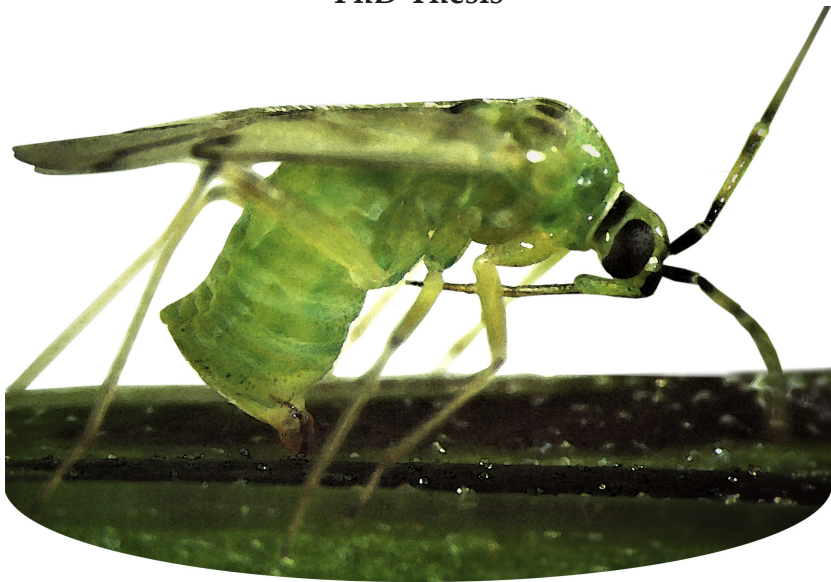# Enhancing Pest Management in Sweet Pepper by the Exploitation of Zoophytophagy

PhD Thesis

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Enhancing Pest Management in Sweet Pepper by the Exploitation of Zoophytophagy

PhD Thesis Presented by **SARRA BOUAGGA** to obtain the degree of Doctoral of Phylosophy

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<div align="right">Moncada, 14/02/2018</div>

Summary

During the last decade, integrated pest management programs based on the augmentation and conservation of zoophytophagous predators have been successfully developed in horticulture greenhouses. In sweet pepper, the release of *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) and *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) has provided effective control of two key pests, the Western flower thrips *Frankliniella occidentalis* Pergande (Thysanoptera: Tripidae) and the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). Despite being a zoophytophagous predator, the phytophagous behaviour of *O. laevigatus* has not been previously explored in depth, nor has the impact of phytophagy on plant physiology. In the **<u>first objective</u>** of this PhD, the hierarchical significance of *O. laevigatus* feeding on sweet pepper was compared with other behaviours. *Orius laevigatus* spent the majority of its time (38%) feeding on apical meristems and apical fresh leaves, which were also preferred residence locations. The phytophagous feeding behaviour of *O. laevigatus* on sweet pepper was shown to trigger defensive responses in the plant. These *O. laevigatus* plant induced defences were then shown to contribute to the repellence or attraction of pests or natural enemies, respectively. Specifically, *O. laevigatus*-punctured sweet pepper plants induced repellency for the whitefly *B. tabaci* and the thrips *F. occidentalis*. In contrast, the whitefly parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) was significantly attracted to *O. laevigatus*-punctured plants. The plant responses to *O. laevigatus* punctures included

the release of an altered blend of volatiles and activation of the jasmonate acid and salicylic acid signalling pathways. These results highlight an interesting facet to the biology of *O. laevigatus*, in which the ability of the predator to induce defensive responses in sweet pepper plants may serve to improve the biological control (BC) of both thrips and whiteflies. Indeed, the results could explain the great success achieved by IPM programs based on the release, establishment and conservation of *O. laevigatus* in sweet pepper crops.

Nevertheless, sweet pepper pest management is still fronting problems because of aphids in the crop. To maintain aphids' population below an economic thresholds, multiples release of specialized natural enemies are needed. This strategy results in a complicated food web due to the large number of species released that which ends up making the release program too expensive for producers. In recent years, looking for alternative aphid's natural enemies which could be integrated with the current and systematically-implemented BC practices in sweet pepper has been one of the research priorities. In this line, the role of predatory mirids (Hemiptera: Miridae) in managing aphids in sweet pepper has been highlighted in several studies. In addition to their services as predators, mirid predators are able to induce plant defences by phytophagy. However, whether this induction occurs in sweet pepper and whether it could be an additional benefit to their role as BC agent in this crop remains unknown. These questions were addressed in the **second objective** of this PhD, and were investigated in two model insects, the mirids *Nesidiocoris tenuis* Reuter and *Macrolophus pygmaeus* Rambur. Plant feeding behaviour was observed in both *N. tenuis* and *M. pygmaeus* on sweet pepper and occupied 33% and 14% of total time spent

on the plant, respectively. The punctures caused by mirid plant feeding induced the release of a blend of Volatile Organic Compounds (VOCs) which repelled the herbivore pests *F. occidentalis* and *B. tabaci*, whereas attracted the whitefly parasitoid *E. formosa*. The repellent effect on *B. tabaci* was observed for at least 7 days after initial exposure of the plant to *N. tenuis*, and attraction of *E. formosa* remained functional for 14 days. Feeding induced plant defences by mirid predators, their subsequent effects on both pests and natural enemy behaviour, and the persistence of these observed effects open the door to new control strategies in sweet pepper crop. Further application of this research is discussed within this chapter, such as the vaccination of plants by zoophytophagous mirids in the nursery before transplantation.

A next step to integrate the possible use of these mirids on sweet pepper BC practices would be to ascertain their potential control on the whitefly *B. tabaci* and the thrips *F. occidentalis*. In the **<u>third objective</u>** of this PhD, a comparative study to measure the efficacy of predation by *N. tenuis*, *M. pygmaeus*, and *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) on the two sweet pepper key pests mentioned above, was conducted. This study was carried out under two greenhouses with different temperatures, 20 ºC and 27 ºC, which simulated the mean temperature registered in the two main crop cycles in Spain (the winter and summer planting period). Both, *N. tenuis* and *M. pygmaeus*, were able to establish on sweet pepper and significantly reduced the number of *F. occidentalis* and *B. tabaci* adults, larvae and nymphs. *Macrolophus pygameus* has the highest density at 20 ºC, whereas *N. tenuis* was more abundant at 27 ºC. In contrast, *D. maroccanus* did not achieve the required level of control and from our observation could not be selected as a biocontrol agent for *F. occidentalis* and *B. tabaci* in this crop.

None of the three mirids were observed to cause any damage on the pepper plant. The implications of these results for the use of mirids in sweet pepper crops were discussed within this chapter.

In the **fourth objective** of this PhD, the practical integration of one of these two predatory mirids with *A. swirskii* to enhance sweet pepper pest management was studied. Firstly, the co-occurrence of both mirid species when released together with *A. swirskii* in presence and absence of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs as alternative prey was investigated. This was compared to the standard release of *O. laevigatus* with *A. swirskii*. Secondly, the efficiency of each combined release (either *N. tenuis*, *M. pygmaeus* or *O. laevigatus* combined with *A. swirskii*) in reducing *F. occidentalis*, *B. tabaci* and the aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) infestations was evaluated under greenhouse conditions. Both, *N. tenuis* and *A. swirskii* were involved in a bidirectional intraguild predation (IGP). Contrary, this interaction (IGP) was apparently unidirectional in the case of *M. pygmaeus* with *A. swirskii* and *O. laevigatus* with *A. swirskii*. Both, *M. pygmaeus* and *O. laevigatus* significantly reduced the abundance of *A. swirskii*. However, in the greenhouse, IGP seemed to be neutralized. Mirids with *A. swirskii* significantly suppressed thrips, whitefly and aphid infestation, suggesting complementarity in the effects of these two predator species. Contrary, the combined use of *O. laevigatus* with *A. swirskii* did not reached a satisfactory control for aphids, despite it reduced effectively the level of thrips and whiteflies. Therefore, our results suggest that the use of mirids, instead of *O. laevigatus*, combined with *A. swirskii*, could result in more efficient and robust BC programs in sweet pepper crops.

In summary, together with the newly discovered ability of predatory mirids to induce plant defences, we expect that future BC of sweet pepper in commercial greenhouse could rely on the release of *A. swirskii* with predatory mirids. What is clear is that the use of mirids in sweet pepper is possible and can be more effective than the current system based on *O. laevigatus*, which motivates me to suggest that mirids deserve more attention in the BC of sweet pepper pests.

Resumen

Durante la última década, los programas de gestión integrada de plagas (GIP) basados en el aumento y la conservación de depredadores zoofitófagos se han desarrollado con éxito en invernaderos hortícolas. En pimiento dulce, la liberación de *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) y *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) ha proporcionado un control efectivo de dos plagas clave, el trips *Frankliniella occidentalis* Pergande (Thysanoptera: Tripidae) y la mosca blanca *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). A pesar de ser un depredador zoofitófago, el comportamiento fitófago de *O. laevigatus* no ha sido explorado previamente en profundidad, ni ha tenido un impacto de fitofagia sobre la fisiología de las plantas. En el **primer objetivo** de esta tesis doctoral, se comparó la importancia jerárquica de la alimentación de *O. laevigatus* con otros comportamientos sobre pimiento. *Orius laevigatus* pasó la mayor parte de su tiempo (38%) alimentándose de meristemo apical y hojas frescas apicales, las cuales también fueron su ubicación preferida. Se ha demostrado que el comportamiento fitófago de *O. laevigatus* en pimiento desencadena respuestas defensivas en la planta, las cuales contribuyen a la repelencia o atracción de plagas o enemigos naturales, respectivamente. Específicamente, las plantas de pimiento picadas con *O. laevigatus* inducen una repelencia a mosca blanca *B. tabaci* y al trips *F. occidentalis*. Por el contrario, el parasitoide de mosca blanca *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) se ve significativamente atraído por plantas picadas con *O. laevigatus*. Las respuestas

de la planta al comportamiento fitófago de *O. laevigatus* incluye la liberación de una mezcla alterada de compuestos volátiles y la activación de las vías de señalización del ácido jasmonato y ácido salicílico. Estos resultados ponen de manifiesto una faceta interesante de la biología de *O. laevigatus*, en la cual la capacidad del depredador para inducir respuestas defensivas en las plantas de pimiento puede servir para mejorar el control biológico (CB) del trips y la mosca blanca. De hecho, los resultados podrían explicar el gran éxito logrado por los programas de GIP basados en la liberación, el establecimiento y la conservación de *O. laevigatus* en el cultivo de pimiento.

Sin embargo, el control de plagas en pimiento todavía tiene problemas debido a los pulgones. Actualmente, para mantener la población de pulgones por debajo del umbral económico, se necesita múltiples liberaciones de enemigos naturales especializados. Esta estrategia resulta en una red trófica complicada debido a la gran cantidad de especies liberadas, además de cara para los productores. En los últimos años, la búsqueda de enemigos naturales alternativos para pulgones y que puedan ser integrados con las prácticas actuales de CB en pimiento ha sido una de las prioridades en la investigación sobre este cultivo. Siguiendo esta línea, el papel de los míridos depredadores (Hemiptera: Miridae) en el manejo de pulgones en pimiento ha sido destacado en varios estudios. Además de sus servicios como depredadores, los miridos zoofitófagos pueden inducir defensas en las plantas debido a su fitofagia. Sin embargo, hasta ahora no se sabe si esta inducción se produce en el pimiento y si podría ser un beneficio adicional a su papel como agente de CB en este cultivo. Estas preguntas se abordaron en el **<u>segundo objetivo</u>** de esta tesis doctoral y estudiaron sobre dos especies de míridos modelo y comerciales actualmente, *Nesidiocoris tenuis* Reuter y

Macrolophus pygmaeus Rambur. El comportamiento de fitofagia sobre la planta de pimiento se observó tanto en *N. tenuis* como en *M. pygmaeus* y ocuparon el 33% y el 14% del tiempo total que estuvieron sobre ésta, respectivamente. Las picaduras alimenticias de los míridos indujeron la liberación de una mezcla de Compuestos Orgánicos Volátiles (COV) que repelieron los herbívoros *F. occidentalis* y *B. tabaci* y atrajeron al parasitoide de mosca blanca *E. formosa*. El efecto repelente sobre *B. tabaci* se observó durante al menos 7 días después de la exposición inicial de la planta a *N. tenuis* y la atracción de *E. formosa* permaneció funcional durante 14 días. Las defensas inducidas en planta debido a las picaduras alimenticias por míridos depredadores y la persistencia de estos efectos abren la puerta a nuevas estrategias de control de plagas en pimiento. La aplicación adicional de esta investigación se analiza en este capítulo, como la vacunación de plantas por míridos zoofitófagos en el vivero antes del trasplante.

Un siguiente paso para integrar el posible uso de estas dos especies de míridos en las prácticas de CB de pimiento fue determinar su posible control sobre la mosca blanca *B. tabaci* y el trips *F. occidentalis*. En el **tercer objetivo** de esta tesis doctoral, se llevó a cabo un estudio comparativo para estudiar la eficacia de depredación por *N. tenuis*, *M. pygmaeus* y *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) sobre las dos plagas clave de pimiento mencionadas anteriormente. Este estudio se llevó a cabo en dos invernaderos con diferentes temperaturas, a 20 ºC y 27 ºC, que simularon la temperatura media registrada en los dos principales ciclos de cultivo en España (el período de siembra de invierno y de verano). Ambos, *N. tenuis* y *M. pygmaeus*, pudieron establecerse en pimiento y redujeron significativamente el número de adultos, larvas y ninfas de *F. occidentalis* y *B. tabaci*. *Macrolophus pygameus*

tiene la densidad más alta a 20 ºC, mientras que *N. tenuis* es más abundante a 27 ºC. Por el contrario, *D. maroccanus* no alcanzó el nivel de control requerido y por tanto no sería seleccionado como agente de CB de *F. occidentalis* y *B. tabaci* en este cultivo. Ninguno de los tres míridos se observó que causa ningún daño en la planta de pimiento. Las implicaciones de estos resultados para el uso de los miridos en los cultivos de pimiento se han discutido en este capítulo.

En el **cuarto objetivo** de esta tesis doctoral, se estudió la integración práctica de uno de estos dos míridos depredadores con *A. swirskii* para mejorar el manejo de plagas en pimiento. En primer lugar, se investigó la coexistencia de ambas especies de miridos cuando se liberaron junto con *A. swirskii* en presencia y ausencia de huevos de *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) como presa alternativa. Esto se comparó con la versión estándar de uso que consta en la liberación de *O. laevigatus* con *A. swirskii*. En segundo lugar, la eficacia de cada liberación combinada (ya sea *N. tenuis*, *M. pygmaeus* u *O. laevigatus* combinada con *A. swirskii*) en la reducción de *F. occidentalis*, *B. tabaci* y el pulgón *Myzus persicae* Sulzer (Hemiptera: Aphididae) fue evaluado en condiciones de invernadero. Ambos, *N. tenuis* y *A. swirskii* estuvieron involucrados en una depredación intragremial bidireccional (DIG). Contrariamente, esta interacción DIG fue aparentemente unidireccional en el caso de *M. pygmaeus* con *A. swirskii* y *O. laevigatus* con *A. swirskii*. Ambos, *M. pygmaeus* y *O. laevigatus* redujeron significativamente la abundancia de *A. swirskii*. Sin embargo, en el invernadero, la DIG pareció estar neutralizada. El uso de miridos con *A. swirskii* suprimió significativamente el trips, la mosca blanca y la infestación de pulgones, sugiriendo complementariedad en los efectos de estas dos especies de

depredadores. Contrariamente, el uso combinado de *O. laevigatus* con *A. swirskii* no alcanzó un control satisfactorio para los pulgones, a pesar de que redujo efectivamente el nivel de trips y moscas blancas. Por lo tanto, nuestros resultados sugieren que el uso de mirids, en lugar de *O. laevigatus*, combinado con *A. swirskii*, podría resultar en programas de CB más eficientes y robustos en el cultivo de pimiento.

En resumen y junto a la capacidad por parte de los míridos zoofitófagos de inducir defensas en plantas, esperamos que el futuro CB de pimiento en invernadero comercial pueda contar con la liberación de *A. swirskii* con míridos predadores. Lo que está claro es que el uso de mirids zoofitófagos en el pimiento es posible y puede ser más efectivo que el sistema actual basado en la liberación de *O. laevigatus*, lo que me motiva a sugerir que los míridos merecen más atención en el CB de plagas en pimiento.

Resum

Durant l'última dècada, els programes de gestió integrada de plagues (GIP) basats en l'augment i la conservació de depredadors zoofitòfags s'han desenvolupat amb èxit en hivernacles hortícoles. En pebrot, l'alliberament de *Amblyseius swirskii* Athias-Henriot (Acari: fitoseid) i *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) ha proporcionat un control efectiu de dues plagues clau, el trips *Frankliniella occidentalis* Pergande (Thysanoptera: Tripidae) i la mosca blanca *Bemisia tabaci* Gennadius (Hemiptera: aleiròdid). Tot i ser un depredador zoofitòfag, el comportament fitòfag d'*O. laevigatus* no ha estat explorat prèviament en profunditat, ni ha tingut un impacte de la fitofagia sobre la fisiologia de les plantes. En el **primer objectiu** d'aquesta tesi doctoral, es va comparar la importància jeràrquica de l'alimentació d'*O. laevigatus* amb altres comportaments sobre pebrot. *Orius laevigatus* va passar la major part del seu temps (38%) alimentant-se del meristem apical i de les fulles fresques apicals, les quals també van ser la seva ubicació preferida. S'ha demostrat que el comportament fitòfag d'*O. laevigatus* en pebrot desencadena respostes defensives a la planta, les quals contribueixen a la repel.lència o l'atracció de plagues o enemics naturals, respectivament. Específicament, les plantes de pebrot picades amb *O. laevigatus* indueixen una repelència a la mosca blanca *B. tabaci* i al trips *F. occidentalis*. Per contra, el parasitoid de mosca blanca *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) es veu significativament atret per plantes picades amb *O. laevigatus*. Les respostes de la planta al comportament fitòfag d'*O. laevigatus* inclou l'alliberament d'una barreja

alterada de compostos volàtils i l'activació de les vies de senyalització de l'àcid jasmonic i l'àcid salicílic. Aquests resultats posen de manifest una faceta interessant de la biologia d'*O. laevigatus*, en la qual la capacitat del depredador per induir respostes defensives en les plantes de pebrot pot servir per millorar el control biològic (CB) del trips i la mosca blanca. De fet, els resultats podrien explicar el gran èxit assolit pels programes de GIP basats en l'alliberament, l'establiment i la conservació d'*O. laevigatus* en el cultiu de pebrot.

No obstant això, el control de plagues en pebrot encara té problemes a causa dels pugons. Actualment, per mantenir la població de pugons per sota del llindar econòmic, es necessita múltiples alliberaments d'enemics naturals especialitzats. Aquesta estratègia resulta en una xarxa tròfica complicada a causa de la gran quantitat d'espècies alliberades, a més de cara per als productors. En els últims anys, la recerca d'enemics naturals alternatius per pugons i que puguin ser integrats amb les pràctiques actuals de CB en pebrot ha estat una de les prioritats en la investigació sobre aquest cultiu. Seguint aquesta línia, el paper dels mírids depredadors (Hemiptera: Miridae) en el maneig de pugons en pebrot ha estat destacat en diversos estudis. A més dels seus serveis com a depredadors, els mírids zoofitòfags poden induir defences a les plantes a causa de la seva fitofagia. No obstant això, fins ara no se sap si aquesta inducció es produeix en el cultiu del pebrot i si podria ser un benefici addicional al seu paper com a agent de CB en aquest cultiu. Aquestes preguntes es van abordar en el **segon objectiu** d'aquesta tesi doctoral i estudiar sobre dues espècies de mírids model i comercials actualment, *Nesidiocoris tenuis* Reuter i *Macrolophus pygmaeus* Rambur. El comportament de fitofagia sobre la planta de pebrot es va observar tant

en *N. tenuis* com a *M. pygmaeus* i van ocupar el 33% i el 14% del temps total que van estar sobre aquesta, respectivament. Les picades alimentàries dels mírids van induir l'alliberament d'una barreja de compostos orgànics volàtils (COV) que van repel·lir els herbívors *F. occidentalis* i *B. tabaci* i van atreure al parasitoid de mosca blanca *E. formosa*. L'efecte repel·lent sobre *B. tabaci* es va observar durant almenys 7 dies després de l'exposició inicial de la planta a *N. tenuis* i l'atracció d'*E. formosa* romandre funcional durant 14 dies. Les defenses induïdes en planta a causa de les picades alimentàries per mírids depredadors i la persistència d'aquests efectes obren la porta a noves estratègies de control de plagues en pebrot. L'aplicació addicional d'aquesta investigació s'analitza en aquest capítol, com la vacunació de plantes per mírids zoofitófagos al viver abans del trasplantament.

Un següent pas per integrar el possible ús d'aquestes dues espècies de mírids en les pràctiques de CB de pebrot va ser determinar la seva possible control sobre la mosca blanca *B. tabaci* i el trips *F. occidentalis*. En el **<u>tercer objectiu</u>** d'aquesta tesi doctoral, es va dur a terme un estudi comparatiu per estudiar l'eficàcia de depredació de *N. tenuis*, *M. pygmaeus* i *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) sobre les dues plagues clau de pebrot esmentades anteriorment. Aquest estudi es va dur a terme en dues hivernacles amb diferents temperatures, a 20 ℃ i 27 ℃, que van simular la temperatura mitjana registrada en els dos principals cicles de cultiu a Espanya (el període de sembra d'hivern i d'estiu). Tots dos, *N. tenuis* i *M. pygmaeus*, van poder establir-se en pebrot i van reduir significativament el nombre d'adults, larves i nimfes de *F. occidentalis* i *B. tabaci*. *Macrolophus pygameus* té la densitat més alta a 20 ℃, mentre que *N. tenuis* és més abundant a 27 ℃. Per contra, *D. maroccanus* no va aconseguir el nivell de control requerit i així

no seria seleccionat com a agent de CB de *F. occidentalis* i *B. tabaci* en aquest cultiu. Cap dels tres mírids es va observar que causa cap dany a la planta de pebrot. Les implicacions d'aquests resultats per a l'ús dels mírids en els cultius de pebrot s'han discutit en aquest capítol.

En el **quart objectiu** d'aquesta tesi doctoral, es va estudiar la integració pràctica d'un d'aquests dos mírids depredadors amb *A. swirskii* per millorar el maneig de plagues en pebrot. En primer lloc, es va investigar la coexistència de les dues espècies de mírids quan es van alliberar juntament amb *A. swirskii* en presència i absència d'ous de *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) com a presa alternativa. Això es va comparar amb la versió estàndard d'ús que consta en l'alliberament d'*O laevigatus* amb *A. swirskii*. En segon lloc, l'eficàcia de cada alliberament combinada (ja sigui *N. tenuis*, *M. pygmaeus* o *O. laevigatus* combinada amb *A. swirskii*) en la reducció de *F. occidentalis*, *B. tabaci* i el pugó *Myzus persicae* Sulzer (Hemiptera: Aphididae) va ser avaluat en condicions d'hivernacle. Tots dos, *N. tenuis* i *A. swirskii* van estar involucrats en una depredació intragremial bidireccional (DIG). Contràriament, aquesta interacció DIG va ser aparentment unidireccional en el cas de *M. pygmaeus* amb *A. swirskii* i *O. laevigatus* amb *A. swirskii*. Tots dos, *M. pygmaeus* i *O. laevigatus* van reduir significativament l'abundància d'*A. swirskii*. No obstant això, a l'hivernacle, la DIG va semblar estar neutralitzada. L'ús de mírids amb *A. swirskii* va suprimir significativament el trips, la mosca blanca i la infestació de pugons, suggerint complementarietat en els efectes d'aquestes dues espècies de depredadors. Contràriament, l'ús combinat d'*O. laevigatus* amb *A. swirskii* no va aconseguir un control satisfactori per als pugons, tot i que va reduir efectivament el nivell de trips i mosques blanques. Per tant, els nostres

resultats suggereixen que l'ús de mirids, en lloc d'*O. laevigatus*, combinat amb *A. swirskii*, podria resultar en programes de CB més eficients i robustos en el cultiu de pebrot.

En resum i junt la capacitat per part dels mírids zoofitòfags d'induir defenses en plantes, esperem que el futur del CB de pebrot en hivernacle comercial pugui comptar amb l'alliberament d'*A. swirskii* amb mírids depredadors. El que està clar és que l'ús de mírids zoofitòfags en el pebrot és possible i pot ser més efectiu que el sistema actual basat en l'alliberament d'*O. laevigatus*, el que em motiva a suggerir que els mírids mereixen més atenció en el CB de plagues en pebrot.

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1 CHAPTER

General Introduction

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General Introduction

1.1. Sweet pepper crop

Sweet pepper, also known as bell pepper, pepper, or capsicum is a cultivar group of the species *Capsicum annuum* L., which is a perennial herbaceous plant from the Solanaceae family (nightshade family). Of the family, the genus *Capsicum* is the second most important vegetable crop in the world, following tomato (Rubatzky and Yamaguchi, 1997a, 1997b). Peppers are native to Mexico, Central America, and northern South America, and have been historically associated with the voyage of Columbus (Heiser, 1976). Pepper seeds were imported to Spain in 1493, since then peppers have spread to other European, African, and Asian countries.

1.1.1. Economic importance

Sweet pepper together with tomato is the crop that occupies most of the area among protected species around the world. Pepper cultivation is almost entirely carried out in open fields, however the extension of protected, greenhouse cultivate peppers has intensively increased. China is leading the world production with 16,000,000 tons (FAOSTAT, 2016). Spain occupies the fifth position with 1,130,340 tons, of which 733,744 tons were exported in 2016, thus, it is ranked as the second exporter in the world after Holland (Hortoinfo, 2017). Last year, the volume of the Spanish

exported pepper represented 22.3% of the total world exportation, with a complete value estimated at 990.5 million Euros, at an average price of 1.35 Euros per kilo (MARM, 2017).

In Spain, over 18,513 hectares (ha) are devoted to the production of peppers, wherein Andalusia is the major production region (Hortoinfo, 2017). Approximately two-thirds of the area dedicated to this crop is inside greenhouses while the remaining one-third is cultivated in open fields (Simón *et al.*, 2016). The provinces with the largest area in 2015-2016 were Almeria (9,611 ha), Murcia (1,450 ha), Malaga (580 ha), Granada (300 ha) and Alicante (250 ha) (Hortoinfo, 2017). The province of Almeria is the biggest producer of sweet pepper for export, focusing its production mainly during the autumn and winter months, from September to March. In contrast, the commercialization of sweet pepper in Murcia begins in March and ends in summer. The Murcia production shares the time period and market destination with the Dutch pepper of traditionally high quality (Fernández-Zamudio *et al.*, 2006).

1.1.2. Pest status in protected sweet pepper

Sweet pepper is susceptible to several pests which can reduce fruit quality and yield (Table 1.1). Insects and mites associated with pepper can cause both direct and indirect damage (Brodsgaard and Albajes, 1999; Weintraub, 2007). Indirect damage occurs mainly when pests are vectors transmitting viruses, whereas direct damage occurs when pests damage roots, stems, flowers and fruit. As an alternative to exclusively treating pest with insecticides, integrated pest management (IPM) based on biological control (BC) tactics have been successfully adopted in various confined and

open production areas around the world. The principle sweet pepper pests and their corresponding natural enemies are summarized in table 1.1.

Since Western flower thrips (WFT) *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Fig.1.1.A) appeared in Europe it has become one of the most serious pest species of sweet pepper, both in greenhouse and in open field (Tavella *et al.*, 1991; Tommasini and Maini, 1995). Thrips generally prefer to live in closed areas such as the flowers, under the calyx of the fruit and in newly opening leaves, which makes them difficult to reach with insecticides (Fig.1.1.D). With their piercing-sucking mouthparts; epidermal and parenchymal cells are punctured by both adults and nymphs, and the contents of the cells sucked out, resulting in silvery chlorotic spots. Direct damage is due to feeding and oviposition on plant leaves, flowers and fruit (Shipp *et al.*, 1998), while indirect damages is caused by virus transmission, of which *tomato spotted wilt virus* (TSWV) is the most economically important (Ullman *et al.*, 1997). Currently, *F. occidentalis* has become resistant to major insecticide groups (Bielza, 2008), therefore IPM based on BC is the alternative strategy for the control of WFT (van Lenteren and Loomans, 1998; van Driesche *et al.*, 1998; Riudavets *et al.*, 1993) (Table 1.1).

Considerable damage to the sweet pepper crop is also attributed to two species of whiteflies, the sweet potato whitefly, *Bemisia tabaci* Gennadius (Fig.1.1.B) and the greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) (van Lenteren and Woets, 1988). Whitefly adults and nymphs feed on the phloem (Fig.1.1.E,F), causing direct damage. While indirect damage is caused from the adult's virus transmission of, mainly, the *Tomato yellow leaf curl virus* (TYLCV) by *B. tabaci* and *Tomato infectious chlorosis virus* (TICV) by *T. vaporariorum*, and/or by sooty mould

which develops on their excreted honeydew, thus, reducing the rate of leaf photosynthesis (Jones, 2003). The developed resistance to several chemical classes of insecticides, including the neonicotinoids which were commonly used for *B. tabaci* control (Elbert and Nauen, 2000), increase the need for BC of this pest. Several natural enemies are currently used for the management of whiteflies in sweet pepper (Gerling *et al*., 2001; Naranjo, 2001; Arnó *et al*, 2010b) (Table 1.1).

Aphids are also among the most problematic pests in protected crops. Several polyphagous species, such as *Myzus persicae* Sulzer (Fig.1.1.C), *Macrosiphum euphorbiae* Thomas, *Aphis gossypii* Glover and *Aulacorthum solani* Kaltenbach (Hemiptera: Aphididae), are reported to induce considerable damage in sweet pepper (Fig.1.1.F,G) (Blom, 2008; Sánchez *et al*., 2011). Aphids are difficult to control due to their high reproductive capability and short developmental period which can quickly lead to resistance to many different insecticide classes. Their BC can be successfully achieved by the augmentative release of natural enemies (Table 1.1) and the introduction of banker plants. This is true for *A. gossypii* and *M. persicae* which can be well controlled in greenhouse sweet pepper; however, the other two species *M. euphorbiae* and *A. solani* are not satisfactory managed (Sánchez *et al*., 2011; Pérez-Hedo and Urbaneja, 2015).
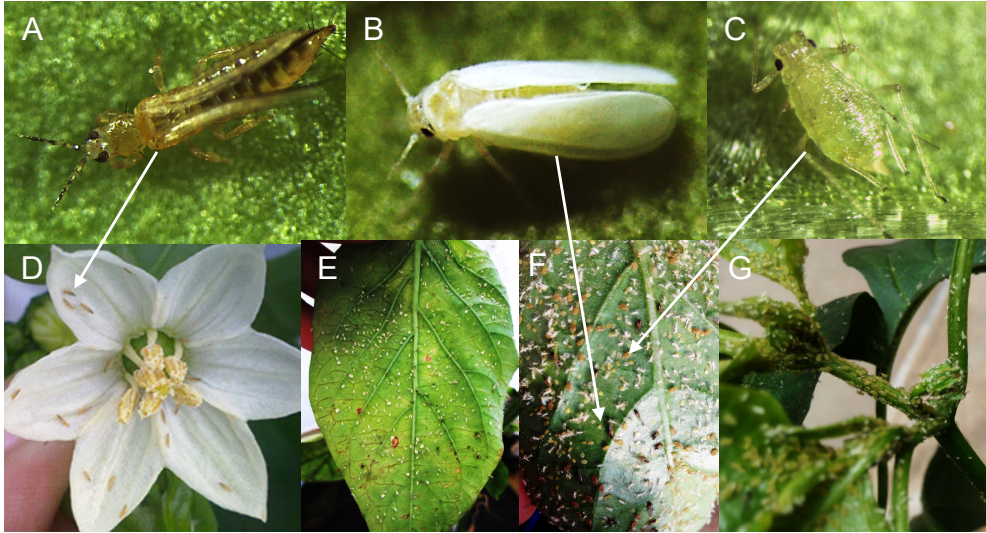
Figure 1.1.

Sweet pepper key pests, *Frankliniella occidentalis* (A, D), *Bemisia tabaci* (B, E, F), *Myzus persicae* (C, F, G).

The two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a common pest in greenhouse pepper (van de Vrie *et al.*, 1972). Damages are related to the pest feeding on the underside of leaves, piercing the chloroplast-containing cells and causing significant injury to the crop. The broad mite, *Polyphagotarsonemus latus* Banks (Acari: Tarsonemidae), is a destructive pest whose attacks cause serious malformations after injecting toxins in the leaf tissue while feeding. It is very small and difficult to detect, thus, the pest is most often only identified when it has already caused severe economic losses (Coss-Romero and Pena, 1998). Phytoseiidae predatory mites are the main component in the commercial BC programs aimed to control *T. urticae* and *P. latus* in sweet pepper (Griffiths, 1999) (Table 1.1).

Several species of leafminers have become serious pests in pepper growing areas (Table 1.1). Direct damage occurs as a consequence of larval feeding inside the leaf and adult feeding punctures on the upper side of the leaf (Dik *et al.*, 1999). A high amount of leaf mines cause a significant decrease in photosynthetic assimilate production that may lead to desiccation and premature leaf fall. Indirect damage is the result of viral disease transmission and pathogen penetration into the leaves via female feeding punctures. Several natural enemies can develop on the larvae and pupae of the *Liriomyza* spp. miners (Diptera: Agromyzidae) (Table 1.1), (Onillon, 1999). Many Lepidopteran species of the family Noctuidae cause considerable damage to pepper crops (Dik *et al.*, 1999). For their control locating them in early stages is very pertinent as they live grouped in very specific areas, hiding during the day and feeding during the night (Table 1.1). The green stink bug, *Nezara viridula* Linnaeus (Hemiptera: Pentatomidae), has recently become one of the main pest in greenhouse pepper cultivation. This bug was easily controlled by insecticide treatments; however, with the increase of BC farming systems, *N. viridula* has become a very destructive pest. This is especially true when high populations are distributed throughout the greenhouse. Egg parasitoids can be used for the BC of this bug, although their use is not common (Table 1.1) (Catalán and Verdú, 2005).

Table 1.1.

Main pests and their associated natural enemies in sweet pepper greenhouses (Source: van Lenteren and Loomans, 1998; Onillon, 1999; Griffiths, 1999; Gerling et al., 2001; Arnó et al., 2010b; Sánchez et al., 2011; van de Velden et al., 2012; van Lenteren, 2012; van Lenteren et al., 2018; Blom, 2017).

Pest	Natural enemies		
	Predators	Parasitoids	Entomopathogenic fungus
Thrips (Thysanoptera: Thripidae) -*Frankliniella occidentalis* Pergande -*Thrips tabaci* Lindeman	-*Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) -*Dicyphus tamaninii* Wagner (Hemiptera: Miridae) -*Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) -*Neoseiulus barkeri* Hughes (Acari: Phytoseiidae) -*Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae) -*Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) -*Orius laevigatus* Fieber (Hemiptera: Anthocoridae) -*Orius albidipennis* Reuter (Hemiptera: Anthocoridae) -*Stratiolaelaps scimitus* Womersley (Acari: Laelapidae)	-*Ceranisus menes* Walker (Hymenoptera: Eulophidae) -*Ceranisus americensis* Girault (Hymenoptera: Eulophidae)	-*Beauveria bassiana* -*Verticillium lecanii*
Whiteflies (Hemiptera: Aleyrodidae) -*Bemisia tabaci* Gennadius -*Trialeurodes vaporariorum* Westwood	-*Amblyseius swirskii* -*Macrolophus pygmaeus* -*Nesidiocoris tenuis* -*Orius laevigatus*	-*Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) -*Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae) -*Eretmocerus eremicus* Rose and Zolnerowich (Hymenoptera: Aphelinidae)	-*Beauveria bassiana* -*Verticillium lecanii* -*Lecanicillium muscarium* -*Isaria fumosorosea*

Pest	Predators and parasitoids	Entomopathogen
Aphids (Hemiptera: Aphididae) -Myzus persicae Sulzer -Aphis gossypii Glover -Macrosiphum euphorbiae Thomas -Aulacorthum solani Kaltenbach	-Adalia bipunctata Linnaeus (Coleoptera: Coccinellidae) -Aphidoletes aphidimyza Rondani (Diptera:Cecidomyiidae) -Chrysoperla carnea Stephens (Neuroptera: Chrysopidae) - Chrysoperla lucasina Lacroix (Neuroptera: Chrysopidae) -Coccinella septempunctata Linnaeus (Coleoptera: Coccinellidae) -Episyrphus balteatus DeGeer (Diptera: Syrphidae) -Macrolophus pygmaeus -Nesidiocoris tenuis -Aphidius colemani Haliday (Hymenoptera: Braconidae) -Aphidius ervi Haliday (Hymenoptera: Braconidae) -Aphelinus abdominalis Dalman (Hymenoptera: Aphelinidae) -Ephedrus plagiator Nees (Hymenoptera: Braconidae) -Lysiphlebus testaceipes Cresson (Hymenoptera: Braconidae)	-Beauveria bassiana
Spider mite (Acari: Tetranychidae) -Tetranychus urticae Koch -Tetranychus turkestani Ugarov and Nikolskii -Tetranychus evansi Baker and Pritchard	-Phytoseiulus persimilis Athias-Henriot (Acari: Phytoseidae) -Amblyseius californicus McGregor (Acari: Phytoseiidae) -Neoseiulus cucumeris -Amblyseius swirskii	-Beauveria bassiana
Broad Mite (Acari: Tarsonemidae) -Polyphagotarsonemus latus Banks	- Neoseiulus cucumeris -Amblyseius swirskii	-Beauveria bassiana
Leaf miners (Diptera: Agromyzidae) -Liriomyza trifolii Burgess -Liriomyza bryoniae Kaltenbach -Liriomyza strigata Meigen -Liriomyza huidobrensis Blanchard	-Coenosia attenuata Stein (Diptera: Muscidae) -Cyrtopeltis modestus Distant (Hemiptera: Miridae) -Nesidiocoris tenuis -Macrolophus pygmaeus - Dacnusa sibirica Telenga (Hymenoptera: Braconidae) - Opius pallipes Wesmael (Hymenoptera: Braconidae) -Diglyphus isaea Walker (Hymenoptera: Eulophidae)	-Steinernema carpocapsae

Pest	Natural enemies	Biopesticides/pathogens
South American tomato pinworm (Lepidoptera: Gelechiidae) -*Tuta absoluta* Meyrick	-*Nesidiocoris tenuis* -*Macrolophus pygmaeus*	-*Trichogramma achaeae* Nagaraja and Nagarkatti (Hymenoptera: Trichogrammatidae) -*Necremnus artynes* Walker (Hymenoptera: Eulophidae)	-*Bacillus thuringiensis*
Caterpillars (Lepidoptera: Noctuidae) -*Helicoverpa armigera* Hubner -*Spodoptera exigua* Hubner -*Spodoptera littoralis* Boisduval	-*Orius laevigatus* -*Chrysoperla carnea* -*Nesidiocoris tenuis* -*Macrolophus pygmaeus* -*Nabis pseudoferus* Remane (Hemiptera: Nabidae)	-*Trichogramma achaeae* -*Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae) -*Telenomus* spp (Hymenoptera: Scelionidae) -*Cotesia* spp (Hymenoptera: Braconidae) - *Hyposoter didymator* Thunber (Hymenoptera: Ichneumonidae)	-*Bacillus thuringiensis* -*Beauveria bassiana* - Nuclear polyhedrosis virus (NPV)
Loopers (Lepidoptera: Noctuidae) -*Trichoplusia ni* Hübner -*Autographa californica* Speyer		- *Hyposoter dydimator* - *Trichogramma evanescens*	- *Bacillus thuringiensis* - *Steinemena carpocapsae*
Bugs (Hemiptera: Pentatomidae) -*Nezara viridula* Linnaeus		-*Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae) - *Trichopoda pennipes* Fabricius (Diptera: Tachinidae) -*Oencyrtus* spp (Hymenoptera: Encyrtidae)	
Bugs (Hemiptera: Miridae) -*Creontiades pallidus* Rambur			

1.1.3. Current status of biological control in sweet pepper

In Europe throughout the last ten years pest control in protected crops has shown noticeable improvement through the replacement of chemical control with BC (Heimpel and Miles, 2017; van Lenteren *et al*, 2018; Barratt *et al*., 2018). The green revolution, the international market demand and the restrictive legislation minimising the number of authorised chemical products have resulted in a quick shift from chemical insecticide dependency to the implementation of IPM programs based on BC for various horticultural crops. In less than 10 years the total greenhouse area grew from 1,400 hectares biologically controlled in 2006 to 26,372 ha in 2014 (Sanchez *et al*., 2014).

One of the most impressive success stories of BC occurred in the sweet pepper crop in Southern Spain (Blom, 2017). Since the end of the last century, attempts to implement BC of sweet pepper started in Campo de Cartagena region. In contrast, in the Almeria region, the leader of sweet pepper production in Spain, BC implementation was delayed mainly due to pest pressure during the summer planting period. However, nowadays, this delay has been overcome and sweet pepper areas under BC program in Almeria have increased from a mere 200 ha in the 2005-2006 season to about 7,500 ha in 2008-2009. Currently the area has surpassed the 10,000 ha in 2015-2016 (Fig.1.2) (Calvo *et al*., 2015; Blom, 2017).

In greenhouse sweet pepper, BC programs are now based on the release of generalist predators, the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) together with the minute pirate bug *Orius laevigatus* Fieber (Hemiptera: Anthocoridae). Both predators are achieving great success in managing the key sweet pepper pests; *B. tabaci* and *F. occidentalis* (Calvo *et al*., 2015).
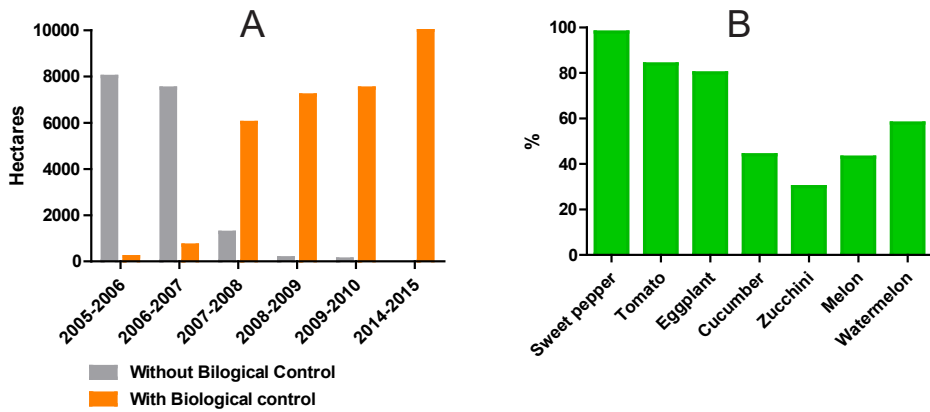
Figure 1.2.

Sweet pepper area (ha) under BC (A); Percentage of farms with BC (B) (Almería region), (Blom, 2017; Calvo *et al.*, 2015).

1.1.3.1. *Amblyseius swirskii*

The predatory mite *A. swirskii* (Fig 1.3) originated in the Mediterranean-Middle east area. Since 2005, it has been reared and commercially available as a BC agent for whiteflies and thrips in different crops (Nomikou *et al.*, 2001, 2002; Belda and Calvo, 2006; Messelink *et al.*, 2008; Calvo *et al.*, 2011, 2015). The ability of *A. swirskii* to establish even before the appearance of the pest, owing to its capability of feeding on alternative food sources, enhances its efficiency as a BC agent. Among these alternative food sources of pollen, nectar, small insects and mites, such as *T. urticae* and *P. latus*, other non-prey food, including eggs of the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) or decapsulated dry cysts of the brine shrimp *Artemia franciscana* Kellogg (Anostraca: Artemiidae) have been reported (Nomikou *et al.*, 2002; van Houten *et al.*, 2005a, 2005b; Messelink *et al.*, 2006, Messelink *et al.*, 2010, van Maanen *et al.*, 2010; Nguyen *et al.*, 2013; Calvo *et al.*, 2015).

Figure 1.3.

Adut *Amblyseius swirskii*.

In Spain during the 2012-2013 season total greenhouse areas under BC using *A. swirskii* exceeded the 18,000 ha (Calvo *et al*., 2015). As mentioned before, releases of this predaceous mite have been extensively used with remarkable success for the control of *B. tabaci* in sweet pepper (Calvo *et al*., 2008, 2009a, 2012a). A rate of 50-75 *A. swirskii*/m² combined with the releases of the parasitic wasp *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae) provided excellent suppression of *B. tabaci* in sweet pepper greenhouses (Calvo *et al*., 2009a).

Amblyseius swirskii mainly preys on eggs and crawlers (Nomikou *et al*., 2001, 2004), whereas *E. mundus* parasitizes nymphal instars of *B. tabaci* but especially the second and third (Urbaneja and Stansly, 2004). Thus, released together, both predators provided satisfactory results. *Amblyseius swirskii* is also an effective predator of thrips in sweet pepper (Belda and Calvo, 2006; Calvo *et al*., 2012a) and other crops. Previous laboratory results on cucumber leaves showed *A. swirskii* to be

more effective than *Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae) which had been previously used to control this pest (Messelink *et al*., 2006). Later, Messelink *et al*. (2008) observed that *A. swirskii* was also able to control the greenhouse whitefly *T. vaporariorum* in cucumber in the presence of *F. occidentalis*. More confirmation in cucumber crops, were observed by Calvo *et al*. (2011) who, at a release rate of 50-75 *A. swirskii*/m^2, adequately managed *F. occidentalis* and *B. tabaci* both alone and simultaneously. Accordingly to the above mentioned results, the adapted release of *A. swirskii* significantly reduced the infestation of *B. tabaci* and *F. occidentalis.* However, in the case of sweet peppers, thrips was not adequately controlled in flowers enough to mitigate the risk of (TSWV) infections (Lacasa and Contreras, 1993; Belda and Calvo, 2006). Therefore, combining the phytoseiid with the anthocorid bug, *O. laevigatus*, satisfies the requirements of the BC of sweet pepper pests (Weintraub *et al*., 2011; Calvo *et al*., 2012a).

1.1.3.2. *Orius laevigatus*

The generalist anthocorid predator *O. laevigatus* (Fig.1.4), is a western Palaearctic species, widespread along Mediterranean and Atlantic coasts. This anthocorid has various characteristics which make it a promising agent of BC. For example, *O. laevigatus* lacks expressed diapauses, presents high fecundity, a long life span and is polyphagous (Tommasini and Nicoli, 1996).
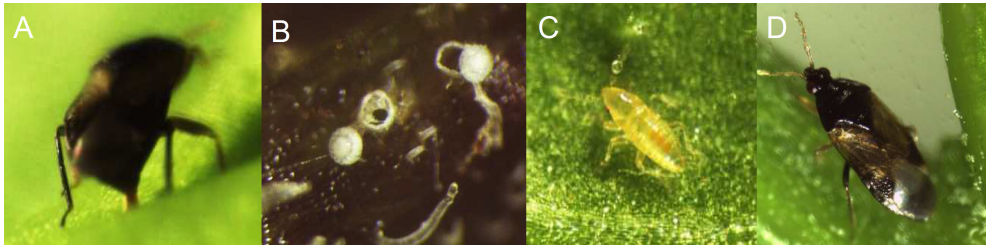
Figure 1.4.

Female *Orius laevigatus* laying eggs (A), Eggs of *O. laevigatus* inserted in sweet pepper stem (B), First instar nymph (N1) (C), Adult (D).

Orius laevigatus is considered to be key predator in the BC of thrips, preying on both larvae and adults. It can also be effective against *B. tabaci* (Chambers *et al.*, 1993; Dissevelt *et al.*, 1995; Frescata and Mexia, 1996; Hamdan and Abu-Awad, 2007, 2008). In addition, *O. laevigatus* can consume other arthropod pests such as aphids and mites (Alvarado *et al.*, 1997; Venzon *et al.*, 2002). Nowadays, this predator is produced by many commercial insectaries (since 1993) and largely used in IPM programs. The number of *Orius* spp. released to control thrips in greenhouse varies in published literature, ranging from 0.4 to 2 individuals per plant (Chambers *et al.*, 1993; Tavella *et al.*, 1996; van Schelt, 1999; Sánchez and Lacasa, 2002; Bosco *et al.*, 2008). The best time for the release of *O. laevigatus* on the pepper crop is about one month after transplanting, when the plants are flowering and fully grown (Bosco *et al.*, 2008).

Like many other anthocorids, *O. laevigatus* is primarily predaceous; however, this predator is also able to exploit plant resources including pollen and plant juices (Frescata and Mexia, 1996; Cocuzza *et al.*, 1997; Armer *et al.*, 1998; Lattin, 1999; Shakya *et al.*, 2009). Plants fulfil several roles throughout the life cycle of *Orius* bugs. They represent an egg-laying substrate but also serve as a source of moisture and supplementary nutrients (Coll, 1996; Lattin,

1999). Using radio labelling experiments, Armer *et al*. (1998) observed adults of *O. insidiosus* Say (Hemiptera: Anthocoridae) feeding on the mesophyll of soya bean plants by ingesting small amounts of sugars, starches and amino acids. It can also obtain water from xylem of the same plant. Besides the feeding behaviour of adults, Lundgren *et al*. (2008) reported that neonate *O. insidiosus* were able to feed on nutritious phloem, allowing them to survive on plant materials for several days. This phytophagous characteristic of anthocorid bugs results in important behaviour which helps predator establishment and population maintenance during the absence of protein-rich food. This alternative feeding, thus, maintains the predator on the crop. Therefore, plant feeding by *Orius* bugs is an interesting aspect which still has yet only been slightly explored. Plant feeding is generally associated with chemical and physiological interactions to fine-tune the predatory attack (section 1.3). Therefore, we hypothesized that plant feeding could result in physiological changes on sweet pepper plants (such as induction of plant defences) which might constitute an additive effect for pest management. Therefore, we focused **Chapter 2** of this thesis on studying the feeding behaviour of this predator and its ability to induce plant defence in sweet pepper.

1.1.4. Gaps in the biological control of aphids

Despite the success of the current BC program in sweet peppers, good management of aphids on the sweet pepper crop is still hard to achieve (Blom, 2008; Bloemhard and Ramakers, 2008). Aphid populations can develop so quickly that the introductions of natural enemies are often unsuccessful (Rabasse and van Steenis, 1999). Habitually, aphids

are controlled through multiple releases of natural enemies (Blom, 2008). The parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae), the specialist predatory midge *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) (Blümel, 2004) and other generalist predators, such as those of the chrysopidae, syrphidae, and coccinellids (Pineda and Marcos-García, 2008; Messelink *et al.*, 2011b, 2013) are regularly used for managing aphids in sweet pepper. *Myzus persicae* and *A. gossypii* can be sometimes controlled with the use of banker plants infested with the cereal aphid *Rhopalosiphum padi* Linnaeus (Hemiptera: Aphidiidae) (Huang *et al.*, 2011). Introductions of natural enemies are done on these banker plants so their populations increase considerably before aphid move to the crop (Calvo and Urbaneja, 2004b). The specialised predatory midge is mainly released for controlling high densities of aphids. The generalist predators (chrysopidae, syrphidae, or coccinellids) are also frequently released against the two aphid species. However, their failure to establish in the absence of aphids lowers the efficacy of their use (Messelink *et al.*, 2011b; Messelink *et al.*, 2013). Overall, these augmentative releases are not always sufficiently effective, not only because the release of parasitoids can be disrupted by the abundance of hyperparasitoids in south eastern Spain (Belliure *et al.*, 2008, Sánchez *et al.*, 2011), but also because the final cost of the biocontrol program in sweet pepper is considerably elevated (Messelink *et al.*, 2011a). Additionally, the occurrence of intraguild predation (IGP) between generalist thrips and whitefly predators with specialist aphid natural enemies has complicated the management of aphids even more in sweet pepper (Messelink *et al.*, 2012). According to Messelink *et al.* (2011b) the predatory mite *A. swirskii* used for managing thrips and whiteflies in sweet pepper can seriously disrupt the BC of aphids by preying on the eggs of the predatory midges.

Christensen *et al.*, (2002) also observed how *Orius* spp. bugs can prey on eggs and larvae of *A. aphidimyza* and, therefore, act as an intraguild predator (Alvarado *et al.*, 1997).

Thus, the BC control of aphids in sweet pepper requires improvement. Facing this need, several reports recently highlighted the positive role of zoophytophagous mirid predators in managing aphids in sweet pepper and other crops (Perdikis and Lykouressis, 2004, Messelink *et al.*, 2011a; Messelink *et al.*, 2015; Pérez-Hedo and Urbaneja, 2015; De Backer *et al.*, 2015). Hence, according to these previous encouraging results we focused **chapters 4** and **5** of this thesis, on studying the efficiency of predatory mirids in managing sweet pepper pests including thrips, whitefly and aphid.

1.2. Zoophytophagous predators

Increased attention has recently been directed at the role of generalist omnivores as regulators of insect herbivore populations in agricultural ecosystems. Zoophytophagous predators are a special case of omnivory defined as predators that feed on prey and plant during the same developmental stage (Wheeler, 2001). Their ability to switch between zoophagy and phytophagy enhanced their establishment and sustained population densities. In turn the population was capable of controlling incipient pest outbreaks early in the season following the initial colonization of the crop by pests or later in the season following a period of pest scarcity (Sánchez and Lacasa, 2008; Castañé *et al.*, 2011). In this group, predatory mirid bugs (Hemiptera: Miridae) such as *Nesidiocoris tenuis* Reuter (Fig.1.5) and *Macrolophus pygmaeus* Rambur (Fig.1.6) are important predators of several agriculture pests.
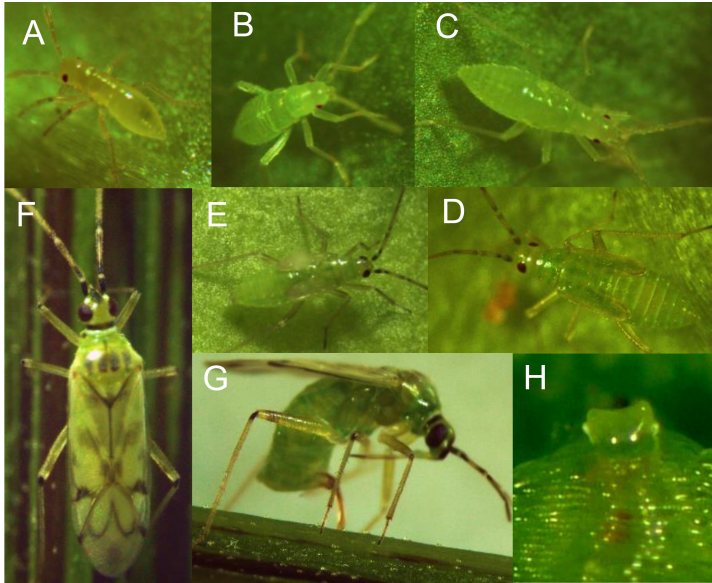
Figure 1.5.

Developmental stages of *Nesidiocoris tenuis*, 1st instar (N1) (A), 2nd instar (N2) (B), 3rd instar (N3) (C), 4th instar (N4) (D), 5th instar (N5) (E), Adult (F), Female laying eggs (G), Egg of *N. tenuis* inserted in sweet pepper stem (H).
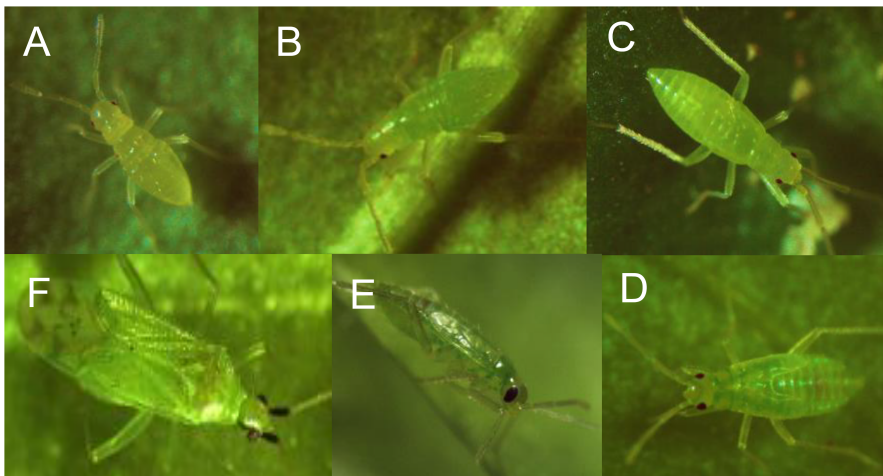
Figure 1.6.

Developmental stages of *Macrolophus pygmaeus*, 1st instar (N1) (A), 2nd instar (N2) (B), 3rd instar (N3) (C), 4th instar (N4) (D), 5th instar (N5) (E), Adult (F).

1.2.1. Mirids as biocontrol agents

Currently, *N. tenuis* and *M. pygmaeus* are widely used as BC agents to manage some major greenhouse pests including, whiteflies, thrips, leafhoppers, leaf miners, spider mites and Lepidoptera species in greenhouses (Perdikis and Lykouressis, 2002; Calvo *et al.*, 2009a; van Lentereen, 2012; Urbaenja *et al.*, 2012; Pérez-Hedo and Urbaneja, 2015). *Macrolophus pygmaeus* has been commercially available since 1994 and its use as a generalist predator has steadily increased in European tomato greenhouses with a medium commercial value (10.000-100.000 individuals sold per week). However, the use of *N. tenuis* started in 2003 with a large commercial value (hundreds of thousands to millions of individuals sold per week) (Malausa and Trottin-Caudal, 1996; van Lenteren, 2003, 2012; Cock *et al.*, 2010). Both predators feed by inserting the stylet and sucking out the body contents of the prey. Two distinctive strategies may be adopted for the use of these predators as BC agents depending on the crop and pest pressure:

- Conservation: Spontaneous appearance.
- Augmentation: Either an inoculative release after transplantation or a nursery release on seedlings.

Conservation BC is based on the fact that both *N. tenuis* and *M. pygmaeus* are two endemic natural enemies that appear spontaneously in several crops of the Mediterranean Basin (Alómar *et al.*, 2002; Sánchez *et al.*, 2003, Calvo and Urbaneja, 2003, Stansly *et al.*, 2004, Urbaneja *et al.*, 2005). Their ability to colonize alternative plants

as mentioned below (Section 1.2.2) (Table 1.2) allows the conservation of both predators and then their establishment during the crop season. However, the appearance of mirid predators in the greenhouses can be too late to guarantee pest control (Perdikis *et al.*, 2011). Therefore, conservation BC should be directed to identify which non-crop host plants support high numbers of these predators early in the season (Gabarra *et al.*, 2004). For example, in tomato unheated greenhouses, Arnó *et al.* (2000) showed tobacco plants to serve as a refuge for *M. pygmaeus* during winter, enhancing the timely and efficient colonization of the following spring tomato crop. Overall, the successful implementation of conservative BC, requires future research efforts to focus on the investigation of various aspects, such as, the correct identification of the mirid species and the non-crop plant, the evaluation of the dispersal ability on the non-crop plant and guarantee the early colonization of the target crop (Perdikis *et al.*, 2011).

As mentioned above, both *N. tenuis* and *M. pygmaeus* are mass-reared and commercially available to release in augmentative BC programs against different pest (Gerling *et al.*, 2001, Calvo and Urbaneja, 2004a, Alómar *et al.*, 2002; Calvo *et al.*, 2009a, Urbaneja *et al.*, 2012). Augmentative BC is based on the inoculation of the crop with the predator before or after pest outbreaks. In greenhouse tomato, inoculation with *N. tenuis* at a rate of 1-2 individual(s)/m², was a common practice usually performed 4-5 weeks after transplantation which effectively controlled whiteflies. This strategy allowed a reduction of 81%-96% in *B. tabaci* abundance (Calvo *et al.*, 2009b). The same management practise achieved great success against *T. absoluta* on tomato crops. The release of 1-2 *M. pygmaeus* or *N. tenuis* adult(s) per m² reduced leaflet and fruit

infestation of *T. absoluta* by up to 97% and 100% with *N. tenuis* and 76% and 56% with *M. pygmaeus*, respectively (Mollá *et al.*, 2009). On infested sweet pepper plants, 1 reproductive couple of *N. tenuis*, *M. pygmaeus* and/or *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) per plant, successfully reduced *M. persicae* infestation nearly 100%, for both predators (Pérez-Hedo and Urbaneja, 2015). However, the success of this latter strategy requires a certain predator density in the crop (Calvo *et al.*, 2009a). The problem is that reaching a sufficient mirid population level takes 5-8 weeks, and in many occasions pests could appear earlier in the crop which could result in serious damage. When established, the infestation may not be able to be solved by predators (Mollá *et al.*, 2011; Urbaneja *et al.*, 2012). Another weak point in this strategy in late crop cycles without heating, is the mirid reproduction rate is not high enough to reach the desired population levels (Urbaneja *et al.*, 2012). Therefore, the nursery release of predatory mirids, before transplanting, could be an alternative strategy, that shortens the establishment period and improves predator distribution in the crop. This technique was used initially with *M. pygmaeus* (Lenfant *et al.*, 2000; Trottin-Caudal *et al.*, 2012). Adult predators were released on the seedlings, and initially fed eggs of the fastidious prey *E. kuehniella*. The eggs laid by *M. pygmaeus* hatch very soon after transplantation, allowing population growth to start earlier (Trottin-Caudal *et al.*, 2012). The preplant release rate of *N. tenuis* at 0.5-1 *N. tenuis*/plant, satisfactorily manage *B. tabaci* and *T. absoluta* populations, without evident damages caused by the phytophagy of *N. tenuis*. In this strategy, *E. kuehniella* eggs are used as substitute prey to enable predator establishment and to increase its abundance prior to pest outbreaks, which might accelerate the response

of the predator and considerably reduce pest incidence (Calvo *et al.*, 2012a, 2012b). The fact that *N. tenuis* females need to mate periodically in order to maintain a sufficient sperm supply to fertilize their eggs (Franco *et al.*, 2011) and predator populations are more concentrated when released before planting are well known. This might result in a higher encounter rate, giving more opportunities for adults to mate and to fertilize maturing eggs, which would increase the number of progeny, and therefore predator abundance in the subsequent generation. Also, fecundity, longevity and preimaginal survival of *N. tenuis* were observed to increase when the predator was fed *E. kuehniella* eggs (Urbaneja *et al.*, 2005). Thus, addition of *E. kuehniella* eggs during the first weeks after release, a normal procedure in use, should also have the positive effect of enhancing establishment of *N. tenuis* and *M. pygmaeus*. The prior release of *M. pygmaeus* with a weekly application of supplemented food (*E. kuehniella* eggs + decapsulated cysts of the brine shrimp *A. franciscana*), was reported to increase the population of the predator to very high densities which favoured the control of *M. persicae* (Messelink *et al.*, 2011a; Messelink *et al.*, 2015). Five weeks after predator release the aphid density remained below 1 aphid per plant, while it exceeded the 1000 aphids per plant in untreated greenhouse plots. These results again suggest that a high predator/prey ratio is needed to achieve sufficient control. Aphids multiply extremely fast by viviparous parthenogenetic reproduction, resulting in relative growth rates on sweet pepper of 0.36 females per female per day (Dewhirst *et al.*, 2012). Aphids will quickly outnumber mirid predatory bugs which have a relatively low reproduction rate (Perdikis and Lykouressis, 2004). Thus, the numerical response of the predators is far too slow to control aphids.

Preventive releases of predators can solve this problem, thus, prior to pest establishment releases can result in relatively high initial predator/pest ratios which are enough to control aphids. Therefore, inoculative release of predatory mirids prior to planting is the best strategy for their successful use as BC agents. This strategy can, however, have some negative sides effects mainly related to tomato crops, but perhaps, not for sweet pepper. In any case, the monitoring of the predator population density is mandatory to avoid any possible damages induced by the plant feeding behaviour of mirid predators.

1.2.2. Phytophagous behaviour of mirid predators

Thanks to their adaptation to living on plant surfaces covered with non-glandular and/or glandular trichomes (Voigt *et al.*, 2007; Voigt and Gorb, 2010), *N. tenuis* and *M. pygmaeus* have been reported on several plants species including wild and cultivated host plants (Table 1.2). *Macrolophus pygmaeus* offers the advantage of obtaining carbohydrates and other nutrients by feeding directly on plant tissues making it independent of nectar and prey availability (Hansen *et al.*, 1999; Perdikis and Lykouressis, 2000, 2002; Portillo *et al.*, 2012). It was reported to survive 3.67 days without any food and 25.67 days by feeding exclusively on eggplant young leaves (Mohd Rasdi *et al.*, 2009). Immature development by *M. pygmaeus* was observed to be completed only when tomato, eggplant, cucumber, pepper, green bean, broad bean or melon were available (Perdikis and Lykouressis, 1999, 2000; Lykouressis *et al.*, 2001). In the absence of prey, the order of suitability for *M. pygmaeus* longevity, fecundity, and population increase is eggplant

then tomato and finally sweet pepper. The lower suitability of pepper sap compared to that of eggplant or tomato may positively affect the predation rate of *M. pygmaeus* on pepper because omnivorous predatory bugs may have a stronger tendency to search for and feed on prey on low quality host plants rather than on high-quality plants (Abrams, 1987). Nymphs with access to a water source (moistened cotton), can successfully reach adulthood when fed exclusively bee pollen and on stamens cut from fully bloomed flowers of the weed *Ecballium elaterium* L. (Cucurbitaceae), without any other plant or prey material (Perdikis and Lykouressis, 2000). The same authors also showed when bee pollen pellets were added to a diet of eggplant leaves, the development period of *M. pygmaeus* was significantly shortened and completed within a period similar to that when it was fed on eggplant leaves with the aphid prey, *M. persicae*. In addition to cultivated plants, *M. pygmaeus* was reported to complete development on *Solanum nigrum* L. (Solanaceae) without prey and can also reproduce when infested by its main native aphid pest (Lykouressis *et al.*, 2008). Whereas, plant feeding seems important for *M. pygmaeus*, its performance as predator depends on the plant food. However, its best development was always observed when it fed on prey (Lykouressis *et al.*, 2001, 2008; Perdikis and Lykouressis, 2004; Ingegno *et al.*, 2011). On the contrary, Lykouressis *et al*. (2014) and Maselou *et al*. (2014) supported the idea that exclude a negative effect between plant feeding and predator functional response. In fact, the predation rate of *M. pygmaeus* did not differ on leaves of four host plants (tomato, pepper, eggplant and black nightshade) of varying suitability for development and/or reproduction.

The range of plant species on which *N. tenuis* has been recorded is large (Table 1.2). This predator can feed on all the aerial parts of tomato plants but has a strong preference for the three uppermost leaves and the apical bud, which is where up to 80% of the predator population is found (El-Dessouki *et al*., 1976; Castañé *et al*., 2011). Contrarily to *M. pygmaeus*, a diet exclusively derived from vegetable substrate (tomato, sweet pepper and eggplant) is insufficient for the development of *N. tenuis* (Urbaneja *et al*., 2005). Although, tomato seems to favour its development compared to eggplant and pepper, a supplemental food (*E. kuehniella* eggs) is mandatory for *N. tenuis* to complete its development, when prey are absent. According to Urbaneja *et al*. (2005), sweet pepper was the least suitable host plant, allowing only 10% of the nymphs to survive through the first instar, survivorship was the lowest, and nymphal development time was the longest. In another series of experiments studying the functional response of *N. tenuis* on various host plants, tomato was also the most suitable plant for predation activity on *T. vaporariorum* (Hassanpour *et al*., 2016), when compared to cucumber and eggplant. *Solanum indicum* L. (Solanaceae) is also reported to be a suitable host plant for *N. tenuis* development and oviposition (Biondi *et al*., 2016). Sesame is very rich in protein, carbohydrates, and minerals so it probably provides the protein fraction needed for *N. tenuis* to continue its development (Mbaebie *et al*., 2010).

Table 1.2.

Host plant species on which *Nesidiocoris tenuis* and *Macrolophus pygmaeus* were found.

Family	Species	Common name	N. tenuis	M. pygmaeus	Sources
Amaranthaceae	*Amaranthus blitum* L.	Purple amaranth		x	Roditakis *et al.*, 2003
Asteraceae	*Artemisia vulgaris* L.	Mugwort		x	Ingegno *et al.*, 2009
	Calendula officinalis L.	Pot marigold	x	x	Tavella and Goula, 2001; El-Dessouki *et al.*, 1976
	Dimorphotheca aurantiaca DC.	Glandular Cape marigold	x	x	Roditakis *et al.*, 2003; El-Dessouki *et al.*, 1976
	Dittrichia viscosa L.	Woody fleabane	x	x	Cano *et al.*, 2009 ; Gabarra *et al.*, 2004
	Gerbera spp.	Gerbera	x	x	Nucifora and Calabretta, 1986; Castañé *et al.*, 2011
Cistaceae	*Cistus creticus* L.	Pink Rock-Rose		x	Gabarra *et al.*, 2004
Cucurbitaceae	*Cucurbita pepo* L.	Zucchini	x	x	Castañé *et al.*, 2003; El-Dessouki *et al.*, 1976
	Cucumis melo L.	Melon	x	x	Alómar *et al.*, 2006a; El-Dessouki *et al.*, 1976
	Cucumis sativus L.	Cucumber	x	x	Perdikis and Lykouressis, 2000; El-Dessouki *et al.*, 1976
	Ecbalium elaterium L.	Squirting cucumber		x	Perdikis and Lykouressis, 2000
	Lagenaria siceraria S.	Calabash	x		Ranganath *et al.*, 2015
Fbaceae	*Ononis natrix* L.	Yellow Restharrow	x	x	Alómar *et al.*, 2006b; Cano *et al.*, 2009
	Phaseolus vulgaris L.	Green beans	x	x	Tavella and Arzone, 1996
	Vicia faba L.	Broad beans	x	x	Perdikis and Lykouressis, 1997
Geraniaceae	*Pelargonium* spp.	Geraniums	x	x	Cano *et al.*, 2009
Lamiaceae	*Ballota hirsuta* B.	Ballote hirsute		x	Martinez-Cascales *et al.*, 2006
	Rosmarinus officinalis L.	Rosemary	x	x	Cano *et al.*, 2009
	Stachys sylvatica L.	Hedge woundwort		x	Wagner, 1964
Pedaliaceae	*Sesamum indicum* L.	Sesame	x	x	Nakaishi *et al.*, 2011; Biondi *et al.*, 2016
Solanaceae	*Capsicum annuum* L.	Pepper	x	x	Perdikis and Lykouressis, 2004
	Datura stramonium L.	Devil's snare		x	Roditakis *et al.*, 2003
	Nicotiana tabacum L.	Tobacco	x	x	Torreno and Magallona, 1994; Lykouressis *et al.*, 2000
	Solanum lycopersicum L.	Tomato	x	x	Goula and Alómar, 1994; Alómar *et al.*, 2002
	Solanum melongena L.	Eggplant	x	x	Perdikis and Lykouressis, 2000; Urbaneja *et al.*, 2005
	Solanum nigrum L.	Black nightshade	x	x	Tavella and Goula, 2001; Ingegno *et al.*, 2009
	Solanum tuberosum L.	Potato	x	x	Gabarra *et al.*, 2004; CABI, 2017
Urticaceae	*Parietaria officinalis* L.	Pellitory-of-the-wal		x	Gabarra *et al.*, 2004

1.2.3. Plant damage by mirid predators

Phytophagous behaviour of some mirid predators have been reported to induce plant damage which affects growing plant tissues, stems, leaves and fruit (El-Dessouki *et al.*, 1976; Castañé *et al.*, 2011). To date, no evidence has been found identifying the exact vascular tissue on which *N. tenuis* and *M. pygmaeus* insert their stylet; however, according to Wheeler (2001), mirid predators are classified as phloem feeders of stem and petioles. During the seventies, the microscopic examination of tomato parts damaged by *N. tenuis* through disections of stem and petiole, illustrated obvious destruction of tissues (El-Dessouki *et al.*, 1976). Cell walls were found to be broken in most of the cells, thus, indicating that *N. tenuis* sucks out the intracellular juice with its stylet. The predator destroys the epidermis cell, then it rolls its stylet inside the cortex tissue and extends it to break up the endodermal layer and some of the xylem, phloem and pith cells (El-Dessouki *et al.*, 1976).

Therefore, plant feeding by *N. tenuis* was reported to cause plant damage, thus, this predator was habitually considered to be a pest in different parts of the World. In tomatoes, repetitive insertion of the stylet on vascular tissue induces necrotic rings on stems, leaves or flower petioles that may reduce plant vigour or cause flower abortion (El-Dessouki *et al.*, 1976). Recently, Sánchez *et al.* (2016), observed feeding activity of *N. tenuis* to produce a significant variation in the concentration and distribution of assimilates (i.e. amino acids and sugars) in tomato stems. This chemical change in tomato was observed to inhibit the transfer of nutrients in the phloem and negatively affect the fitness of nymphs that develop on those parts of the plant (Sánchez *et al.*, 2016). The intensity of injuries on tomato crops is proportional to the availability of the prey (Arnó *et al.*, 2010a; Calvo

et al., 2009b). Describing the damages produced by *N. tenuis* on greenhouse tomato, Sánchez (2008) found the highest damage incidence (5 necrotic rings on the upper 20 cm of the plant shoot) was produced when about 3 *N. tenuis* individuals per leaf occurred in a period when the number of the whitefly population was low. Thus, this predator is following the feeding model 1 proposed by Gillespie and McGregor (2000). According to this model, a decrease in the availability of prey increases the phytophagy, which would only occur when the prey is scarce and the predator is forced to change his feeding habits. Nevertheless, according to Calvo *et al*. (2012b), *N. tenuis* can also follow the feeding model 2, which means phytophagy increases when zoophagy increases (Gillespie and McGregor, 2000). These authors observed necrotic rings on tomato plants when the predator had abundant *E. kuehniella* eggs and whitefly nymphs, they hypothesized that *N. tenuis* tended to increase its feeding behaviour in the presence of the prey due to the necessity of obtaining water for their extra-oral digestion (Calvo *et al*., 2012b). Hence, as concluded by Castañé *et al*. (2011), these models are appropriate for explaining the behaviour of individual predators and are not mutually exclusive: individuals could behave according to one model or another depending on crop circumstances and plant feeding is essential rather than facultative. Overall, phytophagous behaviour of *N. tenuis* seems to affect the final number of fruit per plant and may lead to some economic loss (Sánchez, 2009; Arnó *et al*., 2010a). However, according to Sánchez and Lacasa (2008), such loses might be compensated by the greater weight of the remaining fruit. Indeed, these authors did not observe yield when population levels of *N. tenuis* ranged from 0.53 to 35.2 individuals per plant.

In contrast to *N. tenuis*, *M. pygmaeus* has been generally considered as safe and plant damages have been observed only with very high predator

densities on tomato, zucchini, and gerbera under experimental conditions, but no such damage has been observed under cropping conditions (Castañé *et al.*, 2011). With high predator densities and low prey availability, *M. pygmaeus* was observed to cause dimples, pits and distortion on fruit under laboratory conditions (Castañé *et al.*, 2011). In a field survey conducted in the UK Sampson and Jacobson (1999) reported distorted tomato leaf growth, necrotic spots on leaves, scars on fruit and fruit drop with high predator densities (50-300 individuals per plant) and very low prey abundance. Recently, Moerkens *et al.* (2016, 2017), reported that *M. pygmaeus* can cause economic feeding damage on tomato fruit at high population densities and severity of crop damage is significantly enhanced when plants are infected with pepino mosaic virus PepMV. This virus causes, from a few dimples to yellowish discoloration and deformed fruits. The same authors suggest the minimum economic density threshold to be estimated at 0.32 *M. pygmaeus* per leaf.

In general, crop damage is often the result of complex interactions between the morphological, physiological and behavioural traits of the natural enemy, plant, crop type, and certain environmental factors. Castañé *et al*. (2011) examined scanning electron microscopy images of the mandibular and maxillary stylets of *D. tamaninii* Wagner (Hemiptera: Miridae), *N. tenuis* and *M. pygmaeus* with from which, they concluded great similarly in their shape. The mandibular stylets of all three species had between 10 and 12 teeth and their maxillary stylets had at least 4 strongly curved teeth. Therefore, the differences observed in the types and intensities of the damage produced by *N. tenuis* and *M. pygmaeus* does not seem be directly related with the morphologies of their respective stylet bundles. Indeed, damages are not primarily produced mechanically but are the result of a chemical process

by which the affected cells are killed by salivary enzymes (El-Dessouki *et al*., 1976; Halitschke *et al*., 2011).

Whereas, the relation between mirids and plants seems to be profound (as described above); studies on plant defensive response to the phytophagous behaviour of these groups of predators have been rare. Recently, the phytophagous behaviour of the mirid predators *(N. tenuis, M. pygmaeus* and *D. maroccanus*) and also the oviposition behaviour of the anthocorid bug *O. laevigatus* was investigated and provides clear evidence of inducing plant responses as strictly herbivores do in tomato plants (De Puysseleyr *et al*., 2011; Pérez-Hedo *et al*., 2015a, 2015b, Pappas *et al*., 2015, 2016; Naselli *et al*., 2016). These finding might provide an explanation for the great success recently achieved by the use of mirids as key biocontrol agents for tomato crop (Pérez-Hedo *et al*., 2017a).

1.3. Plant defence mechanisms

In nature plants are in continuous interaction with the ecosystem. Plants avoid unfavourable conditions thanks to their defence mechanisms (Maekawa *et al*., 2011). The occurrence of abiotic stress (i.e. drought, flooding, salinity, extreme temperatures or intense light), enhance plants to develop several mechanisms to protect themselves from disturbance involving the activation of different biochemical pathways leading to the production of either defensive compounds or morphological changes (Bartels and Souer, 2004; Krishna, 2004). Mechanical damages also induce stress responses and the activation of plant defence mechanisms. However, plant tissue damage can be caused by either noticeable or invisible lesions. Some lesions, with small diameters are not detected visually; nonetheless, they might contribute

to the activation of wound-responses to prevent further damage (Pullin and Gilbert, 1989). Besides the described stressful conditions, plants are also faced with a plethora of attackers including herbivorous arthropods and plant pathogens, where insects are among the most important invaders. To survive, plants have developed a stunning array of structural, chemical, and protein-based defences designed to detect invading organisms and stop them before they are able to cause extensive damage (Karban and Baldwin, 1997; Walling, 2000; Heil and Ton, 2008; Ponzio *et al.*, 2013; Kessler, 2017). Plant defence strategies can be generalized with two categories: constitutive defences, which include physical and chemical barriers that exist even before insect attack, and inducible defences, which are activated after detection of insect attack, therefore minimizing the metabolic cost of defence for the plant (Dicke and van Poecke, 2002; Agrawal and Heil, 2012). Two types of inducible defences have been observed in plants: direct defences and indirect defences (Fig. 1.7).
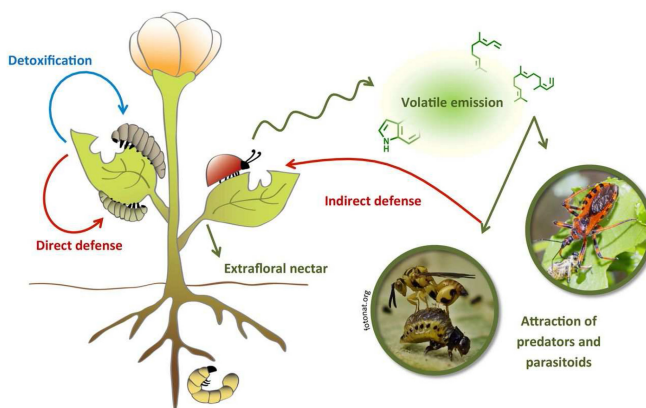
Figure 1.7.

Model of plant-insect herbivore interactions and associated chemical defence mechanisms. Feeding insects above and below ground face direct chemicals defence responses. Indirect defences are represented by extrafloral nectar that can attract ants or natural enemies to defend the plant by the emission of volatile organic compounds (Mithöter and Boland, 2016).

Direct defences rely on the plant structure by the construction of a physical barrier either through the formation of a waxy cuticle (Agrawal *et al.*, 2009; Hanley *et al.*, 2007), and/or the development of spines, setae, thorns, and trichomes, (Sharma *et al.*, 2009; He *et al.*, 2011). Structural defences includes morphological and anatomical traits that confer a fitness advantage to the plant by directly deterring the herbivores from feeding (Agrawal *et al.*, 2009) and range from prominent protuberances on a plant to microscopic changes in cell wall thickness as a result of lignifications and suberizations (He *et al.*, 2011; Hanley *et al.*, 2007). The second direct line of defence is the chemical one which includes secondary plant metabolites, protein inhibitors of insect digestive enzymes, proteases, lectins, amino acid deaminases and oxidases. These chemical lines are used by the plant to offset attackers via the production of toxins, along with anti-digestive and anti-nutritive compounds (War *et al.*, 2012, Fürstenberg-Hügg *et al.*, 2013). In addition to direct defence, plants also benefit from indirect defence through the recruitment of natural enemies (i.e. parasitoids or predators) that actively reduce the number of herbivores. Indirect defences generally start with the recognition of insect elicitors, then plants emit and/or receive chemical information from other plants that plays an important role in behavioural choices of individuals, and consequently influences the spatial distribution of populations on larger scales (Vet, 2001; Lof *et al.*, 2008; Vinatier *et al.*, 2011). The combined action of mechanical damages and elicitors from attacking herbivores induces the production of secondary metabolites (Iason *et al.*, 2012) which result in the emission of specific volatile organic compounds (VOCs) called herbivore induced plant volatiles (HIPVs) (Price *et al.*, 1980; Kessler and Baldwin, 2001; Dicke

and Baldwin, 2010; Hare, 2011). Recently plants have been documented to respond to herbivore oviposition by releasing oviposition-induced plant volatiles (OIPVs) which can recruit egg parasitoids of insect herbivores (Hilker and Meiners, 2010). In some studied cases, a combination of oviposition and feeding activity of the herbivore host was required to trigger natural enemy attraction (Colazza *et al.,* 2010; Conti and Colazza, 2012). These plant volatiles are classified as synomones because they benefit both the emitting plant as well as the responding natural enemy (Vet *et al.,* 1991).

In short, after being attacked by herbivores, plants quickly generate herbivore-specific signals, and through complicated networks, these signals are further converted to large scale biochemical and physiological changes. In addition to these changes in attacked plant parts, certain signals are also conveyed to different parts of the plant where they activate "systemic" defences (Li *et al*., 2002). Cell membrane depolarization, ion flux, mitogen-activated protein kinase (MAPK) activation, phytohormone modulation, production of reactive oxygen species (ROS) and probably nitric oxide (NO) are all associated with herbivore attack and the activation of these responses (Reymond *et al*., 2004; De Vos *et al*., 2005; Bodenhausen and Reymond, 2007; Wu *et al*., 2007).

The network of phytohormones that plants have is crucial not only for regulating plant growth, development and reproduction, but also for the above mentioned induced defences (production of VOCs). It has been well established that jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) play pivotal roles in the regulation of the signal-transduction pathways that lead to the activation of different sets of

defence related genes (von Dahl and Baldwin, 2007; Howe and Jander, 2008). In general, defences against biotrophic pathogens and phloem-feeding herbivores are regulated by the phytohormone SA, while defences against necrotrophic pathogens and chewing herbivores are regulated by JA, in particular jasmonic acid-isoleucine (JA-Ile) (Glazebrook, 2005; Erb *et al*., 2012). Complex hormonal interaction networks enable the tailoring of defences, often via cross-talk, and modulate growth/defence tradeoffs (Pieterse *et al*., 2012; Huot *et al*., 2014). Although, abscisic acid (ABA), auxins, gibberellins (GB), cytokinins (CK) and brassinosteroids (BR) have received less attention as potential factors that modulate herbivore resistance, in the recent literature various reports also indicate these hormones to play an important role in mediating specificity in herbivore-induced defence responses (Erb *et al*., 2012).

1.3.1. Herbivore induced plant volatiles (HIPVs)

Herbivore attack results in the induction of plant defences affecting changes in plant quality. However, the level of this induction is not always the same; many factors interact to produce the final response of the damaged plant. This response was shown to differ with herbivore specie (Voelckel and Baldwin, 2004); and also between herbivore strain (Kant *et al*., 2008). In addition, this response varies subsequently with multiple herbivore species or a singular attack (Voelckel and Baldwin, 2004; Poelman *et al*., 2008). The order of arrival of different species on the same plant may also determine the defence that is induced, and affect the outcome of interactions among herbivore species (Kessler and Baldwin, 2004; Erb *et al*., 2011). It has been suggested that species from the same

feeding guild or sub-guild (Soler *et al.*, 2012) affect each other negatively through the stimulation of the same defensive pathways; whereas, species from different feeding guilds may affect each other positively or not at all due to the negative cross-talk between the different defensive pathways which they induce (Howe and Jander, 2008). Finally, this induced defence can be suppressed by the action of a second attacker (Alba *et al.*, 2011, 2015). Although this is to the benefit of the individuals that suppress plant defences, it may also have positive effects on competitor species (Kant *et al.*, 2015; Godinho *et al.*, 2016).

As described above, when a plant is attacked by an herbivore it emits of a specific blend of volatile compounds known as HIPVs. In the 1980s, the first studies reported the ecological effects of HIPVs when released from damaged plants and target external organisms in a resistance-related context (Fig.1.8) (Heil, 2014). HIPVs are complex blends that can be composed of up to several hundred individual compounds (Pichersky and Gershenzon, 2002; Dudareva *et al.*, 2006; Pichersky *et al.*, 2006). The following definition of HIPVs was suggested by Dicke and Baldwin (2010): "volatiles that a plant produces in response to herbivory. These can either be compounds that a plant does not biosynthesize unless it is damaged or compounds that are also synthesized by undamaged plants but normally in smaller amounts. Because the information content of volatile blends is in the mixture of the compounds, it is also important to consider compounds that are produced by plants but repressed when plants are attacked".
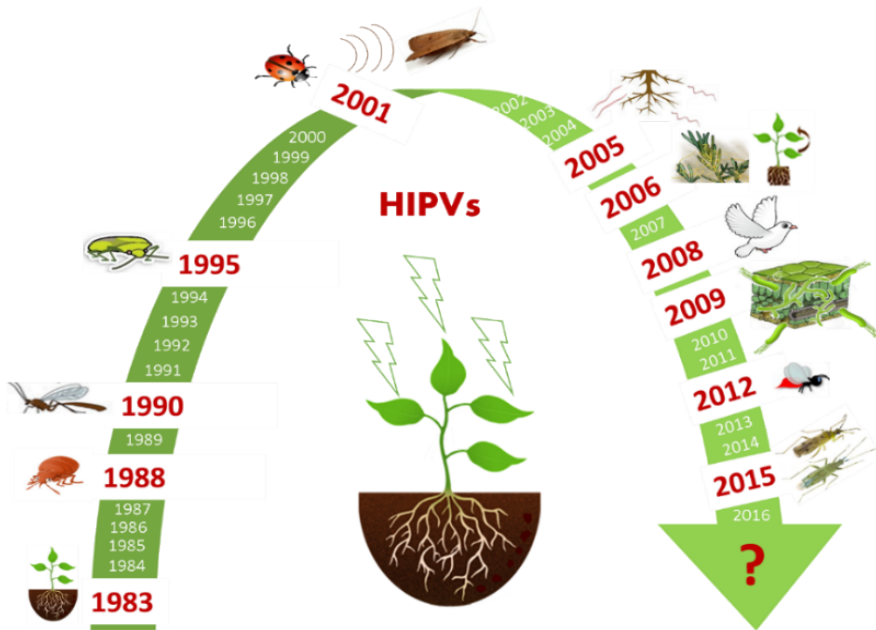
Figure 1.8.

Illustration of milestone articles that depicts the chronology in the discovery of the herbivore induced volatile organic compound (HIPVs) releases that act on the receivers: interplant communication (Baldwin and Schultz, 1983; Rhoades, 1985), predatory mites (Dicke and Sabelis, 1988), parasitoid wasps (Turlings *et al.*, 1990), predatory bugs (Drukker *et al.*, 1995), predatory lady beetles (Ninkovic *et al.*, 2001), herbivorous moths, which are repelled (de Moraes *et al.*, 2001), parasitic plants (Runyon *et al.*, 2006), nematodes (Rasmann *et al.*, 2005), systemic parts of the same plant (Karban *et al.*, 2006), predatory birds (Mäntylä *et al.*, 2008), resistance to pathogens (Yi *et al.*, 2009) and hyperparasitoid (Poelman *et al.*, 2012). Predatory bugs (*N. tenuis* and *M. pygmaeus*) induced VOCs via plant feeding behaviour (Pérez-Hedo *et al.*, 2015a; Pappas *et al.*, 2015), adapted from (Heil, 2014).

With more than 1700 currently known compounds (Dudareva *et al.*, 2006), volatiles play an important role in the mechanisms of plant interactions within the environment. Numerous functions are allocated to HIPVs: firstly, they are reported in the concept of "plant-plant communication" via the transfer of information between and within plants. This communication is done through the airborne transport of signals that lead to the priming

or expression of defences in neighbouring plants or in distal parts of the emitting plant (Baldwin and Schultz, 1983; Rhoades, 1985; Heil and Karban, 2010; Karban *et al.*, 2014). In addition, HIPVs are generally known to mediate tritrophic interactions by attracting herbivore natural enemies, which may confer protection to the plant, referred to as "a cry for help" (Turlings *et al.*, 1990; Dicke *et al.*, 1990; Drukker *et al.*, 1995; Ninkovic *et al.*, 2001). It was also reported that even hyperparasitoids use HIPVs to locate their parasitoid hosts (Poelman *et al.*, 2012). These volatiles can also be used by herbivores to locate suitable hosts or to avoid resistance-expressing plants (repellence effect) (de Moraes *et al.*, 2001; Ballhorn *et al.*, 2013), to inhibit pathogen colonization (Goodrich-Tanrikulu *et al.*, 1995; Zhang *et al.*, 2006), and as reported more recently, to attract herbivores (Bedoya-Perez *et al.*, 2014). In summary, HIPVs aid in the host-location behaviour of diverse animals and enhance the presence of natural enemies that can benefit the emitting plant.

Herbivore induced plant volatiles are released from the disrupted cellular compartments, where they were stored. Such VOCs are released from the damaged sites immediately. They are released through leaf stomata and, in case of the lipophilic volatiles such as terpenes, also through the membrane of epidermal tissues and from other structures, such as trichomes (Baldwin, 2010). Such VOCs can also be emitted from the undamaged sites of plants with some time delay from the moment of the attack (Holopainen and Blande, 2012). VOCs can also be released as newly, "de novo", synthesized compounds. This usually occurs as part of the systemic defence response. The blend of volatiles may vary quantitatively and qualitatively depending on both biotic and abiotic factors, and are specific to each plant-herbivore association and to the nature of the damage (feeding and/or oviposition) (Ingegno *et al.*, 2011; Silva *et al.*, 2017). Plants react distinctly when they are

attacked by a leaf chewer or by a phloem feeder or when attacked by more than one organism (Dicke *et al.*, 2009; Gosset *et al.*, 2009; Zhang *et al.*, 2009; Zhang *et al.*, 2013). Such specificities and diversity are very important for mediating specific interactions of plants with herbivores, carnivores and other plants (Sabelis *et al.*, 2007; McCormick *et al.*, 2012). Similar to above-ground interactions, below-ground ones can be specific at both the plant and herbivore levels (Dudareva *et al.*, 2006; Rasman and Turling, 2007).

Volatile blends are generally dominated by two major classes of compounds: terpenoids and fatty acid derived green leaf volatiles (GLV) (Arimura *et al.*, 2009; Mumm and Dicke, 2010). Terpenoids represent the largest class of secondary metabolites in plants (Dudareva *et al.*, 2006) and are synthesized via two independent alternative pathways from the five-carbon compound, isopentenyl diphosphate (IDP) and its allylic isomer dimethyl allyl diphosphate (DMAPP). While, green leaf volatiles (GLVs) which are six-carbon compounds including the alcohols, aldehydes and their esters, are formed via the hydroperoxidelyase pathway of oxylipin metabolism (Matsui, 2006). Green leaf volatiles are mainly released immediately upon wounding, while others, including terpenoids, are newly synthesized "de novo" and released from several hours up to several days after attack (Paré and Tumlinson, 1997; Turlings *et al.*, 1998).

1.3.2. Zoophytophagous predator induced plant defence

Response of arthropod predators to volatile chemicals emanating from plants infested by their prey has been demonstrated for predatory bugs since 1995 (Drukker *et al.*, 1995; Kessler and Baldwin, 2001). Such evidence has also been obtained for *N. tenuis*, *M. pygmaeus* and *O. laevigatus*, who responded to

the odour emitted from prey infested plants (Moayeri *et al.*, 2006; Ingegno *et al.*, 2011; Lins *et al.*, 2014; De Backer *et al.*, 2015; Rim *et al.*, 2015, 2017; Vaello *et al.*, 2017). Recently, the activity (feeding behaviours and oviposition) of zoophytophagous predators was observed to induce HIPVs (De Puysseleyr *et al.*, 2011; Pérez-Hedo *et al.*, 2015a, 2015b; Pappas *et al.*, 2015, 2016; Naselli *et al.*, 2016). As explained above, these HIPVs can influence the response of phytophagous pests and their natural enemies either by attraction or by repellence (Paré and Tumlinson, 1997). According to Raman *et al.* (1984), plant feeding of the predatory mirid *N. tenuis* on tomato causes biochemical changes in wounded tissue, leading to increased levels of oxidative enzymes and phenolic compounds which may result in the production of volatile compounds. Halitschke *et al.* (2011), observed an induction by feeding of mirid *Tupiocoris notatus* Distant (Hemiptera: Miridae), that renders the native tobacco plant *Nicotiana attenuata* S. (Solanaceae) more resistant to more damaging herbivores such as hornworms. This special case of induced cross-resistance, termed 'plant vaccination', leads to a net benefit for mirid-damaged tobacco plants in their native habitats when multiple herbivore species are present (Kessler and Baldwin, 2004). More recently, Pérez-Hedo *et al.* (2015a) demonstrated that a 24h contact between tomato plant and *N. tenuis* affects the response of the whitefly, *B. tabaci*. The continuous feeding activity of this predator activates the abscisic acid (ABA) and jasmonic acid (JA) signalling pathways in tomato plants; which turn makes it less attractive to *B. tabaci*, while more attractive to the whitefly parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae). Interestingly, the same authors found *N. tenuis* activated tomato plants are able to prime neighbouring plants. Certainly, a blend of volatile organic compounds is released in the atmosphere assuring the communication between predatory activated plants and non-activated

ones, thus, sending out an alert. More confirmation of inducing plant defence in tomato thanks to the phytophagy behaviour of mirids was observed by Pérez-Hedo *et al*. (2015b). *N. tenuis*, *M. pygmaeus* and *D. maroccanus* tested females differ in their activation potential in tomato after having had contact for 24h. *Nesidiocoris tenuis* induced, as mentioned above, repellence of *B. tabaci* and *T. absoluta*, while *E. formosa* was attracted to the volatiles emitted by plants previously contacted by *N. tenuis*. In contrast, tomato plants exposed to *M. pygmaeus* and *D. maroccanus* for 24h were not able to repel *B. tabaci* and, interestingly, became more attractive to *T. absoluta*. On the other hand, *E. formosa* was always attracted to volatiles emitted from predator associated plants. Both studies provide a possible explanation for the great success of these mirids as BC agents in tomato. Their ability to activate the plant defence mechanisms will undoubtedly improve BC. More evidence was obtained in a recent study conducted by Naselli *et al*. (2016), showing all motile stages of *N. tenuis* to trigger defensive responses in tomato plants. In fact, young nymphs (2nd-3rd nymphal instars), mature nymphs (4th-5th nymphal instars), males and females all activate ABA and JA pathways, resulting in reduced attractiveness for *B. tabaci* and enhanced attractiveness for *E. formosa*, relative to undamaged plants with a slight moderation between the considered stages. In addition, Pappas *et al*. (2015, 2016) in agreement with Pérez-Hedo *et al*. (2015b), demonstrated *M. pygmaeus*′ ability to induce plant defence in tomato crops. These authors showed the exposure of tomato plants to adult *M. pygmaeus* females, fifth instar nymphs and unmated females (virgin), increased locally and systematically an accumulation of transcripts and activity of proteinase inhibitors. These inhibitors are known to be involved in plant responses, resulting in reduced performance of the subsequently infesting spider mite herbivore, *T. urticae*, but not of the greenhouse whitefly

T. vaporariorum. More interestingly, Pappas *et al*. (2015), showed *M. pygmaeus* tomato induction vaccinates the plant for a period of two weeks. Therefore, when predators were released in the nursery prior to pest infestation, the defence mechanism was activated with the newly emerged nymphs possibly renewing the induction (Naselli *et al*., 2016).

Besides the feeding behaviour of the mirid predator, the endophytic oviposition of *O. laevigatus* was shown to increase plant resistance to feeding damage of *F. occidentalis* in tomato. The predator elicits a JA mediated wound response, resulting in reduced thrips feeding. A strong accumulation of hydrogen peroxide (H_2O_2), a molecule involved in different parts of the wound response, in leaf tissue surrounding the predator eggs or oviposition puncture sites was observed (De Puysseleyr *et al*., 2011).

Overall, zoophytophagous organisms form community structure by both their predation on herbivores and their phytophagy, resulting in a tomato crop system which is ready to withstand herbivore attack. Despite the importance of this facet of research, literature emphasizing this focus is rare and has begun to be published only recently. Therefore, **Chapters 2 and 3** of this thesis focus on the implication of the phytophagous behaviour of *O. laevigatus*, *N. tenuis* and *M. pygmaeus* in inducing defence responses in sweet pepper. The additional effect of defence mechanism stimulation by this predator together with the predacious potential are expected to enhance BC in this crop **(Chapters 4 & 5)**.

1.4. Thesis outline

The main aim of my PhD thesis is to enhance sweet pepper pest management through the exploitation of zoophytophagy.

To achieve this goal, four objectives were planned:

• To study sweet pepper plant feeding behaviour by the predatory anthocorid, *O. laevigatus* and determine whether phytophagous behaviour can induce plant defences by modulating pest and natural enemy behaviour: attraction and repellence.

• To quantify sweet pepper plant feeding behaviour by the predatory mirids, *N. tenuis* and *M. pygmaeus*, and determine whether this phytophagous habit can elicit direct defences: antixenosis and attraction by parasitoids.

• To determine the pest management potential of three predatory mirids, *N. tenuis*, *M. pygmaeus* and *D. maroccanus*, against thrips and whiteflies in sweet pepper under two different temperatures.

• To study the impact of species co-occurrence on population dynamics of predatory bugs, *O. laevigatus*, *N. tenuis* and *M. pygmaeus*, with *A. swirskii* and elucidate the potential of combined release for the management of sweet pepper pests.

2

Orius laevigatus Strengthens its Role as a Biological Control Agent by Inducing Plant Defences

Orius laevigatus Strengthens its Role as a Biological Control Agent by Inducing Plant Defences

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Abstract

Orius laevigatus is a generalist predator that is widely used in augmentative strategies against the key pest of sweet pepper, *Frankliniella occidentalis*. Despite being a zoophytophagous predator, the phytophagy behaviour of *O. laevigatus* has not been previously explored in depth, nor has the impact of phytophagy on plant physiology. Here, the hierarchical significance of *O. laevigatus* feeding on sweet pepper is compared with other behaviours. *Orius laevigatus* spends the majority of its time (38%) feeding on apical meristems and apical fresh leaves, which were also preferred residence locations. Here, the phytophagous feeding behaviour of *O. laevigatus* on sweet pepper is shown to trigger defensive responses in the plant. These *O. laevigatus* plant induced defences are then shown to contribute to the repellence or attraction of pests or natural enemies, respectively. Specifically, *O. laevigatus*-punctured sweet pepper plants induce repellency for the whitefly *Bemisia tabaci* and the thrips species *F. occidentalis.* In contrast, the whitefly parasitoid *Encarsia formosa* was significantly attracted to *O. laevigatus*-punctured plants. The plant responses to *O. laevigatus* punctures include the release of an altered blend of volatiles and activation of the jasmonate acid and salicylic acid signalling pathways. These results highlight an interesting facet to the biology of *O. laevigatus,* in which the ability of the predator to induce defensive responses in sweet pepper plants may serve to improve the biological control of both thrips and whiteflies.

Key words: minute pirate bug, behaviour, plant response, herbivore induced plant volatiles, sweet pepper.

2.1. Introduction

Many species of the genus *Orius* Wolff, 1811 are considered to be important beneficial insects for a variety of agroecosystems (Hernández and Stonedahl, 1999). *Orius* species are highly polyphagous, yet show preferential tendencies for immature and adult thrips (Chambres *et al.,* 1993; Ruidavet, 1995; Frescata and Mexia, 1996) and to a lesser extent eggs, nymphs and adult whiteflies (Gerling *et al.,* 2001; Arnó *et al.,* 2008). As a result of these prey preferences, some species of *Orius* are commercially produced for augmentative release as biological control agents in various crops worldwide (Chambers *et al.,* 1993; Sánchez and Lacasa, 2002; van Lenteren and Bueno, 2003; van Lenteren *et al.,* 2018). In Europe, the minute pirate bug *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) has become the most successful *Orius* species for biological control, and it is hence widely used in augmentative release programs. The prey range of *O. laevigatus* is broad and includes agricultural pests such as aphids, whiteflies, lepidopteran eggs, mites, and thrips with a distinct preference for the latter group of pests (Venzon *et al.,* 2002; van Lenteren and Bueno, 2003). In south eastern Spain, the release of *O. laevigatus* together with the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) is key to successful pest management in more than 10,000 ha of protected sweet pepper crops (Blom *et al.,* 1997; Sánchez *et al.,* 2000; Calvo *et al.,* 2009a; Blom *et al.,* 2009).

The genus *Orius* can also feed on plants, for example on the pollen and sap, which contributes to population survival in the absence of a primary source of protein-rich food in the crop (Cocuzza *et al.,* 1997; Armer *et al.,* 1998). Indeed, *O. laevigatus* can complete its development by feeding exclusively on fresh pollen from sweet pepper (Vacante *et al.,*

1997). Armer *et al.* (1998) demonstrated that *Orius* species can also feed on xylem and mesophyll contents, which consists of mostly water with small amounts of sugars, starch and amino acids. The presence of amylase in *O. insidiosus* Say (Hemiptera: Anthocoridae) indicates that it can digest starch and therefore can take advantage of feeding directly on the plant (Zeng and Cohen, 2000). Plants are not only a source of nutrients but also a substrate for oviposition. *Orius* females insert their eggs underneath the cuticle in the epidermal to subepidermal cell layers, which enhances offspring survival (Lundgren *et al.*, 2008). During the early developmental stages, *O. insidiosus* can take nutrients from phloem tissue and survive solely from plant material for several days (Lundgren *et al.*, 2008). All this supports that feeding on plant material is common and ecologically relevant for omnivorous *Orius*.

Whereas plant-mediated interactions among herbivores have been relatively well-studied, the effect of phytophagy by omnivorous predators on plant defences and the subsequently induced insect behaviour have not been investigated until recently. De Puysseleyr *et al.* (2011) showed that increased tomato plant resistance to *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) feeding was produced via the jasmonic acid (JA)-mediated wound response during *O. laevigatus* ovipositing. More recently, several papers have demonstrated the capacity of different species of zoophytophagous species (plant-feeding carnivore), including mirid bugs (Hemiptera: Miridae), to induce plant responses in tomato plants (Pérez-Hedo *et al.*, 2015a, 2015b; Pappas *et al.*, 2015, 2016).

It is hence hypothesised that *O. laevigatus* would be able to induce plant responses in sweet pepper as has been demonstrated in other plant-zoophytophage systems. As a first step to better understand the interaction between *O. laevigatus* and sweet pepper, the behaviour of *O. laevigatus* on

the plants was studied and plant feeding behaviour quantified to compare general behaviours. A series of experiments were then conducted to determine whether *O. laevigatus* feeding punctures on sweet pepper induce plant defence responses and whether these in turn lead to behavioural responses in pest and natural enemy species. In parallel, targeted gene-expression analysis was used on plants previously exposed to *O. laevigatus* to ascertain which signaling pathways could be involved in plant defensive responses. Finally, the volatile compounds released as part of the plant response to *O. laevigatus* feeding punctures were characterized.

2.2. Material and methods

2.2.1. Insects and Plants

Adult *O. laevigatus*, the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) were supplied by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain), all were less than 5 days old. *Frankliniella occidentalis* adults were obtained from a colony established at Instituto Valenciano de Investigaciones Agrarias (IVIA) in 2010 and originally collected from Campo de Cartagena (Murcia, Spain). Thrips colonies were maintained on the common bean (*Phaseolus vulgaris* L. Fabales: Fabaceae) and housed in a climatic chamber at 25 ± 2 ºC, 65 ± 10% RH and a 14:10 h (L:D) photoperiod at IVIA.

Pesticide-free sweet pepper seedlings *cv* ('Lipari') [*Capsicum annuum* (Solanaceae)] (Dulce italiano, Mascarell semillas S.L, Valencia, Spain) were individually transplanted to plastic pots (8 x 8 x 8 cm) containing a mixture

of natural soil with local peat moss and housed undisturbed in climatic chamber at 25 ± 2 ºC, 60-80% RH and 14:10 h (L:D) photoperiod at IVIA. Once the sweet pepper plants had approximately 6 fully developed leaves (approximately 15 cm in height) they were used for the study of *O. laevigatus* behaviour, older plants of approximately 20 cm in height were used for the rest of the experiments.

To obtain *O. laevigatus*-punctured plants, four intact sweet pepper plants were enclosed for 24 hours in a 60 x 60 x 60-cm plastic cage (BugDorm-2 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan) and exposed to 100 *O. laevigatus* adults, all less than 4 days old (sex ratio 1:1). All individuals were removed from the plants before the experiment. Prior to their use, *O. laevigatus* were released into a plastic cage (30 x 30 x 30 cm) (BugDorm-1 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan) with water supplied on soaked cotton plugs, and starved for twenty-four hours.

2.2.2. Behaviour of *O. laevigatus* on sweet pepper

The behaviours of both male and female *O. laevigatus* on sweet pepper plants in the absence of prey items was observed during 30 minute assays under laboratory conditions (T= 25 ± 2 ºC, RH= 60-80%). Less than 4-day-old males and presumably mated females of *O. laevigatus* were individually placed inside a plastic 5-ml vial and starved for 24 h before use. Water was supplied on soaked cotton plugs.

A clean sweet pepper plant was placed inside a 60 x 60 x 60 cm plastic cage (BugDorm-2 insect tents). A single predatory *O. laevigatus* (male or female) was gently released and observations began once the individual walked freely onto the plant for the first time. The different observed

behaviours were recorded continuously for 30 minutes. Behavioural observations were made by three researchers using a hand held magnifying glass after first quantifying their reliability. The sweet pepper plant was replaced before the start of each behavioural assay. Observations were repeated until 20 replicates for each sex had been observed.

Locations were defined as off plant (plastic cage, plastic pot or soil) and on plant (distinguishing between apical and basal regions). The apical region was considered the first 5 cm of the plant formed by the apical stem, young leaves and 2 fully developed leaves. Conversely, the rest of the plant, approximately 10 cm with 4 fully developed leaves, basal stem and cotyledons, represented the basal region. Seven behavioural states were defined as follows:

• Grooming (G): The predator's forelegs are used to clean mouthparts or another part of the body.

• Feeding (F): The predator inserts its stylet into the plant and stylet movements can be observed.

• Oviposition (O): The predator presses its whole abdomen onto plant and the ovipositor is inserted into the plant.

• Resting (R): The predator stands motionless.

• Antennating (A): The predator is at rest but moves its antennae.

• Walking (W): The predator walks on different regions of the plant without moving its antennae.

• Walking-Antennating (WA): The predator walks on different regions of the plant and moves its antennae at the same time.

Because the main interest was in quantifying the feeding behaviour compared to other behaviours, only the analysis of the timed behaviourals grouped in the apical and basal regions of the plant are presented.

2.2.3. Plant selection mediated by *O. laevigatus*

A Y-tube olfactometer experiment was conducted to test the olfactory responses of *B. tabaci*, *F. occidentalis* and *E. formosa* females to sweet pepper plants that were previously punctured by *O. laevigatus* relative to intact plants. The Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) consisted of a 2.4 cm diameter Y-shaped glass tube with a 13.5-cm long base and two arms each 5.75-cm long (Pérez-Hedo and Urbaneja, 2015). Both side arms were connected via high density polyethylene (HDPE) tubes to two identical glass jars (5 l volume) each of which connected to an air pump that produced a unidirectional humidified airflow at 150 ml min^{-1} (Pérez-Hedo and Urbaneja, 2015). Four 60-cm fluorescent tubes (OSRAM, L18W/765, OSRAM GmbH, Germany) were positioned 40 cm above the horizontally disposed Y-shaped glass tube. The light intensity registered 2,516 lux over the Y-tube and was measured using a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc. Pullman, WA, USA). All Y-tube experiments were conducted under the following environmental conditions, 23 ± 2 ºC, 60 ± 10% RH.

A single individual female of *B. tabaci*, *F. occidentalis* or *E. formosa* was introduced into the tube (entry array) and observed until she had walked at least 3 cm up one of the arms or until 15 min had elapsed. A minimum of 33 valid replicates from each species were recorded for each pair of odor sources. Females that did not choose a side arm within 15 min were recorded as 'no-choice' and were excluded from data analysis. After recording 5 responses, the Y-tube was rinsed with soap water and acetone and left to dry for 5 min. The odor sources were subsequently switched between the left and right side arms to minimize any spatial effect on choice. Both types

of plants (intact and punctured) were used only once to test the response of 10 females and then were replaced with new plants.

2.2.4. Plant gene expression analysis

The apical region of the sweet pepper plants, as defined above, were subjected to targeted gene expression analysis to detect: (i) *ASR1* (abscisic acid stress ripening protein 1) a marker gene for ABA, (ii) *PIN2* (wound-induced proteinase inhibitor II precursor) a marker gene for JA and (iii) *PR1* (basic PR-1 protein precursor) a marker gene for the SA signalling pathway. Samples of the sweet pepper apical region were collected from both intact and *O. laevigatus*-punctured plants. After homogenization in liquid nitrogen, total RNA was extracted using Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions (Pérez-Hedo *et al.*, 2015a; Naselli *et al.*, 2016). After homogenizing the sample with TRIzol™ Reagent, chloroform was added to separate RNA of protein and DNA, and subsequent isopropanol and 1.2 Mm NaCl was added to precipitate the RNA. The RNA pellets resulting from the precipitation were washed twice with 70% ethanol, dried at room temperature and eluted in water. The RNA was quantified and then treated with the Turbo DNA-free DNase kit (Applied Biosystems) to eliminate any traces of genomic DNA, according to the manufacturer's protocol. cDNA was synthesized by adding to the samples (1 µg/µl), RT buffer, 10 µM Oligo dT and prime script™ RT reagent kit (perfect real time) (TAKARA Bio, CA, USA). The reaction mixture was then incubated in the thermo-cycler for 15 min at 37 ºC and for 5 s at 85 ºC. Real-time PCR amplifications were performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, MA, USA). *Capsicum annuum* forward and reverse

specific primers (0.5 µl) were designed and added to 5 µl of Syber green/ROX qPCR MM and 1 µl of cDNA and then brought to 10 µl total volume with Milli-Q sterile water. PCR reactions were run in duplicate according to manufacturer recommendations. Quantitative PCR was carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland) and the protocol consisted in 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s, 56 ºC for 30 s and 72 ºC for 30 s. Melting curve analysis was performed at 95 ºC for 5 s, 60 ºC for 1 min and then a continuous increase of temperature until 95 ºC finalizing the process. Data acquisition and calculation were performed with the thermal cycler's software and then were collected and analyzed in Microsoft Excel. Each qPCR data point is the average of 8 independent experiments. EF1 (elongation factor-1) was used as a standard control gene for normalization. The nucleotide sequences of the gene-specific primers are described in Table 2.1.

Table 2.1.

Forward and reverse sequences of *ASR1, PIN2, PR1*, and the constitutive gene *EF1*.

Primers	Forward	Reverse
ASR1	5'-TGTGCAATTTGTCTTGTGGAA-3'	5'-CGGACATGACGAGTTCGATA-3'
PIN2	5'-CTTGCCCCAAGAATTGTGAT-3'	5'-GCCCTAGCGTATTACGGAGA-3'
PR1	5'-ACGTCTTGGTTGTGCTAGGG-3'	5'-CCATACGGACGTTGTCCTCT-3'
EF1	5'-CCTGGACAGATTGGAAATGG-3'	5'-GACCACCTGTCGATCTTGGT-3'

2.2.5. Composition of volatile blends

Volatile compounds were collected by means of solid phase microextraction (SPME) and separated and detected by means of gas chromatography coupled to mass spectrometry (GC/MS). Volatiles were adsorbed in a 65-µm PDMS/DVB SPME fiber (polydimethylsiloxane/ divinylbenzene; Supelco, Bellefonte, PA, USA). The adsorbent-coated fiber was mounted on an SPME fiber holder, and injected through the first septum of the sample container. Agitation of the atmosphere inside the container was achieved by pumping at 5 ml/min using an injected syringe through the second septum of the sample container.

Each sample was performed for 3 hours. Four replicates for the intact plant and three replicates for *O. laevigatus*-punctured plant treatments were conducted. After collection, the fiber was retracted into the needle and the SPME device was removed from the container for GC-MS analysis.

Desorption was performed by means of a CombiPAL autosampler (CTC Analytics) at 250 ºC for 1 min in splitless mode in the injection port of a 6890N gas chromatograph coupled to a 5975B mass spectrometer (Agilent Technologies). To prevent cross-contamination, fibers were cleaned after desorption in an SPME fiber conditioning station (CTC Analytics) at 250 ºC for 5 min under helium flow. Chromatography was performed on a DB-5ms (60 m, 0.25 mm, 1.00 µm) column with helium as carrier gas, at a constant flow of 1.2 ml/min. The GC interface and MS source temperatures were 260 ºC and 230 ºC, respectively. Oven programming conditions were 40 ºC for 2 min, 5 ºC/min ramp until 250 ºC, and a final hold at 250 ºC for 6 min. Data were recorded in the 35-300 m/z range at 5 scans/s, with electronic

impact ionization at 70 eV. Untargeted analysis of the chromatograms was performed with MetAlign software (WUR, http://www.metalign.nl).

Kovats retention indexes (KIs) were calculated for the compounds. Differentially emitted compounds were first tentatively identified by the comparison of their mass spectra with those in the NIST 05 Mass Spectral Library. When available, identity was confirmed by coelution with pure standards (Sigma-Aldrich). To quantify the selected compounds, one specific ion was selected for each compound, and the corresponding peak area from the extracted ion chromatogram was integrated by means of the ChemStation E.02.02 software (Agilent Technologies). The criteria for ion selection were the highest signal-to-noise ratio and specificity to that particular region of the chromatogram to provide good peak intergration.

2.2.6. Data analysis

The different behaviours of *O. laevigatus* on sweet pepper plants and the predator location were analyzed using three-way analysis of variance (ANOVA), taking as factors, sex, observer researcher and either behaviour or location. Tukey's test was used for mean separation at $\alpha < 0.05$. Since only three of the 20 tested female *O. laevigatus* oviposited on plants, this behaviour was excluded from the analysis. The data from the olfactory responses were analyzed using a χ^2 goodness of fit test based on a null model where the odor sources were selected with equal frequency. Individuals who did not make a choice were excluded from the statistical analysis. The data from gene expression analyses and volatile profiling were analyzed using one-tailed Student's *t*-test ($P < 0.05$). For volatile profiling, discriminant compounds

were determined by the analysis of the data obtained from the untargeted chromatogram and the results expressed as the means ± standard error.

2.3. Results

2.3.1. Behaviours and locations of *O. laevigatus* on sweet pepper

Orius laevigatus showed preference to the apical region of the sweet pepper plants where the majority of the observed behaviours were performed ($F_{2,110}$= 108.33; *P*< 0.0001) (Fig.2.1). Neither sex nor the observer researcher was found to be significant ($F_{1,110}$= 0.11; *P*= 0.7415 and $F_{2,110}$= 0.06; *P*= 0.9374, respectively).
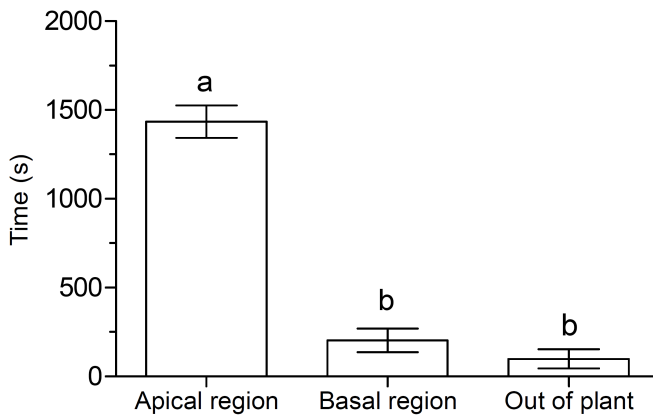
Figure 2.1.

Residence time (mean ± SE) of *O. laevigatus* adults on sweet pepper plants. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test *α*< 0.05) (n=40).

Orius laevigatus spent 38% of their time feeding on plant tissues, which was significantly higher than the time spent on all other activities ($F_{5,221}$= 21.01; *P*< 0.0001) (Fig.2.2). Neither the sex nor the observer were significant in the statistical analysis of the behaviours, ($F_{1,221}$= 0.12; *P*= 0.724 and $F_{2,221}$= 0.48; *P*= 0.6211, respectively).
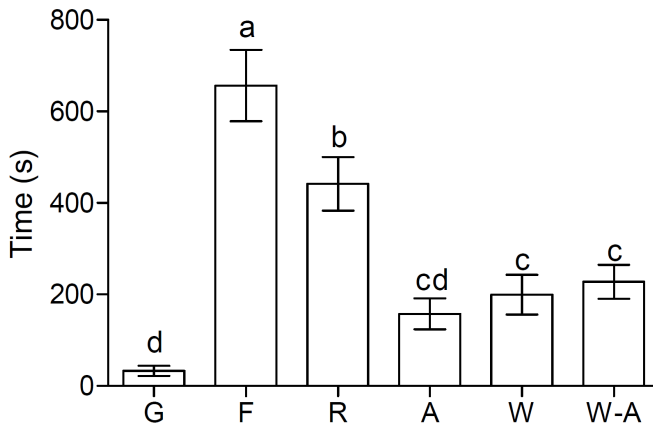
Figure 2.2.

Time (mean ± SE) spent exhibiting different behaviours by *O. laevigatus* adults on sweet pepper plants during a period of 30 min. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test *α*< 0.05) (n=40).

2.3.2. Plant selection mediated by *O. laevigatus*

In the Y-tube experiment, females of *F. occidentalis* showed preference for the odor emitted from intact plants over that from *O. laevigatus*-punctured plants (χ^2= 19.931; *P*< 0.0001) (Fig.2.3). In the case of *B. tabaci*, tested females were also attracted to the odor emitted by intact sweet pepper plants in comparison to *O. laevigatus*-punctured plants (χ^2= 17.071; *P*< 0.0001) (Fig.2.3). In contrast to both phytophagous insects, the parasitoid *E. formosa* significantly chose *O. laevigatus*-punctured plants over intact plants in the olfactometer assay (χ^2= 6.250; *P*= 0.0124) (Fig.2.3).
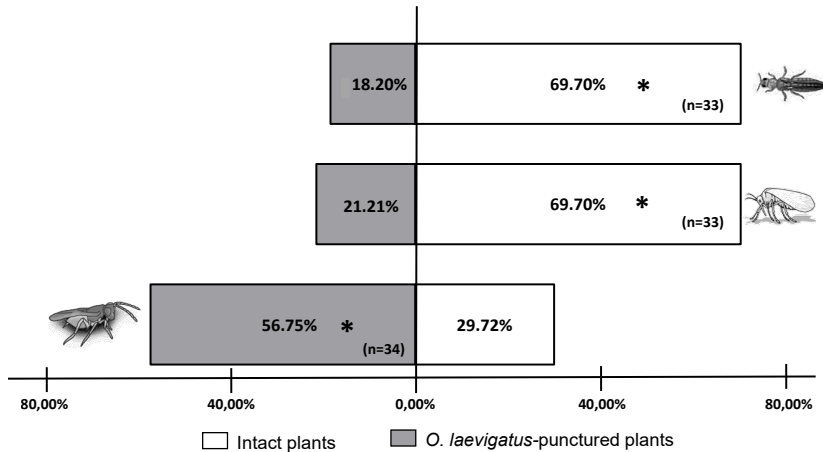
Figure 2.3.

Response in Y-tube olfactometer of females of *F. occidentalis* (n=33), *B. tabaci* (n=33) and *E. formosa* (n=34) to the odor emitted by intact sweet pepper and by sweet pepper plants previously exposed to *O. laevigatus*. Significant differences based on a χ2-test are marked using * (*P*< 0.05).

2.3.3. Plant gene expression analysis

Transcriptional analysis showed that plant feeding by *O. laevigatus* on the apical portion of sweet pepper plants increased the expression of the *PIN2* gene (JA pathway) (*t*= 2.161, *P*= 0.019) and the *PR1* gene (SA pathway) (*t*= 1.835, *P*= 0.039) (Fig. 2.4.a,b). In contrast, the *ASR1* gene (ABA pathway) was not affected in *O. laevigatus*-punctured plants (*t*= 0.113, *P*= 0.455) (Fig.2.4.c).

a)

PIN2

b)

PR1

c)
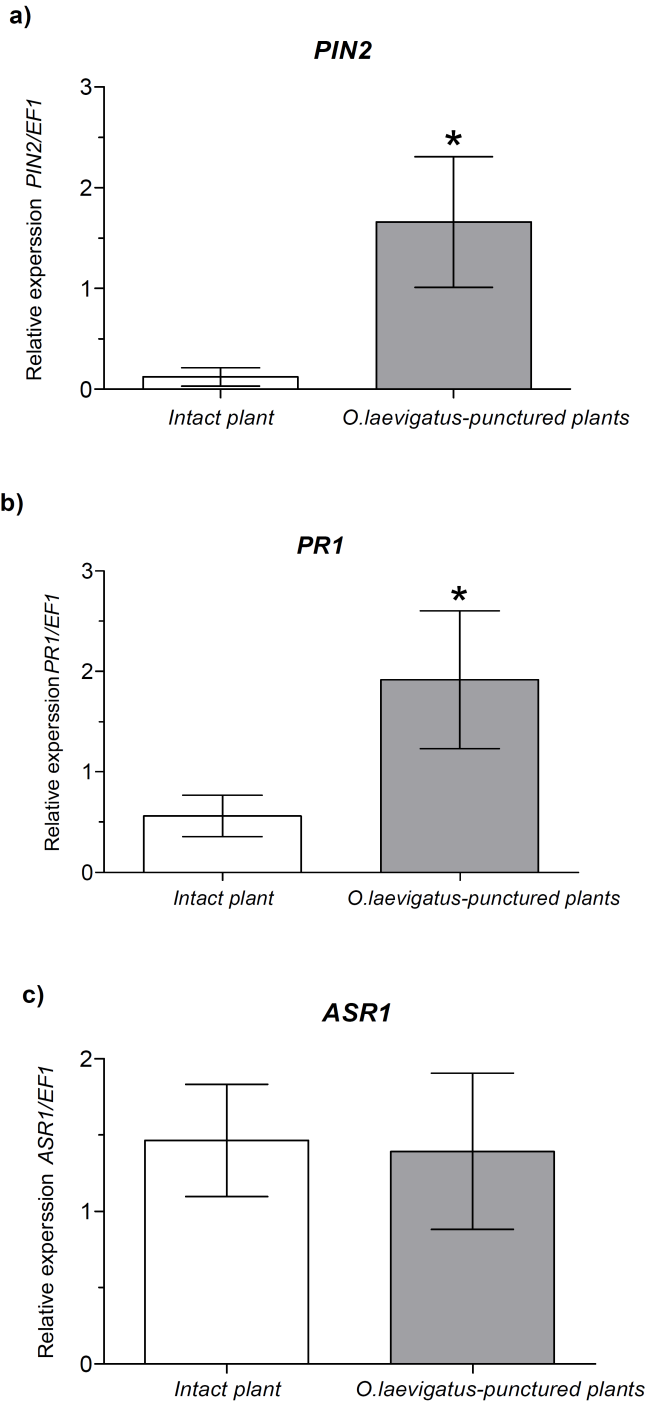
ASR1

Figure 2.4.

Transcriptional response of the defensive genes *ASR1* (**a**), *PIN2* (**b**) and *PR1* (**c**) responsible for the change in level of the phytohormones ABA, JA, and SA, respectively, in *O. laevigatus-* punctured plants. Data are presented as the mean of eight independent analyses of transcript expression relative to a housekeeping gene ± SE (n=8). Significant differences based on *t-* test are marked with (*) (*P*< 0.05).

2.3.4. Composition of volatile blends

Quantitative differences were recorded in the herbivore-induced plant volatiles (HIPVs) profile of *O. laevigatus*-punctured and intact sweet pepper plants. Untargeted analysis of the volatiles emitted allowed the identification of ten compounds with significantly increased levels in punctured plants, while no compounds with decreased levels were identified. The emission of discriminant compounds increased in the range of 2 to 100-fold and corresponded to terpenoids (1 monoterpenoid, 4 sesquiterpenoids and 1 norisoprenoid), a set of two (Z)-3-hexenyl esters, methyl salicylate and another unknown compound (Table 2.2).

Table 2.2.

Significant relative levels (fold changes) of the volatiles emitted by *O. laevigatus* punctured plants relative to intact plants. Student's *t*-test (P< 0.05).

Type	Compound	Kovats RI	Fold change	*t*	*P*
Terpenoids: monoterpenoids	Linalool*	1103	6.42	2.5353	0.0327
Terpenoids: sesquiterpenoids	Sesquiterpene[1]	1418	33.80	4.593	0.0029
	Sesquiterpene[2]	1459	4.30	6.739	0.0005
	(E)-nerolidol*	1574	7.55	7.036	0.0004
	Sesquiterpenoid	1583	97.95	2.557	0.0254
Terpenoids: norisoprenoids	Unknown[3]	1117	4.30	2.71	0.0211
Green leaf volatile esters	(Z)-3-hexenyl propanoate*	1096	3.04	2.353	0.0327
	(Z)-3-hexenyl benzoate*	1596	3.10	5.722	0.0011
Systemic acquired resistance (SAR)	Methyl salicylate*	1215	17.01	3.47	0.0089
Other	Unknown[4]	1664	2.52	2.151	0.0421

*Unequivocal identification (confirmed with a pure standard); Tentative identification based on mass spectra: [1] beta-elemene, [2] alpha-bergamotene, [3] norisoprenoid (C11H18) and [4] methyl dihydrojasmonate

2.4. Discussion

Here, for the first time *O. laevigatus* induced defensive response in sweet pepper plants due to the described phytophagy behaviour. This is of particular interest as the predator *O. laevigatus* has been one of the most studied and successfully used augmentative biological control agents in sweet pepper (van Lenteren *et al.,* 2018). Furthermore, the modulated behaviour of both pest species (*B. tabaci* and *F. occidentalis*) and a natural enemy (*E. formosa*) associated with sweet pepper, in response to an induced plant response by the zoophytophage *O. laevigatus*, is documented for the first time.

Previous studies on the behaviour of several species of *Orius* under different conditions (availability of prey and/or pollen, quality of the plant as a feeding and/or oviposition substrate, architecture of the plant) have been reported (Fritshe and Tamó, 2000; Yano *et al.,* 2005; Jonathan and Fergen, 2006; Lundgren *et al.,* 2008). However, to our knowledge, no other study has quantified the time that a specific *Orius* species spends feeding on sweet pepper. Here, *O. laevigatus* feeding behaviours are described and quantified, and reveal both females and males feed on plant tissue by inserting their stylets, a behaviour which represents 38% of time spent on the plant. When comparing this plant feeding behaviour with the other observed behaviours under experimental conditions, plant feeding was the preferred activity by *O. laevigatus*, occupying the greatest proportion of time. Individuals tested in the behavioural experiment were starved 24 hours, with access to just water, before their release into the experimental arenas. The reason for both female and male *O. laevigatus* plant feeding behaviour upon their release could well be due to the need to obtain nutrients from the plant and not so

much because of the need for hydration. Indeed, the sequential behaviour of importance was searching for prey (the sum of the Antennating-A and "Walking-Antennating-WA behaviour). The experimental conditions (starved *O. laevigatus*, the size of sweet pepper plants and the absence of prey after release) were standardised according to conditions *O. laevigatus* would encounter in the field when released in augmentative biological control programs. The next step would be to quantify the plant feeding behaviour in the presence of prey in sweet pepper plants, and whether under this situation, defensive plant responses are also activated.

It is known that predators avoid laying eggs where prey are scarce or absent as in our study (Evans and Dixon, 1986; Hemptinne *et al.*, 1992). Nakashima and Hirose (2002) observed that females of *O. sauteri* Poppius (Hemiptera: Anthocoridae) oviposited at higher rates in prey-rich patches than in prey-deficient patches. *O. laevigatus* individuals used in this study were subjected to a very similar procedure before their use (starvation with access to water for 24 h) as in Nakashima and Hirose (2002), and only three of the 20 tested females of *O. laevigatus* oviposited on plants. The time represented by oviposition occupied just 0.2% of total observation time. These results suggest that plant defence response can be induced by plant feeding behaviour in addition to the oviposition behaviour described by De Puysseleyer *et al*. (2011) in tomatoes. Recently, Naselli *et al.* (2016) demonstrated that all motile stages of the mirid *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) can induce plant responses in tomato plants, although such responses may differ slightly depending on the stage considered. Similarly, knowing if immature *O. laevigatus* individuals can trigger defensive responses in sweet peppers would also be of interest.

Most of the plant defence mechanisms against insects are activated by signal transduction pathways mediated by JA, SA and ethylene (Shivaji *et al.*, 2010; Gill *et al.*, 2010). Little had previously been described on the subject of plant responses to the feeding of *O. laevigatus* before this study. It is shown here that activation of the JA and SA pathways in *O. laevigatus*-punctured plants is associated with aversion of both arthropod pests, *B. tabaci* and *F. occidentalis* and by contrast, the whitefly parasitoid *E. formosa* was significantly attracted to *O. laevigatus*-punctured plants. Pérez-Hedo *et al.* (2015a) observed that the feeding activity of the predatory mirid *N. tenuis* on tomato plants activated the ABA and JA signaling pathways, which made plants exposed to mirids less attractive to *B. tabaci* and more attractive to *E. formosa*. Additionally, Pérez-Hedo *et al.* (2015b) showed that three different zoophytophagous predators [*N. tenuis*, *Macrolophus pygmaeus* Rambur and *Dicyphus maroccanus* Wagner (Hemiptera: Miridae)] had different capacities to induce specific responses in tomato plants. Tomato plants exposed to *N. tenuis* were less attractive to *B. tabaci* as mentioned above but also to the lepidopteran *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae). By contrast, tomato plants exposed to *M. pygmaeus* and *D. maroccanus* were not able to repel *B. tabaci* and, more interestingly, became more attractive to *T. absoluta*. All three zoophytophagous mirid predators activated the JA signaling pathways, which resulted in the attraction of the parasitoid *E. formosa* to punctured tomato plants.

Pappas *et al.* (2015, 2016) observed that the performance of *Tetranychus urticae* Koch (Acari: Tetranychidae) decreased as a result of the prior exposure of tomato plants to *M. pygmaeus*. This was accompanied by a locally and systematically increased accumulation of transcripts and activity of proteinase inhibitors that are known to be involved in plant responses.

Similarly, a further step in the research with *O. laevigatus* will be to investigate the performance, by evaluating different life history traits, of *B. tabaci* and *F. occidentalis* on *O. laevigatus*-punctured plants.

It is well documented that non-consumptive effects triggered by arthropod predators can modulate behaviour, physiology, development and morphological traits of a subsequently infesting herbivore (Werner and Peacor, 2003). This non-consumptive effect relies on the ability of prey to perceive chemicals and visual cues directly emitted by predators, before being preyed. Escape, avoidance, reduced oviposition, reproduction and reduced feeding are some of the solutions prey employ to avoid predation risk (Nomikou *et al.,* 2003; Sendoya *et al.,* 2009; Ninkovic *et al.,* 2013; Wasserberg *et al.,* 2013; Lee *et al.,* 2014). No chemical compound has yet been isolated which could be related to an *O. laevigatus* chemical cue, since all differentially identified chemical compounds were emitted from the plant. Therefore, the responses of *B. tabaci*, *F. occidentalis* and *E. formosa* were a direct consequence to the odor emitted from the *O. laevigatus*-punctured sweet pepper plants.

The results presented here showed increased emission of HIPVs in sweet pepper punctured by *O. laevigatus* compared to intact plants. These volatiles belong to already identified volatile groups activated in plants being injured by true phytophagy, the green leaf volatiles group (GLVs), terpenoids and methyl salicylate (Kessler and Baldwin, 2001; Kessler and Baldwin, 2002; War *et al.,* 2011). It is known that HIPVs are released by most plant species (McCormick *et al.,* 2014). However, each species of plant could emit a specific blend of volatiles and that the relative amounts of HIPVs could vary widely between species and with the type of damage (Kigathi *et al.,* 2009; Ponzio *et al.,* 2014; Ardanuy *et al.,* 2016). In this chapter, it has

been shown that the particular volatile profile emitted by the *O. laevigatus*-punctured plant repelled *B. tabaci* and *F. occidentalis* and attracted *E. formosa*.

In summary, these results show that the effectiveness of *O. laevigatus* as a biological control agent in sweet pepper is due to not just its predatory role but also to its ability to induce plant defensive responses. These results open the doors to new management methods for *B. tabaci* and *F. occidentalis*. Once the volatile(s) responsible for the repellency of both pests have been identified, new repellent products can be developed. In parallel, plant breeding programs aimed at obtaining plants with an ability for greater emission of these volatiles could be implemented.

Zooohytophagous Mirids Provide Pest Control by Inducing Direct Defences, Antixenosis and Attraction to Parasitoids in Sweet Pepper Plants

3

Zoophytophagous Mirids Provide Pest Control by Inducing Direct Defences, Antixenosis and Attraction to Parasitoids in Sweet Pepper Plants

Sarra Bouagga, Alberto Urbaneja, José L. Rambla, Víctor Flors, Antonio Granell, Josep A. Jaques, Meritxell Pérez-Hedo

Abstract

In addition to their services as predators, mirid predators are able to induce plant defences by phytophagy. However, whether this induction occurs in sweet pepper and whether it could be an additional benefit to their role as biological control agent in this crop remains unknown. Here, these questions are investigated in two model insects, the mirids *Nesidiocoris tenuis* and *Macrolophus pygmaeus*.

Plant feeding behaviour was observed in both *N. tenuis* and *M. pygmaeus* on sweet pepper and occupied 33% and 14% of total time spent on the plant respectively. The punctures caused by mirid plant feeding induced the release of a blend of Volatile Organic Compounds (VOCs) which repelled the herbivore pests *Frankliniella occidentalis* and *Bemisia tabaci* and attracted the whitefly parasitoid *Encarsia formosa*. The repellent effect on *B. tabaci* was observed for at least 7 days after initial exposure of the plant to *N. tenuis,* and attraction of *E. formosa* remained functional for 14 days.

Feeding induced plant defences by mirid predators, their subsequent effects on both pests and natural enemy behaviour, and the persistence of these observed effects open the door to new control strategies in sweet pepper crop. Further application of this research is discussed, such as the vaccination of plants by zoophytophagous mirids in the nursery before transplantation.

Key words: *Nesidiocoris tenuis, Macrolophus pygmaeus,* phytophagy, HIPV's, plant response, vaccination.

3.1. Introduction

Predatory mirid bugs (Hemiptera: Miridae) have been extensively studied in the last few decades for their ecological significance and role as predators of agricultural pests (Schaefer *et al.*, 2000; Wheeler, 2001). In recent studies, their importance as biocontrol agents in sweet pepper has been highlighted (Messelink *et al*, 2011a, 2015; Pérez-Hedo and Urbaneja, 2015).The use of generalist natural enemies in sweet pepper crops is widely common and has been proven successful (Sánchez and Lacasa, 2002; van Lentren, 2012; van Lentren *et al.*, 2018). If properly managed, the release and the conservation of the predatory mite, *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) together with the anthocorid *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) can successfully manage the population of the key pepper pests; sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), greenhouse whitefly, *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) and western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Calvo *et al.*, 2012a, Weintraub, 2011). Moreover, the polyphagous behaviour of *A. swirskii* and *O. laevigatus* contribute to the management of secondary pests, such as spider mites and Lepidoptera (Park *et al.*, 2010; van Maanen *et al.*, 2010). In this system aphids manage to evade the control of both predators (Belliure *et al.*, 2008, Sánchez *et al.*, 2011) and so the multiple release of natural enemy species is usually practiced, which can have considerable implications in the final cost of the biocontrol program in this crop (Blom *et al.*, 2008; Messelink *et al.*, 2011a). Alternative biocontrol strategies in which mirid predators are included have hence been recently explored. *Nesidiocoris tenuis* Reuter, *Macrolophus pygmaeus* Rambur and *Dicyphus maroccanus* Wagner (Hemiptera:

Miridae) were shown to effectively control the aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) on sweet pepper (Pérez-Hedo and Urbaneja, 2015). Furthermore, *M. pygmaeus* was found to be the most effective agent for the control of *M. persicae* in sweet pepper when compared with three other mirid species, *Dicyphus errans* Wolff, *D. tamaninii* Wagner and *Deraeocoris pallens* Reuter (Hemiptera: Miridae) (Messelink *et al.*, 2015). Under combined release, intraguild interactions between *M. pygmaeus* and *O. laevigatus* did not result in population imbalances of either predatory species, but a better control strategy for both thrips and aphids on sweet pepper resulted (Messelink and Janssen, 2014).

Within the mirids, omnivory is common (Wheeler, 2001) and they are able to exploit both plant and prey resources during the same developmental stage (Castañé, 2011; Sánchez *et al.*, 2016). This flexibility in their behaviour increases survival rates by taking advantage of plant resources when prey is either less abundant or completely absent (Sánchez *et al.*, 2004; Urbaneja *et al.*, 2005; Biondi *et al.*, 2016). As in herbivores, the phytophagous behaviour of mirid predators may also induce indirect plant defences (Pérez-Hedo *et al.*, 2015a, 2015b; Pappas *et al.*, 2015, 2016; Naselli *et al.*, 2016). It is well known that plants can respond to the damage induced by phytophagous insects, involving several signal transduction pathways that are mediated by phytohormones. Jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) are the main targeted components and their accumulation in the plant activates signalling cascades that regulate transcriptional response (Burce and Pickett, 2007; How and Jander, 2008; Bari and Jone, 2009; Karban, 2011; Dicke and van Loon, 2014). Indeed, plants damaged by herbivores often produce a blend of volatiles, commonly referred to as herbivore induced plant volatiles (HIPV's) (Takabayashi and

Dicke, 1996; Paré and Tumlinson, 1999; Mumm and Dicke, 2010; Nasseli *et al.*, 2017). These HIPV's consist of a mixture of the so-called green-leaf volatiles (C_6 aldehydes, alcohols and esters), terpenes (monoterpenes, sesquiterpenes, homoterpenes) and aromatic compounds among others (Arimura *et al.*, 2009; Pichersky, 2006). Consequently, natural enemies use the change in the composition and concentration of these released volatiles as a cue for the presence of potential prey or hosts (Sabelis *et al.*, 1999; Dicke and van Loon, 2000; Verkerk, 2004).

In this work, the potential of *N. tenuis* and *M. pygmaeus* to induce plant defences in sweet pepper is investigated and whether this could be an additional benefit to their role as a biological control agent in this crop. The behaviour of *N. tenuis* and *M. pygmaeus* on sweet pepper was first explored in order to quantify feeding activity on the crop. Secondly, the level of the phytohormones involved in the plant defence and the expression of several marker genes was evaluated, both in intact plants (without mirids punctures) and in mirid-punctured plants. A non-targeted analysis of the volatile compounds differentially released by mirid-punctured and intact plants was then performed by means of headspace solid phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS). Thirdly, the response of two key sweet pepper pests, *F. occidentalis* and *B. tabaci*, and the whitefly parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) to the odour emitted by mirid-punctured and intact plants was tested under dual choice experiments. Finally, the persistence of the plant response induced by *N. tenuis* was observed.

3.2. Material and Methods

3.2.1. Plants and insects

Pesticide free *Capsicum annuum* (cv. Lipari) (Dulce italiano, Mascarell semillas S.L, Valencia, Spain) seedlings were used for the study. Two weeks after germination, seedlings were transplanted into a mixture of soil and local peat moss in plastic pots (8 x 8 x 8 cm), housed in climatic chambers at 25 ± 2 ºC, 60-80% RH and 16:8 h (L:D) photoperiod at Instituto Valenciano de Investigaciones Agrarias (IVIA). Plants with 6 fully-developed leaves (approximately 15 cm in height) were used for the study of *N. tenuis* and *M. pygmaeus* behaviour and for the rest of the experiments plants were used once 10 leaves had fully-developed (approximately 20 cm in height). Two sweet pepper plant treatments were required, mirid-punctured plants and intact plants (control plants free from any arthropod contact). Mirid-punctured plant were obtained by exposing sweet pepper plants to 25 adult *N. tenuis* or *M. pygmaeus* (sex ratio 1:1) for 24 hours in a 30 x 30 x 30-cm plastic cage (BugDorm-1 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan).

Nesidiocoris tenuis, *M. pygmaeus* and *B. tabaci* adults, and *E. formosa* pupae, were provided by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). Cultures of *N. tenuis* and *M. pygmaeus* were maintained in climatic chamber at 25 ± 2 ºC, 60-80% RH and 16:8 h (L:D) photoperiod 25 ± 2 ºC, 60-80% RH and 16:8 h (L:D) photoperiod at IVIA. Both mirid cultures were separately caged on sweet pepper plants with access to *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Entofood®; Koppert B.S.) as supplemented food until their use in the bioassays. Five day old adult *N. tenuis* and *M. pygmaeus* were used in

all the experiments. Newly emerged adult *B. tabaci* (less than 2 day old) were similarly reared on sweet pepper plants caged in 60 x 60 x 60-cm BugDorm-2 insect tents. In the case of *E. formosa*, pupae were enclosed in a Petri dish (9 cm diameter) and allowed to emerge under ambient laboratory conditions (25 ± 2 °C), with a small drop of honey provided as food. Females *E. formosa* were used at less than two days old all experiments.

Frankliniella occidentalis adults were obtained from a culture established at IVIA in 2010, originally collected from Campo de Cartagena (Murcia, Spain). The thrips culture was maintained on bean plants (*Phaseolus vulgaris* L.; Fabales: Fabaceae) and housed in a climatic chamber at 25 ± 2 °C, 65 ± 10% RH and a 14:10 h (L:D) photoperiod at IVIA. All female *F. occidentalis* used for experimentation were less than five days old.

3.2.2. Mirid behaviour on sweet pepper

Direct observations for both male and female *N. tenuis* and *M. pygmaeus* behaviour were carried out on intact sweet pepper plant for 30 minutes under a hand magnifying glass (5cm of diameter and a magnification of 2.5x-5x) (Entomopraxix, Barcelona, España). The experimental arena consisted an intact sweet pepper plant inside a plastic cage 60 x 60 x 60-cm (BugDorm-2 insect tents), (T= 25 ± 2 °C, RH= 60-80%). A single mirid predator (male or female) was then released onto the plant. Recording began when the first behavioural activity was observed (typically: walking, though any of the other recorded behaviours were also considered). Duration of each behaviour and the corresponding location on the plant was noted. For each assay (species and sex), twenty replications were carried out and the sweet pepper plant replaced by new intact plant for each of the subsequent observations.

Observed behaviours were the following: Walking (W): Predator walking behaviour on the different regions of the plant. Antennating (A): Stationary searching activity, characterised by moving the antennae. Walking-Antennating (W-A): Non-stationary searching activity characterised by moving the antennae and walking. Feeding (F): The predator uses labium to probe the feeding sites and then inserts the stylet vertically into the plant. Oviposition (O): The predator firstly probes the oviposition site with the labium, then the whole abdomen is pressed onto the plant and the length of the ovipositor inserted into the plant, egg deposition is visible. Grooming (G): Cleaning mouthparts with forelegs and/or cleaning another part of the body. Flying (Fl): Flying movement typically from the plant to the cage walls or the opposite. Resting (R): The predator is at rest, stationary and not carrying out any other described behaviour.

The plant locations visited by the predator during the observation were defined. One location off-plant (plastic cage, plastic pot or soil) and two locations on-plant (apical region and basal region) were defined. The apical region of sweet pepper plant was defined as the first 5 cm of the plant formed by apical stem, young developing leaves and 2 fully developed leaves. The basal region was the rest of the plant, approximately 10-12 cm with 4 developed leaves, basal stem and cotyledons.

3.2.3. Phytohormone analysis and plant gene expression

In order to identify the phytohormone profile of 1) *N. tenuis*-punctured plants, 2) *M. pygmaeus*-punctured plants and 3) sweet pepper intact plants, the hormones, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and JA-isoleucine (JA-Ile) were analysed by ultra-performance

liquid chromatography coupled with mass spectrometry (UPLC-MS) (Flors *et al*., 2008, Forcat *et al*., 2008; Pérez-Hedo *et al*., 2015a).The apical region of the pant, as defined previously, from each treatment was removed and stored at -80 ºC until analysis. Five replicates were collected for each treatment. Analyses were carried out using an Acquity ultra-performance liquid chromatography system (UPLC; Waters, Mildford, MA, USA) and the chromatograph interfaced to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). MassLynx NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and the plant samples. The calibration curves were obtained by using solutions containing increasing amounts of ABA, SA, JA and JA-Ile commercial standards (Sigma-Aldrich, http://www.sigma-aldrich. com).

Expression of (i) *ASR1* (abscisic acid stress ripening protein 1) a marker gene for ABA, (ii) *PIN2* (wound-induced proteinase inhibitor II precursor) a marker gene for JA, and (iii) *PR1* (basic PR-1 protein precursor) a marker gene for the SA signalling pathway, were quantified for each of the three plant treatment samples taken from the apical region of the sweet pepper plants. Samples were cut and then ground in liquid nitrogen and a portion used for RNA extraction. Total RNA (1.5 µg) was extracted using Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions (Pérez-Hedo *et al*., 2015a; Naselli *et al*., 2016). Samples were homogenized with TRIzol™ Reagent and then chloroform was added to separate protein RNA and DNA. RNA was precipitated with the addition of isopropanol and 1.2 Mm NaCl. After quantification, the RNA was treated with the Turbo DNA-free DNase kit (Applied Biosystems) to eliminate any traces of genomic DNA, according to the manufacturer's protocol. cDNA was then synthesized using prime script™ RT reagent kit (perfect real time)

(TAKARA Bio, CA, USA). The reaction mixture was then incubated in the thermo-cycler for 15 min at 37 ºC followed by 5 s at 85 ºC. Real-time PCR amplifications were performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, MA, USA). *Capsicum annuum* specific forward and reverse primers (0.5 µl) were designed and added to 5 µl of Syber green/ROX qPCR MM and 1 µl of cDNA and then brought to 10 µl total volume with Milli-Q sterile water. PCR reactions were run in duplicate, in accordance with manufacturer recommendations. Quantitative PCR was carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland), under the following amplification conditions, 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s, 56 ºC for 30 s and 72 ºC for 30 s. Melting curve analysis was performed at 95 ºC for 5 s, 60 ºC for 1 min and then a continuous increase of temperature up to 95 ºC to finalise the process. Data acquisition and calculation were performed with the thermal cycler's software and were then collected and analysed in Microsoft Excel. Each qPCR data point is the average of 8 independent experiments. EF1 (elongation factor-1) was used as a standard control gene for normalization. The nucleotide sequences of the gene specific primers are the same used in chapter 2 (Table 2.1).

3.2.4. Determination of plant volatile compounds

Volatile compounds emitted from 1) *N. tenuis*-punctured sweet pepper plants, 2) *M. pygmaeus*-punctured plants and 3) intact plants were collected using 5 L volume glass jars as used in Y-tube olfactometer described below (Analytical Research Systems, Gainesville, FL) (Pérez Hedo and Urbaneja, 2015). One sweet pepper plant was introduced (either intact or mirid-punctured) into each jar. After closing the jar, an adsorbent-coated

SPME fibre (PDMS/DVB (65 μm polydimethylsiloxane/Divinylbenzene; Supelco, Bellefonte, PA, USA) was mounted on the fibre holder, and injected through the first septum (top of the jar). Agitation of the atmosphere inside the container was achieved by pumping at 5 ml/min using a syringe injected through the second septum (bottom of the jar). The three jars were maintained in the Y-tube olfactometer table at a light intensity of 2516 lx. The volatiles emitted were captured over a 3 hours period, and 5 replicates per treatment were performed. After collection, the fibre was retracted into the needle and the SPME device was removed from the container. The compounds absorbed in the SPME fibre were separated and detected by means of GC-MS.

Desorption was performed by means of a CombiPAL autosampler (CTC Analytics) at 250 ºC for a duration of 1 min in splitless mode in the injection port of a 6890N gas chromatograph coupled to a 5975B mass spectrometer (Agilent Technologies). To prevent cross-contamination, fibres were cleaned after desorption in an SPME fibre conditioning station (CTC Analytics) at 250 ºC for 5 min under helium flow. Chromatography was performed on a DB-5ms (60 m, 0.25 mm, 1.00 μm) column with helium as carrier gas at a constant flow of 1.2 ml/min. GC interface and MS source temperatures were 260 ºC and 230 ºC respectively. Oven programming conditions were 40 ºC for 2 min, 5 ºC/min ramp until 250 ºC, and a final hold at 250 ºC for 6 min. Data were recorded in the 35-300 m/z range at 5 scans/s, with electronic impact ionization at 70 eV. Untargeted analysis of the chromatograms was performed by means of the MetAlign software (WUR, http://www.metalign.nl).

Kovats retention indices (KI) were calculated for each of the compounds. Differentially emitted volatiles were first tentatively identified by comparing their mass spectra with those in the NIST 05 Mass Spectral

Library. When available, identity was confirmed by coelution with the pure standards (Sigma-Aldrich). For quantitation of the selected compounds, one specific ion was selected for each compound, and the corresponding peak area from the extracted ion chromatogram was integrated by means of the ChemStation E.02.02 software (Agilent Technologies). The criteria for ion selection were the highest signal-to-noise ratio and being specific enough in that particular region of the chromatogram in order to provide good peak integration.

3.2.5. Response to induced sweet pepper plants

The olfactory response of the sweet pepper pests *F. occidentalis* and *B. tabaci* and the whitefly parasitoid *E. formosa,* to both mirid punctured plants and intact plants was firstly investigated in the Y-tube olfactometer. The Y-tube olfactometer consisted of two 5 L volume jars connected with a 2.4 cm diameter Y-shaped glass tube (Pérez-Hedo and Urbaneja, 2015), with a 13.5-cm long base and two arms each 5.75 cm long. Both side arms were connected via high density polyethylene (HDPE) tubes to the two identical glass jars. Each glass jar was connected to an air pump that produced a unidirectional humidified airflow at 150 mL min^{-1} (Pérez-Hedo and Urbaneja, 2015). Four 60-cm fluorescent tubes (OSRAM, L18W/765, OSRAM GmbH, Germany) were positioned 40 cm above the horizontal Y-shaped glass tube. The light intensity registered 2,516 lux over the Y-tube and was measured using a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc. Pullman, WA, USA). All Y-tube experiments were conducted under the following environmental conditions, 23 ± 2 ºC, 60 ± 10% RH.

In the entrance of the Y-tube we individually introduced each of the tested females of either *B. tabaci*, *F. occidentalis* or *E. formosa.* The result of the choice test was only recorded once the female had walked at least 3 cm up one of the arms or the assay was terminated after 15 minutes had elapsed and excluded from the data analysis. A total of 30-40 valid replicates from each species were recorded for each pair of odour sources. For each 5 collected responses the Y-tube was rinsed with soap, water and acetone and then left for 5 minutes to dry. Odour sources were switched between the left and right side arms to minimize any spatial effect on choice. All test plants were replaced after recording 10 responses.

To confirm the Y-tube observation a second choice experiment was conducted using 16 plastic cages (60 x 60 x 60 cm) (BugDorm-2 insect tents) maintained in a climatic chamber at 25 ± 2 ºC, 60-80% RH and 14:10 h (L:D) photoperiod. Inside each cage, three intact plants and three mirid-punctured plants (either by *N. tenuis* or *M. pygmaeus*) were arranged alternately in a circle. One hundred *F. occidentalis* or 100 *B. tabaci* adults were released separately in the centre of the circle of plants. *Frankliniella occidentalis* and *B. tabaci* were allowed to freely forage within the cage for 24 hours, the number on each plant treatment group (intact or mirid-punctured) were counted. The experiment was replicated four times for both *F. occidentalis* and *B. tabaci* to test their response to *N. tenuis*-punctured plants and *M. pygmaeus*-punctured plants.

3.2.6. Persistence of plant induction

In the Y-tube olfactometer we evaluated the persistence of the attraction or antixenosis induced by *N. tenuis* in order to induce indirect defences. The response of *B. tabaci* and *E. formosa* were tested at 4, 7 and

14 days after exposure to *N. tenuis*. Twenty-four hours after activation, *N. tenuis* adults were removed and punctured plants were left in enclosed plastic cages (30 x 30 x 30 cm) (BugDorm-1 insect tents) where the experiment was conducted. A total of 30 responses were recorded.

RNA extraction and gene expression (*ASR1*, *PIN2* and *PR1*) (Table 2.1; Chapter 2) was conducted to confirm Y- tube results. Eight apical regions from intact plants and from *N. tenuis*-punctured plants were collected. The same protocol as described above for quantitative PCR reaction was followed. According to the olfactometer results, the relative expression of defensive genes was performed 14 days after exposure to *N. tenuis* in comparison to intact plants.

3.2.7. Statistical analysis

Mirid behaviour on sweet pepper was analysed using two-way analysis of variance (ANOVA) to differentiate between predator species and sex, followed by comparison of means (Bonferroni post-tests) at $\alpha < 0.05$. One-tailed Student's *t*-test ($P < 0.05$) was performed to compare oviposition behaviour between the two mirid species. To compare between intact plants, *N. tenuis*-punctured plants and *M. pygmaeus*-punctured plants, the volatile profile from mirid-punctured plants and intact plants, phytohormone profile and defensive gene expressions were normalized using a logarithmic transformation and then analysed using a one way analysis of variance (ANOVA), followed by comparison of means (Tukey's test) at $\alpha < 0.05$. In the no-choice experiment, the number of thrips and whiteflies was compared between intact plant assays and mirid-punctured plant assays using a one-tailed Student's *t*-test ($P < 0.05$). To evaluate the persistence of plant defence induction, a one tailed *t*-test

($P< 0.05$) was performed to compare the quantified expression of defensive genes between intact plants and *N. tenuis*-punctured-plants over the time increments. Chi-square (χ^2) goodness of fit tests based on a null model were used to analyse data collected from the olfactory responses where the odour sources were selected with equal frequency. Individuals which did not make a choice were excluded from the statistical analysis. Results were expressed as the mean ± standard error.

3.3. Results

3.3.1. Mirid behaviour on sweet pepper

Both mirid species were found to spend the most time feeding on the plant (Table 3.1), with *N. tenuis* spending significantly more time feeding than *M. pygmaeus* ($F_{1,76}$= 22.37, $P< 0.0001$). Feeding behaviour between the sexes was not significantly different ($F_{1,76}$= 0.09, $P= 0.75$). However, a significant interaction between sex and species was found ($F_{1,76}$= 4.57, $P= 0.03$) with *N. tenuis* males tending to feed on the plant for a longer duration than females, whereas the contrary was observed for *M. pygmaeus*. Time duration of walking activity was higher for *M. pygmaeus* than that of *N. tenuis* ($F_{1,76}$=8.46, $P= 0.0048$) and males of both species spent significantly more time walking than females ($F_{1,76}$= 9.137, $P= 0.0034$). In contrast, females of both species spent significantly more time walking-antennating (walking accompanied by exploratory behaviour of the antennae) than males ($F_{1,76}$= 4.034, $P= 0.0481$), with no significant differences between species observed ($F_{1,76}$= 1.55, $P= 0.22$). For all other observed behaviours (antennating, grooming and flying), both

mirid species and sexes behaved similarly, with no significant difference observed (Table 3.1 and Table S1).

Residency on different plant localities was found to vary between species. *Nesidiocoris tenuis* was found to spend a significantly longer duration of time in the apical region of the sweet pepper plant than *M. pygmaeus* ($F_{1,76}$= 6.80, P= 0.01), while the opposite occurred in the basal region ($F_{1,76}$= 4.7, P= 0.03). No differences were found in residency between the sexes either in the apical ($F_{1,76}$= 3.739, P= 0.05) or in the basal regions ($F_{1,76}$= 0.80, P= 0.37). Males of both species remained for significantly longer time periods off-plant than the females ($F_{1,76}$= 4.75, P= 0.03) with no significant difference observed in time spent off-plant between the two mirid species ($F_{1,76}$= 6.21, P= 0.01) (Table 3.1).

Table 3.1.

Time in seconds (mean ± SE) spent by males and females of *N. tenuis* and *M. pygmaeus* in eight different behavioural states on sweet pepper plant, and the resident time (mean ± SE) of each predator on the three designed locations. Observation were performed during 30 minutes.

Behaviours	*Nesidiocoris tenuis*		*Macrolophus pygmaeus*	
	Female (n= 20)	Male (n= 20)	Female (n= 20)	Male (n= 20)
Grooming	105.1 ± 25.7 A	72.5 ± 27.3 A	72.7 ± 28.5 A	31.8 ± 11.2 A
Feeding	492.0 ± 74.1 A	660.6 ± 86.9 A	313.8 ± 74.1 B	187.9 ± 46.7 B
Flying	2.3 ± 1.0 A	4.4 ± 2.0 A	2.5 ± 1.5 A	5.4 ± 1.8 A
Ovipositing	28.4 ± 10.5 A	-	78.6 ± 30.4 A	-
Resting	504.9 ± 51.2 A	555.6 ± 34.9 A	605.4 ± 106.3 A	728.3 ± 105.1 A
Antennating	144.3 ± 41.5 A	90.6± 24.0 A	270.6 ± 76.0 A	175.3 ± 59.8 A
Walking	84.6 ± 20.7 B	201.2 ± 49.8 B*	193.6 ± 45.3 A	483.8 ± 114.7 A*
Walk.- Antenn.	338.0 ± 66.4 A	186.5 ± 62.6 A*	231.7 ± 63.6 A	145.6 ± 40.3 A*
Locations				
Apical region	1407 ± 114.4 A	1232 ± 159.7 A	1134 ± 149.8 B	749.7 ± 150.4 B
Basal region	154.7 ± 83.4 B	185.6 ± 89.9 B	482.2 ± 131.7 A	321.7 ± 113.5 A
Outside	132.7 ± 85.2 A	346.3 ± 117.5 A*	136.6 ± 68.7 A	677.3 ± 167.8 A*

Values followed by different letters and * in each row were significantly different between predator species and sexes, respectively (ANOVA *P*< 0.05)

3.3.2. Mirids impact both ABA and JA signaling pathways

The feeding behaviour of both mirid predators significantly altered the hormonal profile of sweet pepper plants (Fig.3.1). *Nesidiocoris tenuis* and *M. pygmaeus* feeding behaviour significantly increased the levels of ABA, JA and JA-Ile when compared to intact plants ($F_{2,14}$= 20.27, P< 0.0001 for ABA; $F_{2,14}$= 20.14; P< 0.0001 for JA; $F_{2,14}$= 9.36; P= 0.004 for JA-Ile) (Figs.3.1.a, c, d). Furthermore, the level of ABA was significantly higher following inoculation with *N. tenuis* which may suggest a higher impact on plant's metabolism than *M. pygmaeus* feeding behaviour. In the case of SA, plants punctured by feeding behaviour of either mirid species displayed increased concentrations of this hormone, but these differences were not significantly different between species ($F_{2, 14}$= 3.26; P= 0.074).
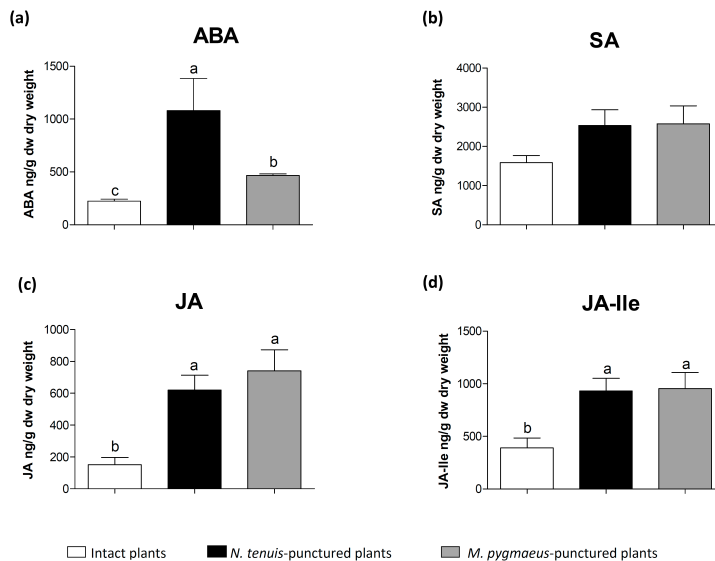
Figure 3.1.

Comparison between the phytohormone levels in the apical regions of intact sweet pepper plants, *N. tenuis*-punctured plants and *M. pygmaeus*-punctured plants, **(a)** ABA, **(b)** SA, **(c)** JA and **(d)** JA-Ile. The presented results are the mean hormone level of five independent analyses ± SE (n=5). Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test α< 0.05).

The quantification of *ASR1*, *PIN2* and *PR1* gene expression displayed upregulation of the *ASR1* and *PIN2* genes in plants punctured by either mirid species ($F_{2, 21}$= 10.10, *P*= 0.001 for *ASR1* and $F_{2, 21}$= 15.27, *P*= 0.0005 for *PIN2*), whereas only *N. tenuis* was able to upregulate the gene *PR1* ($F_{2, 21}$= 10.29; *P*= 0.0017) (Fig.3.2) when compared with the intact sweet pepper plants.
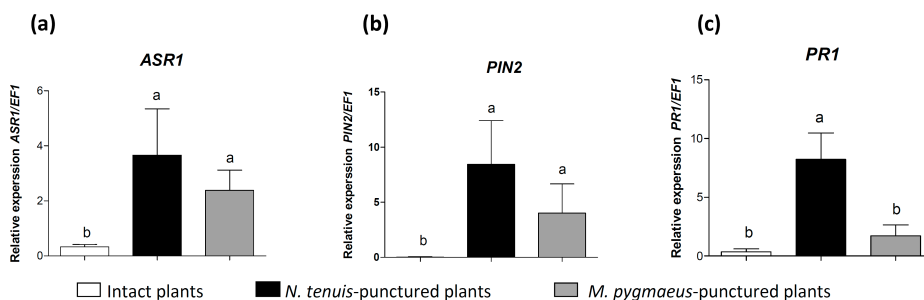
Figure 3.2.

Quantification of defensive genes in the apical regions of intact sweet pepper plants, *N. tenuis*-punctured plants and *M. pygmaeus*-punctured plants, **(a)** *ASR1*, **(b)** *PIN2* and **(c)** *PR1*. Data are presented as the mean of eight independent analyses of transcript expression relative to a housekeeping gene ± SE (n=8). Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test *α*< 0.05).

3.3.3. Mirids significantly altered the volatile blend following inoculation

The untargeted analysis of the volatiles emitted by the tomato plants facilitated the identification of 14 compounds differentially emitted between mirid-punctured and intact plants (Table 3.2) based on their mass spectra and coelution with pure standards, when available. Compounds were identified as terpenoids (2 monoterpenoids, 3 sesquiterpenoids and 1 norisoprenoid),

green leaf volatiles (5 esters ((Z)-3-hexenyl acetate, (Z)-3-hexenyl propanoate, (Z)-3-hexenyl butanoate, (Z)-3-hexenyl 3-methylbutanoate and (Z)-3-hexenyl benzoate) and their common precursor (Z)-3-hexenol), and two further compounds methyl salicylate and octyl acetate. Octyl acetate was only detected in *M. pygmaeus* punctured plants (Table 3.2). All of the identified compounds showed significantly increased emission in punctured plants when compared to intact, ranging from 9-fold to 130-fold.

3.3.4. Mirid infestation triggers parasitic wasp attraction and induces pest antixenosis

In the Y-tube olfactometer, the phytophagous species *F. occidentalis* and *B. tabaci* displayed a significant positive response towards the odour source emitted by intact sweet pepper plants when compared to either *N. tenuis*-punctured plants (χ^2= 10.90; *P*= 0.001 and χ^2= 6.67; *P*= 0.0098, respectively) or *M. pygmaues* punctured plants (χ^2= 10.45; *P*= 0.0012 and χ^2= 10.45; *P*= 0.0012, respectively) (Fig.3.3.a, b). In contrast, *E. formosa* displayed a significant attraction towards the sweet pepper plants punctured by *N. tenuis* (χ^2= 6.48; *P*= 0.01) and *M. pygmaeus* (χ^2= 11.08; *P*= 0.0009) relative to intact plants (Fig.3.3).

Table 3.2.

Significant relative levels (fold changes) of the volatiles emitted by N. tenuis and M. pygmaeus punctured sweet pepper plants relative to intact plants. One way ANOVA, Tukey's multiple comparison test α< 0.05.

Type	Compound	Kovats RI	Fold change		$F_{2,12}$	P
			Nesidiocoris tenuis vs control	Macrolophus pygmaeus vs control		
Terpenoids: monoterpenoids	Linalool*	1103	9.10	22.90	33.3	**0.0002**
	Monoterpene	1050	15.12	-	5.5	**0.0242**
Terpenoids: sesquiterpenoids	Sesquiterpene[1]	1418	96.52	130.61	10.7	**0.0033**
	(E)-nerolidol*	1574	10.50	14.48	18.9	**0.0004**
	Sesquiterpenoid	1583	120.96	85.34	18.7	**0.0004**
Terpenoids: norisoprenoids	Unknown[2]	1098	46.51	54.40	40.6	**P<0.0001**
	(Z)-3-hexenol*	857	-	21.09	6.5	**0.0156**
	(Z)-3-hexenyl acetate*	1002	12.15	84.90	16.8	**0.0006**
	(Z)-3-hexenyl propanoate*	1096	17.07	12.54	5.5	**0.0238**
Green leaf volatile: Esters	(Z)-3-hexenyl butanoate*	1184	48.27	36.19	6	**0.0195**
	(Z)-3-hexenyl 3-methylbutanoate*	1236	54.81	35.75	12.7	**0.0018**
	(Z)-3-hexenyl benzoate	1596	-	24.43	5.1	**0.0297**
Systemic acquired resistance (SAR)	Methyl salicylate*	1215	28.89	34.50	34	**P<0.0001**
Other	Octyl acetate	1206	-	**	-	-

Tentative identification based on mass spectra: 1 beta-elemene, 2norisoprenoid C11H18.
*Unequivocal identification (confirmed with a pure standard)
** only detected in M. pygmaeus-punctured plant
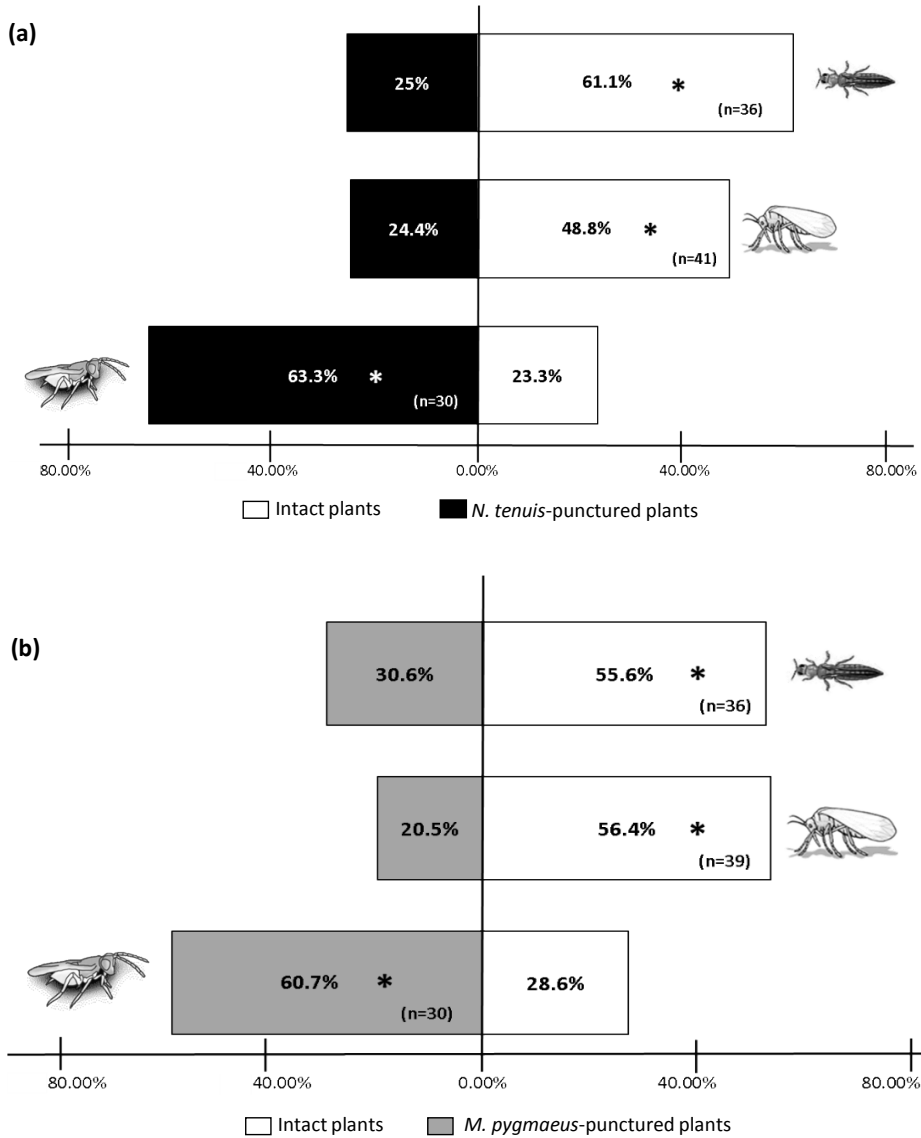
Figure 3.3.

Olfactory response of the selected insects to mirids-punctured plants in comparison to intact plants. **(a)** Response of *F. occidentalis* (n=36), *B. tabaci* (n=41) and *E. formosa* (n=30) in the Y-tube olfactometer when exposed to intact sweet pepper plants and *N. tenuis*-punctured plants. **(b)** Response of *F. occidentalis* (n=36), *B. tabaci* (n=39) and *E. formosa* (n=30) in the Y-tube olfactometer when exposed to intact sweet pepper plants and *M. pygmaeus*-punctured plants. Significant differences based on a χ2-test are marked using * (*P< 0.05*).

In the cage experiments containing both *N. tenuis* punctured and intact plants a significantly lower number of *F. occidentalis* (t= 5.55; P= 0.0007) and *B. tabaci* (t= 3.60; P= 0.006) were found on *N. tenuis*-punctured plants than the control plants. In cage experiments containing *M. pygameus*-punctured plants and intact plants, again significantly lower numbers of *F. occidentalis* and *B. tabaci* individuals were found on *M. pygmaeus*-punctured plants (t= 5.07; P= 0.0011; t= 5.68; P= 0.0006, respectively) (Figs.3.4.a, b).
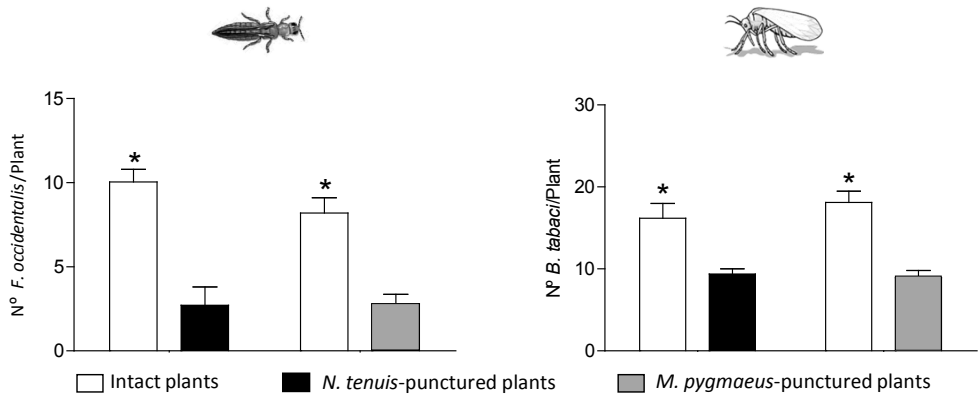
Figure 3.4.

Herbivores choice between mirid punctured plants and intact plants. **(a)** Number of *F. occidentalis* adults per plant (X ± SE) captured 24 hours after releasing 100 *F. occidentalis* in the centre of a cage containing 3 intact plants and 3 *N. tenuis*/*M. pygmaeus*-punctured plants. **(b)** Number of *B. tabaci* adults per plant (X ± SE) captured 24 hours after releasing 100 *B. tabaci* in the centre of a cage containing 3 intact plants and 3 *N. tenuis*/*M. pygmaeus*-punctured plants. Both mirid species were in contact with the plants only 24 h and then removed. Significant difference resulting from a one tailed *t*-test are marked with (*) (P< 0.05).

3.3.5. Indirect defences triggered by *N. tenuis* last for several weeks

The parasitoid, *E. formosa*, was significantly attracted to *N. tenuis*-punctured plants which were previously activated by the mirid, *N. tenuis*, 4, 7 and 14 days before (χ^2= 9.60; *P*= 0.0019; χ^2= 6.25; *P*= 0.0124; χ^2= 4.27; *P*= 0.04 for 4, 7 and 14 days, respectively). In contrast, the phytophagous pest species *B. tabaci* was significantly repelled to *N. tenuis*-punctured plants, but only those plants activated 4 and 7 days before (χ^2= 9.80; *P*= 0.0017; χ^2= 4.27; *P*= 0.04, respectively). This repellent effect was not observed at day 14, where both plant treatments induced similar attraction response in *B. tabaci* (χ^2= 0.26; *P*= 0.60) (Fig. 3.5.a). The relative expression of the genes *ASR1*, *PIN2* and *PR1* quantified at day 14 after activation showed that the three genes were upregulated in *N. tenuis*-punctured plants when compared to intact plants (*t*= 4.51, *P*= 0.004; *t*= 4.101, *P*= 0.006 for *ASR1*, *PIN2* and *PR1*, respectively) (Fig. 3.5.b).

(a)

(b)

☐ Intact plants   ■ 14 days after exposure to *N. tenuis* punctures ended
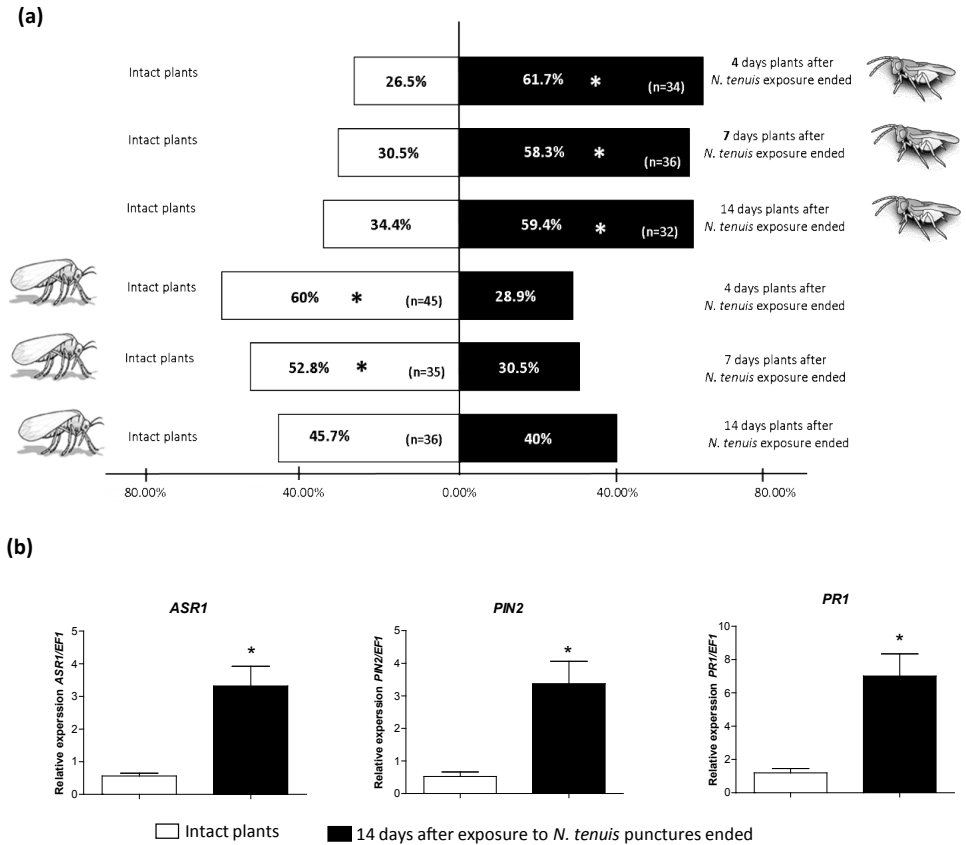
Figure 3.5.

Persistence of sweet pepper induction following *N. tenuis* punctures. **(a)** Response of *E. formosa* and *B. tabaci*, respectively to *N. tenuis*-punctured plants *vis* intact plants after 4 days, 7 days and 14 days exposure ended. Significant differences based on a χ^2-test are marked using * (*P*< 0.05). **(b)** Relative expression of defensives genes *ASR1*, *PIN2* and *PR1* in intact sweet pepper plants with comparison to *N. tenuis*-punctured plants, 14 days after exposure ended. Data are presented as the mean of eight independent analyses of transcript expression relative to the constitutive *EF1* gene ± SE (n=8). Significant difference from a one tailed *t*-test are marked with (*) (*P*< 0.05).

3.4. Discussion

In this study, feeding activity by the zoophytophagous mirid predators, *N. tenuis* and *M. pygmaeus* has been shown to induce defensive responses in sweet pepper plants for the first time. Both predatory mirid species spent significantly more time feeding than any other activity on sweet pepper plant, an important behaviour known to facilitate establishment of the predator in the crop and maintain a population in periods of prey scarcity (Perdikis and Lykouressis, 2000; Urbaneja *et al.*, 2005). When released after 24 hours of starvation, (Montserrat *et al.*, 2000; Durate *et al.*, 2014) *N. tenuis* feeding behaviour was observed at 33% of total observed activity, more than double that of *M. pygmaeus* (14%). Both species displayed a preference for feeding on the apical region of the sweet pepper plant, though the strongest preference was observed in *N. tenuis*, with 93% of feeding activity occurring in this region opposed to 64% in *M. pygmaeus.* These observations are in line with earlier studies which showed that both predatory species occupy different strata of the tomato plant when cohabitating the same plant, with *N. tenuis* spending significantly more time on the uppermost region of the plant and *M. pygmaeus* on the lower leaves of apical region (Perdikis *et al.*, 2014).

Despite the large amount of time spent by both species of mirids feeding on the sweet pepper plants, as of yet, neither of the two species have been described producing crop damage which could affect yield (Urbaneja *et al.*, 2005). Indeed, *M. pygmaeus* is considered a safe and efficient candidate to be used for sweet pepper IPM strategies in Northern Europe to supplement aphid control (Messelink *et al.*, 2015; Pérez-Hedo and Urbaneja, 2015). The use and conservation of *N. tenuis* as a biocontrol agent in sweet pepper

is uncommon in Europe, particularly in the Mediterranean basin where this mirid is naturally abundant. However, in other pepper producing regions such as greenhouses in Kochi Prefecture, Japan, *N. tenuis* is considered a key natural enemy against whiteflies, aphids and thrips, where despite reaching high populations it has not been described to cause damage through plant feeding (Komi, 2016).

Despite significant differences in plant feeding behaviour between the two mirid species, the level of cell wounding was sufficient in both species to activate the defence mechanisms in sweet pepper, as has been described in tomato plants (Pérez-Hedo *et al.*, 2015a, 2015b; Pappas *et al.*, 2015, 2016; Naselli *et al.*, 2016). A significant increase in ABA and JA signalling pathways was found in both *N. tenuis* and *M. pygmaeus*-punctured plants which are co-regulated in response to wounding. This was in accordance with the results of the relative expression of the target defensive genes, *ASR1* and *PIN2*, respectively. Nevertheless, the levels of the phytohormone SA, which has been described as an herbivore repellent in previous studies, (Erb *et al.*, 2012; Shi *et al.*, 2016; Liu *et al.*, 2016) were not significantly different between mirid-punctured plants and intact plants, although there was a tendency for it to be higher in punctured plants. Furthermore, the related gene *PR-1* was upregulated for *N. tenuis*-punctured plants but not for *M. pygmaeus*-punctured plants. *PR-1* has been recognised as a SA marker gene, but it is also responsive to external stimuli and internal signals such as azelaic acid or pipecolic acid which were not determined in the present study (Jung *et al.*, 2009; Návarová *et al.*, 2012). It is therefore likely that mirid inoculation enhances the levels of other internal stimuli. In addition, MeSA, a compound which plays an antagonistic role with free SA levels and a synergistic role

with JA signalling (Boachon *et al*., 2014), is significantly increased following inoculation by either mirid species.

The results confirmed that the release of VOCs by punctured-sweet pepper induces the observed repellency and attractiveness to the tested phytophage and natural enemy species. Indeed, plants exposed to *N. tenuis* and *M. pygmaeus* feeding were associated with repellence of both arthropod pests, *F. occidentalis* and *B. tabaci*, and attraction of the parasitoid *E. formosa*. Similarly, the feeding activity of *N. tenuis* in tomato plants have been found to be responsible for the repellence of *B. tabaci* and *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), and for the attraction of *E. formosa* (Pérez-Hedo *et al*., 2015a, 2015b). However, unlike the induced plant response to *N. tenuis* feeding activities, those induced by *M. pygmaeus* and *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) were found not to repel *B. tabaci* and became attractive to *T. absoluta* (Pérez-Hedo *et al*., 2015b).These results in tomato were found to be related to the upregulation of ABA and JA signalling pathways (Pérez-Hedo *et al*., 2015a), and suggest that *M. pygmaeus* causes a distinct response in tomato and pepper and are consequently capable of emitting different blends of volatiles.

The HIPVs identified in this work were classified in three important groups, green leaf volatiles (GLVs) involving the fatty acid/lipoxygenase biosynthesis pathway, terpenes (the isoprenoid pathway) and methyl salicylate, MeSA, (the shikimic acid pathway). A future step would be to identify the role of each HIPV within the blend and their capacity to repel and/or attract different sweet pepper pests. Of the identified volatile compounds, octyl acetate was only recorded in *M. pygmaeus*-punctured plants. Octyl acetate has been described as a specific compound acting as sexual pheromone emitted by females on some species of the Miridae family

such as *Phytocoris* spp (Millar *et al.*, 1997; Millar and Rice, 1998; Zhang and Aldrich, 2008). It could be that this compound was emitted by *M. pygmaeus* and traces were left on the plant. In any case, the role of this volatile on *M. pygmaeus* deserves further investigation.

Under cage conditions, choice experiments showed that *B. tabaci* and *F. occidentalis* were both less likely to reside on mirid-punctured plants than on intact plants. This lower preference might be a consequence of indirect defence induction mediated by mirids. VOCs inside the box might be mixed-up, hence the consequence of unequal distribution of both pests may be attributed to the contact and feeding upon the plants with high content on JA, which can be a feeding deterrent for arthropod pests (Santamaria *et al.*, 2013; Zhurov *et al.*, 2014). *Macrolophus pygmaeus*-punctured tomato plants were observed to increase locally and systematically the accumulation of transcripts and activity of protease inhibitors that are known to be involved in plant responses, resulting in the decreased life history traits of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (Pappas *et al.*, 2015). In the case of sweet pepper, further research should be done to elucidate these direct defence effects on subsequent herbivore development and reproduction.

Tomato plants exposed to *M. pygmaeus* with all individuals subsequently removed, as in this study, were previously described to remain vaccinated for up to two weeks (Pappas *et al.*, 2015). The impact of *N. tenius* in sweet pepper is demonstrated to remain active for 7 to 14 days. The latter finding would be useful for growers applying a nursery release of *N. tenuis* as a vaccine, adopting such a practice on sweet pepper crops might increase resilience to pest attacks. This would be an added benefit of *N. tenuis* and *M. pygmaeus* in order to effectively manage the key sweet pepper pests,

B. tabaci and *F. occidentalis*. After vaccination, mirids could become established in the crop so they could further contribute to the management of sweet pepper pests. However, the efficacy of *N. tenuis* and *M. pygmaeus* preying upon a mixed diet of sweet pepper pests and their compatibility with other natural enemies already adapted to sweet pepper, such as *A. swirskii* or *O. laevigatus* warrant further investigation. Another application derived from this study would be the ability to manipulate the attractant and repellent capacity of sweet pepper by exposure to HIPVs. As an example, the use of volatile dispensers to emit regular concentrations of one or a blend of these volatiles could result in saturated repellent and attractant environments for pests and natural enemies, respectively. Exploring the capacity to activate plant defences in intact sweet pepper by exposing the plants to these volatiles or volatile blends, would open the door to new ways of pest control in sweet pepper as successfully demonstrated in tomato plants (Pérez Hedo *et al.*, 2015a).

Supporting Information

Table S.1.

Statistics (P, F and degree freedom values) for the two-way ANOVA comparison of time spent by males and females of *N. tenuis* and *M. pygmaeus* in eight different behavioural states on sweet pepper plant, and the resident time of each predator and sex on the three designed locations.

Behaviours	Species	Sex	Species * Sex
Grooming	$F_{1,76} = 2.28$, $P= 0.13$	$F_{1,76}= 2.30$, $P= 0.13$	$F_{1,76}= 0.03$, $P= 0.86$
Feeding	$F_{1,76} = 22.37$, $P < 0.0001$	$F_{1,76}= 0.10$, $P= 0.76$	$F_{1,76}= 4.58$, $P= 0.03$
Flying	$F_{1,76} = 0.11$, $P= 0.74$	$F_{1,76}= 2.30$, $P= 0.13$	$F_{1,76}= 0.07$, $P= 0.78$
Ovipositing	$t_{1,38} = 1.56$, $P= 0.13$	/	/
Resting	$F_{1,76} = 2.85$, $P= 0.09$	$F_{1,76}= 1.15$, $P= 0.29$	$F_{1,76}= 0.20$, $P= 0.66$
Antennating	$F_{1,76} = 3.82$, $P= 0.05$	$F_{1,76}= 1.90$, $P= 0.17$	$F_{1,76}= 0.15$, $P= 0.70$
Walking	$F_{1,76} = 8.46$, $P= 0.0048$	$F_{1,76}= 9.14$, $P= 0.003$	$F_{1,76}= 1.67$, $P= 0.20$
Walk.- Antenn.	$F_{1,76} = 1.55$, $P= 0.22$	$F_{1,76}= 4.03$, $P= 0.05$	$F_{1,76}= 0.31$, $P= 0.58$
Locations			
Apical region	$F_{1,76} = 6.81$, $P= 0.01$	$F_{1,76}= 3.47$, $P= 0.06$	$F_{1,76}= 0.52$, $P= 0.47$
Basal region	$F_{1,76} = 4.75$, $P= 0.03$	$F_{1,76}= 0.81$, $P= 0.37$	$F_{1,76}= 0.37$, $P= 0.54$
Outside	$F_{1,76} = 2.61$, $P= 0.11$	$F_{1,76}= 4.75$, $P= 0.03$	$F_{1,76}= 6.28$, $P= 0.014$

Comparative Biocontrol Potential of Three Predatory Mirids when Preying on Sweet Pepper Key Pests

4

Comparative biocontrol potential of three predatory mirids when preying on sweet pepper key pests

Sarra Bouagga, Alberto Urbaneja, Meritxell Pérez-Hedo

Abstract

Pest management in protected sweet pepper crops mainly rely on biological control strategies. Recently, the zoophytophagous predatory mirids, *Nesidiocoris tenuis*, *Macrolophus pygmaeus*, and *Dicyphus maroccanus*, proved to be effective in the control of aphids on sweet pepper, for which the current biological control strategies have been meagre. The next step to integrate the possible use of these mirids in sweet pepper biological control practices would be to ascertain their potential control on other sweet pepper pests. In this research, a comparative study to assess the establishment and the efficacy of *N. tenuis*, *M. pygmaeus*, and *D. maroccanus* on the two sweet pepper key pests; the thrips, *Frankliniella occidentalis*, and the whitefly, *Bemisia tabaci*, was conducted. This study was carried out with two different temperatures regimes, 20 ºC and 27 ºC, which simulated the mean temperature registered in the two main crop cycles in Spain (the winter and summer planting period). Both, *N. tenuis* and *M. pygmaeus*; were able to establish on sweet pepper and significantly reduced the number of *F. occidentalis* and *B. tabaci* adults, larvae and nymphs. *Macrolophus pygmaeus* had the highest density at 20 ºC, whereas *N. tenuis* was more abundant at 27 ºC. In contrast, *D. maroccanus* was less abundant under both temperatures studied; and did not reduce neither *F. occidentalis* nor *B. tabaci* infestations in this crop. None of the three mirids were observed to cause any damage to the pepper plant. The implications of these results applied to the use of mirids in sweet pepper crops are discussed.

Key words: *Nesidiocoris tenuis*, *Macrolophus pygmaeus*, *Dicyphus maroccanus*, biological control, temperature regime.

4.1. Introduction

Protected sweet pepper (*Capsicum* spp.) is one of the most strategic horticulture crops, widely cultivated in the South-eastern Spain. During the last twenty years, the ever demanding standards for healthy, residue-free products have pushed the growers to explore and adopt environmentally friendly strategies to manage sweet pepper pests (Calvo *et al.*, 2009a; van Lenteren, 2012). The western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Tripidae), is one of the most serious pests of sweet pepper, both in the greenhouse and in the open field (Tommasini and Maini, 1995; Van Driesche *et al.*, 1998). In addition to the direct damages, such as fruit abortion or fruit scarring, caused by *F. occidentalis*, it may cause important indirect damages through its role as a vector of the *tomato spotted wilt virus* (TSWV) (Lacasa *et al.*, 1991; Peters, 1996). Additionally, the sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), is considered to be a secondary pest; which causes direct injury as a result of sap removal, honeydew build-up with sooty mould, physiological disorders, and the transmission of plant viruses (De Barro *et al.*, 2011; Fortes *et al.*, 2012). Therefore, the biological control of both pests in sweet pepper has become a priority in South-eastern Spain. Since the end of the last century, many natural enemies of thrips and whiteflies have been reported and evaluated for their efficacy (Sánchez *et al.*, 2000, Sánchez and Lacasa, 2002; Urbaneja *et al.*, 2001, 2002; Stansly *et al.*, 2005; Blom *et al.*, 2003). All these studies have led to the current pest management strategy in sweet pepper, which is based on the release of the generalist biocontrol agents, the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) and the minute pirate bug *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) (Calvo *et al.*,

2009a). The augmentative release of both predators successfully manages two of the key pests of this crop, *B. tabaci* and *F. occidentalis* (Blom, 2008, Calvo *et al*., 2009a).

Despite the success of the current biological control-based management program in sweet pepper, better control of aphids in this crop is still needed (Bloemhard and Ramakers, 2008; Belliure *et al*., 2008, Sánchez *et al*., 2011). Traditionally, aphids have been managed through the release of a combination of specialized predators, mainly the predatory midge *Aphidoletes aphydimiza* Rondani (Diptera: Cecidomyiidae) and parasitoids, mainly *Aphidius Colemani* Viereck (Hymenoptera: Braconidae) (Blom, 2008). However, the need for multiple releases of natural enemies increases the final cost of the biocontrol program (Messelink *et al*., 2011a). In addition, these releases can be disrupted by the abundance of hyperparasitoids (Sánchez e*t al*., 2011), and by predator interference, where *A. swirskii* and *O. laevigatus* display intraguild predatory behaviour on the eggs of the midge (Messelink *et al*., 2011b; Hosseini *et al*., 2010). Therefore, there is a continuous need of investigations to identify and evaluate alternative natural enemies that can be used for the management of aphids in this crop.

Generalist predators are widely known to contribute to the management of a wide range of agricultural pest, including aphids, around the world (Perdikis and Lykouressis, 2004, Messelink *et al*., 2011a, 2015; Pérez-Hedo and Urbaneja, 2015; Sylla *et al*., 2016). Previous studies have demonstrated that under laboratory conditions the zoophytophagous mirid predators, *Nesidiocoris tenuis* Reuter and *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), successfully feed upon the most commonly occurring sweet pepper aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae), also known as the green peach aphid (Perdikis and Lykouressis 2002, 2004;

Valderrama *et al.*, 2007; Fantinou *et al.*, 2009). More recently, under semi-field conditions, Pérez-Hedo and Urbaneja (2015) observed how *N. tenuis*, *M. pygmaeus*, and *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) significantly reduced the number of *M. persicae* in sweet pepper plants, reaching a level of aphid reduction close to 100%. Furthermore, Messelink *et al.* (2015) and De Backer *et al.* (2015) showed the release of *M. pygmaeus* and *D. tamaninii* Wagner (Hemiptera: Miridae) prior to infestation in combination with the application of supplemented food enhanced the management of *M. persicae* on sweet pepper. These results suggest the use of mirids in sweet peppers may just be the alternative needed in aphid management. However, the capacity of mirids to reduce thrips and whiteflies in sweet pepper crops has not been completely characterized.

To this end, we evaluated the efficacy of *N. tenuis*, *M. pygmaeus* and *D. maroccanus* as predators of thrips and whiteflies and also assessed the establishment of the mirids, on sweet pepper plants under two different temperature regimes (20 ºC and 27 ºC). Both selected temperatures simulated the registered means in the two main sweet pepper planting cycles in the Southeast of Spain.

4.2. Material and methods

4.2.1. Insects and Plants

Nesidiocoris tenuis, M. pygmaeus, and the whitefly, *B. tabaci* were obtained from a commercial supplier (NESIBUG® and MYRICAL®; Koppert Biological Systems, S.L., Águilas, Murcia, Spain). Each mirid species was provided in plastic bottles containing 500 individuals (mature

nymphs and young adults), approximately 3-day-old specimens (FJ Calvo, Koppert BS; Personal Communication). Each predator species was released separately on sweet pepper plants "var. Lipari" (Dulce italiano, Mascarell semillas S.L, Valencia, Spain) until used, inside 60 × 60 × 60-cm plastic cages (BugDorm-2 insect tents, MegaView Science Co., Ltd., Taichung, Taiwan) and supplied with *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Entofood®; Koppert B.S.) as additional food. *Bemisia tabaci* adults were kept on sweet pepper plants (the same cultivar, as described above) inside plastic tents in 30 × 30 × 30-cm plastic cages (BugDorm-1 insect tents, MegaView Science Co., Ltd., Taichung, Taiwan), until their use. The individuals of the third mirid species, *D. maroccanus*, were obtained from a laboratory colony on pesticide free tomato seedlings "var. Optima" which had already been established in the Instituto Valenciano de Investigaciones Agrarias (IVIA), as described in Pérez-Hedo and Urbaneja (2015).

The *F. occidentalis* adults, were also obtained from a colony previously established at IVIA in 2010 originally collected from Campo de Cartagena (Murcia, Spain). The thrips colony was raised on bean plants (*Phaseolus vulgaris* L., Fabales: Fabaceae). All stock colonies described above were housed in climatic chambers at 25 ± 2 ºC, 65 ± 10% RH under a 14:10 h (L:D) photoperiod at IVIA.

The pesticide-free sweet pepper seedlings (*Capsicum annuum* L. var. "Lipari") were transplanted into plastic pots (8 × 8 × 8 cm) containing a mixture of natural soil with local peat moss and were housed in a climatic chamber under the same environmental conditions as described above. Plants (approximately 25 cm in height) with 12 fully developed leaves were used for the experiments described below.

4.2.2. Experimental design and sampling

The experiment was conducted in plastic screened cages (60 × 60 × 60-cm BugDorm-2, as described above), which were maintained in two identical cabinets of a glasshouse located at IVIA, under two different temperature regimes, 20 ± 2 ºC and 27 ± 2 ºC. The relative humidity was 65 ± 10% and the natural photoperiod was used. Temperature and relative humidity were maintained during the entire duration of the experiment by the climate controller Ambitrol 500 (Sistemes Electrònics Progrès SA, Bellpuig. Spain). The experiment was carried out during the eleven weeks; between mid-March to the beginning of June, 2015.

To evaluate the efficiency of *N. tenuis*, *M. pygmaeus*, and *D. maroccanus* to control a mixed infestation of *F. occidentalis* and *B. tabaci* on sweet pepper a randomized complete block design with four treatments (release of *N. tenuis*, release of *M. pygmaeus*, release of *D. maroccanus* and a control without predator release) replicated four times each (cage=replicate) were used in both of the two glasshouse cabinets. Mirids were released stimulating the strategy of predator in first (pre-plant release) (Calvo *et al.*, 2012b). This strategy entails mirids being released in the nursery 5-7 days before transplanting the sweet pepper plants upon which mirid individuals had already laid eggs; therefore, small nymphs and adults were still present. In each replicate, eight healthy sweet pepper plants (25 cm high) and four couples (male/female) of each species of mirid were introduced on the same day (1 adult/plant). Each mirid species was separately released in quadruplets. The control treatment did not receive mirid releases. During the first two weeks of the experiment, approximately 0.1 g / plant of frozen eggs of *E. kuehniella* were equally distributed on the plants by

manually sprinkling. The addition of this alterntative food facilitated mirid establishment and oviposition (Urbaneja-Bernat *et al.*, 2015). The plants were irrigated twice a week throughout the experiment.

Two weeks after the release of mirids, 8 couples (male/female) (5 adults/m²) of *F. occidentalis* and 16 couples (10 adults/m²) of *B. tabaci* were introduced per cage eight times (1 infestation per week). Both pests were separately introduced into Petri dishes that were then left open at the base of the plant in each of the cages. These selected rates were chosen to simulate a strong and early whitefly and thrips infestation.

One week after the first pest infestation, the samplings were started in both of the glasshouse cabinets at 20 ± 2 ºC and 27 ± 2 ºC. From four randomly selected plants per replicate; three leaves (one from the upper, one from middle, and one from the lower part of the plant) were inspected and the number of live *F. occidentalis* and *B. tabaci* adults, larvae, and nymphs were recorded. The same whole plants were also inspected to count the total number of mirid adults and nymphs. Eight evaluations (one per week) were conducted. Special care was always taken to sample the control cages first and then the cages containing the predators, to reduce the risk of accidental contamination among the treatments.

4.2.3. Statistical analysis

The total number of *F. occidentalis* and *B. tabaci* per sampled leaf and the total number of mirids per plant were log (x+1) transformed prior to analysis using Generalized Linear Mixed Models (GLMM). Treatment was considered to be a fixed factor while time in weeks, a random one. Each GLMM used a normal distribution and identity link function.

Untransformed values are presented in the figures. Whenever a significant difference was found, pairwise comparisons of the fixed factor levels were performed with the least significant difference (LSD) post-hoc test ($P < 0.05$). To calculate the percentage of *F. occidentalis* and *B. tabaci* reduction; Abbott's formula was applied (Abbott, 1925) using the number of *F. occidentalis* and *B. tabaci* accumulated at the end of the experiment (week 8; the area under the duration of the experiment incidence curve) (Calvo *et al.*, 2009b). To know whether differences between the percentages of reduction existed, data were log transformed then a one-way analysis of variance (ANOVA) followed by comparison of means (Tukey's test) at $\alpha < 0.05$ or one-tailed Student's *t* test ($P < 0.05$) was performed. The results were expressed as the means ± standard error.

4.3. Results

4.3.1. *Frankliniella occidentalis* management

In the glasshouse at 20 ºC, the three species of mirids significantly reduced the populations of *F. occidentalis*, when compared to the control (Fig. 4.1.a) (Table 4.1). However, the number of *F. occidentalis* was significantly lower in the cages where *N. tenuis* and *M. pygmaeus* were released when compared to the cages in which *D. maroccanus* was released.

Table 4.1.

P values for the pairwise comparison of the number of *F. occidentalis* (adults + larvae) per leaf which received a release of *D. maroccanus* (*Dm*), *M. pygmaeus* (*Mp*), *N. tenuis* (*Nt*) and control without release (C). Values in bold correspond to significant differences between the treatments.

Treatments	20 ºC		27 ºC	
	t_{124}	P	t_{124}	P
C vs. *Dm*	**2.881**	**0.005**	**3.914**	**< 0.001**
C vs. *Mp*	**6.572**	**< 0.001**	**6.677**	**< 0.001**
C vs. *Nt*	**5.950**	**< 0.001**	**6.601**	**< 0.001**
Dm vs. *Mp*	**3.691**	**< 0.001**	**2.763**	**0.007**
Dm vs. *Nt*	**3.070**	**0.003**	**2.687**	**0.008**
Mp vs. *Nt*	0.621	0.536	0.076	0.940

No significant difference in the percentage of *F. occidentalis* reduction was found between the sweet pepper plants that received releases of *N. tenuis* or *M. pygmaeus*. At week 8, *N. tenuis* and *M. pygmaeus* reduced the infestation of *F. occidentalis* by 82% and 87%, respectively. In contrast, *D. maroccanus* only achieved a 33% reduction, which was significantly lower when compared to the reduction achieved by *M. pygmaeus* and *N. tenuis* (F_{2-11} = 12.42; *P*= 0.003) (Fig.4.1.b). Similar results were recorded from cages maintained in the glasshouse at 27 ºC (Table 4.1). *Nesidiocoris tenuis* and *M. pygmaeus* were more voracious and reduced the infestation of *F. occidentalis* by 89% and 90%, respectively, at week 8 (F_{2-11} = 16.95; *P*= 0.001). *Dicyphus maroccanus* was less efficient and reduced 45% of the *F. occidentalis* population when compared to control treatment (Fig.4.1.d). At this temperature, no differences were observed between the percentages reduction of *F. occidentalis* in sweet pepper, which received releases of *N. tenuis*, and those which received *M. pygmaeus* (Table 4.1).

20 °C

27 °C

Figure 4.1.

(a) Number (mean ± SE) of *F. occidentalis* (adults + larvae) per sweet pepper leaf at 20 ºC, **(b)** percentage reduction (Abbott) (mean ± SE) of *F. occidentalis* individuals at 20 ºC, **(c)** number (mean ± SE) of *F. occidentalis* (adults + larvae) per sweet pepper leaf at 27 ºC, and **(d)** percentage reduction (Abbott) (mean ± SE) of *F. occidentalis* individuals at 27 ºC in a glasshouse experiment comparing the effectiveness of the mirid predators, *N. tenuis*, *M. pygmaeus*, and *D. maroccanus* at different time intervals under the two temperature regimes mentioned above. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test *α* < 0.05).

4.3.2. *Bemisia tabaci* management

The number of *B. tabaci* per sampled leaf in the cages maintained in the glasshouse at 20 ºC was low for all the treatments. The release of *M. pygmaeus* and *N. tenuis* significantly reduced the *B. tabaci* infestation when compared to the *D. maroccanus* and control cages. No significant differences were found between the numbers of *B. tabaci* counted in *D. maroccanus* and control cages (Fig.4.2.a) (Table 4.2).

Table 4.2.

P values for the pairwise comparison of the number of *B. tabaci* (adults + nymphs) per leaf which received a release of *D. maroccanus* (*Dm*), *M. pygmaeus* (*Mp*), *N. tenuis* (*Nt*) and control without release (C). Values in bold correspond to significant differences between the treatments.

Treatments	20 ºC		27 ºC	
	t_{124}	*P*	t_{124}	*P*
C vs. *Dm*	1.736	0.085	**2.509**	**0.013**
C vs. *Mp*	**5.413**	**< 0.001**	**5.257**	**< 0.001**
C vs. *Nt*	**4.456**	**< 0.001**	**5.262**	**< 0.001**
Dm vs. *Mp*	**3.677**	**< 0.001**	**2.748**	**0.007**
Dm vs. *Nt*	**2.720**	**0.007**	**2.753**	**0.007**
Mp vs. *Nt*	0.957	0.340	0.005	0.996

The infestation by *B. tabaci* at week 8 was reduced by 82% and 65% by *M. pygmaeus* and *N. tenuis,* respectively (t_6 = 2.263; *P*= 0.108) (Fig.4.2.b). A similar trend was observed when the experiment was conducted at 27 ºC; however, under this temperature the three mirid species significantly reduced the *B. tabaci* infestation when compared to the control. The number of *B. tabaci* was significantly lower in the *N. tenuis* and *M. pygmaeus* cages

when compared to that in the *D. maroccanus* cages (Fig.4.2.c) (Table 4.2). A reduction of 96% of the infestation was obtained by *M. pygmaeus* and *N. tenuis*, whereas, *D. maroccanus* was less efficient and only reduced the *B. tabaci* populations by 46% (F_{2-11}= 7.06; *P*= 0.015) (Fig.4.2.d).

20 °C

27 °C
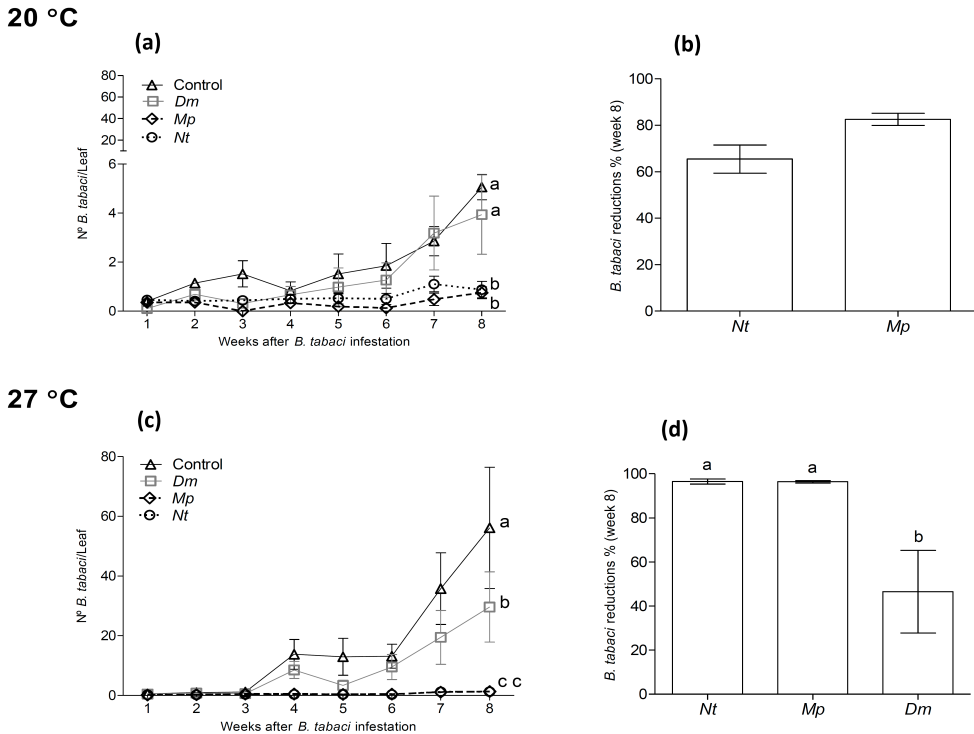
Figure 4.2.

(a) Number (mean ± SE) of *B. tabaci* (adults + nymphs) per sweet pepper leaf at 20 °C, **(b)** percentage reduction (Abbott) (mean ± SE) of *B. tabaci* individuals at 20 °C **(c)** Number (mean ± SE) of *B. tabaci* (adults + nymphs) per sweet pepper leaf at 27 °C, and **(d)** percentage reduction (Abbott) (mean ± SE) of *B. tabaci* individuals at 27 °C in a glasshouse experiment comparing the effectiveness of the mirid predators, *N. tenuis, M. pygmaeus,* and *D. maroccanus* at different time intervals under the two temperature regimes mentioned above. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test *α*< 0.05).

4.3.3. Mirid populations

At 20 ºC, *M. pygmaeus*, with an average of 1.6 ± 0.2 individuals/plant, was significantly more abundant than *N. tenuis* (1.15 ± 0.3), which in turn was higher than *D. maroccanus* (0.3 ± 0.2) (Fig.4.3.a) (Table 4.3). In contrast, when the experiment was conducted at 27 ºC, *N. tenuis* was the most abundant predator with an average of 2.1 ± 0.4 individuals/plant, which was significantly higher than both the *M. pygmaeus* and *D. maroccanus* populations (Table 4.3). Significant differences were obtained between the *M. pygmaeus* (1.2 ± 0.2) and *D. maroccanus* (0.25 ± 0.05) population numbers, with the latter being very low throughout the entire experiment (Fig.4.3.b) (Table 4.3).

Table 4.3.

P values for the pairwise comparison of the number of mirids (adults + nymphs) per plant that received *D. maroccanus* (*Dm*), *M. pygmaeus* (*Mp*), and *N. tenuis* (*Nt*) releases. Values in bold correspond to significant differences between the treatments.

Treatments	20 ºC		27 ºC	
	t_{124}	*P*	t_{124}	*P*
Dm vs. *Mp*	**7.292**	**< 0.001**	**3.992**	**< 0.001**
Dm vs. *Nt*	**4.886**	**< 0.001**	**7.754**	**< 0.001**
Mp vs. *Nt*	**2.406**	**0.018**	**3.762**	**< 0.001**

20 °C **(a)**

27 °C **(b)**
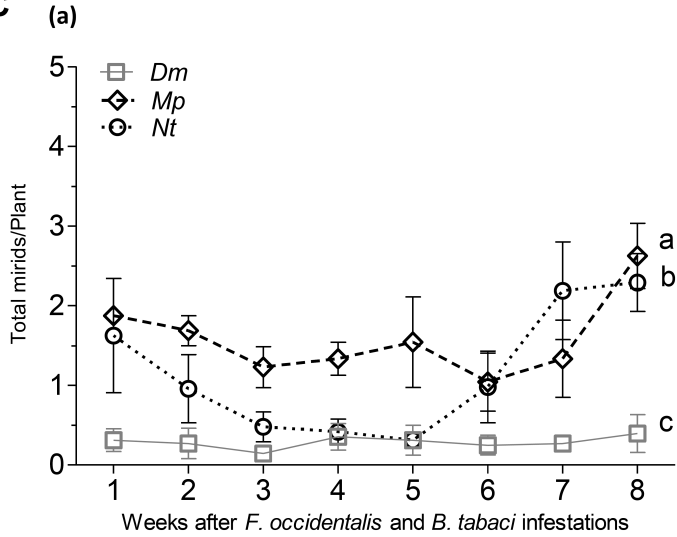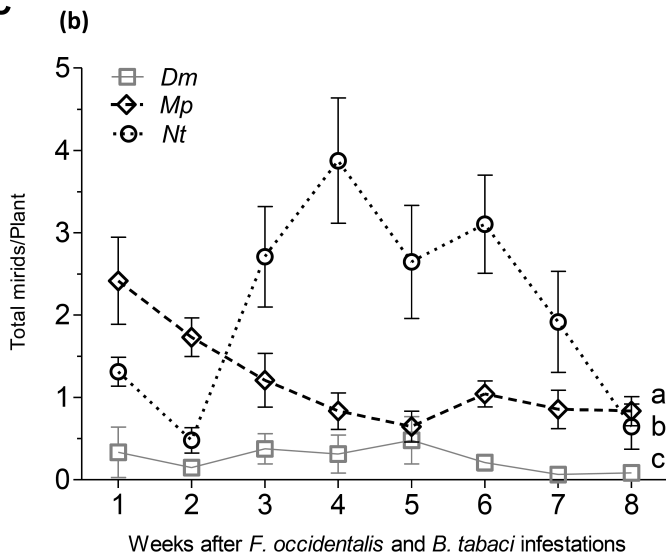
Figure 4.3.

Number (mean ± SE) of *N. tenuis*, *M. pygmaeus*, and *D. maroccanus* (adults + nymphs) per sweet pepper plants at different time intervals under two temperature regimes **(a)** 20 ºC and **(b)** 27 ºC.

4.4. Discussion

Our results show that the pre-plant release strategy of the three selected predatory mirids, supported by the addition of *E. kuehniella* eggs, successfully established on sweet pepper plants although the number of mirids per plant was much lower in the case of *D. maroccanus*. Accordingly, the number of individuals established by the release and augmentation of *N. tenuis* or *M. pygmaeus* before the infestation of thrips and whiteflies resulted in continuous suppression of both pests. The low abundance of *D. maroccanus* was relative to the level of control reached by both pests, which remained around 40% in both temperature regimes.

Although, the members of Miridae are common predators in Mediterranean agroecosystems and spontaneously colonize various agricultural crops, including sweet pepper (Perdikis and Lykouressis, 1996; Tavella *et al.*, 1997; Castañé *et al.*, 2004; Gabarra *et al.*, 2004), their commercial use in this crop is underrepresented. The mirid predators are known to be generally associated to hairy plants (Wheeler, 2001), but our results and previous studies have shown their establishment and reproduction to also be possible in plants lacking hair and or trichomes (Perdikis and Lykouressis, 2004; Urbaneja *et al.*, 2005; Barbara *et al.*, 2011; Messelink *et al.*, 2015; Pérez-Hedo and Urbaneja, 2015).

Our results illustrated differences in the abundance of each predator species in the two temperatures tested. *Macrolophus pygmaeus* showed strong preference for the lower temperature tested where it was significantly more abundant in all the released cages when compared to *N. tenuis* and *D. maroccanus*. Conversely, *N. tenuis* was the most abundant at 27 ºC. These trends linked to temperature are in accordance with the observations made

by Sánchez *et al*. (2003), who reported that *M. pygmaeus* was more abundant than *N. tenuis* in the spring and early summer in horticultural crops in inland areas of South-eastern Spain, whereas *N. tenuis* was the predominant species found naturally in the coastal areas where temperatures were higher. Previous studies confirmed that *N. tenuis* is more thermophilous than any of the other Dicyphinae members, such as *M. pygmaeus*, *M. caliginosus* Wagner, and *D. tamaninii* Wagner (Hemiptera: miridae) (Sánchez *et al.*, 2009). The optimum temperature required by *N. tenuis* ranges between 20 and 30 ºC, with a high fertility rate observed at 30 ºC, that allows this mirid better adaptation to high temperatures (Sánchez *et al.*, 2009; Hughes *et al.*, 2009, 2010). Indeed, in this research we observed how *N. tenuis* was able to double its population at 27 ºC.

Temperature is not the only factor that could have influenced the distinct development of the mirids. The availability of prey could also have influenced the population dynamics obtained. Prey scarcity was more evident at 20 ºC, especially in the case of *B. tabaci*, which is better adapted to higher temperatures (Bonato *et al.*, 2007; Naranjo *et al.*, 2010). *Macrolophus pygmaeus* can continue its immature development by feeding exclusively on the plant tissue although its survival is highly affected (Perdikis and Lykouressis, 1996, 2000; Portillo *et al.,* 2012). However, *N. tenuis* is prey dependent (Urbaneja *et al.*, 2005), which could have accentuated the thermal differences between both species even more. Prey preference and switching behaviour to feeding on the most preferred prey are additional characters to be considered when evaluating the potential of polyphagous predators in multi-pest agroecosystems. However, in the case of Hemipteran predators, developmental rate and fecundity are usually enhanced when they are fed multi-prey diets (Wheeler, 2001), which may indicate that even

in the case of strong preferences for one prey, the necessity to diversify the diet can be very influential. Mixed diets are known to have a positive effect on reproduction in some predator species (Evans *et al.*, 1999). For example, in greenhouse cucumber, better control was achieved by the predatory mite *A. swirskii* due to the positive effect of a mixed diet of thrips and whiteflies (Messelink *et al.*, 2008).

Macrolophus pygmaeus* being slightly less abundant at 27 ºC than at 20 ºC was more effective at 27 ºC than at 20 ºC. The strong reduction in *B. tabaci* populations in the presence of *F. occidentalis* and *vice versa* could be due to an increased response by *M. pygmaeus* to higher prey availability (Holt, 1977; Holt and Lawton, 1994). Several previous studies support the idea of numerical responses by *M. pygmaeus* depending on the number of available prey (Fauvel *et al.*, 1987; Alómar *et al.*, 2002). In accordance with our results, Fantinou *et al.* (2008), observed an increase in the predation rate of *M. pygmaeus* on *M. persicae* at 30 ºC where the amount of susceptible prey was higher than at lower temperatures where the availability of prey was lower. The reasons for this shift in the predator's behaviour when foraging at higher temperatures may be related to increased metabolic rates and behavioural changes of the predator and/or prey, which could result in more successful predatory searches (lower search time) or less effective defence by the prey (Fantinou *et al.*, 2008).

The third tested mirid species, *D. maroccanus*, failed to successfully establish throughout the length of the experiment at both temperatures studied. In previous studies, *D. maroccanus* was observed to successfully establish itself and prey on *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) on tomato crops (Abbas *et al.*, 2014), and on *M. persicae* on sweet pepper plants (Pérez-Hedo and Urbaneja, 2015). The reasons that could explain its failure

to establish itself remain unclear, but perhaps thrips and whiteflies were not suitable prey species for this predator. As a result of the low abundance of *D. maroccanus*, neither of the two pests were adequately suppressed, despite the lower population levels of them when compared to the control cages. All the plants treated with *D. maroccanus* appeared unhealthy at the end of the experiment, with no visual differences observed between them and the control cages.

Our results are not the first to describe the efficiency of *N. tenuis* and *M. pygmaeus* in managing thrips and whiteflies; this has already been seen in several agricultural crops (Castañé *et al.*, 1996; Riudavets and Castañé, 1998; Blaeser *et al.*, 2004; Bonato *et al.*, 2006; Sánchez and Lacasa, 2008; Calvo *et al.*, 2012b; Malo *et al.*, 2012). However, in the case of sweet pepper, this is the first study that evaluates the effectiveness of both mirids when pre-plant release was done without combining them with other natural enemies. Indeed, Calvo *et al.* (2009a) evaluated the potential predation of *N. tenuis* combined with that of the parasitoid, *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae), and *A. swirskii*. In that study, the combined release of *N. tenuis* with *A. swirskii* did not improve the biological control of *B. tabaci* on sweet pepper. However, Messelink and Janssen (2014) confirmed the combination of *M. pygmaeus* and *O. laevigatus* to enhance the biological control of *F. occidentalis* and *M. persicae* in sweet pepper crops.

Overall, our results, together with those of previous studies (regarding the efficiency of mirids in managing aphids on sweet pepper, could help to develop new strategies for pest management in commercial sweet pepper crops using generalist predators. Moreover, the efficacy of *N. tenuis* and *M. pygmaeus* preying upon a mixed diet of sweet pepper pests including aphids warrants further research. Biological control agents are

frequently used in combination; however, the success of a biological control program can be disrupted by direct and or indirect interactions, such as competition, apparent competition, intraguild predation, and behavioural interference, between natural enemies. Hence, we have planned a study on the compatibility of *N. tenuis* and *M. pygmaeus* with other natural enemies already adapted to sweet pepper, such as *A. swirskii*, to evaluate their combined efficacy in this crop.

Combined Use of Predatory Mirids with *Amblyseius swirskii* to Enhance Pest Management in Sweet Pepper

Combined use of predatory mirids with *Amblyseius swirskii* to enhance pest management in sweet pepper

Sarra Bouagga, Alberto Urbaneja, Meritxell Pérez-Hedo

Abstract

The combined release of *Orius laevigatus* with *Amblyseius swirskii* provides effective control of sweet pepper key pests, such as thrips and whiteflies. However, the management of the aphids can still be improved. Recently, the predatory mirids *Nesidiocoris tenuis* and *Macrolophus pygmaeus* have been found to be effective in the control of aphids, thrips and whiteflies when tested alone. Hence, integrating one of these two mirids with *A. swirskii* might enhance sweet pepper pest management. In this work, we began by investigating the co-occurrence of both mirid species when released together with *A. swirskii*. This was compared to the standard release of *O. laevigatus* with *A. swirskii*. *Nesidiocoris tenuis* and *A. swirskii* were involved in a bidirectional intraguild predation (IGP). On the contrary, this interaction (IGP) was apparently unidirectional in the case of *M. pygmaeus* with *A. swirskii* and *O. laevigatus* with *A. swirskii*. Both, *M. pygmaeus* and *O. laevigatus* significantly reduced the abundance of *A. swirskii*. Secondly, in a greenhouse experiment, where the same release combinations were tested (either *N. tenuis, M. pygmaeus* or *O. laevigatus* combined with *A. swirskii*), IGP seemed to be neutralized. Mirids with *A. swirskii* significantly suppressed thrips, whitefly and aphid infestations. Contrarily, the combined use of *O. laevigatus* with *A. swirskii* did not reached a satisfactory control for aphids, despite the reduction in thrips and whitefly densities. Therefore, our results suggest that the use of mirids combined with *A. swirskii* could result in more efficient and robust biological control programs in sweet pepper crops.

Key words: Intraguild predation, *Nesidiocoris tenuis*, *Macrolophus pygmaeus*, *Orius laevigatus,* biological control.

5.1. Introduction

Biological control (BC) programs, especially in greenhouse crops, are increasingly based on releases of several species of generalist predators against common greenhouse pests (Calvo *et al*., 2009a, 2012a; van Lenteren, 2012, van Lenteren *et al*., 2018). One of the most impressive success stories of BC was observed is sweet pepper crops in southeast Spain (Calvo *et al*., 2015). This success was achieved thanks to the combined release of the anthocorid bug *Orius laevigatus* Fieber (Hemiptera: Anthocoridae), and the predatory mite, *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) which can control two important sweet pepper pests, the thrips species, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) and the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). The release of *O. laevigatus* is mainly focused for controlling thrips, but when well-established it can also contribute to the management of whiteflies (Arnó *et al*., 2008), aphids (Alvarado *et al*., 1997), and spider mites (Venzon *et al*., 2002). Complementary, *A. swirskii* is released for controlling whiteflies (Nomikou *et al*., 2002; Calvo *et al*., 2009a) but it can also manage thrips (Messelink *et al*., 2006), broad mites (van Maanen *et al*., 2010) and to a lesser extent, spider mites (Messelink *et al*., 2010). In spite of the broad diet range of both predators, the BC system is still challenged due to aphids. The management of aphids requires the release of a combination of specialized parasitoids and predators (Blom, 2008; Messelink *et al*., 2011b, 2013). However, the abundance of hyperparasitoids in southeastern Spain (Belliure *et al*., 2008, Sánchez *et al*., 2011) and the interference occurring among generalist thrips predators and specialist aphid natural enemies (Messelink *et al*., 2011b), have negatively affected the outcomes of this BC program in sweet pepper. To make matters

worse, the program can end up being extremely expensive (Messelink *et al.*, 2011a).

In recent years a line of research to improve the BC of aphids has been focusing on identifying the generalist zoophytophagous predators able to be established in the sweet pepper crop prior to aphid arrival. This could result in rapid responses to new aphid infestations and would prevent aphid establishment (Messelink *et al.*, 2011a). The effectiveness of zoophytophagous mirid predators (Hemiptera: Miridae) such as *Macrolophus pygmaeus* Rambur, *Nesidiocoris tenuis* Reuter, *Dicyphus maroccanus* Wagner and *D. tamaninii* Wagner has been explored in sweet pepper crops. Either, their inoculation or their release after pest outbreaks resulted in effective aphid management in this crop (Messelink *et al.*, 2015; Pérez-Hedo and Urbaneja, 2015). In addition, some of these mirid species, such as *N. tenuis* and *M. pygmaeus* can also be effective to control whiteflies and thrips in sweet pepper under different temperatures (chapter 4). Therefore, the next step would be to know whether the pre-pest establishment of both species of mirids in sweet pepper could manage the populations of the three pests, whiteflies, thrips and aphids. For this, the combined use of either *N. tenuis* or *M. pygmaeus* and *A. swirkii* to reduce the populations of *F. occidentalis*, *B. tabaci* and *Myzus persicae* Sulzer (Hemiptera: Aphididae) was evaluated under greenhouse conditions. The contribution of *A. swirskii*, in the end, is currently so notably positive for BC in sweet pepper that a hypothetical use of mirids would inexorably be linked to the release of *A. swirskii*. Both alternative strategies were compared with the standard release of *O. laevigatus* and *A. swirskii.*

Nonetheless, generalist zoophytophagous predators do not only feed on pests or plant-provided food, but also on other natural enemies (Rosenheim *et al.*, 1995; Rosenheim, 1998). This feeding on other natural enemies can be classified as intraguild predation (IGP) when the competitors share prey and thus contend for it (Polis *et al.*, 1989; Holt and Polis, 1997; Rosenheim *et al.*, 1995). Therefore, the combined release of a pair of generalist predators could result in a negative outcome on BC of the target pest (Rosenheim *et al.*, 1995; Rosenheim and Harmon, 2006; Janssen *et al.*, 2006). As mentioned above, both predatory mirids and *A. swirskii* are true omnivores (Coll and Guershon, 2002) feeding on several trophic levels such as plant materials (pollen, nectar, plant sap), herbivores and other natural enemies, which can increase the probability of IGP. Indeed, Messelink *et al.* (2011b) showed that the predatory mite *A. swirskii* can seriously disrupt BC of aphids by preying on the eggs of the predatory midges, *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae). *Orius* bugs can also prey on eggs and larvae of *A. aphidimyza*, and therefore act as an intraguild predator (Christensen *et al.*, 2002). However, this outcome could also results positive for BC. Messelink and Janssen (2014), observed that IGP between *M. pygmaeus* and *O. laevigatus* did not affect BC of thrips and aphids, at contrary the combined augmentative release of both predator increased pest suppression.

Therefore, a laboratory experiment was conducted in order to study the interaction between predatory mirids and *A. swirskii* in combined release, in comparison to the standard release of *O. laevigatus* with *A. swirskii*. In this caged-experiment whether the intensity of IGP (if any) affects the abundance of the predatory bugs (*N. tenuis*, *M. pygmaeus* and *O. laevigatus*) or the predatory mite (*A. swirskii*) when co-occurring on sweet pepper plants provided or deprived of eggs of the shared prey, *Ephestia kuehniella* Zeller (Lepidoptera:

Pyralidae) was studied. Then, under greenhouse conditions we evaluated the efficacy of each the mixed releases (*N. tenuis* with *A. swirskii*; *M. pygmaeus* with *A. swirskii* and *O. laevigatus* with *A. swirskii*) in reducing *F. occidentalis*, *B. tabaci* and *M. persicae* infestation in sweet pepper. Implications of these results for the future of BC of sweet pepper pests are discussed.

5.2. Materials and Methods

5.2.1. Plants and insects

The plants used in all experiments were pesticide free-sweet pepper seedlings [*Capsicum annuum* (Solanaceae)] *cv* ('Lipari') (Dulce italiano, Mascarell semillas S.L, Valencia, Spain). Two weeks after germination seedlings were transplanted in plastic pots (8 × 8 × 8 cm) containing a mixture of natural soil with local peat moss and were housed in a growth chamber at 25 ± 2 ºC, 60-80% RH and 14:10 h (L:D) photoperiod at the Instituto Valenciano de Investigaciones Agrarias (IVIA).

Nesidiocoris tenuis, *M. pygmaeus*, *O. laevigatus* and *A. swirskii* adults, were obtained from a commercial supplier (NESIBUG®, MYRICAL®, THRIPOR®, SWIRSKI-MITE®, Koppert Biological Systems, S.L., Águilas, Murcia, Spain). Each predatory bug was provided in plastic bottles containing 500 adult individuals approximately 3-days-old (FJ Calvo, Koppert BS; Personal Communication). *Amblyseius swirskii* was supplied in bottle or in sachet according to the experimental requirements. Frozen *E. kuehniella* (Entofood®; Koppert B.S.) eggs were used as additional food to enhance mirids establishment in sweet pepper. *Frankliniella occidentalis* adults were obtained from a colony established at IVIA initiated in 2010 with

individuals originally collected from Campo de Cartagena (Murcia, Spain). The thrips colony was reared on bean plants (*Phaseolus vulgaris* L.; Fabales: Fabaceae) and housed in a growth chamber at IVIA, at the same conditions mentioned above. The *M. persicae* (green phenotype) colony was obtained from a laboratory insect culture established on sweet pepper plants in IVIA, as described in Pérez-Hedo and Urbaneja (2015).

5.2.2. Co-occurrence between predatory bugs and *A. swirskii*

Under laboratory conditions, we studied the co-occurrence of predatory bugs' *N. tenuis, M. pygmaeus, O. laevigatus* and the predatory mite, *A. swirskii* on sweet pepper plants provided or deprived of *E. kuehniella* eggs. Four different treatments were assayed per predatory bug: 1) Predatory bug alone, 2) Predatory bug + *E. kuehniella*, 3) Predatory bug + *A. swirskii* and 4) Predatory bug + *A. swirskii* + *E. kuehniella*. In addition two treatments with *A. swirskii* were also conducted 1) *A. swirskii* alone and 2) *A. swirskii* + *E. kuehniella*. There were four replicates of each treatment. For this, 56 screened plastic cages (30 x 30 x 30 cm) (BugDorm-1 insect cages; MegaView Science Co., Ltd., Taichung, Taiwan) were maintained in a climatic chamber at IVIA (same conditions mentioned above). In each cage (replicate), one sweet pepper plant with 6 fully-developed leaves (approximately 15 cm in height) was placed inside. Two couples (male and female) of each predatory bug (*N. tenuis, M. pygmaeus* or *O. laevigatus*) and 50 *A. swirskii* individuals were released at the same time. The number of adults and nymphs of each predatory bug species and the number of *A. swirskii* individuals (adults, protonymphs, deutonymphs and larvae) per plant was weekly recorded using a manual magnifying glass. The experimental period lasted six consecutive weeks.

5.2.3. Greenhouse efficacy experiment

The experiment was carried out in a greenhouse equipped with drip irrigation system located at IVIA in Moncada (Valencia, Spain). The greenhouse was divided into 24 experimental cages with access through an isolating double mesh door. Cages were screened with "anti-thrips" polyethylene mesh of 220 x 331 µm interstices and had concrete floors. Each experimental compartment measured 4 x 3 x 3 m (length × height × width) and were equipped with five hydroponic substrate containers (growbag). Each cage was accessed by a separate door secured with a zipper.

The climatic conditions including temperature and relative humidity were recorded using a data-logger (model TESTO 175-H2, Amidata S.A. Pozuelo de Alarcón, Madrid), that was placed in the central cage. The average temperature during the experiment ranged between 24.9 ºC on May 4th, 2016 and 32.7 ºC on June 9, 2016 with a minimum and maximum temperature of 21.1 ºC and 43.5 ºC, respectively. The average relative humidity varied between 55.4% on May 4th, 2016 and 98.6% on June 9, 2016 with absolute minimum and maximum values of 33.7% and 99.7%, respectively.

Four different treatments were considered: 1) *N. tenuis* + *A. swirskii*, 2) *M. pygmaeus* + *A. swirskii*, 3) *O. laevigatus* + *A. swirskii* and 4) control, without any predators released. Four replicates per treatment were made. Each replicate was assigned to one cage, hence 16 cages were used. The experiment started by inoculating sweet pepper seedlings in the nursery with either *N. tenuis* or *M. pygmaeus* at a release rate of one predator per plant (Fig.5.1). To help predator establishment and oviposition, frozen eggs of the factitious prey *E. kuehniella,* approximately 0.1 g per plant were supplied as alternative food (Urbaneja-Bernat *et al.*, 2015). One week later, either inoculated

or non-inoculated sweet pepper plants (depending on the treatment) were transplanted and moved to the greenhouse. In each cage, 20 plants were transplanted to hydroponic substrates at a rate of 4 plants per growbag. The day following transplantation one sachet of *A. swirskii*, containing around 125 mites of mixed ages, was hung on the first plant node on every three sweet pepper plant. One week after transplantation the infestation of the plants with weekly releases of *F. occidentalis* (5 adults/m^2, 40 adults/cage), *B. tabaci* (10 adults/m^2, 80 adults/cage) and *M. persicae* (30 aphid of mixed age/plant) was initiated. Six releases were conducted for *F. occidentalis* and for *B. tabaci*. In the case of *M. persicae* only three releases were made due to the rapid proliferation of aphids in the control treatment. These release rates and their frequency were chosen to simulate a strong and early pest attack. Four weeks after transplantation, sweet pepper plants began flowering and *O. laevigatus* individuals were released in the cages corresponding to "*O. laevigatus + A. swirskii*" treatment at the rate of 1 adult/plant. Two releases of *O. laevigatus* were done at an interval of 7 days. One week later the pest infestation samplings were initiated and continued until the end of the experiment which was 6 weeks later. For this, five random plants per cage were selected and the number of *F. occidentalis*, *B. tabaci*, *M. persicae* and the number of predators (immature and adults) per plants were recorded. Special care was always taken to count the replications in the control compartment first and then the cages with the predators to reduce risk of accidental contamination among treatments. The full experiment ran for nine weeks, from beginning of May to the end of June 2016. The experiment was stopped after the sixth data collection date due to the high attack in the control cages which resulted in the total collapse of the control plants. Plants were irrigated twice a week throughout the experiment. A graphical scheme summarizing the release calendar is shown in Fig.5.1.
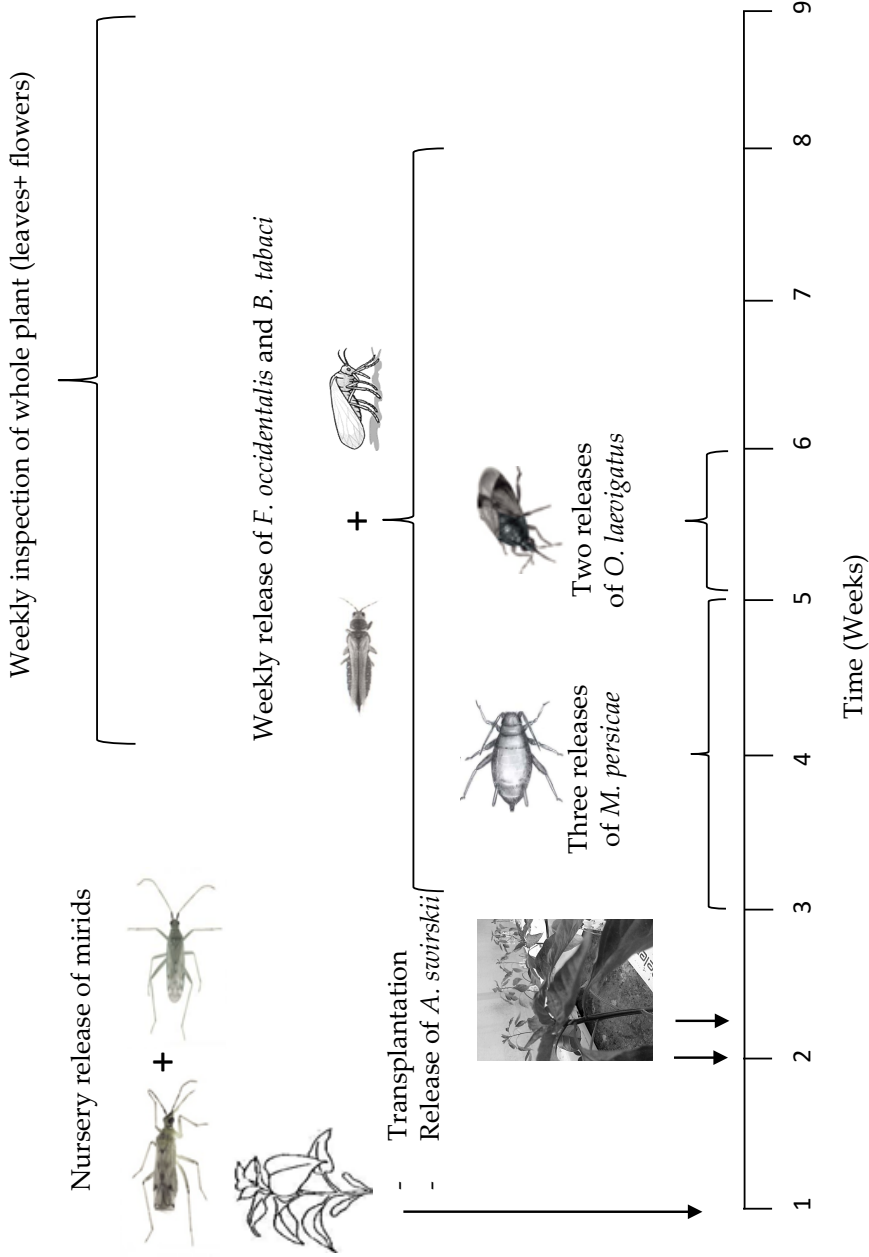
Figure 5.1.

Timeline representing mirid inoculation, *A. swirskii* and *O. laevigatus* introduction, pest infestation and insect abundance, data collection during the greenhouse experiment.

5.2.4. Statistical analysis

In the co-occurrence experiment, the accumulated number of predatory bugs (adults + nymphs) and predatory mites (nymphs, protonymphs, deutonymphs and adults) during the course of the trial were calculated. The resulting estimates of insect per days (=area under the weekly incidence curve) was log transformed and then subjected to a one-way analysis of variance (ANOVA) joined with a Tukey's test for mean separation ($\alpha < 0.05$). Treatments with zero values during the 6 weeks of the analysis were excluded from the analysis. Therefore, in *N. tenuis* treatment, one-tailed Student's *t*-test ($P< 0.05$) was conducted since only two treatments were compared. For the greenhouse experiment, the total number of *F. occidentalis*, *B. tabaci*, *M. persicae* and predators per sweet pepper plants were log (x+1) transformed prior to analysis using the generalized Linear Mixed Models (GLMM). Treatment was considered to be a fixed factor and time (weeks) as a random one. Each GLMM used a normal distribution and identity link function. Whenever a significant difference was found, pairwise comparisons of the fixed factor levels were performed with the least significant difference (LSD) post-hoc test ($P< 0.05$). Untransformed value are presented in figures. To calculate *F. occidentalis*, *B. tabaci* and *M. persicae* percentage of reduction, Abbott's formula was applied, *100 × [1- (treated/control)]* (Abbott, 1925) using the accumulated number of *F. occidentalis*, *B. tabaci* and *M. persicae*. To know whether differences between the percentages of reduction existed, data were log transformed and then a one way ANOVA followed by comparison of means (Tukey's test) at $P< 0.05$ was conducted. The results were expressed as the means ± standard error.

5.3. Results

5.3.1. Dynamics of predatory bugs when co-occurred with *A. swirskii*

The population dynamics of the three predatory bugs was significantly different among the four release treatments (released alone, released together with *A. swirskii*, released with the addition of *E. kuehniella* eggs and released together with *A. swirskii* with the addition of *E. kuehniella* eggs (*N. tenuis*: $t_{1,7}$= 2.51, *P*= 0.046; *M. pygmaeus*: $F_{3,15}$= 296.8, *P*< 0.001 and *O. laevigatus*: $F_{2,11}$= 230.7 *P*< 0.001) (Fig.5.2). The highest population density for the three predators was observed in treatments receiving *E. kuehniella* eggs as additional food. Both *N. tenuis* and *O. laevigatus* were unable to survive and reproduce in the absence of *E. kuehniella* eggs. Nevertheless, *M. pygmaeus* was able to survive 7 weeks after its release on sweet pepper plants deprived from alternative prey, although at a very low density (Table 5.1; Fig.5.2). When predatory bugs were released together with the predatory mite on those plants provided with the addition of *E. kuehniella* eggs, *N. tenuis* significantly decreased its population density (Fig.5.2.a). However, the density of *O. laevigatus* and *M. pygmaeus* was not negatively affected when released with *A. swirskii* in the presence of *E. kuehniella* eggs (Figs.5.2.b,c).
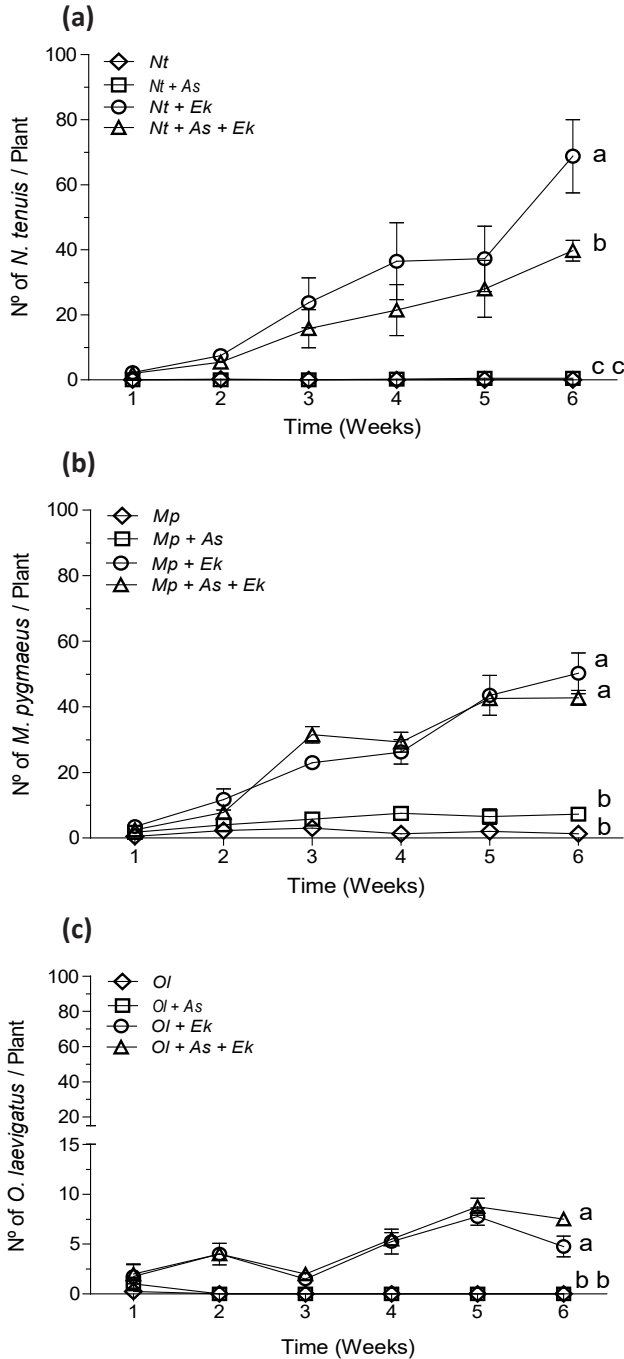
Figure 5.2.

Variation in the abundance of predatory bug **(a)** *N. tenuis* (*Nt*), **(b)** *M. pygmaeus* (*Mp*) and **(c)** *O. laevigatus* (*Ol*) (adults + nymphs, mean number ± SE) per sweet pepper plant in single species treatment and in mixed treatment with *A. swirskii* (*As*) when the shared prey *E. kuehniella* (*Ek*) eggs were and were not provided. Different letters indicate significant differences of predator bug numbers of the same species between treatments.

Table 5.1.

Average of predatory bugs (*N. tenuis*, *M. pygmaeus*, *O. laevigatus*) (± SE) and predatory mites (± SE) accumulated per day when released alone or when combined on plant deprived of or provided with eggs of the alternative shared prey, *E. kuehniella*. Means in the same row followed by the same letter are not significant different (Tukey, $P< 0.05$; *t-test, $P< 0.05$).

Predatory bug	*Nesidiocoris tenuis**	*Macrolophus pygmaeus*	*Orius laevigatus*
Predatory bug alone	-	3.7 ± 0.2 b	-
Predatory bug + *A. swirskii*	-	5.2 ± 0.6 b	0.3 ± 0 b
Predatory bug + *E. kuehniella*	45.7 ± 5.5 a	38.0 ± 1.6 a	6.7 ± 0.5 a
Predatory bug + *E. kuehniella* + *A. swirskii*	26.5 ± 5.3 b	38.5 ± 1.4 a	7.4 ± 0.8 a

Predatory mite	Without *E. kuehniella*	With *E. kuehniella*
A. swirskii	24.2 ± 2.5 a	91.5 ± 5.4 a
A. swirskii + *N. tenuis*	23.1 ± 2.1 a	46.1 ± 8.8 b
A. swirskii + *M. pygmaeus*	21.5 ± 2.1 a	48.7 ± 3.4 b
A. swirskii + *O. laevigatus*	20.5 ± 2.0 a	20.6 ± 2.1 c

5.3.2. Dynamics of *A. swirskii* when co-occurred with predatory bugs

The number of *A. swirskii* per plant was not different when comparing the population dynamics of the treatment where *A. swirkii* was released alone with the population dynamics of those treatments where *A. swirskii* was released with one of the three predatory bugs tested ($F_{3,15}$ = 0.52, *P*= 0.68) (Fig.5.3.a). In spite the general trend, the populations of *A. swirksii* decreased from the date they were released. This predatory mite was able to survive in the sweet pepper plants without access to prey during the 7 weeks of the experiment, independent of the presence or absence of a predatory bug. Nevertheless, the population dynamics of *A. swirskii* were significantly different between the four treatments where the releases of *A. swirskii* were supplemented with eggs of *E. kuehniella* ($F_{3,15}$ = 24.02, *P*< 0.001) (Fig.5.3.b). The combined release of any of the three predatory bugs with *A. swirskii* in the presence of *E. kuehniella* significantly reduced *A. swirskii* populations when compared to the treatment when *A. swirskii* was released with *E. kuehniella* alone. Indeed, the combined release with *O. laevigatus* was the one that was significantly more detrimental for the phytoseiid populations (Fig.5.3.b).
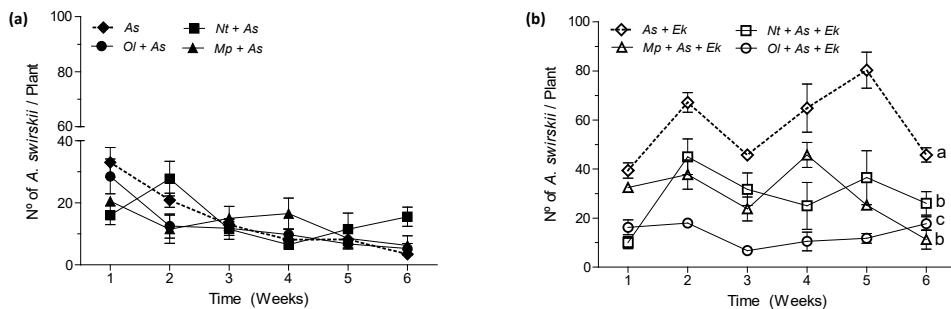
Figure 5.3.

Variation in the abundance of *A. swirskii* (*As*) (adults + deutonymph + protonymph + nymphs, mean number ± SE) per sweet pepper plant in single specie treatment and in mixed treatment with predatory bugs when the shared prey *E. kuehniella* (*Ek*) eggs were **(a)** and were not provided **(b)**. Different letters indicate significant differences of *A. swirskii* numbers between treatments.

5.3.3. Predators abundance under greenhouse conditions

The three predatory bugs established themselves on sweet pepper plants when released together with *A. swirskii* and remained active until the end of the experiment, although significant differences were observed among their abundances ($F_{2,69}$= 13.10, *P*< 0.001) (Fig.5.4.a) (Table 5.2). *Nesidiocoris tenuis* was significantly the most abundant predator in comparison to the number reached by *M. pygmaeus* and *O. laevigatus* (Table 5.2; Fig.5.4.a). On the contrary, the number of *A. swirskii* per plant was similar when released in a mixed treatment with either *N. tenuis*, *M. pygmaeus* or *O. laevigatus* ($F_{2,69}$= 1.90; *P*= 0.16) (Table 5.2; Fig.5.4.b). However, it should be mentioned that the number of *A. swirskii* per plant was abruptly reduced after the release of *O. laevigatus* (Fig.5.4.b), although when compared to the number of *A. swirskii* in the other two predatory bug treatments, no differences were found.

Table 5.2.

P-values for the pairwise comparison of the number of *A. swirskii* (*As*) and predatory bug (*O. laevigatus, N. tenuis, M. pygmaeus*) per sweet pepper plant in the following treatments (*N. tenuis + A. swirskii* (*Nt+As*), *M. pygmaeus + A. swirskii* (*Mp+As*) and *O. laevigatus + A. swirskii* (*Ol+As*)). Values in bold correspond to significant differences between treatments.

Treatments	Predatory mite		Predatory bug	
	t_{69}	*P*	t_{69}	*P*
Nt+As vs Mp+As	0.31	0.76	**3.50**	**0.001**
Nt+As vs Ol+As	1.50	0.14	**4.98**	**<0.001**
Mp+As vs Ol+As	1.82	0.07	1.48	0.14

(a)

(b)
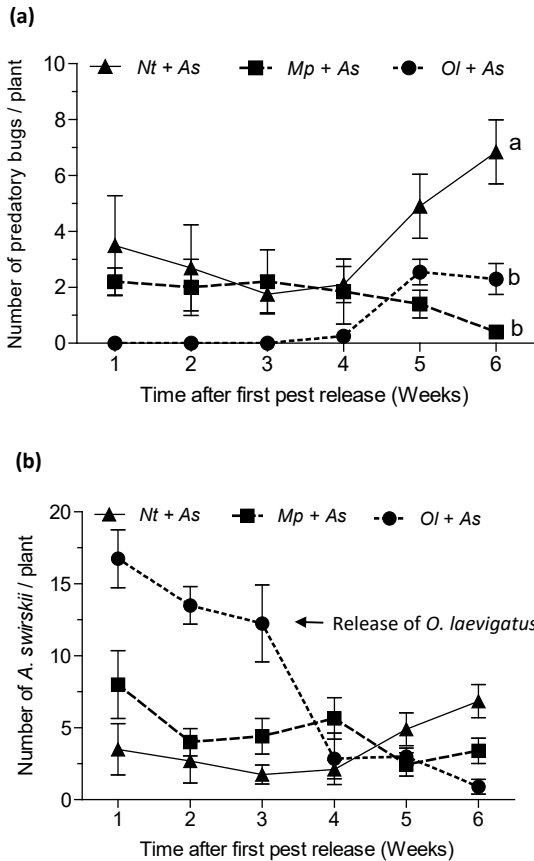
Figure 5.4.

(a) Dynamics of the predatory bugs' *N. tenuis* (*Nt*), *M. pygmaeus* (*Mp*) and *O. laevigatus* (*Ol*) (adults + nymphs, mean number ± SE) per sweet pepper plant when released combined with *A. swirskii*. **(b)** Dynamics of *A. swirskii* (*As*) (adults + deutonymph + protonymph + nymphs, mean number ± SE) per sweet pepper plant in different combined treatments with predatory bugs under greenhouse experimental conditions. Different letters indicate significant differences of predators' numbers between treatments.

5.3.4. Pest management

The number of *F. occidentalis* per sampled plant was continuously suppressed at significant levels in the three treatments which received releases of predators (*Nt+As*, *Mp+As* and *Ol+As*) when compared to the control ($F_{3,92}$= 13.66; P< 0.001) (Table 5.3; Fig.5.5.a). The infestation by *F. occidentalis* was significantly reduced to 97%, 95% and 75%, in the *Nt+As*, *Mp+As* and *Ol+As* treatments, respectively, without differences between

them (Fig.5.5.b). In a similar way, *B. tabaci* was significantly reduced in the three treatments with predator releases (*Nt+As*, *Mp+As* and *Ol+As*) when compared to the control ($F_{3,92}$= 22.475; *P*< 0.001) (Table 5.3; Fig.5.5.c). The percentage of whitefly reduction was not significantly different between treatments and was 95%, 82% and 89% in *Nt+As*, *Mp+As* and *Ol+As* treatments, respectively (Fig.5.5.d). Aphid densities significantly differed among treatments ($F_{3,92}$= 15.66; *P*< 0.001) (Table 5.3). Sweet pepper plants managed with either *Nt+As* or *Mp+As*, harbored significantly lower density of aphids when compared to *Ol+As* and to the control. The abundance of aphids was also significantly lower in *Ol+As* when compared to the control (Fig.5.5.e). Aphid infestation was significantly reduced in *Nt+As* (90%) and *Mp+As* (77%) in comparison to *Ol+As* (35%) (Fig. 5.5.f).

Table 5.3.

P-values for the pairwise comparison of the number of *F. occidentalis*, *B. tabaci* and *M. persicae* per sweet pepper plant that received a release of *N. tenuis + A. swirskii* (*Nt+As*), *M. pygmaeus + A. swirskii* (*Mp+As*), *O. laevigatus + A. swirskii* (*Ol+As*) and control without release (C). Values in bold correspond to significant differences between treatments.

Treatments	*Franliniella occidentalis*		*Bemisia tabaci*		*Myzus persicae*	
	t_{92}	*P*	t_{92}	*P*	t_{92}	*P*
C vs Nt+As	**5.37**	**<0.001**	**6.54**	**<0.001**	**6.36**	**<0.001**
C vs Mp+As	**5.55**	**<0.001**	**6.80**	**<0.001**	**4.60**	**<0.001**
C vs Ol+As	**4.54**	**<0.001**	**6.76**	**<0.001**	**2.03**	**0.04**
Nt+As vs Mp+As	0.18	0.85	0.26	0.80	1.77	0.08
Nt+As vs Ol+As	0.82	0.41	0.23	0.82	**4.33**	**<0.001**
Mp+As vs Ol+As	1.00	0.312	0.03	0.97	**2.55**	**0.01**
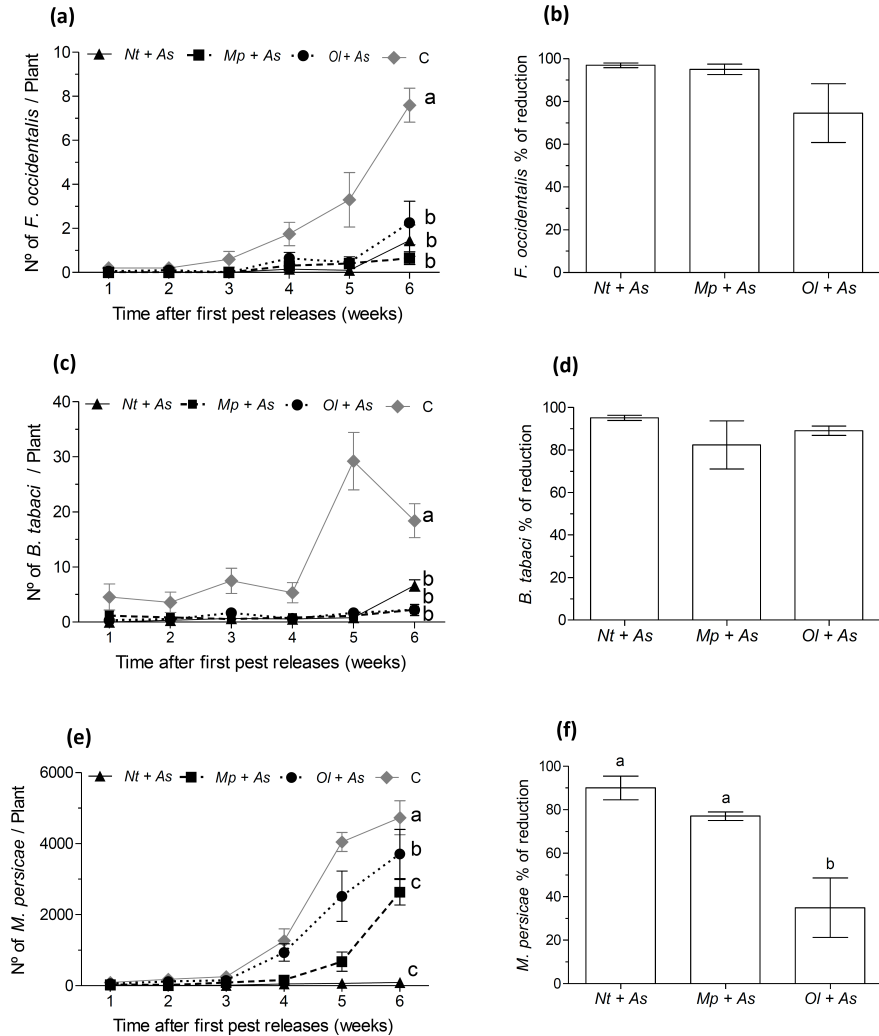
Figure 5.5.

(a) Number (mean ± SE) of *F. occidentalis* (adults + nymphs) per sweet pepper plant, **(b)** percentage reduction (Abbott) (mean ± SE) of *F. occidentalis* individuals **(c)** Number (mean ± SE) of *B. tabaci* (adults + nymphs) per sweet pepper plant, **(d)** percentage reduction (Abbott) (mean ± SE) of *B. tabaci* individuals, **(e)** Number (mean ± SE) of *M. persicae* (adults + nymphs) per sweet pepper plant, **(f)** percentage reduction (Abbott) (mean ± SE) of *M. persicae* individuals in a greenhouse experiment comparing the effectiveness of predatory bug (*N. tenuis* (*Nt*), *M. pygmaeus* (*Mp*) and *O. laevigatus* (*Ol*)) when released in mixed treatment with *A. swirskii* (*As*) each, at different time intervals. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test *α*< 0.05).

5.4. Discussion

Under laboratory conditions, sweet pepper plants maintained *M. pygmaeus* at a very low developmental level during approximately two months. In contrast, *N. tenuis* and *O. laevigatus* were not able to build up a significant population when prey was absent on the plant. The ability of *M. pygmaeus* to continue its immature development by feeding exclusively on plant tissue is already known (Perdikis and Lykouressis, 2000; Portillo *et al.*, 2012), while *N. tenuis* is prey dependent (Urbaneja *et al.*, 2005) and *O. laevigatus* survival and reproduction are dependent on the presence of either prey or pollen (Cocuzza *et al.*, 1997). Interestingly, we observed that even when one of the three predatory bugs co-existed on the same plant with *A. swirskii*, despite the need of *N. tenuis* and *O. laevigatus* for a protein source, the three predators ignored the predatory mite and *vice versa*. On the contrary, when *E. kuehniella* eggs were provided as a shared prey, only *N. tenuis* was negatively affected by *A. swirskii*. The population levels of *A. swirskii* were negatively affected by the three predatory bugs and significantly more affected by the presence of *O. laevigatus* than that the presence of mirids (Fig.5.3.b). Urbaneja *et al.* (2003) previously observed *O. laevigatus* preying on *Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae) in sweet pepper greenhouses. Because *N. cucumeris* was released in large quantities in a period of prey scarcity, prior to the release of *O. laevigatus*, these authors pointed out that the availability of *N. cucumeris* (intraguild prey) facilitated the establishment of *O. laevigatus* (intraguild predator). In our experiment, it seems that when *E. kuehniella* eggs were provided as shared prey, which is a high quality food for both of the predatory bugs (Cocuzza *et al.*, 1997; Castañé *et al.*, 2006) and the predatory mite (Nguyen *et al.*, 2014), competition

occurred. However, reasons why *A. swirskii* only negatively affected the stability of *N. tenuis* is still an open question.

Intraguild predation among predators is widespread and both unidirectional and bidirectional. IGP appears to be common and associated with natural enemies used in greenhouse production systems (Rosenheim *et al.*, 1995). Based on theory, IGP is not expected to benefit BC (Rosenheim *et al.*, 1995), and could be a determining factor in the abundance and distribution of BC agents (Lucas and Alómar, 2001; Perdikis *et al.*, 2014). However, in practice, results are diverse and the potential risk of IGP disrupting BC appears to be low in many cases (Janssen *et al.*, 2006; Messelink and Janssen, 2014). This is also the case of the current study, where, despite being involved in IGP, the mixed release of *N. tenuis* and *A. swirskii*, which were involved in a bidirectional IGP in the laboratory experiment, was virtually the best combination in the suppression of thrips, whiteflies and aphids under our greenhouse conditions. Availability and variability of prey (thrips, whiteflies and aphids) and food provided by the plant (pollen, nectar, plant sap) might be the reason for this non-aggressive coexistence. Indeed, an increase in extraguild prey density has been suggested to decrease the likelihood of predation events occurring among members of the predator guild (Polis *et al.*, 1989, Lucas *et al.*, 1998; Holt and Huxel, 2007). The within plant distribution and patch occupation by both predators, might have also reduced the encounter rate. *Amblyseius swirskii* on sweet pepper is generally found on the tuft domatia of the vein axils, a special structure of the sweet pepper leaves, which constitute a refuge for this mite and therefore might reduce the intensity of IGP under field conditions (Walter, 1996; Schmidt, 2014). The treatment involving *M. pygmaeus* with *A. swirskii* was as effective in pest suppression as *N. tenuis* with *A. swirskii*, however *M. pygmaeus* was

significantly less abundant than *N. tenuis*. It is well known that *M. pygmaeus* is less thermophilous than *N. tenuis* (Sánchez *et al*., 2009), hence the high temperatures registered (mean of 30 ºC) during the experimental period could have hampered the development of this predator. The combined release of *O. laevigatus* with *A. swirskii* successfully reduced *F. occidentalis* and *B. tabaci* populations at levels similar to those that provided treatments based on predatory mirids. However, in the cages that received the combined release of *O. laevigatus* and *A. swirskii*, aphids were far from being controlled, which resulted in the collapse of all the plants.

In this experiment, we adopted the pre-plant release strategy for mirids suggested by Calvo *et al*. (2012b) for tomatoes. This strategy is widely used today in greenhouse tomato crops in Southeastern Spain (Pérez-Hedo and Urbaneja 2016; Pérez-Hedo *et al*., 2017a). At transplantation, mirids had already laid eggs and the establishment was easier thanks to their ability to feed on plant tissue along with the addition of *E. kuehniella* eggs.

Overall, in order to extrapolate our experimental conditions to field conditions the availability of prey must be high. This high availability of prey is necessary to maintain the population of the mirids after their pre-plant release. In this sense, this strategy could be valid for summer plantings when the level of prey is high when the transplant occurs. However, in late winter plantations where prey availability is low, it is necessary to supplement the pre-plant release of mirids with the alternative food source. This could possibly make the system more expensive. In any case all these conditions have to be evaluated before making a decision regarding the use of mirids under true field conditions.

In conclusion, our study provides further evidence that the release of natural enemies involved in IGP does not necessary have negative effects

on BC. Additional success by mirids in managing sweet pepper pests was confirmed throughout this study. Together with the newly discovered ability of predatory mirids to induce plant defence (Bouagga *et al.*, 2017, 2018), we expect the future BC of sweet pepper in commercial greenhouse could rely on the release of *A. swirskii* with predatory mirids. What is clear is, the use of mirids in sweet pepper is possible and can be more effective than the current system based on *O. laevigatus*, which motivates us to suggest that mirids deserve more attention in the BC of sweet pepper pests.

6 CHAPTER

General Discussion and Conclusions

6

General Discussion and Conclusions

The awareness of BC as a principle strategy for managing sweet pepper pests has increased tremendously in Spanish greenhouses. Since 2007, the willingness of consumers to pay for pesticide free fresh produce and the restrictive legislation minimising the number of authorised chemical products has resulted in a shift from insecticide use to the implementation of integrated pepper management programs based on the introduction of natural enemies in an extensive area (Blom *et al.*, 2008; Calvo *et al.*, 2009a, 2012a; van Lenteren, 2012). This shift was quick, subsequently BC in sweet pepper became outstandingly successful thanks to the selection and release of BC agents native to the Mediterranean basin. Nowadays, in South-eastern Spanish sweet pepper greenhouses the use of the anthocorid bug *O. laevigatus* and the phytoseiid predatory mite *A. swirskii* is done in augmentative releases to control the key pests, thrips and whiteflies (Blom *et al.*, 2008; Calvo *et al.*, 2012a; Calvo *et al.*, 2015).

Omnivorous predators, including *O. laevigatus*, are known to supplement their carnivorous diet by plant feeding, consuming sap, pollen, and nectar (Cocuzza *et al.*, 1997; Armer *et al.*, 1998; Lundgren *et al.*, 2008). Feeding on a wide range of prey was widely described for *O. laevigatus*. The piercing-sucking mouth parts allow the predator to inject saliva into a prey substrate by a specialized salivary canal in an elongated maxillary stylet. Salivary components pierce and liquefy the solid substrates of its prey before ingestion through the maxillary food canal (Walker, 2003).

Studying gene expression in the salivary glands of *O. laevigatus* Baek and Lee (2014), determined the presence of a hemolysin-like protein known to destruct cells; digestive enzymes and antimicrobial proteins were also identified. Genes related to homeostasis, antioxidation, anticoagulation and neuropeptide or peptide hormone processing were also found to be transcribed in the salivary gland, likely facilitating *O. laevigatus* prey feeding. However, as mentioned above, *O. laevigatus* can also puncture the plant and inject its toxic saliva which consequently is expected to induce plant damages. In **the second Chapter** of this thesis, we showed **plant feeding to be a common and important behaviour for *O. laevigatus***. We clearly observed how *O. laevigatus* inserts its stylet into sweet pepper tissue to pump out essential nutrients. This phytophagous behaviour was quantified (38% of the observation time) and was classified as the preferred behaviour for this predator in both males and females in comparison to the rest of the activities which we also recorded during our observations. Consequently, our results concluded that mechanical damages caused by stylet insertion, perhaps with the injection of salivary secretion and regurgitates (Herbivore Associated Molecular Patterns, HAMPs) of *O. laevigatus* resulted in the induction of plant defences as those which generally occur in response to herbivore attacks (Karban and Baldwin, 1997; Kessler and Baldwin, 2001).

Against herbivores, plants deploy varying mechanism to overcome their attacks. Such defences can be displayed constitutively or can be induced. In general, constitutive defences are always present, and induced plant defences are activated by herbivory (Karban and Baldwin 1997; Kant *et al.*, 2015). Omnivorous predators also induce plant defence thanks to their phytophagy behaviour; however, information describing this mechanism of defence is scarce. In this second chapter, using a test model consisting of

sweet pepper plants, *O. laevigatus*, *F. occidentalis*, *B. tabaci* and the whitefly parasitoid, *E. formosa*, we observed that the exposure of sweet pepper plants to the activities (mainly feeding) of this predatory bug, increased resistance to sweet pepper pests directly by the up regulation of defensive genes (JA and SA pathways) and indirectly by the increased emission of HIPVs, which have modulated insects' behaviours. The volatile blends from *O. laevigatus*-punctured plants and intact sweet pepper plants differed quantitatively. This quantitative change constitutes a repellence effect for thrips and whiteflies, while an attraction for *E. formosa*. In plants the two major resistance mechanisms against herbivorous insects are antixenosis (interference with insect behaviour) and antibiosis (interference with insect physiology) (War *et al*, 2012). Here, for the first time we are confirming the **phytophagous behaviour of *O. laevigatus* induce antixenosis in sweet pepper**. This result reinforces and explains the potential use of *O. laevigatus* in sweet pepper greenhouse and illuminates how the predator efficiency is not only due to its predation, but also to its ability to induce plant defences. This novelty can be further explored to develop new approaches for pest management.

Despite the success of the current BC program in sweet pepper (combined release of *O. laevigatus* with *A. swirskii* against thrips and whiteflies) aphid management is still difficult to accomplish (Messelink *et al*., 2012). Therefore, much attention has been given during the last lustrum to find alternative natural enemies which could satisfy aphid management in this crop (Pérez-Hedo and Urbaneja, 2015; Messelink *et al*., 2015). Investigations focused on this theme stated how the use of mirid predators might significantly improve BC of aphids (Messelink *et al*., 2011a). Mirid predators combine zoophagy and phytophagy to complete their development and reproduction. Hence, with such predators, establishment can be prior to pest

infestation. The highest density achieved by these kinds of predators before pest outbreak is key to their final successful use as BC agents (Arnó *et al*., 2010a, Urbaneja *et al*., 2012; Pérez-Hedo and Urbaneja, 2015; Messelink *et al*., 2015). In addition, mirid predators were recently observed to induce an ecological change in tomato after feeding. This change was described by Pérez-Hedo *et al*. (2015a, 2015b) as an induced defensive response in tomato plants and later on, confirmed by Pappas *et al*. (2015, 2016). In the **third chapter** of this thesis, we observed plant feeding to result in an important behaviour for *N. tenuis* **and** *M. pygmaeus*; **this phytophagy habit elicits plant defence in sweet pepper**. The quantification of plant feeding by both male and female predators turned out to be an important activity when compared to the rest of the described behaviours. Interestingly, we observed that *N. tenuis* (33%) spent twice as much time feeding on sweet pepper than *M. pygmaeus* (14%). However, both predators were able to induce plant injuries that activate the mechanisms of defence. Punctured sweet pepper plants by either *N. tenuis* or *M. pygmaeus*, resulted in activating the phytohormonal routes of ABA and JA. Similar results were recently obtained by Zhang *et al*. (2018); both phytohormones were accumulated in sweet pepper leaves exposed to *M. pygmaeus*. To further confirm plant defences, we have identified the HIPVs that produced sweet pepper following mirid feeding. The quantitative change in the blend of volatiles induced by either *N. tenuis* or *M. pygmaeus* incites plant resistance in punctured plants by modulating pest behaviour (attraction of *E. formosa* and repulsion of *F. occidentalis* and *B. tabaci*). The major differential peaks on the chromatogram of the volatile blend obtained from our results compared to the volatile blend recently obtained by Pérez-Hedo *et al*., (2017b) on tomato plants, concluded similarity in the response of both plants. Four GLV ((Z)-3-hexenol, (Z)-3-hexenyl

acetate, (Z)-3-hexenyl propionate, (Z)-3-hexenyl butyrate) and methyl salicylate, were induced by both predators on both plants and interestingly were found to repel *B. tabaci* and attract *E. formosa* (Pérez-Hedo *et al.*, 2017b). Methyl salicylate is one of the main components of the volatile blends released by damaged plants. It is well known that this compound can have repellent and deterrent effects on phytophages (Groux *et al.*, 2014). Application of this compound on bean or cucumber leaves decreased the time spent by adult thrips on the lamina, reduced the number of eggs laid and the degree of damage (Koschier *et al.*, 2007). Methyl salicylate is also reported to be a general attractant for natural enemies of pest species (James and Price, 2004; Mallinger *et al.*, 2011; Gadino *et al.*, 2012). Besides, quantitative changes in the volatile blend between mirid-punctured plants and intact plants, a qualitative change was also observed. Octyl-acetate was only emitted from *M. pygmaeus*-punctured plants. In accordance with our results, Pérez-Hedo *et al.* (2017b) detected octyl-acetate and characterized this volatile to be specific to *M. pygmaeus*, suggesting its possible contribution in the management of the tomato key pest, *T. absoluta,* in relation to previous results by the same authors, describing *T. absoluta´s* reaction to *M. pygmaeus*-punctured tomato plants (Pérez-Hedo *et al.*, 2015b). Overall, we concluded that new approaches for pest management can be explored from the obtained results. The use of powerful chemical pest repellents applied on the crop is an innovative approach that has not yet been properly investigated and certainly not developed into an effective commercial product. Establishment of natural enemy attractant dispensers in the areas of pest concentration would reduce predator search time and prevent the migration of the predator in the case of low prey density. This technique would probably increase the effectiveness of BC agents in the presence of the pest or preventively,

before the appearance of the pest. It would be expedient to, both, find other highly attractive volatiles among the natural and synthetic compounds and to improve the methods of application of the most promising compounds. Another possible application of our results is the integration of plant breeding programs in selecting commercial varieties with higher emission rates of one of most active volatile organic compounds. Among our results, we have observed that once sweet pepper is activated by the feeding punctures of *N. tenuis*, plants are still protected for a period of two weeks. With the feeding puncture of *M. pygmaeus* it was also observed perseverance of the effect during two weeks (Pappas *et al.*, 2015). The persistence of this plant defence encourages the strategy of the pre-plant release (see below, chapter 4). Nursery inoculation will ensure plant robustness before pest outbreak and will delay the infestation due to the repellent effect.

Besides, the above described mechanism of plant defences induced by *N. tenuis* and *M. pygmaeus*, mirids are essentially predators, and have been successfully used for managing various pests (Urbaneja *et al.*, 2012; Zappalá *et al.*, 2012, 2013, Calvo *et al.*, 2012b, 2012c; van Lenteren *et al.*, 2018; Pérez-Hedo *et al.*, 2017a). In particular, unlike other predatory bugs, the *Orius* genus is not hampered by glandular trichomes (Coll and Ridgway, 1995), tomato plants represent a very suitable host for mirids (Riudavet and Castañé, 1998; Tavella and Goula, 2001; Urbaneja *et al.*, 2005). Therefore, inoculation with *N. tenuis* in Southern Europe and *M. pygmaeus* in Northern Europe and conservation of their natural populations have been very effective for controlling key tomato pests, including *B. tabaci* and *T. absoluta* (Arnó *et al.*, 2010b; Calvo *et al.*, 2012c; Gabarra *et al.*, 2008; Gerling *et al.*, 2001; Urbaneja *et al.*, 2012). Unlike tomato, sweet pepper plants have smooth, waxy leaf surfaces with trichomes clustered in domatia only on the abaxial side

(Ferreira *et al.*, 2008); this lack of trichomes could be a disadvantage for hairy plant associated bugs like *N. tenuis* and *M. pygmaeus* and might explain the scarce use of mirids in sweet pepper crops (Tavella and Goula, 2001; Sánchez *et al.*, 2003). However, even if sweet pepper is not the preferred host plant (Urbaneja *et al.*, 2005), in the **fourth chapter** of this thesis, we have observed that *N. tenuis* **and** *M. pygameus* **were successfully established on sweet pepper and effectively managed thrips and whiteflies.** The strategy of the pre-plant releases or "predator in first" (Calvo *et al.*, 2012b), enhanced by the addition of alternative food of the factitious prey *E. kuehniella* was crucial to ensure predator establishment and to assure an adequate predator: prey ratio which positively affects BC. However, provision of alternative food increases BC costs; the price of *E. kuehniella* fresh eggs is approximately \$400 per kg (Urbaneja-Bernat *et al.*, 2015). Therefore, the development of inexpensive alternative food sources is one of the major opportunities and challenges for enhancing BC in greenhouse crops in the near future. Many artificial diets have been tested with the aim of developing less expensive mass rearing techniques (Castañé and Zapata, 2005; Bonte and de Clercq, 2008; Nguyen *et al.*, 2013; Urbaneja-Bernat *et al.*, 2015), but these diets are currently not applied to support predator populations in commercial crops. In addition to the importance of alternative diet in the success of mirids in sweet pepper, another criterion that should be considered during the selection of predators is the thermal requirement of each. Among the fourth chapter results, we have observed how *N. tenuis* was more abundant and more effective at a higher temperature (mean of 27 ºC), whereas *M. pygmaeus* was more abundant at a cooler temperature (mean of 20 ºC), but was still effective for both temperatures. Despite, the observed difference among predator abundance, both of them successfully reduced the mixed infestation of

thrips and whiteflies. *Macrlophus pygmaeus* showed an interesting behaviour that could benefit its use as a BC agent. The low abundance of this predator at 27 °C did not negatively affect the management of the mixed diet, on the contrary, we observed an increase in pest suppression. Overall, our results along with previous studies (Messelink and Janssen, 2014; Pérez-Hedo and Urbaneja, 2015; Messelink *et al.*, 2015) expect the future of BC in sweet pepper to possibly be based on mirids. Paying special attention to mirid families may result in the selection of other efficient predators for the BC of horticulture crops. Although, we did not obtain encouraging results for the use of *D. maroccanus* in sweet pepper, many species from the genus *Dicyphus* are promising BC agents.

As we have seen above (**chapters 2 and 3**), *N. tenuis* and *M. pygmaeus* increase resilience in sweet pepper, both by inducing defensive responses and by predation. However, the best control strategies are mainly based on the combined releases of multiple natural enemies, the current success of the BC in sweet pepper (*O. laevigatus* and *A. swirskii*) attests this approach. Nevertheless, the use of different natural enemies for the BC of diverse pest species results in the creation of complex artificial food webs in agricultural crops. This implies how pest densities are not only managed by their natural enemies, but also by direct and indirect interactions with other pests and other natural enemies; thus, such interactions can affect BC (Rosenheim *et al.*, 1995; Janssen *et al.*, 1998). Intraguild predation (IGP) has been described for many natural enemies that are used for BC in greenhouse crops (Rosenheim *et al.*, 1995). Although, in theory we do not expect IGP to benefit BC, several examples in the literature have mentioned lacks of negative effects (Janssen *et al.*, 2006). In the **fifth chapter** of this thesis, this approach was supported and our results reveal that, **despite being involved in a bidirectional**

IGP, the combined release of *N. tenuis-A. swirskii* under semi-field conditions resulted in a more effective suppression of thrips, whiteflies and aphids in sweet pepper. Among our results, we have also observed an apparently unidirectional IGP, where ***M. pygmaeus* and *O. laevigatus* intraguild prey on *A. swirskii* when shared food (*E. kuehniella*) was added to the plant**. Diverse results were obtained from laboratory and semi-field conditions. The population of *A. swirskii* was observed to be similar in the three mixed release combinations (predatory bugs with predatory mites). Possible coexistence between predatory bugs and predatory mites was resolved under field conditions by the availability of a mixed diet (thirps, whiteflies and aphids) and an increase in plant density which may have offered refuge and the avoidance of possible interactions among predators.

The infestation by thrips and whiteflies were effectively managed in the three treatments; however, where *O. laevigatus-A. swirskii* were released, aphids destroyed the plants and resulted in lower affectivity. Although, in the first chapter we observed *O. laevigatus* to induce plant defence in sweet pepper, we assume that the strategy employed in its release on flowering pepper may not benefit plants from the induced defence. One of the possible reasons is having plants which are already infested by herbivores that are also inducing plant defences. Thrips have a different feeding mode from whiteflies and aphids; hence, different defensive pathways are induced in the plant on which they feed (Wetering *et al.*, 1998; Walling, 2008). Probably, the pathways induced by both feeding modes can negatively interfere with the pathways induced by *O. laevigatus* feeding or oviposition (De Puysseleyr, 2011) and the induced response will be suppressed. Howe and Jander (2008), mentioned that negative cross-talk between distinct defensive pathways induced by species from different feeding guilds might suppress the induced

defence. On the contrary, in our experiment, mirids were released in the nursery, thus, upon planting, plants were already activated. The persistence of this activation (Chapter 3) motivated the defence response before pest outbreak; thus, delaying infestation which results in better management of pepper pest with either the inoculative release of *N. tenuis* or *M. pygmaeus* with *A. swirskii*. Specific temperature requirements of each predatory bug (Chapter 4) should also be considered for the use of mirids, because from our results, *M. pygmaeus* was less abundant than *N. tenuis* due to the higher registered temperature in the greenhouse (May-June, 30 ºC).

According to the results presented in this PhD thesis, it can be concluded that **pest management in sweet pepper could be enhanced by the exploitation of zoophytophagy. The anthocorid plant bug** and **mirids, are able to induce defence responses in sweet pepper plants**. The information generated in this regard can serve as the basis for future developments in crop protection.

Overall, our results illustrate how mirids can be successfully employed in BC programs in sweet pepper. The results of this thesis support these conclusions: i) Their aptitude to establish on this crop is improved by supplemental food, ii) The safety of their feeding behaviour in sweet pepper is marked by the absence of economic damage, iii) The positive outcome of their phytophagous behaviour induces plant defence eliciting resistance mechanisms that reduce pest infestation, iv) Their polyphagy and their performance on managing sweet pepper key pests including thrips, whiteflies and particularly aphids and v) Their combined release with the predatory mite *A. swirskii* is without negative outcomes on the BC final balance. All together, these are sufficient reasons for an evaluation of mirids in commercial greenhouses that could rely on and reinforce their future commercial use in sweet pepper.

7 CHAPTER

References

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CHAPTER

References

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