UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTIGACIONES EN ECOSISTEMAS Y SUSTENTABILIDAD

MANEJO INTEGRADO DE ECOSISTEMAS

EFECTOS COLATERALES DE LOS PLAGUICIDAS EN LAS INTERACCIONES PLANTA MICROORGANISMO ABEJA CON UN ENFOQUE EN LA SALUD Y NUTRICIÓN DE LA ABEJA *APIS MELLIFERA*

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

M. en C. JOSET TSIRI DÍAZ GUERRERO

TUTOR PRINCIPAL DE TESIS: Dr. John Larsen

IIES-UNAM, Morelia

COMITÉ TUTOR: Dra. Ek del Val

IIES-UNAM, Morelia

Dr. Jorge Contreras Garduño

ENES-UNAM, Morelia

Morelia Michoacán, marzo 2024

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COORDINACIÓN GENERAL DE ESTUDIOS DE POSGRADO
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P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **09 de octubre de 2023** se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del estudiante **DÍAZ GUERRERO JOSET TSIRI,** con número de cuenta **516012076** con la tesis titulada **"EFECTOS COLATERALES DE LOS PLAGUICIDAS EN LAS INTERACCIONES PLANTA MICROORGANISMO ABEJA CON UN ENFOQUE EN LA SALUD Y NUTRICIÓN DE LA ABEJA *Apis mellifera*",** realizada bajo la dirección del DR. JOHN LARSEN, quedando integrado de la siguiente manera:

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Vocal:	DR. RÉMY VANDAME

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 22 de enero de 2024

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

c. c. p. Expediente del alumno

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1. RESUMEN

Las abejas (*Apis mellifera*) son organismos clave de gran relevancia económica, ecológica y social. Se utilizan principalmente para la producción de miel y subproductos, así como para la polinización comercial de cultivos. Su capacidad de vivir en grandes colonias, trasladarse en grandes grupos y adaptarse a diversos climas, regiones y cultivos las convierte en una especie esencial para la producción agrícola mundial y para la seguridad alimentaria a nivel global.

Las abejas, así como otros insectos, enfrentan múltiples retos para su salud, como por ejemplo las plagas y enfermedades, el cambio climático, la pérdida y fragmentación del hábitat y los plaguicidas, entre muchas otras (Goulson et al., 2015). En los sistemas agrícolas, las abejas se ven expuestas a múltiples plaguicidas, con los cuales entran en contacto, tanto de forma directa como de manera indirecta. De forma directa, se puede atribuir la muerte de miles de colonias de abejas al uso de plaguicidas, causando efectos letales o subletales, disminuyendo su capacidad de defensa contra parásitos y patógenos y afectando su nutrición y reproducción. De manera indirecta, las abejas pueden estar expuestas a los plaguicidas a través del consumo de fuentes de alimento o agua contaminadas, lo que multiplica las posibles vías de exposición y las concentraciones de estos compuestos en las abejas.

Sin embargo, hace falta información sobre los efectos indirectos de los plaguicidas en la salud de las abejas. Estos efectos pueden ser causados por alteraciones en los microorganismos benéficos rizosfera de las plantas que las abejas utilizan como alimento, lo que afecta la fijación de nutrientes y la interacción entre la planta, los microorganismos y

las abejas. Los plaguicidas pueden alterar las complejas interacciones entre los componentes bióticos y abióticos en los ecosistemas naturales y agroecosistemas, lo que tiene un impacto directo en la salud y productividad de las plantas y, de manera indirecta, en la salud y nutrición de las abejas.

En esta tesis, se estudiaron los efectos de los plaguicidas en las interacciones multitróficas entre las plantas, los microorganismos y las abejas. Estos efectos se observaron a través de cambios en la composición y abundancia de los microorganismos asociados a las raíces, lo que modifica la manera en la que las plantas se nutren y, por lo tanto, la nutrición y salud de las abejas.

Los resultados principales mostraron efectos significativos de los plaguicidas sobre los microorganismos beneficiosos de la rizosfera, afectando negativamente a algunos grupos de microorganismos en la raíz y en el suelo, y promoviendo a otros, lo cual tuvo un efecto en la adquisición de nutrientes por parte de la planta e indirectamente en la condición y nutrición de las abejas. Además, los plaguicidas y el manejo convencional tuvieron un efecto negativo sobre los microorganismos presentes en los alimentos de las abejas y en las abejas mismas, afectándolos de formas directas e indirectas a través de cambios inducidos en las plantas.

2. ABSTRACT

Honey bees (*Apis mellifera*) are key organisms of significant economic, ecological, and social importance. They are primarily used for production of honey and related products, and for the commercial pollination of crops. Their ability to live in large colonies, move in large groups, and adapt to diverse climates, regions, and crops makes them an essential species for global agricultural production and food security (Klein et al., 2007).

Honey bees, like other insects, face multiple challenges to their health, such as pests and diseases, climate change, habitat loss and fragmentation, and pesticides, among many others. In agricultural systems, honey bees are exposed to multiple pesticides, coming into contact with them both directly and indirectly. Directly, the use of pesticides can be attributed to the death of thousands of bee colonies (Johnson et al., 2010), causing lethal or sublethal effects, reducing their defense capacity against parasites and pathogens, and affecting their nutrition and reproduction. Indirectly, honey bees can be exposed to pesticides through the consumption of contaminated food sources or water, multiplying the potential pathways of pesticide exposure and their concentrations in honey bees.

Despite the various known direct and indirect effects of pesticides on honey bees, there is a need to understand the collateral effects of pesticides on honey bee health trough alterations in beneficial microorganisms in the rhizosphere, affecting nutrient acquisition and influencing the interaction between plants, microorganisms, and honey bees. Most studies focus on how soil microorganisms affect plant-herbivore interactions, but there is a lack of information about their interaction with pollinators and how this influences plant performance.

The complex interactions between plants, biotic, and abiotic components in natural ecosystems and agroecosystems are susceptible to alterations caused by pesticides, which affect plant health and productivity, indirectly impacting honey bee health and nutrition. In this thesis, we study the effects of pesticides on multitrophic interactions involving plants, microorganisms, and bees, mediated by changes in the composition and abundance of root-associated microorganisms that modify the way plants are nourished, thus influencing honey bee nutrition and health.

3. INTRODUCCIÓN

La abeja mielera o melíefera (*Apis mellifera*) es una especie clave para la producción agrícola mundial de gran relevancia económica, ecológica y social, utilizada principalmente para la producción de miel y subproductos y, además, para la polinización comercial de cultivos. La posibilidad de trasladar colonias de abejas en grandes grupos y la adaptabilidad de *A. mellifera* a una gran variedad de climas, regiones y cultivos, las convierte en una especie clave para la seguridad alimentaria a nivel mundial (Klein et al., 2007).

En los agroecosistemas, las abejas están expuestas a una gran variedad de plaguicidas a través de múltiples vías, ya sea de manera directa o indirecta (Krupke et al., 2012). Se ha atribuido directamente el colapso de miles de colonias de abejas al uso de plaguicidas (Johnson et al., 2010). Sin embargo, existe una falta de información sobre los efectos colaterales indirectos de los plaguicidas en la salud de las abejas, incluyendo su nutrición, reproducción y comportamiento (Goulson et al., 2015).

La aplicación indiscriminada de plaguicidas como el imidaclopid para controlar insectos dañinos, junto con su uso excesivo, la falta de rotación, dosis inadecuadas y aplicaciones fuera de tiempo ha provocado que las plagas desarrollen resistencia. Esto, a su vez, ha generado un incremento desmedido en su utilización (Maggi et al., 2019). Además, los plaguicidas tienen efectos negativos en organismos no objetivo, como los insectos polinizadores, lo que ha provocado un descenso de sus poblaciones (Potts et al., 2010).

La disminución de la diversidad de los insectos y de su abundancia, trae consigo efectos negativos e irreversibles en las cadenas tróficas y en los servicios ecosistémicos que los insectos proveen (Kremen y Chaplin-Kramer, 2007). En algunos países, se ha reportado una disminución en la biomasa de insectos de hasta un 82% (Hallmann et al., 2017). El

insecticida imidacloprid, el herbicida glifosato y el fungicida benomil, son tres de los plaguicidas más aplicados en el mundo para el control de plagas (Alavanja, 2009, Jeschke et al., 2010 y Benbrook, 2016) con diferentes modos de acción, sistémico y de contacto, produciendo efectos letales y sub-letales sobre las abejas y con diferentes tiempos de persistencia en el ambiente.

Los plaguicidas agrícolas de uso común pueden tener efectos colaterales en las plantas al alterar los microorganismos benéficos asociados a la rizósfera. Estos cambios pueden modificar la fijación de nitrógeno o la solubilización del fósforo (Hussain et al., 2009), afectando así la forma en que la planta adquiere nutrientes y cómo los distribuye al polen y al néctar, lo que a su vez altera las interacciones multitróficas entre la planta, los microorganismos y las abejas. Los microorganismos del suelo desempeñan diversas funciones en los ecosistemas, y una de las más importantes es su papel como facilitadores de nutrientes para las plantas (Smith y Read, 1997). Los microorganismos del suelo y los insectos son los principales motores que moldean el desempeño de las plantas, vía relaciones positivas o negativas (Pineda et al., 2010, Pangesti et al., 2013, Sugio et al., 2015).

Las interacciones multitróficas son dinámicas de aprovechamiento de nutrientes multi-especie, en las cuales existe un flujo de energía entre especies que interactúan (Barnes et al., 2018). Esto puede ocurrir parcial o totalmente a través de interacciones mutualistas o antagonistas, o mediante la facilitación de nutrientes para las plantas mediada por microorganismos (Seibold et al., 2018). Los microorganismos del suelo se alimentan de hasta el 25% de la energía producida por las plantas en forma de azúcares, ácidos orgánicos y aminoácidos (Tinker, 1984). A su vez, las plantas se alimentan de minerales facilitados por los microorganismos y del carbono resultante de la descomposición de sus células (Prakash et al., 2015). Directamente, las abejas se alimentan de los microorganismos en el

pan de abeja, aprovechando su energía para satisfacer la demanda metabólica de algunos aminoácidos esenciales. Indirectamente, las abejas se benefician de los nutrientes facilitados por los microorganismos del suelo a través del néctar y polen de las plantas (Dharampal et al., 2019, Liu et al., 2019).

La mayoría de los estudios que abordan las interacciones multitróficas microorganismo-planta-insecto se centran en cómo los microorganismos del suelo afectan las interacciones entre la planta y el herbívoro. Se ha estudiado si estos microorganismos hacen que las plantas sean más atractivas para los herbívoros, lo que podría incrementar su calidad nutricional; si inducen sistemas de defensa, como la atracción de enemigos naturales, el desarrollo de armas químicas o la compensación mediante una mayor producción de biomasa (Bardgett et al., 1998; Kempel y Schädler, 2009; Bezemer et al., 2013; Macias-Rodríguez et al., 2020). Sin embargo, falta información sobre cómo los microorganismos del suelo interactúan indirectamente con los insectos polinizadores, a través de posibles cambios nutricionales en el néctar y polen de las plantas inducidos por estos microorganismos. Finalmente, si estos cambios nutricionales en las plantas benefician o perjudican, al polinizador y si una mayor interacción con los polinizadores, representa una mejor adecuación para la planta.

Las plantas forman parte de un intrincado sistema de interacciones, en ecosistemas naturales y en agroecosistemas, donde se conectan con componentes bióticos y abióticos, interactuando de manera compleja con variaciones espaciales y temporales (Loreau et al., 2001; Kumar et al., 2019). Estas complejas interacciones son susceptibles a alteraciones causadas por los plaguicidas repercutiendo en la salud y productividad de las plantas. Los microorganismos patogénicos y benéficos influencian el comportamiento y desempeño de

sus hospederos, modificando las interacciones entre las plantas y los microorganismos (Biere y Bennett, 2013).

Los microorganismos en el suelo median efectos nutricionales en la planta, así como la emisión de volátiles que pueden atraer, reclutar o repeler, además de influir en su capacidad de defensa y reproducción (Franco et al., 2017; Jacoby et al., 2017). Esto afecta indirectamente a los insectos que se alimentan de ella, ya sean polinizadores o herbívoros, así como a los microorganismos que viven dentro de estos insectos y que deben adaptarse a los cambios en la alimentación del hospedero. Además, los microorganismos del suelo ejercen una influencia indirecta sobre el comportamiento del insecto hospedero, al proporcionar funciones metabólicas para la nutrición o la desintoxicación, lo que permite un aprovechamiento diferencial de los recursos (Frago et al., 2012). Los microorganismos presentes en el suelo, en las plantas y en los insectos desempeñan un papel crucial en las interacciones planta-insecto y son susceptibles a una variedad de efectos causados por los plaguicidas.

Los efectos directos de los plaguicidas sobre la salud humana y el ambiente se han estudiado ampliamente (Rani et al., 2021), sin embargo, hay un faltante de información sobre los efectos indirectos en las cadenas tróficas. Ante la necesidad de generar información básica y aplicada de los efectos indirectos sobre los insectos y los mecanismos por los cuales se ven afectados, se propone como objetivo general de este trabajo evaluar las consecuencias del uso de plaguicidas en las abejas melíferas, las cadenas tróficas y los efectos directos e indirectos de estos. Esta tesis se divide en cuatro capítulos. En el primero, abordamos los efectos directos e indirectos de los plaguicidas en las abejas, los cuales provocan una gran diversidad de efectos letales y subletales. En el segundo, evaluamos los efectos del sistema

de producción y el cultivo en la adquisición de nutrientes por parte de las abejas, tanto en términos proteicos como en ácidos grasos, así como en los microorganismos benéficos asociados a su alimentación. En el tercer capítulo, revisamos los efectos del fungicida Benomil en los microorganismos benéficos del suelo y cómo esto afecta la nutrición de las plantas e indirectamente, a las abejas. En el cuarto y último capítulo, llevamos a cabo un experimento de campo donde evaluamos los efectos de tres de los grupos de plaguicidas más utilizados en el mundo: un insecticida (imidacloprid), un fungicida (benomil) y un herbicida (glifosato), en las interacciones entre plantas, microorganismos y abejas.

4.1 Primer capítulo

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Alterations in bee-plant-soil multitrophic interactions after fungicide soil application

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ABSTRACT

Soil microorganisms play a key role in plant growth and health, and honey bees depend on plant pollen and nectar for their nutrition. Despite this clear above- and belowground connection, our knowledge about bee-plant-soil interactions is yet limited. Also, how these multitrophic interactions are affected by agricultural practices like pesticide applications is not well understood. Here we investigated possible non-target effects of soil application of the fungicide Benomyl on bee-plant-soil interactions in a greenhouse experiment with vetch (*Vicia sativa* L.). When plants were flowering honey bees were confined to their respective experimental soil treatments (untreated control, Benomyl and soil disinfection) in tents made from anti-insect mesh and micro-hives for foraging. At harvest, results showed that Benomyl and soil disinfection strongly affected key root and soil microorganisms essential for plant nutrition, such as mycorrhiza and rhizobia, and Gram-positive and Gram-negative bacteria, which coincided with phenotypic alterations in plant development and subsequently significant effects on honey bee nutrition and health, and finally honey production. In conclusion, our results show that soil application of the fungicide Benomyl strongly impact on bee-plant-soil multitrophic interactions, calling for further investigation on non-target effect of pesticides including multitrophic studies when evaluating environmental impacts of pesticides.

1. Introduction

Above- and below ground plant-microbe-insect interactions strongly affect plant performance in both natural and agricultural ecosystems (Wardle et al., 2004; van der Putten et al., 2013). In this context, root-associated microorganisms such as arbuscular mycorrhizal fungi (AMF) and rhizobia are known to alter their host plant phenotype and associated aboveground insects including herbivores and pollinators (Heinen et al., 2018). Also, it is known that agricultural practices such as pesticide applications can modulate such below- and aboveground interactions (Barber et al., 2013). Indeed, soil application of the fungicide Benomyl has been shown to alter aboveground plant-pollinator interactions linked with disruption of the functioning of mycorrhizal associations (Gange et al., 2005; Cahill et al., 2008). Among plant-pollinator interactions particularly plant-honeybee interactions deserve special attention due to the vital importance of honey bees in agricultural plant production.

Honey bees rely on plants as their main natural source of carbohydrates and proteins (Brodschneider and Crailsheim, 2010) and the quality of bee collected pollen affects bee development, longevity, mortality, foraging behavior, and drone and queen fertility (Haydak, 1970; Rousseau and Giovenazzo, 2016). Additionally, honey bee immune responses are energetically costly and requires carbohydrates and protein for maintenance. Hence, honey bee malnourishment is directly correlated with poor pathogen defense (Alaux et al., 2010) leading to increased susceptibility to destructive pathogens like Varroa and Nosema (Alaux et al., 2011; Azzouz-Olden et al., 2018).

Besides the importance of carbohydrates and proteins in honey bee nutrition, lipids naturally obtained from pollen, are another key component in honey bee nutrition due to their important functions in

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bee metabolism. Oleic, palmitic, stearic, and linoleic are the principal fatty acids in bee body (Manning, 2001). Deficiencies on the essential fatty acids can lead to increased mortality (Corby-Harris et al., 2021) and decreased honey bee nutrition, learning, and reproduction (Arien et al., 2015; Manning, 2001).

On the other hand, to better understand the role of the plant associated microbiota in plant-honeybee interactions, the specificity of certain fatty acids in particular groups of microbiota provide a solid method platform in terms of specific microbial biomarkers in soil, plants, and honey bees (Frostegård et al., 2011; Diaz et al., 2019; Norris et al., 2023), useful when investigating trophic plant-microbe-insect interactions.

Such plant biotic below and aboveground interactions are part of a complex system of multitrophic interactions where plants have developed intricate mechanisms to respond defensively or beneficially to insects and microorganisms shaping plant performance and plant fitness through positive or negative interactions (Pineda et al., 2010; Pangesti et al., 2013; Sugio et al., 2015).

In the belowground section, root associated microorganisms, including mycorrhizal fungi and diazotrophic bacteria, play a key role in plant nutrition, defense, and abiotic stress resilience, in exchange of shelter and organic carbon sugars (Albayrak et al., 2006; Vázquez et al., 2020). Also, free living plant growth promoting Gram-positive and Gram-negative rhizobacteria are known to promote plant nutrition and health. For example, they contribute to host protection against root diseases via nutrient competition or by antibiosis with the production of antimicrobial compounds. Furthermore, they promote plant growth by facilitating nutrient mineralization and uptake, and trough phytohormone production (Francis et al., 2010). Such root microbe plant growth and health promoting effects can cause plant phenotypic alterations, which have been shown to modulate plant-insect interactions in the aboveground section (Heinen et al., 2018).

However, these complex interactions are susceptible to disturbances from different agricultural practices such as soil pesticide application, which can impact the health and productivity of plants by inducing modifications in soil microbial communities, altering not only their abundance but also their functions, diversity, and activity (Haney et al., 2000; Lo, 2010). Multiple negative effects on AMF caused by pesticides, such as Benomyl, have been reported, including the inhibition of phosphorus uptake, enzymatic activity, and root colonization (Larsen et al., 1996; Kling and Jakobsen, 1997; Okiobe et al., 2022). In addition, non-target effects of pesticides like fungicides on nitrogen fixing bacteria have been documented, reducing nodulation and nitrogen uptake (Hussain et al., 2009; Getachew and Abeble, 2021).

Since the production of pollen and nectar is dependent on the facilitation of nitrogen and phosphorus by rhizobium and mycorrhiza (Lau and Stephenson, 1993), such adverse effects of pesticides on beneficial rhizosphere microorganisms could disrupt the functional root symbiosis with these beneficial microorganisms indirectly affecting aboveground plant-honey bee interactions. However, such multitrophic interactions are not considered in standard risk assessment of pesticides on non-target organisms such as honey bees (OECD, 2017).

The pesticide benomyl and its metabolite carbendazim is a systemic fungicide and though banned in many countries for its known adverse effect on human health and crop associated invertebrates and mammals Benomyl is still widely used to control plant fungal diseases in many crops especially in developing countries (Pearson and Miller, 2014).

In this work, we evaluated the effects of Benomyl on plant nutrient acquisition mediated by possible changes in root-associated microorganisms and the overall effect on the bee-plant-soil multitrophic interaction, focusing on honey bee nutrition and health. Our main hypothesis was that Benomyl will have a negative impact on the multitrophic interactions between plants, microorganisms, and honey bees, through induced changes in soil beneficial microorganisms, impacting plant health and nutrient uptake, resulting in a decline in honey bee health and nutrition.

2. Materials and methods

2.1. Experimental design and setup

The experiment was conducted under greenhouse conditions during the winter of 2020–2021, from November 15 to March 3 (Fig. 1), using a clay-loam soil collected from an agricultural plot (19°51′20″N–101°8′4″W), which had 67 mg kg^{-1} soil of inorganic nitrogen, 1.14% of organic matter, and 20 mg kg^{-1} Olsen P. After collection, soil was sun-dried, milled, and sieved through a 4.5 mm mesh. For the soil semi-sterilization treatments, the soil was heated to 94 °C for 24 h. Three treatments were established: 1) untreated control soil, 2) Soil disinfection (semi-sterile) and 3) Benomyl soil application. Each treatment had three replicates each with 100 pots with 5 plants resulting in 500 plants in each replicate. Each microplot was 2.5 × 1.5 m. After soil sieving, 300 kg of soil were disinfected for 24 h at 96 °C for the soil disinfection treatment. For the fungicide treatment, Benomyl 50% w/w was diluted in water to a final concentration of 10 μg g^{-1} and applied during the second week after sowing and weekly for two consecutive weeks. In the control treatment, the agricultural soil was used directly after sieving. During the entire experiment plants remained unfertilized. To prevent honey bees from escaping, an insect net was used to cover each replicate at 15% of blooming. One disease free mini-hive (300 mm long, 300 mm wide, 340 mm high) was introduced, each contained a new queen from a local breeder. Hereafter, a cup of bees (approximately 600 bees) and three frames with organic certified beeswax foundation. Africanized sister honey bees (*Apis mellifera*) were used.

2.2. Harvest and analyses

Prior to installing the micro hives 9 weeks after sowing and in the beginning of flowering 3 random plants in each of the 9 experimental units were selected and analyzed for numbers of flowers, shoot and root dry weight, shoot N and P concentration and fatty acid biomarkers in soil and roots.

2.2.1. Numbers of flowers

Total number of flowers were counted for each plant.

2.2.2. Shoot and root dry weight

Plants were divided into shoot and root parts, which were then dried for 3 day at 80 °C and weighted. Before drying roots were washed in tap water and cut into 5–10 mm pieces and homogenously mixed to take a representative 2 g subsample which were kept in the freezer at −20 °C until further fatty acid analysis.

2.2.3. Shoot phosphorus and nitrogen concentration

To prepare the samples for analysis, 0.25 g of sieved dried shoot material were digested with (1 g $CuSO_4$; 10 g $K2SO_4$), 3 ml of H_2O_2 (30% v/v) and 7 ml of sulfuric acid (H_2SO_4) in 75 ml glass tubes. After 24 h, samples were heated to 375 °C for 3 h in a digester block, in ramps of 50 °C every 20 min (BMD-3050, Novatech). Extracts were filtered through Whatman No. 1 filter and recovered for colorimetrical analysis at 660 nm in a Braun + Luebbe III autoanalyzer (Seal, Inc). Nitrogen was determined following the method of Solórzano (1969) and phosphorus according to Murphy and Riley (1962).

2.2.4. Root and soil microorganisms

For fatty acid analysis, a sample of 5 g of rhizosphere soil and 2 g of roots from each plant was collected and freeze-dried. After freeze drying, a subsample of 1 g of soil and 250 mg of roots was used for the analysis. To determine the abundance of Gram positive bacteria, the fatty acids 11:0iso, 14:0iso, 15:0iso and 15:0anteiso, 16:0iso and 17:0iso and 17:0anteiso were summed. For Gram negative bacteria, the fatty acids 10:0 3OH, 12:0 20H, 16:0 2OH and 16:0 30H were summed.

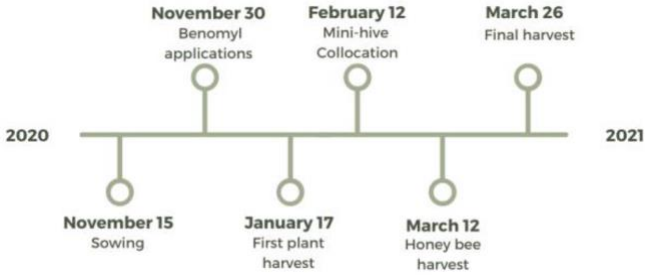

Fig. 1. Experimental timeline.

Rhizobium root colonization was assessed by the fatty acid 17:0 cyclo as biomarker and the fatty acid 16:1ω5 was use as a biomarker of arbuscular mycorrhizal fungi (AMF). The fatty acid analysis consisted in a three-step process. First, saponification with sodium hydroxide, methanol, and water for 30 min at 100 °C. Second, methylation with chlorhydric acid and methanol for 10 min at 80 °C. Finally, extraction with hexane and methyl tert-butyl methyl ether. Fatty acids were detected in a gas chromatograph (Agilent 7890 B) equipped with a fused silica capillary column and analyzed by Sherlock software version 3.1 (MIDI Inc., Delaware, USA). Quantification was performed by adding an internal standard, 19:0 (nonadecanoic methyl ester, Sigma), of known concentration and comparing peak areas.

After flower onset 12 weeks after sowing bees were left foraging for 1 month after which bee fatty acids, protein, prophenoloxidase, peholoxidase and lytic activity, as well as honey production were measured.

2.2.5. Bee fatty acids

Abdomens from nine bees per hive were separated from their head, thorax, legs, and wings. The abdomens were then cold macerated. Fatty acid analysis of bees was performed as mentioned above.

2.2.6. Protein, prophenoloxidase, peholoxidase and lytic activity

To study the indirect effect of Benomyl on the immune response system of honey bees, we evaluated the protein content as well as prophenoloxidase, peholoxidase and lytic activity. After one month of foraging, nine bees per treatment were randomly selected and cold macerated using phosphate-buffered saline (PBS, Sigma). After centrifugation for 10 min at 17000 g and 4 °C the supernatant was diluted in 1.5 ml of saline phosphate buffer and stored at −70 °C until analysis (Varioskan Flash Multimode Reader, Thermo Scientific). Pierce BCA protein kit (Thermo Scientific) was used for protein analysis. In an ELISA microplate reader, micro-wells were loaded with 40 μL of saline phosphate buffer, 150 μL of working reagent, and 10 μL of sample. After incubating the microplate for 15 min at room temperature, the absorbance was measured at 562 nm (Varioskan Flash Multimode Reader, Thermo Scientific). Final concentrations were calculated by comparison with the standard curve. For prophenoloxidase activity micro wells were filled with 50 μL of sample, 38 μL of saline phosphate buffer, 2 μL of α-chymotrypsin, and 10 μL of L- Dopa solution. Also, two controls were added. Absorbance was read each 5 min for 30 min (Varioskan Flash Multimode Reader, Thermo Scientific). Prophenoloxidase activity was calculated with the slope of the kinetic curve in the linear phase of the reaction after absorbance blank subtraction. Phenoloxidase activity was measured adding 40 μL of saline phosphate buffer, 10 μL of L- Dopa solution, and 50 μL of sample. Additionally, two controls samples were included, consisting of 90 μL of PBS and 10 μL of L-Dopa. Absorbance was measured at 490 nm (Varioskan Flash Multimode Reader, Thermo Scientific), every 5 min for a total of 30 min after incubation at for15 min at room temperature, protected from light. For lytic activity, micro-wells were filled with 30 μL of sample, 200 μL of *Micrococcus lysodeikticus* (320 μg/ml) and two blanks with 30 μL of saline phosphate buffer and 200 μL of *Micrococcus lysodeikticus*. Absorbance readings were taken every 5 min during 30 min at 540 nm (Varioskan Flash Multimode Reader, Thermo Scientific). Lytic activity was calculated by the slope of the kinetic curve in the linear phase.

2.2.7. Honey production

Honey from each hive was extracted and centrifuged at 3000 g for 30 min to separate residual pollen and wax. Hereafter the amount of honey was weighed.

At the end of the experiment insect tents were removed and the plants were left to dry in the green house for one month after which total shoot dry weight, number of pods and seed germination were analyzed. The total numbers of pods were harvested and weighted both fresh and on a dry weight basis. To assess the germination percentage, once dry, seeds were extracted from the pods weighted and germinated for five days, using paper towels, and deionized water.

2.3. Statistics

The analyses were conducted using R version 3.4.1 (R Core Team, 2019), and figures were generated using ggplot2 (Wickham, 2016). Generalized linear models (GLMs) were fitted using an exhaustive analysis with the lowest AIC and Post-hoc Tukey's test by least square means package (Emmeans) (Lenth et al., 2018). Structural equation models were used to test direct and indirect effects of Benomyl on root associated microorganisms, plant development, and honey bee nutrition and health selecting the model with the best global goodness of fit using the program piecewiseSEM (Lefcheck, 2016).

3. Results

3.1. Plant growth performance

Plant growth was reduced by the application of Benomyl and soil disinfection. The shoot dry weight in these treatments were significantly lower 8 weeks after sowing (Fig. 2A), and this trend continued until the end of the experiment (Fig. 2B), with the shoot dry weight of the control plants presenting the highest level. Plants exposed to Benomyl produced fewer pods with lower levels of germination as compared with control plants and plants grown in disinfected soil (Fig. 2C). No significant differences were found for seed weight and shoot N concentration (Table 1). However, shoot P concentration was significantly higher in plants grown in control soil as compared to plants exposed to Benomyl (Fig. 2D).

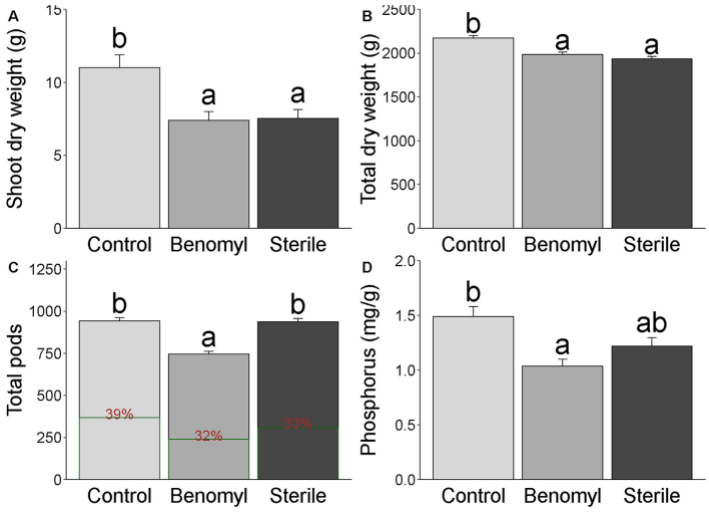

Fig. 2. Shoot dry weight (g), (A), total dry weight (g), (B), total pods and germination percentage, (C) and milligrams of phosphorus per gram of dry shoot tissue, (D). Different letters represent significant differences ($p < 0.05$).

Table 1
p values from one way ANOVA of all plant, honeybee, soil-root microorganisms variables measured ($n = 3$).

Trophic level	Variables	*p* values
Plant	Shoot dry weight	***
	Total dry weight	***
	Total pods	***
	Seed weight	0.42
	Phosphorus	***
	Nitrogen	0.27
Bee	Bee protein	*
	Prophenoloxidase	**
	Phenoloxidase	0.05
	Lytic activity	*
	Honey weight	***
	Palmitoleic fatty acid	***
	Palmitic fatty acid	0.94
	Stearic fatty acid	***
	Arachidonic fatty acid	***
Soil and root microorganisms	Gram negative (root)	*
	Gram positive (root)	***
	HMA (soil)	***
	Rhizobium biomarker (root)	***

$p < 0.0001$ '***'; $p < 0.001$ '**'; $p < 0.01$ '*'.

3.2. Rhizosphere microorganisms

The abundance of Gram positive bacteria in soil was significantly reduced by the application of Benomyl and by the soil disinfection (Fig. 3A). Similarly, Gram negative bacteria was affected by soil disinfection, but not by application of Benomyl (Fig. 3B). AMF in soil were affected by both Benomyl and soil disinfection (Fig. 3C), while Rhizobium biomarkers in roots were only negatively altered by soil disinfection (Fig. 3D).

3.3. Bee performance

Regarding bee performance, the total protein was lower in the treatment with plants grown in disinfected soil, whereas no significant differences were found between control and Benomyl treatments (Fig. 4A). Bees that fed from plants in the control treatments showed significantly higher levels of prophenoloxidase activity when compared with the benpmyl and soil disinfection treatments (Fig. 4B). However, no significant differences were found in phenoloxidase activFty (Table 1), and lytic activity was higher in the treatment with disinfected soil as compared to the control and Benomyl treatments (Fig. 4D). Bees in the control treatments stored more honey than bees in soil disinfection and Benomyl treatments (Figure C). Additionally, palmitoleic, stearic, and arachidonic fatty acids in bee abdomen were significantly lower in and Benomyl soil disinfection treatments (Fig. 5).

3.4. Structural equation model

A structural equation model was created to investigate the relations between soil, plants, and honey bees (*p*-value = 0.77). The results showed that the model explained 39% of the variation in AMF biomarkers, 60% in shoot dry weight, 17% in phosphorus, 25% in flower production, 22% in bee protein, 38% in arachidonic acid, 37% in prophenoloxidase and finally 71% in honey production. Benomyl negatively correlated with both shoot dry weight ($-0.76\ p \leq 0.05$) and with AMF ($-0.62\ p \leq 0.05$). Simultaneously, AMF biomarkers positively correlated with arachinodic acid in bee fat ($0.61\ p \leq 0.05$), honey production ($0.49\ p \leq 0.05$), and phosphorus in shoot tissue. However, AMF were negatively correlated with shoot dry weight ($-0.47\ p \leq 0.05$). At the same time, shoot dry weight was positively correlated with honey production ($0.61\ p \leq 0.05$) and prophenoloxidase in bees. Furthermore, phosphorus was positively correlated with floral display ($0.50\ p \leq 0.05$), while nitrogen was positively correlated with bee protein ($0.46\ p \leq 0.05$) and negatively correlated with prophenoloxidase ($-0.45\ p \leq 0.05$). D-separation test showed no significantly missing paths in the model (Fig. 6).

4. Discussion

Here we report adverse effects of the fungicide Benomyl on bee-plant-soil interactions where inhibition of key root microorganisms

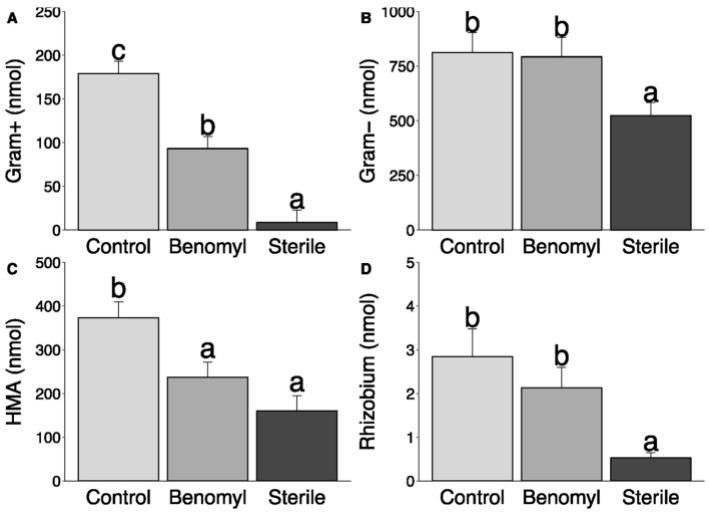

Fig. 3. Fatty acids of gram positive bacteria (nmole g^{-1} root dwt) 11 iso, 14 iso, 15 iso and anteiso, 16 iso and 17 iso and anteiso, (A), fatty acids of gram negative bacteria (nmole g^{-1} root dwt) 10:0 3OH, 12:0 20H, 16:0 2OH and 16:0 30H, (B), fatty acids of AMF in soil (nmole g^{-1} soil dwt) 16:1 w5c 89, (C), fatty acids of rhizobium (nmole g^{-1} root dwt) 17:0 cyclo, (D). Different letters represent significant differences ($p < 0.05$).

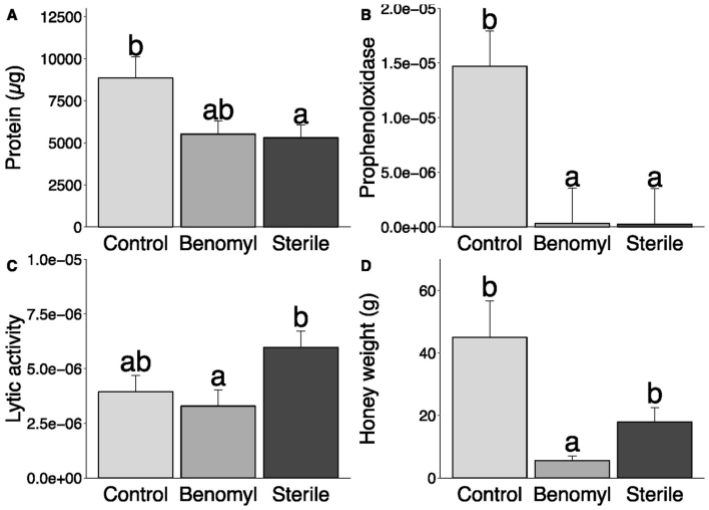

Fig. 4. Honey bee protein concentration (µg/mL), (A), prophenoloxidase activity (slope of the kinetic curve), (B), lytic activity (slope of the kinetic curve), (C) and Honey weight (g), (D). Different letters represent significant differences ($p < 0.05$).

important for plant nutrition, growth, and reproduction resulted in reduced honey bee health and honey production.

The observed decrease in the abundance of root-inhabiting microorganisms such as AMF after Benomyl soil application coincided with a reduction in key plant functions like phosphorus acquisition, floral display, seed production, and germination rate, which is in accordance with other studies on AMF-plant interactions (Parniske, 2008; Bennett and Meek, 2020). Additionally, Benomyl soil application altered the fatty acids of rhizosphere inhabiting Gram positive bacteria. Gram positive bacteria are important for plant development since they are linked to mineral solubilization, phytohormone production, and biocontrol (Francis et al., 2010), altering plant growth and health. In contrast, Gram negative bacteria were not affected by Benomyl soil application, possibly because of the function of some groups of Gram negative bacteria as pesticide degrading microorganism (Zhang et al., 2009; Wattanaphon et al., 2008). Benomyl had no effects on the

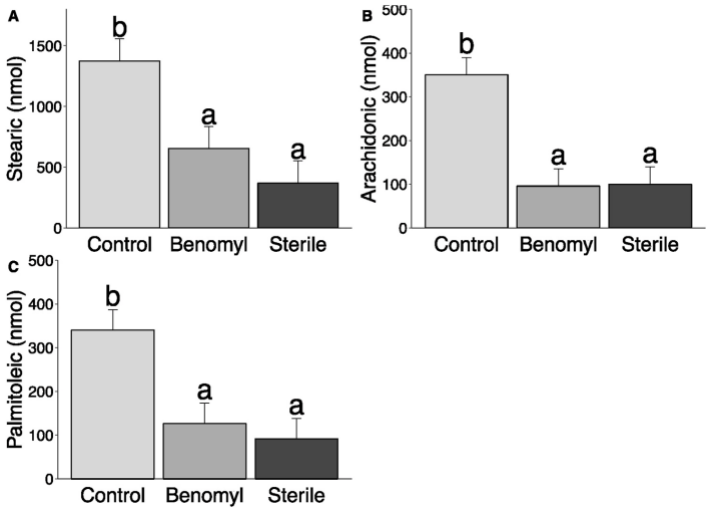

Fig. 5. Fatty acids in honey bee fat (nmole bee^{-1}). (A) Stearic acid (18:0), (B) Arachidonic acid (20:4 w6c) and (C) Palmitoleic acid (16:1 w7c). Different letters represent significant differences ($p < 0.05$).

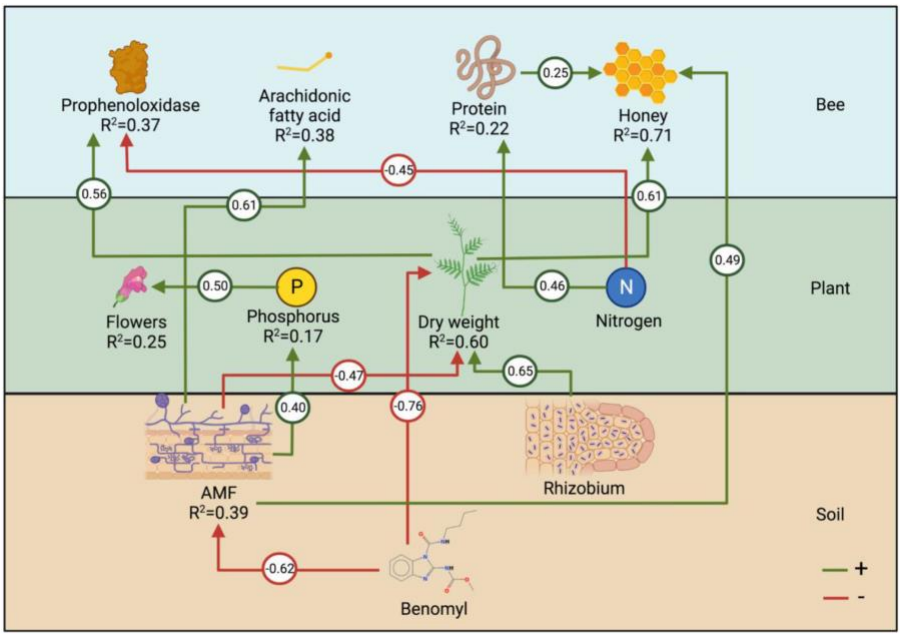

Fig. 6. Structural equation best fitted model (p-value = 0.77) green arrows represent significant positive correlations, red arrows negative correlations, d-separation test did not show any missing significant path. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Rhizobium biomarkers (17:0 cyclo), supporting the finding by Habte (1985) that Rhizobium are resistant to fungicides, which may even promote plant growth due to the elimination of competition with other microorganisms (Hossain and Alexander, 1984).

Benomyl soil application also strongly affected plant performance by decreasing shoot dry weight, total dry weight, shoot phosphorus concentration, total number of pods, and seed germination rate. This maybe related to a possible direct phytotoxic effects of Benomyl due to the *n*-butyl isocyanate byproduct (Petit et al., 2012). However, another explanation of the reduced plant performance after Benomyl soil application maybe indirectly related to the inhibition and disruption of the mycorrhizal vetch association. Here it is important to note that also soil disinfection from soil heating resulted in reduced plant growth, which also coincided with reduced abundance of AMF soil biomass.

In terms of honey bee performance Benomyl soil application resulted in reduced prophenoloxidase activity, as well as honey production, most likely due to the negative effects induced by Benomyl on root associated microorganisms and plant growth. Prophenoloxidase is a key component of the honey bee immune cellular and humoral response against pathogens (Evans et al., 2006). In the Benomyl treatment, prophenoloxidase activity was suppressed, potentially increasing honey bee susceptibility to microbial infections, and decreasing survival probabilities. Prophenoloxidase response is energetically costly and relies directly on the nutritional environment of the insect (Roger et al., 2017). In contrast, no significant differences were observed in phenoloxidase among the different treatments. Prophenoloxidase is an inactive enzyme, activated in response to immune challenges, indicating that the bees in the different treatments were not affected by pathogens. Lytic activity showed only significant differences between the Benomyl and soil disinfection treatments, higher in the treatment with Benomyl and with no differences between the control and soil disinfection treatment. Lytic activity plays an important role in invertebrate immunity (Jiang et al., 2010).

As expected, the fungicide Benomyl reduced AMF biomarkers, which directly and indirectly influenced shoot dry weight. However, AMF root colonization had a negative impact on shoot dry weight due to its high cost for plant development, leading to growth depression (Raya-Hernández et al., 2020). Nonetheless, overall AMF positively affected the plant-pollinator interactions, increasing honey accumulation and the amount of arachidonic fatty acid in the bee abdomen, an essential fatty acid that bees cannot synthetize, involved in growth and immune response of honey bees (Yu et al., 2022).

To obtain carbon from plants, AMF stimulate SWEET transporters, which facilitate the efflux of sugars and increase nectar secretions (Manck-Götzenberger and Requena 2016; Breia et al., 2021). Since no mineral fertilization was used in the present study, root associated microorganism played a crucial role in supplying nutrients to the plant, which are vital for pollen production (Lau and Stephenson, 1993) and subsequent bee assimilation. Our findings demonstrate that AMF alter plant-honey bee interactions are in accordance with previous studies showing that AMF modifies pollinator visitation (Barber et al., 2013), floral traits such as number and size (Gange et al., 2005; Varga and Kytöviita, 2010), and the emission of organic volatile compounds (Kiers et al., 2010). AMF also modulate the fatty acid composition of their host plants (Wu et al., 2019), thereby altering honey bee fatty acid acquisition. Additionally, AMF root colonization have been shown to positively correlate with plant phosphorus, and consequently, flower production (Poulton et al., 2002). On the other hand, Rhizobium showed only indirect effects on honey production and prophenoloxidase activity by promoting shoot dry weight. No direct effects were found between Rhizobium and shoot nitrogen concentration, possibly due to nitrogen dilution resulting from the plant growth promotion effect and reallocation of nitrogen to pollen protein.

Despite the observed negative effects of Benomyl on multitrophic bee-plant-microbe interactions, we acknowledge that Benomyl can be highly effective in controlling plant fungal pathogens. However, considering the clear adverse effects on human health and the environment Benomyl should be substituted with less harmful fungicides or agroecological alternatives. However, it is crucial to always consider the potential negative effects on bees.

Since vetch used in the present study has low fertilization requirements and the capacity of fixing nitrogen from the atmosphere, we did not apply any fertilizer. Nevertheless, we cannot rule out that mineral and/or organic fertilization could mitigate the adverse effects of Benomyl in the bee-plant-soil multitrophic interaction examined. Furthermore, conducting experiments under field conditions and in other crops, with different nutritional requirements is necessary to understand bee-plant-soil interactions in more realistic scenarios.

Besides standard quantitative statistics, the employment of structural equation models in the present provided an additional statistical tool to test the direct and indirect effects of Benomyl on root associated microorganisms, plant development, and honey bee nutrition and health. This allowed us to unravel the intricate causal relationships among multiple variables, revealing indirect effects of the pesticide that may not be visible with conventional statistic tools.

This study provides scientific evidence of the potential non-target effects of pesticides on multi trophic bee-plant-soil interactions. In particular, our results clearly show that pesticides such as Benomyl can significantly impact soil microbial communities, ultimately affecting the health and nutrition of plants and honey bees. Further research is needed to understand the complex interactions between soil microorganisms, plants, and honey bees, as well how these interactions can be altered by the use of pesticides.

Author contributions

JL designed the study, performed the research, designed the figures, provided methods and materials and wrote the manuscript, TD collected data, performed the research, analyzed output data and wrote the manuscript, JC designed the study and provided methods and materials, JM collected data and performed the research, ED was involved in the experimental design and wrote the manuscript, all authors contributed substantially to revisions.

We state that, after manuscript acceptance, the data supporting the results will be archived in an appropriate public repository and is available upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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4.2 Segundo capitulo

Fotografía: Tsiri Díaz Guerrero

Plaguicidas y abejas

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Resumen

Los efectos de los plaguicidas sintéticos en las abejas son altamente perjudiciales, causando, además de una elevada mortandad, una gran variedad de efectos negativos en su salud, comportamiento, reproducción y nutrición. Debido a la actividad agrícola, las abejas están constantemente expuestas a una multitud de plaguicidas los cuales afectan de manera significativa sus poblaciones y los servicios que estas proveen. Es importante conocer los efectos de los plaguicidas en las abejas, ya que estas pueden estar expuestas a ellos por múltiples vías. Directamente a través de la deriva, cuando los plaguicidas se dispersan en el aire y se desplazan más allá del área de aplicación prevista, o de forma indirecta mediante el consumo de fuentes de alimento o agua contaminadas. Además de los efectos en la mortalidad, los plaguicidas pueden provocar efectos subletales, disminuyendo su capacidad de defensa contra parásitos y patógenos, afectando así su nutrición y reproducción. Por lo tanto, es crucial proponer estrategias integrales de protección para las abejas que consideren estos múltiples efectos y vías de exposición.

Palabras clave

abejas, nutrición, plaguicidas, reproducción, sistema inmune

Introducción

Las abejas, se consideran uno de los grupos de insectos de mayor importancia debido a los servicios ecosistémicos de polinización que proveen. En particular la abeja de la miel (*Apis mellifera*) es una especie clave para la producción agrícola mundial de gran relevancia económica y social, generando aproximadamente 200 mil millones de dólares anuales en servicios de polinización, lo que representa el 9.5 % del valor de la producción agrícola mundial (Potts *et al*. 2010).

Debido a los servicios de polinización que proveen, las abejas se ven constantemente expuestas por diferentes vías a una gran variedad de plaguicidas. Se calcula que, a nivel mundial, hasta el 75 % de la miel tiene algún tipo de plaguicida. De acuerdo con la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) se aplican alrededor de cuatro millones de toneladas de plaguicidas al año. Los plaguicidas más utilizados en el mundo pertenecen a tres grandes categorías: herbicidas, fungicidas e insecticidas. La aplicación de estos plaguicidas se hace de manera indiscriminada, y debido a la falta de rotación, dosis inadecuadas y aplicaciones fuera de tiempo, las plagas han generado resistencia, produciendo como consecuencia un incremento desmedido en su uso.

Figura 1. Efectos directos e indirectos de los plaguicidas en las abejas. Elaboración propia

Los plaguicidas tienen efectos negativos sobre otros organismos distintos a las plagas, como los insectos polinizadores, provocando un descenso en sus poblaciones (Potts *et al*. 2010). En el mundo, algunos insecticidas han sido prohibidos debido a su alta toxicidad, permanencia y acumulación en el ambiente. Sin embargo, estos siguen vendiéndose y usándose indiscriminadamente en México, debido a la falta de normativa que los regule (OECD 2021).

Los plaguicidas se pueden clasificar de acuerdo con su selectividad, de amplio y bajo espectro. También se pueden clasificar en plaguicidas sistémicos, si tienen la capacidad de moverse a diferentes partes de la planta, protegiendo los diferentes tejidos o actúan donde son aplicados. Además de las propiedades de los plaguicidas mencionados, su especificidad la determina la dosis utilizada, el tiempo y la forma de aplicación.

Efectos letales

Las abejas son altamente sensibles a una gran variedad de plaguicidas. De manera directa, se puede atribuir la muerte de miles de colonias de abejas al uso de plaguicidas (Johnson *et al*. 2010). Esta sensibilidad, más alta que en otros insectos, es atribuida a que poseen hasta 10 veces menos enzimas de detoxificación de plaguicidas como la glutatión S-transferasa o la citocromo monooxigenasa p450, ambas involucradas en el metabolismo de los plaguicidas (Claudianos *et al*. 2006). Los plaguicidas se consideran altamente tóxicos para las abejas si se requieren menos de 2 microgramos por abeja para matar al 50 % de la población evaluada, medianamente tóxicos de 2 a 11 microgramos y relativamente no tóxicos si se requieren más de 11 microgramos.

Efectos subletales

La asociación simbiótica de las abejas con su microbiota se ha mantenido por periodos largos de tiempo, resultando en una fuerte dependencia mutua. Ya que las abejas dependen fuertemente de los microorganismos simbióticos asociados para su nutrición, la falta de estos conlleva a la pérdida de rutas metabólicas esenciales. Además, los microorganismos intervienen en la detoxificación de los plaguicidas de manera directa ya que cuentan con las rutas metabólicas o de manera indirecta modulando las respuestas metabólicas de detoxificación del insecto. La pérdida o alteraciones de estos microorganismos puede resultar en efectos que no causan directamente la muerte, pero sí consecuencias negativas en su desempeño, aumentando la susceptibilidad a patógenos, mayor sensibilidad a plaguicidas, efectos en su nutrición, defensa, reproducción y comunicación.

Efectos en la nutrición

Los plaguicidas afectan la nutrición de las abejas de diferentes maneras, entre las que se encuentran la reducción de la ingesta de alimento, disminución de la digestibilidad, afectaciones en su metabolismo y la reducción de las posibles fuentes de alimentación en abundancia y diversidad. A su vez, algunos insecticidas neonicotinoides reducen las capacidades de forrajeo, afectando la orientación y la movilidad de las abejas, lo cual repercute negativamente en su nutrición.

Efectos en la reproducción

Las abejas que han sido expuestas a dosis subletales de neonicotinoides muestran efectos adversos en la oviposición, fertilidad y apareamiento. Aunado a esto, la exposición a neonicotinoides afecta el desarrollo de ovarios y la espermateca de las reinas además del conteo, viabilidad y movilidad de espermatozoides de los zánganos.

Efectos en el sistema inmune

El sistema inmune de los insectos es uno de los principales factores de protección contra una variedad de plagas y parásitos. Los plaguicidas tienen efectos variados sobre estos sistemas, afectando las respuestas humorales (inmunes), celulares, oxidativas y de comportamiento resultando en un aumento en la susceptibilidad a plagas y a enfermedades.

Efectos en los sistemas comportamentales

Los efectos subletales de los plaguicidas neonicotinoides en los receptores de la acetilcolina y en la activación y desactivación de los canales de sodio y de cloro conllevan a efectos negativos en el comportamiento y en la memoria de las abejas, tienen efectos de hiper o hipo-sensibilidad, afectando los tiempo y distancias de vuelo, así como su capacidad de navegación, lo cual reduce la cantidad de recursos que pueden recolectar para su colonia y su capacidad para regresar a esta. Afectan el aprendizaje olfativo y visual, lo cual tiene efectos negativos en la selección de fuentes de alimento, decisiones, tareas y evasión de depredadores.

Efectos en los sistemas comportamentales

Los plaguicidas sistémicos se pueden acumular y permanecer en el suelo por largo tiempo. Además, se pueden mover a diferentes partes de la planta como al tallo, raíces, glándulas extraflorales, flores y semillas, causando efectos letales o subletales en el insecto. Los microorganismos del suelo desempeñan un papel clave en el desarrollo de las

plantas, estimulan su crecimiento directa o indirectamente mediante aportes a su nutrición, defensa ante plagas y producción de fitohormonas a cambio de refugio y fotosintatos. Los microorganismos rizosféricos facilitan la toma de fósforo y propician la fijación y asimilación del nitrógeno en las plantas, de los cuales depende indirectamente el desarrollo y nutrición de los insectos que se alimentan de estas (Lau y Stephenson 1994). Estos microorganismos rizosféricos son cruciales para la modulación de las interacciones microorganismo-planta-abeja y son susceptibles a una gran variedad de plaguicidas.

Conclusiones

Se requiere generar información básica y aplicada sobre los efectos directos, indirectos, letales y subletales de los plaguicidas en las abejas, para comprender mejor cómo pueden afectarlas y de esta manera tomar mejores decisiones sobre su uso seguro. Además, estos estudios deben considerar los posibles efectos en las interacciones multitróficas planta-microorganismo-abeja.

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4.3 Tercer capitulo

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Honey bee protein and lipid nutrition in avocado and blueberry agroecosystems with conventional and organic management

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Abstract

Honey bees play a crucial role in agricultural production. Farmers rely on commercial bee pollination to obtain optimal yields, and beekeepers on the income from pollination fees. However, commercial pollination confronts bees with pests and pathogens, pesticides, and

low-quality food, which in many cases do not fulfill the minimal nutritional requirements of honey bees. In this work, we evaluated honeybee nutrition in avocado and blueberry agroecosystems under organic and conventional management, by assessing the nutritional quality of pollen and bee bread based on their protein, fatty acid, and microbial content, and by analyzing honeybee health through the examination of abdominal fatty acid profiles and head protein content. Low protein content in honey bee hemolymph was evident under conventional management, resulting in alterations in bee bread microbial composition. Regardless of management, avocado pollen showed higher protein levels, which were translated into higher honey bee head protein levels. However, higher protein levels in avocado pollen were also translated into reduced fatty acids in bee bread and in honey bee fat. Crop system and beebread microbial composition altered the plant-honey bee nutrition cascades transforming pollen to beebread with increased amount of saturated, unsaturated, and total fatty acids. In conclusion, both crop species and crop systems determine honey bee nutrition through alterations in the pollen transformation, affecting protein and fatty acid assimilation.

Keywords

Honey bee nutrition; Nutrition cascades; Agroecosystems; Fatty acids; Honey bee microorganisms

Statements and Declarations

The authors have no relevant financial and non-financial interest related to the work to declare.

Introduction

Honey bees are among the most important pollinators, playing a crucial role in agroecosystems (Reilly et al. 2020). Semi-domestication of bees allows beekeepers to provide pollination services with hives to farmers at a commercial scale assuring world food supply (Aizen and Harder, 2009). However, agricultural crops do not always fulfill the minimal honey bee nutritional demands in terms of protein and carbohydrates (Colwell et al. 2017). Pollen is the only source of proteins and lipids for honey bees (Corby-Harris et al. 2018). After pollen collection by forager honey bees, the pollen is mixed with honey, inoculated with microorganisms, and stored. After hatching, honey bees start to consume bee bread, which is known to be important in relation to their longevity, body size, ovary development, and larval growth (Vanderplanck et al. 2014). Biochemical transformation of pollen to bee bread is mainly made by yeasts (Haydak 1958) and *Lactobacillu*s spp. (Chevtchik 1950), transforming pollen into a rich and complex food, increasing nutrient bioavailability, carbohydrates, enzymes, vitamins, and lactic acids (Khalifa et al. 2020). Besides pollen pre-digestion, pollen-borne microorganisms play an important role for bee nutrition and deficiencies of microorganisms in their diet have been shown to cause poor development and survival (Dharampal et al. 2019). In hives, microorganisms are both vertically and horizontally transmitted via social contact, and after emergence honey bees are inoculated by older bees. Hence the bee microbiome is well conserved, but susceptible to environmental changes, diseases, feeding habits and pesticide exposition (Yoder et al. 2013; Kwong and Moran, 2016; Diaz et al. 2019**).**

Protein is one of the principal components of honeybee nutrition and is naturally obtained by plant pollen. The nutritional value of pollen is defined by its protein percentage and amino acid composition (Crailsheim, 1990), which fluctuate from 9 to 37% depending on the origin (Liolios et al. 2005). Lipids are another important component for honey bee

nutrition, which are crucial in the production of fat, glycogen, and cell membranes (Graham 1992). Oleic and palmitic fatty acids are two of the principal fatty acids found in honey bees (Manning 2001) and imbalance of these fatty acids may cause honey bee learning deficiencies (Arien et al. 2018; Bennett et al. 2022) and alterations in immune reactions (Bedick et al. 2001). Fatty acids not only can give us insight of the honey bee nutritional status, but also provide qualitative and quantitative information of the microbial community trough specific microbial biomarkers (Liu et al. 2019)

As before mentioned bee bread fermentation is made principally by microorganism like yeast and bacteria. Pesticides like fungicides, herbicides and insecticides are known to have a strong non-target effects on bee bread microorganisms, and in this way indirectly affect honeybee health and nutrition (El Agrebi et al. 2020).

In contrast to other bees, honey bees have been shown to lack the ability to select pollen with good nutritional values (Corby-Harris et al. 2018) and therefore the availability of resources rich in nutrients is critical for their nutrition and health. Diversified floral resources in agroecosystems provide spatial and temporal nutrient supply (Decourtye et al. 2010). In contrast, conventional crop systems with monoculture represent a challenge for honey bee nutrition (Colwell et al. 2017) since pollen and nectar nutritional values differ between crops of which some are lacking essential fatty acids, amino acids, and minimal protein content to fulfill the honey bee nutritional needs to support colony development. In conventional crop systems pesticides adversely affect honey bee nutrition trough intensive herbicide applications which reduce bee food sources. In addition, pesticides can lead to alterations in bee bread microbial composition (El Agrebi et al. 2020). Added to this, in general pesticides negatively effects fatty acid metabolism by suppressing fatty acid

synthesis (Erban et al. 2019) and by reducing food consumption and sugar levels on haemolymph (Tosi et al. 2017).

Avocado (*Persea americana*) and blueberry (*Vaccinium corymbosum*) are two high value crops, which rely on fertilization and pest management products either biological (organic) or as agrochemicals (conventional) like pesticides and mineral fertilizers (Aktar and Chowdhury, 2009). In Mexico, avocado is one of the leading crops produced in large areas as monocultures, while blueberry is commonly produced under closed plastic tunnels limiting natural pollination. The direct effect of agricultural management has been widely studied in terms of honey bee pesticide exposure (Crenna et al. 2020), but knowledge about indirect effects of agricultural management on honey bee nutrition is limited (Sánchez-Bayo et al. 2017).

Here we studied the effects of crop system (organic and conventional) and crop type (avocado and blueberry) on honey bee nutritional cascades focusing on fatty acids and proteins in pollen, beebread and in honey bees. We hypothesized that conventional management would have negative effects on honey bee nutrition cascades reducing potential honey bee food sources. Our prediction was that honey bee nutrition depends on both crop species and management with conventional blueberry systems representing the lowest nutritional value for honey bees.

Materials and methods

Field site location

In central Mexico, newly created nucleus hives were established in avocado and blueberry agroecosystems both with organic and conventional production systems. Organic systems were certified by third-party organizations according with the federal regulatory program for organic produced agricultural products, and conventional sites followed the guidance of the secretary of agriculture and rural development. Sites with similar size plots and characteristics (climate, age of the crop, weed management, pesticide and fertilization schemes) were selected in avocado and blueberry (Blueberry: Organic 19°22'13.3"N 101°28'54.4"W, and conventional 19°22'37.7"N 101°29'21.6"W and avocado: Organic 19°28'16.9"N 102°01'32.3"W, and conventional 19°28'49.1"N 102°01'24.8"W).

45 days before peak blooming, 10 nucleus hives with a newly mated hybrid Italian/Africanized queen obtained from a local supplier and three frames of different age brood and one as reserve (honey and bee bread) were placed in each site. Hives were supplied empty frames with organic certified bee wax foundation. At peak blooming, and after one month of hive placement, three randomly selected hives were sampled per crop, management, and site (n=3). Only colonies showing normal development with Varroa levels below 1% and no visible signs of other diseases were selected for the study. In each hive, a capped frame with pupae ready to emerge was caged with a metallic mesh. Emerged bees were marked daily in the thorax with a color code, and after seven days, ten adult honey bees per hive were sampled for subsequent analysis. Also, five grams of randomly collected beebread, was collected from newly introduced foundation frames, using an alcohol disinfected spatula.

Pollen

Pollen was collected from the anthers of flowers at various sites and crops as mentioned above. Three randomly selected plants per site were used to create a composite sample for

each plant. Pollen was cold macerated using 1 ml of saline phosphate buffer, vortexed 10 secs and the supernatant was separated by centrifugation 1 minute at 3000 g. Hereafter pollen protein from the supernatant was measured using a BCA Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions at 595 nm. Final protein concentration was calculated according to the corresponding standard curve (Degrandi-Hoffman et al. 2015). Fatty acid methyl esters (FAMEs) were extracted from 200 mg of hand collected pollen in a three-step analysis: saponification with sodium hydroxide, methanol and water for 30 minutes at 100 ºC; methylation with chlorohydric acid and methanol for 10 minutes at 80 º C and extraction with hexane and methyl tert-butyl methyl ether. Fatty acids were detected in a gas chromatograph (Agilent 7890B) at 300 ºC equipped with a fused silica capillary column and analyzed by Sherlock software version 3.1 (MIDI Inc. Delaware, USA). Fatty acid identification was made by comparison with an external calibration standard and quantification with the addition of an internal standard 19:0 (nonadecanoic methyl ester, Sigma) of known concentration and comparing peak areas. To determine pollen quality, saturated, unsaturated, and total fatty acid biomarkers were summed and compared in the different treatments.

Bee bread

Floral origin of bee bread was determined by microscopy at 400x magnification in an Olympus BX41 microscope, according with standard procedures adapted for bee bread (Bakour et al. 2019). Identification was made by morphological comparison with pollen collected from the main crop and with the pollen atlas database (pollenatlas.net) of 500 grains per sample. Two hundred milligrams of bee bread per hive were homogenized with a mortar and pestle at 4 °C until no visible intact grains were observed at 400x magnification. Hereafter bee bread pollen diversity and microbial composition was evaluated by FAME

analysis as described above. For each treatment, fatty acid biomarkers were grouped according to taxonomy (eukaryotic and prokaryotic) and type (saturated, unsaturated, and total). To compare the relative contribution of the prokaryotic and eukaryotic fatty acids in the bee bread, we summed iso and ante-iso and monounsaturated fatty acids as biomarkers of prokaryotes, while eukaryotic fatty acids were calculated by summing polyunsaturated fatty acids (Table 1). Protein percentage was measured as before mentioned.

Bee head

After thawing, heads of the bees collected earlier were dissected and crushed in a mortar containing a phosphate buffer solution. The protein was separated from debris by centrifugation at 3000 rpm for 10 minutes and the protein percentage was assessed as before mentioned.

Bee fat body

Fatty acids were extracted from bee recently crushed abdomens according to analysis as described above. Fat body quality was assessed by comparing the amount of saturated, unsaturated, and total fatty acids.

Honey bee gut yeasts and Lactobacillus

In order to identify beneficial microorganisms associated with honey bee guts, nine dissected honey bee midguts per site, three for each hive, were added to two different broths. MRS (Man, Rogosa and Sharpe) to promote Lactobacillus growth and YEPD for yeasts (yeast extract, peptone, and dextrose) acidified to a pH of 4.5 adding HCl 1N as needed. Broth enrichment cultures were incubated for 72 hours at 37 º C for MRS and 30 º C for YEPD. Hereafter, 40 mg of cells from the exponential growth phase were harvested and characterized by biomarker fatty acid analysis as described above.

Statistics

Fatty acids in pollen, bee bread and abdomens, pollen protein and bee head protein were analyzed with Generalized Linear Mixed Model (GLMM) with Gamma distribution, using crop and management as fixed effects and random effect for hive ID was included (Bates et al. 2022). For visualization of the dataset, a Principal component analysis (PCA) was performed in R studio (Team, R. C. 2013 version 1.2.5019) with Factoextra package (Kassambara. 2017). Structural equation models were used to test the direct and indirect effects of pollen protein and fatty acids on honey bee nutrient assimilation, all the plausible variables and their interactions are tested by d-separation test for evaluation of possible missing paths among unconnected variables (Shipley and Douma 2021), overall fit of the network of causality was made by piecewiseSEM and model acceptance based on Fisher´s C statistics (p>0.05) and selecting the model with the best global goodness of fit with the piecewisesem package (Lefcheck, 2016) in R studio (Team, R. C. 2013 version 1.2.5019).

Results

Protein in pollen was significantly higher in avocado than in blueberry, regardless management type (Figure 1A). In the same manner, bee bread protein content showed higher levels in avocado when compared with blueberry (Figure 1B). Neither in pollen protein nor in bee bread significantly interactions were noticed between management and crop.

Saturated fatty acids of pollen from both avocado and blueberry agroecosystems were significantly higher with organic management compared to conventional management. In conventional management, pollen from blueberry had even lower levels of saturated fatty

acids when compared with avocado pollen (Figure 2A). In terms of unsaturated fatty acids, pollen from avocado with conventional management showed the highest levels, whereas conventional blueberry the lowest. The opposite was observed with organic management, where the amount of unsaturated fatty acids was higher in blueberry than in avocado (Figure 2B). In terms of total fatty acids, the only difference observed was that blueberry pollen from conventional management had the lowest amount of total fatty acids (Figure 2C).

Microscopy analysis of bee bread showed that in organic avocado 78% of identifiable pollen belong to the main crop, 83% in conventional avocado, 92% in organic blueberry and 92% in conventional blueberry.

Total fatty acids in bee bread showed significant differences for crop management, with organic management resulting in higher levels of total fatty acids in beebread than conventional management (Figure 3A). Saturated fatty acids of beebread showed higher levels in organic blueberry than in organic avocado, and for conventional management lower levels of saturated fatty acids in bee bread were observed regardless the crop (Figure 3B). In terms of unsaturated fatty acids in bee bread, no significantly differences were found, neither for crop management nor crop (Table 1).

The ratio between eukaryotic and prokaryotic fatty acid biomarkers in bee bread was higher in organic than in conventional management (Figure 4A) and higher in blueberry than in avocado (Figure 4B), while no interaction was observed for these two factors. Yeast biomarkers in bee bread showed no significantly differences neither for crop management nor crop (Table 2). *Lactobacillus* biomarkers in bee bread were higher in organic management when compared with conventional, whereas no significant differences were found for the factor crop (Figure 5). Protein percentage of the bee head was significantly

higher in avocado in both organic and conventional management, whereas bees from conventional blueberry showed the lowest level of head protein percentage (Figure 6). The amount of saturated fatty acids in bee abdomen was highest in organic avocado and lowest in conventional avocado (Figure 7). No differences were observed between treatments for unsaturated fatty acids in honey bee abdomen. First two components of the principal component analysis contributed to the 61.6% of the experiment variance, multivariate analysis showed a separation in the first component (Dim1) between the conventional avocado and blueberry groups. In the first component (Dim 1) conventional blueberry group is in the bottom followed by conventional avocado, organic blueberry and in the top, organic avocado, this separation is caused principally by an increase in saturated fatty acids in bee bread, total fatty acids in bee bread, saturated fatty acids in pollen and unsaturated fatty acids in bee bread (Figure 8).

The structural equation model of honey bee nutrition cascades best fitted model $p=0.27$, explained 68% of the variation in bee bread protein, 67% in bee bread microorganisms, 76% of bee protein, 80% in linoleic fatty acid and 92% on oleic fatty acid. Oleic and linoleic acid in fat were significantly positive correlated with total fatty acid in pollen. Besides, bee bread protein, bee protein, linoleic acid in fat and bee microorganism abundance in pollen were positively correlated with pollen protein. In addition, bee bread microorganisms were positively correlated with total fatty acids in pollen and linoleic fatty acid with unsaturated fatty acids of bee bread. Finally, oleic acid in fat was negatively correlated with bee bread protein and multigroup analysis showed that all paths are constrained to the global models showing no differences depending on the management or crop (Figure 9).

Discussion

Here we show that both crop species and crop system determine honey bee nutrition, though crop species to a higher extent than crop system. Overall, our results clearly show that avocado pollen provides higher quality honey bee nutrition than blueberry pollen. As predicted, crop species and system significantly affected honey bee nutrition with strongest contrast found in conventional blueberry providing lowest values for honey nutrition. Crops and not management define the availability of alternative pollen sources to the main crop, being blueberry the one with the poorest pollen diversity in bee bread, this is in part explained because bees are kept inside plastic macro tunnels. Also, avocado crops in the area, are widely distributed in large extensions in the avocado strip, preventing bees to obtain alternative sources of pollen to the main crop (Saenz-Ceja et al. 2022). In addition, regulatory programs prohibit weeds in crops destined to exportation reducing alternative sources of pollen (De la Federación, D. O. 2002). Our results showing low pollen protein in blueberry is in accordance with Colwell et al. (2017), which has been further shown to lead to poor colony development (Dufour et al. 2020), since such low protein levels do not fulfill the minimal honey bee nutritional requirements (Haydak 1970). Though a slight increase in protein content in beebread compared to pollen was seen for blueberry systems, this was still lower than what was found for avocado systems. Assimilation of protein in honey bee head seems to be linked with both crop species and management, where bees from agroecosystems with low pollen protein percentage showed low levels of protein in the head, which was further underlined in conventional crops. Protein is important for nursing, hypopharyngeal and mandibular glands, which is provided to larvae and young bees by nurse bees storing and disseminating protein (Crailsheim, 1990). After hatching,

honey bee head protein increases in a 92% (Haydak, 1934), crucial for carbohydrate metabolism, antioxidant activities, detoxification and learning (Zaluski et al. 2020). Overall, the amount of fatty acids, saturated as well as unsaturated, was higher in beebread than in pollen, most likely mediated by microbial fermentation and the addition of other pollen sources. However, though avocado pollen from conventional agroecosystems had the highest amount of unsaturated fatty acids, this was not transformed in to higher levels of unsaturated fatty acids in bee fat. Unsaturated fatty acids play an important role in honey bee nutrition (Manning, 2001) and disease prevention (Feldlaufer et al. 1993).

The ratio between eukaryotic (plants and fungi) and prokaryotic (bacteria) fatty acids can provide information about the general microbial composition in the bee bread during pollen fermentation revealing useful information about the relative abundance of microorganism and their fatty acid contribution to honey bee nutrition. Interestingly in the present study this ratio differed both between crop species and management system. Organic management increased the eukaryotic/prokaryotic fatty acid ratio and in terms of crop species, bee bread from avocado agroecosystems showed a better balance between eukaryotic and prokaryotic biomarkers compared to blueberry. Bee bread microbial diversity and abundances is related to pollen origin and nutrients (Vásquez and Olofsson 2009) and can be altered by pesticide exposition (Bartlewicz et al. 2016). Myristic, palmitic and linoleic fatty acids play an important role in honey bee attraction to pollen (Manning. 2001), which in the present study were more abundant in beebread from organic agroecosystems than in the conventional ones.

Contrary to other bees, honey bees seem to lack the ability to select pollen with desirable nutritional characteristics (Corby-Harris et al. 2018), which however, in this study was quantified from corbicular pollen without considering possible effects of pollen-borne

microorganisms. Indeed, it has been shown that pollen-borne microorganisms like yeasts and *Lactobacillus* represent a decision choice for the bees controlled by volatile emissions (Rering et al. 2018). Pollen microorganisms not only release basic nutrients to bees, but also play a direct role in bee nutrition (Dharampal et al. 2019). Principal component analysis showed clear differences in the crop species and management studied, as a result of changes in the fatty acid profiles, protein content of bee bread and pollen and in bee nutrient acquisition.

As an overall synthesis of our work, the structural equation model showed how fatty acid and protein assimilation is mediated by different drivers in the nutrition cascades, which should be taken into consideration, when studying honey bee nutrition. Despite the negative effects of monocultures in the honey bee nutrition, pollen diversity, protein and bee bread microorganisms honey bee nutrition cascades seems to be very well conserved, in concordance with the nutrition pathways in bees (Kunieda et al. 2006).

This study provides novel insights about fatty acid and protein cascades with the main finding that both crop species and management system alter fatty acid and protein assimilation but not the honey bee nutrition cascades. Site replication is labor intensive due to the difficulty of finding sites with similar characteristics, but in future experiments this should be considered thoroughly to improve the robustness of the results. Our findings suggest that pollen quality should not only be addressed with protein content, but also take into consideration fatty acids, which is important in honey bee health and nutrition.

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Figures

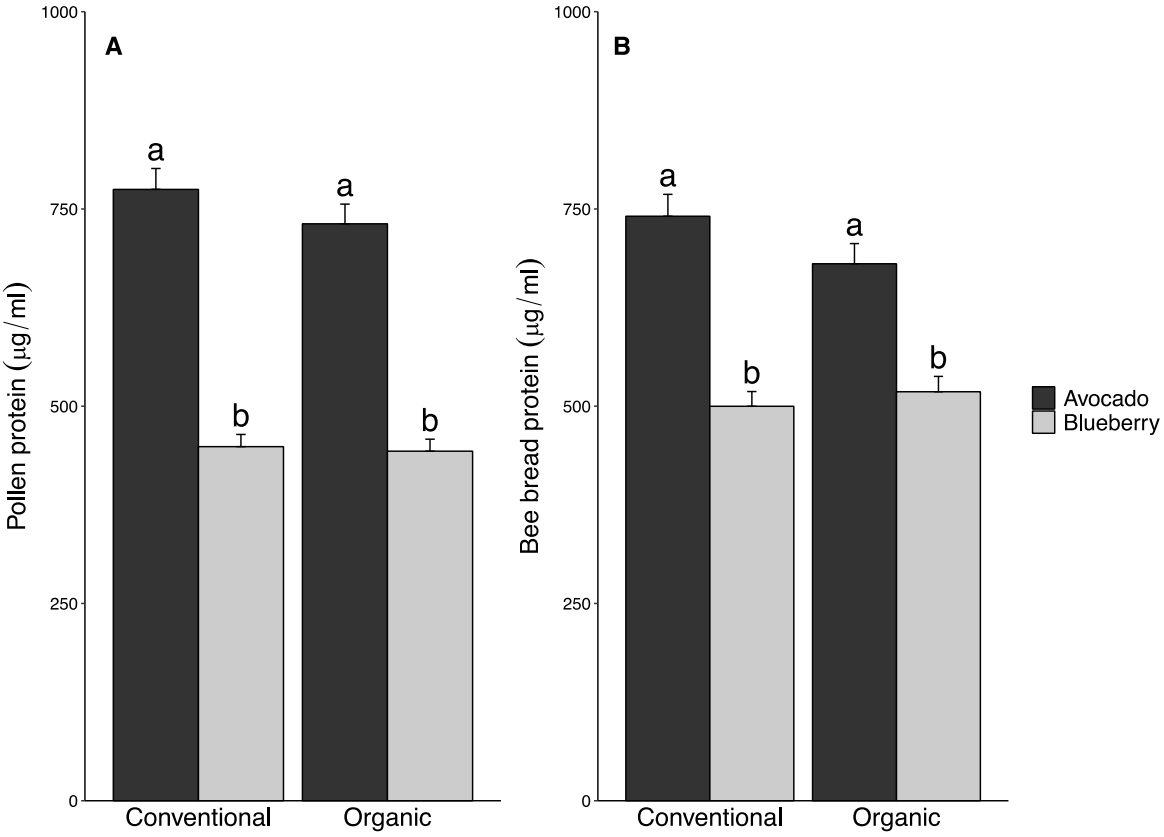


Figure 1. Protein in the pollen (A) and bee bread (B) in avocado and blueberry under organic and conventional management, different letters represent significant differences (*n*=3).

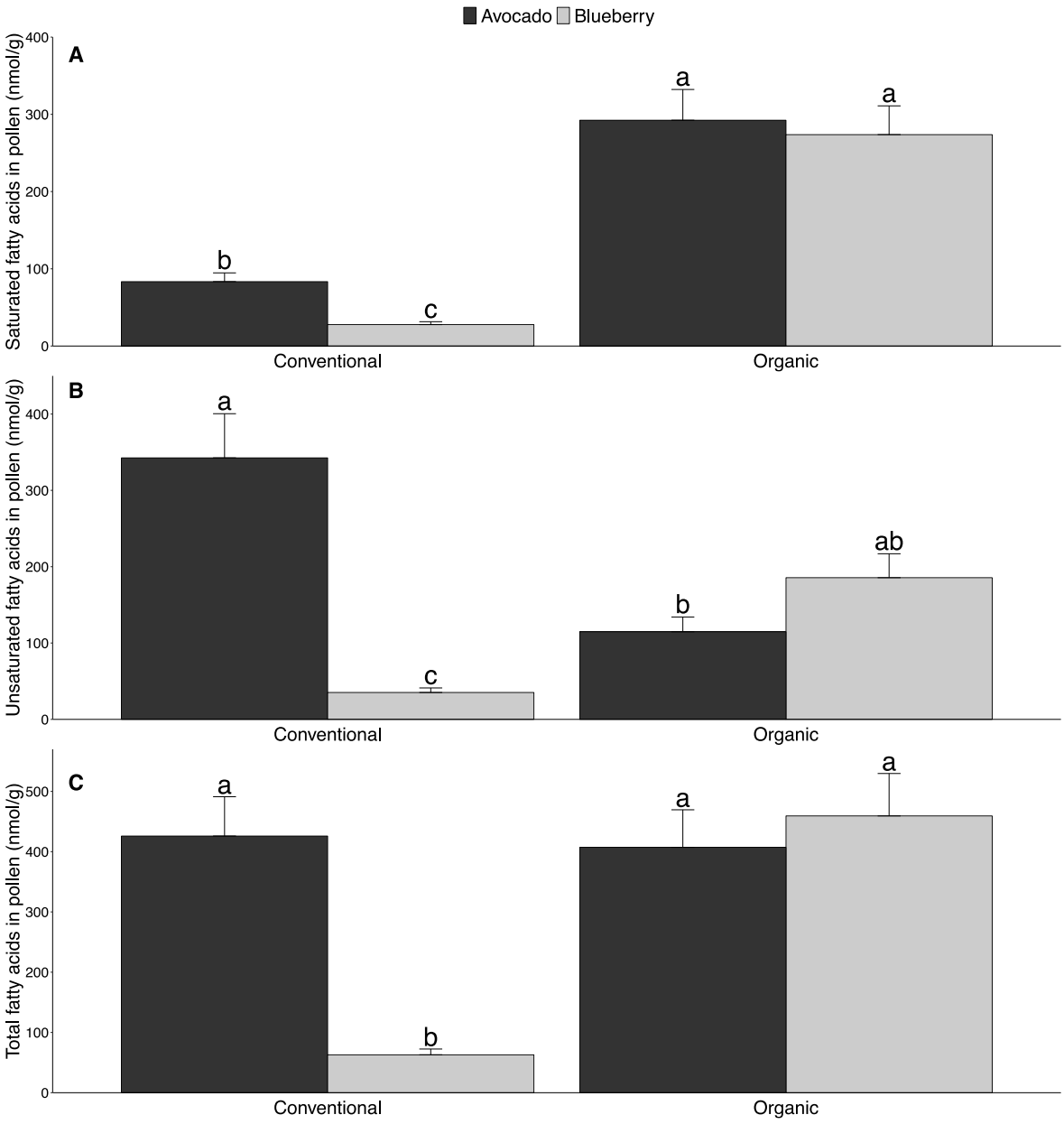

Avocado
Blueberry
A
Saturated fatty acids in pollen (nmol/g)
400
300
200
100
0
b
c
a
a
Conventional
Organic
B
Unsaturated fatty acids in pollen (nmol/g)
400
300
200
100
0
a
c
b
ab
Conventional
Organic
C
Total fatty acids in pollen (nmol/g)
500
400
300
200
100
0
a
b
a
a
Conventional
Organic

Figure 2. Saturated (A), unsaturated (B) and total fatty acids (C) in pollen from avocado and blueberry under organic and conventional management, different letters represent significant differences (*n*=3).

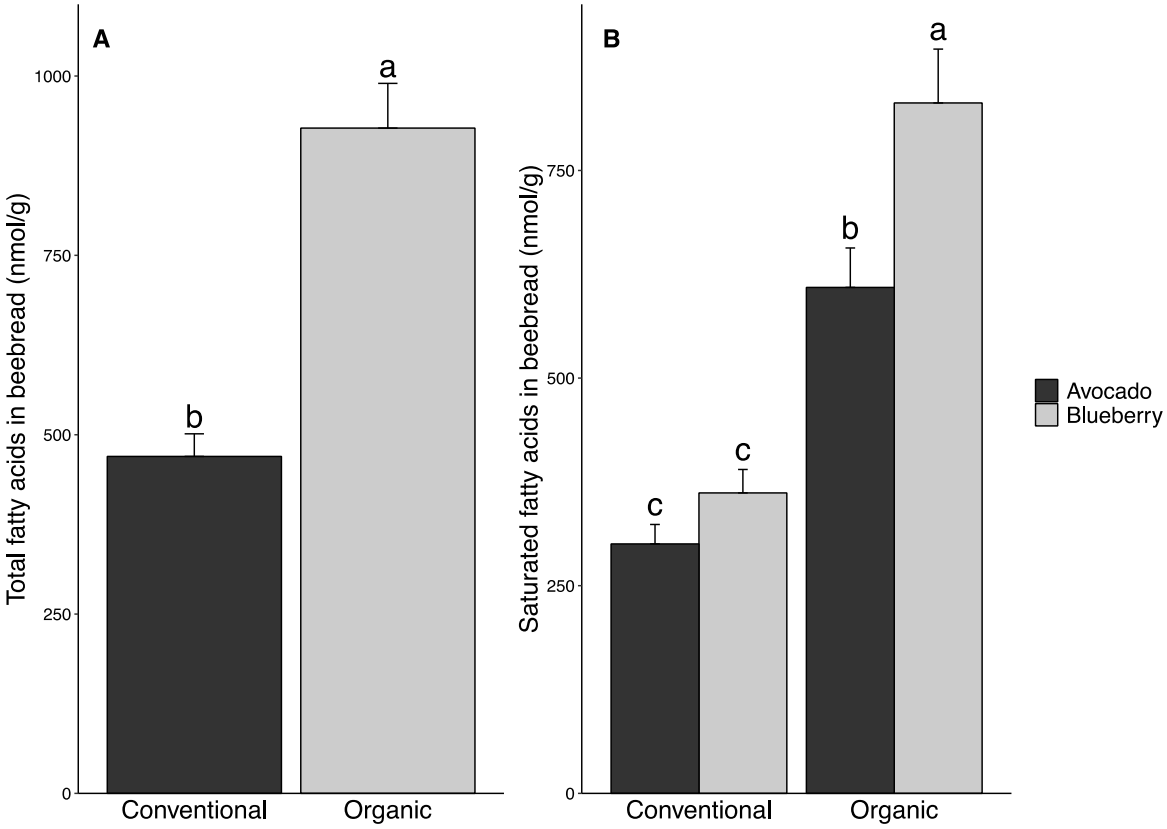


Figure 3. Total fatty acids in different crop system (A, *n*=6) and saturated fatty acids in bee bread from avocado and blueberry under organic and conventional management (B, *n*=3. Different letters represent significant differences.

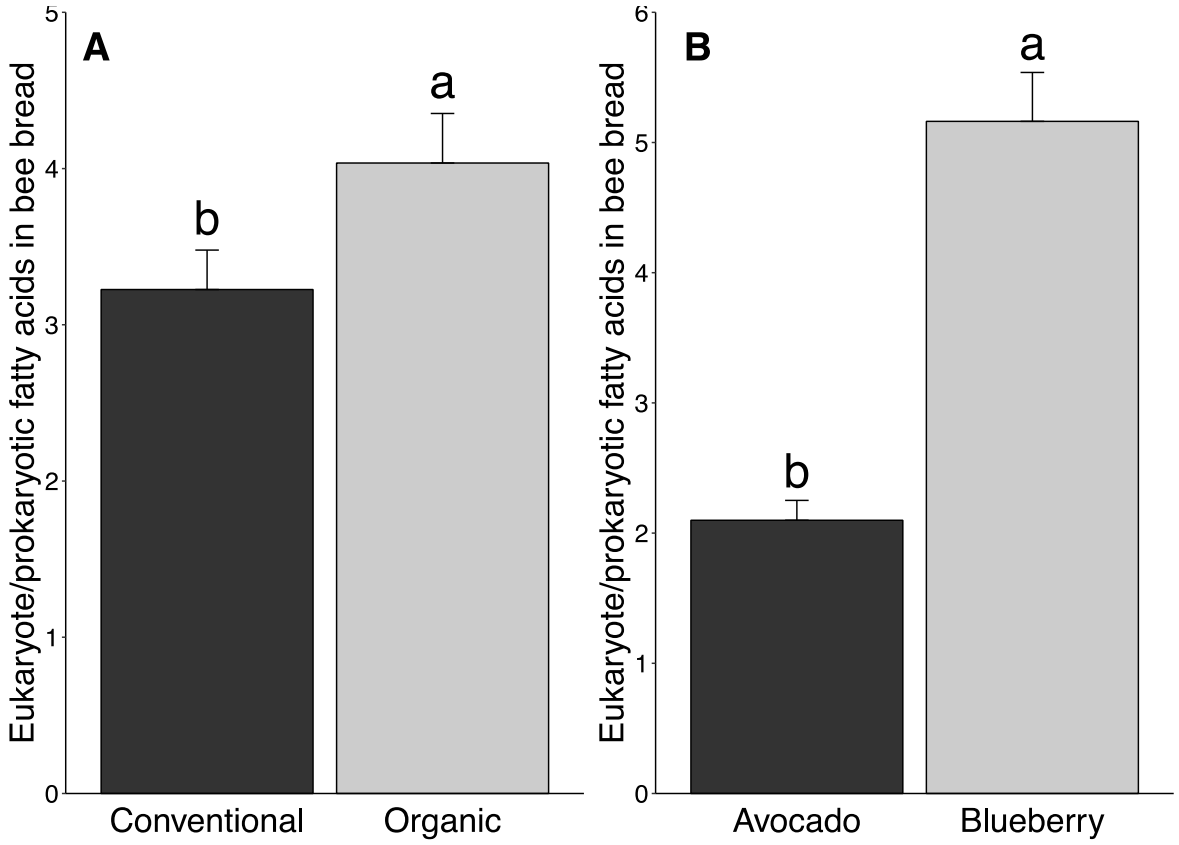


Figure 4. Factor treatment means of crop system (A, *n*=6)) and crop species (B, *n*=6) of the ratio between eukaryotic and prokaryotic fatty acids in bee bread. Different letters represent significant differences.

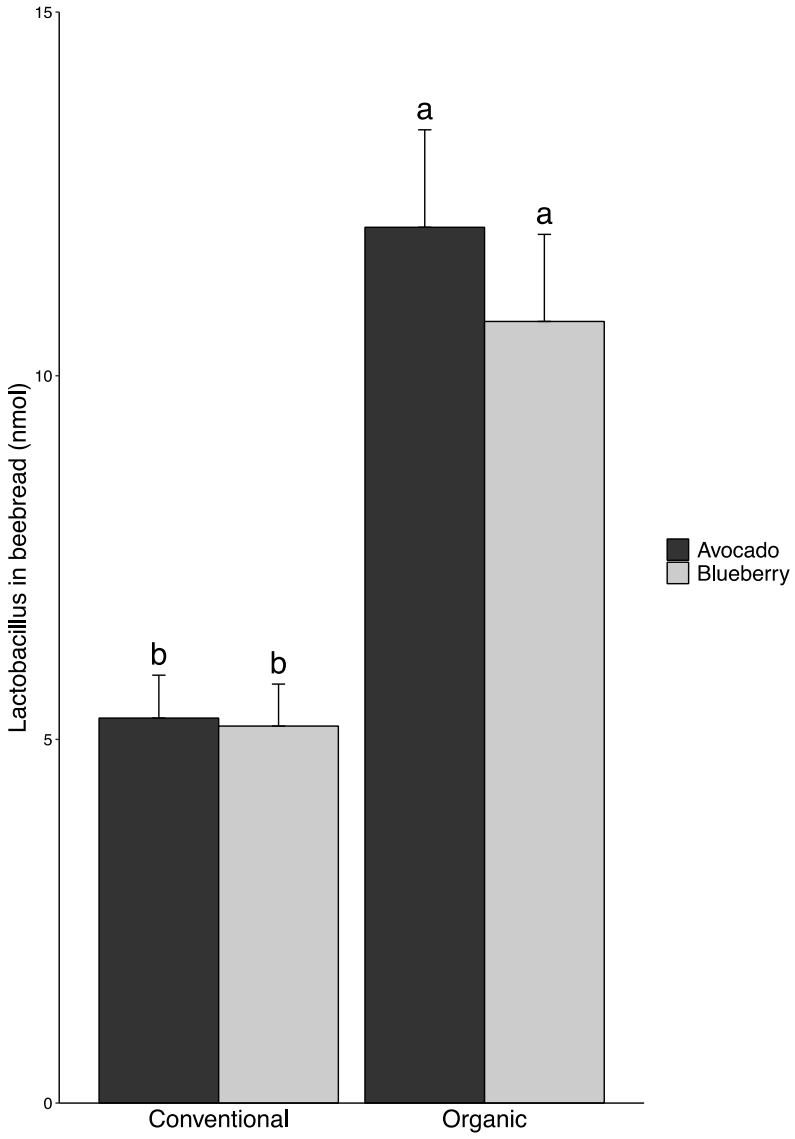


Figure 5. *Lactobacillus* biomarkers in bee bread culture from avocado and blueberry crops under conventional and organic management (*n*=3). Different letters represent significant differences.

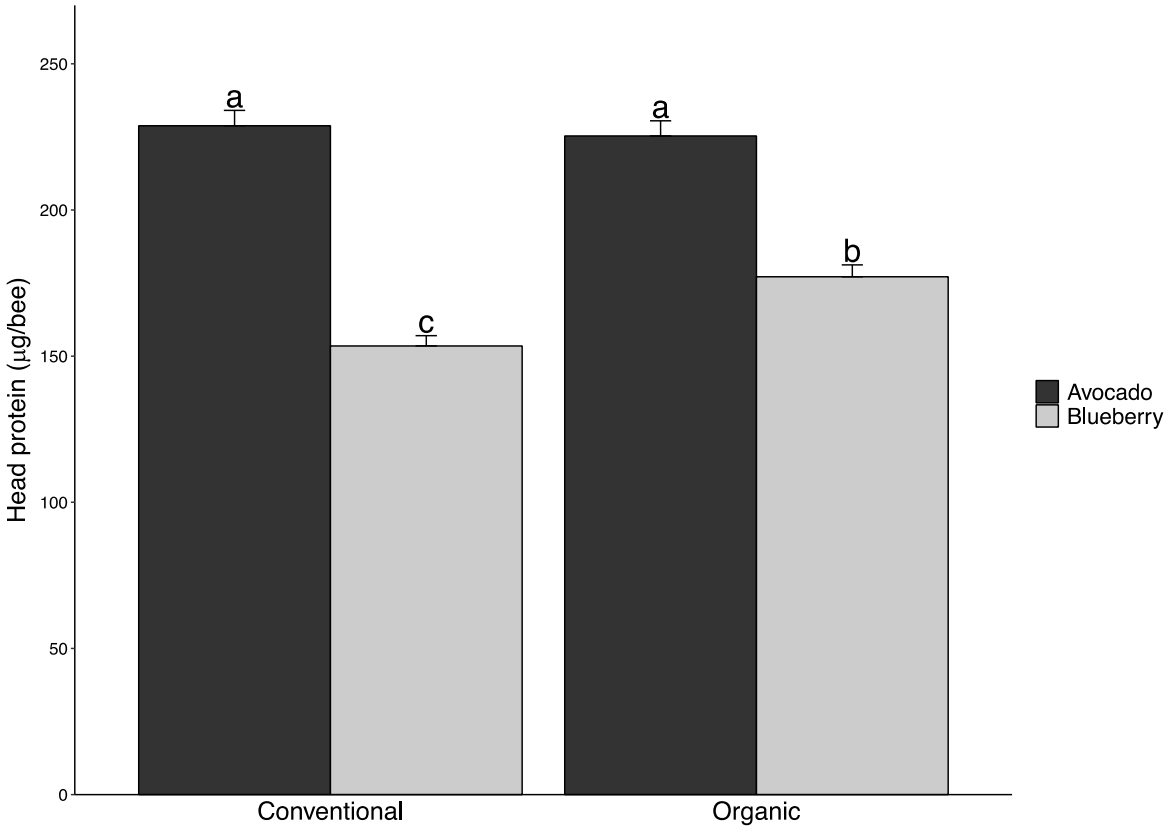


Figure 6. Protein in the bee head in avocado and blueberry crops under organic and conventional management (*n*=3). Different letters represent significant differences.

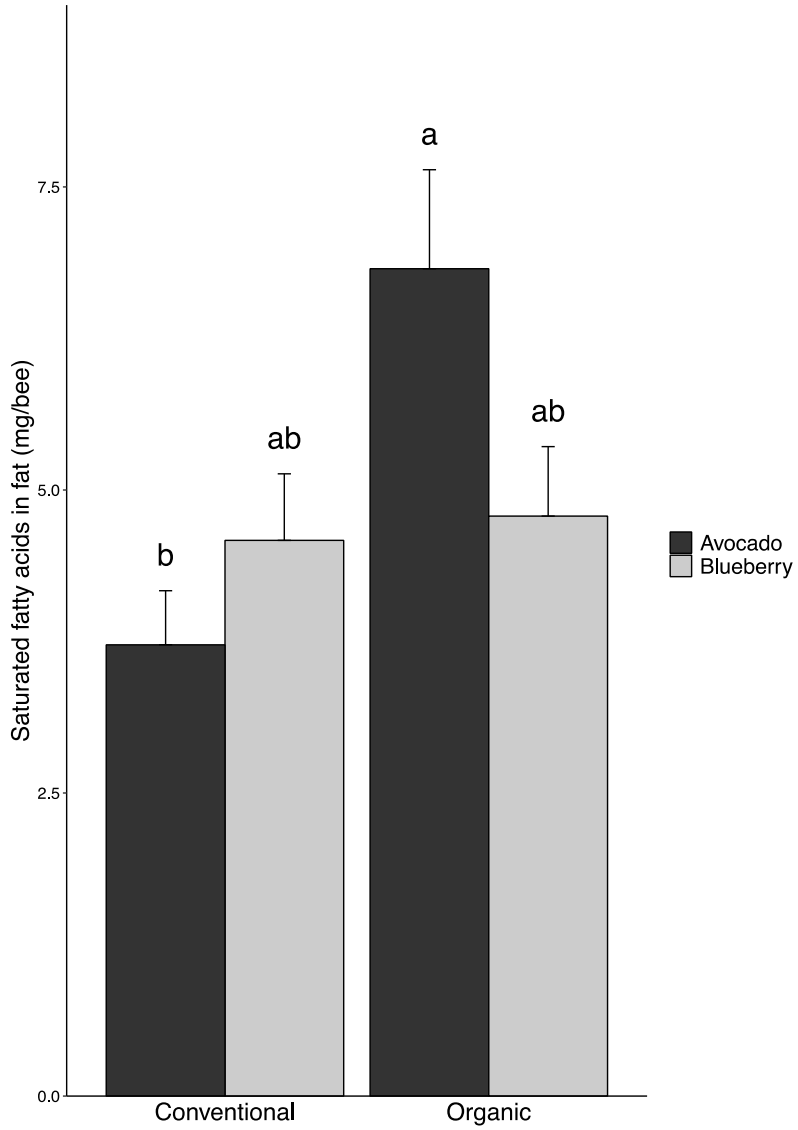


Figure 7. Saturated fatty acids in bee abdomen from avocado and blueberry under organic and conventional management (*n*=3). Different letters represent significant differences.

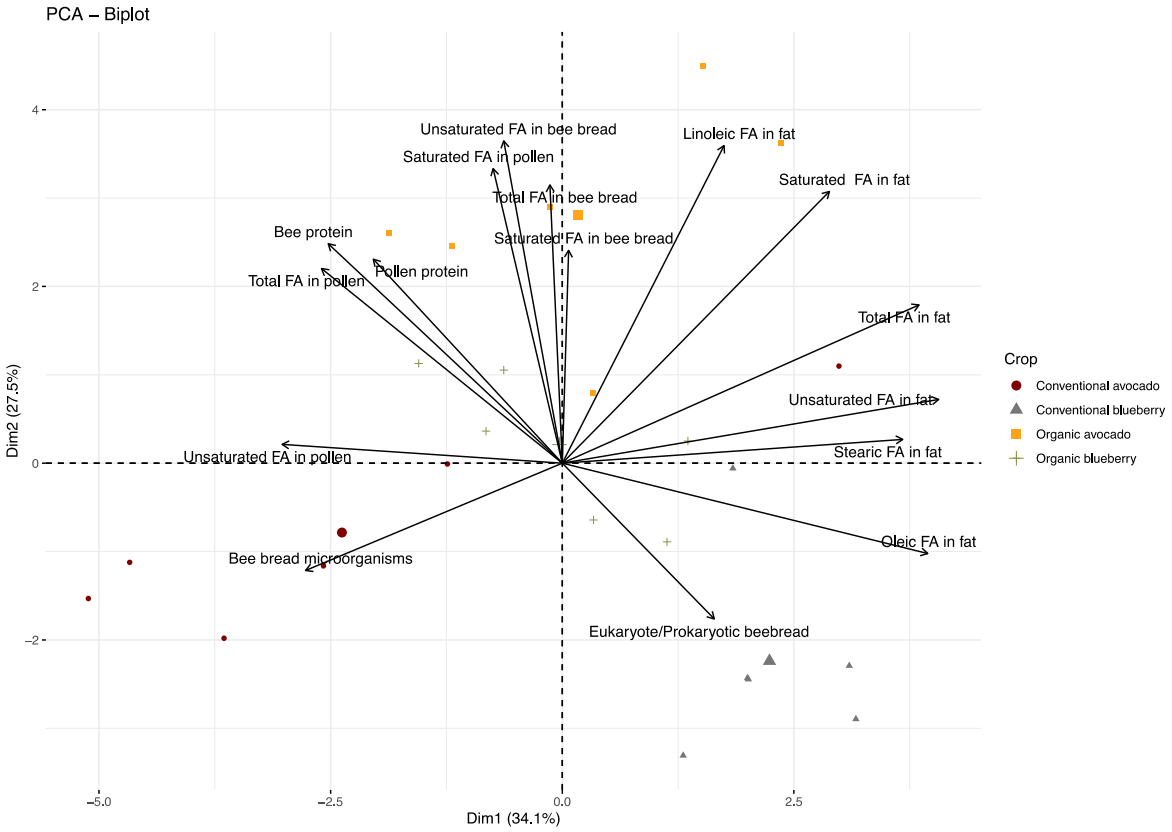


Figure 8. Principal component analysis biplot of all variables examined.

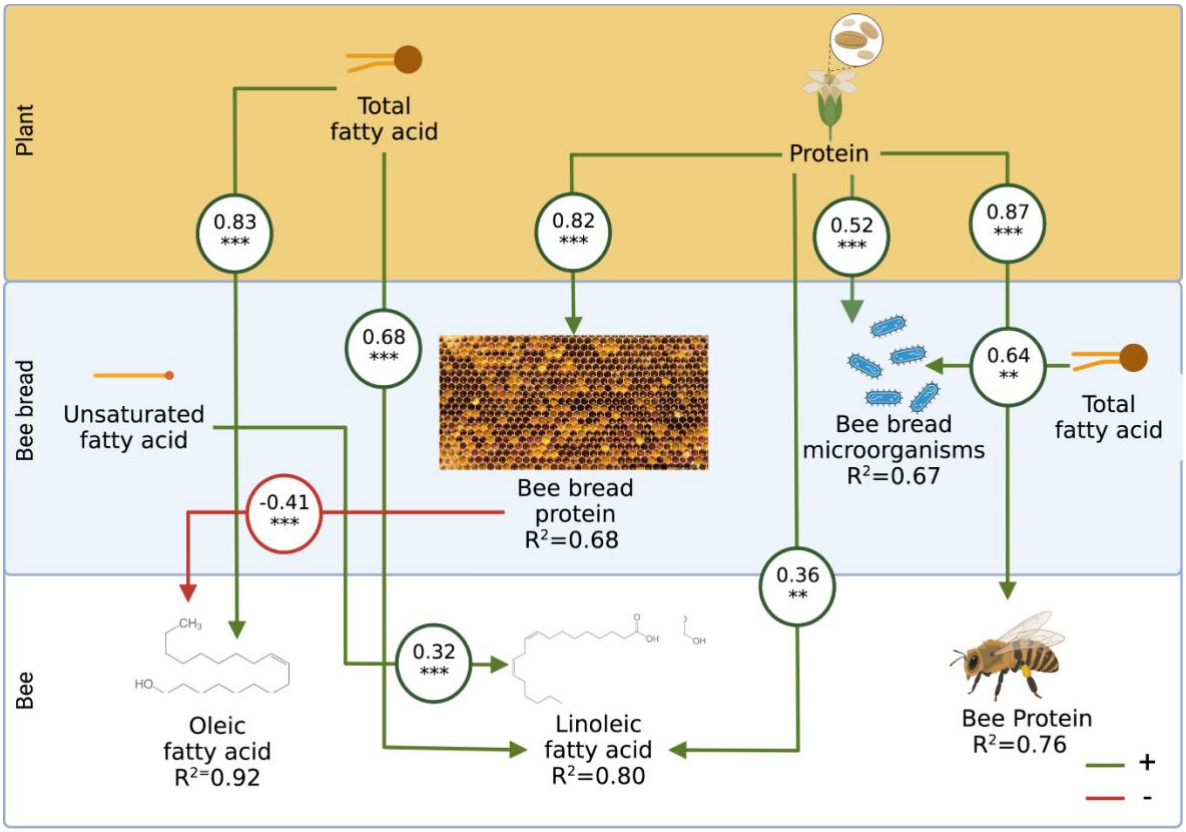


Figure 9. Structural equation best fitted model (p=0.27) of nutrition cascades in honey bees, green and red arrows are significant positive and negative coefficients, only significative regressions are presented, d separation test showed no significative missing paths.

Table 1. Fatty acid used as biomarkers

Biomarker	Fatty acid
Prokaryotes	14:0i, 15:0i,15:0a, 16:0i,17:0i, 17:0a ,10:0 3OH, 12:0 20H, 16:0 2OH and 16:0 30H
Eukaryotes	14:0, 16:0, 18:0, 20:0, 18:1 ω 9c and 18:2 ω6
Lactobacillus	14:0i, 15:0a and16:0i
Saturated	14:0, 16:0, 18:0 and 20:0
Unsaturated	18:1 ω 9c and 18:2 ω6

Table 2. *p* values of ANOVA analyses of the individual factors and their interaction for all variables measured

P values	Management	Crop	Management x crop
Pollen protein	0.35	***	0.39
Bee bread protein	0.36	***	0.13
Saturated in pollen	***	0.17	***
Unsaturated in pollen	0.17	***	***
Total in pollen	***	**	***
Saturated in bee bread	***	**	0.73
Unsaturated in bee bread	0.07	0.05	0.87
Total in bee bread	***	0.34	0.88
Head protein	*	***	***
Saturated in fat	**	0.32	*
Unsaturated in fat	0.60	0.17	0.17
Total in fat	0.18	0.59	0.07
Eukaryotic/prokaryotic	*	***	0.56
Yeast	0.23	0.08	0.06
Lactobacillus	***	0.47	0.85

p values significance codes: 0 '***'; 0.001 '**' 0.01 '*'

4.4 Cuarto capítulo

Non-target effects of soil application of pesticides on plant-microorganism-bee interactions with common vetch under field conditions

Introduction

Common vetch (*Vicia sativa*) is a crop used for livestock feed and as green manure or cover crop. It has the particularity of forming associations with beneficial microorganisms such as mycorrhiza and rhizobium, which provide the plant with phosphorus and nitrogen in the form of polyphosphates and glutamine, in exchange for lipids and carbohydrates such as sucrose and lactose (Iyer et al., 2016; Rich et al., 2017). Additionally, vetch provides insects such as honey bees with pollen and nectar that have desirable nutritional characteristics in terms of sugar and protein content.

Honey bees are essential in agricultural systems due to their pollination contributions for food production; pollination services provided by the honey bee (*Apis mellifera*) generate over 200 billion USD yearly (Meixner., 2010), accounting for 9.5% of the value of agricultural produce worldwide (Potts et al. 2010). In addition, honey bees produce honey, one of the most valuable products in the world, which is appreciated by his medicinal, nutritional, and organoleptic properties (Zafar et al., 2020; El-Senduny et al., 2021). Three of the most frequently used pesticides in the world for pest management are imidacloprid for insect pest control, glyphosate the most common broad-spectrum herbicide, and benomyl to control a wide range of fungal diseases (Alavanja, 2009; Jeschke et al., 2010; Benbrook, 2016). They have various modes of action (systemic and contact) and variable environmental persistence times. Direct effects of these pesticides on honeybees have been widely studied. However, information about indirect impacts of

pesticides on honeybees are limited. According to Hussain et al. (2009), commonly used agricultural pesticides can have negative impacts on plants by changing the rhizosphere's beneficial microbes and impairing nitrogen fixation or phosphorus solubilization. Therefore, they may influence how plants acquire nutrients and distribute them to pollen and nectar, influencing the interactions between plants, microorganisms and bees in a multitrophic system.

The objective of the present study was to evaluate the non-target effects of pesticides on soil microorganisms, roots and honeybees, testing the hypothesis that commonly used pesticides will alter the soil microbial composition, thereby, plant nutrient acquisition and indirectly plant-bee interactions.

Material and methods

Common vetch (*Vicia sativa*) plants were sown, in the autumn-winter 2021 crop cycle. Vetch was cultivated in an agricultural field, using micro-plots measuring 2.5 meters wide by 10 meters long (Figure 8). In each plot and one week after sowing for three consecutively weeks, one of the three pesticides was applied to the soil according to the experimental randomized block design, at concentrations recommended for pest and disease treatment in the field: fungicide (benomyl 1g l^{-1}), insecticide (imidacloprid 2ml L^{-1}), and herbicide (glyphosate 4ml L^{-1}), along with a control without pesticides, each treatment had four replicates.

Plant harvesting was carried out after the plants had grown for four months and had begun to blossom. For each treatment, we selected a one-meter linear section of the vetch plants, including their roots and the surrounding soil. To ensure that bees would feed in the designated treatments and to prevent bee escapes, each plot was covered with an insect

greenhouse constructed from PVC tubes and an insect proof. The greenhouse was designed to be insect-proof while allowing honey bees to fly freely. Additionally, a new beehive was set up for each plot. After installation, the beehives were left for 21 days. After that time, honey, bee bread (pollen fermented by bees before consumption), eggs, adult bees, brood, and larvae were harvested and counted.

For nutrient analysis, plant shoots were dried for seven days until no changes on weight were recorded. After that, 0.25g of the dried shoot material were sieved and digested. After 24 hours, samples were heated to 375 degrees Celsius for 3 hours in a digester block, in ramps of 50 ºC every 20 min (BMD-3050, Novatech). Extracts were filtered through Whatman No. 1 filter and recovered for colorimetrical analysis at 660 nm in a Braun+Luebbe III autoanalyzer (Seal, Inc).

Fatty acid analysis to determine microbial biomarkers and nutrition in soil, roots and bees. Rhizosphere soil samples (5g) were collected. After homogenization, the soil was stored at -80 ºC for subsequent lyophilization. Then, 1g of lyophilized soil was weighed and placed in glass tubes for analysis. Roots were cleaned with tap water, homogenized using dissection scissors, and then frozen at -80º C. After freezing, the roots were lyophilized, pulverized, and placed in glass tubes for posterior analysis. Capped honey and 7-days-old bee bread (1g) were randomly collected using a disinfected spatula from newly introduced frames with new foundation and cells. Following collection, the honey and bee bread were frozen at -80 and then lyophilized. A subsample of 0.2 g was weighted and stored in glass tubes for posterior fatty acid analysis.

The fatty acid analysis consisted in a three-step process; saponification with sodium hydroxide, methanol and water, methylation with chlorhydric acid and methanol and

extraction with hexane and methyl tert-butyl methyl ether. Fatty acids were detected in a gas chromatograph equipped with a flame ionization detector (Agilent 7890B and analyzed by Sherlock software version 3.1 (MIDI Inc., Delaware, USA). Quantification was performed by adding an internal standard, 19:0 (nonadecanoic methyl ester, Sigma), of known concentration. The fatty acid biomarkers used for microbial identification were: Gram positive bacteria: 13:0 anteiso, 13:0 iso, 14:0 iso, 15:0 iso, 16:0 iso, and 17:0 anteiso. Gram negative: 10:0 3OH, 12:0, 12:0 2OH, 16:0 2OH, 15:1 w6c, 17:0 iso 3OH, 17:1 w8c, and 18:1 w5c. Actinomycetes: 19:0 10-methyl. Fungi: SF5 (18:0 anteiso and/or 18:2 w6,9c) As input variables, we tested the effect of imidacloprid, benomyl, and glyphosate on the response variables of soil, root, and honey bee fatty acids, as well as in plant nutrition. For statistical analysis. Generalized linear models (GLMs) were fitted using an exhaustive analysis with the lowest AIC and Post-hoc Tukey´s test by least square means package (Emmeans) (Lenth, 2018) The statistical analyses were conducted using R version 3.4.1 (R Core Team 2019), and figures were generated using ggplot2 (Wickham 2016).

Results

Total shoot dry weight was significantly higher in plants treated with imidacloprid when compared with the control plants (Figure 1). In the same way, total shoot nitrogen and phosphorus were higher in plants grown in soil treated with imidacloprid (Figure 2). Regarding fatty acid biomarkers in soil, the only significant difference was observed between glyphosate, imidacloprid and benomyl treatments. In particular, the glyphosate treatments showed the lowest levels of fatty acid biomarkers. However, no differences were found when compared with control (Figure 3). In relation to root fatty acid biomarkers, imidacloprid treatments were significantly higher when compared with control for both Gram positive and Gram negative fatty acids, with the exception of the 15:1 w6c which was

lower in the imidacloprid treatments when compared with glyphosate (Figure 4). In contrast, Gram negative and actinobacteria biomarkers in bees (18:1 w5c and 19:0 10-methyl) were lower in imidacloprid treatments when compared with control. In addition, for 18:1 w5c, significant differences were found between the control, benomyl and glyphosate treatments, with the control treatment being the highest. Furthermore, the 10:0 3OH fatty acid showed marked significant differences between glyphosates and benomyl. Oleic fatty acid was higher in the imidacloprid treatments followed by glyphosate, control and finally benomyl was the lowest (Figure 5). Regarding bee bread fatty acids, the same trend was observed in the 16:0 anteiso biomarker, imidacloprid treatments were significantly higher when compared with control and benomyl. In contrast, for the 14:0 iso fatty acid, imidacloprid was significantly lower than benomyl and for the 16:0 2OH fatty acid the only observed differences were among the control, benomyl and glyphosate treatments (Figure 6). Finally, regarding fatty acids in honey, the only significant difference was found in terms of total fatty acids, with the control treatment lower than imidacloprid, glyphosate and benomyl treatments (Figure 7).

Discussion

The main results of the experiment show a significant effect of the applied pesticides on microbial biomarkers of soil, roots, bee bread, bees and honey. In addition, a plant growth promotion effect was noticed caused by the application of imidacloprid. This promotion might be attributed to the ability of some microorganisms to utilize imidacloprid as a carbon source (Cycon et al., 2013). Furthermore, the degradation of imidacloprid is slow, taking up to 229 days (Muhammad et al., 2012), providing a longer period for soil microorganisms to degrade and utilize it, which can be reflected in plant nutrition. Imidacloprid has been shown to have stimulatory effects on plant-available nitrogen

through the promotion of arginine deaminase activity. This effect is attributed to the adaptation of certain microorganisms to the repetitive application of pesticides (Singh et al., 2005). Additionally, imidacloprid has demonstrated the ability to promote plant phosphorus acquisition by enhancing the growth of plant growth-promoting bacteria. These bacteria facilitate phosphorus solubilization through the production of gluconic acid (Rajasankar et al., 2013). Despite promoting plant growth and nutrition, imidacloprid is a systemic insecticide that can be transported from the soil to the flowers (Bonmatin et al., 2005). This indirect impact affects the foraging behavior of honey bees and plant-pollinator interactions and, consequently, their pollination services crucial for plant reproduction (Yang et al., 2008).

Soil microorganisms were unaffected by pesticide applications. Pesticides have shown to have diverse effects on soil microbiota, promoting growth in some cases while reducing it in others. Depending on soil characteristics and the dosage use, with negative effects observed at higher doses than the recommended ones. (Chen et al., 2001; Hafez & Thiemann, 2003; Kremer and Means, 2009; Cycoń et al., 2013; Mahapatra et al., 2017). The reported effects of glyphosate on soil microorganisms varies and depend on factors such as soil type, crop, and application timing (Busse et al., 2001). Moreover, in most cases, the impact of pesticides is transient, as the soil has the capacity to restore its normal microbiota over time. Since we only applied pesticides at the beginning of the experiment, the soil had enough time to recover. Interestingly, Benomyl did not show any significant effects on fungal microorganism biomarkers, possibly due to a dilution effect in the soil. Regarding root fatty acid biomarkers, imidacloprid showed a promoting effect on both Gram-positive and Gram-negative groups. A diverse range of microorganisms have demonstrated the ability to metabolize imidacloprid (Singh et al., 2005; Rajasankar et al.,

2013; Muerdter & LeFevre, 2019), thereby altering the normal soil microbiota. This alteration promotes certain functions, such as nitrogen and phosphorus plant acquisition, but may also lead to the loss of other functions, such as microbial mediated plant defense mechanisms.

Oleic fatty acid was significant lower in the honey bees, in benomyl treatments, possible due to negative effects on honey bee fatty acid metabolism. Benomyl it is a systemic fungicide which can move to different plant tissues (Absollhi, 2014) and affect lipids in plants and honey bees trough reactive oxygen species production (Shahid et al., 2018). Additionally, Benomyl has been shown to have phytotoxic effects on plants (Reyes, 1975; Zutshi & Kaul, 1975), directly impacting their nutritional quality. Furthermore, Benomyl exhibited inhibitory effects on honey bee Gram-negative biomarkers but not in soil, possibly due to the microbial sensitivity of honey bees to fungicides (Liu et al., 2022). Moreover, in bee bread, imidacloprid significantly increased the Gram-positive biomarker (16:0a) due to the induced differential effects of the fungicides on some microorganisms (Cycoń et al., 2013), leading to microbial alterations in the normal microbiota of bee bread. Similarly, glyphosate resulted in a decrease in the Gram-negative biomarker (16:0 2OH) in bee bread. The mode of action of the herbicide involves the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is essential for the production of aromatic amino acids. This enzyme is present in some groups of honey bee gut microorganisms, and its inhibition can negatively impact honey bee health trough induced changes in the normal honey bee microbiota (Motta et al., 2018). Finally, all the pesticides showed a significant increase in fatty acids on honey, possible due to the induced changes in honey bee microorganisms. Naturally, fatty acids in honey are minimal (Barman et al., 2023), suggesting the action of some microorganisms on honey decomposition.

This study shows the differential effects of pesticides on root microorganisms, plant nutrition, honey, bee bread, and honeybees, highlighting the importance of integrative studies when assessing the negative effects of a pesticide. The positive effects of some pesticides on soil microorganism, roots, and plant nutrition should be considered with caution due the induced negative effects on honeybee fatty acids and the alterations in bee bread and honey microbial composition. In future studies, these effects should be evaluated with different crops and for longer periods to evaluate the long-term effects. Furthermore, considering that these pesticides are frequently utilized in combination, future studies must address this aspect to understand their combined impacts on soil-plant-honey bee multitrophic interaction.

Figures

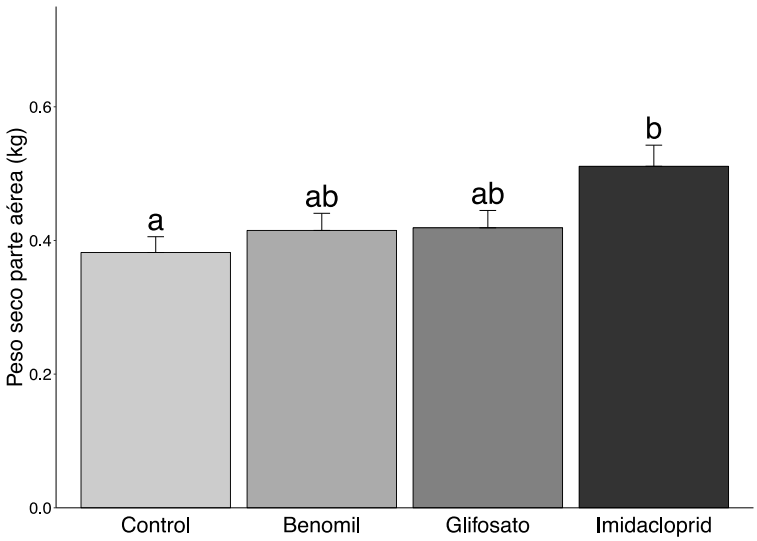


Figure 1. Total shoot dry weight.

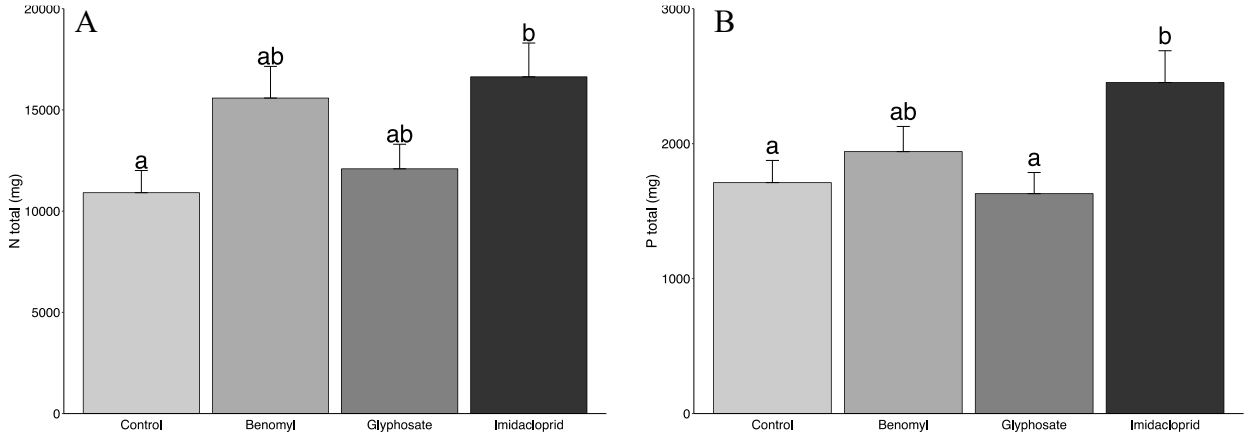
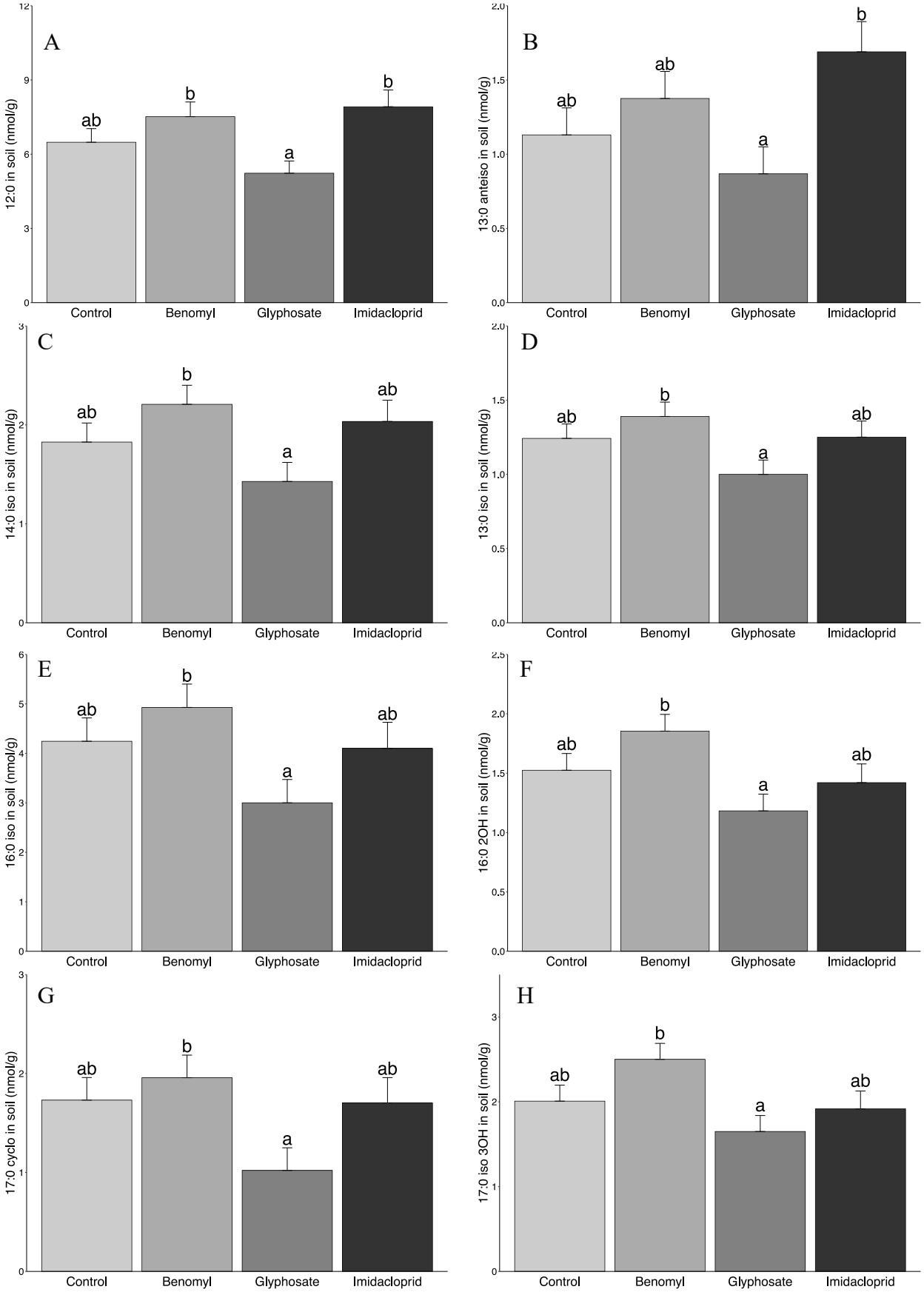


Figure 2. Total nutrients in vegetal tissue; Nitrogen (A) and phosphorus (B).


A
12:0 in soil (nmol/g)
ab
b
a
b
Control
Benomyl
Glyphosate
Imidacloprid
B
13:0 anteiso in soil (nmol/g)
ab
ab
a
b
C
14:0 iso in soil (nmol/g)
ab
b
a
ab
D
13:0 iso in soil (nmol/g)
ab
b
a
ab
E
16:0 iso in soil (nmol/g)
ab
b
a
ab
F
16:0 2OH in soil (nmol/g)
ab
b
a
ab
G
17:0 cyclo in soil (nmol/g)
ab
b
a
ab
H
17:0 iso 3OH in soil (nmol/g)
ab
b
a
ab

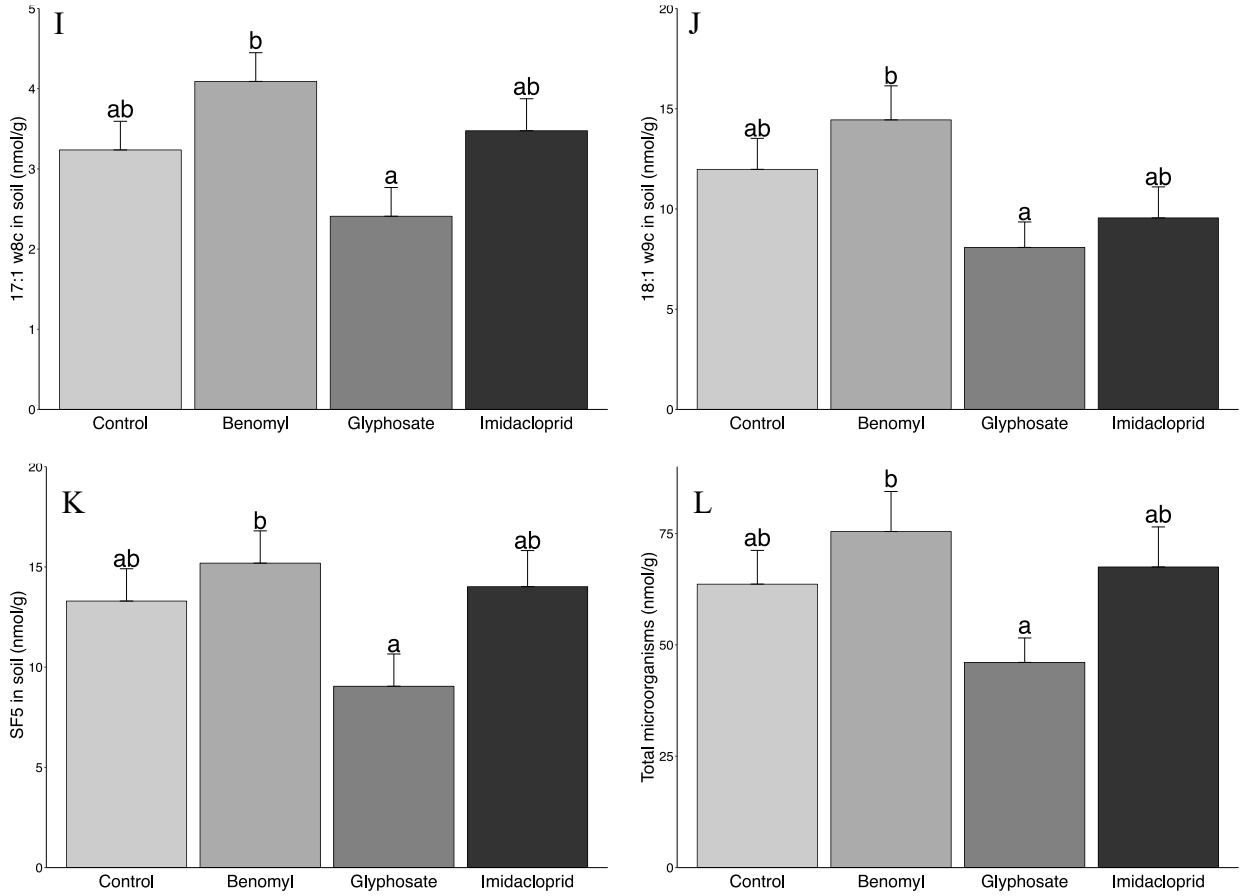


Figure 3. Fatty acid biomarkers in soil; Gram negative (A, B, F, H and I), Gram positive (B, C, D and E), fungi (K), total microorganisms (L) and 18:1 w9c (oleic acid).

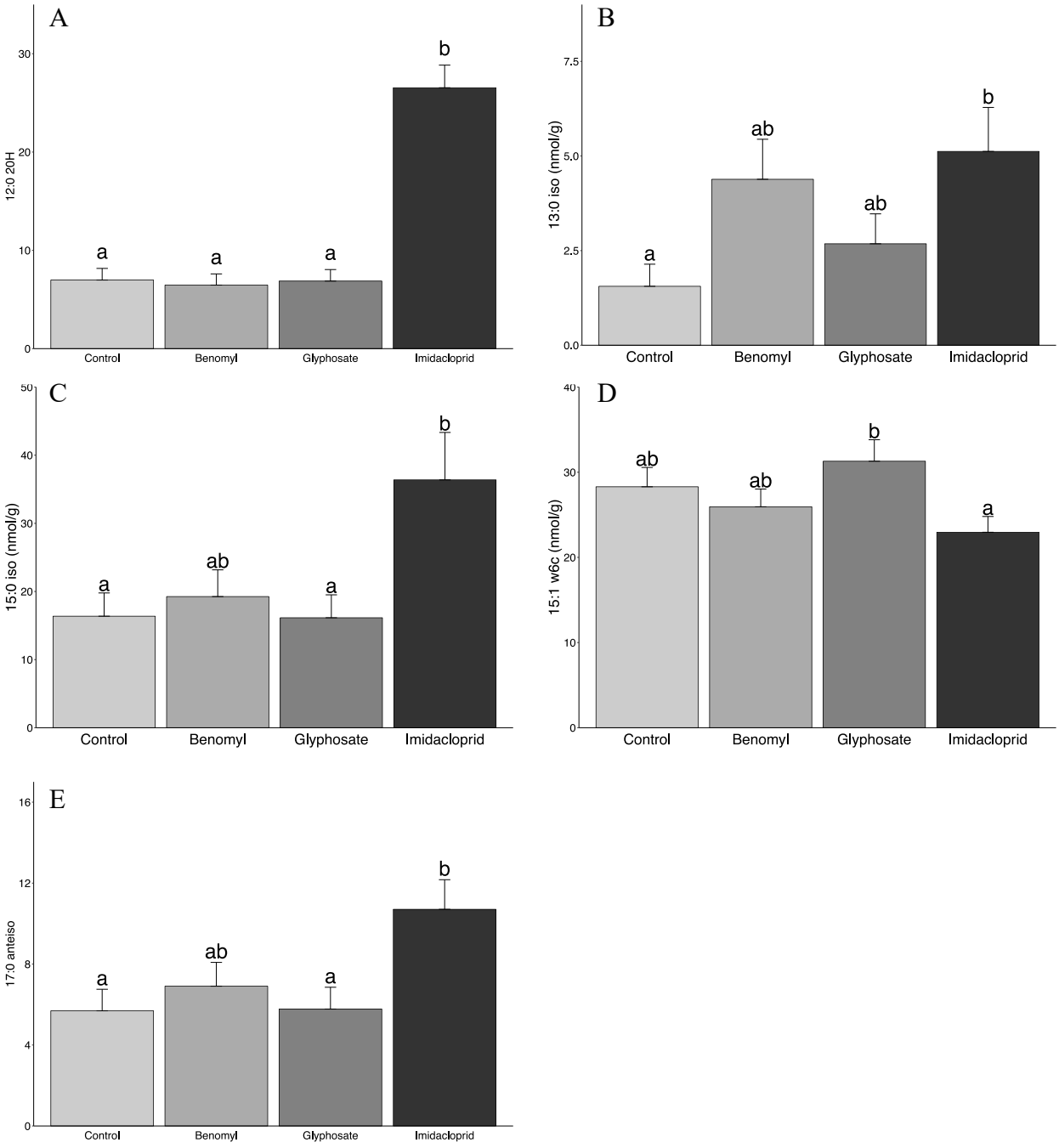


Figure 4. Fatty acid biomarkers in roots; Gram negative (A and D), Gram positive (B, C and E).

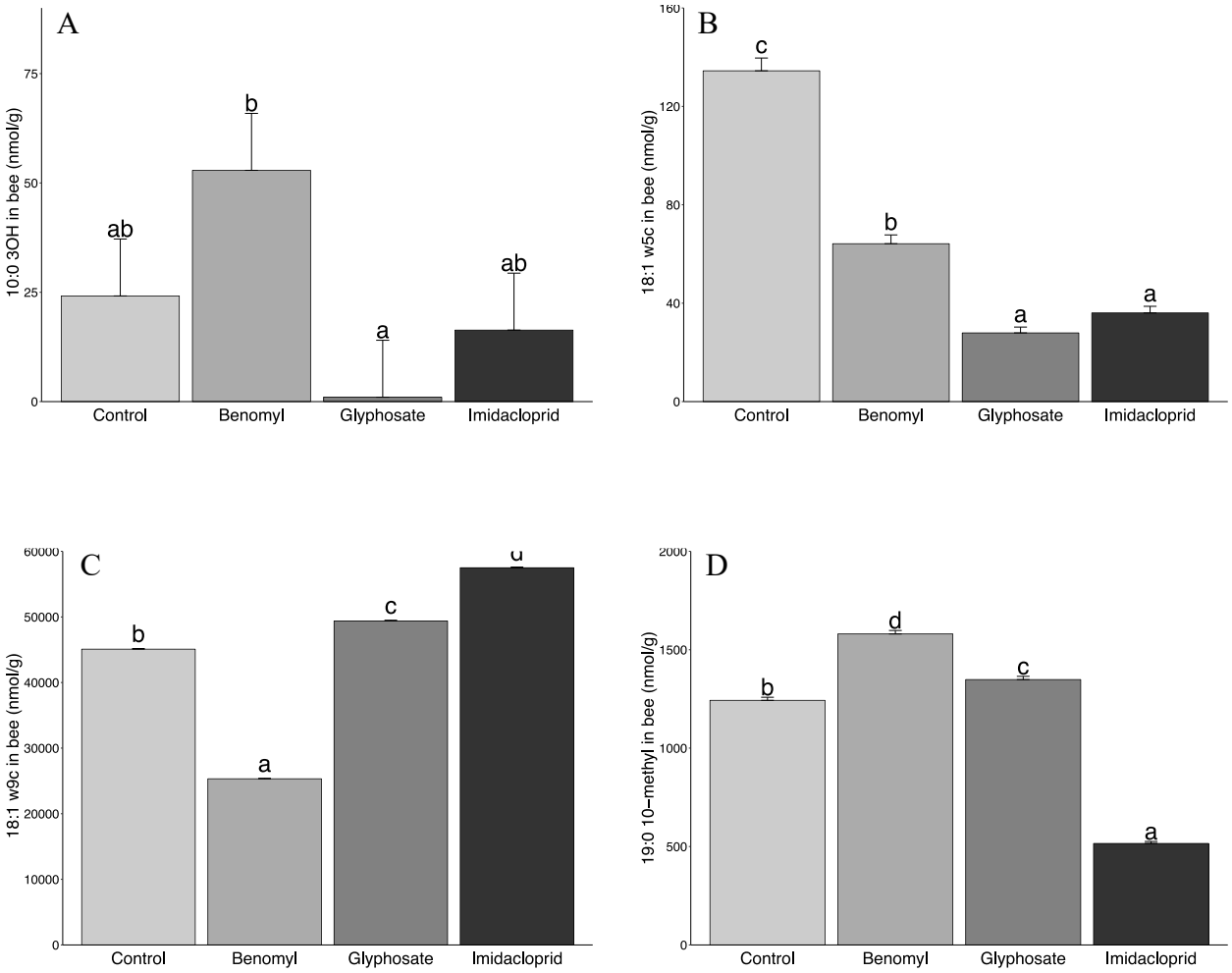


Figure 5. Fatty acid biomarkers in honey bees; Gram negative (A and B), actinomycetes (D) and oleic acid (C).

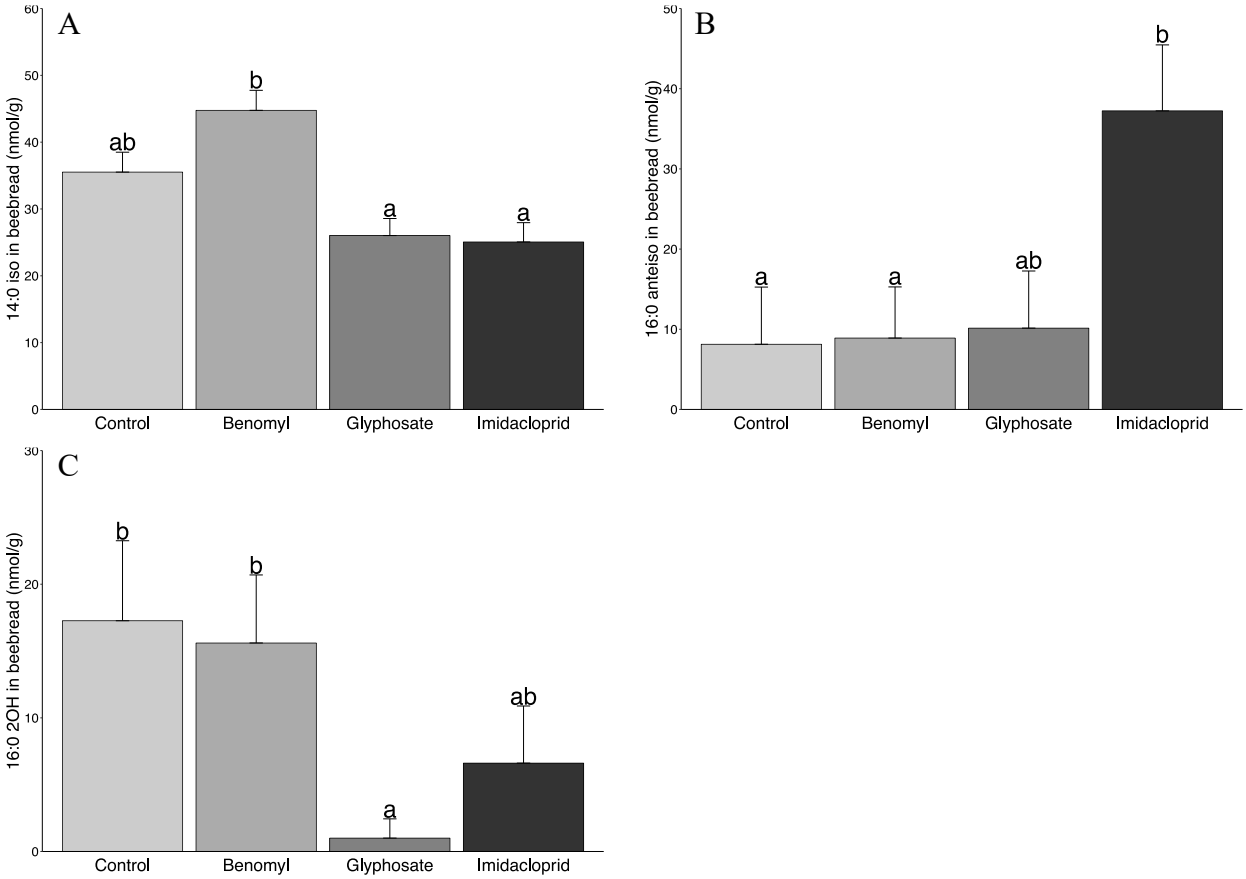


Figure 6. Fatty acid biomarkers in bee bread; Gram positive bacteria (A y B) and Gram negative (C).

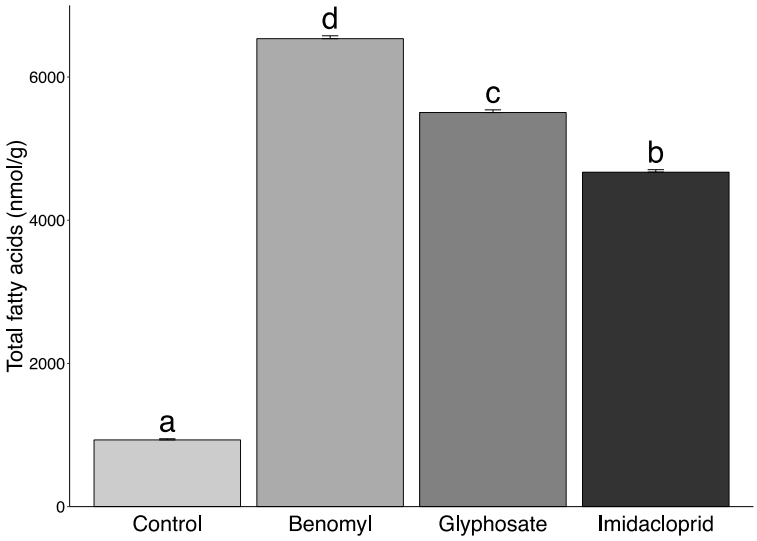


Figure 7. Total fatty acid biomarkers in honey.

Root	***p***	**Bee**	***p***

10:0 2OH	0.8684	10:0 3OH	***
12:0 anteiso	0.7896	12:0	0.6057
12:0	0.6466	14:1 w5c	0.1302
13:0 iso	*	14:0	0.6401
13:0 anteiso	0.935	16:1 w9c	0.45
12:0 2OH	***	17:0 anteiso	0.3916
12:0 3OH	0.3602	18:1 w9c	***
14:0 iso	0.07565	18:1 w5c	***
14:0 anteiso	0.4897	18:1 w7c 11-methyl	0.8184
14:0	0.8175	19:1 iso	0.9914
15:0 iso	*	19:0 10-methyl	***
15:0 anteiso	**	20:0	0.758
15:1 w6c	*	Summed Feature 3	0.6212
16:0 iso	0.08473	Summed Feature 5	0.3945
16:1 w5c	0.8135	Total	0.6166
17:0 iso	0.1085	**Bee bread**	***p***
17:0 anteiso	*	10:0 2OH	0.6548
17:1 w8c	0.7301	12:0 iso	0.8837
17:0 cyclo	0.1783	12:0 2OH	0.6326
16:0 2OH	0.246	14:0 iso	***
18:1 w9c	0.4463	16:0 anteiso	*
17:0 iso 3OH	0.4194	16:1 w9c	0.6692
19:0 cyclo w8c	0.1294	17:0 anteiso	0.3556
Summed Feature 5	0.4797	16:0 2OH	**
Summed Feature 7	0.3811	16:0 3OH	0.5973
Total	0.5557	18:3 w6c	0.1927
Honey	***p***	18:1 w7c 11-methyl	0.3305
Total	***	18:0 3OH	0.197
14:1 w5c	0.8725	Summed Feature 5	0.09039
18:1 w9c	0.4911	Summed Feature 7	0.9239
Summed Feature 5	0.3567	Total	0.2126
Plant	***P***		
PSPA	**		
NTOTAL	**		
PTOTAL	*		

Table 1. *p* values; 0 '***' 0.001 '**' 0.01 '*' 0.05

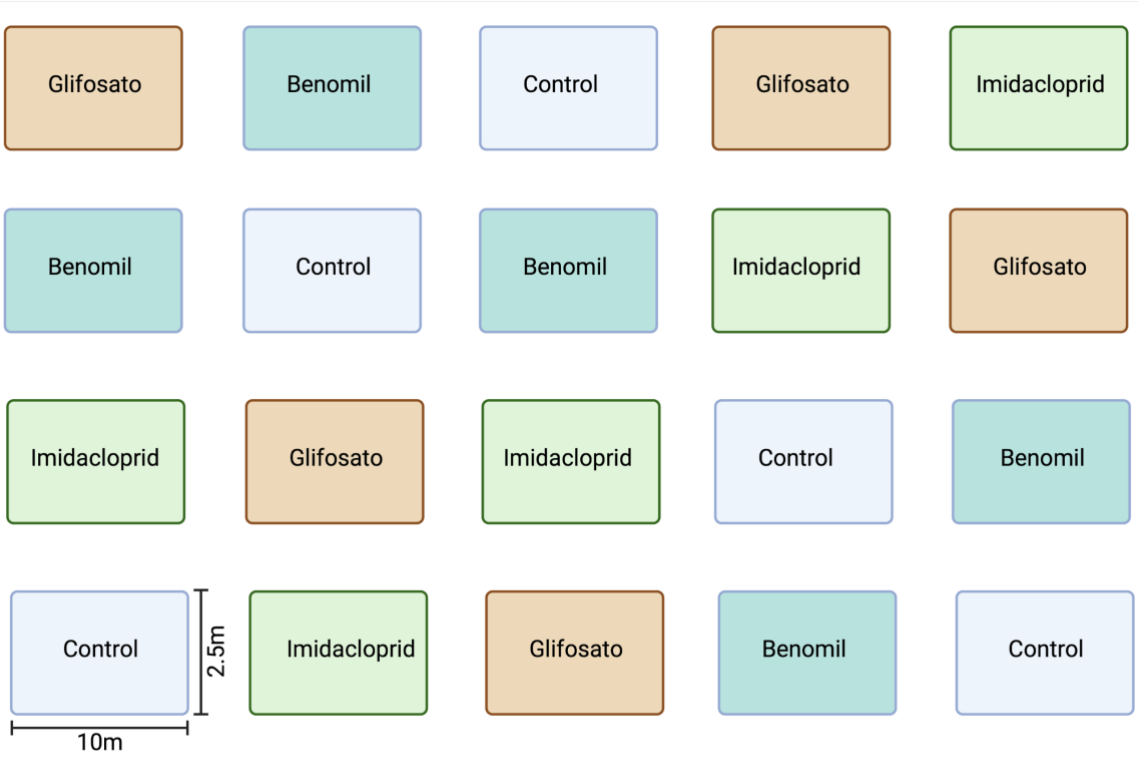


Figure 9. Experimental setup.

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5. DISCUSIÓN Y CONCLUSIONES

El uso de plaguicidas es una de las prácticas más comunes para proteger a las plantas del daño ocasionado por insectos, microorganismos y por la competencia con otras plantas en agroecosistemas. Sin embargo, su aplicación indiscriminada, alta persistencia y ubicuidad en el ambiente han traído consigo efectos no deseados sobre organismos no blanco. En esta tesis, evaluamos los efectos directos e indirectos de los plaguicidas en las abejas, en particular en sus microorganismos asociados, sistema inmunitario, nutrición y desarrollo.

Efectos generales del sistema de manejo agrícola y cultivo:

Encontramos que el manejo y el cultivo afectan a las abejas de diferentes maneras y a través de distintas vías. Directamente el tipo de manejo agrícola y el cultivo afectan las abejas en términos de su nutrición, sistema inmunitario y microrganismos benéficos asociados y de manera indirecta, alteran las interacciones multitróficas entre microorganismos, plantas y abejas.

El manejo convencional y la identidad de la especie cultivada tuvieron un claro efecto en la nutrición de las abejas, resaltando el papel que desempeñan tanto el manejo agrícola como el tipo de cultivo en la transformación y adquisición de nutrientes por parte de las abejas. Estos factores afectan la forma en que las abejas procesan sus alimentos, gracias a la acción de microorganismos como los lactobacilos, y cómo esta transformación altera la asimilación de nutrientes, tanto proteicos como lipídicos, en las abejas. Al contario de otras abejas, la abeja de la miel no tiene la habilidad de seleccionar polen con características nutricionales deseables para su nutrición (Corby-Harris et al. 2018). Por lo tanto, la disponibilidad de recursos de adecuada calidad nutricional es crucial para su salud.

Sin embargo, esto muchas veces no es posible en los agroecosistemas con monocultivos de grandes extensiones. A pesar de esto, observamos que sin importar la calidad del polen y el manejo del cultivo donde se encuentran las abejas, los mecanismos de asimilación de nutrientes por parte de las abejas están bien conservados (Kunieda et al., 2006) los cuales incrementan el contenido de nutrientes en el polen por la acción del metabolismo de algunos microrganismos inoculados por las abejas previo a su consumo (Ghosh & Jung, 2022), compensando los pólenes de baja calidad nutricional.

Efectos indirectos de la aplicación del fungicida benomil en las abejas:

Plaguicidas generalmente considerados seguros para las abejas, como el fungicida benomil, demostraron tener una gran variedad de efectos secundarios. El benomil afecta a grupos específicos de microorganismos benéficos del suelo, como los hongos micorrízicos arbusculares, lo cual tiene consecuencias en el desarrollo y reproducción de las plantas. Este impacto tuvo efectos indirectos en la nutrición de las abejas, particularmente en términos de ácidos grasos, reduciendo la abundancia del ácido graso araquidónico en el abdomen de las abejas, esencial para su nutrición, crecimiento y defensa ante patógenos (Yu et al., 2022). Además, estos plaguicidas afectan la capacidad de defensa de las abejas contra patógenos al reducir la concentración de la enzima de respuesta inmunológica, la profenoloxidasa, y desequilibrar su microbiota benéfica, afectando grupos clave de microorganismos.

La aplicación de fungicidas tiene efectos sobre la composición microbiana del suelo, afectando principalmente la la colonización de hongos micorrízicos arbusculares en las plantas (Sukarno et al., 1993). La inhibición de la colonización de hongos micorrízicos arbusculares tiene efectos significativos en el desarrollo de las plantas y en las interacciones

entre la planta y el polinizador (Gange et al., 2003, Gange et al., 2005). Cuando el fungicida es aplicado en la parte aérea afecta el microbioma del néctar, lo cual, puede tener serias repercusiones en las tasas de atracción de polinizadores (Schaeffer et al., 2017). Los hongos micorrízicos arbusculares juegan un papel clave en la mediación de los efectos "bottom up", modificando las características nutricionales de la planta, promoviendo la adquisición de nutrientes, tolerancia a patógenos y reduciendo estrés hídrico y salino a cambio de fotosintatos (Clarck y Zeto et al., 2000; Azcon-Aguilar et al., 2002; Borkoswska., 2002). Los hongos micorrízicos arbusculares incrementan la actividad fotosintética de las plantas y la producción de tejidos y fotosintatos, de los cuales se alimentan microorganismos e insectos asociados a ésta (Mathur et al., 2018).

Estudios previos han demostrado el efecto positivo de las micorrizas en los insectos polinizadores. Estos hongos contribuyen al aumento del tamaño de las plantas, lo que permite que las plantas más grandes produzcan más ramas. Esto, a su vez, sostiene un mayor número de flores, haciéndolas más atractivas para los polinizadores. (Koltai y Kapulnik., 2010). También se ha reportado un incremento en el número de flores producidas y los tiempos de duración de la floración, debido a cambios inducidos en la producción de etileno y mediante la promoción en la adquisición de fósforo (Lu y Koide., 1994; Besmer y Koide., 1999).

Las micorrizas incrementaron la cantidad de néctar y polen producido por las plantas, lo cual atrae a más visitantes. Este aumento en la cantidad de visitantes tiene un efecto directo en el número de semillas producidas, afectando así la reproducción de las plantas. Por otro lado, las micorrizas pueden inducir cambios en la fenología floral, acelerando el proceso de floración de las plantas (Poulton et al., 2002; Gange y Smith, 2005).

Las plantas pueden transportar algunos plaguicidas como los insecticidas del suelo a la parte aérea a través de sus tejidos por el xilema, afectando a las abejas de maneras directas e indirectas. Directamente, el insecto puede consumir el néctar causando su muerte (Atwood et al., 2018). De manera indirecta, los insecticidas transportados a otros tejidos pueden modificar la composición y abundancia de microorganismos en el polen y néctar afectando la atracción de los polinizadores y por lo tanto, la reproducción de la planta (Ramakrishnan et al., 2021). Además, los plaguicidas influyen en los microorganismos asociados a estos sistemas. Algunos microorganismos presentes en el suelo, la planta y los insectos tienen la capacidad de metabolizar los insecticidas, generando compuestos más o menos tóxicos para el hospedero. Esto tiene efectos significativos en las interacciones entre la planta, los microbios y los insectos, otorgando una ventaja competitiva a los organismos capaces de aprovechar estos compuestos, así como a los insectos y plantas que se asocian con ellos.

(Van et al., 2003).

La comunicación entre plantas e insectos abajo y arriba del suelo es mediada por la emisión de compuestos volátiles. Estos compuestos permiten la transmisión de la información sobre lo que está ocurriendo en los diferentes niveles de las cadenas tróficas, ya sea para inducir o repeler la reproducción herbívoria o depredación (Babicova et al., 2014). Estos compuestos volátiles deben ser cuidadosamente regulados para atraer a los polinizadores y depredadores, al mismo tiempo que repelen a los herbívoros (Jacobsen y Raguso, 2018). Las micorrizas ejercen un efecto significativo en la composición de los volátiles utilizados para atraer a los polinizadores; se ha documentado que el uso de fungicidas altera la colonización micorrízica y, en algunos casos, aumenta la emisión de compuestos volátiles. Esto se debe a que las micorrizas controlan la emisión de volátiles

para modular las interacciones planta-insecto mediante cambios inducidos en la nutrición de la planta (Beckclin et al., 2011). Por otro lado, ciertos plaguicidas pueden volatilizarse desde el suelo o la superficie de las hojas, interrumpiendo estas complejas comunicaciones y los mecanismos de control (Ramakrishnan et al., 2021).

Bajo condiciones de campo, el uso de plaguicidas de uso común, como herbicidas, insecticidas y fungicidas, aplicados al suelo al inicio del cultivo, tienen efectos significativos en la comunidad de microorganismos en general. Estos plaguicidas alteran las comunidades de microorganismos presentes en el suelo, en las plantas, en el pan de abeja, en la miel y en las propias abejas, lo que afecta la adquisición de ácidos grasos esenciales para el desarrollo de las abejas, como el ácido oleico. Lo que inicialmente podría considerarse como un efecto positivo, al promover el desarrollo de las plantas y su nutrición, muestra que tiene efectos negativos de manera indirecta en las abejas, al reducir algunos ácidos grasos esenciales para su nutrición.

Existe una compleja relación entre las plantas y su entorno, ya sea en ecosistemas naturales o agroecosistemas. Sin embargo, esta intrincada red de interacciones se ve amenazada por la presencia y el uso indiscriminado de plaguicidas, los cuales, han causado efectos no deseados en organismos no blanco. Los resultados principales de este trabajo muestran los impactos tanto directos como indirectos de los pesticidas en las abejas, enfocándose en la microbiota asociada a las plantas y a las abejas, en el sistema inmune, en la nutrición y en el desarrollo en general de la colonia de abejas. Directamente, los plaguicidas afectan la nutrición, el sistema inmune y los microorganismos benéficos asociados a las abejas, mientras que indirectamente alteran las complejas interacciones multitróficas entre microorganismos, plantas y abejas.

En general, existe un vacío de información sobre cómo las cadenas tróficas se ven afectadas por los insecticidas, debido a la complejidad de estas, la mayoría de los estudios abordan solo una parte de la cadena e infieren lo demás. Por todo esto, se requieren desarrollar estudios integrativos que consideren los efectos de los pesticidas en estas interacciones. Se requiere generar más información básica y aplicada con trabajos bajo condiciones de campo y laboratorio. Además, el uso de diferentes sistemas de cultivo es necesario para evaluar los efectos indirectos de los pesticidas en las cadenas tróficas. Esto nos permitirá comprender mejor cómo estos efectos puede afectar a las abejas y de esta manera tomar mejores decisiones sobre el uso de los plaguicidas.

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