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Non-target effects of fungicides on nectar-inhabiting fungi of almond flowers

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Summary

Nectar mediates interactions between plants and pollinators in natural and agricultural systems. Specialized microorganisms are common nectar inhabitants, and potentially important mediators of plant-pollinator interactions. However, their diversity and role in mediating pollination services in agricultural systems are poorly characterized. Moreover, agrochemicals are commonly applied to minimize crop damage, but may present ecological consequences for non-target organisms. Assessment of ecological risk has tended to focus on beneficial macroorganisms such as pollinators, with less attention paid to microorganisms. Here, using culture-independent methods, we assess the impact of two widely-used fungicides on nectar microbial community structure in the mass-flowering crop almond (*Prunus dulcis*). We predicted that fungicide application would reduce fungal richness and diversity, whereas competing bacterial richness would increase, benefitting from negative effects on fungi. We found that fungicides reduced fungal richness and diversity in exposed flowers, but did not significantly affect bacterial richness, diversity, or community composition. The relative abundance of *Metschnikowia* OTUs, nectar specialists that can impact pollination, was reduced by both fungicides. Given growing recognition of the importance of nectar microorganisms as mediators of plant-pollinator mutualisms, future research should consider the impact of management practices on plant-associated microorganisms and consequences for pollination services in agricultural landscapes.

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Introduction

Nectar is the primary carbohydrate source for many pollinators and drives both their foraging activity (Nieh *et al*., 2006) and reproductive success (Pelletier and McNeil, 2003). Nectar foraging behavior is often at the heart of beneficial pollination interactions between flower visitors and plants. As a critical ecosystem service, pollination affects approximately 35% of the global food supply (Klein *et al*., 2007). Moreover, as recently as 2009, the economic value attributed to pollination services (both commercial and wild) had been estimated to reach $361 billion globally (Lautenbach *et al*., 2012). Thus, understanding factors that impact nectar resource quality and attractiveness is critical for maintenance of services vital for food production in agricultural systems.

Recent studies have shown that yeasts and bacteria frequently colonize floral nectar (Brysch-Herzberg, 2004; Herrera *et al*., 2008; Fridman *et al*., 2012), compete for nectar resources (Peay *et al*., 2012; Tucker and Fukami, 2014; Vannette and Fukami, 2014), and in some cases, influence plant-pollinator interactions (Herrera *et al*., 2013; Vannette *et al*., 2013; Schaeffer and Irwin, 2014; Schaeffer *et al*., 2014; Vannette and Fukami, 2016). The presence of yeasts in nectar has been shown to increase foraging by bumble bees, key pollinators of many agricultural crops (Herrera *et al*., 2013; Schaeffer and Irwin, 2014; Schaeffer *et al*., 2014). Bumble bee foragers actively seek out, forage longer on, and remove more nectar from flowers colonized by yeasts: behaviors that can have a positive influence on plant reproduction (Galen and Plowright, 1985; Galen and Stanton, 1989) and crop yield. In contrast, studies to date indicate that bumble bees (Junker *et al*. 2014), honey bees (Good *et al*., 2014) and hummingbirds (Vannette *et al*., 2013) can display an aversion to nectar colonized by some bacteria examined.

Although nectar yeasts and bacteria have been found in agricultural systems (Gilliam *et al*., 1983; Fridman *et al*., 2012), their community composition and response to agricultural management practices is poorly understood. For example, managers use a vast array of

agrochemicals to combat pests and pathogens in agricultural systems. Fungicides, a class of pesticide, are commonly applied pre- and post-harvest to protect crops from fungal pathogens (Price *et al.*, 2015). These agrochemicals can contaminate both pollen and nectar, posing an ecological and economic risk for beneficial interactions (Chauzat *et al.*, 2006; Gill *et al.*, 2012; Krupke *et al.*, 2012; Pettis *et al.*, 2013). For instance, exposure to and consumption of fungicides can have detrimental effects on beneficial macroorganisms such as bees, affecting larval development (Mussen *et al.*, 2004), foraging behavior (Sprayberry *et al.*, 2013), and driving mortality through both direct and indirect pathways (Pettis *et al.*, 2013; Bernauer *et al.*, 2015). Although evidence is mounting on the detrimental effects of agrochemicals on macroorganisms, less attention has been paid to non-target microorganisms that may also benefit crop yield (but see Álvarez-Pérez *et al.*, 2016; Bartlewicz *et al.*, 2016).

Here, we assess the impact of two widely used fungicides (metconazole and penthiopyrad) on nectar microbial community structure in the economically important, mass-flowering crop almond (*Prunus dulcis*). Fungicides are applied to almond during bloom to minimize damage caused by pathogens (Adaskaveg *et al.*, 2011). We predicted that flowers exposed to fungicides would have lower fungal richness and diversity compared with flowers not treated. In contrast, we predicted that bacterial richness and diversity would increase, benefiting from negative effects of fungicides on competing fungi. To test these predictions, we sampled nectar from flowers of trees sprayed with either fungicide or those that were not for comparison. Using a culture-independent approach, we used Illumina sequencing of the ITS1 rDNA region and 16S rRNA gene to characterize the richness, diversity, and composition of fungal and bacterial communities respectively that colonize almond nectar and how composition is affected by fungicides (see Supporting Information Text S1 for detailed experimental procedures).

Results and discussion

Across all samples, we obtained 1 946 581 and 462 653 quality sequences for fungi and bacteria respectively. These sequences were classified for a total of 1841 and 2263 unique fungal and bacterial OTUs at the 97% sequence-similarity level across all samples. For fungi, Ascomycota was the dominant phylum, accounting for 93.3% of taxa, while Basidiomycota accounted for 6.4% of OTUs. More specifically, the majority of fungal taxa were identified as *Metschnikowia reukaufii* (79.9% of OTUs), a cosmopolitan, ascomycetous yeast that is a common nectar specialist (Lachance *et al.*, 2001). For bacteria, Proteobacteria accounted for 40.1% of OTUs, while Bacteroidetes (11.2%), Actinobacteria (7.2%), and Firmicutes (5.9%) were also common.

We found that nectar microbial communities from flowers exposed to fungicides had reduced fungal richness (Fig. 1A; $F_{2,39} = 3.92$, $P = 0.03$) and Shannon diversity (Fig. 1C; $F_{2,39} = 3.67$, $P = 0.04$). OTU richness (absolute count of OTUs) and Shannon diversity (accounting for both OTU richness and evenness) of fungi observed in nectar samples exposed to fungicides were reduced by 20% and 50% respectively. Although fungicides reduced fungal richness and diversity, we detected no significant shifts in relative abundance of individual OTUs (DESeq 2 analysis: all tests $P > 0.05$), nor differences in fungal community composition among treatments (Fig. 2A: PERMANOVA: $F_{2,39} = 1.14$, $P = 0.26$). Fungicide effects on fungal OTU richness and diversity were largely driven by loss of OTUs assigned to *M. reukaufii*, with both fungicides having pronounced effects on *Metschnikowia* richness ($F_{2,39} = 4.43$, $P = 0.02$) and diversity ($F_{2,39} = 3.24$, $P = 0.05$). More specifically, metconazole and penthiopyrad reduced Metschnikowiaceae OTU richness by 19% and 21% respectively. Concurrent with decreases in *Metschnikowia* relative abundance (Fig. 3A), Nectriaceae OTUs were found to increase in relative abundance in flowers exposed to both fungicides tested. However, this increase was not statistically significant (Kruskal–Wallis test: $\chi^2 = 3.81$, df = 2, $P = 0.15$), nor does it necessarily imply that they increased in absolute abundance in our samples given the nature of the methodology used. The two most abundant Nectriaceae OTUs were tentatively identified as *Fusarium delphinoides* and an unidentified Nectriaceae species. Many taxa within the Nectriaceae, particularly *Fusarium* species, are known fungal pathogens of many plant hosts, both natural and agricultural. It is unknown whether these taxa are pathogenic to almond, but their increase in relative abundance suggests they may be more resistant to the fungicides tested in our study than *Metschnikowia*. This uncertainty remains to be investigated, as well as additional functional work to determine whether their increase may have been driven partly by competitive release resulting from the fungicide-induced decrease in *Metschnikowia* relative abundance.

Though fungicides affected measures of *alpha* diversity, the two fungicides did not differ in the strength of their effects (*post-hoc* Tukey HSD tests: $P = 0.99$). Both metconazole (triazole) and penthiopyrad (carboxamide) are broad-spectrum fungicides effective against many fungal pathogens that target fruit, vegetable, and nut crops. Recently, a suite of synthetic fungicides widely used in agriculture have also been shown to reduce the performance of nectar-inhabiting yeasts under laboratory conditions (Álvarez-Pérez *et al.*, 2016; Bartlewicz *et al.*,

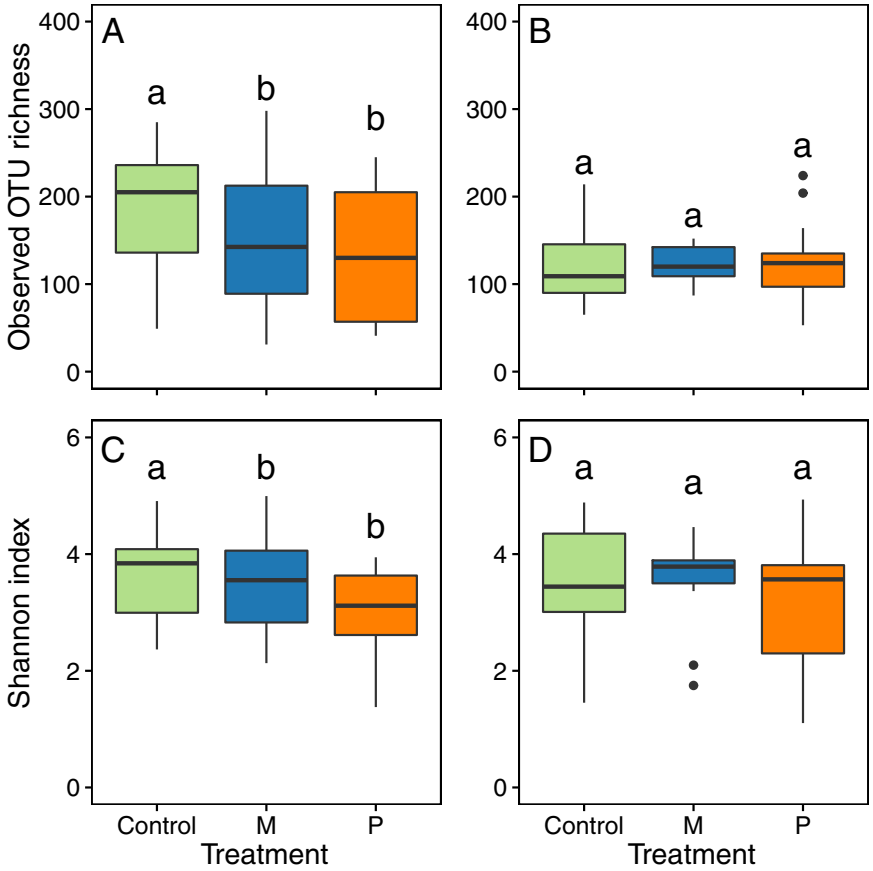

Fig. 1. Differences in (A, B) observed richness and (C, D) Shannon diversity of fungal and bacterial communities respectively in flowers of *P. dulcis* exposed to fungicides (M: metconazole and P: penthiopyrad). For each boxplot, the box represents the interquartile range (IQR), black line the median, whiskers extend to the upper and lower quartiles (±1.5 times the IQR), and dots are outliers. Letters indicate significant differences (alpha = 0.05) between treatments as determined by a *post-hoc* Tukey honestly significant difference test.

2016), including numerous members of the *Metschnikowia* clade. Moreover, experimental application of these fungicides to the flowering plant *Linaria vulgaris* revealed that these effects can also occur *in-situ* (Bartlewicz *et al*., 2016). In our study using field-collected samples from a commercially-important crop, *Metschnikowia* OTUs made up a reduced proportion of sequence pools in fungicide-treated samples compared with nectar samples from unsprayed controls flowers. Although previous work has found that *M. reukaufii* strains are abundant and diverse in other systems (e.g., Herrera *et al*., 2014), we suspect that the nearly 1200 OTUs belonging to *M. reukaufii* identified in our study is an overestimate of actual diversity, and that sequencing errors during amplification of the highly abundant *M. reukaufii* DNA may explain this. Furthermore, although we detected evidence for *M. reukaufii* susceptibility to fungicides, the relative degree of its susceptibility in our study versus strains isolated from natural hosts warrants further investigation. Numerous fungi, largely pathogenic taxa, develop resistance to multiple fungicides utilized in agricultural systems (Brent and Hollomon, 1998; Ma and Michailides, 2005; Price *et al*., 2015). Álvarez-Pérez *et al*. (2016) observed that some *Metschnikowia* strains display a "trailing" phenotype (reduced, but persistent growth) at concentrations above the noted minimum inhibitory concentration observed for synthetic fungicides tested in their study. While this "trailing" phenotype may be a consequence of the assay procedure, it may also suggest inter- and intra-specific variation in susceptibility, which could allow for evolution of resistance. Presumably putatively beneficial taxa can equally develop resistance, although they have not been tested.

Fungicide application had no observable effect on bacterial OTU richness (Fig. 1B: $F_{2,37} = 0.93$, $P = 0.40$), Shannon diversity (Fig. 1D: $F_{2,37} = 1.39$, $P = 0.26$), or community composition (Fig. 2B: PERMANOVA: $F_{2,37} = 0.75$, $P = 0.89$). Moreover, while families Pseudomonadaceae and Thiotrichaceae increased in relative abundance in response to fungicides tested (Fig. 3B), none of these shifts were statistically significant (Kruskal–Walis tests: $P > 0.05$). This lack of an effect could

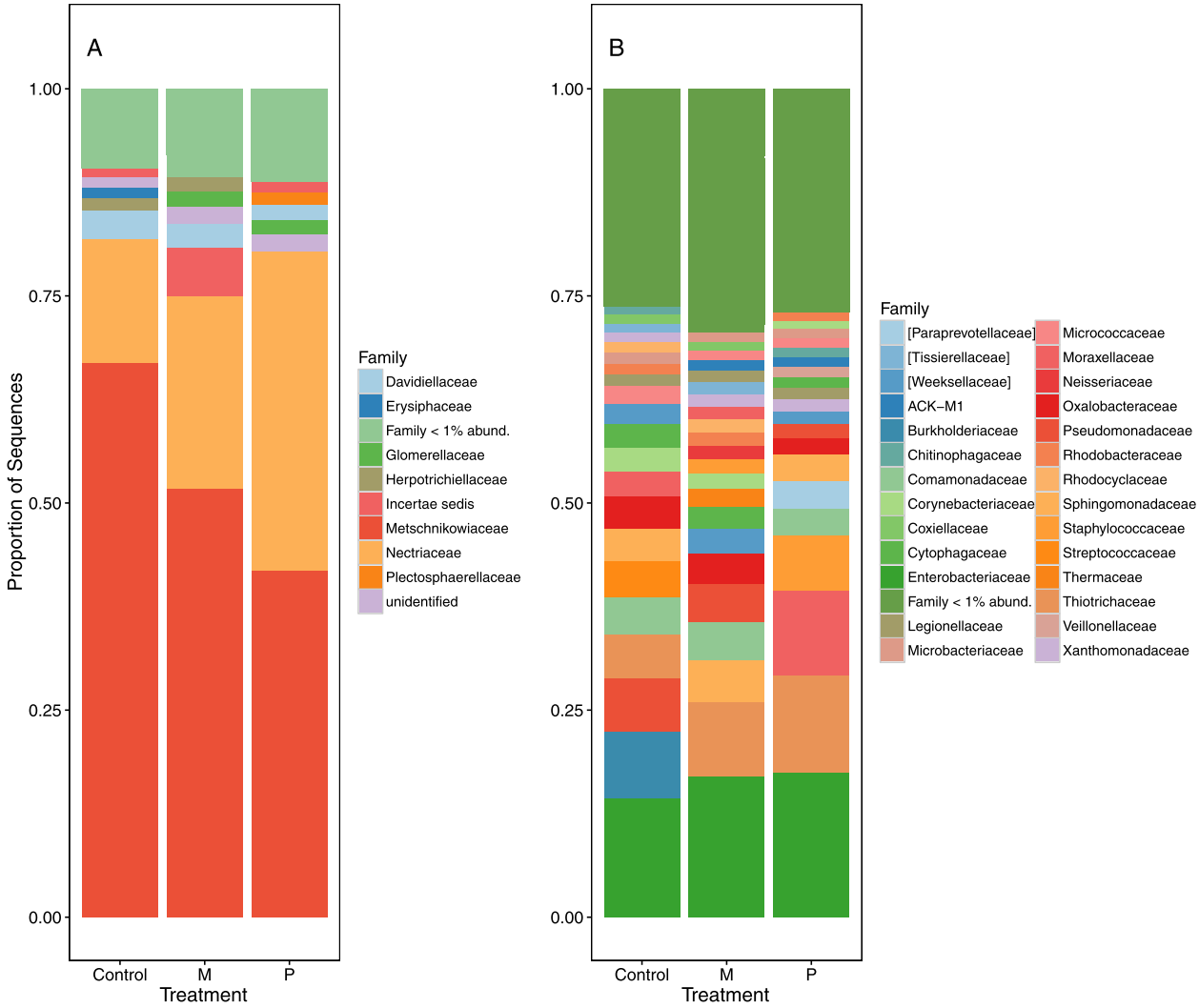

Fig. 2. Relative abundance (proportion of sequences) of (A) fungi and (B) bacteria in flowers of *P. dulcis* exposed to fungicides (M: metconazole and P: penthiopyrad). Each color represents a fungal and bacterial family with individual OTUs assigned to each binned together.

be due to minimal compositional change following fungicide application, especially if bacterial communities are dispersal-driven rather than structured by competitive dynamics. Both bacteria and fungi are frequent colonists of floral nectar in natural and managed agricultural systems and compete for nectar resources (Álvarez-Pérez *et al.*, 2012; Fridman *et al.*, 2012; Tucker and Fukami, 2014). Colonization history (Peay *et al.*, 2012), as well as abiotic conditions (Tucker and Fukami, 2014), can influence the outcome of these interactions. Our initial prediction was that bacterial richness and diversity would increase following fungicide application, resulting from negative effects on competing fungal colonists. Our methods limit our ability to assess fungicide effects on competition between nectar fungi and bacteria or the resulting changes in absolute abundance. Culture-based or culture-independent methods that account for the activity of taxa (e.g., RNA-seq) could further elucidate the impact of fungicides on competitive outcomes between fungi and bacteria in colonized nectar.

If nectar-inhabiting yeasts are influential in agricultural systems, susceptibility of nectar yeasts to fungicides and their loss following application could weaken plant-pollinator mutualisms and crop yield. Nectar yeasts have been shown to have a positive influence on bee pollinators in some natural systems (Herrera *et al.*, 2013; Schaeffer and Irwin, 2014; Schaeffer *et al.*, 2014). Flowers lacking yeasts may be less attractive to foragers, resulting in altered patterns of visitation, with consequences for pollen movement and services in agricultural systems highly dependent on pollinator visitation for yield. Moreover, nectar-inhabiting fungi often compete with bacteria for nectar resources (Tucker and Fukami, 2014). Loss of yeasts may result in open niche

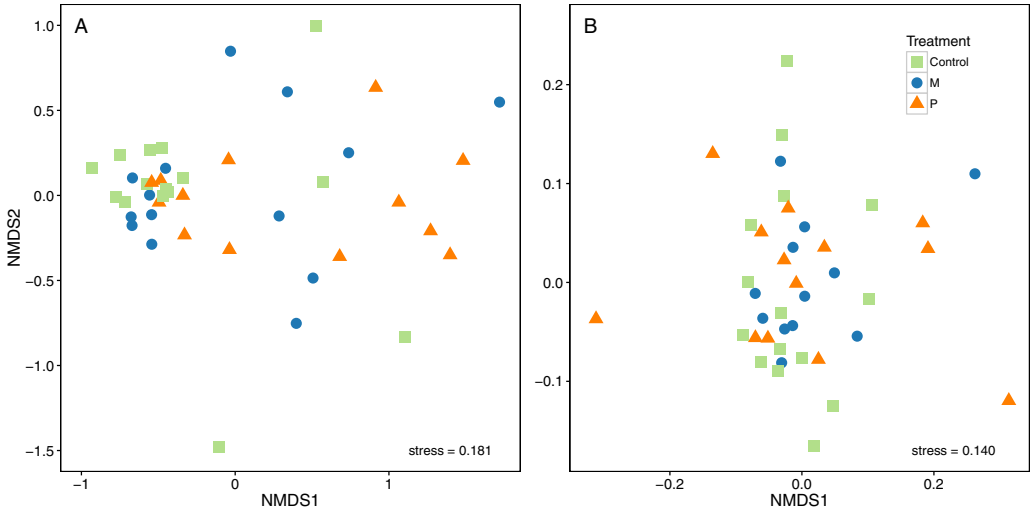

Fig. 3. Differences in composition of (A) fungal and (B) bacterial communities in flowers of *P. dulcis* exposed to fungicides (M: metconazole and P: penthiopyrad). Non-metric multidimensional scaling plots are based on Bray–Curtis and weighted-UniFrac dissimilarities for fungi and bacteria OTUs respectively.

opportunities, allowing competing bacteria to readily proliferate. Bacterial colonists of nectar can deter pollinators (Vannette *et al*., 2013; Good *et al*., 2014; Junker *et al*., 2014), including honey bees and bumble bees, key pollinators of almond flowers. Such deterrence is likely the result of microbial influence on nectar quality, through reductions in nectar sugar concentration or pH (Vannette *et al*., 2013; Good *et al*., 2014; Junker *et al*., 2014). However, effects of nectar inhabiting microbial communities have yet to be examined in agricultural systems.

Conclusions

This study highlights the potential risk of fungicide usage on non-target microorganisms in agricultural landscapes. Given the growing recognition of the importance of nectar microorganisms as mediators of plant-pollinator mutualisms, we hypothesize that these effects may have consequences for the quality of pollination services provided in agricultural landscapes. This warrants further investigation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Fungal (A) and bacterial (B) species (operational taxonomic units) accumulation curves for nectar samples taken from *P. dulcis* flowers exposed to fungicides. Vertical dashed lines represent rarefaction cutoffs (Fungi: 2047; Bacteria: 1760) for analyses.

Text S1. Experimental procedures.