



Universitat d'Alacant  
Universidad de Alicante

**Teresa Vaello López**

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# **Interacciones multitróficas en el cultivo del pimiento**

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Centro Iberoamericano de la Biodiversidad (CIBIO)

Facultad de Ciencias

**Interacciones multitróficas en el cultivo del pimiento**

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*"Es algo sano y necesario volver la mirada a la tierra y,  
al contemplar sus bellezas, reconocer el asombro y la humildad"*

Rachel L. Carson



A mis padres y abuelo



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## Resumen

Las primeras investigaciones sobre control biológico se llevaron a cabo utilizando un enfoque vertical, centrándose en las interacciones tróficas simples entre plantas, herbívoros y enemigos naturales. Más recientemente, este enfoque se ha extendido a las interacciones multitróficas para incluir las interacciones del cuarto nivel trófico. Y en paralelo, hemos conocido que los organismos que viven bajo tierra también pueden interactuar con niveles tróficos más altos. Por lo tanto, las plantas pueden mediar múltiples interacciones entre comunidades heterotróficas, tanto en su parte subterránea como en la aérea. Estas interacciones pueden ser positivas o negativas desde la perspectiva biológica de cada individuo, y pueden fluctuar desde organismos bajo tierra hasta organismos de la parte aérea o viceversa. Esta tesis reúne una serie de estudios que conectan con los diferentes niveles tróficos que encontramos en el **sistema agrícola del pimiento**, desde los microorganismos del suelo hasta los hiperparasitoides que constituyen el cuarto nivel trófico. Estudios sobre ecología química son importantes en el desarrollo de efectivas y exitosas estrategias integradas de manejo de plagas, donde la abundancia y distribución de los enemigos naturales pueden ser manipuladas a través de semioquímicos, mejorando así el control biológico por conservación. Además, las plantas son capaces de modular sus redes de señalización en respuesta al ataque de los insectos, por ese motivo es importante estudiar el papel que juega la doble herbivoría en la respuesta defensiva de la planta en un sistema agrícola. Así, en esta tesis combinamos enfoques tanto ecológicos como aplicados para poder comprender mejor las interacciones entre diferentes niveles tróficos en un sistema agrícola.

Debido a la importancia de la **ecología química** en el control biológico, en el **capítulo 2** estudiamos cómo los enemigos naturales de los herbívoros basan su decisión de búsqueda en las señales de información del segundo nivel trófico (el herbívoro). Evaluamos el papel de la feromona de agregación de *Frankliniella occidentalis* Pergande, 1895 como potencial kairomona para su enemigo natural, el insecto depredador *Orius laevigatus* (Fieber, 1860). Para este propósito, analizamos la respuesta de *O. laevigatus* al acetato de (R) -lavandulilo y neril (S) -2-metilbutanoato [RLA: NMB], los dos componentes principales de la feromona de agregación del trips. Se ofrecieron los compuestos a los insectos depredadores tanto de manera individual como en mezclas específicas, en un experimento con un olfatómetro de tubo de doble elección en Y. El chinche depredador se sintió significativamente atraído por la mezcla 1: 2.3 [RLA: NMB]. Estos resultados sugieren que la mezcla 1: 2.3 [RLA: NMB] de los compuestos de la feromona de agregación pueden ser usados como un atrayente para *O.*

*laevigatus*. Los resultados de este trabajo resaltan la importancia de estudiar las respuestas de los enemigos naturales ante estímulos químicos de sus presas, para comprender mejor su comportamiento de búsqueda.

En redes alimentarias complejas, las plantas son comúnmente atacadas por múltiples especies herbívoras, afectando a la preferencia y al rendimiento de otros insectos herbívoros y de sus enemigos naturales. El **papel de los insectos omnívoros** en los ecosistemas puede ser más complejo debido al consumo de organismos tanto vegetales como animales. Sin embargo, el efecto sobre los herbívoros y sus enemigos naturales ejercido por insectos omnívoros (diferentes a sus presas), ha recibido poca atención. Por lo tanto, el objetivo principal del **capítulo 3** fue investigar si la doble interacción llevada a cabo entre los pulgones y los trips (ambos herbívoros aunque el segundo puede actuar como omnívoro), puede afectar a diferentes niveles tróficos, desde el desarrollo de la planta y del pulgón hasta el desarrollo del tercer nivel trófico, en nuestro caso el sírfido depredador *Sphaerophoria rueppellii* (Wiedemann, 1820). Nuestros resultados muestran cómo la presencia de trips reduce el desarrollo de la planta y del pulgón. Además, la presencia de trips reduce la fecundidad del sírfido, y estos también evitan la oviposición en plantas que contienen tanto ejemplares de trips, como su feromona de agregación. Este estudio revela además cómo la presencia de una especie plaga o de un compuesto semioquímico (emitido por la especie plaga), puede afectar el comportamiento y a la preferencia de oviposición de un enemigo natural de otro insecto plaga presente simultáneamente en la planta.

En el **capítulo 4**, estudiamos la **respuesta defensiva de la planta de pimiento** en un sistema donde interactúan diferentes niveles tróficos. Se sabe que la herbivoría afecta a los herbívoros que llegan más tarde a la planta, principalmente a través de la regulación de las fitohormonas del ácido jasmónico (JA) y del ácido salicílico (SA). Además, los microorganismos del suelo o los parasitoides pueden alterar la respuesta defensiva de las plantas frente a la herbivoría. Sin embargo, se conoce muy poco si los organismos de niveles tróficos distintos al de los herbívoros, tanto por debajo como por encima del suelo, son capaces de alterar las interacciones entre especies de insectos que comparten una planta. En este capítulo investigamos si el parasitoide de áfidos *Aphidius colemani* (Dalman, 1820), así como diferentes comunidades de microorganismos del suelo (creados a través de la retroalimentación planta-suelo), afectan a las vías de señalización del JA y del SA, en respuesta a la herbivoría de pulgones y de trips. Además, se estudia el desarrollo futuro de los trips en plantas previamente infestadas por pulgones. Nuestros resultados muestran que la

expresión del gen *CaPINII* (gen biosintético del JA), está más suprimido por los pulgones sanos que por pulgones parasitados. Sin embargo, la parasitización no afecta la expresión de *CaPALI*, (gen biosintético del SA). Además, la acción fitófaga de los pulgones mejoró el desarrollo de los trips en comparación con las plantas que no estuvieron infestadas con pulgones. No obstante, este resultado no se observó cuando los pulgones estuvieron parasitados. Los suelos condicionados a través de la retroalimentación planta -suelo no afectaron a la respuesta defensiva de la planta ni a la interacción entre los herbívoros. Este estudio muestra cómo los miembros del tercer nivel trófico pueden modificar las interacciones de los herbívoros alterando la fisiología de la planta.

La retroalimentación planta-suelo se refiere a cambios producidos en el suelo por una planta, que consecuentemente puede afectar a futuras plantas que crecen en ese mismo suelo, estos cambios son producidos principalmente por cambios en los microorganismos del suelo. Algunos de esos microorganismos pueden afectar al desarrollo de los herbívoros que viven sobre el suelo. Si bien la mayor parte de este conocimiento proviene de estudios con microorganismos individuales, no se sabe mucho sobre cómo las diferentes comunidades microbianas del suelo pueden afectar al desarrollo de las plantas e insectos asociados. El objetivo del **capítulo 5** fue examinar si los **efectos de la retroalimentación planta-suelo pueden afectar a los organismos de la parte aérea hasta alcanzar el cuarto nivel trófico**. En este capítulo, trabajamos con el sistema complejo del pimiento: el pulgón como herbívoro, un parasitoide así como su hiperparasitoide. Los resultados mostraron cómo el legado que algunas especies de plantas dejan en el suelo puede reducir la germinación del pimiento. Por el contrario, la densidad de pulgones se vio afectada positivamente por el efecto del legado del suelo que estuvo condicionado por la planta *Hypochoeris radicata*. La retroalimentación planta-suelo también afectó negativamente al desarrollo del parasitoide *Aphidius colemani*. Finalmente, el legado del suelo no tuvo efecto en el cuarto nivel trófico, el hiperparasitoide *Dendrocerus aphidum* (Rondani, 1877). Por lo tanto, los resultados de este estudio demuestran que los distintos microorganismos del suelo, pueden influir en el desarrollo de las plantas y alcanzar el tercer nivel trófico.

## Abstract

Research on biological control has been conducted using a vertical approach, focusing on trophic interactions between plants, herbivores and natural enemies. Lately, this approach has been extended to multitrophic interactions to include contributions of the fourth trophic level. Also recently, we have known that organisms that live below-ground may interact with higher trophic levels. Thus, plants mediate multiple interactions between below and above-ground heterotrophic communities. This thesis covers a series of studies connecting the different trophic levels found in a **sweet pepper system**, from soil microorganisms until the fourth trophic level. Studies about chemical ecology are important in the development of effective and successful strategies where abundance and distribution of natural enemies could be manipulated by semiochemicals for improved conservation biological control. Moreover, plants can modulate the plant signalling networks in response of insect attack, thus it is important to study the role of multiple insect attacks in plant defense in an agricultural system. Hence, we combined both ecological and applied approaches, in order to better understand the interactions that connect the trophic levels in an agricultural system.

Due to the importance of **chemical ecology** in biological control, in **chapter 2** we study how herbivore natural enemies base their foraging decision on information cues from the second trophic level (herbivorous prey). We evaluated the role of the aggregation pheromone from *Frankliniella occidentalis* Pergande, 1895 as a potential kairomone for its natural enemy, the predatory bug *Orius laevigatus* (Fieber, 1860). For this purpose, we analysed the response of *O. laevigatus* to (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate [RLA:NMB], the two major components of the thrips aggregation pheromone. We offered the compounds to the predatory bugs both in separate and as specific blends, in experiments involving a dual choice Y-tube olfactometer. The predatory bug was significantly attracted to the blend 1:2.3 [RLA:NMB]. These results suggest that specific blends of these compounds from the aggregation pheromone may be used as an attractant to *O. laevigatus*. The results of this work highlight the importance of studying olfactory responses of natural enemies for a better understanding of their foraging behaviour.

In terrestrial food webs, plants are commonly attacked by multiple herbivorous species, affecting the preference and performance of other herbivores and natural enemies. The **role of omnivorous insects** in ecosystems may be more complex because of the

consumption of both plant and animal organisms. However, the effect on herbivores and their natural enemies through non-prey omnivorous insects, has received little attention so far. Thus, the main goal of the **chapter 3** was to investigate whether the dual herbivore interaction between aphids and thrips (which can act as an omnivore), may affect different trophic levels, from plant and aphid performance until the third trophic level, i.e. the predatory syrphid *Sphaerophoria rueppellii* (Wiedemann, 1820). Our results show how the presence of thrips decreases host plant and aphid performance. Furthermore, thrips presence reduces syrphid fecundity, and syrphids avoid ovipositing on plants containing either thrips or thrips aggregation pheromone. This study reveals how the presence of a non-prey species pest or a non-prey semiochemical compound, can impact the behaviour and performance of a predator.

In **chapter 4**, we study the **defensive response of the sweet pepper** plant in a multitrophic system. It is known that herbivory affects subsequent herbivores feeding on the same plant, mainly regulated by the phytohormones jasmonic (JA) and salicylic acid (SA). Additionally, organisms such as soil microbes or parasitoids, can change plant responses to herbivory. However, it is not much known how organisms of trophic levels other than herbivores, below- and above-ground, alter the interactions between insect species sharing a host plant. Here, we investigated whether the parasitoid *Aphidius colemani* (Dalman, 1820) and different soil microbial communities (created through plant-soil feedbacks) affect the JA and SA signalling pathways in response to aphids and thrips, as well as subsequent thrips performance. Our results show that the expression of the JA-responsive gene *CaPINII* is more suppressed by aphids than by parasitised aphids. However, parasitism does not affect the expression of *CaPALI*, a biosynthetic gene of SA. Furthermore, aphid feeding enhanced thrips performance compared with uninfested plants, but this was not observed when aphids were parasitised. Soils where different plant species were previously grown, did not affect plant responses or the interaction between herbivores. This study shows that members of the third trophic level can modify herbivore interactions by altering plant physiology, something that was not known for phloem feeders.

Plant-soil feedbacks are changes in the soil by a plant that affect subsequent plants, and are mainly driven by changes in soil microorganisms. Some of those soil microbes can affect the performance of above-ground herbivores. While most of this knowledge comes from studies with single microbes, not much is known how different soil microbial communities affect the performance of plants and associated insects. The objective of

**chapter 5** was to examine whether **plant-soil feedback effects can affect the aboveground organisms up to the fourth trophic level**. Here, we worked with a complex sweet pepper system: aphids as herbivores, parasitoids and hyperparasitoids. The results showed how soil legacies can reduce germination of sweet pepper. Aphid population was in contrast positively affected by the soil legacy effect from *Hypochoeris radicata*-conditioned soil. Plant-soil feedbacks also affected parasitoid performance. Finally, soil legacies had no significant effect on the fourth trophic level, the hyperparasitoid *Dendrocerus aphidum* (Rondani, 1877). Thus, this study provides evidence that through changes in the soil, plants can influence up to the third trophic level, i.e. other plants growing in that soil, their herbivores and carnivores feeding on those.

# **SECCIÓN I**

## **Capítulo I**

### **Introducción general**

### **Interacciones multitróficas**

Las plantas y los insectos herbívoros constituyen aproximadamente la mitad de la biodiversidad macroscópica del mundo y representan una de las interacciones dominantes y más complicadas en los ecosistemas terrestres (Strong et al., 1984). Estos ecosistemas se caracterizan por una gran diversidad de especies y una correspondiente diversidad de interacciones entre estas. Sin embargo, los estudios sobre ecología han estado históricamente dirigidos principalmente a las interacciones entre sólo dos niveles tróficos; las plantas y el herbívoro o el depredador y la presa. No obstante, estas interacciones no pueden entenderse completamente sin incorporar en estos estudios al tercer nivel trófico; el de los enemigos naturales de los herbívoros, como son los depredadores y parasitoides (Price, 1980). Los enemigos naturales afectan desde una posición superior de la cadena trófica a los niveles inferiores de esta, afectando a la estructura y dinámica de las comunidades de herbívoros (a través de las interacciones depredador-presa o parasitoide-huésped). En consecuencia, estas interacciones pueden influir en el desarrollo o en la abundancia de las plantas (Schmitz et al., 2000). Del mismo modo, también las plantas pueden mediar efectos que transcurren desde abajo hacia arriba en la red alimentaria, influenciando así la diversidad y dinámica de poblaciones de niveles tróficos superiores (Hunter & Price, 1992). Sin embargo, el nivel trófico de los depredadores o parasitoides, también puede ser atacado por los insectos hiperparasitoides, un cuarto nivel trófico que a su vez, puede ser atacado por sus propios hiperparasitoides facultativos (o terciarios) (Brodeur, 2000). De esta forma, se pone de manifiesto la necesidad de realizar estudios desde un enfoque multitrófico, abordando la complejidad de las redes alimentarias de una manera mucho más realista.

En las últimas décadas ha aumentado el interés por el estudio de las interacciones multitróficas, impulsado por la necesidad de integrar los conocimientos en ecología en campos aplicados como puede ser el de la agricultura sostenible. El tercer nivel trófico es la base del control biológico, donde los depredadores y parasitoides son los encargados de mantener a las plagas de herbívoros bajo control, sin embargo organismos del cuarto nivel trófico (como los hiperparasitoides) pueden interrumpir su eficacia. Esta tesis doctoral pretende abarcar y enlazar estos dos campos del conocimiento. A través de los resultados obtenidos en estudios ecológicos sobre interacciones multitróficas, se ponen en práctica mecanismos y técnicas que pueden en un futuro próximo aumentar la resistencia de los cultivos a plagas de artrópodos. El conocimiento del comportamiento y desarrollo de los

diferentes agentes de control biológico junto con el conocimiento global de las interacciones que se producen entre ellos, servirán para la mejora del control biológico de las plagas.

### **Ecología química**

Todos los organismos dentro de un ecosistema están interconectados unos con otros por medio de cadenas alimentarias y redes tróficas (Hunter & Price, 1992), y la comunicación entre ellos es facilitada por señales químicas presentes en el medio ambiente (Vet & Dicke, 1992; Karban & Baldwin, 1997; Turlings & Wäckers, 2004). Estas interacciones ocurren frecuentemente entre organismos sobre y bajo el suelo y son mediadas principalmente por compuestos químicos, procedentes de las plantas y de los propios insectos, los cuales pueden afectar positiva o negativamente tanto a los receptores como a los emisores de estas señales químicas (Masters & Brown, 1997; Van der Putten et al., 2001; Wyatt, 2011).

Una parte de esta tesis se centra en el estudio de los compuestos químicos implicados en la comunicación entre insectos, los llamados semioquímicos. El término semioquímico hace referencia a una sustancia de señalización inter o intraespecífica de un organismo (Mori, 1989), y se puede dividir en dos grupos principales (Whittaker & Feeny, 1971):

1. Las feromonas: implicadas en la comunicación intraespecífica.
2. Los aleloquímicos: implicados en la comunicación interespecífica, y se dividen en cuatro tipos: (i) Kairomonas (benefician al receptor), (ii) Alomonas (benefician al emisor), (iii) Sinomonas (benefician a ambos) y (iv) Antimonas (no benefician a ninguno).

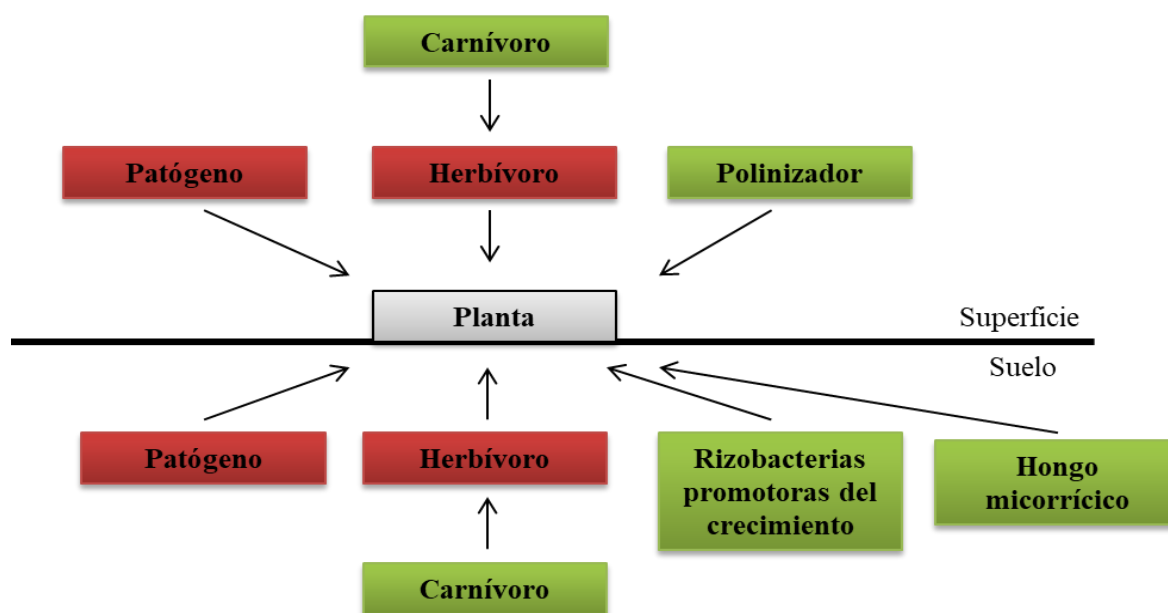
Es importante destacar que un mismo compuesto puede actuar como feromona o aleloquímico, así como una alomona también puede actuar como kairomona al mismo tiempo, estos cambios dependen del organismo que libera o recibe la señal.

El hecho de que las feromonas sean sustancias responsables de la comunicación entre insectos, las ha convertido en una potente herramienta en las estrategias de control de plagas en agricultura sostenible. Durante esta tesis, en los capítulos 2 y 3, nos centramos en el estudio de las feromonas, en concreto en la feromona de agregación del thrips *Frankliniella occidentalis* Pergande, 1895 (Thysanoptera: Thripidae), el cual es considerado como una de las peores plagas en agricultura a nivel mundial (Kirk & Terry, 2003). En el estudio de Hamilton et al., (2005), se descubrieron dos principales compuestos volátiles emitidos por el

macho adulto de *F. occidentalis*, y ausentes en las hembras adultas. Estos compuestos fueron identificados como (R)-lavandulilo acetato y neryl (S)-2- methylbutanoato, y fueron catalogados como los componentes principales de la feromona de agregación de *F. occidentalis*. Una vez descubiertos estos compuestos, se han ido utilizando en el control de plagas, en formato de trampas pegajosas impregnadas con la feromona, de manera que los trips llegan a ellas y quedan adheridos a la trampa (Covaci et al., 2012). En esta tesis hemos estudiado qué papel juega esta feromona en la atracción de enemigos naturales de diferentes plagas, con la finalidad de ofrecer información sobre posibles nuevas estrategias “de origen natural”, mediante las cuales podamos manipular el comportamiento de los enemigos naturales, y así mejorar las estrategias de control de plagas agrícolas.

### Defensas de las plantas

En la naturaleza, las plantas interactúan con una gran variedad de organismos, algunos de los cuales son dañinos (patógenos o insectos herbívoros), mientras que otros son beneficiosos (rizobacterias promotoras de crecimiento, hongos micorrícicos, depredadores o parasitoides de herbívoros y polinizadores) (Pieterse et al., 2007) (Figura 1).



**Figura 1.** Las plantas son miembros de una comunidad compleja, donde interactúan con organismos dañinos (rojo) y beneficiosos (verde). Las defensas de las plantas son activadas en respuesta al conjunto de los organismos. Así que las defensas se superponen, lo que indica que la regulación de la respuesta adaptativa de la planta está equilibrada entre la protección contra agresores y la adquisición de beneficios.

Las plantas han desarrollado estrategias de defensa altamente sofisticadas para defenderse de los organismos dañinos, como son las plagas de herbívoros y los patógenos. Estas defensas pueden ser: i) constitutivas; lo que significa que están presentes en todo momento, independientemente de la presencia o ausencia de herbívoros, o ii) inducidas; que aparecen en respuesta al ataque o daño de los herbívoros (Kessler & Baldwin, 2002). Las defensas inducidas, pueden ser a su vez directas o indirectas, y desempeñan un papel crucial en la protección de las plantas contra las plagas de herbívoros (Schoonhoven et al., 2005; Stotz et al., 2000).

Las defensas directas son cualquier rasgo vegetal físico (espinas y tricomas) o químico (metabolitos primarios o secundarios) que afectan a la vulnerabilidad del herbívoro o al rendimiento del herbívoro atacante. Las defensas directas se inducen a través de la herbivoría y se caracterizan por su modo de operación. Pueden actuar como anti-digestivos al inhibir las enzimas digestivas en el intestino del herbívoro, mientras que otros mecanismos incluyen la liberación de compuestos tóxicos como alcaloides, terpenoides y fenoles que actúan reduciendo los nutrientes y cualidades de la planta, reduciendo su digestibilidad (revisado en Kessler & Baldwin, 2002). Por otro lado, las defensas indirectas tienen como objetivo reducir la presión de las plagas atrayendo a enemigos naturales y promoviendo su eficiencia (Karban, 2011). Una forma de lograrlo es mediante la emisión de compuestos orgánicos volátiles que atraen a los enemigos naturales de niveles tróficos más altos hacia la planta dañada (Vet & Dicke, 1992).

Estas respuestas de defensa inducidas se regulan mediante la activación de la señalización de rutas controladas por las fitohormonas ácido jasmónico (JA), ácido salicílico (SA), ácido abscísico (ABA) y etileno (ET) (Pieterse et al., 2014). Las plantas responden a los diferentes ataques de herbívoros activando vías de señalización de hormonas específicas, pero la principal ruta defensiva es la del JA. Sin embargo se cree que algunos herbívoros inducen otras rutas para contrarrestar por medio de interacciones negativas (cross-talk) la activación de la ruta del JA. Por ejemplo, el ataque de herbívoros que se alimentan del floema, como los áfidos, da como resultado una mayor activación de la vía dependiente del SA (Moran & Thompson, 2001). Por el contrario, los insectos masticadores y los que se alimentan de células, como el caso de los trips, activan principalmente la vía del JA (Pieterse et al., 2012). En los últimos años, se ha descubierto que las respuestas defensivas de las plantas que se desencadenan a través del ataque de los herbívoros, tienen consecuencias en el desarrollo de herbívoros que se alimentarán más tarde de esa misma planta, y este fenómeno

se lleva a cabo a través de la regulación de las fitohormonas (Rodríguez-Saona et al., 2010; Soler et al., 2012). Sin embargo, hasta el momento no hay mucha información de cómo los organismos del tercer nivel trófico son capaces también de modular la respuesta defensiva de la planta y en tal caso, de influir también en el desarrollo de futuros herbívoros (Poelman et al., 2011).

### **Microbioma del suelo**

La naturaleza del suelo, conformada por sus propiedades químicas, físicas y biológicas, juega un papel clave en la determinación del crecimiento, productividad y éxito reproductivo de las plantas, así como la composición y productividad de comunidades de plantas. Al mismo tiempo, las plantas pueden influir también en las propiedades del suelo, a través de insumos de compuestos químicos y materia orgánica. Las plantas, afectan a los procesos hidrológicos y a la temperatura de la superficie del suelo, proporcionando también hábitats o recursos para organismos tanto microscópicos como macroscópicos (Bardgett & Wardle, 2010). La influencia de las plantas en las propiedades bióticas y abióticas del suelo puede alterar la capacidad del suelo para sustentar a estos mismos individuos, a otros individuos de la misma especie o de especies diferentes de plantas. Estos cambios en las propiedades del suelo causados por las plantas, que a su vez pueden influir en el desarrollo y rendimiento de futuras plantas que se desarrollan en este mismo suelo, se denominan “retroalimentación planta-suelo” (“Plant-soil feedback”, PSF por sus siglas en inglés) (Bever et al., 1997; Wardle, 2002; Ehrenfeld et al., 2005; Kulmatiski & Kardol, 2008; Van der Putten et al., 2013).

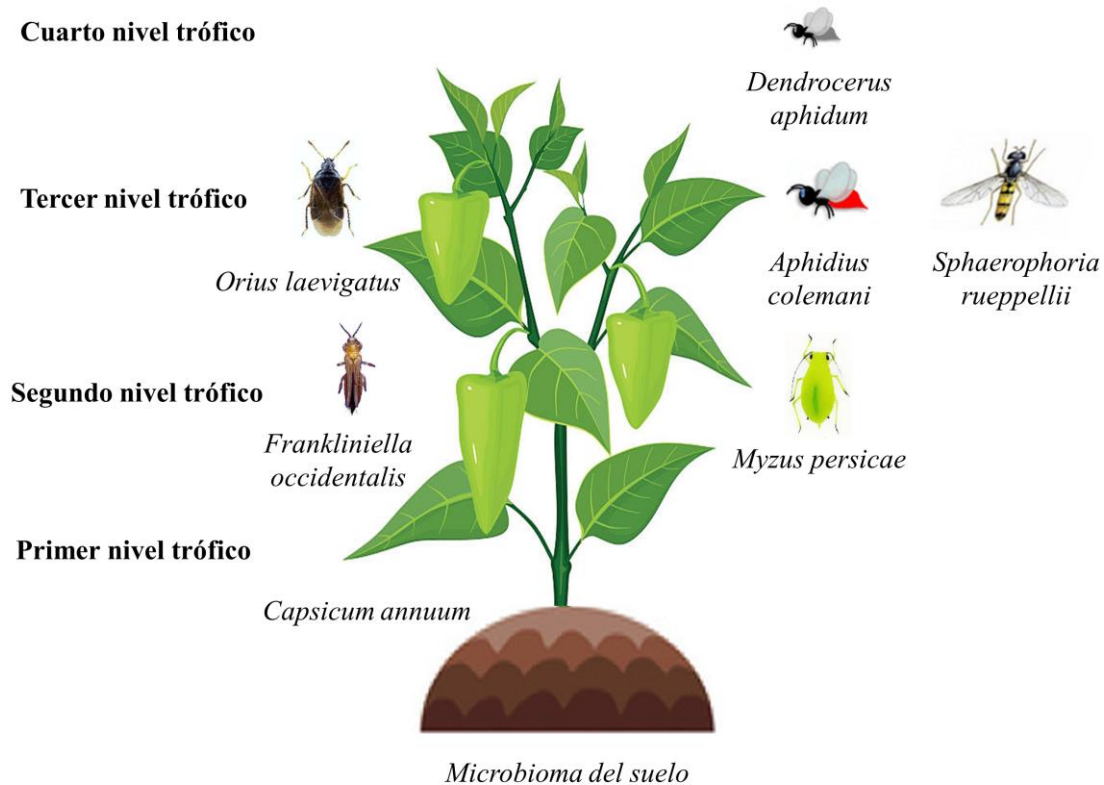
La retroalimentación planta-suelo de una especie de planta, que afecta al mismo individuo o a otros individuos de la misma especie, se conoce como directa, intraespecífica o específica, mientras que cuando la retroalimentación planta-suelo ocurre entre diferentes especies se denomina indirecta, interespecífica o heteroespecífica (Van de Voorde et al., 2011). La retroalimentación planta-suelo se considera positiva cuando mejora el rendimiento de la siguiente planta y aumenta la probabilidad de que esa especie monopolice localmente su hábitat. A la inversa, esta retroalimentación planta-suelo se considera negativa cuando hace que el suelo sea menos adecuado para el crecimiento y desarrollo de futuras plantas, al aumentar la probabilidad de que esa especie de planta sea reemplazada por otras especies que se adapten mejor a las nuevas condiciones del suelo. Debido a que una sola especie puede influir en el suelo a través de una variedad de mecanismos bióticos y abióticos, es la suma de

todos estos efectos, o la retroalimentación neta planta-suelo, lo que determina si el rendimiento de la planta aumenta o disminuye (Van der Putten et al., 2013).

La mayoría de los estudios de retroalimentación planta-suelo se centran en cómo los cambios ocasionados por microorganismos del suelo afectan al desarrollo de la planta, sin embargo, estos cambios en la planta pueden afectar consecuentemente al desarrollo de los insectos herbívoros que se alimentan de ella (Wurst & Ohgushi, 2015). Muchos estudios muestran como la adición de microorganismos específicos de la biota del suelo, como pueden ser hongos micorrícicos, rizobacteria o nemátodos, pueden influir en el desarrollo de herbívoros que se encuentran en la parte aérea de la planta (Koricheva et al., 2009; Pineda et al., 2010). Estos estudios, demuestran el gran potencial que tienen ciertos microorganismos específicos en modular tanto la respuesta de las plantas así como la de los insectos herbívoros. Sin embargo, cabe esperar que el efecto que tendría la retroalimentación planta-suelo sería mucho mayor, debido a que interactúa no sólo una especie de microorganismo sino el total de la microbiota (Pineda et al., 2017; Kaplan et al., 2018). Sin embargo, como ocurre en el apartado anterior, se sabe muy poco sobre cómo la retroalimentación planta-suelo puede modificar la respuesta defensiva de las plantas y cómo podría afectar al desarrollo no sólo de herbívoros que se alimentan de la planta, sino también a las interacciones entre los herbívoros y al desarrollo de insectos del tercer y cuarto nivel trófico.

### **Sistema de estudio**

Esta tesis se centra en las interacciones de la planta con múltiples niveles tróficos; herbívoros con diferentes modos de alimentación (pulgones que se alimentan del floema y trips que se alimentan de las células de la planta), depredadores tanto del pulgón como del trips, un parasitoide del pulgón y, por último, un hiperparasitoide que dificulta la acción del parasitoide del pulgón como agente de control biológico. Además también se tiene en consideración el papel del microbioma del suelo en las relaciones insecto-planta. Todo ello bajo el marco de estudio del cultivo del pimiento (Figura 2).



**Figura 2.** Niveles tróficos estudiados en la tesis

### *Primer nivel trófico*

*Capsicum annuum* L. (Solanaceae), conocido popularmente en España como pimiento dulce o pimiento morrón, es una especie de cultivar agrícola que tiene una gran importancia económica a nivel mundial. La extensión de los pimientos de cultivo de invernadero protegidos ha aumentado de manera intensiva en los últimos años (Ramakers, 2004), la mayoría de los cuales ha sufrido una transformación hacia el control integrado de plagas, aumentando así el cultivo ecológico de pimiento en Europa. El cultivo del pimiento sufre varias plagas de difícil control (como el pulgón y el trips), por lo que nuevas estrategias en agricultura sostenible son necesarias. Una de esas estrategias podría ser potenciar las defensas de la planta de pimiento. Previos estudios han desarrollado esta técnica en plantas como *Arabidopsis* o en cultivos como el tomate (una solanácea como el pimiento) (Pieterse et al., 2000; de Vos et al., 2007; Rodríguez-Saona et al., 2010; Martínez-Medina et al., 2013), sin embargo no existe mucha información sobre la inducción de defensas en plantas de

pimiento. Estudios recientes, han aportado información sobre genes que pueden estar envueltos en la respuesta defensiva del pimiento frente al ataque de herbívoros, como *CaLOX2* y *CaPINII* presentes en la ruta del ácido jasmónico (JA) y *CaPALI* y *CaPRI* de la ruta del ácido salicílico (SA) (Sarde et al., 2018a, 2018b). Por lo que estudios sobre la inducción de la respuesta defensiva del pimiento son muy novedosos.

### *Segundo nivel trófico*

*Frankliniella occidentalis* Pergande, 1895 (Thysanoptera: Thripidae), conocido popularmente como el trips de las flores, es considerado una plaga de insectos polívoros en todo el mundo. Los trips se alimentan generalmente del tejido celular de la hoja o del polen de las plantas, sin embargo pueden alimentarse también de huevos de ácaros depredadores (Faraji et al., 2002; Janssen et al., 2003), huevos de ácaros fitófagos (Agrawal & Klein, 2000) o de mosca blanca (Van Maanen et al., 2012). Así que tanto herbívoros como depredadores que cohabitan con trips en las plantas, no sólo deben competir por el recurso alimentario compartido sino que también deben enfrentarse a la depredación por parte del trips, lo que ocasiona una disminución de la eficiencia por parte de los depredadores al controlar las plagas. Los trips son reconocidos también por causar daños indirectos, ya que son vectores de tospovirus (Ullman et al., 1997; Belliure et al., 2005). Además, esta especie es difícil de controlar en los cultivos debido a su resistencia hacia los insecticidas, su comportamiento tigmocinético (que ocupa grietas estrechas dentro o entre las partes de las plantas), el corto tiempo de generación, la alta fecundidad y el sistema de reproducción de haplodiploides (Reitz & Funderburk, 2012). Por todos estos motivos, esta especie es una de las plagas más graves en muchos cultivos ornamentales y de hortalizas, incluido el pimiento.

*Myzus persicae* (Sulzer 1776) (Hemiptera: Aphididae), conocido como pulgón verde, es otra de las plagas más dañinas en los cultivos tanto del pimiento como de otros hortícolas, siendo una de las plagas más generalistas que existen. Su ciclo de vida tiene una alternancia de fases anfigónicas y partenogénicas. La elevada tasa de crecimiento poblacional de las fases partenogénicas junto con la eficaz ingestión de savia, son los principales factores que los convierten en plaga. Los pulgones ocasionan un daño directo a través de su modo de alimentación, ya que producen una succión de la savia y la inyección, junto de la saliva, de enzimas tóxicas para la planta, causando el debilitamiento de esta. Los daños indirectos se deben a la producción de grandes cantidades de melaza, que queda sobre frutos y hojas

proporcionando el crecimiento de hongos (conocido como negrilla), que limitan la capacidad fotosintética, y por otra parte, los pulgones pueden actuar como vectores de virus, que pueden ocasionar importantes daños fisiológicos a la planta y producir así efectos devastadores en la producción (Rabasse & Steenis, 2002; Blümel, 2004).

### *Tercer nivel trófico*

*Orius laevigatus* (Fieber 1860) (Hemiptera: Anthocoridae), es un chinche depredador normalmente utilizado con éxito en el control biológico de trips en diversos cultivos hortícolas y ornamentales protegidos (Urbaneja et al., 2005), destacando su utilización en el cultivo del pimiento, al ser el principal depredador de *F. occidentalis*. Respecto a sus hábitos alimenticios, tanto en los estadios ninfales como adultos, son conocidos por ser depredadores polípagos que pueden alimentarse de un amplio rango de presas: trips, mosca blanca, pulgones, ácaros o incluso pequeñas larvas de lepidóptero. Además pueden alimentarse también de polen, lo cual es una gran ventaja para su mantenimiento en el cultivo en periodos de ausencia de presa (Alvarado et al., 1997; Urbaneja et al., 2005). En cuanto a su comportamiento de búsqueda y dispersión en el cultivo, detecta a la presa principalmente mediante el sentido del olfato o del tacto y no la vista, localizándose sus estructuras olfativas en las antenas.

*Sphaerophoria rueppellii* (Wiedemann 1830) (Diptera: Syrphidae), es un sírfido afidófago, cuyas larvas se alimentan de pulgones. Presenta tres estadios larvarios antes de su pupar y todos ellos depredan sobre un rango amplio de homópteros, principalmente sobre diversas especies de áfidos. Los sírfidos son muy voraces, llegando a consumir hasta 1000 pulgones durante su ciclo larvario (Tenhumberg, 1995; Rojo et al., 1996), que dura una semana aproximadamente, por lo que son un importante agente de control biológico. La fase adulta es antófila, alimentándose de néctar y de polen (Schneider, 1969). Las hembras realizan la puesta cerca de colonias incipientes de pulgones, característica que unida a la movilidad del adulto, voracidad de sus larvas y polivoltismo, les convierte en excelentes agentes biológicos para el control del pulgón en cultivos de pimiento así como otros cultivos hortícolas..

*Aphidius colemani* (Dalman, 1820) (Hymenoptera, Braconidae), es un endoparasitoide muy efectivo para el control de las poblaciones de *M. persicae*. Se trata de una avispa parasítica, dotada de una especializada capacidad de búsqueda para localizar sus presas. La

hembra de *A. colemani*, una vez que detecta la colonia de pulgones, los palpa con sus antenas y una vez comprobado que el pulgón es del tamaño adecuado y no ha sido previamente parasitado, oviposita dentro del pulgón. La larva nacida del huevo, pasa por cuatro estadios y se desarrolla por completo dentro del cuerpo del pulgón, el cual queda inmovilizado hasta quedar fijo en la planta convirtiéndose en la típica “momia” de color dorado. Una vez que el parasitoide completa su desarrollo dentro del pulgón, surge como un nuevo adulto listo para parasitar nuevos pulgones (Rodríguez et al., 2003).

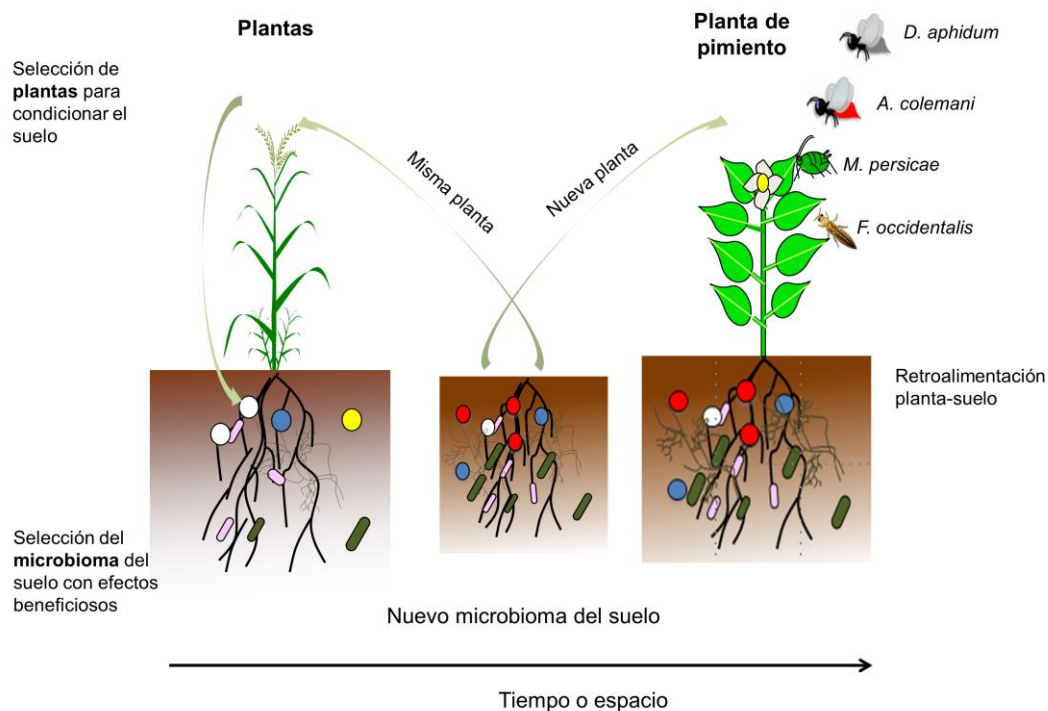
#### *Cuarto nivel trófico*

*Dendrocerus aphidum* (Rondani, 1877) (Hymenoptera: Ceraphronoidea), es un parasitoide secundario o hiperparasitoide. Se trata de un parasitoide idiobionte (el huésped no se alimenta tras la parasitación) que parasita prepupas y pupas. Recientemente se ha convertido en el foco de estudio de trabajos sobre desarrollo y comportamiento, debido a que es uno de los principales problemas en el manejo de las plagas de pulgón, puesto que puede llegar a parasitar a la mayoría de pupas de *A. colemani*, lo que ocasiona una disminución de la eficiencia del parasitoide como agente de control biológico.

#### *Microbioma del suelo*

El suelo es la mayor fuente de diversidad de microorganismos, los cuales determinan el microbioma asociado a cada planta. Como se ha explicado en un apartado anterior, los microorganismos del suelo no sólo son cruciales para mejorar la supervivencia y desarrollo de las plantas o la tolerancia al estrés, sino que también inducen resistencia sistémica a las plantas que están siendo atacadas por insectos herbívoros o patógenos. Así que el microbioma del suelo ha emergido como un elemento clave en los estudios sobre la inmunidad de las plantas (Pineda et al., 2017). Por esta razón, en la realización de esta tesis se tuvieron en cuenta los microorganismos del suelo para realizar un estudio integrado de las relaciones multitróficas en el cultivo del pimiento, aportando nuevos conocimientos sobre las interacciones microorganismo-insecto-planta en un cultivo de interés agrícola. Para la creación de diferentes microbiomas del suelo, nos basamos en el concepto de retroalimentación planta-suelo (PSF) (Figura 3) (Kos et al., 2015). Las especies silvestres elegidas en esta tesis fueron las herbáceas *Achillea millefolium* (L.) e *Hipochaeris radicata* (L.), y los pastos *Lolium perenne* (L.) y *Festuca ovina* (L.). Los criterios de selección de estas

especies para crear los diferentes microbiomas fueron su frecuente uso en los estudios de ecología sobre retroalimentación planta-suelo, así como en los resultados obtenidos de estas especies en el crecimiento y resistencia a insectos en el cultivo hortícola del crisantemo (Bezemer et al., 2006, Wubs et al., 2016, Ma et al., 2017).



**Figura 3.** Manipulación del microbioma del suelo a través de la técnica de retroalimentación planta-suelo. El nuevo microbioma puede afectar al crecimiento de la planta y a su resistencia frente al ataque de herbívoros, pudiendo alcanzar estos efectos a niveles tróficos superiores. En esta tesis se estudia el efecto de la retroalimentación planta-suelo en la planta de pimienta, en el trips *Frankliniella occidentalis*, el pulgón *Myzus persicae*, su parasitoide *Aphidius colemani* y en el hiperparasitoide *Dendrocerus aphidum*. Figura modificada de Pineda et al., 2017 Trends in Plant Science.

### Objetivo principal y preguntas planteadas

El objetivo principal de esta tesis doctoral fue estudiar las relaciones insecto-planta en el cultivo del pimienta, otorgando especial importancia al estudio de las relaciones complejas en las que intervienen herbívoros de diferentes gremios de alimentación y diferentes tipos de enemigos naturales. Además como novedad en los estudios de interacciones en sistemas

agrícolas, investigamos también el efecto del microbioma (aplicando el concepto de “retroalimentación planta-suelo”) como potencial inductor de resistencia en plantas de pimiento. De esta forma, se pretende proporcionar una visión global sobre cómo modelos de investigación más realistas, con interacciones multitróficas que van desde suelo hasta el cuarto nivel trófico, pueden enriquecer nuestra comprensión sobre los sistemas biológicos y así promover nuevas líneas de investigación en el área del control biológico.

Para poder llevar a cabo esta investigación, las siguientes preguntas fueron realizadas:

1. ¿Es capaz un chinche depredador (*Orius laevigatus*), utilizado para el control del trips, de percibir señales olfativas de su presa?
2. Teniendo en cuenta la omnivoría del trips *Frankliniella occidentalis*, ¿hasta qué punto puede afectar su presencia a la eficacia de un depredador afidófago (el sírfido *Sphaerophoria rueppellii*) controlando las poblaciones de pulgón?
3. ¿Puede el hecho de que un herbívoro (*Myzus persicae*) esté parasitado (por *Aphidius colemani*) modular las defensas de inducción de la planta? Y si esto fuese posible, ¿tendría consecuencias en el desarrollo de futuros herbívoros (trips en este caso) alimentándose de esa planta?
4. ¿Podría la retroalimentación planta-suelo influir en la respuesta defensiva de la planta? ¿Hasta qué nivel trófico pueden escalar los efectos de la retroalimentación planta-suelo?

## Esquema de la tesis

Los enemigos naturales realizan la búsqueda de localización de presas principalmente a través de señales de información química, procedentes de olores de plantas (primer nivel trófico). Sin embargo, los insectos herbívoros (segundo nivel trófico) también pueden emitir señales químicas que pueden ser utilizadas por sus depredadores para localizarlos. En el **capítulo 2** se estudia el papel de la feromona de agregación de *F. occidentalis* como una posible kairomona para su enemigo natural *O. laevigatus*. Para este propósito, se analizó la respuesta de *O. laevigatus* a (R)-lavandulilo acetato y neril (S)-2-metilbutanoato, los dos componentes principales de la feromona de agregación de trips. Estos compuestos se

ofrecieron tanto de manera individual como en mezclas específicas, a ninfas y adultos del depredador. El ensayo se llevó a cabo a través de un olfatómetro de dos brazos. Los resultados de este trabajo resaltan la importancia de estudiar las respuestas olfativas de los enemigos naturales para comprender mejor su comportamiento de búsqueda de presas.

En el capítulo 3, extendemos la influencia de esta misma feromona a un enemigo natural de otro herbívoro, un sírfido afidófago, y como puede mediar las interacciones entre trips y sírfidos. En la naturaleza, las plantas son atacadas normalmente por múltiples especies de herbívoros, lo que afecta a la preferencia y al desarrollo de un segundo herbívoro así como de los enemigos naturales. El papel de los insectos omnívoros en los ecosistemas puede ser más complejo, debido a que pueden alimentarse tanto de plantas como de otros insectos. Sin embargo, el efecto que puede ocasionar un insecto omnívoro en el desarrollo de otro herbívoro y/o de su enemigo natural, no ha recibido mucha atención hasta el momento. El objetivo principal en este capítulo fue investigar si la interacción entre un insecto omnívoro (el trips) y un herbívoro (el pulgón) puede afectar a los diferentes niveles tróficos encontrados en el sistema de estudio del cultivo del pimiento. Para ello se analizó el efecto de la presencia del trips en el desarrollo de la planta y del pulgón, así como la preferencia y desarrollo del depredador afidófago *S. rueppellii*. Además, también se observó el efecto de la feromona de agregación del trips en la respuesta de oviposición del depredador del pulgón. En este capítulo se discute cómo la existencia de una especie plaga (o de un compuesto semioquímico de esta) puede afectar al comportamiento y desarrollo de un depredador de una especie plaga diferente.

Los herbívoros que se alimentan de una planta pueden afectar al desarrollo de futuros herbívoros que se alimenten de la misma planta, tema abordado en el capítulo 3. Esto es debido a la regulación de las defensas de la planta; principalmente a través de las fitohormonas del ácido jasmónico (JA) y del ácido salicílico (SA). Los organismos tales como los microorganismos del suelo o los parasitoides que se desarrollan dentro de sus hospedadores herbívoros, pueden alterar la respuesta defensiva de las plantas. Sin embargo, no hay mucha información sobre cómo organismos de niveles tróficos distintos de los herbívoros, tanto de la microbiota del suelo como de la superficie, podrían alterar las interacciones entre especies de insectos que comparten una misma planta huésped. En el **capítulo 4** se investiga si el parasitoide *A. colemani* así como diferentes comunidades microbianas del suelo (creadas a través de la retroalimentaciones planta-suelo) pueden afectar a las vías de señalización del JA y del SA en respuesta al ataque del pulgón *M. persicae* y del

trips *F. occidentalis*. Además también se analiza si estos posibles cambios en las defensas de la planta tienen consecuencias en el futuro desarrollo del trips alimentándose de la misma planta. En resumen, en este capítulo se discute cómo miembros del tercer nivel trófico pueden modificar las interacciones de los herbívoros alterando la fisiología de las plantas.

Las comunidades microbianas del suelo pueden afectar el rendimiento de los herbívoros. La mayor parte de los estudios que conectan microbiota del suelo con herbívoros provienen de estudios realizados con microorganismos individuales, sin embargo no se sabe mucho sobre cómo el conjunto de comunidades microbianas del suelo pueden afectar el desarrollo de las plantas y los insectos asociados a estas. En el **capítulo 5** investigamos si diferentes comunidades microbianas del suelo (creadas a través de retroalimentaciones planta-suelo) pueden afectar diferentes niveles tróficos, abarcando desde el primer nivel trófico (con el estudio del desarrollo de la planta de pimiento), hasta alcanzar el cuarto nivel trófico (con el estudio del desarrollo del hiperparasitoide)

Finalmente, en el **capítulo 6**, los resultados de esta tesis son discutidos de manera integrada con una orientación especial hacia la mejora de las estrategias de control biológico. En este capítulo se discute sobre el papel de la ecología química en el control biológico, el papel de los herbívoros y enemigos naturales en las relaciones multitróficas, la importancia del suelo y su conexión con los diferentes niveles tróficos así como la aplicación de conceptos ecológicos en cultivos agrícolas. En el **capítulo 7** se exponen las conclusiones extraídas de todos los resultados y observaciones obtenidas en esta tesis.

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## SECCIÓN 2

### Capítulo 2

**Olfactory response of the predatory bug *Orius laevigatus* (Hemiptera: Anthocoridae) to the aggregation pheromone of its prey, *Frankliniella occidentalis* (Thysanoptera: Thripidae)**

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## Chemical Ecology

## Olfactory Response of the Predatory Bug *Orius laevigatus* (Hemiptera:Anthocoridae) to the Aggregation Pheromone of Its Prey, *Frankliniella occidentalis* (Thysanoptera: Thripidae)

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### Abstract

Herbivore natural enemies base their foraging decision on information cues from different trophic levels but mainly from plant odors. However, the second trophic level (i.e., the herbivorous prey) may also provide reliable infochemical cues for their natural enemies. We have evaluated the role of the aggregation pheromone from *Frankliniella occidentalis* (Pergande) as a potential kairomone for its natural enemy, the predatory bug *Orius laevigatus* (Fieber). For this purpose, we have analyzed the response of *O. laevigatus* to (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate, the two major components of the thrips aggregation pheromone. These compounds have been offered to *O. laevigatus* adult females and nymphs of the predatory bugs both in separate and as specific (1:1 or 1:2.3) blends, in experiments involving a dual choice Y-tube olfactometer. None of the compounds attracted adults or nymphs when they were individually supplied. Conversely, they were significantly attracted to both adults and nymphs when offered as a blend. A 1:2.3 (*R*)-lavandulyl acetate:neryl (*S*)-2-methylbutanoate blend was attractive to both nymphs and adults, while a 1:1 blend elicited response only in nymphs. These results suggest that specific blends of these compounds from the aggregation pheromone may be used as an attractant to *O. laevigatus*. The results of this work highlight the importance of studying olfactory responses of natural enemies for a better understanding of their foraging behavior. Potential uses of these results in future studies are discussed.

**Key words:** Adults, aggregation pheromone compounds, kairomone, nymphs, Y-tube olfactometer

The western flower thrips, *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae), is a polyphagous insect pest worldwide. This species is probably one of the most serious pests in many ornamental and vegetable crops not only because of direct damage caused by feeding and oviposition but also by indirect damage as a vector of tospoviruses (Ullman et al. 1997, Belliure et al. 2005). This species is difficult to control because of its resistance to insecticides, thigmokinetic behavior (occupying narrow crevices within or between plant parts), short generation time, high fecundity, and haplodiploid breeding system (Jensen 2000, Reitz and Funderburk 2012). Thus, the major progress in the control of this pest in crops such as sweet pepper in Europe during the last decade has come from integrated pest management (IPM) strategies: the use of blue sticky traps baited with the thrips aggregation pheromone, and the release

of the predators *Amblyseius swirskii* (Athias-Henriot 1962) (Acari: Phytoseiidae) and *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae) (Van Der Blom 2008, Sampson and Kirk 2013).

The pirate bug *O. laevigatus* is one of the most effective thrips predators both in greenhouses and in open field crops (Tommasini and Nicoli 1993, Blaaser et al. 2004). Locating efficiently their hosts is crucial for generalist predators such as *Orius*, both to fulfil its nutritional requirements and to find suitable oviposition sites for their offspring. Both adults and nymphs occur simultaneously on the same plant enabling both stages to localize their prey. However, the trophic connections between an immature predator and its prey have been studied only occasionally (Juen and Traugott 2007), and this in spite of the observed fact that adults and nymphs may produce defensive secretions or alarm pheromones of different composition

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## Introduction

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release of the predators *Amblyseius swirskii* (Athias-Henriot 1962) (Acari: Phytoseiidae) and *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae) (Van Der Blom, 2008; Sampson and Kirk, 2013).

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It is generally accepted that *Orius* locate their prey by means of olfactory cues emitted by prey-infested plants (Van Laerhoven et al., 2000; Aldrich et al., 2007; Arab et al., 2007; Mochizuki and Yano, 2007), but much less is known about the potential attraction exerted by prey pheromones. Predators also can use pheromones from herbivorous insects as prey-finding kairomones (Grégorie et al., 1992; Aldrich, 1999; Boone et al., 2008; Cottrell et al., 2014). Several pheromones are known to play important roles in the chemical ecology of thrips: sex, trail, alarm, contact recognition, or aggregation pheromone, this last being the only commercially available for pest management (Covaci et al., 2012; Kirk, 2014). The two major compounds identified in the thrips aggregation pheromone are (*R*)-lavandulyl acetate (RLA) and neryl (*S*)-2-methylbutanoate (NMB), emitted at a ratio ranging from 1:0.8 to 1:5 (Hamilton et al., 2005; Zhang et al., 2011). This ratio differs even between species as was described in males of *Frankliniella intonsa* (Trybom, 1895) and *F. occidentalis* (Zhang et al., 2011). Previous studies about the attraction to the synthesized compounds from the aggregation pheromone were tested with thrips, where the blend 1:1 (RLA:NMB) were equally attractive to thrips than NMB tested alone (Hamilton et al., 2005). Thus the blue or yellow sticky traps, which are currently used in biological control, can be baited with NMB to significantly increase thrips catch without directly affecting key natural enemies (Broughton and Harrison, 2012; Sampson et al., 2012; Sampson and Kirk, 2013). However, the potential attractiveness of RLA and NMB from the aggregation pheromone to natural enemies is however largely unknown.

The aim of this study was to investigate whether the predatory bug *O. laevigatus* recognized as attractants the major components of the aggregation pheromone of *F. occidentalis* (RLA and NMB). We tested two working hypotheses: (i) the thrips aggregation pheromone is used as a prey-finding kairomone by its predator *O. laevigatus*, and (ii) there are differences between adults and nymphs of *Orius* in their ability to recognize volatile clues. For this, we investigated the olfactory response of adults and nymphs of *O. laevigatus* to RLA and NMB. These compounds were offered both as single compounds and as 1:1, which was found to be attractive to *F. occidentalis* (Hamilton et al., 2005), and 1:2.3 (RLA:NMB) blends, this last closer to the natural presence of both compounds in the aggregation pheromone of the genus *Frankliniella* (Hamilton et al., 2005; Zhang et al., 2011). The potential use of these chemicals in future studies applied in conservation biological control to preserve natural enemies in the crops is also discussed.

## Material and methods

### *Plant and insect rearing*

Sweet pepper plants (*Capsicum annuum* L., cv. California Wonder) were grown in plastic pots (5.5 cm in diameter, 7 cm in height) in a climatic chamber ( $24 \pm 1$  °C,  $70 \pm 10$  % relative humidity (RH) and 16 h L:8 h D photoperiod). Apical leaves from four-week old plants were cut and used in the olfactometer experiments.

A colony of *O. laevigatus* was initiated from commercially available individuals. The colony was maintained in a climate room at the same conditions at  $24 \pm 1$  °C,  $70 \pm 10$  % RH, 16 h L: 8 h D photoperiod. Predatory bugs were reared in plastic containers (5 L) with two holes (5 cm in diameter) on top covered with fine nylon gauze, and were provisioned with buckwheat (*Fagopyrum esculentum* Moench) as a walking substrate and lined with crumpled tissue to provide hiding places for the juveniles to reduce cannibalism. An artificial diet consisting of eggs from the Mediterranean flour moth *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae) were supplied. Both, the colony of *O. laevigatus* and eggs of *E. kuehniella* were supplied by Agrobio S.L., Almería, Spain. Fresh green beans (*Phaseolus vulgaris* L.) were used as the oviposition substrate. Three times a week, beans with eggs of *O. laevigatus* were collected and placed in a new container with flour moth eggs to obtain cohorts of *Orius* of a similar age. Females (between 2-5 days in the adult stage) and third and

fourth instar nymphs (between 7-10 days after hatching) were used in the olfactometer experiments. The insects were starved for 2 h prior to their release in the olfactometer.

#### *Application of compounds*

RLA and NMB were synthesized according to Hamilton et al. (2005) and Zada and Harel (2004), respectively. In order to prepare the odour source for the olfactometer experiments, test compounds were individually diluted (1:1, v/v) in paraffin oil (Sigma-Aldrich, USA). From these solutions, RLA and NMB were used in separate and also two 1:1 and 1:2.3 (RLA:NMB) blends were prepared. Single compounds (1 µl) or the appropriate blend (2 µl) were applied on a piece of filter paper (1x1 cm) that was placed on to a detached pepper leaf in order to mimic plant background odour.

#### *Olfactometer tests*

The double-choice bioassays were conducted in a Y-tube olfactometer similar to that described by Takabayasi and Dicke (1992). The olfactometer was a piece of glass of 60 mm in length (stem) with a stem-angle of 45°, 50 mm in length (arms), and an inner diameter of 10 mm. Both arms of the Y-tube were tilted upwards at an angle of 30°, connected to a two 0.5-l glass container holding the odour source. Air was filtered through activated charcoal at a flow rate of around 170 ml/min prior to be pumped into the glass bottles containing the odour cues. All connections were made with Teflon tubes. Choice tests were conducted between 10:00 am and 13:00 pm in a darkened, air-conditioned room ( $25 \pm 1^\circ\text{C}$ ) and the olfactometer was illuminated from above by a fluorescent lamp providing 600 lux light intensity. Clean air was drawn through the Y-tube for at least 15 min before introducing the first odour source and afterwards, 15 min before testing the first predatory bug at the beginning of each bioassay in order to allow the odour to reach a constant release rate (De Kogel et al. 1999).

For the Y-tube olfactometer bioassay, we tested first if sweet pepper leaves could be an attraction cue for the predatory bug. For this, we tested a pepper leaf against clean air as odour source. The leaf did not attract adults or nymphs of *O. laevigatus* (Fig. 1), consequently, a pepper leaf was used as control throughout the rest of the experiments and it was also present with the test compounds as a background odour. The treatments tested were: (i) clean air vs. sweet pepper leaf, (ii) control vs. 1 µl RLA, (iii) control vs. 1 µl NMB, (iv) control vs. 2 µl from 1:1 (RLA:NMB), (v) control vs. 2 µl from 1:2.3 (RLA:NMB).

In total, 262 adults and 233 nymphs of *O. laevigatus* were tested in the olfactometer, where four sets of plants were tested per each treatment on different day. Ten to 15 bugs per

plant and day were used. Each bug was used only once. Bugs were individually placed at the stem of the Y-tube with a fine brush and their movement towards one of the arms of the Y-tube was timed using a chronometer. The maximum observation period was 5 min, and individuals which did not make a choice within this interval were scored as “no choice”, and consequently excluded from the statistical analysis. After five individuals tested, Y-tube and glass containers were alternated to avoid influences of positional effect in the set-up. The apparatus was cleaned before each test by rinsing with acetone.

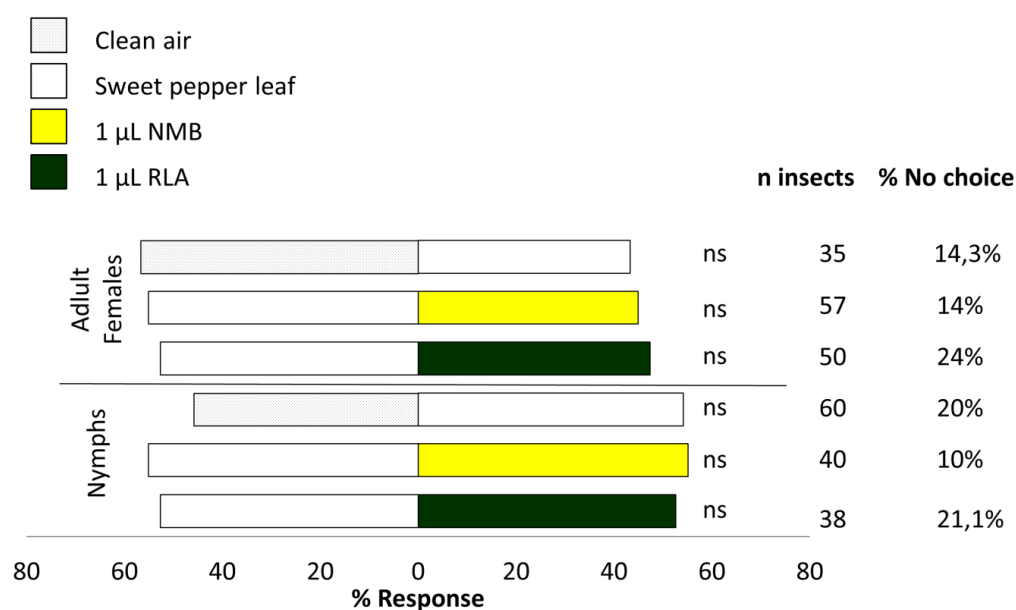
### *Statistical analysis*

Two-side binomial tests were carried out to determine whether choices of the predatory bugs differed from a 50:50 distribution ( $p = q = 0.5$ ,  $\alpha = 0.05$ ). Data were analysed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

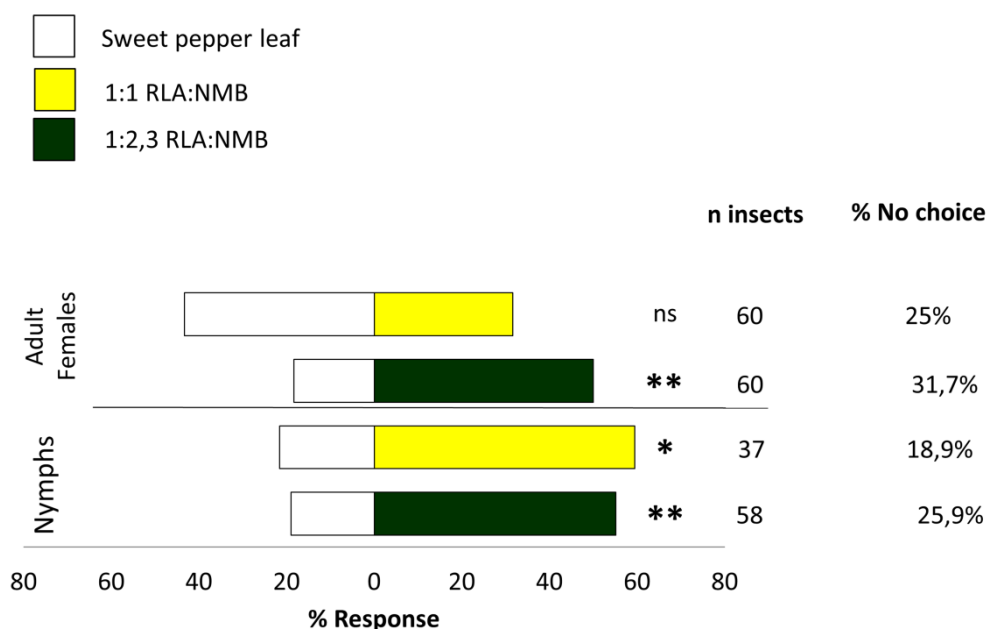
## **Results**

RLA and NMB were firstly tried in separate for their potential attractiveness to *O. laevigatus* using a Y-shape double-choice olfactometer. RLA had no significant effect on the attraction of adults ( $P = 0.871$ ) or nymphs ( $P = 0.585$ ) of the bug. A similar lack of attraction ( $P = 0.585$  for adults and  $P = 0.868$  for nymphs) was found with NMB (Fig. 1).

In view of this failure of the two components of the thrips aggregation pheromone to significantly attract either nymphs or adults of *O. laevigatus*, we repeated the choice tests using mixtures of the two compounds at a different ratio. Thus, we prepared a 1:1 and 1:2.3 (RLA:NMB) blend, as described in Materials and Methods. The results obtained (Fig. 2) showed that adults of *O. laevigatus* did not show preference ( $P = 0.371$ ) for the 1:1 blend although nymphs did ( $P = 0.016$ ). However, both adults ( $P = 0.004$ ) and nymphs ( $P = 0.002$ ) were significantly attracted to the 1:2.3 blend.



**Figure. 1** Response of adults and nymphs of *O. laevigatus* to the individual compounds of the thrips aggregation pheromone. The total number of tested individuals (n insects) and percentage of individuals that not made a choice (% no choice) are given on the right side of the bars. NS: Not significant ( $P > 0.05$ , binomial test).



**Figure. 2.** Response of adults and nymphs of *O. laevigatus* to the blends 1:1 and 0.3:0.7 (RLA:NMB) compounds of the thrips aggregation pheromone. The total number of tested individuals (n insects) and percentage of individuals that not made a choice (% no choice) are given on the right side of the bars. Asterisks indicate significant differences within a choice test ( $*P < 0.05$ ;  $**P < 0.01$ ), whilst non-statistically significant differences are indicated by *ns* ( $P > 0.05$ ; binomial test).

## Discussion

There is an increasing knowledge that pheromones from herbivores may also serve as semiochemicals in the upper trophic level represented by the herbivore natural enemies. It has been reported that the predators *Orius tristicolor* White (Hemiptera: Anthocoridae) and *Amblyseius cucumeris* (Acarina: Phytoseiidae) use the sex pheromone from its prey *F. occidentalis* as a prey-finding kairomone (Teerling et al., 1993). *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae) uses some components of its host's sex pheromone to locate sites that may carry hosts (Boo and Yang, 2000). Many other parasitoids and predators have been reported to use host pheromones as kairomones (Boo and Yang, 2000 and references therein). However, if this is also the case with the thrips aggregation pheromone is still largely unknown. In the present research, we have conducted different olfactometer assays to test whether *O. laevigatus* is able to identify the major compounds released in the aggregation pheromone of its prey *F. occidentalis*, without the presence of prey. Our results show that the predatory bug effectively responded to the aggregation pheromone of its prey. Furthermore, both adults as nymphs of *O. laevigatus* were attracted to specific blends of RLA and NMB but not to single compounds. This ability to discriminate blends and single compounds as odour clues for insects has been described elsewhere. Reddy et al. (2002) found that single compounds from the sex pheromone of the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae) did not elicit responses in its predator *Chrysoperla carnea* (Neuroptera: Chrysopidae) but a blend did. Interestingly, this is not always like this. The parasitoids *T. chilonis* or *Cotesia plutellae* (Hymenoptera: Braconidae) were attracted to a synthetic pheromone blend but also to single compounds from the sex pheromone of *P. xylostella* (Reddy et al. 2002). Hamilton et al. (2005) observed that NMB alone or a 1:1 (RLA:NMB) blend were attractive to *F. occidentalis*. The fact that the predator *O. laevigatus* only however recognizes the blend of the two components as odour clue is relevant because NMB is the only compound present in the thrips aggregation pheromone currently used in biological control of this pest (Broughton and Harrison, 2012; Broughton et al., 2015). Davidson et al. (2015) described a joint attraction of both prey and their predator by means of a semiochemical. In this case the compound was not a pheromone but a 4-pyridyl carbonyl compound named methyl-isonicotinate which when baited over blue sticky or white water traps caused significant more captures of thrips and also of *O. laevigatus* than in absence of the lure. In view that neither adults nor nymphs of *O. laevigatus* are attracted by NMB, the use of sticky traps baited with this compound to capture thrips can be considered as safe from

a predator's point of view, confirming previous observations (Broughton and Harrison, 2012; Sampson et al., 2012). Thus, in accordance with our first working hypothesis, the blend of the two major compounds from the thrips aggregation pheromone is used as a prey-finding kairomone by the predator *O. laevigatus*.

It was also remarkable that adults and nymphs of *O. laevigatus* responded differently to blends of RLA and NMB, confirming our second working hypothesis. Thus, adults only recognized an unbalanced blend while nymphs were also attracted to a 1:1 blend. This finding was in accordance with other reports in which specific mixtures also became as odour clues for some insect species: males of the oriental fruit moth *Grapholita molesta* (Busks, 1916) (Lepidoptera: Tortricidae) only responded to a specific ratio from the two major sex pheromone components; (E)-8-dodecenyl acetate and (Z)-8-dodecen-1-ol (Linn and Roelofs, 1983). Female orange wheat blossom midges, *Sitodiplosis mosellana* (Géhin, 1857) (Diptera: Cecidomyiidae) showed high attraction to a blend of the six compounds released from panicles of their host plant, wheat, but only when these volatiles were presented in natural ratio (Birkett et al., 2004). Up to our knowledge, this is the first study however that shows these differences in the olfactory attraction between adults and nymphs from the same species. The possible reasons for these differences are undoubtedly challenging. One reason may be a shift in the foraging behaviour between both stages. In this sense, Harwood et al. (2009) found differences between adults and nymphs from *Orius insidiosus* (Say, 1832) (Hemiptera: Anthocoridae); whereas *Aphis glycines* (Matsumara) (Hemiptera: Aphididae) constituted a greater proportion of the diet of immature *O. insidiosus*, *Neohydatothrips variabilis* (Beach) (Thysanoptera: Thripidae) DNA was found in greater frequency in adults. In our case, both stages use the same trophic resource, *F. occidentalis*, but however their different type of mobility (adults walk and fly while nymphs just walk) may account for a higher selectivity of adults in odour perception. Moreover, adults have to make decisions about feeding and oviposition in order to guarantee the offspring survival. Thereby, the fact that adults discriminated 1:1 from 1:2.3 (LA:NMB) blend may be due to *Frankliniella* species emits an unbalanced blend of LA and NMB as aggregation pheromone, in which a major proportion of NMB was generally observed (Zhang et al., 2011). Interestingly, we have found differences in the structure of the antennae between adults and nymphs of *O. laevigatus* (unpublished data) which may account for this change in "sensitivity" in odour clues, although this assumption still awaits further research. In addition, these results bring a new record to the list infochemicals used by beneficial organisms and may be considered an innate response in that the predators used in the trials had not previously been in contact with

the prey (Turling et al., 1993; Van Laerhoven et al., 2000; Steidle et al., 2003; Amorós-Jimenez et al., 2015).

In summary, the importance of thrips attraction by conspecific pheromones and its application in IPM strategies to capture thrips is well-documented (Hamilton et al., 2005; Harbi et al., 2013; Akella et al., 2014; Elimem et al., 2014). The present work extends the scope of this strategy because the potential effect of these pheromones on the natural enemies is also taken into account. The results show that the blend from the major components of the thrips aggregation pheromone can be used as attractant to both nymphs and adults of *O. laevigatus* and consequently they may be used as prey kairomones. Fundamental knowledge of the chemical ecology of pest insects and their natural enemies is now being applied to enhance the biological control of pests. Simpson et al. (2011) conducted a novel biological control approach, “attract and reward” which combines uses synthetic herbivore-induced plant volatiles to improve immigration of beneficial taxa into crops and nectar plants to maintain their population. Since *O. laevigatus* is expensive to release and slow to establish within the crops, particularly when flowers are scarce (Bennison et al., 2011), we suggest that future research in biological control should consider the use of the blend (RLA:NMB) to focus the attraction of *O. laevigatus* towards specific crops thus providing news opportunities in the use of prey pheromones as modulators of predator searching behaviour.

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### **Author contribution statement**

TV, JLC, MAMG, IdA conceived and designed the research. IdA synthesized the test compounds. TV conducted the experiments and wrote the draft. TV, AP analysed the data. All authors read and approved the manuscript.

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## SECCIÓN 2

### Capítulo 3

#### **Role of thrips omnivory and their aggregation pheromone on the multitrophic interaction between sweet pepper plants, aphids and hoverflies**

Role of thrips omnivory and their aggregation pheromone on the multitrophic interaction between sweet pepper plants, aphids and hoverflies (2019). Accepted in *Frontiers Ecology and Evolution*. doi: 10.3389/fevo.2018.00240.



# Role of Thrips Omnivory and Their Aggregation Pheromone on Multitrophic Interactions Between Sweet Pepper Plants, Aphids, and Hoverflies

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In complex food webs, plants are commonly attacked by multiple herbivorous species, affecting the preference and performance of other herbivores and natural enemies. The role of omnivorous insects in ecosystems may be more complex because of the consumption of both plant and animal organisms. However, the effect of omnivorous insects on herbivores and natural enemies, has received little attention so far. The main goal of this study was to investigate whether the dual herbivore interaction between aphids and thrips, an omnivorous pest, on a sweet pepper system, may affect different trophic levels, from plant and aphid performance until the third trophic level, i.e., preference and performance of the predatory hoverfly *Sphaerophoria rueppellii*. Additionally, we tested whether the aggregation pheromone of the thrips *Frankliniella occidentalis*, could disturb the oviposition behavior of the predatory hoverfly. Our results show that the presence of thrips decreases host plant and aphid performance. Furthermore, despite not affecting syrphid larval performance, thrips presence reduces fecundity of the adults that emerge from those larvae. Additionally, we observed that syrphids avoid ovipositing on plants with either thrips or thrips aggregation pheromone. The present study reveals how the presence of thrips or a semiochemical compound related to thrips, can impact the behavior and performance of an aphidophagous predator.

**Keywords:** dual attack, *Frankliniella occidentalis*, *Myzus persicae*, *Sphaerophoria rueppellii*, omnivore-herbivore interaction, pheromone

## INTRODUCTION

In nature, plants are commonly attacked by multiple herbivorous species. Plant responses to feeding by single herbivores can impact the preference and performance of other herbivores (Stam et al., 2014; Pineda et al., 2017; Vaello et al., 2018), affecting the attractiveness and performance of natural enemies (Shiojiri et al., 2002; Ponzio et al., 2014; Stam et al., 2017). Many of multi-attack interactions include omnivorous arthropods which are capable of exploiting both plant and prey resources, ensuring their survival in the absence of one resource (Coll and Guershon, 2002). Thus, in dual attack situations, herbivores that co-exist with omnivores on the same plant compete for

## Abstract

In complex food webs, plants are commonly attacked by multiple herbivorous species, affecting the preference and performance of other herbivores and natural enemies. The role of omnivorous insects in ecosystems may be more complex because of the consumption of both plant and animal organisms. However, the effect of omnivorous insects on herbivores and natural enemies, has received little attention so far. The main goal of this study was to investigate whether the dual herbivore interaction between aphids and thrips, an omnivorous pest, on a sweet pepper system, may affect different trophic levels, from plant and aphid performance until the third trophic level, i.e. preference and performance of the predatory hoverfly *Sphaerophoria rueppellii*. Additionally, we tested whether the aggregation pheromone of the thrips *Frankliniella occidentalis*, could disturb the oviposition behaviour of the predatory hoverfly. Our results show that the presence of thrips decreases host plant and aphid performance. Furthermore, despite not affecting syrphid larval performance, thrips presence reduces fecundity of the adults that emerge from those larvae. Additionally, we observed that syrphids avoid ovipositing on plants with either thrips or thrips aggregation pheromone. The present study reveals how the presence of thrips or a semiochemical compound related to thrips, can impact the behaviour and performance of an aphidophagous predator.

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## Introduction

In nature, plants are commonly attacked by multiple herbivorous species. Plant responses to feeding by single herbivores can impact the preference and performance of other herbivores (Stam et al., 2014; Pineda et al., 2017; Vaello et al., 2018), affecting the attractiveness and performance of natural enemies (Shiojiri et al., 2002; Ponzio et al., 2014; Stam et al., 2017). Many of multi-attack interactions include omnivorous arthropods which are capable of exploiting both plant and prey resources, ensuring their survival in the absence of one resource (Coll and Guershon, 2002). Thus, in dual attack situations, herbivores that co-exist with omnivores on the same plant compete for plant food but also have a potential risk of omnivore predation. For example, negative performance and preference were observed in

the herbivore whitefly *Trialeurodes vaporariorum* (Westwood 1856) (Hemiptera: Aleyrodidae) in co-occurring system with the omnivorous thrips *Frankliniella occidentalis* Pergande 1895 (Thysanoptera; Thripidae) (Pappas et al., 2018). Moreover, these omnivore-herbivore interactions may cascade up to other trophic levels. For example, thrips control by *Amblyseius swirskii* (Athias-Henriot 1962) (Acari: Phytoseiidae) was delayed in the presence of the whitefly *T. vaporariorum*, whereas the control of this whitefly was improved in the presence of thrips (Messelink and Janssen, 2008; Messelink et al., 2010).

Most of the studies about the responses of predators to dual herbivory are mainly focused on the behaviour of predatory mites (De Boer et al., 2008; Zhang et al., 2009; Lima et al., 2017) or predatory bugs (Moayeri et al., 2007), whereas predator performance, and effects of omnivorous arthropods, have received little attention so far. Predatory hoverflies (Diptera: Syrphidae) are important aphid natural enemies, which are often studied in the context of biological control (Brewer and Elliott, 2004; Pineda and Marcos-García, 2008; Almohamad et al., 2009; Amorós-Jiménez et al., 2014, 2015; Amiri-Jami et al., 2017), being commercially used as biological control agents in greenhouse crops. Gravid syrphid females, searching for plants on which they lay eggs, are able to detect and avoid potential intra- or interspecific competition (Hindayana et al., 2001; Fréchette et al., 2007; Pineda et al., 2007; Amorós-Jiménez et al., 2015). Thus, oviposition behaviour is the first step in a successful biological control program, especially for natural enemies with larval stages much less mobile than the adult stage, such as the case of hoverflies. Although a previous study demonstrated syrphid's vulnerability to predation by other generalist predators (Fréchette et al., 2007), to our knowledge no information is available about syrphid behaviour, under dual herbivore attack.

In sweet pepper crops, but also in many other crops worldwide, the phloem feeder aphid *Myzus persicae* (Sulzer 1776) (Hemiptera: Aphididae) and the thrips *F. occidentalis* often co-occur on a same plant (Messelink et al., 2013). Thrips usually feed on leaf tissue or plant pollen, but may also feed from eggs of predatory mites (Faraji et al., 2002; Janssen et al., 2003), eggs of spider mites (Agrawal and Klein, 2000) or crawlers of the whitefly *Trialeurodes vaporariorum* (Westwood 1856) (Hemiptera: Aleyrodidae) (Van Maanen et al., 2012). Nowadays, aphids are a major problem in biological control programs on sweet pepper (Bloemhard and Ramakers, 2008), and thrips have been described to disturb and even prey on aphid predators and other natural enemies (Magalhães et al., 2005; Messelink et al., 2013). The predatory hoverfly *Sphaerophoria rueppellii* (Wiedemann 1830) (Diptera: Syrphidae) is commonly released in greenhouse crops to control, among other aphid species,

the green peach aphid *M. persicae* (Amorós-Jiménez et al., 2012, 2015), however the potential disruption of syrphids through thrips presence is not known.

Thus, in this study, we investigated how the interaction between aphids and omnivorous thrips may affect different trophic levels, from plant and aphid performance to the third trophic level, assessing preference and performance of a predatory hoverfly. Previous studies have shown that dual herbivory by insects belonging to different feeding guilds can affect plant performance (Ponzio et al., 2016), and how through plant-mediated interactions, the performance of future herbivores feeding on those plants can be decreased (Erb et al., 2011; Pappas et al., 2018). Based on those studies, we hypothesize that in presence of *F. occidentalis* (omnivorous and plant cell-content feeder), sweet pepper growth and aphid (plant phloem feeder) performance will be negatively affected. Regarding the effects of thrips presence on syrphids, we expect a reduced number of eggs oviposited on plants with dual herbivory compared with only aphids presence, and associated to this, a negative syrphid performance. This hypothesis is based on previous studies where *F. occidentalis* preyed on eggs from its natural enemy *Iphiseius degenerans* (Berlese 1889) (Acari: Phytoseiidae) (Faraji et al., 2002; Janssen et al., 2003) and also where non-prey insects such as ants were able to reduce the performance and oviposition of the hoverfly *Episyrphus balteatus* (de Geer 1776) (Diptera: Syrphidae) (Amiri-Jami et al., 2017). Moreover, we expected that the aggregation pheromone of *F. occidentalis* ((R)-lavandulyl acetate: neryl (S)-2-methylbutanoate), could be used by *S. rueppellii* to discriminate between plants with or without thrips. This hypothesis is based on our previous results showing that the thrips aggregation pheromone was attractive to a thrips natural enemy, the predatory bug *Orius laevigatus* (Fieber 1860) (Hemiptera: Anthocoridae) (Vaello et al., 2017).

## Material and methods

### *Plants and Insects*

The study system consisted of sweet pepper (*Capsicum annuum* L., var. California wonder), a cultivar commonly used in greenhouse crops, the aphid *M. persicae*, the omnivorous thrips *F. occidentalis*, and the hoverfly *S. rueppellii*. Sweet pepper plants were grown from seeds in plastic pots (5.5 cm in diameter, 7 cm in height) with a mix of soil and vermiculite 1:1, in a climatic chamber at 24 °C, 60 % relative humidity (RH) and a 16 h light and 8 h dark photo regime. Insects were reared at the CIBIO, University of Alicante, Spain. *M. persicae* was

reared on *C. annuum* plants for multiple generations and *F. occidentalis* was reared on fresh green beans *Phaseolus vulgaris* L. (Fabaceae) as described by Espinosa et al., (2002), under the same conditions as above. The predator *S. rueppellii* was provided by BioNostrum Pest Control S.L. (Alicante, Spain) in three different life stages: eggs, pupae and adults (10±1 day old). *S. rueppellii* was maintained as described by Amorós-Jimenez et al. (2012).

#### *Performance of Sweet Pepper and Myzus persicae*

Aphid population growth was measured in the presence of single or dual herbivory. We established two treatments: (i) single herbivory: 20 sweet pepper plants (five weeks old) were infested with 10 individuals of *M. persicae* adults (8±1 days old) on the second fully expanded leaf of each plant, during 3 days (ii) dual herbivory: 20 sweet pepper plants were arranged as for the treatment of single herbivory, but 3 days before each plant was infested with 10 *F. occidentalis* larvae (2 days old) on the second fully expanded leaf of each plant, allowing the colonization and feeding on the plant. All the plants were individually enclosed in plastic cages with two windows with anti-thrips gauze to prevent the escape of insects and ensure ventilation. Twice a week, the location of the cages with plants was randomized, to minimize positional effects. After 7 days of infestation, the total number of *M. persicae* individuals was counted in both treatments. Plant height was measured before and after infestation, and above and belowground biomass was collected at the end of the experiment and oven dried (40 °C during 5 days) before measuring dry weight.

#### *Performance of Sphaerophoria rueppellii*

To assess the performance of *S. rueppellii*, we used the same treatments as explained above in a new experiment. After 3 days of aphid infestation in single herbivory and dual herbivory (and after 6 days of thrips infestation in dual herbivory treatment), three *S. rueppellii* larvae (2 days old since eggs hatched) were transferred to each of the infested plants with a fine paint brush. In total 120 larvae were used (20 plants x 2 treatments x 3 larvae per plant). Larval development was monitored daily, and larval mass was recorded on the second instar (L2), third instar (L3) and pupal stage. Newly emerged adults were kept in a net cage (60 x 60 x 30 cm) separately for each treatment. Adults were provided with water, sucrose, multi-flower bee pollen and an aphid-infested pepper plant as oviposition stimulus to allow ovaries maturation. Then 9 days old *S. rueppellii* females were allowed to lay eggs individually for 48 h in cages (60 x 60 x 30 cm) with an aphid-infested sweet pepper plant (infested with 10

individuals of *M. persicae* adults ( $8 \pm 1$  days old) on the second expanded leaf, during 3 days). The number of females used were: 21 for the treatment with aphids and 17 for the treatment with aphids and thrips. Fertility was measured by counting the number of eggs laid by females 24 h and 48 h after release. A new aphid-infested plant was offered after 24 h. Fecundity was measured counting the hatched first instar (L1) larvae two days after syrphid oviposition. After the oviposition assay, wing length was determined for males and females separately as a surrogate for total body size in syrphids and as a good indicator for adult fitness (Stubbs and Falck, 1983, Kingsolver and Huey, 2008; Amorós-Jiménez et al., 2014). Morphological measurements were always done using the left wing, to avoid any effect due to asymmetry. We used an Image Analyzer LEIKA (M205C/DFC425, Cambridge, United Kingdom) for wing length measurements. All the bioassays were performed in climate chamber at 24 °C, 60 % relative humidity (RH) and a 16 h light and 8 h dark photo regime.

#### *Oviposition Preference of S. rueppellii*

Oviposition preference was determined in a two-choice experiment. An insect-proof cage (30 x 30 x 60 cm) was placed inside a climatic chamber. Two sweet pepper plants (5 weeks old) were placed in two different corners of the cage to test the oviposition preference for plants infested with single (aphids) or dual herbivory (aphids and thrips). Plant position was randomised for every new replicate. To study the oviposition response, a total number of 60 individuals of syrphid females (9 days old), obtained from pupae from BioNostrum Pest Control S.L., were tested. The number of eggs after 2 h and 24 h were recorded individually per female syrphid. The oviposition preference bioassay was performed in climate chamber at 24 °C, 60% relative humidity (RH) and a 16 h light and 8 h dark photo regime.

In a new experiment we tested the syrphid oviposition response to single herbivore (aphids) versus a component of *F. occidentalis* aggregation pheromone (R)-lavandulyl acetate:neryl (S)-2-methylbutanoate [RLA:NMB] (Hamilton et al., 2005). RLA and NMB were synthesized according to Hamilton et al. (2005) and Zada and Harel (2004), respectively. The purity of the compounds was analyzed by gas chromatography - mass spectrometry (GC-MS) integration, and the optical purity was estimated from the corresponding specifications of the starting materials (RLA 99.2% purity, 98.7% enantiomeric excess (ee) and NMB 91.5% purity, 98.0% ee). In order to prepare the odour source, tested compounds were individually diluted (1:1, v/v) in paraffin oil (Sigma-Aldrich, USA). The ratio used in this experiment was

selected according to previous results testing the attraction of the natural enemy *O.laevigatus* to *F. occidentalis* aggregation pheromone (Vaello et al., 2017). Parafilm oil (2 µl) (used as control) and 1RLA:2.3NMB blend (2 µl) were applied on a piece of filter paper (1 × 1 cm) that was placed on the second expanded leaf of the sweet pepper plant. After 15 min, the female adults were released (the filter paper was kept in the plant during all the oviposition experiment). The treatments used in this case were: (i) Aphid treatment: 10 *M. persicae* (8±1 days old) were placed on the second fully expanded leaf from each sweet pepper plant, and after three days we added 2 µl of paraffin oil on the plant, as described above. (ii) Aphid-pheromone treatment: 10 *M. persicae* (8±1 days old) were placed on the second fully expanded leaf from each sweet pepper plant, and after three days we added to the plant 2 µl of 1 RLA: 2.3NMB ratio from *F. occidentalis* aggregation pheromone. The oviposition cage consisted of an insect-proof cage (30 x 30 x 60 cm) which was placed in a climatic chamber. A total number of 84 syrphid female individuals (9 days old obtained from BioNostrum Pest Control S.L.) were tested in this experiment. The parameters used to study ovipositional response were: number of eggs laid at 2 h and 24 h after syrphid release. The oviposition preference bioassay was performed at 24 °C, 60% relative humidity (RH) and a 16 h light and 8 h dark photo regime.

### *Thrips Predation*

To assess how herbivores can affect predator performance, potential predation on syrphid eggs by omnivore thrips was recorded in two experiments. In the first experiment we observed the predation and/or damaged eggs with a Petri dish bioassay of two treatments: (i) Aphid treatment: 10 aphids (8±1 days old) were placed in a sweet pepper leaf with 10-15 syrphid eggs in a Petri dish and (ii) Aphid and thrips treatment: 10 aphids (8±1 days old) and 10 second instar thrips larvae (3±1 days old) were placed on a sweet pepper leaf with 10-15 syrphid eggs in a Petri dish. Syrphid eggs were obtained by confining 15-20 mature females in an insect-proof cage (30 x 30 x 60 cm) with aphid infested sweet pepper plants as stimulus; after 2 h we selected leaves with syrphid eggs for the experiment and aphids were removed carefully with a paint brush. After 24 h of introducing aphids and thrips in the Petri dish, eggs were observed under a binocular microscope, to record egg damage, and after 48 h, survival rate was followed in time to observe egg hatching.

A second experiment was conducted with entire sweet pepper plants (5 weeks old). Each treatment included 20 sweet pepper plants that were infested with 10 individuals of *M.*

*persicae* adults ( $8\pm 1$  days old) on the second fully expanded leaf of each plant for 3 days. All the plants were individually enclosed in plastic cages with two windows with anti-thrips gauze to prevent the escape of insects and ensure ventilation. Afterwards, one adult female of *S. rueppellii* was allowed to oviposit for 24 h on each plant. Eggs were counted and we left on the plant a number of 10-15 eggs (to standardize the numbers of eggs per plant). From the results of the Petri dish experiment, we selected two treatments with different thrips life stages, with the aim to assess the effect of thrips life stage on predation behaviour: (i) Larval treatment: 10 second instar thrips were transferred on the plant with a fine paint brush. (ii) Adult treatment: 10 adults ( $15\pm 1$  days old) were released on the plant with a fine paint brush. Egg damage, hatching and larval survival at 48 h were observed. Both experiments were performed in a climate chamber at 24 °C, 60 % relative humidity (RH) and a 16 h light and 8 h dark photo regime.

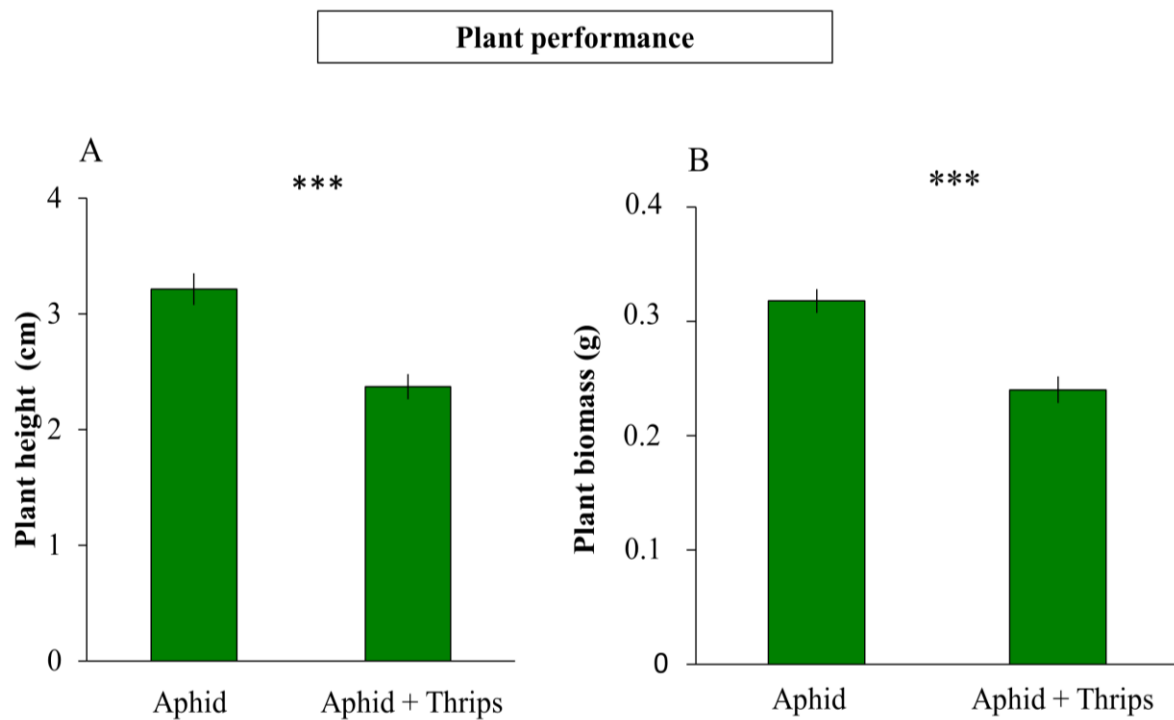
### *Statistical Analyses*

Plant performance (plant height and plant biomass) was analysed with a t-test. Aphid density was analysed with Generalized Linear Models (GLM) with Poisson distribution and dispersion parameter estimated. Regarding data on syrphid performance; weight and wing length measures were analysed with t-test, fecundity was analysed using a GLM with Poisson distribution and fertility with a GLM with binomial distribution. Results from the oviposition experiment were analysed with the Wilcoxon test. All statistical tests were performed using SPSS 15.0.; SPSS Inc., Chicago, IL, USA.

## **Results**

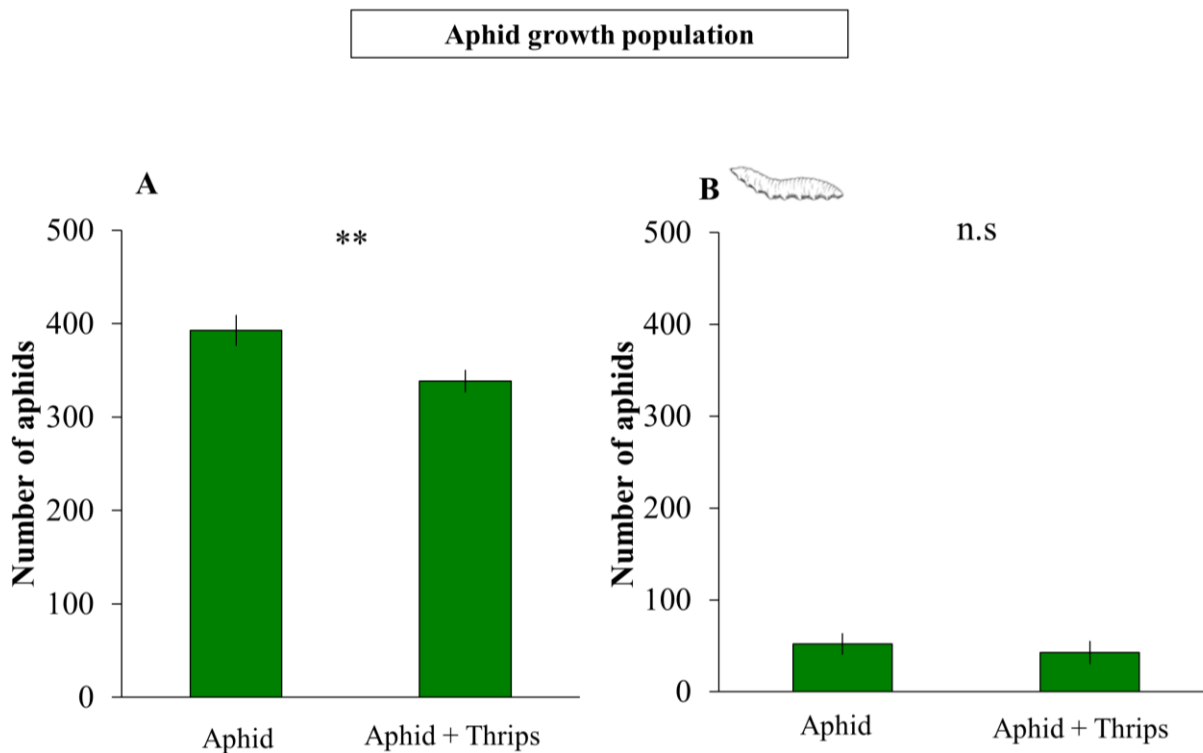
### *Thrips Presence Decreases Plant Performance and Aphid Population*

Plant height was measured before and after infestation, and the height between these two time points was reduced when thrips were present in the system compared to only aphids ( $F_{1, 38} = 1.325$ ;  $P < 0.001$ ; Figure 1A). Similarly, plant biomass was lower when thrips and aphids were together on the same plant compared to aphids alone ( $F_{1, 38} = 0.312$ ;  $P < 0.001$ ; Figure 1B).



**Figure 1.** Plant performance of 6-week-old sweet pepper after one week of herbivory: (A) plant height difference (before and after infestation with aphid versus aphid + thrips); (B) total plant biomass. Bars represent means  $\pm$  SE (n=20 replicates). Asterisks indicate significant differences between infestation treatments: \*\*\*  $P < 0.001$  (t-test).

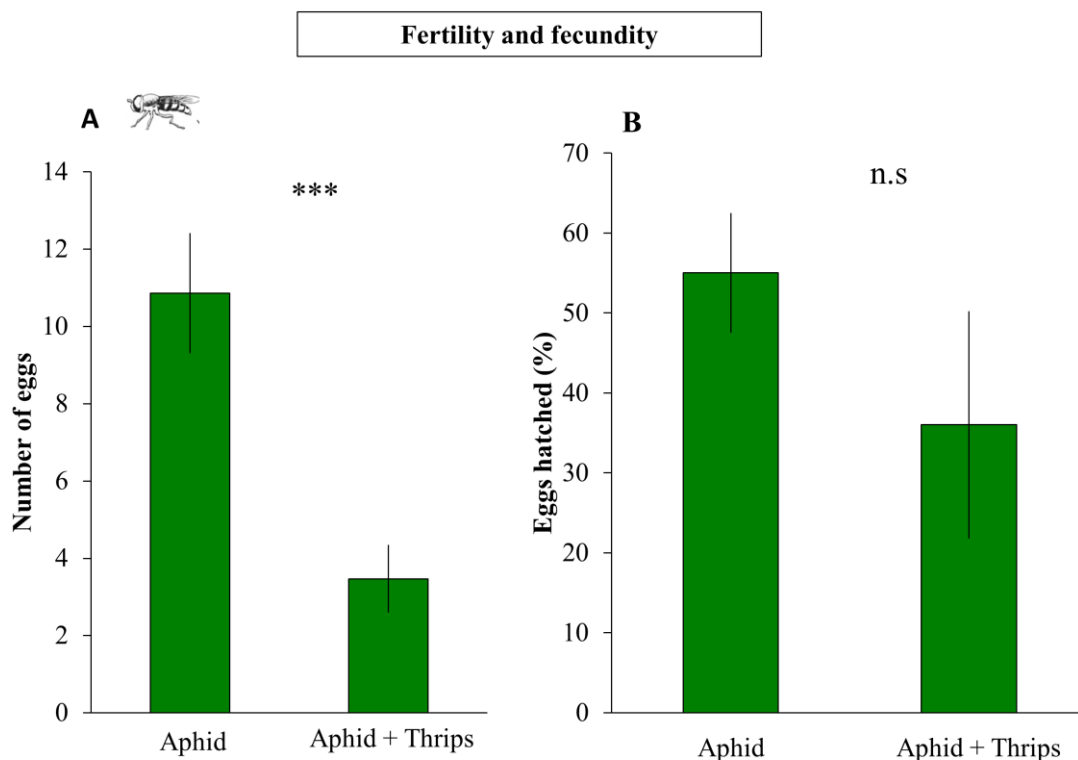
After 7 days of herbivore infestation, the population of aphids was higher in plants without thrips ( $F_{1, 38} = 7.261$ ;  $P = 0.007$ ; Figure 2A). We also measured plant performance when *S. rueppellii* was present. The difference in plant height between single or dual herbivory in the presence of syrphid larvae was not significant ( $F_{1, 38} = 0.999$ ;  $P = 0.166$ ), whereas plants had lower biomass when thrips were also present ( $F_{1, 38} = 0.346$ ;  $P = 0.046$ ). The population of aphids when syrphids were present after 7 day-infestation, did not show differences between single or dual herbivory ( $F_{1, 38} = 0.339$ ;  $P = 0.561$ ; Figure 2B).



**Figure 2.** Number of aphids on plants infested with aphid vs aphid + thrips after: (A) one week of herbivore infestation; (B) development of *S. rueppellii* larvae feeding on infested plants until pupal stage. Bars represent means  $\pm$  SE (n=20 replicates). Asterisks indicate significant differences between infestation treatments: \*\*  $P < 0.01$ , n.s not significantly different ( $P > 0.05$ , GLM Poisson).

#### *Thrips presence decreases fecundity but not fertility of S. rueppellii*

*S. rueppelli* larval development, was not affected by the presence of thrips in our study system. Parameters such as larval and pupal biomass, adult survival and wing length were not significantly affected by single or dual herbivory (Table 1). However, in the adults that developed from larvae feeding on an aphid colony with thrips presence, fecundity was negatively affected by thrips, with a lower number of eggs laid by female adults, both at 24 h ( $F_{1,36} = 11.648$ ;  $P = 0.001$ ) and 48 h ( $F_{1,36} = 15.057$ ;  $P < 0.001$ ; Figure 3A) after female adults release. In contrast, fertility was not affected by the presence of thrips, with similar egg hatching ( $F_{1,28} = 1.435$ ;  $P = 0.231$ ; Figure 3B), nor was the mortality of the emerging first instar larvae (L1) ( $F_{1,28} = 0.360$ ;  $P = 0.549$ ).



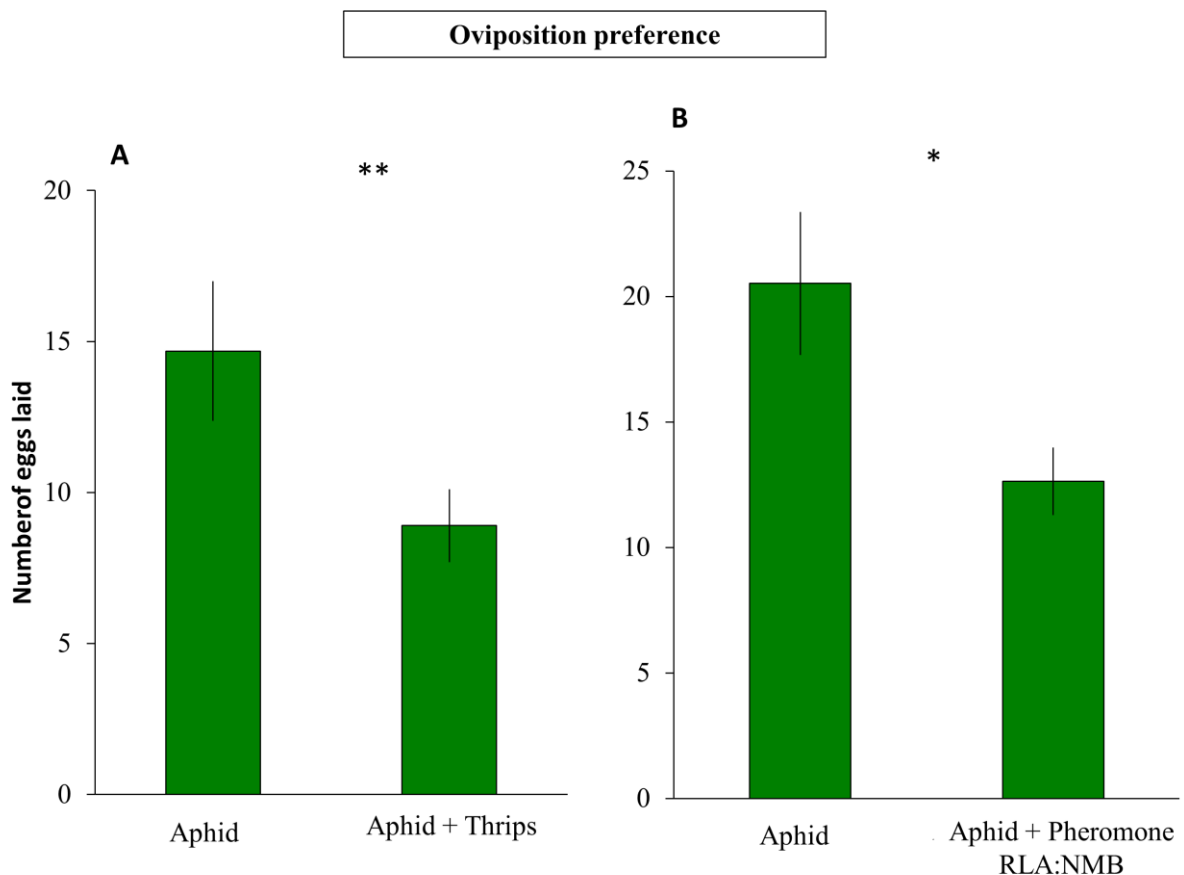
**Figure 3.** Fecundity and fertility of *S. rueppellii* female adults after completing their life cycle feeding on aphid-infested plants (n= 21 replicates) and on aphid + thrips-infested plants (n=17 replicates). (A) Number of syrphid’s eggs laid (Fecundity) and (B) number of viable eggs hatching (Fertility). Bars represent means  $\pm$  SE. Asterisks indicate significant differences between infestation treatments: \*\*\*  $P < 0.001$ , n.s not significantly different  $P > 0.05$  (GLM).

**Table 1.** Morphological traits of *S. rueppellii* when fed on *M. persicae* with and without the presence of *F. occidentalis*. Measures represent means  $\pm$  SE. Data here presented were not significantly different between the two treatments (t-test analyses).

Measure	Thrips absent	Thrips present
L2 weight (mg)	1.342 $\pm$ 0.081	1.417 $\pm$ 0.160
L3 weight (mg)	11.834 $\pm$ 0.454	11.738 $\pm$ 0.303
Pupa weight (mg)	9.595 $\pm$ 0.141	10.728 $\pm$ 1.980
Wing length males (mm)	3.619 $\pm$ 0.024	3.656 $\pm$ 0.034
Wing length females (mm)	3.881 $\pm$ 0.024	3.843 $\pm$ 0.041

*Syrphid Females Avoid Ovipositing on Plants with Thrips or their Aggregation Pheromone*

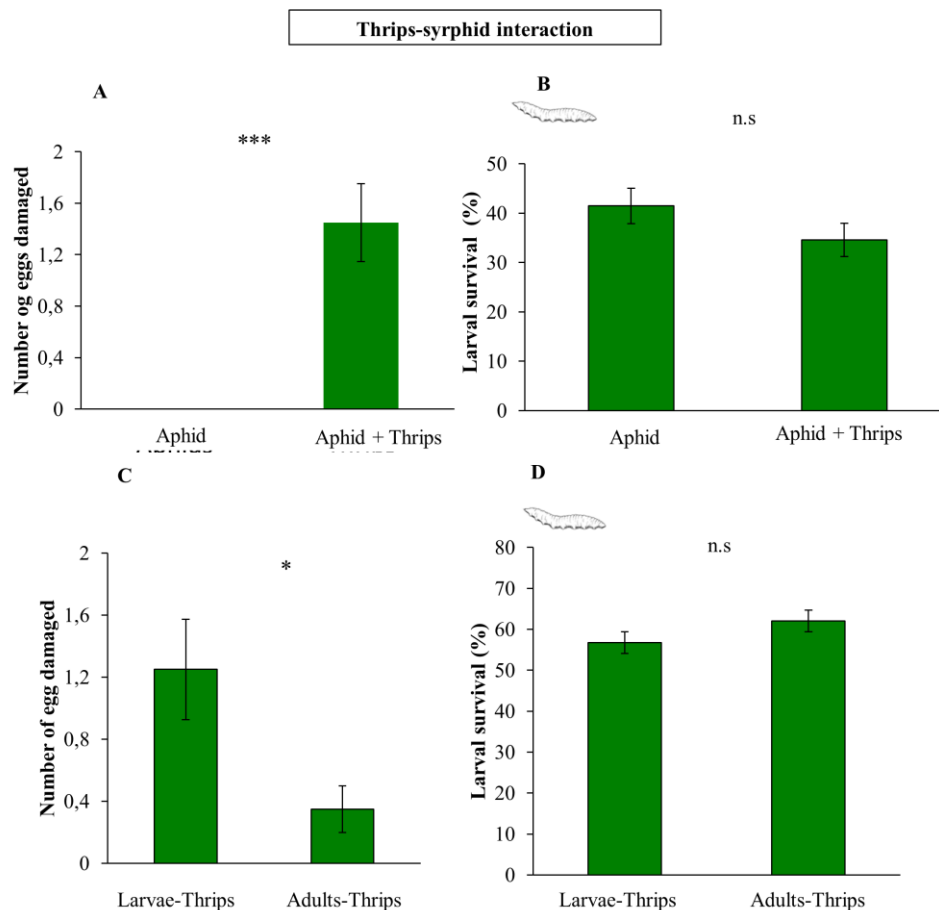
The presence of *F. occidentalis* in an aphid colony did not affect the decision of *S. rueppellii* to lay their eggs in the first 2 hours after syrphid release ( $Z = -1.550$ ;  $P = 0.121$ ). However, after 24 h, the number of eggs recorded on plants infested with aphids and thrips was less than on plants without thrips ( $Z = -3.178$ ;  $P = 0.001$ ; Figure 4A). In the second experiment where we tested the oviposition behaviour in the presence of a synthetic infochemical of the thrips aggregation pheromone, we observed similar results. Females did not show significant differences selecting an oviposition place between plants with aphids versus plants with aphids and thrips aggregation pheromone on the first 2 h ( $Z = -1.299$ ;  $P = 0.194$ ). However after 24h, the number of eggs on plants with aphids and thrips aggregation pheromone was lower than in plants with only aphids ( $Z = -2.992$ ;  $P = 0.003$ ; Figure 4B).



**Figure 4.** Oviposition of *S. rueppellii* on sweet pepper plants after 24 h (A) Number of syrphid eggs laid on aphid vs aphid+thrips-infested plants ( $n=60$  replicates) and (B) number of syrphid eggs laid on aphid-infested plants vs aphid-infested plants plus  $2 \mu\text{l}$  of 1 RLA: 2.3NMB *F. occidentalis* aggregation pheromone ( $n=42$  replicates). Bars represent means  $\pm$  SE. Asterisks indicate significant differences between infestation treatments: \*\*  $P \leq 0.001$ , \*  $P < 0.05$  (Wilcoxon test).

*Thrips-predator Interaction does not affect Final Syrphid Survival*

In a first Petri dish assay we tested whether thrips would damage hoverfly eggs. We observed that *F. occidentalis* second instar larvae damage syrphid eggs, causing loss of turgor on its surface (F<sub>1, 58</sub>= 15.661; P< 0.001; Figure 5A, 6), although no effect on survival rate was recorded (F<sub>1, 58</sub>= 1.964; P= 0.161; Figure 5B). In a second experiment, we tested if the developmental stage of thrips affects differently egg damaging in an experiment with whole plants. The results showed that adult thrips caused lower syrphid egg damage than second instar thrips larvae (F<sub>1, 38</sub>= 6.843; P= 0.009; Figure 5C), and for the Petri dish experiment, this damage did not affect syrphid survival (F<sub>1, 38</sub>= 0.285; P= 0.594; Figure 5D).



**Figure 5.** Direct effects of the presence of thrips on syrphid performance. (A) *S. rueppellii* eggs damaged by prey in a Petri dish experiment; (B) *S. rueppellii* larval survival after prey presence in a Petri dish experiment; (C) *S. rueppellii* eggs damaged by larvae vs adults thrips on entire plants and (D) *S. rueppellii* larval survival in the presence of second instar larvae and adults thrips on entire plants. Bars represent means  $\pm$  SE (n=20 replicates). Asterisks indicate significant differences between infestation treatments: \*\*\* P < 0.001, \* P < 0.05 and n.s not significant differences (P > 0.05, GLM).

## Discussion

The present study demonstrated that the effects of the omnivorous insect *F. occidentalis*, in dual herbivore interaction with aphids, could scale up to the third trophic level. Thrips affected plant growth as well as aphid population growth but also the ovipositional preference and performance of the aphidophagous hoverfly *S. rueppellii*. We also provide the first evidence of thrips predation on syrphid eggs, although without affecting syrphid juvenile survival in our study. Moreover we showed how specific chemical cues from the aggregation pheromone of *F. occidentalis* could disrupt syrphid ovipositional behaviour.

Thrips can affect plant-mediated interactions with arthropods such as spider mites or whiteflies (Pallini et al., 1998; Agrawal et al., 1999; Pappas et al., 2018). However, although the simultaneous presence of thrips and aphids is common in many agricultural crops, few studies have focused on their interactions. Despite that in our study the number of thrips was much lower than the number of aphids (10 thrips versus more than 300 aphids per plant), thrips presence reduced the growth of sweet pepper plants, as it has been shown for a range of vegetable crops (Welter et al., 1990; Shipp et al., 1998). This reduction in plant performance is probably due to the induction of plant defences by thrips in early stages of plant development (five weeks old), producing an energetic cost associated with plant defense (Agrawal, 1998). In a previous study, we showed that the performance of *F. occidentalis* on *M. persicae* pre-infested plants was enhanced (Vaello et al., 2018). In this study, we observed the opposite direction of this interaction showing a negative effect of thrips on aphids. Hence, our results show both direct effects of thrips herbivory in sweet pepper growth (with reduced host-plant quality) and indirect effects on aphid performance (with lower aphid population).

Due to these effects on aphids, we hypothesized that the performance of the predator *S. rueppellii* could be negatively influenced by thrips presence indirectly via the reduced population of prey (aphids) in the presence of thrips. Our results show that the fecundity of *S. rueppellii* was reduced, although larval development was not affected by thrips presence. This type of effects has been shown for the hoverfly *E. balteatus*, whose larval survival was not different when feeding on aphids on two different plant species, in contrast to its fecundity (Amiri-Jami et al., 2017). Similar results were found between thrips and predatory mites, where the net reproduction of *Neoseiulus californicus* (McGregor 1954) (Acari: Phytoseiidae) was reduced in the presence of thrips (Choh et al., 2017). However, the fact that in the presence of *S. rueppellii*, aphid population was not affected by the presence of

thrips indicates that thrips do not interfere with the hoverfly success as biological control agent.

Moreover, in this study we also demonstrate that thrips presence can have direct effects on predators. We observed that thrips larvae could damage the surface of the chorion of syrphid eggs (Figure 6), without however affecting juvenile survival. The lack of effect on survival rate may be explained by the fact that the hatching rate of *S. rueppellii*'s eggs was less than 60 %, and probably the effect of egg damage by thrips was diluted, although we still observed a trend. Probably in natural conditions with plants experiencing higher infestation of thrips, the survival rate of syrphids would be lower. Regarding the opposite direction, although thrips are a rare prey for syrphids, a previous study already reported *Sphaerophoria quadrituberculata* (Bezzi, 1915) (Diptera: Syrphidae) feeding on thrips *Cercothrips afer* (Priesner, 1925) (Thysanoptera: Phlaeothripidae) (Callan, 1955). In previous assays we observed that less than 10% of *S. rueppellii* larvae were able to feed and complete their life cycle until adulthood, feeding exclusively on thrips (Marcos-García, pers. comm.). These findings suggest that syrphids developing in co-occurrence conditions predate preferably on aphids but they could occasionally also consume non-preferred prey such as thrips (Figure 7). Feeding on a non-optimal prey may decrease predator performance, as occurred with the predator *N. californicus* in the presence of thrips (Choh et al., 2017). One possibility is therefore that syrphid larvae feed with thrips to protect themselves, but this behaviour needs to be further tested.



**Figure 6.** Egg of *S. rueppellii* damaged by a *F. occidentalis* second instar larva.



**Figure 7.** First instar of *S. rueppellii* larva feeding on *F. occidentalis* second instar larva.

Moreover we suggest that another direct effect of thrips on hoverflies could be related to causing stress on their larvae with a later effect on fecundity. In a recent study, the presence of ants in aphid colonies reduced the performance and oviposition of *E. balteatus* through disturbing syrphid predation or with ants preying on syrphid larvae (Amiri-Jami et al., 2017). In a similar way, here we observed reduced oviposition in aphid-infested plants with thrips presence, and a reduced performance associated to that behaviour. Hoverflies show an oviposition preference for plant-aphid species combinations that maximize their performance, either avoiding heterospecific competitors (Almohamad et al., 2009), intraguild interactions (Pineda et al., 2007), or a risk to their offspring (Almohamad et al., 2009). The “intimidation” by omnivorous herbivores to predators may induce the ecology of fear (Clinchy et al., 2013), triggering stress to predators, decreasing the number of eggs laid and preventing optimal oviposition searching.

Syrphids exploit various chemical blends from plants and insects when searching for oviposition sites, such as the semiochemical (E)- $\beta$ -farnesene (E $\beta$ F), the main component of the alarm pheromone of most aphid species (Francis et al., 2005; Verheggen et al., 2008). However limited information exists about syrphids response to semiochemicals from non-preferred prey. Our results show that the aggregation pheromone of *F. occidentalis* leads to oviposition avoidance by *S. rueppellii*, indicating that predators are able not only to rely on semiochemicals from a non-preferred prey, but also using it to distinguish unsuitable oviposition places. Although not evaluated in the current study, one possibility is that syrphid females retain their eggs in the absence of suitable oviposition sites, as occurred for *Eupeodes corollae* (Fabricius, 1794) (Diptera: Syrphidae) in the absence of aphids (Almohamad et al.,

2009). In contrast, the predatory bug *O. laevigatus*, a natural enemy of *F. occidentalis*, responded positively to the aggregation pheromone of its prey (Vaello et al., 2017). Thus, the aggregation pheromone RLA:NMB seems to act both as an attractant for a thrips predator such as *O. laevigatus* and as repellent for *S. rueppellii*. Moreover, the fact that *S. rueppellii* prefers to oviposit on plants without thrips, could be due to the detection of fewer aphids in colonies with thrips presence as we observed. Further possibilities to be tested are whether *S. rueppellii* avoids competition or intraguild predation with other predators with this behaviour (such as *O. laevigatus*), or how the presence of thrips may alter the volatile blend produced by plants infested with aphids, affecting in the end the oviposition behaviour of syrphids.

In conclusion, this study shows that although the presence of omnivorous insects can affect three trophic levels, the efficiency of the biological control may not be compromised, as was shown for *S. rueppellii* that controlled the aphid population in both the presence and the absence of thrips. This study also raised awareness about how an aggregation insect pheromone may act as a repellent for natural enemies. Hence, in a biological control context, it is crucial to analyse the effect of co-occurring pest species interactions to predict the effectiveness of natural enemies, especially for oviposition site selection and subsequent predator performance.

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### **Author contribution**

T.V, A.P and M.A.M.G conceived and designed the research. T.V. conducted the experiments. T.V and A.P analysed the data. T.V, A.P and M.A.M.G interpreted the results and wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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## **SECCIÓN 2**

### **Capítulo 4**

#### **Modulation of plant-mediated interactions between herbivores of different feeding guilds: Effects of parasitism and belowground interactions**

Modulation of plant-mediated interactions between herbivores of different feeding guilds: Effects of parasitism and belowground interactions (2018). *Scientific Reports* 8:14424 doi: 10.1038/s41598-018-32131-9

# SCIENTIFIC REPORTS

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## Modulation of plant-mediated interactions between herbivores of different feeding guilds: Effects of parasitism and belowground interactions

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Herbivory affects subsequent herbivores, mainly regulated by the phytohormones jasmonic (JA) and salicylic acid (SA). Additionally, organisms such as soil microbes belowground or parasitoids that develop inside their herbivorous hosts aboveground, can change plant responses to herbivory. However, it is not yet well known how organisms of trophic levels other than herbivores, below- and above-ground, alter the interactions between insect species sharing a host plant. Here, we investigated whether the parasitoid *Aphidius colemani* and different soil microbial communities (created through plant-soil feedbacks) affect the JA and SA signalling pathways in response to the aphid *Myzus persicae* and the thrips *Frankliniella occidentalis*, as well as subsequent thrips performance. Our results show that the expression of the JA-responsive gene *CaPIN1* in sweet pepper was more suppressed by aphids than by parasitised aphids. However, parasitism did not affect the expression of *CaPAL1*, a biosynthetic gene of SA. Furthermore, aphid feeding enhanced thrips performance compared with uninfested plants, but this was not observed when aphids were parasitised. Soils where different plant species were previously grown, did not affect plant responses or the interaction between herbivores. Our study shows that members of the third trophic level can modify herbivore interactions by altering plant physiology.

Plants have evolved sophisticated strategies to defend themselves against pathogens and herbivorous insects. Plants regulate defence signalling pathways mainly through the production of the phytohormones jasmonic acid (JA) and salicylic acid (SA). The JA-signalling pathway is mainly induced by necrotrophic pathogens and insects that inflict cellular damage such as chewing or cell-content feeding herbivores, whereas the SA-signalling pathway is induced by biotrophic pathogens and phloem-feeding insects<sup>1–3</sup>. In recent years, new advances have shown how the plant response to one attacker can influence the performance of herbivores sharing the same host plant, a phenomenon that is driven by positive or negative interactions between these two signalling pathways<sup>4–7</sup>. For example, previous herbivory from phloem feeders that induce a SA response, often facilitates the later performance of herbivores that induce a JA response in the plant, via negative cross-talk of these signalling pathways<sup>4–9</sup>. However, it remains unknown how organisms of trophic levels other than herbivores interacting with the same plant will affect these often-facilitative interactions between herbivorous insects from different feeding guilds inducing different signalling pathways.

Aboveground, herbivorous insects can be attacked by parasitoids whose larvae develop inside their hosts. It is well established that plants can influence parasitoid performance<sup>8,10</sup>, but it has only recently been demonstrated that parasitoids can also affect plant responses to herbivory. Plants that are attacked by parasitised caterpillars show altered herbivore-induced plant responses, such as the emission of volatiles or the expression of certain

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## Abstract

Herbivory affects subsequent herbivores, mainly regulated by the phytohormones jasmonic acid (JA) and salicylic acid (SA). Additionally, organisms such as soil microbes belowground or parasitoids that develop inside their herbivorous hosts aboveground, can change plant responses to herbivory. However, it is not yet well known how organisms of trophic levels other than herbivores, below- and above-ground, alter the interactions between insect species sharing a host plant. Here, we investigated whether the parasitoid *Aphidius colemani* and different soil microbial communities (created through plant-soil feedbacks) affect the JA and SA signalling pathways in response to the aphid *Myzus persicae* and the thrips *Frankliniella occidentalis*, as well as subsequent thrips performance. Our results show that the expression of the JA-responsive gene *CaPIN II* in sweet pepper was more suppressed by aphids than by parasitised aphids. However, parasitism did not affect the expression of *CaPAL1*, a biosynthetic gene of SA. Furthermore, aphid feeding enhanced thrips performance compared with uninfested plants, but this was not observed when aphids were parasitised. Soils where different plant species were previously grown, did not affect plant responses or the interaction between herbivores. Our study shows that members of the third trophic level can modify herbivore interactions by altering plant physiology.

## Introduction

Plants have evolved sophisticated strategies to defend themselves against pathogens and herbivorous insects. Plants regulate defence signalling pathways mainly through the production of the phytohormones jasmonic acid (JA) and salicylic acid (SA). The JA-signalling pathway is mainly induced by necrotrophic pathogens and insects that inflict cellular damage such as chewing or cell-content feeding herbivores, whereas the SA-signalling pathway is induced by biotrophic pathogens and phloem-feeding insects (Walling, 2000; De Vos et al., 2005; Pieterse et al., 2012). In recent years, new advances have shown how the plant response to one attacker can influence the performance of herbivores sharing the same host plant, a phenomenon that is driven by positive or negative interactions between these two signalling pathways (Rodriguez-Saona et al., 2010; Soler et al., 2012a; Ali et al., 2014; Pineda et al., 2017). For example, previous herbivory from phloem feeders that induce a SA response, often facilitates the later performance of herbivores that induce a JA response in the plant, via negative cross-talk of these signalling pathways (Rodriguez-Saona et

al., 2010; Soler et al., 2012; Ali et al., 2014; Ponio et al., 2014; Pineda et al., 2017). However, it remains unknown how organisms of trophic levels other than herbivores interacting with the same plant will affect these often-facilitative interactions between herbivorous insects from different feeding guilds inducing different signalling pathways.

Aboveground, herbivorous insects can be attacked by parasitoids whose larvae develop inside their hosts. It is well established that plants can influence parasitoid performance (Ode, 2006; Gols, 2014), but it has only recently been demonstrated that parasitoids can also affect plant responses to herbivory. Plants that are attacked by parasitised caterpillars show altered herbivore-induced plant responses, such as the emission of volatiles or the expression of certain defence genes (Poelman et al., 2011b; Zhu et al., 2014, 2015; Ode et al., 2016). Even more interesting is that the altered plant responses due to caterpillar parasitism can also affect other insects, such as hyperparasitoids (Zhu et al., 2015), moths species (Poelman et al., 2011b), and parasitoids developing in another herbivore (Poelman et al., 2011a). At present, our knowledge on how the third trophic level affects interactions between multiple plant attackers via the plant's response is still limited to brassicaceous plants and associated caterpillars, thus it remains unknown whether these findings can be extended to other groups of plants and herbivores, especially those that induce different plant signalling pathways (such as phloem feeders). Thus, the possibility of aphid parasitoids as modulators of plant defences, or how these changes may influence other feeding guilds, such as cell-feeding insects, is still unknown.

Belowground, plants interact with soil microbes, such as mycorrhizal fungi and plant growth promoting rhizobacteria, that can enhance plant growth and induce systemic resistance against different attackers (Yang et al., 2009; Pineda et al., 2010; Jung et al., 2012; Pieterse et al., 2014). There is increased awareness that interactions with the full community of soil microbes, the so-called soil microbiome, contribute to a plant's extended phenotype, and can thereby affect herbivorous insects. For example, soil microbial communities can influence primary and secondary plant metabolite concentration (Bezemer et al., 2005a; Erb et al., 2009; Pangesti et al., 2016), affecting above-ground insects (Soler et al., 2012b; Bezemer et al., 2005b; Kostenko et al., 2012; Bezemer et al., 2013). In turn, plants can also shape the biotic (e.g. microbes) and abiotic characteristics of the soil they grow in, and these changes can affect the performance of plants that grow in that soil subsequently. This phenomenon has been termed "plant-soil feedbacks" (PSF) (Ehrenfeld et al., 2005; Bartelt-Ryser et al., 2005; Kos et al., 2015). The concept of PSF can be applied to create distinct soil

microbiomes by growing different plant species in a given soil, which results in different effects on above-ground herbivores that feed on plants that are subsequently grown in these soils (Bezemer et al., 2013; Van der Putten et al., 2013). To date, there are no studies of PSF-mediated plant responses on herbivorous insects in a dual-attack situation.

In this study, we investigated the induction of the JA and SA-signalling pathways and herbivore interactions in a community context, where plants interact with two herbivore species of different feeding guilds, an aboveground parasitoid, and different belowground soil communities. We used a model system of *Capsicum annuum* var. *maranello* (sweet pepper), the phloem feeding aphid *Myzus persicae* (Sulzer, 1776) (Sternorrhyncha, Aphididae), which is commonly used as a model of SA-inducer,–and the cell content feeding thrips *Frankliniella occidentalis* Pergande, 1895 (Thysanoptera, Thripidae), which is known to induce and be sensitive to JA-regulated defences in Arabidopsis and tomato (Abe et al., 2008,2009; Kawazu et al., 2012; Bravo-Escobar et al., 2017). Both aphids and thrips, are generalist insects and major pests in sweet pepper plants, but also in many other crops worldwide. At the third trophic level, we used the parasitoid *Aphidius colemani* (Dalman, 1820) (Hymenoptera, Braconidae), parasitising the aphid *M. persicae*, in the described plant-herbivore system.

Previous studies have shown that aphid feeding facilitates caterpillar performance, associated with an strong induction of the SA signaling pathway by aphids (although aphids also induce JA signaling) and a suppression of the JA pathway induced by caterpillars (Rodriguez-Saona et al., 2010; Ali et al., 2014; Ponzio et al., 2014; Pineda et al., 2017). Based on those studies, and on the fact that parasitism can enhance the induction of JA by caterpillars (Poelman et al., 2011b), we hypothesized that parasitized aphids would induce a stronger JA signaling compared to healthy aphids, interfering with the facilitation of aphids towards thrips. Similarly, we expected that PSF would enhance the JA-plant response to aphids, and therefore resistance to thrips. This hypothesis is based on the fact that PSF are mainly driven by soil microbes (Bezemer et al., 2013; Heinen et al., 2018), and that different soil microbes can prime plants for a stronger JA-responses (Pieterse et al., 2012,2014; Pineda et al., 2017). As a consequence, we expected that PSF would also enhance the effects of parasitism on plant signalling (since both above- and belowground factors can enhance a JA-response), with aphids having a more negative effect on thrips than in sterile soil. In order to test these hypothesis, we addressed two main research questions: (i) Does parasitism of aphids or PSF influence the induction of marker genes of the JA and SA defensive signalling pathways in pepper plants?; (ii) Do these effects of PSF and/or parasitism on plant responses

affect the later performance of *F. occidentalis*? By using a model system of agricultural interest we highlight the potential relevance of parasitism at modulating plant responses to aphids and their interaction with thrips.

## Materials and methods

### *Plants and insects*

The study system consisted of Sweet pepper (*Capsicum annuum* var. *maranello*), an organically certified cultivar commonly used in organic greenhouse crops, the generalist phloem feeding aphid *M. persicae*, the cell-content feeding thrips *F. occidentalis* and *A. colemani* as the parasitoid of *M. persicae*. For the soil conditioning, we selected the forb *Achillea millefolium* L. (Asteraceae) and the grass *Lolium perenne* L. (Poaceae), species that in a previous study on chrysanthemum led to a thrips-suppressive plant-soil feedback effect (Pineda et al., in prep.).

Insects were reared at NIOO-KNAW, Wageningen, The Netherlands. *Myzus persicae* was reared on *C. annuum* plants for multiple generations, *A. colemani* was provided by Koppert Biological System and *F. occidentalis* was reared on fresh green beans *Phaseolus vulgaris* L. (Fabaceae), in climate chambers at 22 °C ± 2°C, 40% relative humidity (RH) and a 16 h light and 8 h dark photo regime.

### *Soil preparation*

To create distinct soil microbiomes, living soil collected from a grassland from the national park The Hoge Veluwe (The Netherlands) was conditioned by the wild plants *A. millefolium* and *L. perenne*, as described in Kos *et al.* (2015). One seedling per pot was transplanted into this soil (12 replicates per each wild plant), and pots were randomly located inside a greenhouse (21/16 °C day/night, 16h photoperiod). Plants were watered three times per week. Natural daylight was supplemented by 400 W metal halide lamps (225 μmol m<sup>-2</sup> s<sup>-1</sup> PAR). After 8 weeks of conditioning phase, soil inocula were collected, keeping each soil replicate separately.

Then the soil inocula were mixed with sterilized bulk soil (10% soil inocula: 90% sterilized soil) and for control soil only sterilized bulk soil was used. To obtain sterilized bulk soil, the same field soil was sterilized by gamma irradiation (> 25 KGray: Isotron, Ede, The

Netherlands). Pots (13 x 13 x 13cm) were filled with 1 kg of mixed soil in total. The mixing of inocula with sterilized soil reduces differences in the abiotic characteristics of the soil, while the biotic component can recolonize the sterile soil (Kostenko et al., 2012). To ensure that at least one seedling of sweet pepper survived, we used two seeds per pot. The seeds were surface-sterilized (1 min in 0.1% sodium chloride solution and rinsed with water) and germinated directly in the pots. After two weeks, only one seedling of sweet pepper was kept per pot. Plants were grown in a greenhouse under the same conditions as described above. Plants were watered three times per week, supplied with nutrient solution (Hoagland). In total, there were 288 pots (4 herbivore treatments x 3 soil treatments x 12 replicates x 2 time points).

#### *Insect infestation and harvesting*

Five weeks after germination, plants were randomly allocated to one of the following treatments (i) uninfested, (ii) aphid-infested, (iii) parasitised aphid-infested and (iv) thrips-infested. All treatments were replicated 12 times per soil treatment and two groups were labelled as 24h and 48h. All the plants were individually covered with gauze cages to prevent the escape of insects, and uninfested plants were covered as well to standardise conditions. For the treatment of aphid-infested plants, thirty three-day-old nymphs of *M. persicae* were placed on the second expanded leaf from each plant. For the treatment of parasitised aphid-infested plants, a colony of *A. colemani* was allowed to parasitise approximately 1500 two-day-old nymphs of *M. persicae* during 24h. Then, thirty of these three-day-old newly parasitised aphids were placed on each plant (99% of parasitism rate was confirmed after material collection). Parasitized aphids become mummies and stop feeding at the fourth instar or adult stage (Perdikis et al., 2004), which occurs around 10 days after parasitoid oviposition (Zamani et al., 2007). Thus during this experiment parasitized aphids were feeding, since they were parasitized only 4 and 5 days before. For the thrips-infested plants treatment, ten three-day-old nymphs of *F. occidentalis* were enclosed in a clip cage on the plant, to ensure that thrips did not escape through the gauze. Results of gene expression from thrips-infested plants were analysed separately (see suppl. Materials) because of the use of clip cages in that single treatment, which can damage superficially the leaf and therefore interfere in the gene expression results compared with the control plants.

At 24 and 48h after insect infestation, one leaf disc of 1cm diameter was harvested per plant, using the second expanded leaf. In the plants with aphid infestation, we carefully

removed their exuviae with a fine paintbrush prior to the collection. Uninfested plants were harvested similarly to the infested plants. Four separate biological replicates were arranged per treatment, and each replicate consisted of a pool of three leaf discs from three individual plants (randomly pooled). The collected material was labelled as 24h or 48h harvested and immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation.

*RNA extraction, cDNA synthesis and quantitative RT-qPCR reaction*

Total RNA extraction and purification was done following the protocol of Isolate II RNA Plant Kit (Bioline, London, United Kingdom). After purification, the RNA concentration and purity were measured using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer (all samples with  $OD_{260/280} = 1.9-2.1$ ), and RNA integrity was confirmed by gel electrophoresis. Isolated RNA was converted into cDNA using the iScript cDNA synthesis Kit (Biorad, Hercules, CA, USA), and diluted 1:20 with RNase free water.

Quantitative RT-qPCR analysis was used to evaluate the expression profiles of two genes involved in the JA-signalling pathway (*CaLOX2* and *CaPIN II*) and two genes involved in the SA-pathway (*CaPAL1* and *CaPRI*), in which are appropriated markers in sweet pepper plants and common markers of the SA and JA pathways in other systems (Sarde et al., in prep) (see Supplementary information Table S1 for primer sequences). In addition, the expression of the reference genes *CaUEP* and *CaACTIN* was assessed for normalization (see further methods in Supplementary information).

*Thrips performance experiment*

Nymphs of *F. occidentalis* were allowed to develop until the adult stage while feeding on sweet pepper plants with previous herbivory (either from thrips, aphids or parasitised aphids), and growing on the different soils. From the same plants that were infested for 48h and a sample was taken for molecular analyses, the fourth entire leaf was used for the performance bioassay (see supplementary methods: Thrips performance). The leaf petiole from each plant was inserted in 2 ml 1.5% plant agar in a 90 mm Petri dish, to maintain leaf freshness. Using a fine paintbrush, five two-day-old nymphs of *F. occidentalis* were transferred to each Petri dish. In total, there were 144 plates (4 herbivore treatments x 3 soil treatments x 12 replicates) and 720 individuals of thrips (5 nymphs x 144 samples). The thrips were monitored daily, starting 4 days later and until they became adults ( $\pm 7$  days monitoring).

Survival and length of adult body-size, measured from head until the last part of the abdomen by a digital microscope (SZX12 Olympus; Tokyo, Japan), was recorded (due to differences between males and females, body size measurements were analyzed separately for each sex). The bioassay was performed in a growth chamber at 22 °C, 40% relative humidity (RH) and a 16 h light and 8 h dark photo regime.

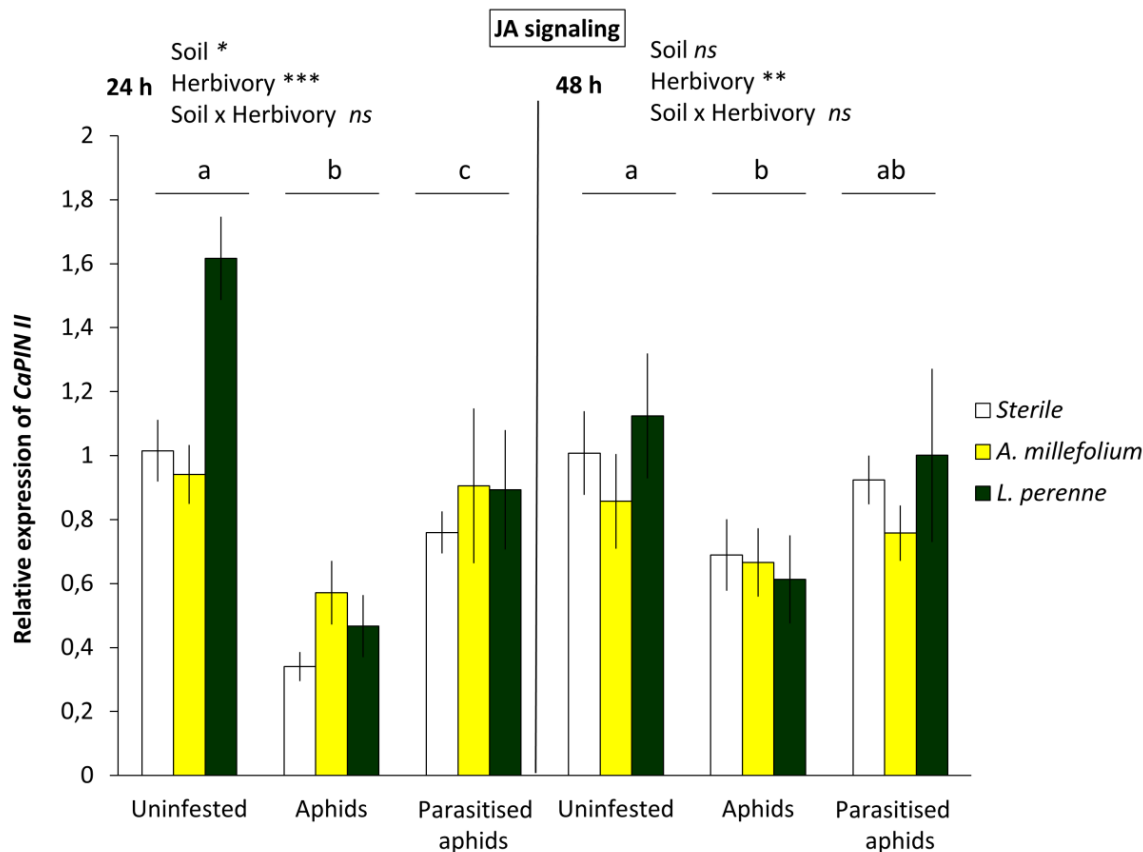
### *Statistical analyses*

After confirmation of the assumptions of normality and homogeneity of variances, differences in gene expression levels between previous herbivory attack and plant-soil feedbacks were analysed using two-way ANOVA's, where herbivory and soils were set as fixed factors. Analysis were done separately for the different time points. The same two-way ANOVA models were applied to analyse the differences in thrips body size, with separate analysis for males and females, after averaging measurements from individuals from each replicate (Petri dish). To analyse whether induction by PSF and/or sequential herbivory affected thrips survival we used generalised linear models (GLM), with logit link function and binomial distribution, and the dispersion parameter estimated to correct for over-dispersion. All pairwise comparisons were done with the post-hoc protected LSD test (SPSS 15.0.; SPSS Inc., Chicago, IL, USA).

## **Results**

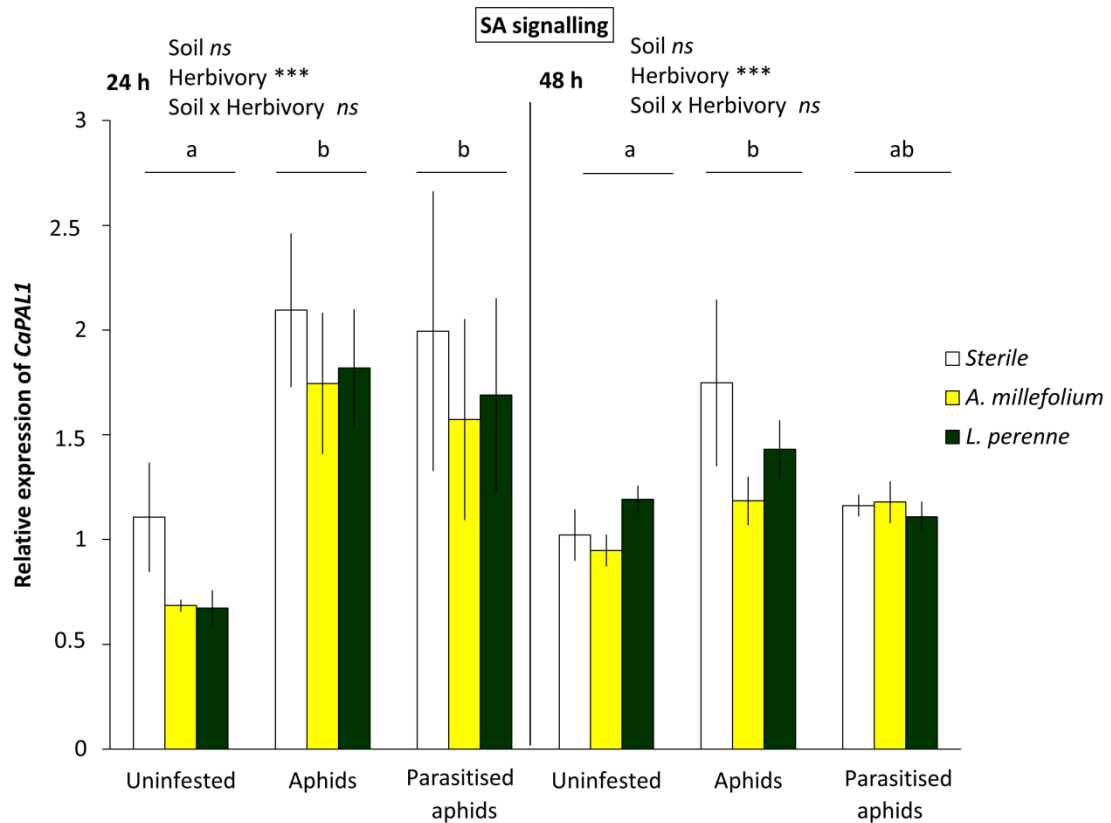
### *Parasitism alters plant defence responses*

The transcript levels of the JA-responsive gene *CaPIN II* showed a strong down-regulation upon aphid feeding at 24h and 48h after infestation (Fig.1). However, the expression of *CaPIN II* was significantly less suppressed by parasitised aphids than by unparasitised ones at 24h after insect infestation (2-way ANOVA;  $F= 21.533$ ;  $df= 2, 33$ ;  $P < 0.001$ ; LSD,  $P < 0.05$ ; Fig.1). At 48h after infestation, healthy aphids still significantly suppressed *CaPIN II* expression compared to levels in uninfested plants, while levels in plants with parasitised aphids were similar to both other treatments (2-way ANOVA;  $F= 3.698$ ;  $df= 2, 34$ ;  $P = 0.039$ ; LSD,  $P > 0.05$ ; Fig.1).



**Figure 1.** Expression levels of *CaPIN II* in *C. annuum* in uninfested, aphid-infested, or parasitised aphids- infested plants, each grown in sterile soil, or inoculated with living soil conditioned by the plants *A. millefolium* or *L. perenne*. Bars represent mean *CaPIN II* expression levels normalised as  $2^{-\Delta\Delta Ct}$  with standard error bars (n = 4). Bars marked with different letters are significantly different (LSD,  $P < 0.05$ ), with separate analysis for the two time points (24 and 48 h after aphid infestation).

In contrast to *CaPIN II*, the transcript levels of *CaPAL1* (SA-biosynthetic gene) were up-regulated upon feeding by both aphids and parasitised aphids, compared with uninfested plants after 24h of insect infestation (2-way ANOVA;  $F= 5.684$ ;  $df= 2, 33$ ;  $P =0.009$ ; LSD,  $P<0.05$ ; Fig. 2). However, at 48h after insect feeding, only the treatment with unparasitised aphids maintained significantly induced levels of *CaPAL1*, whereas the expression level in plants with parasitised aphids did not differ from uninfested and aphid-infested plants (2-way ANOVA;  $F= 4.673$ ;  $df= 2, 34$ ;  $P =0.018$ ; LSD,  $P>0.05$ ; Fig.2). In contrast to *CaPAL1* and *CaPIN II*, the expression of *CaLOX2* (JA-biosynthetic gene) and *CaPRI* (SA-responsive gene) were not up- or downregulated after infestation with (parasitised) aphids (see Supplementary Fig. S5 and S6,  $P>0.05$ ).



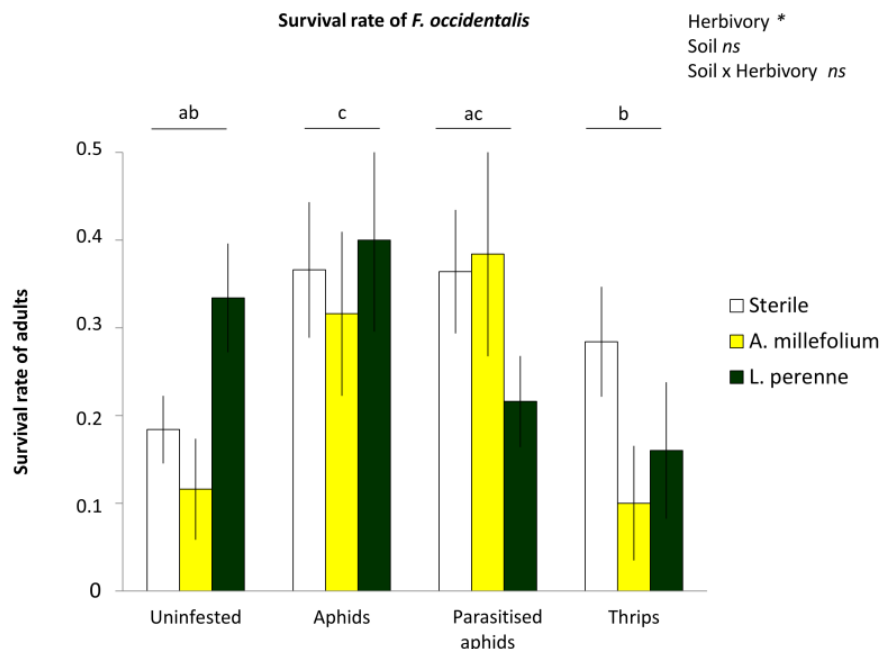
**Figure 2.** Expression levels of *CaPAL1* in *C. annuum* in uninfested, aphid-infested, or parasitised aphids- infested plants, each grown in sterile soil, or inoculated with living soil conditioned by the plants *A. millefolium* or *L. perenne*. Bars represent mean *CaPAL1* expression levels normalised as  $2^{-\Delta\Delta Ct}$  with standard error bars (n = 4). Bars marked with different letters are significantly different (LSD, P < 0.05), with separate analysis for the two time points (24 and 48 h after aphid infestation).

#### *Plant-soil feedbacks (PSF) do not affect plant defensive hormonal pathways*

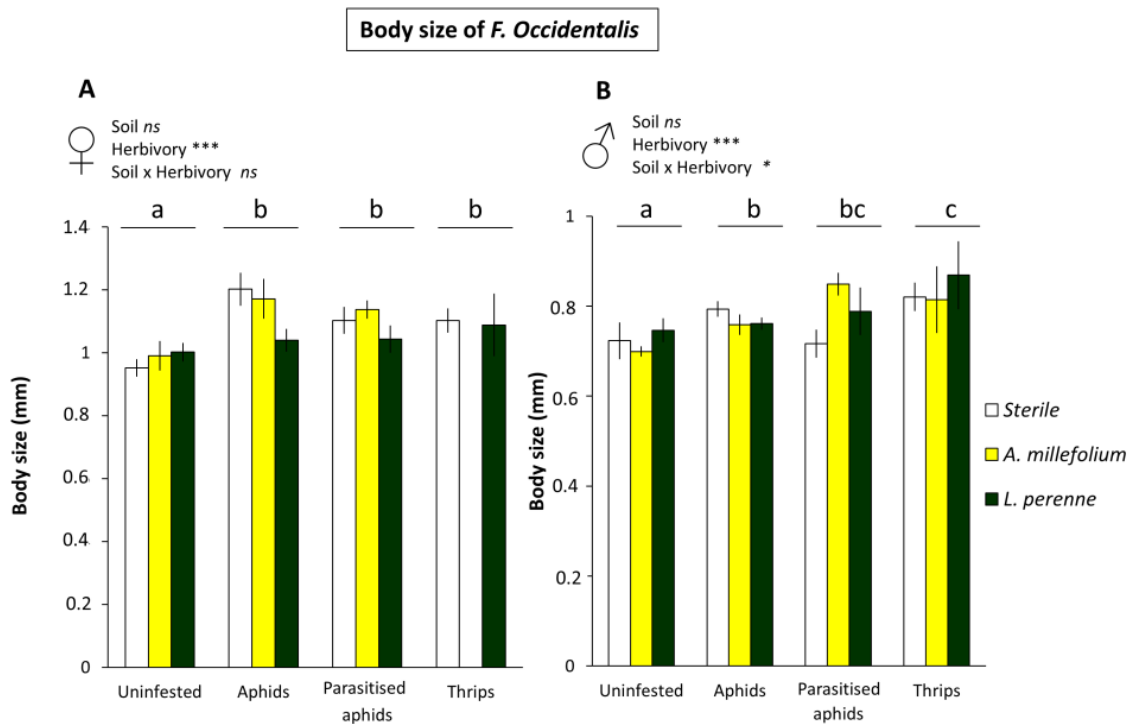
We investigated the potential effect of three different soil communities on the induction of JA and/or SA defensive genes in sweet pepper plants. The soils selected for the experiment were: soil conditioned with *Achilea millefolium* and *Lolium perenne* and sterile soil as control (see Materials and Methods). No main effect of soil type was found on the expression of *CaLOX2* (JA-biosynthetic gene) (P>0.05), or SA-marker genes (*CaPAL1* and *CaPRI*), neither at 24h nor 48h after insect infestation (P>0.05) (Fig.2, Fig. S5 and S6). In contrast, the expression of *CaPIN II* (JA-responsive gene) was up-regulated comparing PSF effects in undamaged plants, where soil conditioned by *L. perenne* led to a stronger response than sterile soil at first time point (24h) (see suppl. statistical results) (2-way ANOVA; F= 3.532; df= 2, 33; P =0.045; Fig.1). However, the soil effect on the expression of *CaPIN II* was no longer observed at 48h (2-way ANOVA; F= 0.758; df= 2, 34; P = 0.479; Fig.1).

*Aphid herbivory facilitates thrips performance, but not if aphids are parasitised*

Thrips survival from two-day-old nymphs until adult stage and length of adult body size were measured as performance parameters of *F. occidentalis*. Thrips were growing on detached leaves from sweet pepper plants previously treated as described above for gene expression analyses. Thrips survival was highest when feeding on leaves from plants that were previously infested by unparasitised aphids (GLM, binomial test;  $F=9.491$ ;  $df= 3, 125$ ;  $P =0.023$ ; Fig. 3), whereas no differences in survival rates were found for thrips feeding on plants previously infested by parasitised aphids or thrips, compared with uninfested plants. Moreover, a strong effect was observed on the body size of thrips that reached the adult stage. Both females (2-way ANOVA;  $F=5.017$ ;  $df= 3, 69$ ;  $P =0.004$ ; Fig. 4A) and males (2-way ANOVA;  $F=4.470$ ;  $df= 3, 106$ ;  $P =0.006$ ; Fig. 4B) were significantly larger when feeding and developing on leaves previously infested by either parasitised or unparasitised aphids, or thrips, compared with uninfested plants. The effect of plant-soil feedbacks was also analysed on thrips performance; but no effect was found on thrips survival nor adult size ( $P>0.05$ ). However, a significant interaction was found between soil and herbivory on the body size of thrips males (2-way ANOVA;  $F=2.264$ ;  $df= 6, 106$ ;  $P =0.044$ ; Fig. 4B), but this interaction was not found in females (2-way ANOVA;  $F=1.008$ ;  $df= 5, 69$ ;  $P =0.421$ ; Fig. 4A).



**Figure 3.** Performance of *F. occidentalis*, number that reach the adult stage (out of 5 initial individuals) on *C. annuum* for four different treatments: a) uninfested plants, b) aphid-infested plants, c) parasitised aphid-infested plants, d) thrips-infested plants, on three different soil types: a) sterile soil, b) *A. millefolium*, c) *L. perenne*. Bars represent means  $\pm$  SE (n=12 replicates). Different letters indicate significant pairwise differences between infestation treatments ( $P<0.05$ ).



**Fig. 4.** Performance of *F. occidentalis*, length of body size in adult stage for female (A) and male (B) thrips on *C. annuum* for four different treatments: a) uninfested plants, b) aphid-infested plants, c) parasitised aphid-infested plants, d) thrips-infested plants, on three different soil types: a) sterile soil, b) *A. millefolium*, c) *L. perenne*. Bars represent means  $\pm$  SE (n=12). No surviving females were found in thrips-infested plants grown on *A. millefolium* soil. Different letters indicate significant pairwise differences between infestation treatments ( $P < 0.05$ ).

## Discussion

This study shows that the plant response to aphid herbivory can be altered by parasitism, but not by legacies left in the soil by previous plants. This pattern also scales-up into the consequences for the survival of a subsequent herbivore feeding on those plants. There is an increasing awareness about how parasitism can alter the plant-defence signalling network and how these changes may have cascading effects at the insect community level. Caterpillar parasitism can increase the feeding damage done by caterpillars, increasing thus the induction of chemical plant defenses with a fitness cost for the plant (Ode et al., 2016). Moreover, parasitism of caterpillars can alter both plant gene expression and metabolomics, affecting the foraging decisions made by subsequent herbivores, parasitoids and even hyperparasitoids at the fourth trophic level (Poelman et al., 2011b, Zhu et al., 2015). Our findings demonstrate such differences in plant responses due to parasitism of phloem feeders, which not only have

a completely different biology, but also elicit different plant responses. Since parasitoids are indirectly affected by the plant on which their host is feeding (Botrell et al., 1998) and are mostly immobile inside their hosts, parasitoids may be expected to alter the plant-defence mechanisms for their own benefit. Although it is a not-yet tested hypothesis, a possibility is that parasitoids may alter herbivore induced plant responses to deter other predators that can kill the parasitoids when ingesting their herbivorous prey. This phenomenon of intraguild predation is common between aphid parasitoids and predators, and in a previous study we showed that hoverflies reduce their oviposition on plants colonized by parasitised aphids (Pineda et al., 2007). In contrast, although not yet known for phloem feeders, hyperparasitoids of caterpillar parasitoids were more attracted to plants infested by parasitised than unparasitised caterpillars (Zhu et al., 2015). Future studies including multiple members of the different trophic levels may unravel the costs and benefits of the plant responses altered by parasitoids.

Our results show that heterospecific herbivory aboveground by aphids facilitates thrips performance, but not when aphids were parasitised, or when plants were previously infested by conspecific thrips. Several studies with phloem feeders that induce the SA signalling pathway and leaf chewers that induce the JA pathway in a similar way as thrips do, have observed facilitative interactions, mainly associated with cross-talk of these two signalling pathways (Stout et al., 1997; Rodriguez-Saona et al., 2005; Soler et al., 2012a; Li et al., 2014). Here, at the plant signalling level, we also observed that aphids suppressed the JA-marker gene *CaPIN II*, and associated with this, thrips performance on aphid-infested plants was enhanced. Linked to this result, parasitism mitigated the suppression of *CaPIN II*, and also the aphid-triggered facilitation of thrips performance. Further work with pepper plants that have the JA signalling pathway impaired (Sarde *et al.*, in prep) could confirm the role of JA pathway in shaping the interactions between multiple attackers in the presence of a member of the third trophic level.

A yet unresolved, question is which mechanisms in the herbivores are triggering the observed differences between parasitised and unparasitised aphids. In our experiments, we have repeatedly observed that the honeydew covering the plants colonized by parasitised aphids show small white spots that are not present on plants with unparasitised aphids. Aphid honeydew was shown to play a significant role suppressing JA accumulation in *Vicia faba* plants (Schwartzberg and Tumlinson, 2004). Parasitised aphids may release a higher amount of honeydew than healthy aphids (Völkl, 1992), and therefore a stronger JA suppression by

parasitised aphids would be expected. In contrast, our results show a weaker suppression of the JA response in plants colonized by parasitised compared to unparasitised aphids. Thus, we suggest that altered plant responses due to parasitism may be due to changes in honeydew composition rather than quantity. An alternative hypothesis is that the different plant response may be due to changes in aphid saliva, which plays a key role in plant-aphid interactions (Mutti et al., 2008; De Vos and Jander, 2009). In caterpillars, parasitism changes the herbivore oral secretions, and this alters plant responses such as the emission of herbivore-induced plant volatiles compounds (HIPVs), which allows parasitoids to discriminate between parasitised and unparasitised hosts, but also allows hyperparasitoids to locate their hosts (Poelman et al., 2001a, 2012; Ma et al., 2017). At present, there is no information about differential composition of saliva and honeydew comparing parasitised with healthy aphids.

We did not find evidence of soil treatments altering the relationships between those herbivores nor the plant response (gene expression in JA and SA signalling). In our study, we created different soil microbiomes by applying the concept of plant-soil feedbacks (Ehrenfeld et al., 2005; Van der Putten et al., 2013), with a methodology that reduces the potential differences in abiotic soil properties (see materials and methods). The selected plants (*L. perenne* and *M. millefolium*) used to condition the soil were selected according to previous results of PSF effects on plant performance and pathogen resistance in chrysanthemum (*Dendranthema X grandiflora*) (Ma et al., 2017), and resistance against thrips (Pineda et al., in prep.). However, no effect of PSF on induced resistance nor on molecular plant responses were observed in this study with sweet pepper. Plant-soil-insect feedbacks and their underlying mechanisms is a so-far unexplored field, and the effects on insects may vary depending on factors such as plant species, plant communities, or insect feeding guild (Bezemer et al., 2013; Heinen et al., 2018). Further studies on these and other factors affecting plant resistance to herbivores will not only increase our understanding of how plants and insects interact in nature, but also of how to apply the concept of PSF to protect agricultural crops.

The vast majority of studies on plant defences have focused on the responses to the attack of single microbes or herbivores. However, in nature, plants interact with multiple species of attackers and beneficial organisms, calling for increased complexity of the study systems. Using an agriculturally important plant species, our work highlights the role of parasitism at modulating plant defences and heterospecific insect interactions.

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### **Author contribution**

T.V., M.A.M.G., J.G.d.B and A.P. conceived and designed the research. S.S. designed the molecular primers. T.V. and A.P. conducted the experiments and analysed the data. T.V., M.A.M.G., S.S. and A.P. interpreted the results and wrote the manuscript. All authors contributed to revise the manuscript.

### **Additional information**

Competing interest: The authors declare no competing interests.

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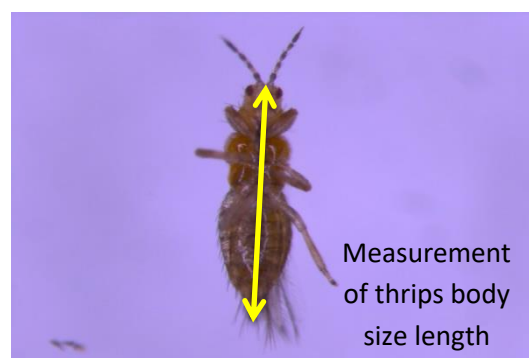
## Supplementary information

**Modulation of plant-mediated interactions between herbivores of different feeding guilds: Effects of parasitism and belowground interactions**

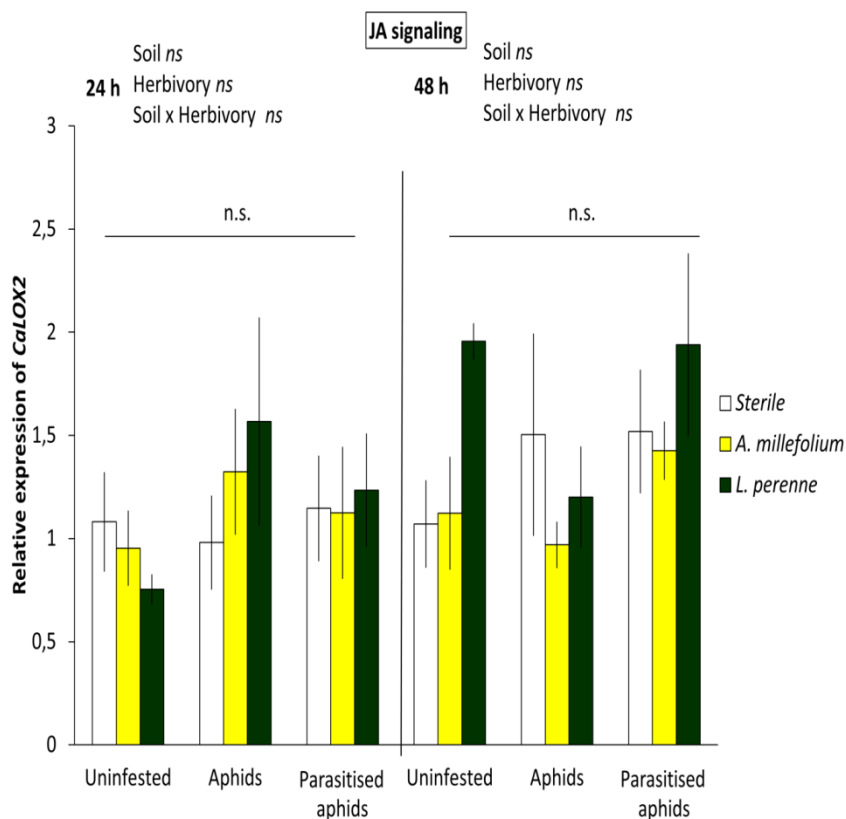
The following Supporting Information is available for this article:

**Supplementary methods***Thrips performance on 2<sup>nd</sup> and 4<sup>th</sup> leaf*

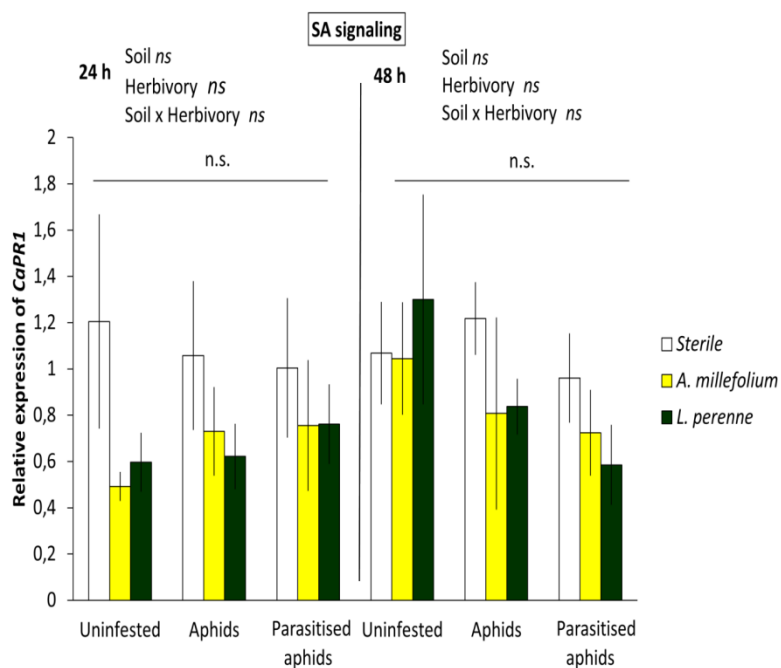
A previous experiment was done to observe the performance of *F. occidentalis* on different sweet pepper leaves. We use the same conditioned soil that in the main manuscript (*A.millefolim*, *L.perenne* and Sterile soil). Nymphs of *F. occidentalis* were allowed to develop until adult stage while feeding on leaves from previous uninfested conditioned-soil sweet pepper plants. Second and fourth expanded leaf, were used for the bioassay. The leaf petiole from each plant was inserted in 2 ml 1.5% plant agar in a 90 mm petri dish, to maintain leaf freshness. Using a fine paintbrush, five two-day-old nymphs of *F. occidentalis* were transferred to each petri dish. The thrips were then monitored daily starting 4 days later and until they became adults ( $\pm 7$  days monitoring). Survival and length of adult body-size was recorded. In total, there were 90 replicates (3 soil treatments x 12 soil replicates x 2 leaf stage) and 450 individuals of thrips observed (5 nymphs x 90 replicates). The bioassay was performed in a growth chamber at 22 °C, 40% relative humidity (RH) and a 16 h light and 8 h dark photo regime.



Supplementary results



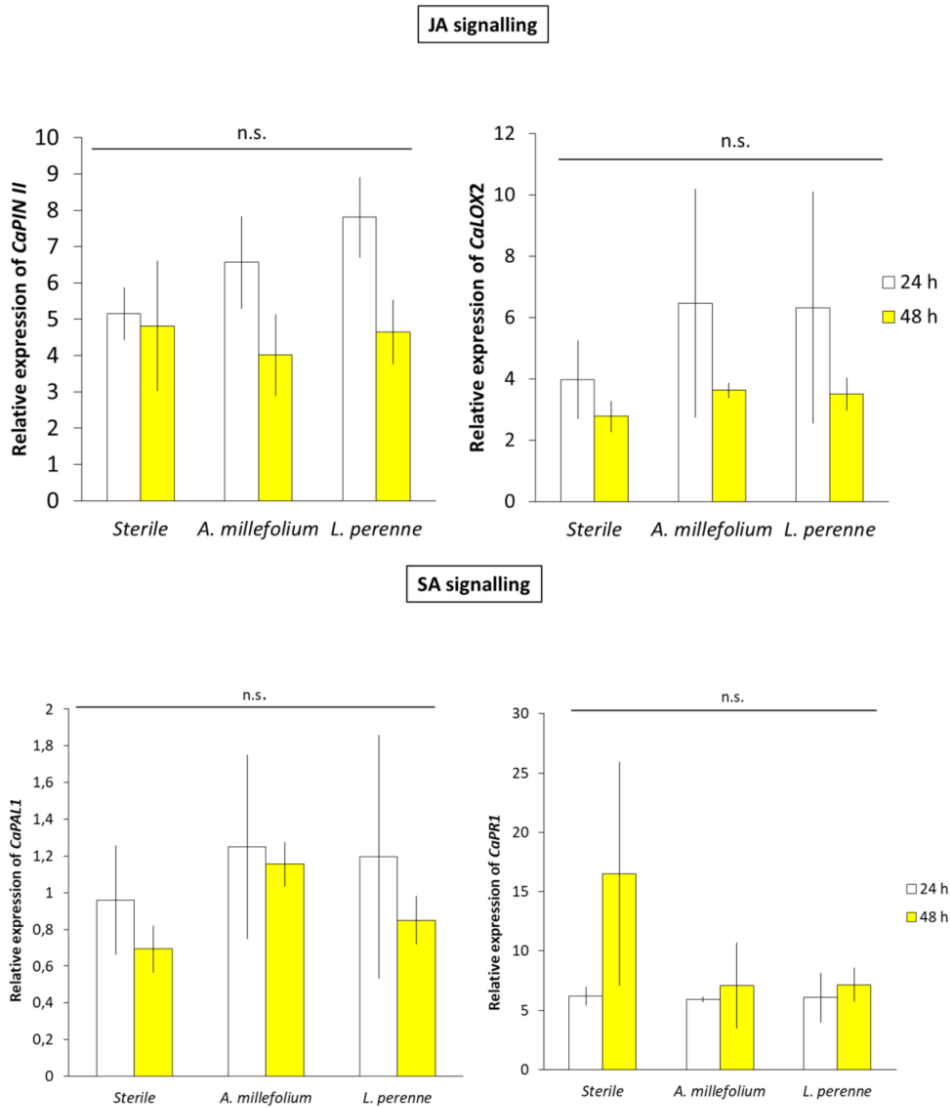
**Figure S5.** Expression levels of *CaLOX2* in *C. annuum* in a) uninfested plants, b) aphids infested plants, c) parasitised aphids infested plants, in three different soil treatments a) sterile soil, b) *A. millefolium*, c) *L. perenne*; for 24 and 48H. Bars represent mean *CaLOX2* expression levels normalised (by geometrically averaging the Ct values from the genes *CaUEP* and *CaACTIN*, and referred to the control uninfested treatment) as  $2^{-\Delta\Delta Ct}$  with standard error bars (n = 4). Bars marked with ns are non significantly different (P > 0.05).



**Figure S6.** Expression levels of *CaPRI* in *C. annuum* in a) uninfested plants, b) aphids infested plants, c) parasitised aphids infested plants, in three different soil treatments a) sterile soil, b) *A. millefolium*, c) *L. perenne*; for 24 and 48H. Bars represent mean *CaPRI* expression levels normalised (by geometrically averaging the Ct values from the genes *CaUEP* and *CaACTIN*, and referred to the control uninfested treatment) as  $2^{-\Delta\Delta Ct}$  with standard error bars (n = 4). Bars marked with n.s. are non significantly different (P > 0.05).

**Fig. S7. PSF did not affect the induction of marker genes upon thrips attack**

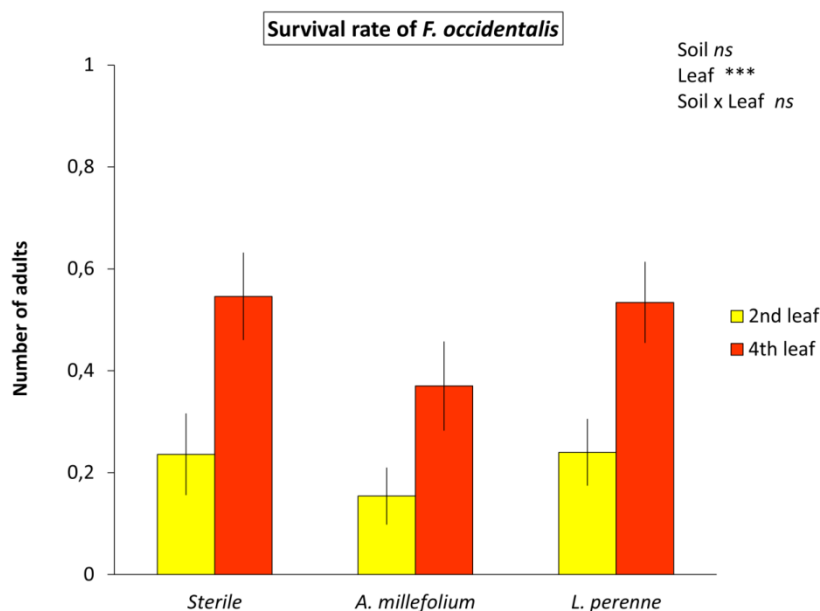
We analysed separately the results of plant defence response with thrips because of the use of clipcages, which can damage superficially the leaf and therefore interfere in the gene expression results compared with the control plants. PSF not had effects on the regulation in any of the responsible genes of SA or JA signalling pathway ( $P > 0.05$ ).



**Fig. S8. PSF effect on survival rate of *F. occidentalis***

To evaluate whether PSF and/or the effect of younger or older leaves may had in *F. occidentalis* performance, the survival of thrips from nymphs to adults stage and their final body size on sterile soil, *A.millefolium* and *L.perenne* soil, was tested. The results show how *F. occidentalis* had a lower survival rate when fed on the second leaf (young leaf) from sweet pepper plant compared with individuals which were fed on fourth leaf (old leaf) (GLM; binomial test; F: 14.868; df: 1; P <0.001). However, no effects of PSF were found on the survival rate, (GLM; binomial test; F: 1.442; df:2 ; P =>0.05)

We did not found body size effects neither on males nor females (P =>0.05). Thus, we decided to use the fourth leaf, for the main experiment, due to the higher survival rate results.

**Supplementary qPCR reaction information**

All qPCR reactions were performed in a Biorad CFX Thermocycler (Biorad, Hercules, CA, USA). The amplification reactions were performed in 20 µl final volume containing 10 µl SensiFAST SYBR No-ROX mix (Bioline, London, United Kingdom), 0.8 µl forward primer and reverse primer [concentration see Supporting Information TableS1], 3.4 µl RNase free water and 5 µl cDNA. The following PCR protocol was used for all PCR analyses with exception of reactions containing the primer for *CaLOX2*: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 30 s at 60 °C. For reaction mixes containing *CaLOX2* primers the same protocol was used with 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 30 s

at 62 °C. All reactions were performed in duplicate and average values were used in the analyses. Reactions efficiencies and Ct values were calculated using the LinRegPCR software (version 2014.7). The most stable reference was calculated from the two reference genes by repeated pair-wise correlation analysis using the Excel-based tool BestKeeper (Pfaffl et al. 2004). Duplicate samples were averaged and expression was normalized by subtracting the BestKeeper reference. The gene expression was further normalized against the control treatment of the respective pool of treatment replicate using the comparative Ct-method (also known as  $2^{-\Delta\Delta Ct}$  method) (Livak and Schmittgen 2001).

**Table S1: Primer sequences for RT-qPCR**

Sequences of all primers used in RT-qPCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Concentration (μM)
<i>CaUEP</i>	CCGACTACAACATCCAGAAG	CACACTCAGCATTAGGACAC	7.5
<i>CaACTIN</i>	CCCAGATTATGTTTGAGACC	GCAAAGCATAACCCTCATAG	7.5
<i>CaPAL1</i>	GGAAATGGCTGCTGAATCAT	GCTCCAATTTAACCCACAA	7.5
<i>CaLOX2</i>	GCCATTTCTGGATCGGATTA	GCATCAACAGGTGGTGTGAC	7.5
<i>CaPRI</i>	CCTTACGGGGAAAACCTAGC	ACCCTAGCACAACCAAGACG	7.5
<i>CaPIN II</i>	TGGCTGTTCCCAAAGAAGTT	GGTCAGACTCTCCTTCACAA	7.5



## **SECCIÓN III**

### **Capítulo 5**

#### **Plant-soil feedback effects beyond the third trophic level**

## Abstract

Changes in soil properties that are caused by plants, and that influence the performance of plants that grow later in that soil, are termed ‘plant-soil feedbacks’ and can be driven by changes in soil microorganisms, such as bacteria, mycorrhizal or pathogenic fungi. Some of those soil microbes can affect the performance of above-ground herbivores. While most of this knowledge comes from studies with single microbes, not much is known how different soil microbial communities affect the performance of plants and associated insects. The objective of this study was to examine whether plant-soil feedback effects can affect the aboveground organisms up to the fourth trophic level. Here, we worked with the pepper plant *Capsicum annuum* (first trophic level), the phloem feeder *Myzus persicae* (second trophic level), its parasitoid *Aphidius colemani* (third trophic level), and the hyperparasitoid *Dendrocerus aphidum* (fourth trophic level). Soil legacies from the plant species *Festuca ovina*, *Achillea millefolium* and *Hypochaeris radicata* reduced germination of sweet pepper compared to sterile soil inocula and inocula from *Lolium perenne*. Aphid population was in contrast positively affected by soil inocula from *H. radicata*. Plant-soil feedbacks also affected the third trophic level, where parasitoid performance was lower on soils inoculated by *L. perenne* and *H. radicata*. Finally, soil legacies had no effect on the fourth trophic level. Thus, this study provides evidence that through changes in the soil, plants can influence up to the third trophic level, i.e. other plants growing in that soil, their herbivores and carnivores feeding on those. Moreover, we discuss the implications of these findings in sustainable agriculture.

**Keywords:** *Aphidius colemani*, biological control, *Dendrocerus aphidum*, fourth trophic level, multitrophic interactions, *Myzus persicae*, plant-soil feedbacks.

## Introduction

Changes of the biotic and abiotic characteristics of the soil induced by a plant, that influence other plants growing in the same soil are termed ‘plant–soil feedbacks’ (PSF) (Bartelt-Ryser et al., 2005; Ehrenfeld et al., 2005; Kulmatiski and Kardol, 2008; Van del Putten et al., 2013). Plants can influence soil properties by releasing root exudates and taking up nutrients that affect soil microbes, such as mycorrhizal fungi and plant growth promoting rhizobacteria.

Several studies have observed how specific soil microorganisms can enhance plant growth and induce systemic resistance against different attackers above ground (Yang et al., 2009; Pineda et al., 2010; Jung et al., 2012; Pieterse et al., 2014). However, relatively little is known about how the full soil community, such that created by PSF, can cascade up to higher trophic levels.

The consequences of legacy effects of plants on the soil microbiome and then on plant growth, not only can affect herbivores but also could affect the third trophic level, such as parasitoids that develop inside their host. Microbe-plant interactions can alter the fitness and success of natural enemies, such predators and parasitoids, predominantly by variations in indirect plant defences; such as changes in plant and vigour, altering plant metabolism or through microbial release of volatiles organic compounds (VOC's) that interfere with plants signalling (Rasmann et al., 2017). For instance, previous studies have shown that vigorous plants can support larger pest populations which in turn promote larger parasitoid or predator populations (Cornelissen et al., 2008; Kher et al., 2014), or how increased levels of defense metabolites in plant tissues can reduce the immune capacity of herbivore hosts, enhancing the chances of successful parasitization (Schmid-Hempel, 2009; Smilanich et al., 2011). Thus, although soil legacy effects created by aboveground and belowground herbivory can affect the performance of herbivores feeding in those plants and consequently their parasitoids (Bezemer et al., 2005, 2013; Kostenko et al., 2012), not much is known about how legacy effects created by different plant species may affect the third trophic level.

The field of how belowground interactions affect the fourth trophic levels, such as hyperparasitoids has not advanced much yet, and nothing is known whether specific microbial strains or different soil communities can affect the fourth trophic level. Hyperparasitoids are secondary parasitoids that develop inside or on the body of primary parasitoids and ultimately kill their host, commonly occupying the fourth trophic level in plant-insect food chains (Sullivan and Volkl, 1999). Hyperparasitoids may exert a significant negative effect on plant-fitness by removing parasitoids and reducing the efficacy of biological control (Sullivan and Volkl, 1999). A previous study testing the cascading effect from belowground herbivory to the fourth trophic level, demonstrated that the root herbivore *Delia radicum* L. (Diptera, Anthomyiidae), through changes in primary and secondary plant compounds, affected negatively the performance of the hyperparasitoid *Lysibia nana* (Gravenhorst, 1829) (Hymenoptera, Ichneumonidae) (Soler et al., 2005). This cascading

effect via changes in the plant and reaching until higher trophic levels was also found when *Pieris brassicae* L. (Lepidoptera, Pieridae) oviposit on *Brassica nigra* L. Here, the induced plant response, affected also the fourth trophic level, by increasing the hyperparasitising rate and reducing hyperparasitoid performance (Pashalidou et al., 2015). Hence, PSF effects that affect succeeding plants, herbivores and their parasitoids, are likely to also affect the fourth trophic level.

In the present study we investigated whether the effects of soil legacies left in the soil by different plant species cascade up to the fourth trophic level. Our model system was sweet pepper plants, *Capsicum annuum* (L) (first trophic level), the aphid *Myzus persicae* (Sulzer, 1776) (Hemiptera, Aphididae) (second trophic level), the endoparasitoid of aphids *Aphidius colemani* (Dalman, 1820) (Hymenoptera, Braconidae) (third trophic level) and its secondary hyperparasitoid *Dendrocerus aphidum* (Rondani, 1877) (Hymenoptera, Ceraphronoidea) (fourth trophic level). This system has a high interest in agriculture since *M. persicae* is a highly generalist pests that affects many crops, and its biological control is based on the parasitoid *A. colemani*. However, in recent years aphid biological control involving these species is failing due to the hyperparasitoid here tested. With this work we aimed to address whether the ecological concept of PSF can be implemented for a more sustainable agriculture at a multitrophic level.

The hypotheses tested in this study were the following: first we expected that the magnitude of PSF effects on *C. annuum* growth depends on the plant species that conditioned the soil. This hypothesis is based on a previous study showing that certain plant species that conditioned soil had a positive effect on chrysanthemum plants (*Dendranthema X grandiflora*) and resistance to a pathogen (Ma et al., 2017). Moreover, due to the fact that soil microbes and changes in soil microbial communities through PSF can affect phloem feeders above-ground (Pineda et al., 2012; Kos et al., 2015a), we expected that the conditioned soils with positive effects on plant growth would increase aphid growth population. Finally, based on previous studies which linked the effect of soil organisms to the third and fourth trophic level (Soler et al., 2005; Hempel et al., 2009), we hypothesised that the performance of the parasitoid *A. colemani* and their hyperparasitoid *D. aphidum* would be related to PSF mediated changes in the parasitoid host *M. persicae*. Finally, we discuss the potential role of PSF as new strategy to improve plant growth and to enhance biological control.

## Material and methods

### *Plants and insects*

The focal plant in our study was the Sweet pepper *C. annuum* (L.) var. *maranello*, an organically certified cultivar commonly used in organic greenhouse crops. For the conditioning phase we selected four species, including grasses: *Lolium perenne* (L.) and *Festuca ovina* (L.), and forbs: *Achillea millefolium* (L.) and *Hipochaeris radicata* (L.), which in previous studies showed to have plant-soil feedback effects on other plants (Bezemer et al., 2006; Ma et al., 2017). Seeds were surface sterilized (1 min in 1% sodium hypochlorite solution and rinsed with water afterwards) and germinated on glass beads in a climate chamber at 20 °C. To ensure that all species were of comparable size at the start of the experiment, 1-week-old seedlings were placed at 4 °C until transplanting.

The herbivore *Myzus persicae* was reared on *C. annuum* plants for multiple generations. The parasitoid *A. colemani* was provided by Koppert Biological Systems (Berkel en Roderijs, The Netherlands) and the hyperparasitoid *D. aphidum* was collected from sweet pepper greenhouse crops in the Netherlands, and cultured on pre-pupae and pupae of *A. colemani*. All insects were then reared at the NIOO-KNAW (Wageningen, The Netherlands) in separate climate chambers per each insect species at 22 °C ± 2°C, 40% relative humidity (RH) and a 16 h light and 8 h dark photo regime.

### *Experimental set-up*

To create distinct soil microbiomes, living soil collected from a grassland (Mossel, Ede, The Netherlands) was conditioned by the wild plants *L. perenne*, *F. ovina*, *A. millefolium* and *H. radicata*, as described in Kos *et al.* (2015a). Two seedling per pot was transplanted into this soil to ensure growing, and after one week only one seedling was kept per pot (5 replicates per each wild plant), and pots were randomly located inside a greenhouse (21/16 °C day/night, 16h photoperiod). Plants were watered three times per week. Natural daylight was supplemented by 400 W metal halide lamps (225  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR). After 8 weeks of conditioning phase, soil inocula were collected, keeping each soil replicate separately.

Then the soil inocula were mixed with sterilized bulk soil (10% soil inocula: 90% sterilized soil) and for control soil only sterilized bulk soil was used. To obtain sterilized bulk soil, soil from the the same field was sterilized by gamma irradiation (> 25 KGray: Isotron, Ede, The Netherlands). Pots (13 x 13 x 13cm) were filled with 1 kg of mixed soil in total. The mixing of inocula with sterilized soil reduces differences in the abiotic characteristics of the soil, while the biotic component can recolonize the sterile soil (Kos et al., 2015a). To ensure that at least one seedling of sweet pepper survived, we used two seeds per pot. The seeds were surface-sterilized (1 min in 0.1% sodium chloride solution and rinsed with water) and germinated directly in the pots. After two weeks, only one seedling of sweet pepper was kept per pot. Plants were grown in a greenhouse under the same conditions as described above. Plants were watered three times per week, supplied with nutrient solution (Hoagland). In total, there were 225 pots (3 insect treatments x 5 soil treatments x 15 replicates).

#### *Sweet pepper performance*

Seed germination was assessed for each individual plant after one week. Plant height from all plants was measured on the fourth week after transplanting the sweet pepper. Seven weeks after transplanting, plants were harvested, above ground biomass of each plant was clipped and roots were carefully washed from the soil. Plant material was oven dried (60°C) and root and shoot dried weigh of each plant was determined.

#### *Insect performance*

Four weeks after germination, plants were randomly allocated to one of the following treatments (i) uninfested, (ii) aphid-infested and (iii) parasitised aphid-infested, as we describe here below. All treatments were replicated 15 times per soil treatment. All the plants were individually covered with gauze cages to prevent the escape of insects, and uninfested plants were covered as well to standardise conditions. The whole experiment was performed in the same greenhouse conditions as described above.

For the treatment of aphid-infested plants, two newly born nymphs of *M. persicae* (<24 h old) were placed on the second expanded leaf from each plant successfully germinated from each soil inocula. Then, starting 5 days later, plants were checked daily and when the

first nymphs were detected, only one adult was kept on each plant. Aphid development was monitored for a maximum of 19 days, where on the 13<sup>th</sup> day all the number of aphids was counted at each plant. The following performance parameters were measured: (i) pre-reproductive period; (ii) effective fecundity, recorded after a period equivalent to the pre-reproductive time (calculated for each individual aphid), and (iii) the intrinsic rate of increase,  $r_m = 0.74 (\ln Md)/d$  (Wyatt and White, 1977), where  $Md$  = effective fecundity and  $d$  = pre-reproductive time.

For the parasitoid treatment, 10 *M. persicae* adults ( $8 \pm 1$ -day old) were placed in 4 weeks old-sweet pepper plants and were allowed to produce nymphs during 24h. Then 35 newly-born aphids were left per plant, removing manually the adults and exceeding nymphs. Then a naïve female (without previous contact with neither plants nor aphids) of *A. colemani* was released in each individual gauze net during 24h (and afterwards removed). Plants were regularly checked and from the day the first mummy (aphid containing a parasitoid pupa) appeared, plants were checked daily and total number of mummies was counted per each plant. Mummies were carefully placed with a paint brush into vials and divided in two, and half of the mummies were used to observe the parasitoid performance: (i) egg-to-mummies development time, (ii) survival rate, (iii) egg-to-adults development time and (iv) adult dry mass.

From the other half of the mummies, a total of 5 mummies were randomly selected to be parasitized by a naïve female of the hyperparasitoid *D. aphidum*. For that, a female was released in the vial and its oviposition behaviour was observed during the full day. Parasitism was considered successful when females were on a mummy for a period longer than 20 min. A single droplet of honey on the inside the glass vial was provided per each hyperparasitoid to allow feeding. When the five mummies per vial were parasitized, these were stored individually, and checked daily until hyperparasitoid emergence. Different performance parameters were observed: (i) survival rate, (ii) egg-to-adults development time and (iii) adults dry mass. Both parasitoids and hyperparasitoids mummies were maintained in greenhouse climate conditions (21/16 °C day/night, 16h photoperiod).

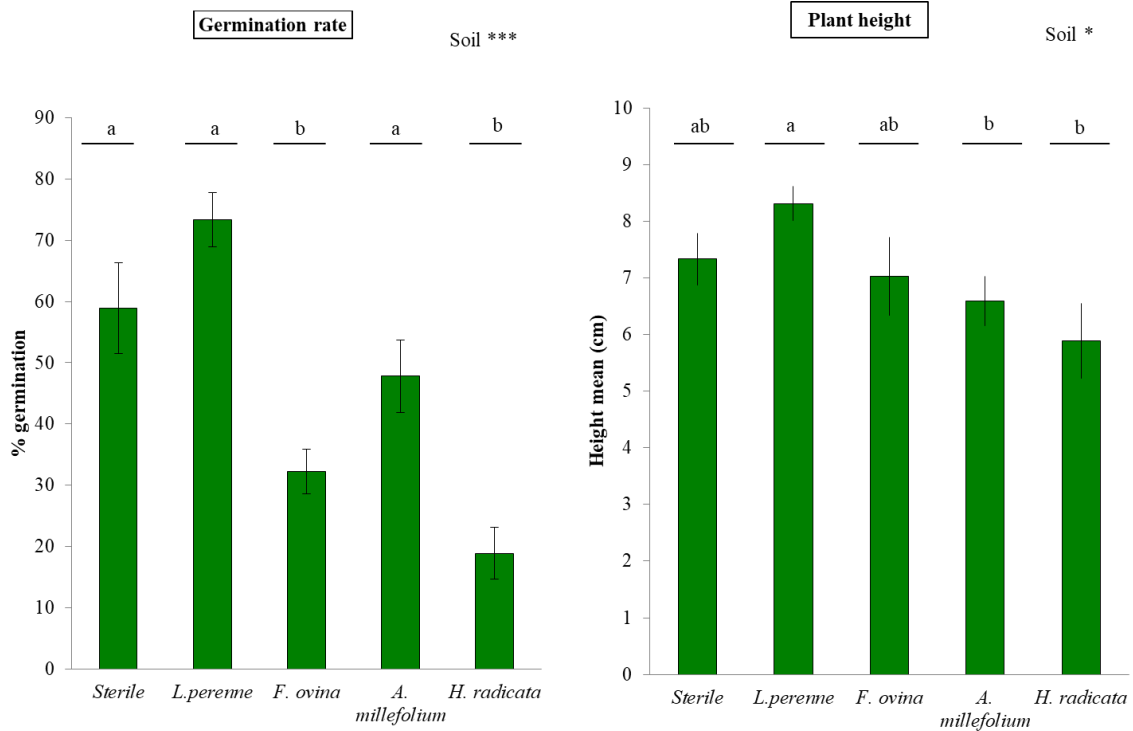
### *Statistical analysis*

To analyse the plant soil feedback effects on plant and insect performance, all the data were averaged per soil replicate. Germination rate was analysed with a generalized linear model (GLM) with a binomial distribution and dispersion parameter estimated. Height and plant biomass were analysed with one-way ANOVA. Total number of aphids was analysed with generalized linear model (GLM) with Poisson distribution. One-way ANOVA was used to analyse aphid intrinsic rate, as well as developmental times and dried weight of the parasitoids and hyperparasitoids. Number of parasitoid mummies and survival of parasitoids and hyperparasitoids was analysed with GLM with a binomial distribution and dispersion parameter estimated. All post-hoc tests were performed with protected LSD test. All the statistical tests were performed using SPSS 15.0, SPSS Inc., Chicago, IL, USA.

## **Results**

### *Plant-soil feedbacks affect seed germination and plant growth*

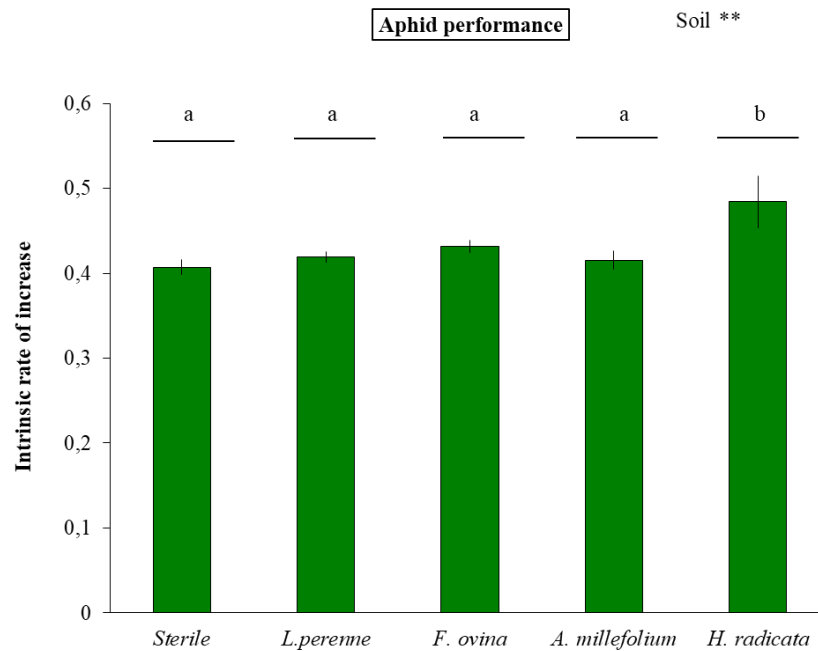
There was a strong effect of the conditioned plant inocula on germination rate of *C. annuum* ( $F= 69.2$ ;  $df= 4, 40$ ;  $P<0.001$ ; Figure 1A), with a reduced germination of seeds that were sown on soil with inocula from *F. ovina* and *H. radicata*, compared to sterile soil or those inoculated with soil from *L. perenne* and *A. millefolium*. Four weeks after sowing, plants also showed differences in their height depending on the soil inocula ( $F: 2.8$ ;  $df= 4, 69$ ;  $P= 0.034$ ; Figure 1B), with the lowest height when growing with soil inocula from the forbs *A. millefolium* and *H. radicata*, intermediate in sterile soil and soil from *F. ovina*, and highest in soil from *L. perenne*. Aboveground biomass was also measured at the end of the experiment, and in the treatment of uninfested plants (five weeks-old) PSF had no effect ( $F= 4.8$ ;  $df= 4, 18$ ;  $P= 0.301$ , Figure S1A). However in aphid-infested plants (seven weeks-old) PSF had an effect ( $F= 11.3$ ;  $df= 4, 18$ ;  $P= 0.023$ ; Figure S1B), with increased biomass on *L. perenne* compared with *A. millefolium* and *F. ovina* and reduced biomass on *A. millefolium* compared with sterile soil.



**Figure 1.** Plant-soil feedback effects on sweet pepper performance: (A) Germination rate and (B) Plant height. Bars represent means  $\pm$  SE ( $n = 5$  soil replicates, each averaged for 15 plants). Different letters indicate significant pairwise differences between conditioned soils ( $P < 0.05$ , LSD).

#### *Plant soil feedbacks can increase aphid population*

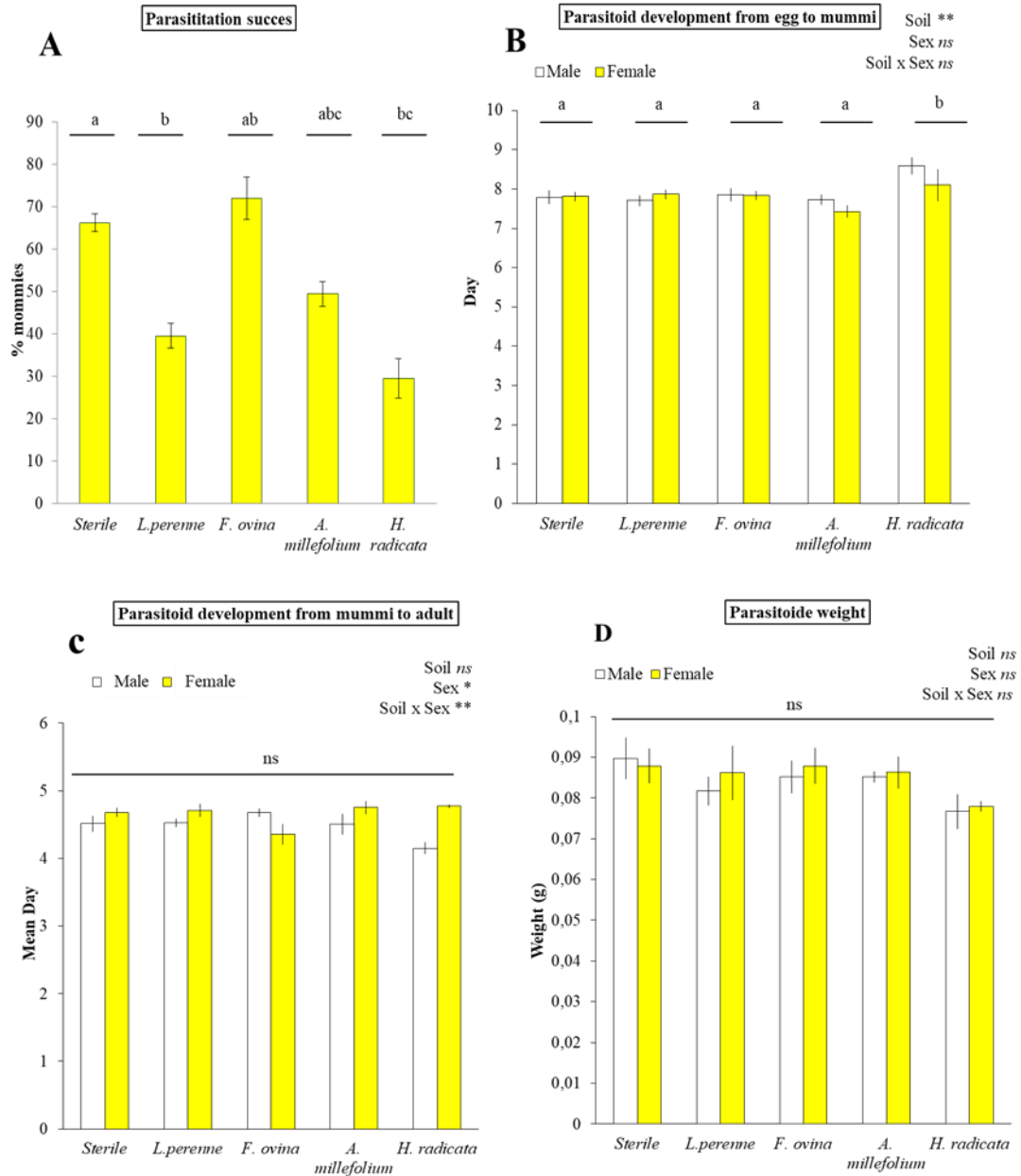
Aphid growth was also measured through the intrinsic rate of population increase ( $r_m$ ). Here, PSF had a strong effect on aphid performance ( $F = 3.8$ ;  $df = 4, 24$ ;  $P = 0.018$ ; Figure 2), increasing the aphid population intrinsic rate when *H. radicata* conditioned the soil compared to other soils. The developmental time from aphid to adult stage (day that start the progeny) was not affected by PSF ( $F = 0.9$ ;  $df = 4, 51$ ;  $P = 0.941$ ; Figure S2A). The total number of aphids 13 days after infestation showed a trend but was not significantly affected by PSF ( $F = 8.2$ ;  $df = 4, 20$ ;  $P = 0.083$ ; Figure S2B).



**Figure 2.** Plant-soil feedback effects on the generalist *Myzus persicae*. Intrinsic rate of increase:  $rm = 0.74 (\ln Md) / d$ , where  $Md$ , effective fecundity and  $d$ , pre-reproductive time (Wyatt & White 1977). Bars represent means  $\pm$  SE ( $n = 5$  soil replicates, each averaged for 15 plants). Different letters indicate significant pairwise differences between conditioned soils ( $P < 0.05$ ).

#### *Plant soil feedbacks can reduce parasitoid performance*

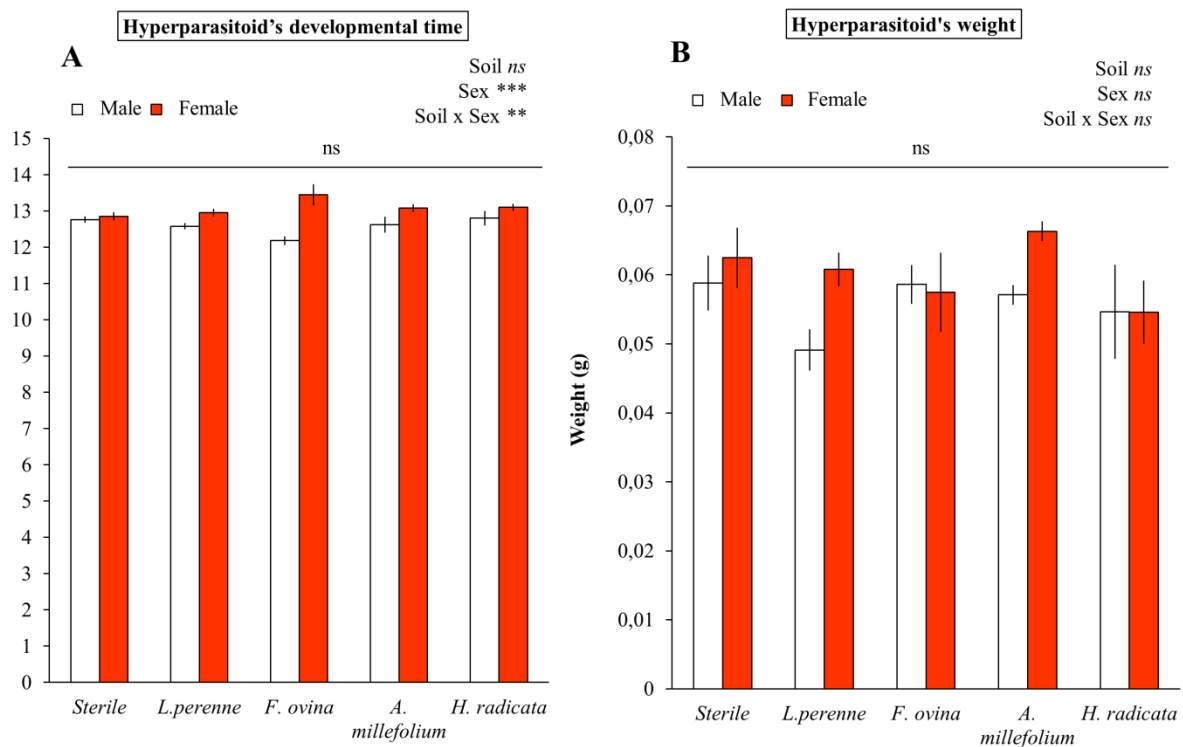
To study whether PSF effects cascade up to the third trophic level, several parameters of the parasitoid *A. colemani* were measured. Parasitoid survival (number of mummies relative to the introduced aphids) was influenced by the plant species that conditioned the soil inocula ( $F = 10.9$ ;  $df = 4, 17$ ;  $P = 0.027$ ; Figure 3A) with a reduced number of mummies on plants that were grown with inocula from *L. perenne* and *H. radicata* compared to sterile soil. Moreover, the time from parasitization to mummy formation of the parasitoid was significantly longer (therefore lower performance) in soil with a legacy effect from *H. radicata* in comparison with the sterile soil and the other soils ( $F = 4.6$ ;  $df = 4.43$ ;  $P = 0.005$ ; Figure 3B). In contrast, no differences were found in the developmental time from mummy formation to adult emergence ( $F = 0.7$ ;  $df = 4, 43$ ;  $P = 0.579$ ; Figure 3C). This developmental time was dependent on the parasitoid sex ( $F = 6.7$ ;  $df = 1, 43$ ;  $P = 0.014$ ; Figure 3C) and we observed an interaction between sex and soil treatment ( $F = 4.2$ ;  $df = 4, 43$ ;  $P = 0.0079$  Figure 3C). Parasitoid adult weight was not affected neither by soil legacy effects ( $F = 1.4$ ;  $df = 4, 43$ ;  $P = 0.256$ ; Figure 3D) nor sex ( $F = 0.3$ ;  $df = 1, 43$ ;  $P = 0.589$ ; Figure 3D).



**Figure 3.** Plant-soil-feedback effects on the parasitoid *Aphidius colemani*. (A) Parasitization success. (B) Parasitoid development from egg to mummy (C) Parasitoid development from mummy to adult (D) Parasitoid weight. Bars represent means  $\pm$  SE (n = 5 soil replicates, each averaged for total number of parasitoids for averaged plant, with a total number of 495 individuals). Different letters indicate significant pairwise differences between conditioned soils (P < 0.05). Bars marked with ns are non significantly different (P > 0.05).

*PSF effects do not cascade up to the fourth trophic level*

We did not observe differences between soil legacies on the number of hyperparasitoids adults emerged ( $F= 0.2$ ;  $df= 4, 17$ ;  $P= 0.930$ ). Moreover, the time the hyperparasitoid needed to develop from egg to adult was not affected by PSF either ( $F= 0.3$ ;  $df= 4, 32$ ;  $P= 0.859$ ; Figure 4A), but we found differences between males and females developmental time ( $F= 25.6$ ;  $df= 1, 31$ ;  $P<0.001$ ; Figure 4A). Plant legacy effects did not affect hyperparasitoid weight ( $F= 1.1$ ;  $df= 4, 32$ ;  $P= 0.364$ ; Figure 4B).



**Figure 4.** Plant-soil feedbacks effects on the hyperparasitoid *Dendrocerus aphidum*. (A) Hyperparasitoid developmental time. (B) Hyperparasitoid weight. Bars represent means  $\pm$  SE ( $n = 5$  soil replaces, each averaged for total number of hyperparasitoids for averaged plant, from a total number of 156 individuals). Bars marked with ns are non significantly different ( $P > 0.05$ ).

## Discussion

Our study demonstrates that PSF can influence plant-insect interactions up to the third trophic level. We show how soil legacies left by a plant species, influences germination and height of another plant species growing in that soil, aphids feeding on that second plant, and parasitoids that develop inside those aphids. However, as a Russian doll, hyperparasitoids that develop inside those parasitoids were not affected by the soil legacies of this study.

Although PSF has become a key concept in terrestrial ecology, past studies have mainly addressed plant biomass production. However, plant performance comprises a sequence of different life stages, beginning from seed germination and ending on the production of a viable progeny. Thus, the colonization of a plant by beneficial microbes occurs at an early stage during germination and establishes beneficial associations that last throughout the entire life cycle of the plants (Santhanam et al., 2015). Here our study showed that even using a little amount of conditioned soil as inoculum (10%), its feedback effect could be transferred across species, reducing the germination rate in sweet pepper. Previous studies testing germination in PSF environment, observed that seedling stages were particularly susceptible to mortality caused by pathogens (Packer and Clay, 2000; Hersh et al., 2012). Moreover, some rhizobacterial species may inhibit seed germination, such as the isolated strain *Pseudomonas aeruginosa*, which inhibits maize and wheat germination (Tiwari et al., 2017). However, whether we can use PSF for enhanced germination remains to be tested. Thus, greater understanding of the characteristic PSF associated microbes will provide insights into strategies for more effective seed germination.

The soil legacy of *H. radicata* resulted to have the longest cascading effect, from seed germination, over aphid growth population up to parasitoid performance. *H. radicata* is a common plant in grassland fields and has been used in previous PSF experiments (Bezemer et al., 2006; Kos et al., 2015a; Wubs et al., 2016), showing negative effect on future plant community establishment or in chrysanthemum plant growth (Jing et al., 2015, Ma et al., 2017). A previous study demonstrated that *H. radicata* has an allelopathic effect on gramineous forage crops, inhibiting their growth (Kim et al., 2005), as occurred in our results, where an inhibitory effect on sweet pepper germination was observed. Interestingly, the highest aphid growth population and lowest plant growth observed in this study was in *H. radicata* conditioned soil. Contrary as occurred in other studies, where plant growth and

aphid densities are correlated (Kos et al., 2015a, 2015b) our study did not show this correlation. Fungal activity and plant products, as exudates, may play a role in insect-plant interactions (Saikkonen et al., 2013). Thus we suggest that plant compounds from *H. radicata* may suppress the plant defense in sweet pepper plants. Although previous studies demonstrated no effect on induction of plant defense through PSF effects in sweet pepper plants (Vaello et al., 2018), we suggest that future works may increase the range of conditioned soils including plants with potentially allelopathic effect.

Although several studies have demonstrated the effectiveness of soil microorganism enhancing parasitoid performance and preference (Guerrieri et al., 2004; Hempel et al., 2009), little is known about how plant soil legacies created by different plant species can affect the parasitoids performance. In this study we demonstrate that soil inocula from *H. radicata* led to a reduced *A. colemani* performance (by increasing their developmental time and reducing survival). Several mechanisms could explain these results. First, since aphid performance was enhanced in this treatment, a possibility is that a strong host may defend better against its parasitoids (Rothacher et al., 2016). Second, plant defences leading to the production of toxins may interfere with the development of parasitoid larvae either through direct exposure inside their host, or by modulating host immune function (Gols, 2014; Kaplan et al., 2016). Third, plants can also accumulate certain toxins present in the soil, and that could also affect herbivores and their parasitoids (Hartl and Baldwin, 2006). Which of these mechanisms are here explaining the plant soil feedback effects on parasitoids is not known, but a fascinating area for future studies.

Primary parasitoids can be parasitized by secondary parasitoids that attack the primary parasitoid larva inside the still-living aphid or, as is the case of *Dendrocerum* spp., attacking the parasitoid prepupae and pupae inside the aphid mummy (Sullivan and Völk, 1999). These differences in hyperparasitoid attacking host may have consequences in their development. Moreover, primary parasitoids and larval hyperparasitoids can potentially influence the final size of the aphid host, whereas mummy parasitoids cannot (Cohen et al., 2005). Thus, the diluted effect of PSF that we observed in this study on the fourth trophic level, may be due to the fact that *D. aphidum* parasitize mummy parasitoids, and their final size are closely related to the size of their primary parasitoid, which in our study was not affected by PSF (we observed no effect of PSF on *A. colemani* weight). Hence, we suggest that effects which

potentially affect size and weight of aphid mummy parasitoids would be likely related to the size and weight of its hyperparasitoids. However, this relationship between hyperparasitoids and their host depends on the identity of the plant, herbivore host and its primary parasitoid. For example in a brassicaceus-caterpillar system, hyperparasitoid performance of *L. nana* was related to its parasitoid host, *Cotesia glomerata* L. (Hymenoptera, Braconidae) in *Brassica nigra* plants (Soler et al., 2005). In contrast, the hyperparasitoids *L. nana* were influenced by *Brassica oleracea* plant toxins but not by the size of its parasitoid host *C. glomerata* (Harvey et al., 2003). Studies of belowground interactions on the fourth trophic level are very limited (Soler et al., 2005; Meyer et al., 2009), and future studies with different primary and secondary parasitoids living styles, as well as other herbivore hosts will contribute to understand how belowground interactions may cascade up to the fourth trophic level.

Advances in natural systems have improved our understanding of linkages and feedbacks between plants and soil organisms, which can be used for the development of more sustainable agricultural practices (Kaplan et al., 2018; Mariotte et al., 2017). Taking into account the importance of the soil in agriculture at promoting crop yield, one more step in sustainable agriculture is to consider the soil microbiome as an ally to protect crops against pests (Pineda et al., 2017) and/or enhancing the performance natural enemies in biological control. Moreover, due the high levels of hyperparasitism observed in aphid-parasitoid complexes in agroecosystems (Schooler et al., 2011) which can strongly disturb the efficacy of aphid biological control (Gómez-Marco et al., 2015), studies on hyperparasitoids performance are strongly needed. In summarize, this study insight about how PSF effects can cascade up until the third trophic level, and we encourage that the next challenge in PSF studies is to find out inoculos or an approach that can be implemented in sustainable agriculture.

## **Acknowledges**

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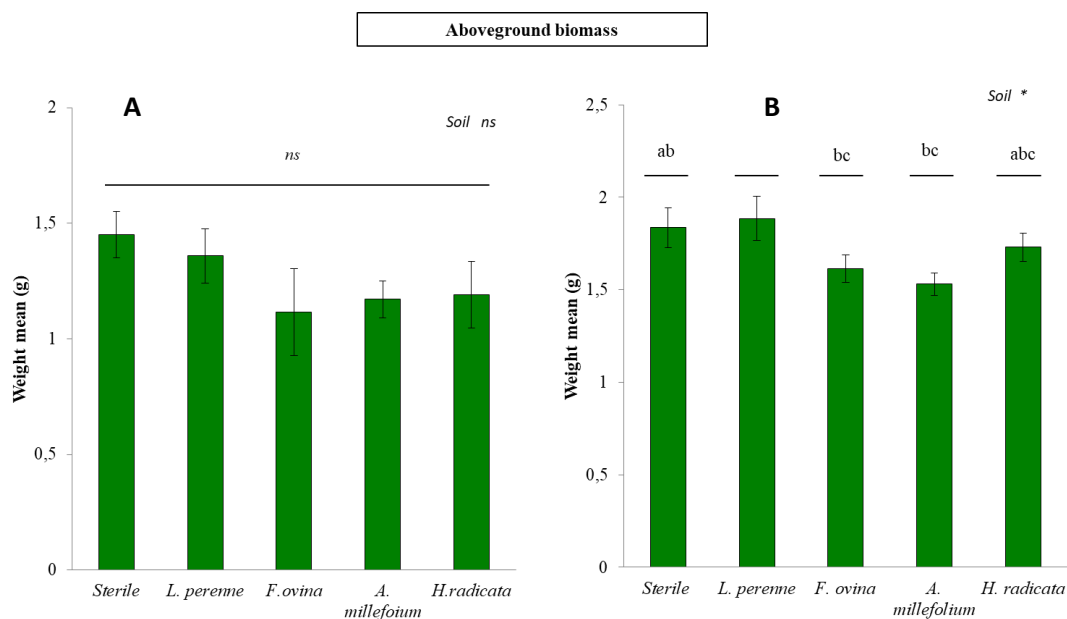
## Supplementary Information

**Plant-soil feedback effects beyond the third trophic level**

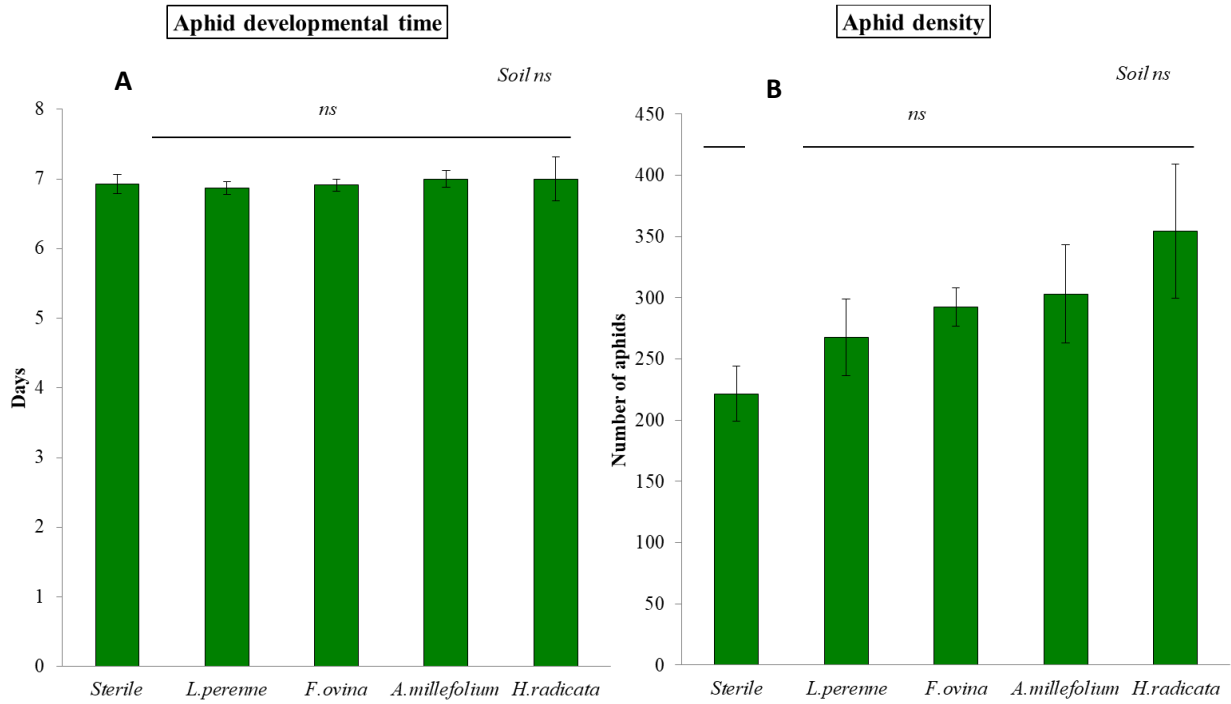
The following Supporting Information is available for this article:

**Supplementary results***Plant-soil feedbacks affect seed germination and plant growth*

A second height was measured on the fifth week where either unparasitized or parasitized aphids had been feeding on the plants during one week. Here, the soil effect on plant height disappeared both for parasitized aphid-infested ( $F= 2.095$ ;  $df= 4.69$ ;  $P= 0.094$ ) and aphid-infested plants ( $F= 2.344$ ;  $df= 2.69$ ;  $P=0.105$ ).



**Figure S1.** Plant-soil feedback effects on sweet pepper performance: (A) Aboveground biomass in uninfested plants (5 weeks old) and (B) Aboveground biomass in aphid infested plants (7 weeks old). Bars represent means  $\pm$  SE ( $n = 5$  soil replicates, each averaged for 15 plants). Different letters indicate significant pairwise differences between conditioned soils ( $P < 0.05$ , LSD). Bars marked with ns are non significantly different ( $P > 0.05$ ).



**Figure S2.** Plant-soil feedback effects on the generalist *Myzus persicae*. (A) Aphid developmental time to adult stage and (B) Aphid density, number of aphids counted the 13<sup>th</sup> day after infestation. Bars represent means  $\pm$  SE ( $n = 5$  soil replicates, each averaged for 15 plants). Bars marked with ns are non significantly different ( $P > 0.05$ ).

## **SECCIÓN IV**

### **Capítulo 6**

#### **Discusión general**

## **Introducción**

La presente tesis ha estado dedicada al estudio de las interacciones multitróficas en el cultivo del pimiento. Esta elección obedece a que las dos principales especies plagas en este cultivo son el pulgón *Myzus persicae* y el trips *Frankliniella occidentalis* (Ramakers, 2007) y sin embargo, hasta el momento no se habían estudiado en profundidad las interacciones de estos insectos con agentes bióticos de otros niveles tróficos. Los diferentes estudios llevados a cabo en esta tesis, tienen un valor relevante tanto en agricultura como en el campo de la ecología, ya que utilizamos un sistema complejo de estudio que incluye hasta cuatro niveles tróficos (subterráneos y aéreos) y aplicamos conceptos ecológicos que pueden servir, en un futuro, para mejorar las estrategias de control biológico utilizadas en la actualidad. En este capítulo se discute la importancia y trascendencia de los resultados obtenidos en esta tesis.

## **Importancia del conocimiento de ecología química en las estrategias de control biológico**

La Organización Internacional del Control Biológico (IOCB) define el control biológico como el uso de organismos vivos, de sus recursos o de sus productos, con el fin de prevenir o reducir las pérdidas o daños causados por organismos plaga. Es considerado actualmente como el método de control de plagas más seguro desde el punto de vista medioambiental y más beneficioso económicamente (van Lenteren, 2012). Las investigaciones que se llevan a cabo en muchos sistemas agrícolas están enfocadas normalmente a la mejora de las estrategias de control para suprimir o disminuir el efecto de las plagas en los cultivos. Sin embargo, existen dos aspectos relativos a este tema que necesitan ser atendidos en las investigaciones sobre control biológico: i) la atracción de insectos beneficiosos durante las fases iniciales de los cultivos, y ii) el mantenimiento de estos insectos durante la mayor parte del desarrollo del cultivo (Khan et al., 2008). Así, el control biológico por conservación, busca preservar y mantener las poblaciones de enemigos naturales ya presentes en los cultivos, para mejorar su abundancia y actividad controladora, particularmente a través de técnicas culturales. Es en este punto donde el control biológico se puede beneficiar de los conocimientos de la ecología química.

Los insectos son químicos analíticos por excelencia, perciben el mundo a través de los olores (principalmente a través de las sensilas ubicadas en las antenas y piezas bucales) y son

capaces de discriminar feromonas naturales de moléculas con cambios estructurales mínimos (Leal, 2005). De este modo, la ecología química utiliza las estructuras quimiorreceptoras de los insectos como biosensores para la identificación de feromonas y otros semioquímicos. En el capítulo 2 y parte del capítulo 3, esta tesis estudia el efecto de la feromona de agregación del trips en diferentes enemigos naturales, con el objetivo de ofrecer información sobre su potencial uso en agricultura sostenible.

En el capítulo 2, se demuestra cómo la mezcla de los dos principales componentes de la feromona de agregación del trips *F. occidentalis*, (R)-lavandulilo acetato y neryl (S)-2-methylbutanoato [RLA:NMB] (Hamilton et al., 2005), mezclados en un ratio específico es capaz de actuar como una kairomona, atrayendo al enemigo natural del trips, el chinche depredador *Orius laevigatus*. Sin embargo, cuando los compuestos se ofrecen por separado, no resultan ser un estímulo para el depredador. Además se demuestra que aunque ninfas y adultos de *O. laevigatus* son capaces de reconocer la mezcla de estos dos compuestos en las combinaciones aquí evaluadas, los adultos sólo son atraídos en la ratio 1:2.3 [RLA:NMB], que se considera la más cercana a la ratio natural de emisión del trips (Hamilton et al., 2005; Zhang et al., 2011), mientras que las ninfas son atraídas tanto a la ratio 1:1 como 1:2.3 [RLA:NMB]. Por lo tanto se demuestra que ninfas y adultos de *O. laevigatus* tienen capacidades olfativas diferentes. Curiosamente, en el capítulo 3, se obtienen unos resultados completamente opuestos al capítulo anterior, donde el depredador del pulgón *Sphaerophoria rueppellii* se ve repelido el estímulo olfativo procedente de la mezcla 1:2.3 [RLA:NMB] evitando la oviposición en plantas de pimiento que contengan este estímulo. Estos resultados son interesantes porque demuestran que el depredador del pulgón es capaz de reconocer feromonas procedentes de insectos diferentes a su presa común (el pulgón), y que además utiliza esa información para su beneficio, evitando la oviposición en plantas que contengan ese estímulo olfativo (en el mismo capítulo se demuestra que la presencia de trips es perjudicial para el sírfido).

Los resultados extraídos en estos dos capítulos son de gran relevancia para el control biológico de conservación. Por ejemplo, en cultivos donde la producción de polen por parte de las plantas no es constante, es muy difícil que ciertos enemigos naturales que se alimentan de polen permanezcan en los cultivos durante estas épocas. Por este motivo, se ponen en práctica estrategias como las estaciones de polen, para permitir así el establecimiento de los depredadores (Adar et al., 2014). Por lo tanto la posible combinación de estos “comederos”

junto con la aplicación de la feromona de agregación podría ser una solución a la permanencia de los depredadores de trips en los cultivos.

### **Efecto de la doble herbivoría en el cultivo del pimiento**

En la naturaleza, las plantas están expuestas al ataque de múltiples herbívoros al mismo tiempo. El daño ocasionado por un insecto influye directamente no sólo sobre la planta sino que también tiene consecuencias en el conjunto de la comunidad asociada a esta (Ohgushi, 2008; Dicke & Baldwin, 2010; Poelman et al., 2011). En la actualidad, estudios relacionados con las interacciones entre diferentes herbívoros y otros niveles tróficos están recibiendo mayor atención por parte de los investigadores, debido a la necesidad de comprender el conjunto de las interacciones en un sistema más real. En esta tesis se estudia el efecto de la doble herbivoría por parte del pulgón y del trips en un sistema multitrófico, donde participan los principales enemigos naturales del pulgón; el depredador *S. rueppellii* y el parasitoide *Aphidius colemani*.

En el capítulo 3 demostramos como la presencia del trips afecta negativamente a los tres niveles tróficos estudiados en este capítulo. Se demuestra que el trips afecta al desarrollo de la planta, la cual no es capaz de crecer al mismo ritmo que cuando el trips no está. Además, el trips es capaz de afectar al desarrollo del pulgón, reduciendo el crecimiento de sus poblaciones. Por último, la presencia de trips es capaz de afectar negativamente al desarrollo y preferencia de puesta del enemigo natural del pulgón, el sírfido *S. rueppellii*. Los resultados muestran como los sírfidos que se desarrollan en un ambiente con trips, ven reducida su fecundidad y además, las hembras adultas evitan oviponer en plantas de pimiento que tengan tanto presencia de trips, como su feromona de agregación. Los motivos por los cuales el sírfido podría evitar la oviposición en presencia de trips son: i) asegurar su descendencia (demostramos que el trips es capaz de dañar los huevos del sírfido), ii) asegurar una población elevada de pulgón a su descendencia (ya que en presencia de trips, la densidad de pulgón disminuye). Por otra parte, en el capítulo 4 se demuestra también como la herbivoría previa del pulgón en la planta de pimiento, aumenta la supervivencia y desarrollo del trips. Además, observamos que este efecto del incremento de la supervivencia del trips en plantas que han tenido previamente áfidos, se ve menguado cuando los pulgones se encuentran parasitados por *A. colemani*, o cuando la planta ha estado previamente infestada

por el propio trips. A pesar de que es habitual que los pulgones y los trips convivan normalmente en los cultivos, hasta el momento no se había estudiado el efecto de sus interacciones en un sistema multitrófico.

Por otra parte, es importante destacar la importancia de estos resultados para el control biológico de plagas. Por ejemplo, el sírfido *S. rueppellii* demuestra su eficacia controlando las plagas de pulgón reduciendo sus poblaciones tanto en presencia como en ausencia del trips. También hay que destacar que aunque su fecundidad (número de huevos puestos por el sírfido) se ve reducida en presencia del trips, no lo hace su fertilidad (número de larvas emergidas de los huevos), por lo que el control de pulgones, por parte de futuras generaciones de sírfidos, no se ve afectado negativamente. Comprobamos además como la presencia de pulgones parasitados, reduce la supervivencia de los trips, demostrándose así que el control de las plagas de pulgón a través del uso de parasitoides tiene un efecto directo también en el control de los trips. Para poder explicar estos resultados, a continuación discutimos el papel de las defensas de las plantas en este tipo de interacciones.

### **Enemigos naturales como moduladores de las defensas de las plantas**

Las plantas han desarrollado una serie de estrategias específicas que les permite prevenir o reducir el ataque de herbívoros o patógenos. El tipo de alimentación y el nivel de especialización de los herbívoros son factores importantes que determinan la naturaleza de la respuesta defensiva de la planta (Karban & Baldwin, 1997; Voelckel & Baldwin, 2004; Ali & Agrawal, 2012). En esta tesis estudiamos la respuesta de la planta frente al ataque de dos herbívoros con diferentes tipos de alimentación (el pulgón y el trips). En concreto, nos centramos en el estudio de las rutas de señalización que están reguladas por fitohormonas, estas rutas terminan con la síntesis de compuestos defensivos. Las fitohormonas que juegan un papel importante en las defensas de la planta son principalmente el ácido salicílico (SA) y el ácido jasmónico (JA) (Pieterse et al., 2009). Recientemente, se ha demostrado que los organismos del tercer nivel trófico son capaces de modular la respuesta defensiva de la planta (Fatouros et al., 2005; Poelman et al., 2011; Ode et al., 2016). Sin embargo, estos estudios están enfocados al sistema de estudio de brasicáceas y orugas masticadoras. En esta tesis estudiamos la posible modulación de las defensas por parte de un parasitoide a través del

pulgón. Así, en el capítulo 4 demostramos como además de la capacidad de la planta a responder tanto al ataque del pulgón como del trips (alterando la respuesta defensiva del SA y del JA), se demuestra por primera vez la capacidad de modulación de la respuesta defensiva de la planta por parte de la parasitización de *M. persicae* por el parasitoide *A. colemani*. En los resultados de este capítulo se observa cómo la expresión del gen *CaPINII* (responsable de la inducción del JA) es más suprimido por pulgones que por pulgones parasitados. La parasitización no afectó a la expresión de *CaPALI* (gen responsable de la inducción del SA). Estos resultados son novedales en parte porque la mayoría de estudios que exploran mecanismos moleculares y químicos de defensas se basan en plantas modelo, y aquí utilizamos pimiento, una planta de gran importancia económica que no ha sido prácticamente estudiada en el campo de interacciones insecto-planta. Además de ello, estos resultados son relevantes en el campo de la ecología, debido a que hay un incremento en la necesidad de comprender cómo la parasitización puede alterar la señalización de las defensas de la planta y como estos cambios pueden afectar a los insectos a nivel comunidad. Por ejemplo, estos cambios en la supresión de las defensas podría tener efectos en organismos del cuarto nivel trófico, como los hiperparasitoides (Poelman et al., 2012; Zhu et al., 2015).

### **Importancia del suelo en un sistema multitrófico**

El suelo es una de las mayores fuentes de diversidad de microorganismos, los cuales determinan el microbioma asociado a una planta. Los microorganismos del suelo no sólo son cruciales para la mejora del crecimiento de la planta y la tolerancia al estrés abiótico sino que también son capaces de inducir resistencia frente a patógenos e insectos tanto en el suelo como en la parte aérea (Martínez-Medina et al., 2017; Pineda et al., 2017). Por esta razón, estudios sobre el microbioma del suelo han emergido como un componente principal para la inmunidad de las plantas y se han convertido en el punto de mira de muchos ecólogos y agrónomos.

En los capítulos 4 y 5 de esta tesis añadimos a nuestro sistema de estudio diferentes microbiomas del suelo generados a través de la técnica retroalimentación planta suelo (Van der Putten et al., 2013; Kos et al., 2015), con el objetivo de comprobar si estos suelos son capaces de inducir resistencia a la planta de pimiento o si sus efectos son capaces de llegar a niveles superiores de la cadena trófica. Desafortunadamente, en el capítulo 4 no pudimos

demostrar que los suelos aquí estudiados proporcionaran a la planta de pimiento una mayor, o menor, inducción de defensas. Las plantas utilizadas para condicionar los diferentes tipos de suelo fueron seleccionadas a través de los resultados obtenidos en un estudio previo, sobre el efecto de la retroalimentación planta-suelo en el cultivo de crisantemo (Ma et al., 2017). Teniendo en cuenta que el legado de efectos que deja una planta en el suelo es específico para cada planta (Kos et al., 2015), haciendo un cribado previo de suelos y observando sus efectos en el cultivo de pimiento, podríamos haber seleccionado plantas que, quizá, potenciasen un cambio en las defensas de la planta.

Aunque en el capítulo 4 no comprobamos ningún efecto en las defensas de la planta por parte de los suelos utilizados, en el capítulo 5 si observamos como los efectos de la retroalimentación planta-suelo alcanzan el tercer nivel trófico, afectando al desarrollo del parasitoide *A. colemani*. Estos efectos se diluyen al llegar al cuarto nivel trófico, donde no se encuentra ningún efecto en el desarrollo del hiperparasitoide del pulgón *Dendrocerus aphidum*. En este capítulo 5 se extraen además varios resultados interesantes. Uno de ellos fue demostrar cómo el efecto del suelo juega un papel importante en la germinación de las semillas; donde con sólo un 10 % de inóculo de suelo condicionado por *H. radicata*, se redujo significativamente la germinación de la planta de pimiento. Sería muy interesante, en investigaciones futuras, comprobar el efecto de la retroalimentación planta suelo en la supresión de la germinación de malas hierbas (siempre y cuando no afecte al cultivo). Otro resultado fue observar como en plantas desarrolladas en suelo de *H. radicata*, el pulgón se desarrolló mucho mejor. Este resultado nos ha parecido llamativo, puesto que en investigaciones previas, los pulgones siempre se desarrollan mejor en plantas que han tenido un efecto beneficioso a través de los microorganismos del suelo (Pineda et al., 2012; Kos et al., 2015). Por ese motivo, hubiese sido interesante estudiar la respuesta defensiva de la planta en suelos condicionados por *H. radicata*, y comprobar así la posible supresión de las defensas de la planta. Finalmente, y siguiendo con el estudio del suelo condicionado por *H. radicata*, observamos que el tiempo de desarrollo del parasitoide *A. colemani*, se vio incrementado en este suelo. Cuanto más tarda en desarrollarse un parasitoide, mayor es el riesgo de ser depredado, además como especie deben desarrollarse lo más rápido posible, para hacerse pronto adultos y tener descendencia. Así, que el incremento demostrado en el desarrollo de *A. colemani*, significa un efecto negativo en el control biológico. Desafortunadamente, ninguno de los suelos estudiados en esta tesis presentó un efecto

positivo en el sistema multitrófico del pimiento. Sin embargo, hay que destacar que los estudios de retroalimentación planta-suelo son muy novedosos en el ámbito de la agricultura, (hasta dónde sabemos, sólo el estudio previo de Ma et al., 2017 y los de esta tesis relacionan este concepto ecológico con cultivos hortícolas). Esperamos que en un futuro, al igual que se utilizan microorganismos específicos para condicionar el suelo (como *Bacillus* o *Trichoderma*) lleguemos a utilizar el conjunto del microbioma para manipular y mejorar los cultivos, a través de la mejora del crecimiento de la planta y de su resistencia a las plagas.

### **Aplicación de conceptos ecológicos en cultivos agrícolas**

Las plagas provocan cada año importantes pérdidas en agricultura, por eso es importante lograr nuevos métodos de control que sean más eficaces, y más respetuosos con el medio ambiente, permitiendo así el desarrollo de una agricultura sostenible. Durante el desarrollo de esta tesis, se ha tenido en cuenta la importancia de los estudios ecológicos y su integración en sistemas de estudio aplicados en la agricultura. Esta tesis ha tenido como objetivo comprender el funcionamiento de las interacciones multitróficas entre el suelo, la planta, las especies plaga y sus enemigos naturales, y esperamos que en un futuro este conocimiento pueda ser utilizado para mejorar las estrategias de control de plagas en agricultura sostenible. Los sistemas de agricultura moderna se deben beneficiar de la aplicación de conceptos y modelos extraídos a partir de estudios de ecología aplicada. Así, tanto la ecología química como el estudio de las interacciones sobre el nivel del suelo, son conceptos ecológicos que se han estudiado en profundidad, y que a día de hoy se aplican ya en la agricultura sostenible. Uno de los retos con los que nos vamos a encontrar en un futuro cercano es la manipulación del microbioma del suelo para su utilización en agricultura. Así, como hemos visto en esta tesis, uno de los retos en este campo va a ser predecir que especies de plantas pueden ser usadas para condicionar y mejorar los suelos agrícolas. Estos ensayos acompañados de estudios moleculares donde se observe la inducción de las defensas de las plantas y el efecto en los diferentes niveles tróficos, serán necesarios para comprender y poner en práctica estos conocimientos en el diseño de nuevas estrategias de control en agricultura sostenible.

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## **SECCIÓN IV**

### **Capítulo 7**

#### **Conclusiones generales**

## Conclusiones

- 1) Ninfas y adultos de *Orius laevigatus* responden positivamente a la feromona de agregación (R)-lavandulilo acetato y neryl (S)-2-methylbutanoato [RLA:NMB] procedente de su presa, el trips *Frankliniella occidentalis* cuando es presentada como una mezcla de los compuestos en un ratio de 1:2.3 [RLA:NMB], mientras que las ninfas también son capaces de reconocer el ratio 1:1 [RLA:NMB]. Sin embargo, tanto ninfas como adultos, no son capaces de reconocer el estímulo olfativo cuando los compuestos de la feromona son presentados de manera individual.
- 2) La feromona de agregación del trips *Frankliniella occidentalis*, en el ratio 1:2.3 [RLA:NMB], es capaz de actuar de atrayente para el chinche depredador *Orius laevigatus* (actuando como kairomona) y como repelente para los adultos del sírfido afidófago *Sphaerophoria rueppellii* (actuando como alomona).
- 3) La presencia del insecto omnívoro *Frankliniella occidentalis* es capaz de afectar a diferentes niveles tróficos. Desde reducir el crecimiento de la planta (primer nivel trófico) y el crecimiento poblacional del pulgón *Myzus persicae* (segundo nivel trófico) hasta finalmente afectar al desarrollo y comportamiento del sírfido *Sphaerophoria rueppellii* (tercer nivel trófico), así como dañando los huevos de este depredador. Sin embargo, aunque el trips es capaz de perturbar al sírfido, la eficacia del sírfido como agente controlador de plagas de pulgón no se ve afectada.
- 4) El parasitoide *Aphidius colemani* es capaz de modular la respuesta defensiva de la planta de pimiento a través de la parasitización del pulgón. Se observa una diferencia en la respuesta defensiva de la planta en relación a la supresión del gen *CaPINII* (responsable de la inducción de la ruta del ácido jasmónico (JA), ya que las plantas con pulgones parasitados por *A. colemani* emiten una respuesta menos supresiva del JA que plantas con pulgones no parasitados.

- 5) Los cambios ocasionados por la herbivoría de pulgones en la respuesta defensiva de la planta tiene un efecto beneficioso en la supervivencia y desarrollo del herbívoro *Frankliniella occidentalis*, mientras que la previa herbivoría tanto por pulgones parasitados como por trips, no lo tiene.
- 6) El efecto del legado del suelo llevado a cabo a través de la retroalimentación planta suelo por las especies *Achilea millefolium* y *Lolium perenne*, no influye en la respuesta defensiva de la planta de pimiento, en relación a la expresión de varios genes de las rutas del JA y/o del SA, tanto en presencia como en ausencia de herbivoría.
- 7) La retroalimentación planta-suelo influye en las relaciones planta-insecto hasta alcanzar el tercer nivel trófico. El legado del suelo procedente de *Hypochaeris radicata*, influye negativamente en la germinación y desarrollo de la planta de pimiento, en el crecimiento poblacional del pulgón (habiendo más pulgones), así como en el desarrollo del parasitoide. Sin embargo los efectos de la retroalimentación planta-suelo se diluyen a medida que aumentamos de nivel trófico, sin afectar al desarrollo del hiperparasitoide *Dendrocerus aphidum* que se desarrolla dentro de *Aphidius colemani*.

