia + Mentor (WP)	*Bacillus subtilis* + Propiconazole (50 µg/ml)	4.4 + 0.116 g/liter	x	–
Theia + PropiStar (EC)	*Bacillus subtilis* + Propiconazole (50 µg/ml)	4.4 g/liter + 0.119 ml/liter	x	–
Theia + Quash (WDG)	*Bacillus subtilis* + Metconazole (50 µg/ml)	4.4 + 0.111 g/liter	–	x
Theia + Caramba (EC)	*Bacillus subtilis* + Metconazole (50 µg/ml)	4.4 g/liter + 0.581 ml/liter	–	x

[z] WP = wettable powder; EC = emulsifiable concentrate; and WDG = water-dispersible granule.

Effect of propiconazole on vegetative cell growth

Propiconazole affected the vegetative cell growth of *B. subtilis* depending on the concentration and formulation (Fig. 2). The OD_{600} values for the technical grade showed an increase in turbidity after 24 h for all test concentrations. However, turbidity was lower at 100 and 150 µg/ml compared with 0, 10, and 50 µg/ml (Fig. 2A). After 48 h, the OD_{600} values were similar for all concentrations. There were no significant differences between the CFU/ml values after 24 h of incubation in NB ($P = 0.125$) (Fig. 2B). Only after 48 h were the CFU/ml values in the concentration of 150 µg/ml significantly lower ($P < 0.001$) compared with 0, 10, and 50 µg/ml. An evaluation of the regression analysis ($P = 0.135$) and the Pearson correlation coefficient ($r = 0.279$) showed a weak correlation and no significant relationship between OD_{600} and CFU/ml values for the technical grade of propiconazole.

For Mentor (WP), an increase in OD_{600} values was measurable after 24 h for all concentrations. However, the OD_{600} values for 50, 100, and 150 µg/ml propiconazole were lower compared with 0 and 10 µg/ml (Fig. 2C). The CFU/ml values after 24 h of incubation for 100 µg/ml were significantly lower compared with 0 µg/ml (Fig. 2D). All other concentrations did not differ significantly from each other. After 48 h, there were also no significant differences between the concentrations for the CFU/ml values in Mentor (WP) ($P = 0.351$). There was no significant relationship ($P = 0.309$) and only a weak correlation ($r = 0.192$) between OD_{600} and CFU/ml values for Mentor (WP).

For PropiStar (EC), clear differences were observed between the concentrations for the vegetative cell growth of *B. subtilis*. An increase in OD_{600} values after 24 h was detectable for 0, 10, and 50 µg/ml (Fig. 2E). However, this was not the case for the higher concentrations of 100 and 150 µg/ml. Even after 48 h, no turbidity was measurable at these concentrations. This was consistent with the CFU/ml values. The CFU/ml values for 0, 10, and 50 µg/ml were significantly higher after 24 h ($P = 0.013$) and 48 h ($P > 0.001$) compared with 100 and 150 µg/ml (Fig. 2F). The regression analysis showed a significant correlation ($P > 0.001$), and a moderate Pearson correlation ($r = 0.669$) between CFU/ml and OD_{600} values could be calculated for the EC formulation.

We conducted an additional experiment to uncover the influence of Mentor (WP) and PropiStar (EC) on *B. subtilis* cell growth during the first 24 h of incubation (Fig. 3). An increase in optical density was clearly measurable starting at 8 h for the control and the Mentor

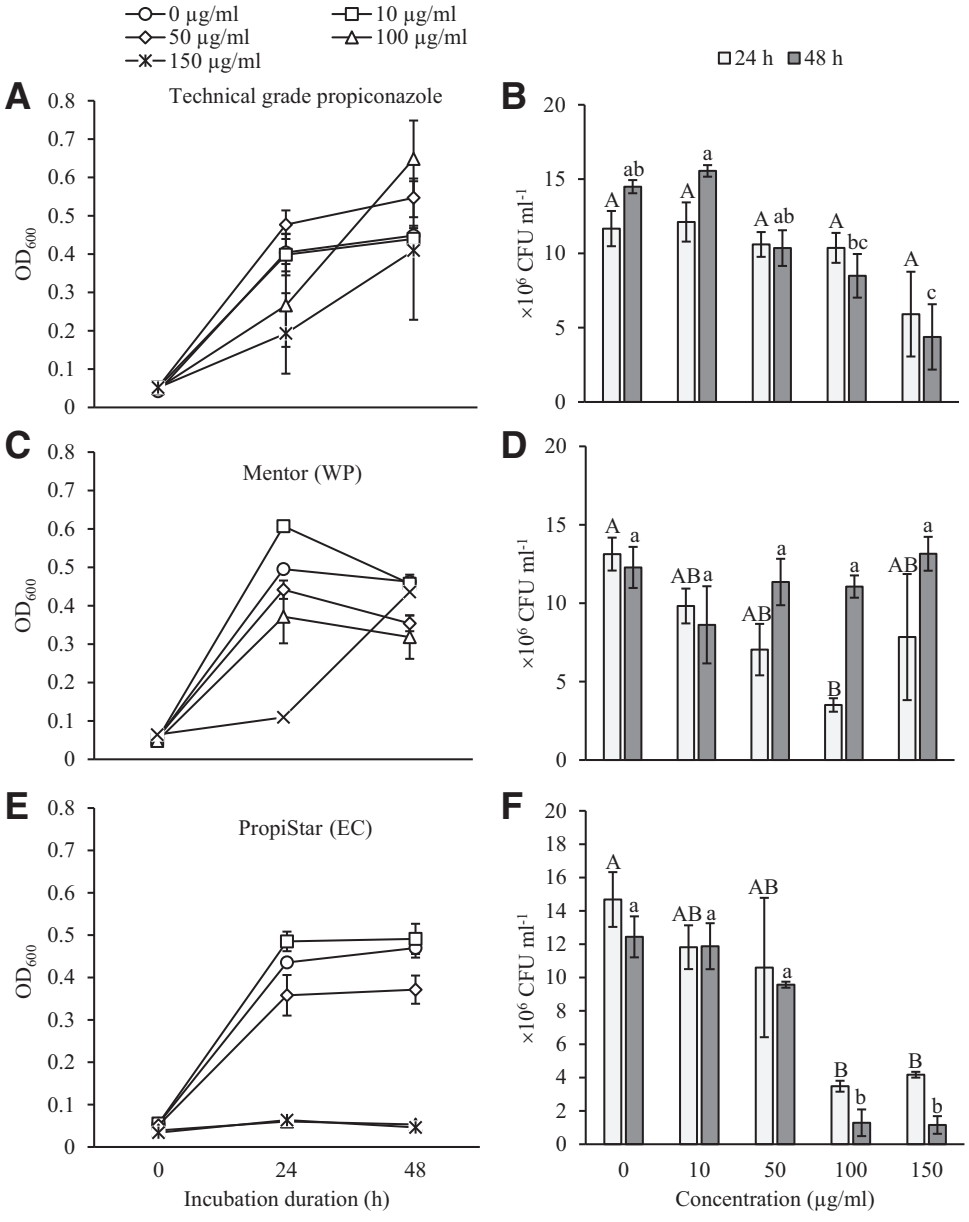

Fig. 2. Influence of **A and B,** technical-grade propiconazole, **C and D,** formulated propiconazole (Mentor [wettable powder, WP]), and **E and F,** formulated propiconazole (PropiStar [emulsifiable concentrate, EC]) on the vegetative cell growth of *Bacillus subtilis*. Means of three independent experimental replicates ± standard error; Tukey, $P < 0.05$; capital letters show a significant difference between treatments for 24 h and lowercase letters for 48 h. OD_{600}, optical density at 600 nm.

(WP) treatment. The timeline between 8 and 14 h revealed steady cell growth in the Mentor (WP) treatment that was almost as strong as the cell growth of the control treatment. Cell growth in the PropiStar (EC) treatment did not increase to detectable levels for the first 22 h of incubation (Fig. 3). This experiment could not be conducted for metconazole formulations because the EC formulation did not allow for any measurable *B. subtilis* growth at a dose of 50 µg/ml.

Effect of metconazole on vegetative cell growth

Like propiconazole, metconazole also affected the vegetative cell growth of *B. subtilis* depending on the concentration and formulation. When *B. subtilis* was cultured in technical-grade metconazole, an increase in optical density was measurable after 24 h for all concentrations except 150 µg/ml (Fig. 4A). The OD_{600} value for the concentrations of 50 and 100 µg/ml was lower compared with the control and 10 µg/ml, indicating slower growth. After 48 h of incubation, a small increase in the OD_{600} value at 150 µg/ml was measurable. There were no significant differences between CFU/ml values after 24 h, except for 100 µg/ml, which was significantly lower compared with the untreated control ($P > 0.001$) (Fig. 4B). After 48 h, the CFU/ml values for all concentrations were at a similar level, and there were no significant differences ($P = 0.42$). The evaluation of the regression analysis showed no significant correlation ($P = 0.08$) and a weak correlation for the Pearson correlation coefficient ($r = 0.473$).

For Quash (WDG), an increase in turbidity (OD_{600}) was measurable at concentrations of 0, 10, and 50 µg/ml after 24 h, but not for 100 and 150 µg/ml (Fig. 4C). After 48 h, the OD_{600} values were similar for all test concentrations. Thus, vegetative cell growth at concentrations of 100 and 150 µg/ml only started after 24 h. There were no significant differences between the CFU/ml values after 24 h of incubation ($P = 0.719$) (Fig. 4D). After 48 h, the CFU/ml values of the control were significantly higher compared with 50, 100, and 150 µg/ml ($P > 0.001$). Again, there was no significant relationship ($P = 0.167$) and only a weak correlation ($r = 0.259$) between OD_{600} and CFU/ml values.

The OD_{600} values for the EC formulation (Caramba) showed a clear inhibition of cell growth for all concentrations, except for the lowest concentration of 10 µg/ml after 24 h of incubation (Fig. 4E). Even after 48 h, no increase in turbidity was measurable for 50, 100, and 150 µg/ml. This was consistent with the CFU/ml values. The CFU/ml values for 0 and 10 µg/ml were higher but only significantly higher at 48 h ($P > 0.001$) compared with the other concentrations (Fig. 4F). The regression analysis ($P > 0.001$) and the Pearson correlation coefficient ($r = 0.723$) showed a significant and strong correlation between OD_{600} and CFU/ml values.

Effect of experimental treatments on *M. fructicola* lesion development and brown rot incidence on apple

For the disease development studies on detached fruit, the 50 µg/ml concentration was selected because it was the highest concentration that allowed for uninhibited *B. subtilis* cell growth in mixtures with non-EC formulations. The evaluation 5 days after inoculation with *M. fructicola* isolate TF005 showed significant differences between the treatments for lesion radius (mm) ($P > 0.001$) and brown rot incidence (%) on apple ($P > 0.001$) (Fig. 5A and B). The inoculated control and Mentor (WP) at a concentration of 50 µg/ml showed the highest disease incidence and lesion radius. Theia showed a significant reduction in lesion radius compared with the inoculated control but no significant difference for disease incidence. PropiStar (EC) solo treatment at a concentration of 50 µg/ml significantly reduced both lesion growth and brown rot incidence. Theia plus Mentor (WP) and Theia plus PropiStar (EC) reduced lesion growth and disease incidence more than Mentor (WP) and Theia by themselves, and the strongest effects were observed for Theia plus Mentor (WP). The observed control efficacy for the latter combination was 85.5% (Table 3) with a synergistic effect value of 24.4. The combination of Theia and PropiStar (EC) achieved an observed control efficacy of 75.3%, and an antagonistic effect (−7.8) was observed due to a calculated expected control efficacy of 83.1%.

Effect of experimental treatments on *C. siamense* lesion development and anthracnose incidence on cherry

Cherries sprayed with the different treatments and inoculated with *C. siamense* showed significant differences in lesion radius and anthracnose disease incidence 5 dpi. The largest lesion radius was measured for the inoculated control and was significantly higher than those of other treatments (Fig. 6A; $P < 0.001$). The smallest lesion radius was measured for the combination of Theia and Quash (WDG), which was statistically different from Theia, Caramba (EC), and the combination of Theia and Caramba (EC). Disease incidence for the combination of Theia and Quash (WDG) was lower compared with the inoculated control, Theia, and Caramba

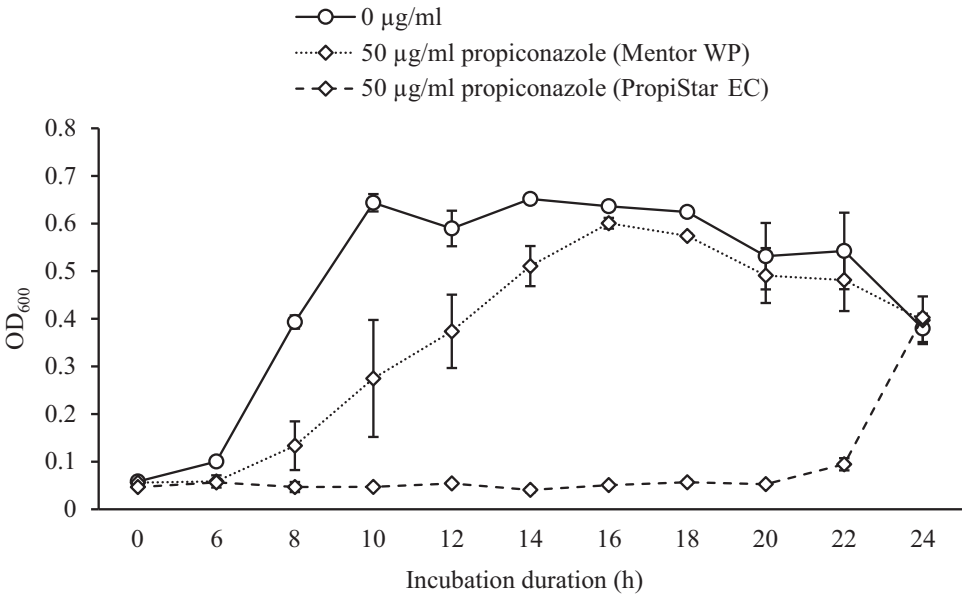

Fig. 3. Influence of 50 µg/ml of propiconazole formulated as Mentor (wettable powder [WP]) or PropiStar (emulsifiable concentrate [EC]) on the vegetative cell growth of *Bacillus subtilis* over the first 24 h. Means of three independent experimental replicates ± standard error. OD_{600}, optical density at 600 nm.

(EC) (Fig. 6B; $P < 0.001$). The observed control efficacy for Theia plus Quash (WDG) was 81.8%, and the expected control efficacy was 82.2%. Thus, a difference of −0.4 was calculated, and no synergistic effect was found (Table 4). The observed control efficacy for Theia plus Caramba (EC) was 52.7%, but the expected control efficacy was 65.9%, which resulted in a calculated antagonistic effect value of −13.2.

Effect of experimental treatments on *Botrytis cinerea* lesion development and gray mold incidence on cherry

All treatments showed significantly reduced lesion growth 5 days after inoculation with *Botrytis cinerea* compared with the inoculated control (Fig. 7A; $P < 0.001$). The lesion radius of the combination of Theia and Quash (WDG) was significantly lower compared with the inoculated control and Theia. However, gray mold disease incidence was not different among treatments (Fig. 7B; $P < 0.001$). The observed control efficacy for the combination of Theia and Quash (WDG) was 86.7%, and the expected control efficacy was 92.0%, which indicated an antagonistic effect (−5.3) (Table 5). For the combination of Theia and Caramba (EC), a control efficacy of 73.7% against *Botrytis cinerea* was observed. However, the expected control efficacy was 86.2%, showing a difference of −12.5 and an antagonistic effect between the two fungicides.

Discussion

Mixtures between BCAs and synthetic fungicides at lower concentrations are an interesting approach to decreasing pesticide risk by increasing the effectiveness of BCAs (Brannen and Kenney 1997; Kondoh et al. 2001; Peng et al. 2014). However, mixing partners should not negatively influence each other when expected to generate synergistic effects against plant diseases. Based on OD_{600} and CFU/ml values, propiconazole and metconazole strongly inhibited the vegetative cell growth of *B. subtilis* at the concentration commonly used in the field (about 150 µg/ml). Lower concentrations of 50 µg/ml of propiconazole or metconazole did not slow the growth of *B. subtilis* in liquid culture when applied as non-EC formulations. Liu et al. (2023) showed a similar inhibitory effect of the DMI fungicide tebuconazole on *B. subtilis* strain H158: Vegetative cell growth was unaffected at concentrations below 25 µg/ml. In

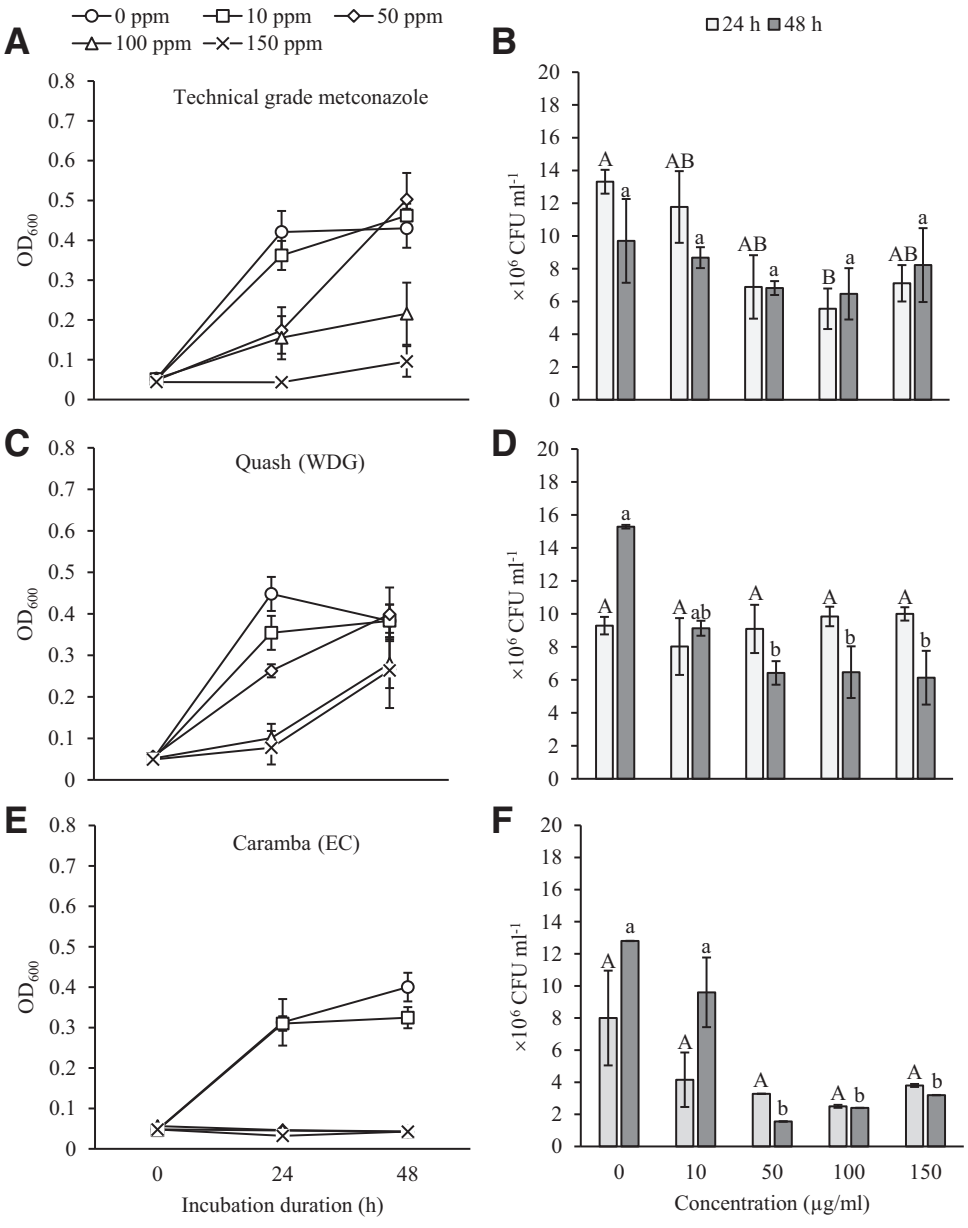

Fig. 4. Influence of **A and B,** technical-grade metconazole, **C and D,** formulated metconazole (Quash water-dispersible granule [WDG]), and **E and F,** formulated metconazole (Caramba emulsifiable concentrate [EC]) on the vegetative cell growth of *Bacillus subtilis*. Means of three independent experimental replicates ± standard error; Tukey, $P < 0.05$; capital letters show a significant difference between treatments for 24 h and lowercase letters for 48 h. OD_{600}, optical density at 600 nm.

contrast, other studies showed high tolerance of *B. amyloliquefaciens* (Xu et al. 2022) and *B. subtilis* (Peng et al. 2014) to the active ingredient difenoconazole at levels commonly used in the field. It is conceivable that there are differences in susceptibility to DMI fungicides among *Bacillus* species and even among strains of the same species.

TABLE 3. Effect of the combination of Theia with different propiconazole formulations against *Monilinia fructicola* lesion formation on apple

Treatment[x]	Observed control (%)[y]	Expected control (%)[z]	Difference
Theia	53.8 b	–	–
Mentor (WP)	15.8 a	–	–
PropiStar (EC)	63.5 bc	–	–
Theia + Mentor (WP)	85.5 d	61.1	24.4
Theia + PropiStar (EC)	75.3 cd	83.1	–7.8

[x] For Mentor (WP) and PropiStar (EC), the final concentration of propiconazole was 50 µg/ml. WP = wettable powder; EC = emulsifiable concentrate.
[y] Means of 10 fruits per experiment and three total experimental repeats; different letters indicate significant differences between treatments for the observed control (Tukey, $P > 0.001$).
[z] Colby (1967).

Our experiments were designed to study the impact of DMI fungicides on the ability of *B. subtilis* to multiply, which influences their ability to compete for space and nutrients and to produce antifungal metabolites under field conditions. Under practical conditions, the BCA and DMI fungicide would not interact in the same way as simulated in this study. The mixture would be suspended in water and applied within hours, and their interaction on the plant surface could be different due to cycles of product drying and rewetting influencing cell count and due to chemical degradation over time, among other things. BCAs may establish on the plant surface over time (Bellamy et al. 2021; Oliver and Beckerman 2022), and follow-up studies in the field could shed light on whether DMIs at concentrations that are suppressive in vitro influence BCA establishment. Despite the potential interference of cell growth by DMIs, other biologically active compounds may still be present in the formulation and reduce disease. For example, *B. subtilis* metabolites such as Iturin A had inhibitory effects on *B. cinerea* and *Penicillium digitatum* on fruits (Ambrico and Trupo 2017). Other studies showed that a spore suspension of *B. subtilis* significantly reduced *P. digitatum* establishment on citrus fruit when applied 24 h before inoculation (Leelasuphakul et al. 2008). In the same study, the metabolites of *B. subtilis* alone showed a significantly better effect when applied at the same time as the pathogen (Leelasuphakul et al. 2008).

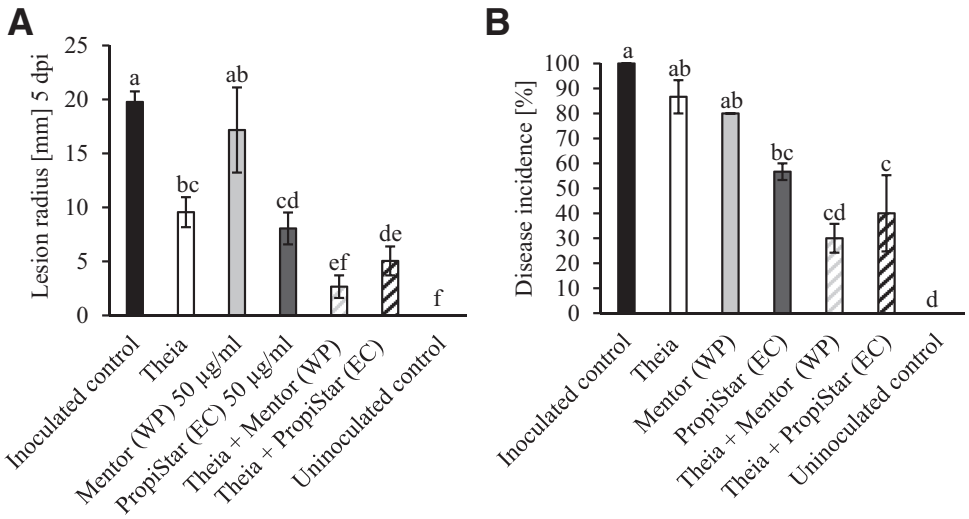

Fig. 5. A, Lesion radius (mm) of *Monilinia fructicola* and **B,** mean disease incidence on 'Gala' apples 5 days postinoculation (dpi; means of three independent experimental replicates ± standard error). For Mentor (wettable powder [WP]) and PropiStar (emulsifiable concentrate [EC]), the final concentration of propiconazole was 50 µg/ml. Different letters indicate significant differences between treatments (Tukey, $P < 0.05$).

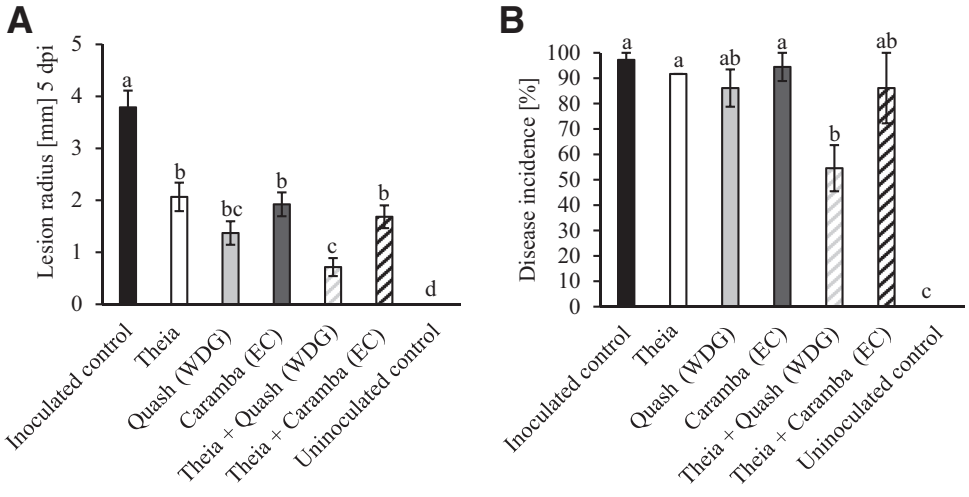

Fig. 6. A, Lesion radius (mm) of *Colletotrichum siamense* and **B,** disease incidence on cherries 5 days postinoculation (dpi; means of three independent experimental replicates ± standard error). For Quash (water-dispersible granule [WDG]) and Caramba (emulsifiable concentrate [EC]), the final concentration of metconazole was 50 µg/ml. Different letters indicate significant differences between treatments (Tukey, $P < 0.05$).

Determining inhibition of *B. subtilis* growth after 24 and 48 h using OD_{600} values in the presence of the fungicide allowed for early detection of BCA/fungicide interactions. The methodology for assessing the compatibility of bacterial BCAs with the mixing partners varies between published studies. Some studies base the assessment solely on CFU values (Ji et al. 2019; Peng et al. 2014) and others just on OD_{600} values (Anand et al. 2010; Liu et al. 2018, 2023). For example, Peng et al. (2014) assessed the compatibility of *B. subtilis* and practical concentrations of difenoconazole using only surviving bacterial cells (CFU values) on wheat seeds. Other work by Liu et al. (2018) shows differences between quinone outside inhibitors azoxystrobin and trifloxystrobin based on OD_{600} and CFU values. In that same study, growth of *B. subtilis* (OD_{600}) after 12 h of incubation showed the highest turbidity (OD_{600}) for the combination with azoxystrobin, whereas trifloxystrobin appeared to be most compatible with the BCA on rice plants based on CFU values (Liu et al. 2018). Our results showed no significant correlations between OD_{600} and CFU/ml values, except for the EC formulation. Slowed cell growth between *B. subtilis* and DMI fungicides was only detected using OD_{600} values, indicating that CFU/ml unit counts are not appropriate to detect such early interactions.

B. subtilis forms endospores that survive when environmental conditions are unfavorable for cell growth (Nicholson 2002). This ability makes *B. subtilis* a good candidate for mass production, extended shelf life, and commercialization as a biorational fungicide (Bonterra et al. 2022). When the spores are released into the environment and favorable conditions are present, rapid germination occurs, and thus, vegetative cell growth is initiated (McKenney et al. 2013). The spores constantly monitor their environment with the help of a series of receptors embedded in the inner spore membrane to be able to end dormancy in a targeted manner (McKenney et al. 2013). The structure of the spore is complex, consisting of an exposome, outer membrane, inner membrane, cortex, and core, which contains the DNA (Setlow 2006). In general, spores of *B. subtilis* are considered resistant to ultraviolet radiation, chemicals, extreme heat, and other stresses (McKenney et al. 2013; Setlow 2006). The factors important for the chemical resistance of spores depend mainly on the chemical itself (Setlow 2006).

We observed at concentrations high enough to slow down but not arrest cell growth (Figs. 2A [100 and 150 µg/ml] and C [150 µg/ml] and 3A [50 µg/ml] and B [100 and 150 µg/ml]) a subsequent recovery of cell growth development. That suggests the presence of a detoxification mechanism that, upon activation, may enable cells, upon initial delay, to multiply with no or very little inhibition. In general, a BCA should not only survive environmental stresses but also establish itself quickly on the host plant (Bellamy et al. 2021). Any delay of colonization due to the presence of inhibitors in the tank mixtures or due to the presence of inhibitory pesticide residues on the plant surface could therefore impair the efficacy of a BCA.

In our investigations, we found that not only did the concentration of DMI fungicide influence the vegetative cell growth of *B. subtilis*, but the formulation did as well. EC formulations inhib-

TABLE 4. Effect of the combination of Theia with different metconazole formulations against *Colletotrichum siamense* lesion formation on cherries

Treatment[x]	Observed control (%)[y]	Expected control (%)[z]	Difference
Theia	48.9 a	–	–
Quash (WDG)	65.2 a	–	–
Caramba (EC)	48.9 a	–	–
Theia + Quash (WDG)	81.8 a	82.2	–0.4
Theia + Caramba (EC)	52.7 a	65.9	–13.2

[x] For Quash (WDG) and Caramba (EC), the final concentration of metconazole was 50 µg/ml. WDG = water-dispersible granule; EC = emulsifiable concentrate.
[y] Means of 10 fruits per experiment and three total experimental repeats; different letters indicate significant differences between treatments for the observed control (Tukey, $P = 0.056$).
[z] Colby (1967).

TABLE 5. Effect of the combination of Theia with different metconazole formulations against *Botrytis cinerea* lesion formation on cherries

Treatment[x]	Observed control (%)[y]	Expected control (%)[z]	Difference
Theia	58.1 a	–	–
Quash (WDG)	80.8 a	–	–
Caramba (EC)	67.0 a	–	–
Theia + Quash (WDG)	86.7 a	92.0	–5.3
Theia + Caramba (EC)	73.7 a	86.2	–12.5

[x] For Quash (WDG) and Caramba (EC), the final concentration of metconazole was 50 µg/ml. WDG = water-dispersible granule; EC = emulsifiable concentrate.
[y] Means of 12 fruits, three experimental replicates; different letters indicate significant differences between treatments for the observed control (Tukey, $P = 0.197$).
[z] Colby (1967).

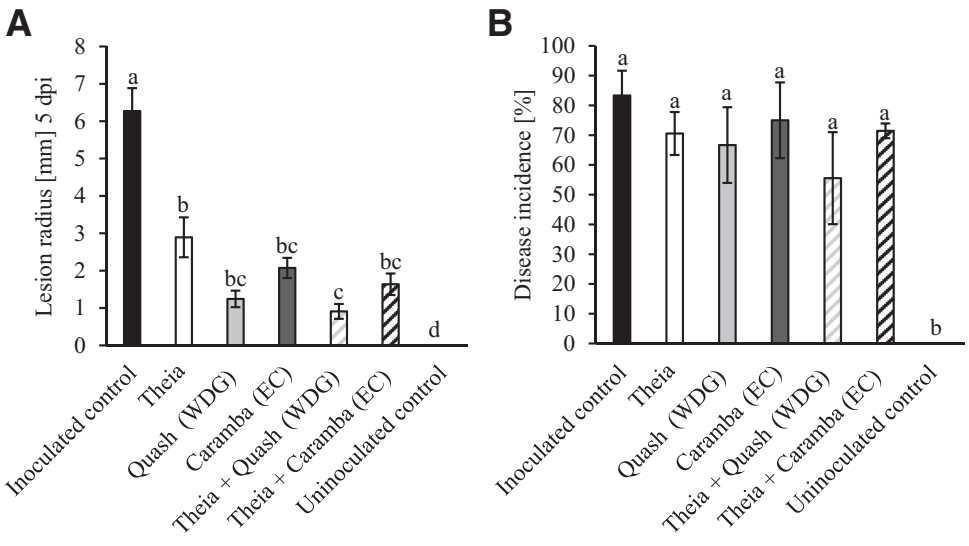

Fig. 7. A, Lesion radius (mm) of *Botrytis cinerea* and **B,** disease incidence on cherries 5 days postinoculation (dpi; means of three independent experimental replicates ± standard error). For Quash (water-dispersible granule [WDG]) and Caramba (emulsifiable concentrate [EC]), the final concentration of metconazole was 50 µg/ml. Different letters indicate significant differences between treatments (Tukey, $P < 0.05$).

ited bacterial growth more strongly than WP or WDG formulations. For example, at 50 μg/ml of propiconazole, the *B. subtilis* cell growth was faster for Mentor (WP; OD_{600} value of 0.45 at 24 h) compared with PropiStar (EC; OD_{600} value of 0.35 at 24 h). The difference at that concentration was even more pronounced for metconazole. At 50 μg/ml, the cell growth of *B. subtilis* was completely arrested for Caramba (EC), whereas the cell growth for Quash (WDG) was only reduced at 24 h but completely recovered at 48 h. The exact composition of the fungicide formulations is usually not disclosed by the chemical companies, which makes an explanation of the observed results difficult. EC formulations are oil-based, and studies have investigated the influence of oils on *Bacillus* spp. It has been reported that essential plant oils altered membrane and cell wall properties in *B. subtilis*, increasing membrane permeability (Bouyahya et al. 2019; Zhu et al. 2020). It has also been shown that essential oils have an inhibitory effect on both the vegetative cell growth and spore germination of *B. subtilis* (Ribes et al. 2021; Voundi et al. 2015). Non-ionic surfactants are also added to most EC formulations (Knowles 2008; Oliver and Beckerman 2022), which could further facilitate the penetration of the active ingredient into the bacterial cell or spore. This could be a possible explanation for the delayed vegetative cell growth by EC formulations. The growth pattern between the technical grade of both DMI fungicides and WP or WDG formulation was largely similar, and the solvent used for the technical-grade fungicides did not impact cell growth. This reinforces the hypothesis discussed above and points toward a stronger inhibition of cell growth by oil-based EC formulations. Perhaps contributing to the better compatibility of *B. subtilis* cells with WP or WDG formulations are inert ingredients commonly found in such formulations. WP formulations contain 20 to 80% finely ground minerals such as bentonite, talc, or kaolinite to improve the fungicide durability (Backman 1978). These could provide additional nutrients that can be utilized by the bacteria, promoting earlier onset of vegetative cell growth. Granules such as WDGs are also known to be some of the few formulations that slow down the release of the active ingredient (Backman 1978). This lower release of the active ingredient may also contribute to the different formulation-dependent levels of inhibition of *B. subtilis* vegetative cell growth.

The observed differences between fungicide formulations on the vegetative cell growth of *B. subtilis* were also reflected in our detached fruit studies. In all three detached fruit experiments, the combination of Theia with either the WP (propiconazole) or the WDG (metconazole) formulation showed superior control effects compared with Theia plus EC formulations. In the experiments with *C. siamense* and *Botrytis cinerea* on cherry, the Theia plus Caramba EC formulations had stronger antagonistic effects compared with the WDG formulation. There were even clearer differences in the experiment with *M. fructicola* on apple, showing a synergistic effect only for the combination of Theia and Mentor (WP) but not for Theia and PropiStar (EC). This indicates a correlation between uninhibited *B. subtilis* cell growth in the early stages of interaction with the fungicides and the formulation-dependent response in detached fruit studies. Whether 50 μg/ml is the optimal concentration to achieve synergy is unknown. The optimal concentration may be greater than 50 μg/ml but will be less than 100 μg/ml for both propiconazole and metconazole concentrations. It is also possible that a concentration less than 50 μg/ml of a DMI fungicide would lead to similar beneficial effects in disease control if the interaction was solely due to the BCA gaining a competitive advantage over the pathogen due to pathogen development suppression.

In conclusion, DMI fungicides can inhibit the cell growth of BCAs such as *B. subtilis*, and this negative interaction should be considered when attempting to use these fungicides in tank mixtures or alternations for disease management. Mixtures of BCAs and DMIs at concentrations that do not suppress cell growth are an interesting approach that may be explored for disease management of fruit crops.

Literature Cited

AgBiome. 2023. A full-spectrum approach to crop protection. https://agbiome.com/crop-protection/theia/

Ambrico, A., and Trupo, M. 2017. Efficacy of cell free supernatant from *Bacillus subtilis* ET-1, an Iturin A producer strain, on biocontrol of green and gray mold. Postharvest Biol. Technol. 134:5-10.

Anand, T., Chandrasekaran, A., Kuttalam, S., Senthilraja, G., and Samiyappan, R. 2010. Integrated control of fruit rot and powdery mildew of chilli using the biocontrol agent *Pseudomonas fluorescens* and a chemical fungicide. Biol. Control 52:1-7.

Backman, P. A. 1978. Fungicide formulation: Relationship to biological activity. Annu. Rev. Phytopathol. 16:211-237.

Beckerman, J., Palmer, C., Tedford, E., and Ypema, H. 2023. Fifty years of fungicide development, deployment, and future use. Phytopathology 113:694-706.

Bellamy, S., Xu, X., and Shaw, M. 2021. Biocontrol agents to manage brown rot disease on cherry. Eur. J. Plant Pathol. 161:493-502.

Bonterra, A., Badosa, E., Daranas, N., Francés, J., Roselló, G., and Montesinos, E. 2022. Bacteria as biological control agents of plant diseases. Microorganisms 10:1759.

Bouyahya, A., Abrini, J., Dakka, N., and Bakri, Y. 2019. Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. J. Pharm. Anal. 9:301-311.

Brannen, P. M., and Kenney, D. S. 1997. Kodiak®—A successful biological-control product for suppression of soil-borne plant pathogens of cotton. J. Ind. Microbiol. Biotechnol. 19:169-171.

Breeden, S., Sanders, W., Rios, T., Cheh, A., and Brannen, P. M. 2021. Evaluation of copper and biological treatments for control of bacterial spot of peach in Georgia 2021. Plant Dis. Manag. Rep. 16:PF029.

California Department of Pesticide Regulation. 2023. Sustainable pest management: A roadmap for California. https://www.cdpr.ca.gov/docs/sustainable_pest_management_roadmap/spm_roadmap.pdf

Chen, K., Tian, Z., He, H., Long, C.-a., and Jiang, F. 2020. *Bacillus* species as potential biocontrol agents against citrus diseases. Biol. Control 151:104419.

Cline, W. O. 2022. Evaluation of organic fungicides for control of fruit rots on blueberry in North Carolina 2022. Plant Dis. Manag. Rep. 17:PF008.

Colby, S. R. 1967. Calculating synergistic and antagonistic responses of herbicide combinations. Weeds 15:20-22.

Gilardi, G., Manker, D. C., Garibaldi, A., and Gullino, M. L. 2008. Efficacy of the biocontrol agents *Bacillus subtilis* and *Ampelomyces quisqualis* applied in combination with fungicides against powdery mildew of zucchini. J. Plant Dis. Prot. 115:208-213.

Fernández-Ortuño, D., Grabke, A., Li, X., and Schnabel, G. 2015. Independent emergence of resistance to seven chemical classes of fungicides in *Botrytis cinerea*. Phytopathology 105:424-432.

Gura, W. P., Gelain, J., Sikora, E. J., Vinson, E. L., Brannen, P. M., and Schnabel, G. 2024. Re-evaluation of sensitivity of *Monilinia fructicola* isolates to the DMI fungicide propiconazole in the Southeastern United States and investigation of the genetic element Mona. Plant Dis. 108:375-381.

Hu, M.-J., Grabke, A., Dowling, M. E., Holstein, H. J., and Schnabel, G. 2015. Resistance in *Colletotrichum siamense* from peach and blueberry to thiophanate-methyl and azoxystrobin. Plant Dis. 99:806-814.

Ji, X., Li, J., Meng, Z., Zhang, S., Dong, B., and Qiao, K. 2019. Synergistic effect of combined application of a new fungicide fluopimomide with a biocontrol agent *Bacillus methylotrophicus* TA-1 for management of gray mold in tomato. Plant Dis. 103:1991-1997.

Knowles, A. 2008. Recent developments of safer formulations of agrochemicals. Environmentalist 28:35-44.

Kondoh, M., Hirai, M., and Shoda, M. 2001. Integrated biological and chemical control of damping-off caused by *Rhizoctonia solani* using *Bacillus subtilis* RB14-C and flutolanil. J. Biosci. Bioeng. 91:173-177.

Leelasuphakul, W., Hemmanee, P., and Chuenchitt, S. 2008. Growth inhibitory properties of *Bacillus subtilis* and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Postharvest Biol. Technol. 48:113-121.

Liu, L., Liang, M., Li, L., Sun, L., Xu, Y., Gao, J., Wang, L., Hou, Y., and Huang, S. 2018. Synergistic effects of the combined application of *Bacillus subtilis* H158 and strobilurins for rice sheath blight control. Biol. Control 117:182-187.

Liu, L., Zhao, K., Cai, L., Zhang, Y., Fu, Q., and Huang, S. 2023. Combination effects of tebuconazole with *Bacillus subtilis* to control rice false smut and the related synergistic mechanism. Pest Manag. Sci. 79:234-243.

Marin, M. V., Baggio, J. S., Martin, R., and Peres, N. A. 2022. Evaluation of products for the control of *Phytophthora* crown rot in annual strawberry, 2021-2022. Plant Dis. Manag. Rep. 16:PF053.

McKenney, P. T., Driks, A., and Eichenberger, P. 2013. The *Bacillus subtilis* endospore: Assembly and functions of the multilayered coat. Nat. Rev. Microbiol. 11:33-44.

Nicholson, W. L. 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. 59:410-416.
Oliver, R. P., and Beckerman, J. L. 2022. Fungicides in Practice. CABI Digital Library. https://doi.org/10.1079/9781789246926.0007
Ongena, M., and Jacques, P. 2008. *Bacillus* lipopeptides: Versatile weapons for plant disease biocontrol. Trends Microbiol. 16:115-125.
Ons, L., Bylemans, D., Thevissen, K., and Cammue, B. P. A. 2020. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. Microorganisms 8:1930.
Peng, D., Li, S., Chen, C., and Zhou, M. 2014. Combined application of *Bacillus subtilis* NJ-18 with fungicides for control of sharp eyespot of wheat. Biol. Control 70:28-34.
Raaijmakers, J. M., De Bruijn, I., Nybroe, O., and Ongena, M. 2010. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: More than surfactants and antibiotics. FEMS Microbiol. Rev. 34:1037-1062.
Ribes, S., Ruiz-Rico, M., and Barat, J. M. 2021. Efficient reduction in vegetative cells and spores of *Bacillus subtilis* by essential oil components-coated silica filtering materials. J. Food Sci. 86:2590-2603.
Setlow, P. 2006. Spores of *Bacillus subtilis*: Their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101:514-525.
Vijay, K. K. K., Reddy, M. S., Kloepper, J. W., Yellareddygari, S. K. R., Lawerence, K. S., Zhou, X. G., Sudini, H., Miller, M. E., Appa, R. P., Surendranatha, R. E. C., Niranjana, S. R., and Chandra, N. S. 2011. Plant growth-promoting activities of *Bacillus subtilis* MBI 600 (Integral®) and its compatibility with commonly used fungicides in rice sheath blight management. Int. J. Microbiol. Res. 3:120-130.
Voundi, S. O., Nyegue, M., Lazar, I., Raducanu, D., Ndoye, F. F., Stamate, M., and Etoa, F.-X. 2015. Effect of essential oils on germination and growth of some pathogenic and spoilage spore-forming bacteria. Foodborne Pathog. Dis. 12:551-559.
Xu, X., Wang, Y., Lei, T., Sohail, M. A., Wang, J., and Wang, H. 2022. Synergistic effects of *Bacillus amyloliquefaciens* STDB009 and difenoconazole on Fusarium wilt of tomato. Plant Dis. 106:2165-2171.
Yadav, U., Bano, N. Bag, S., Srivastava, S., and Singh, P. C. 2022. An insight into endophytic bacterial community of tomato after spray application of propiconazole and *Bacillus subtilis* strain NBRI-W9. Microbiol. Spectr. 10:e01186-22.
Zhu, H.-l., Chen, G., Chen, S.-n., Wang, R.-q., Chen, L., Xue, H., and Jian, S.-p. 2020. Changes in cell membrane properties and phospholipid fatty acids of *Bacillus subtilis* induced by polyphenolic extract of *Sanguisorba officinalis* L. J. Food Sci. 85:2164-2170.