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Floral Microbes Suppress Growth of *Monilinia laxa* with Minimal Effects on Honey Bee Feeding

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Abstract

Management of *Monilinia laxa*, the causal agent of brown rot blossom blight in almond (*Prunus dulcis*), relies heavily on the use of chemical fungicides during bloom. However, chemical fungicides can have nontarget effects on beneficial arthropods, including pollinators, and select for resistance in the pathogen of concern. Almond yield is heavily reliant on successful pollination by healthy honey bees (*Apis mellifera*); thus, identifying sustainable, effective, and pollinator-friendly control methods for blossom blight during bloom is desirable. Flower-inhabiting microbes could provide a natural, sustainable form of biocontrol for *M. laxa*, while potentially minimizing costly nontarget effects on almond pollinators and the services they provide. As pollinators are sensitive to floral microbes and their associated taste and scent cues, assessing effects of prospective biocontrol species on pollinator attraction is also necessary. Here, our objective was to isolate and identify potential biocontrol microbes from an array of agricultural and natural flowering hosts and test their efficacy in suppressing *M. laxa* growth in culture. Out of an initial 287 bacterial and fungal isolates identified, 56 were screened using a dual culture plate assay. Most strains reduced *M. laxa* growth in vitro. Ten particularly effective candidate microbes were further screened for their effect on honey bee feeding. Of the 10, nine were found to both strongly suppress *M. laxa* growth in culture and not reduce honey bee feeding. These promising results suggest a number of strong candidates for augmentative microbial biocontrol of brown rot blossom blight in almond with potentially minimal effects on honey bee pollination.

Keywords: biocontrol, *Monilinia laxa*, necrotrophic pathogen, pollinator, *Prunus dulcis*

Monilinia spp. are necrotrophic fungal pathogens that result in pre- and postharvest brown rot in a wide range of stone fruit crops globally. *Monilinia laxa*, the causal agent of brown rot blossom blight, causes severe damage to untreated almond (*Prunus dulcis*) orchards in California. Infection occurs at bloom, when the highly susceptible stigma, anthers, and petals become exposed as the flowers open. Once infection occurs, the fungus will grow into the floral tube, peduncle, and eventually, the shoot of the tree (Martini and Mari 2014). Brown rot causes infected blossoms to turn dark brown and wilt, and can be accompanied by fruit rot and reduced crop yields. When the fungus reaches the shoot of the tree, a canker can form that provides an overwintering site and source of secondary inoculum the following year, as spores are released and subsequently transferred to flowers and other tissues through wind or rain events (Martini and Mari 2014).

Management strategies for control of *M. laxa* (hereafter, *Monilinia*) rely heavily on use of chemical fungicides that primarily act as demethylation (sterol) or quinone outside inhibitors (Haviland et al. 2021). The typical spray regimen practiced during almond bloom is one fungicidal application at full bloom during years with low rainfall or two fungicidal applications, at pink bud (5 to 10% bloom) and full bloom, when *Monilinia* risk factors are high, i.e., a wet spring or severe infections during the previous growing season (Haviland et al. 2021). In addition, 77% of the world-market for fungicides consists of six classes with single-target–site activity. The heavy use of fungicides with limited modes of action in pest management practices can lead to increased resistance in pathogen strains. Resistance to these major classes of fungicides has already been observed across several major crop pathogens (Fones et al. 2020). *Monilinia* isolates exhibiting varying levels of resistance to fungicides have been found in field tests of almond and other stone fruits (Egüen et al. 2016; Ma et al. 2005; Malandrakis et al. 2012; Ogawa et al. 1984).

Not only can pathogens develop resistance to fungicides, but fungicide application can also negatively affect the health of farm workers, pesticide applicators, consumers, and the environment (Oruc 2010). For example, there is growing evidence that fungicide use has negative effects on commercial and natural pollinator populations, upon which almond production is heavily reliant. Fungicide exposure has been shown to alter the composition of fungi associated with bee bread in honey bees (Yoder et al. 2013), disrupt larval or pupal development, and ultimately reduce colony size (Mussen et al. 2004; Steffan et al. 2017). Fungicides can also act in synergy with other pesticides, increasing toxicity toward pollinators as well as increase pollinator susceptibility to disease (Fisher et al. 2017; Iverson et al. 2019; Johnson et al. 2013; Pettis et al. 2013; Wade et al. 2019). These concerns have led to a growing interest in more sustainable forms of pathogen management, including microbial biocontrol.

Microbial biocontrol involves the use of endophytic and/or epiphytic microorganisms to enhance plant resistance to diseases (Busby et al. 2016; De Silva et al. 2019). Potential biocontrol agents can act to reduce damage from plant pathogens through a variety of mechanisms, including resource competition, antimicrobial production, mycoparasitism, and induction of plant defense systems (De Silva et al. 2019; Köhl et al. 2019; Latz et al. 2018). Microbial biocontrol

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can be used both pre- and postharvest to combat pathogens and contaminants that can have detrimental effects in crop production, thereby reducing reliance on chemical pesticides. In addition, some microbial biocontrol agents have the potential to provide benefits to the plants and increase crop success (Sellitto et al. 2021). Biocontrol studies of *Erwinia amylovora*, causal agent of fire blight in pome fruits, suggest that preharvest application of biocontrol agents to blossoms during early bloom provides the best opportunity for control of *E. amylovora* and other floral pathogens (Johnson and Stockwell 1998). As antagonistic microbes colonize susceptible plant tissues, their establishment and competition for nutrients can prevent pathogen invasion, an important primary mechanism of combatting necrotrophic pathogens (Busby et al. 2016). Similar biocontrol methods have also been used to combat diseases caused by other *Monilinia* species. For example, *Bacillus subtilis* and *Pantoea agglomerans* have been shown to suppress mummy berry disease in blueberries, caused by *Monilinia vaccinii-corymbosi* (Ngugi et al. 2005; Scherm et al. 2004; Thornton et al. 2008). Successful use of antagonists in pome and blueberry flowers suggests that biocontrol microbes should be examined for control of *Monilinia* in almond blossoms.

Despite the potential for pathogen suppression, application of biocontrol agents during bloom has the potential to impact pollination services, as microbes have been shown to influence pollinator foraging behaviors (Vannette 2020). Honey bees, the primary pollinators of almond orchards, can be deterred from visiting food sources by the growth of some, but not all, microbial species in nectar (Good et al. 2014; Rering et al. 2018, 2020). The behavior of other floral visitors, including bumble bees which also pollinate almonds, can be affected by microbial colonization of flowers (Herrera et al. 2013; Junker et al. 2014; Russell and Ashman 2019; Schaeffer et al. 2019). Behavioral responses are driven in part by chemical cues emitted from plants and/or microbes and can vary based on sensory modality (i.e., gustatory versus olfactory; Schaeffer et al. 2019; Sobhy et al. 2018) or context (Schaeffer et al. 2020). Assessing the impact of microbe presence on flower attractiveness to pollinators is therefore vital for understanding how application of biocontrol agents affects pollinator systems; however, effects on pollinator foraging preferences are rarely examined.

To test the hypothesis that flower-inhabiting microbes can act as a form of sustainable biocontrol against the causal agent of brown rot blossom blight, our objectives were to isolate and identify potential biocontrol agents from natural and agricultural flower populations, test candidate isolates for efficacy in suppressing *Monilinia* growth in culture, and screen candidate biocontrol agents for their impacts on pollinator (honey bee) behavior and potential attractiveness using a laboratory assay. Here, we identify microbial species that reduce the growth of *Monilinia* with no reduction in honey bee feeding preference.

Materials and Methods

Isolation and identification of flower-inhabiting microbes. Almond flowers were collected during the spring of 2016 from 15 almond orchards distributed across multiple counties of the San Joaquín Valley of California, including orchards managed using conventional and organic practices. Flower tissues (e.g., anther, stamen, petals) were dissected under clean laboratory conditions, plated on potato dextrose agar (PDA) medium, and maintained at 25°C for 5 to 10 days. Fungal and bacterial colonies were transferred to new PDA plates to obtain pure cultures and conserved in sterile distilled water. A representative *M. laxa* isolate was obtained from flowers exhibiting blossom rot symptoms. Additional agricultural isolates of potential antagonists were collected from apple and pear orchards in Yolo and Solano counties in California. To increase the diversity of microbial isolates tested, and address whether agriculturally isolated microbes may act as better suppressors of *Monilinia* than novel, naturally occurring microbial isolates were also collected from the native plant species *Epilobium canum* (Onagraceae) and *Mimulus aurantiacus* (Phyrmaceae) as well as from floral visitors including honey bees and bumble bees in Yolo and Solano counties in California. Microbial isolation from apple, pear, and native flowers used the same process of flower dissection used in the collections from almond blossoms above, but plated on yeast media agar, Reasoner's Agar supplemented with 20% sucrose, and Luria-Bertani (LB) agar to isolate fungi and bacteria, respectively. Additional strains that colonize plant surfaces or flower-visiting insects were also sourced from the Phaff Yeast Culture Collection (https://phaffcollection.ucdavis.edu/; Table 1).

A subset of the microbial strains isolated from almond, which were morphologically identified as similar to known biological control agents, were selected for subsequent *in situ* analysis and sequencing via amplification of a conserved rRNA region by PCR. Additionally, all isolates from nonagricultural flowers and apple and pear orchards were sequenced for species identification. For fungi, the internal transcribed spacer (ITS) region was amplified and sequenced using primers ITS1 and ITS4 (Mitchell et al. 1994). Bacterial 16S regions were amplified using the 27F and 907R or 1492R primer pair (Lane et al. 1985; Mao et al. 2012). In short, microbial isolates were suspended in PCR grade water and 1 µl of microbial suspension was placed into a PCR tube containing Choice Taq Blue Mastermix (1.5 mM $MgCl_2$, 2×), primers for the associated target (as listed above), and nuclease-free PCR-grade water. The PCR reactions were run in a thermocycler (Bio-Rad, Berkeley, CA) and programmed according to isolation target. PCR of bacterial isolates was programmed for initial denaturation at 95.0°C for 3 min, followed by 30 amplification cycles including denaturation at 95.0°C for 30 s, annealing of primers at 51.0°C for 30 s, extension at 72.0°C for 2 min, and ending with a final extension at 72.0°C for 10 min. PCR of yeast isolates was programmed for initial denaturation at 94.0°C for 3 min, followed by a touchdown procedure with 10 cycles consisting of denaturation at 94.0°C for 30 s, annealing of primers at 56.5°C for 30 s (decreasing in 0.5°C increments from 56.5 to 51.5°C), and elongation at 72.0°C for 45 s, followed by 20 cycles of amplification with denaturation at 94.0°C for 30 s, annealing at 51.5°C for 45 s, and extension at 72.0°C for 45 s, with a final extension at 72.0°C for 10 min. Finally, PCR of filamentous fungal isolates was programmed for initial denaturation at 94.0°C for 1 min, followed by 35 cycles of amplification with denaturation at 94.0°C for 30 s, primer annealing at 51.0°C for 20 s, and extension at 72.0°C for 1 min, followed by a final extension at 72.0°C for 8 min. All PCR products were held at 4°C before sequencing and storage at –20°C. Amplicons were sequenced using Sanger sequencing and BLAST searches were used to identify isolates (Supplementary Table S1). Strains for subsequent experiments were chosen to maximize the taxonomic diversity typically found on flower surfaces and in nectar.

Antagonist activity. A subset (56) of the identified microbial isolates (Table 1) from 15 different genera were selected to be tested for antagonistic activity. Multiple isolates were chosen from the *Aureobasidium*, *Bacillus*, *Epicoccum*, and *Penicillium* genera, based on their prevalence on almond trees and their use as biocontrol agents in other crop species (De Cal et al. 2009; Fan et al. 2017; Guijarro et al. 2019; Holb and Kunz 2013; Madrigal et al. 1994; Mari et al. 2007, 2012; Pusey et al. 1988). Additional isolates were chosen to increase genera diversity and have relatively equivalent distribution between agricultural and natural isolates. These included microbes isolated from the stigma of blossoms, the initial site of *Monilinia* infection (Martini and Mari 2014), as well as from common insect visitors to almond blossoms, which would be hypothesized to have limited nontarget effects on visiting pollinators. Antagonistic activity of selected bacteria and fungi (Table 1) was tested using a dual culture technique in Petri dishes filled with PDA medium. To establish the pathogen, a mycelial plug (5 mm in diameter) of *M. laxa* was placed in the center of a 90-mm Petri dish, then two plugs of each putative antagonist were placed opposite of each other, such that there was 30 mm between each antagonist plug and the pathogen. Plates inoculated with only the pathogen served as controls. Filamentous fungi, including *M. laxa*, were allowed to grow for 8 to 12 days before use in the assay to ensure active growth. Bacteria and yeast isolates were allowed to grow overnight and then suspended in a phosphate-buffered saline solution and replated as "lawns" that were allowed to grow for 24 to 48 h before plugs were taken for use in the assay. Dual culture assays were incubated at 25°C for up to 14 days. All species were replicated a minimum of four times, with higher replicates performed for species with variable growth (Table 1).

After incubation, plates were photographed and *Monilinia* growth area was measured in both control and experimental dishes using the ImageJ program FIJI (Schindelin et al. 2012). Percent *Monilinia* growth inhibition caused by each candidate antagonist was calculated by dividing the difference between the treatment growth area and the average control growth area by the average growth area in the paired control treatment. Additionally, for each experimental dish the inhibition halo was measured by subtracting the diameter of the *Monilinia* culture from the total distance between the two antagonist cultures.

Pollinator feeding preference. The effect of candidate microbes on honey bee feeding behavior was assessed using a capillary feeder (CAFÉ) assay (Supplementary Fig. S1; Reade et al. 2016). Ten candidate isolates were chosen from the seven species that resulted in >50% *Monilinia* growth reduction, with high performing isolates (e.g., Ba114) selected for species in which more than one isolate was tested and at least one isolate selected from each species to increase species level representation when examining effects of pollinator preference. Microbial suspensions (optical density [OD] = 0.1) were prepared in a 30% sucrose solution with the exception of *Epicoccum nigrum* isolate KARE781, for which a mycelial plug was placed in the sucrose solution. We chose to use a 30% sucrose solution, which represents a higher sugar concentration than most almond nectar, to maximize honey bee feeding attempts in this lab assay. Suspensions were incubated at 25°C for 48 to 72 h before use in the CAFÉ assay. Honey bee foragers were collected during flight as they returned to hives at the UC Davis Laidlaw Honey Bee Research Facility (Davis, CA). Individual bees were restrained in microcentrifuge tubes (Supplementary Fig. S1a), fed ad libitum with a 30% sucrose solution, and starved overnight for 16 h in the dark at 25°C. Starved bees were tested for a proboscis extension response to the 30% solution before being used in the assay by tapping the antenna with the feeding solution, so that only bees that responded to the stimuli were used in the behavioral assays. Honey bees were then placed in vials (diameter = 26 mm; height = 50 mm) with perforated lids to allow for flow of oxygen. Two 100-µl capillary tubes, which were bent at ~25 and 30 mm to create a U-shape with another bend at 55 mm, were placed into holes in the bottom of the vial spaced 12 mm apart and equidistant from the edge of the vial (Supplementary Fig. S1b). One capillary tube contained a control 30% sucrose solution, while the other contained the microbial suspension. Honey bees were allowed to feed for 3 h and the volume of sugar solution consumed was measured every half-hour, with feeding solutions refilled as needed. Evaporation controls were set up in the same manner, except that they lacked introduction of a honey bee. This allowed us to account for potential reductions in volume over the course of the 3-h assay stemming from evaporation. Trials were run until there was a minimum of 10 replicate bees for each microbe (n = 10 to 14). Extra replicates were set up to account for potential loss of replicates from bees dying during experiment, failing to feed, or bodily contact with the capillary tube leading to reduction in volume not associated with feeding. Any replicate that had the same or less combined feeding solution loss as the evaporation control (resulting from bee death, consistent lethargy, or other conditions as described above) was excluded from analysis. Treatment orientation and filling order (control versus treatment) were randomized for each trial and also recorded, but neither variable affected honey bee consumption ($P >$ 0.05), and therefore were excluded from final analysis.

Statistical analysis. All statistical analyses were performed in the program suite R Studio using R v.3.6.2 (R Core Team 2019).

Antagonist activity. To determine if any factors (i.e., microbial kingdom, microbial source [i.e., agricultural versus natural], genus,

Table 1. Candidate biocontrol agents tested for antagonist effect against *Monilinia laxa*, the causal agent of brown rot blossom blight in almond[a]

Genus	Species[b]	Kingdom	Strain ID(s)	Isolation category	Isolation source
Acinetobacter	*nectaris* (n = 12)	Bacteria	EC_033	Natural	*Epilobium canum*
Aureobasidium	*pullulans* (n = 5)	Fungi	A17, A23, A27, A33, A51	Agricultural	*Prunus dulcis*
	Unknown (n = 7)	Fungi	A1, A7, A13, A37, A39, A41, A45	Agricultural	*P. dulcis*
Bacillus	*subtilis* (n = 15)[c]	Bacteria	Ba76, Ba95, Ba114, Ba123, Ba125	Agricultural	*P. dulcis*
Candida	*bombi* (n = 17)	Fungi	**UCDFST 02-329**	Natural	*Apis mellifera*
	magnoliae (n = 17)	Fungi	**UCDFST 02-257**	Natural	*A. mellifera*
	rancensis (n = 17)	Fungi	**UCDFST 02-252**	Natural	*A. mellifera*
Cryptococcus	*carnescens* (n = 16)	Fungi	EC_072	Natural	*E. canum*
	victoriae (n = 19)	Fungi	Muller_Con_Int_N_Y1	Agricultural	*P. dulcis*
Epicoccum	*nigrum* (n = 48)[c]	Fungi	KARE627, KARE661, KARE661R, KARE685, KARE702, KARE721, KARE723, KARE758, KARE758R, KARE768, KARE769, KARE780, KARE781, KARE802, KARE806, KARE839	Agricultural	*P. dulcis*
Hanseniaspora	*uvarum* (n = 15)	Fungi	Asa_Int_AS_Y1	Agricultural	*P. dulcis*
Lachancea	*thermotolerans* (n = 10)	Fungi	**UCDFST 68-140**	Natural	Apidae
Metschnikowia	*koreensis* (n = 14)	Fungi	EC_061	Natural	*E. canum*
	pulcherrima (n = 14)	Fungi	Asa_Edge_AS_Y2	Agricultural	*P. dulcis*
	reukauffii (n = 14)	Fungi	EC_052	Natural	*E. canum*
Meyerozyma	*guilliermondii* (n = 10)	Fungi	**UCDFST 02-286**	Natural	*A. mellifera*
Neokomagataea	*thailandica* (n = 16)	Bacteria	EC_112	Natural	*E. canum*
Penicillium	*brevicompactum* (n = 4)	Fungi	KARE755	Agricultural	*P. dulcis*
	chloroleucon (n = 4)	Fungi	EC_084	Natural	*E. canum*
	polonicum (n = 8)[d]	Fungi	KARE754 (M1), KARE754 (M2)	Agricultural	*P. dulcis*
Pseudomonas	*fluorescens* (n = 16)	Bacteria	P8_P_B3	Agricultural	*Pyrus communis*
	graminis (n = 10)	Bacteria	P2_P_B2	Agricultural	*P. communis*
	migulae (n = 12)	Bacteria	P8_P_B1	Agricultural	*P. communis*
	veronii (n = 10)	Bacteria	MAV_P_B1	Agricultural	*Malus* × *domestica*
Starmerella	*bombicola* (n = 10)	Fungi	**UCDFST 02-305**	Natural	*A. mellifera*
Zygosaccharomyces	*rouxii* (n = 13)	Fungi	**UCDFST 68-105**	Natural	*A. mellifera*

[a] Candidates are a subset of all microbial strains identified in preliminary isolation efforts. Strain IDs in bold were sourced from the Phaff Yeast Culture Collection at UC Davis (UCDFST).
[b] More replicates were performed on strains that exhibited variable levels of growth.
[c] Each *B. subtilis* (5) and *E. nigrum* (13) strain was replicated three times and overall effects across all strains as well as effects of individual strains on *Monilinia* growth were examined.
[d] Each *P. polonicum* (2) strain was replicated four times and overall effects across both strains on *Monilinia* growth were examined.

species, and experimenter) had a significant effect on *Monilinia* growth inhibition, a stepwise linear regression was used for model selection (function 'stepAIC' in the software package "MASS"; Venables and Ripley 2002). After species was found to be the only significant factor, the stepwise linear regression was followed by a linear regression model with species as the explanatory variable (function 'lm' in the software package "stats"; R Core Team 2019). To assess which species significantly reduced *Monilinia* growth, the effect of each species was assessed by comparing *Monilinia* growth area in dual culture assays to control growth area using a *t* test followed by a Bonferroni corrections to correct for multiple testing (function 'p.adjust' in the program package "stats").

For each species, we assessed if inhibition halo measurements were significantly greater than zero using a one-tailed *t* test (function 't.test' in the program package "stats"). Bonferroni corrections were applied to correct for multiple comparisons.

For species in which multiple isolates were replicated (*B. subtilis* and *E. nigrum*), among-isolate variation in percent growth inhibition and inhibition halo measurements was examined using one-way analysis of variance followed by a posthoc Tukey-Honest Significant Difference test (functions 'aov' and 'TukeyHSD', in the program package "stats").

Pollinator feeding preference. To assess if microbial candidates affected honey bee consumption of a 30% feeding solution, the effect of microbial presence was assessed by comparing consumption of the microbial suspension to the control sucrose solution using a paired Student's *t*-test. Bonferroni corrections were applied to correct for multiple testing. To examine if there was a significant difference in overall consumption in the CAFÉ assay across microbial candidates, a one-way analysis of variance was used followed by a posthoc Tukey honestly significant difference test.

Results

Isolation and identification of flower-inhabiting microbes. A total of 287 microorganism isolates were collected from agricultural and natural flowering populations. Microbial isolates isolated from almond flowers included 88 *E. nigrum*, 57 *Aureobasidium*, three *Penicillium*, three *Cryptococcus* (*Vishniacozyma*), one *Hanseniaspora*, one *Metschnikowia*, and 73 putative *Bacillus* isolates. Additional agricultural isolates were collected from apple and pear orchards and included isolates from the genera *Acinetobacter*, *Aureobasidium*, *Bacillus*, *Cryptococcus* (*Vishniacozyma*), *Erwinia*, *Metschnikowia*, *Meyerozyma*, *Pantoea*, *Penicillium*, *Pseudomonas*, and *Rhodotorula*. Fungal genera represented by natural microbial isolates included single isolates of *Aspergillus*, *Aureobasidium*, *Candida*, *Cladosporium*, *Cryptococcus*, *Debaryomyces*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Naganishia*, *Penicillium*, *Starmerella*, and *Zygosaccharomyces*, while bacteria were represented by the genera *Acinetobacter*, *Bacillus*, *Micrococcus*, *Neokomagataea*, and *Rosenbergiella*.

Antagonist activity. *Percent growth inhibition.* The majority of isolates tested (Table 1), from both natural and agricultural flower populations, reduced the growth of *Monilinia* on plates but varied substantially in the extent of suppression (Fig. 1; Supplementary Fig. S2). A linear regression revealed that microbial species ($F_{25,338}$ = 18.36, P < 0.0001) differed in their effects on *Monilinia* percent colony growth, while other factors such as genera and isolation source did not significantly contribute to variation in growth. Sixteen species (61.5% of total tested) significantly reduced *Monilinia* growth. Species from both agricultural and natural sources within the genera *Metschnikowia* and *Penicillium* significantly reduced *Monilinia* growth (Table 1; Fig. 1). Notably, within some microbial genera, species varied to a great extent in percent growth inhibition (e.g., *Pseudomonas*), whereas in others (e.g., *Cryptococcus* and *Penicillium*), microbial effects on *Monilinia* growth were similar across species (Fig. 1).

Inhibition halo. In contrast to percent growth reduction, only three species (11.5% of total tested) produced significant inhibition halos (Fig. 2). The largest inhibition halos averaged over 1 cm and were produced by *E. nigrum* and *B. subtilis* isolates (Fig. 2; Supplementary Fig. S3). Of these, only the *Penicillium* species isolated from a nonagricultural source created significant inhibition halos. Notably, some of the isolates that were most effective at reducing colony growth on plates, including *Pseudomonas veronii* and *E. nigrum* isolates KARE768 and KARE769, did not produce inhibition halos (Figs. 1 and 2; Supplementary Figs. S2 and S3).

Variation within species. Isolates of *E. nigrum* varied in effects on *M. laxa* colony growth inhibition (Supplementary Fig. S2) and

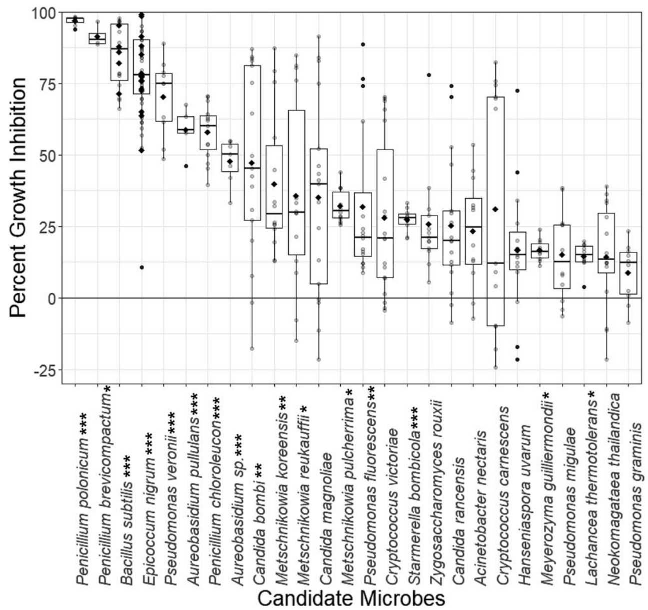

Fig. 1. Percentage of relative growth inhibition of *Monilinia laxa* imposed by candidate microbial species. Significant differences in *M. laxa* growth between control and dual culture plates were analyzed using a Student's *t*-test and adjusted for multiple testing using Bonferroni corrections (*P < 0.05, **P < 0.01, ***P < 0.001). The black diamond indicates the mean value for individual isolates within a species. All other points indicate individual replicates including multiple isolates tested within a species.

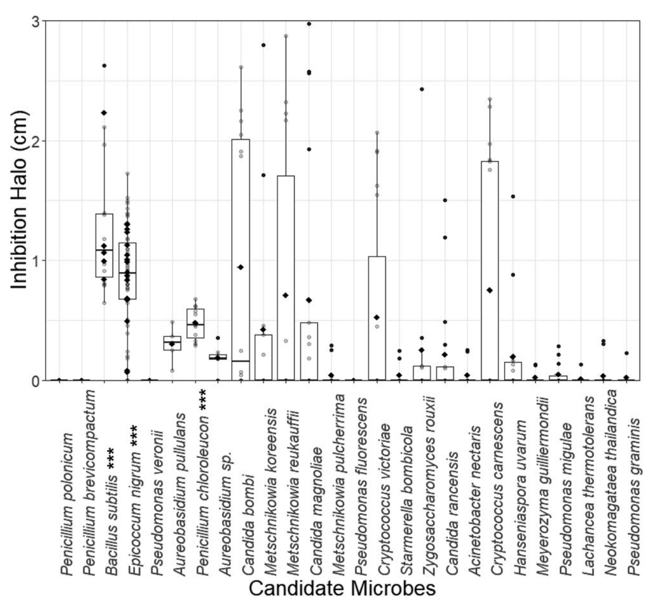

Fig. 2. Growth inhibition halo measurements of candidate microbial isolates against *Monilinia laxa* (microbe ordering consistent with Fig. 1). Significant differences in inhibition halo measurement from zero were analyzed using a Student's *t*-test and adjusted for multiple testing using Bonferroni corrections (*P < 0.05, **P < 0.01, ***P < 0.001). The black diamond indicates the mean value for individual isolates within a species. All other points indicate individual replicates including multiple isolates tested within a species.

isolates of both *E. nigrum* and *B. subtilis* varied in inhibition halo (Supplementary Fig. S3). Isolate Ba95 produced the largest inhibition halo, averaging a little over 2 cm, and was significantly different from the other *B. subtilis* isolates that all exhibited inhibition halos ~1 cm.

Pollinator feeding preference. Of the 10 candidate isolates tested, only *Aureobasidium pullulans* (A17) significantly affected honey bee feeding preference and elicited an aversive response to the microbe-containing feeding solution (Fig. 3). Additionally, no candidate microbes significantly increased or decreased overall feeding rates (Supplementary Fig. S4).

Discussion

Here, we identify flower-inhabiting microbes that have significant antagonistic effects on the almond pathogen *Monilinia* using in vitro assays. Of particular note are *B. subtilis* (Ba95) and *E. nigrum* (KARE661, KARE792, KARE780, and KARE781), which produced large inhibition halos, suggesting that these isolates may produce antimicrobial compounds that limit the growth of *Monilinia* (Supplementary Fig. S3). *B. subtilis* has been previously shown to produce a wide range of antimicrobial compounds that can be used in combatting plant pathogens (Caulier et al. 2019; Fan et al. 2017; Ongena and Jacques 2008), including Bacillomycin D, which has exhibited antifungal properties against *Aspergillus flavus* on corn crops (Gong et al. 2014). Additionally, the known ability of *B. subtilis* to produce biofilms makes it an excellent candidate for use in biological control (Branda et al. 2001). A 2017 review suggests that the ability to produce biofilms may make certain microbes more suitable for biocontrol as biofilms are stress-tolerant, adept at microbial communication, competitive for resources, capable of upregulating antimicrobial production, and can induce beneficial responses in plants in either defense or stimulated growth (Pandin et al. 2017). In vitro tests are often useful screening methods and can identify effective strains for further commercial development, but additional field trials will be required to confirm efficacy under realistic field conditions (Bouaichi et al. 2019; Gerami et al. 2013; Mercier and Lindow 2001).

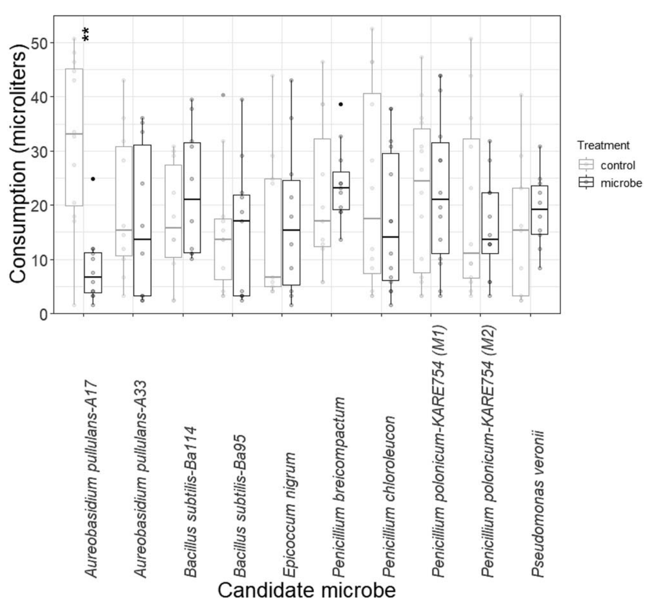

Fig. 3. Choice test between 30% sucrose control and 30% sucrose inoculated with microbial antagonist: *Aureobasidium pullulans* (A17 and A33), *Bacillus subtilis* (Ba95 and Ba114), *Penicillium chloroleucon* (EC84), *P. polonicum* (KARE754 [M1] and KARE754 [M2]), *P. brevicompactum* (KARE755), *Epicoccum nigrum* (KARE781), and *Pseudomonas veronii* (MAV_P_B1). Significant differences in feeding between control and microbe solutions were analyzed using a paired Student's *t*-test and adjusted for multiple testing using Bonferroni corrections (** $P < 0.01$).

While the strains outlined above significantly suppressed *Monilinia* growth and produced inhibition halos, some isolates only suppressed *Monilinia* growth (*P. veronii*: MAV_P_B1, and *E. nigrum*: KARE754(M1), KARE754(M2), KARE755, KARE768, and KARE769; Figs. 1 and 2; Supplementary Figs. S2 and S3). The lack of inhibition halos suggests that these strains may act as substrate competitors instead of directly suppressing *Monilinia* via antimicrobial production. Supporting either substrate or space competition, we observed that these antagonist strains exhibited faster growth and greater plate colonization than *Monilinia* in the dual culture assay. In previous work, *Pseudomonas* species have been shown to be effective competitors for limiting nutrients. Indeed the production of iron-sequestering siderophores can reduce the growth of several plant pathogens in *in vitro* (Gupta et al. 1999), soil- (Elad and Baker 1985) and plant-based trials (Cabrefiga et al. 2007; Duijff et al. 1993). Notably, competition for nutrients has been proposed as a better method of suppression of necrotrophs that utilize resources from dead plant tissues, like *Monilinia*, than other antagonistic actions such as antibiosis or mycoparasitism, which may work better at suppressing biotrophic pathogens, which parasitize living cells (Busby et al. 2016). This could explain why we observed significant growth inhibition among many isolates tested, despite few producing an inhibition halo, and suggests that strains that did not produce an inhibition halo could be included in future field trials. Comparing modes of action among species and the relative efficacy of multiple modes of action in field trials may be a promising future direction.

To date, most microbes targeted for biocontrol have originated from agricultural systems. In our study, agricultural isolates produced the highest percent growth inhibition, but natural isolates tested were also effective, accounting for 37.5% of the isolates that produced a significant reduction in *Monilinia* growth. Additionally, there was also no significant effect of isolation source on antagonistic activity in our analysis. This suggests that naturally occurring microbes could be an untapped resource for biocontrol strategies. However, our sampling strategy was limited in its ability to test this hypothesis fully. The genera *Bacillus* and *Aureobasidium* were particularly effective but isolates from agricultural rather than natural populations were used to prioritize taxonomic breadth rather than extensive examination of among-isolate variation. As a result, we suggest that additional sampling and experimental assays, including additional species and isolates within species, would be required to provide a more complete test of this hypothesis. In addition, field trials are also required to test the prediction that microbes isolated from orchard environments are better able to establish and persist in agricultural environments with associated chemical applications.

Our study employed the novel use of the CAFÉ assay to predict potential nontarget effects of microbes on pollinator food choice. Our results suggest that most candidate microbes effective in reducing *Monilinia* growth have minimal effects on honey bee feeding. In contrast, one *Aureobasidium* isolate reduced honey bee feeding, as documented in Rering et al. (2018), so we suspect that use of *Aureobasidium* could reduce honey bee foraging and pollination. However, relating bee behavior to pollination services is complex as pollination success is the result of series of factors including pollinator preference and aversion to a variety of signals, so field trials to examine pollination outcomes would also be useful to confirm this prediction. One additional consideration in the use of biocontrol agents, particularly those that may be considered for bee vectoring (Mommaerts and Smagghe 2011; Shipp et al. 2008), is direct effects of candidate isolates on pollinator survival and reproduction. For microbial species in our study, our preliminary observations suggest that *Penicillium* isolates do not grow in culture at 35°C (data not shown), the approximate temperature inside honey bee hives, suggesting that even if introduced to the hive they would experience little to no growth and therefore would have minimum impacts on brood health. However, for any biocontrol agents for which field efficacy is established, an assessment of effects on pollinator health is recommended.

In conclusion, our results revealed several species with strong antagonistic abilities to combat *M. laxa in vitro* and minimal impact on adult honey bee preference. The ability of some isolates, like

B. subtilis Ba114, to produce both an inhibition halo and a large reduction in *Monilinia* growth suggest that it may be a strong candidate as a biological control agent. Additionally, three of the most effective species have previously been examined as effective biocontrol against *Monilinia* species in different crop species (sour cherry, peaches, nectarines) and applied at different life stages (blossom, pit hardening, postharvest), further suggesting that they are ideal candidates for control of *Monilinia* in almond (De Cal et al. 2009; Holb and Kunz 2013; Larena et al. 2005; Madrigal et al. 1994; Mari et al. 2012, 2007; Pusey et al. 1988). Thus, we suggest that field trials with these species may be promising and provide an opportunity to move toward more sustainable control of orchard pathogens.

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