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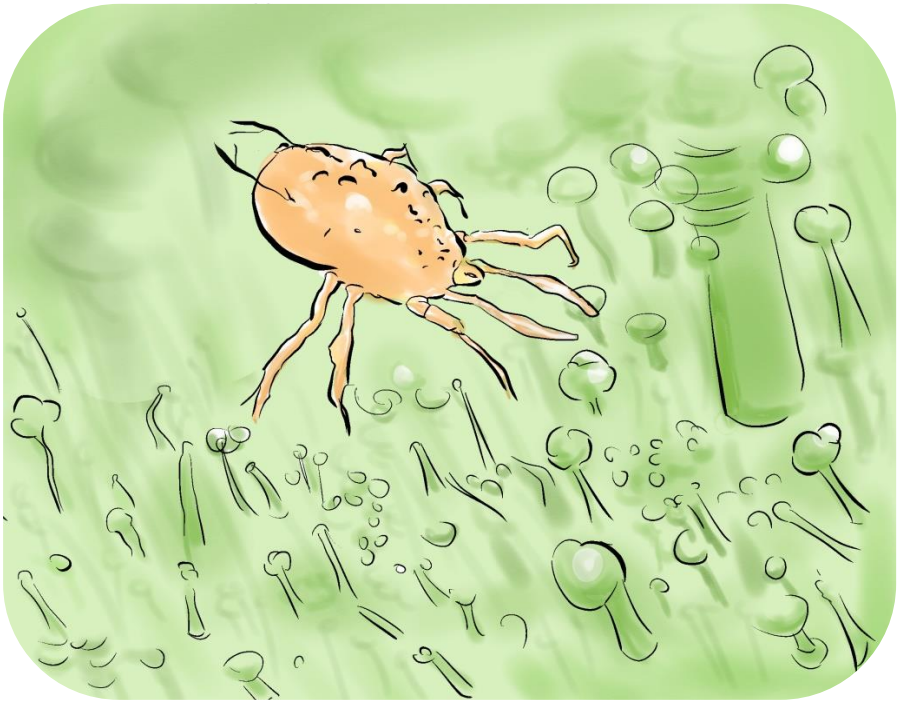
Living on an unfriendly plant host: impact of tomato on the predatory mite *Amblyseius swirskii*



ANGELIKI PASPATI

Supervisors

Alberto Urbaneja

Joel González-Cabrera

November 2019

Doctoral Programme in Sciences

Universitat Jaume I Doctoral School

Living on an unfriendly plant host: impact of tomato on the predatory mite *Amblyseius swirskii*

Report submitted by **ANGELIKI PASPATI** in order to be eligible for a doctoral degree awarded by the

Universitat Jaume I

Angeliki Paspati

Supervisors

Alberto Urbaneja

Joel González-Cabrera

Castelló de la Plana, November 2019

Funding

This work was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 641456.

To the ones that inspire us

Acknowledgments

First of all, I would like to express my gratitude to my supervisors Alberto Urbaneja and Joel González-Cabrera for giving me the opportunity to do this PhD and guiding me and encouraging me during this long journey. Many thanks to Dr. Antonio Granell, Dr. José Luis Rambla and Dr. María Pilar Lopez Gresa from the Institute for Plant Molecular and Cellular Biology "IBMCP" for their support during our collaboration on the identification of acyl sugars. Also, I am very thankful to Jóse, Miquel, Carolina, Maria and Giorgos for their technical support and their valuable help with experiments. Also, I am very grateful that I had the opportunity to be part of the Entomology Group of IVIA, a group of wonderful colleagues to make research with, discuss ideas, receive advice and gain knowledge. Special thanks to Milena Chinchilla, a very good companion and friend the past four years, with whom, I was more than lucky to participate in the same project and share the most exciting experiences of this journey. I would like to thank my colleagues and good friends, Jessi, Alice, Azu, Paco, Francesc, Juan Pedro, Marta, Miguel, Omar, Sara, Carlos, Carolina and Maria that now I feel like my family in Valencia, for bringing joy, laughters, true companionship, affection and lots of support in my daily life at work, all those years. Many thanks to Angelos for his art, that decorates the cover of this book. Last, my grateful thanks to all my good friends and family for their moral support, encouragement and infinite love.

Summary

Integrated pest management programs based on the augmentation and conservation of phytoseiid predators, natural enemies of agricultural pests, are available for many crops worldwide. A valuable player in biological control of key greenhouse pests, such as the whiteflies *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), is the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae). However, on tomato crops, the release of *A. swirskii* does not provided effective control of the pests, because it cannot become established. The impact of the plant traits on *A. swirskii* biological parameters has not been explored in depth and the tomato metabolites affecting the phytoseiid establishment are not known.

The first objective of this PhD was to investigate the biological parameters of *A. swirskii* which are mostly affected on tomato plants and the tomato trichomes's secondary metabolites with the highest negative impact on the establishment of this predatory mite. It was found, that the survival of adult mites attempting to disperse on the tomato plant was significantly reduced, due to the impact of trichome secondary metabolites, present in high concentration on the stems. Among the secondary metabolites detected in tomato trichomes, the acyl sugars were most likely involved in mite mortality. Moreover, this study showed the attachment and allocation of the acyl sugars on the body of the phytoseiids, after walking on the plants. Altogether, these results open perspectives to further investigate the mechanisms underlining the detrimental effect of tomato acyl sugars on phytoseiids and their prey, the herbivores.

Currently, cultivars used for tomato production are often selected for high density of trichomes, since they show increased tolerance to herbivore attacks; however, they can be harmful to predatory mites, as shown in this study. The second objective of this PhD was to investigate the dispersal ability of *A. swirskii* on three tomato mutants with different trichome composition: plants without type I trichomes (hairs absent), plants with excess of type I

trichomes (woolly), and plants with all types of trichomes distorted (hairless). Moreover, in the second objective of this study, the efficiency of *A. swirskii* and *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) to control respectively, two tomato key pests, the whiteflies and the spider mites on hairless tomato plants was addressed as well. On the hairless plants, the dispersal ability of *A. swirskii* was higher than on wild-type plants, however, the negative effect of the trichome exudates was still present. The herbivore pests, whiteflies and spider mites were found to perform better on hairless tomato plants than on wild- type plants. The efficiency of the predatory mites, *A. swirskii* and *P. persimilis* on hairless tomato plants was poor, since they were not able to suppress the pest. The findings highlight the effect of the plant physiology on biological control based on phytoseiids, and also, the importance of taking this effect into account by tomato plant-breeding programs and addressing new lines of research for selecting more 'friendly' plants to phytoseiids and other natural enemies.

It is known that the tomato defences induce gene responses to herbivores; however, the responses of phytoseiids to tomato trichomes remain unknown. As third objective of this PhD, the gene response of *A. swirskii* to the tomato trichome exudates and also, three major detoxification gene sets, the cytochromes P450 (CYPs), the glutathione S-transferases (GSTs) and the carboxyl/cholinesterases (CCEs) were identified and characterized. Thirty-nine of *A. swirskii* genes were found differentially expressed after transfer to tomato leaves, when compared to pepper leaves, a favourable host plant for *A. swirskii*, and some of the expressed genes were associated with the metabolism of tomato exudates. Moreover, it was shown that many members of the detoxification gene sets CYPs, GSTs and CCEs are present in *A. swirskii*, but do not play a significant role when in contact with the tomato exudates. In addition, more than 45,000 unigenes of *A. swirskii* were successfully annotated, based on known genes, after the *de novo* transcriptome assembly was conducted. These results suggest

that the gene responses of the phytoseiid mites are very different from what has been found for the herbivore mites.

The impact of the conditions in mass rearing facilities, on the genetic diversity of commercial biocontrol agents, such as the phytoseiids, is not known. The forth objective of this PhD was to investigate the effect of long-term mass rearing on the genetic diversity and differentiation of commercial *A. swirskii*. Eight wild and one commercial population of *A. swirskii* were compared and the results indicated that the commercial population is less heterozygous and has higher genetic differentiation than their wild counterparts. The implications of reduced genetic variation in this biocontrol agent in their performance when released to control pests were discussed within this chapter.

In summary, in this study it is concluded that the acyl sugars produced by the tomato trichomes are detrimental for the phytoseiid *A. swirskii*, and this information can serve as the basis for future research in plant-breeding programs, aiming to successful application of phytoseiids for tomato crop protection. Also, the demonstration of the impact of current mass rearing practices on the genetic variation of *A. swirskii*, suggest the revision and innovation of these practices, in order to achieve utmost performance of biocontol agents in pest management.

Resumen

Los programas integrados de manejo de plagas basados en el aumento y la conservación de depredadores fitoseidos, enemigos naturales de las plagas agrícolas, están disponibles para muchos cultivos en todo el mundo. Un enemigo natural clave en el control biológico de plagas en cultivos de invernadero, como la mosca blanca *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), es el ácaro depredador *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae). Sin embargo, en los cultivos de tomate, la liberación de *A. swirskii* no proporciona un control efectivo de las plagas, ya que no puede establecerse. El impacto de las defensas de la planta en los parámetros biológicos de *A. swirskii* no se ha explorado en profundidad y se desconocen los metabolitos del tomate que afectan el establecimiento de los fitoseidos.

El primer objetivo de este doctorado fue investigar los parámetros biológicos de *A. swirskii* que se ven afectados principalmente en las plantas de tomate, y encontrar los metabolitos secundarios de los tricomas del tomate con el mayor impacto negativo en el establecimiento de este ácaro depredador. Se descubrió que la supervivencia de los ácaros adultos que intentaban dispersarse en la planta de tomate se redujo significativamente, debido al impacto de los metabolitos secundarios de los tricomas, presentes en altas concentraciones en los tallos. Entre los metabolitos secundarios detectados en los tricomas de tomate, los acil azúcares probablemente estuvieron involucrados en la mortalidad de los ácaros. Además, en este estudio pudimos mostrar como los acil azúcares se pegan y distribuyen en el cuerpo de los fitoseidos, después de caminar sobre las plantas. En conjunto, estos resultados ofrecen perspectivas para investigar más a fondo los mecanismos que subyacen en el efecto perjudicial de los acil azúcares del tomate en los fitoseidos y sus presas, los herbívoros.

Actualmente, los cultivares utilizados para la producción de tomate se seleccionan a menudo para la alta densidad de tricomas, ya que muestran una mayor tolerancia a los ataques de herbívoros; sin embargo, esta selección puede ser dañina para los ácaros depredadores, como se muestra en este estudio. El segundo objetivo de este doctorado fue investigar la capacidad de dispersión de *A. swirskii* en tres mutantes de tomate con diferente composición de tricomas: plantas sin tricomas tipo I ("hairs absent"), plantas con exceso de tricomas tipo I ("wooly") y plantas con todos los tipos de tricomas distorsionados ("hairless"). Además, en el segundo objetivo de este estudio, también se abordó la eficiencia de *A. swirskii* y *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) para controlar, respectivamente, dos plagas clave del tomate, las moscas blancas y las arañas rojas en plantas "hairless". En las plantas "hairless", la capacidad de dispersión de *A. swirskii* fue mayor que en las plantas de tipo silvestre, sin embargo, el efecto negativo de los exudados de tricomas todavía estaba presente. Se descubrió que las plagas herbívoras, las moscas blancas y las arañas rojas se reproducen mejor en las plantas de tomate "hairless", que en las plantas de tipo silvestre. La eficiencia de los ácaros depredadores, *A. swirskii* y *P. persimilis* en plantas de tomate "hairless" fue baja, ya que no pudieron suprimir la plaga. Estos resultados muestran la importancia del efecto de la fisiología de las plantas en el control biológico basado en fitoseidos, y también, enfatizan la necesidad de tener en cuenta este efecto en los programas de fitomejoramiento de tomate para poder abordar nuevas líneas de investigación con el objeto de seleccionar plantas más 'amigables' para fitoseidos y otros enemigos naturales.

Se sabe que las defensas del tomate inducen respuestas genéticas a los herbívoros; sin embargo, las respuestas de los fitoseidos a los tricomas de tomate siguen siendo desconocidas. Como tercer objetivo de este doctorado, se determinó la respuesta génica de *A. swirskii* a los exudados de tricomas de tomate. También se identificaron y caracterizaron tres conjuntos principales de genes de desintoxicación, los citocromos P450 (CYP), las glutatión S-

transferasas (GST) y las carboxil / colinesterasas (CCE). Treinta y nueve genes de *A. swirskii* se encontraron expresados diferencialmente cuando este fitoseido se encontraba en hojas de tomate, en comparación con las hojas de pimiento, una planta huésped favorable para *A. swirskii*. Algunos de estos genes expresados diferencialmente se asociaron con el metabolismo de los acil azúcares. Además, se demostró que muchos miembros de los conjuntos de genes de desintoxicación CYP, GST y CCE están presentes en *A. swirskii*, pero no juegan un papel importante cuando los fitoseidos están en contacto con los exudados de tomate. Además, tras realizar el ensamblaje *de novo* del transcriptoma, más de 45,000 unigenes de *A. swirskii* consiguieron anotarse con éxito, en base a genes conocidos. Estos resultados sugieren que las respuestas genéticas de los ácaros fitoseidos son muy diferentes de lo que se ha encontrado para los ácaros herbívoros.

Se desconoce el impacto de las condiciones de cría en masa, en la diversidad genética de los agentes comerciales de biocontrol, como los fitoseidos. El cuarto objetivo de este doctorado fue investigar el efecto de la cría en masa a largo plazo sobre la diversidad genética y la diferenciación de *A. swirskii* comercial. Se compararon ocho poblaciones salvajes de *A. swirskii* y una comercial, y los resultados indicaron que la población comercial es menos heterocigótica y tiene una diferenciación genética más alta que sus contrapartes salvajes. En este capítulo se discuten las implicaciones de la reducción de la variación genética en este agente de biocontrol en su rendimiento para controlar plagas.

En resumen, en este estudio se concluye que los acil azúcares producidos por los tricomas de tomate son perjudiciales para el fitoseido *A. swirskii*, y esta información puede servir como base para futuras investigaciones en programas de fitomejoramiento, con el objetivo de una aplicación exitosa de fitoseidos para la protección de cultivos de tomate. Además, la demostración del impacto de las prácticas actuales de cría en masa sobre la variación genética

de *A. swirskii*, sugiere la revisión e innovación de estas prácticas, con el fin de lograr el máximo rendimiento de los agentes de biocontol en el manejo de plagas.

Resum

Els programes de maneig integrat de plagues basats en l'augment i la conservació de fitoseids depredadors, enemics naturals de les plagues agrícoles, estan disponibles per a molts cultius a tot el món. Un enemic natural clau en el control biològic de plagues de cultius d'hivernacle, com la mosca blanca *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), és l'àcar depredador *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae). No obstant això, en els cultius de tomàquet, l'alliberament d' *A. swirskii* no proporciona un control efectiu de plagues, ja que no pot arribar a establir-se. L'impacte de les defenses de la planta de tomàquet en els paràmetres biològics d' *A. swirskii* no s'ha explorat en profunditat i es desconeixen els metabòlits del tomàquet que afecten l'establiment dels fitoseids.

El primer objectiu d'aquest doctorat va ser investigar els paràmetres biològics d' *A. swirskii* que es veuen afectats principalment en les plantes de tomàquet, i trobar els metabòlits secundaris dels tricomes del tomàquet amb el major impacte negatiu en l'establiment d'aquest àcar depredador. Es va descobrir que la supervivència dels àcars adults que intentaven dispersar-se a la planta de tomàquet es va reduir significativament, a causa del impacte dels metabòlits secundaris dels tricomes, que estaven presents en altes concentracions en les tiges. Entre els metabòlits secundaris detectats en els tricomes de tomàquet, els acil sucres probablement van estar involucrats en la mortalitat dels àcars. A més, aquest estudi va mostrar com els acil sucres se pegaven i distribuïen pel cos dels fitoseids, després de caminar sobre les plantes. En conjunt, aquests resultats obren perspectives per investigar més a fons els mecanismes subjacents de l'efecte perjudicial dels acil sucres del tomàquet en els fitoseids i les seves preses, els herbívors.

Actualment, els cultivars utilitzats per a la producció de tomàquet es seleccionen sovint per l'alta densitat de tricomes, ja que aquestos mostren una major tolerància als atacs d'herbívors;

però per contra, poden ser perjudicials per als àcars depredadors, com es mostra en aquest estudi. El segon objectiu d'aquest doctorat va ser investigar la capacitat de dispersió d' *A. swirskii* en tres mutants de tomàquet amb diferent composició de tricomes: plantes sense tricomes tipus I ("hairs absent"), plantes amb excés de tricomes tipus I ("wooly") i plantes amb tots els tipus de tricomes distorsionats ("hairless"). A més, en el segon objectiu d'aquest estudi, també es va abordar l'eficiència d' *A. swirskii* i *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) per controlar, respectivament, dues plagues clau del tomàquet, la mosca blanca i l'aranya roja en plantes "hairless". En les plantes "hairless", la capacitat de dispersió d' *A. swirskii* va ser major que en les plantes de tipus silvestre, però, l'efecte negatiu dels exsudats de tricomes encara era present. Es va descobrir que ambdós herbívors, la mosca blanca i l'aranya roja se van desenvolupar millor en les plantes de tomàquet "hairless", que en les plantes de tipus silvestre. L'eficiència dels àcars depredadors, *A. swirskii* i *P. persimilis* en plantes de tomàquet "hairless" va ser baixa, ja que no van poder controlar la plaga. Estos resultats ressalten l'efecte de la fisiologia de les plantes en el control biològic basat en fitoseids, i també, la importància de tenir en compte aquest efecte en els programes de fitomillorament de tomàquet per poder abordar noves línies de recerca per seleccionar plantes més 'amigables' per fitoseids i altres enemics naturals.

Se sap que les defenses del tomàquet indueixen respostes genètiques als herbívors; però, les respostes dels fitoseids als tricomes de tomàquet segueixen sent desconegudes. Com a tercer objectiu d'aquest doctorat, la resposta gènica d' *A. swirskii* als exsudats de tricomes de tomàquet va ser estudiada. També, se va identificar i caracteritzar tres conjunts principals de gens de desintoxicació, els citocroms P450 (CYP), les glutatió S-transferases (GST) i les carboxil / colinesterases (CCE) . Trenta-nou gens d' *A. swirskii* es van trobar expressats diferencialment després de la transferència a les fulles de tomàquet, en comparació amb les fulles de pebrot, una planta hoste favorable per *A. swirskii,* i alguns dels gens expressats es

van associar amb el metabolisme dels acil sucres. A més, es va demostrar que molts membres dels conjunts de gens de desintoxicació CYP, GST i CCE són presents en *A. swirskii*, però no juguen un paper important quan els fitoseids estan en contacte amb els exsudats de tomàquet. A més, se va realitzar l'acoblament *de novo* del transcriptoma anotant amb èxit més de 45.000 unigens d' *A. swirskii,* basant-se en gens coneguts. Aquests resultats suggereixen que les respostes genètiques dels àcars fitoseids són molt diferents del que s'ha trobat per als àcars herbívors.

Per als agents comercials de biocontrol, com els fitoseids, es desconeix l'impacte de les condicions de cria en massa en la seua diversitat genètica. El quart objectiu d'aquest doctorat va ser investigar l'efecte de la cria en massa a llarg termini sobre la diversitat genètica i la diferenciació d' *A. swirskii* comercial. Es van comparar vuit poblacions salvatges d' *A. swirskii* i una comercial, i els resultats van indicar que la població comercial és menys heterozigòtica i té una diferenciació genètica més alta que els seus contraparts salvatges. Les implicacions de la reducció de la variació genètica en aquest agent de biocontrol en el seu rendiment quan s'alliberen per controlar les plagues es van discutir en aquest capítol.

En resum, en aquest tesi doctoral es conclou que els acil sucres produïts pels tricomes de tomàquet són perjudicials per al fitoseid *A. swirskii*, i aquesta informació pot servir com a base per a futures investigacions en programes de fitomillorament, amb l'objectiu d'una aplicació exitosa de fitoseids per a la protecció de cultius de tomàquet. A més, el demostrar l'impacte de les pràctiques actuals de cria en massa sobre la variació genètica d' *A. swirskii,* suggereix la revisió i innovació d'aquestes pràctiques, amb la finalitat d'aconseguir el màxim rendiment dels agents de biocontol en el maneig de plagues.

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Chapter 1

Introduction

1.1. Biological control

Although chemical pesticides are often cheap and very effective, they do cause many problems, including human health effects, water and soil contamination and non-target effects, reducing biodiversity (Fantke *et al*. 2012). In addition pesticide resistance often develops after multiple applications of a pesticide in the same area and it is a result of the underlying natural genetic variation in the pest population. Pests gradually evolve resistance to pesticides by delaying the pesticide absorbance into their body, increasing the detoxification of the chemical toxin or losing the sensitivity at the site of action of the chemical (Samways 1981, Roush and Tabashnik 1991). Pesticide application also kills a broad range of natural enemies and can lead to resurgence of the pest population, if a large proportion of natural enemies are destroyed (Prischmann *et al*. 2005). Moreover, the elimination of natural enemies can leave room for organisms, normally present at low numbers that do not cause economic damage, to become major pests due to a decrease of the predation and competition pressures, resulting eventually, in secondary pest outbreaks (Vassiliou 2004). After the development of large scale pesticide resistance, particularly in greenhouse crops in the 1950s and the increased awareness regarding the impact of the pesticide on the natural environment, an alternative was needed and the first natural enemies for biological control of greenhouse pests were used in Europe in the 1960s (van Lenteren 2000).

Biological control or biocontrol is the action to suppress a pest population density by manipulating other organisms which are natural enemies of the pest (De Bach, 1964). Natural enemies, also known as biological control agents, can be native or non-native species of parasitoids, predators and pathogens which reduce the population density of a pest. The interactions between the pests and their natural enemies are dynamic, they are affected by the

the organisms traits involved in the interaction, and also they are affected by environmental changes (Van den Bosch and Messenger, 1973). The aim of biological control application is to suppress a pest organism to a level that no longer poses economic damage and less often, even to eliminate it from a local area (Eilenberg, 2006). The first biological control approaches were used by Chinese farmers who sold and applied native predatory ants on citrus and date trees in order to protect them from their insect pests (Peng, 1983). The first success of a biological control program was the introduction of the predaceous vedalia beetle, *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae) in California from Australia, in 1888, for the management of the cottony cushion scale insect, *Icerya purchasi* (Maskell) (Hemiptera: Margarodidae), a destructive pest of citrus orchards and fruit plantations (Waage et al., 1988). This program was very effective and within one year the cottony cushion scale population had already declined. The great success of this program boosted the use of natural enemies, and laid the foundations for the inclusion of biological control as a tool in the control of pests in other countries, including Spain (Jacas *et al.*, 2006).

Nowadays, biological control is used in pest management of organic crops, and conventional agriculture, as well. It is usually combined with other sustainable methods of pest control, such as the use of resistant plant cultivars, traps with pheromones and other attractive cues, or the application of selective chemical pesticides, within a multi-strategy approach called Integrated Pest Management (IPM). The goal of IPM is to minimize losses associated with pests and diseases and its fundamental concepts are the economic injury level and the economic threshold. The economic injury level is the pest density (or amount of injury) that cause yield losses equal to the pest management costs and the economic threshold is the point at which management intervention is required to prevent pest increase at the economic injury level. In biological control, the biocontrol agent, ideally, suppress the pest in a density-dependent manner, as the pest population increase (Coll *et al.*, 2007).

There are three types of biological control described, the classical biological control, the conservation biological control and the augmentative biological control (Van den Bosch *et al.*, 1982, Driesche and Bellows, 1996). Classical biological control is the control of an invasive pest by introducing and releasing a non-native natural enemy of usually the same origin of the pest, which will eventually become established in the new ecosystem and will suppress the invasive pest population (Figure 1.1). Conservation biological control is the sustainable manipulation of the ecosystem in a way that will conserve and enhance the existing natural enemies in order to control the pest populations, which can be either native or non-native but naturalized (Figure 1.1). Augmentative biological control involves the release of large number of natural enemies, usually laboratory-reared, in open field or greenhouse crops for immediate reduction of the pest populations, and their presence in the ecosystem is temporal. There are two forms of augmentative control, the inundative release and the seasonal, inoculative release. During the inundative method rapid pest control is obtained from the mass released natural enemies, which later disappear from the crop (van Lenteren 2000) (Figure 1.1). One example of this method is the mass release of *Trichogramma* spp against the corn borer in maize in Europe (Wajnberg *et al.* 1994). The inoculation involves the release of small amounts of natural enemies at time intervals throughout the pest period and it is usually used in greenhouse crops, where control is obtained for a number of generations of the pest (Figure 1.1). Well known natural enemies used in augmentative releases in greenhouses are the phytoseiid mites of the acari subclass; *Phytoseiulus persimilis* (Athias-Henriot), a specialist spider mite predator or *Amblyseius swirskii* (Athias-Henriot), a generalist predator of whiteflies, thrips and mites (van Lenteren *et al.* 2012).

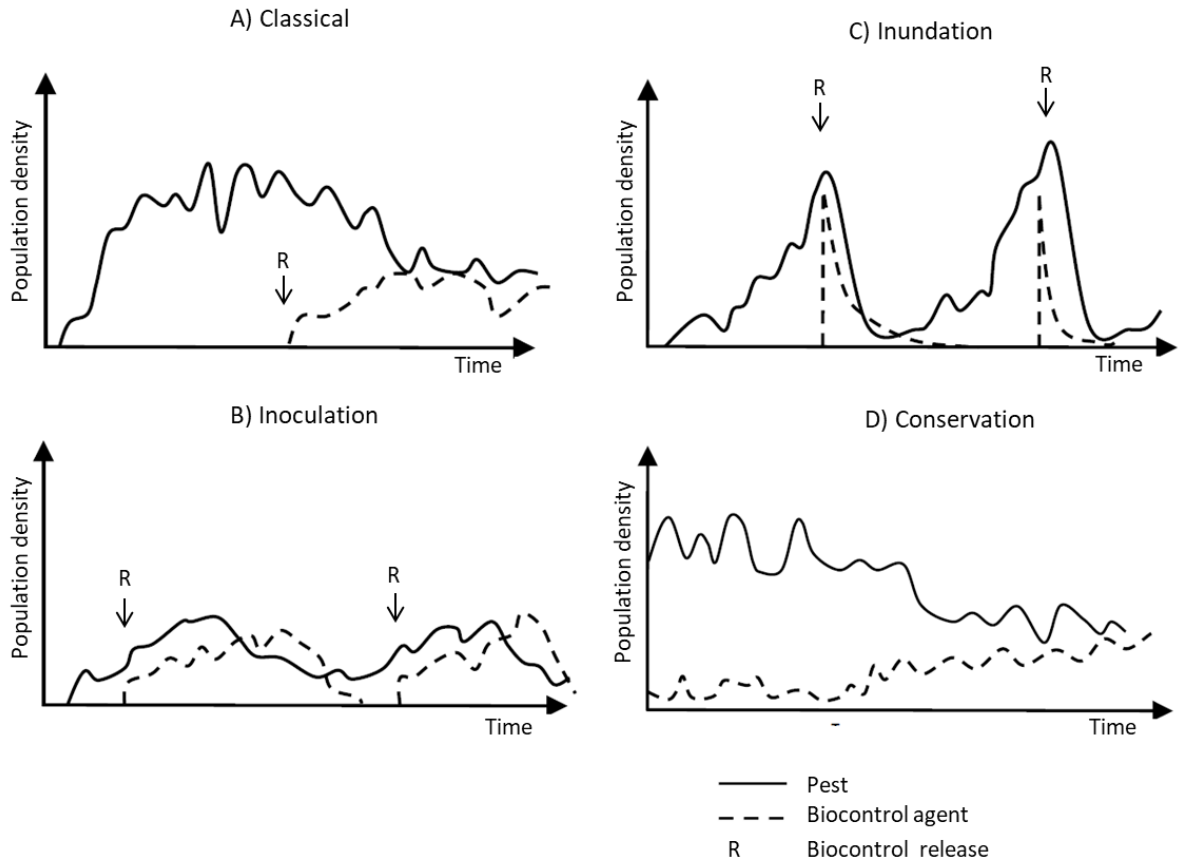


Figure 1.1. Pest and biocontrol agent population density through time in four biological control strategies: A) Classical biological control, B) Inoculation biological control, C) Inundation biological control and D) Conservation biological control. Figure adapted from Eilenberg (2006).

1.2. The tomato crops: pests and natural enemies

The cultivated tomato (*Solanum lycopersicon* Miller) is the horticultural product with the greatest economic importance worldwide, as 182,301,395 tonnes of tomatoes were produced in 2017 (FAOSTAT, 2019). Spain is the second largest producer after Italy, in the European Union and the eighth worldwide (FAOSTAT, 2019). During 2017, a total of 60,852 ha were planted in Spain, of which 5,163,466 tons of tomatoes were yielded (FAOSTAT, 2019).

Among the 150 phytophagous arthropods which attack the tomato crops, 19 species cause severe yield losses in the four most important tomato production countries in the European

Union (Spain, France, Italy and the Netherlands) (Pérez-Hedo *et al*. 2017). These phytophagous species are the following: the lepidopterans *Helicoverpa armigera* (Hübner), *Chrysodeixes chalcites* (Esper), *Spodoptera littoralis* (Boisduval), *Spodoptera exigua* (Hübner), *Autographa gamma* (L.) and *Agrotis segetum* Denis & Schiffermüller (Lepidoptera: Noctuidae) and the South American pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae); the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) that transmits the tomato spotted wilt virus (TSWV); the leafminers of the genus *Liriomyza*, *L. trifolii* (Burgess), L. bryoniae (Spencer) and *L. huidobrensis* (Blandchar) (Diptera: Agromyzidae); the aphids *Myzus persicae* (Sulzer), *Macrosiphum euphorbiae* (Thomas) and *Aulacorthum solani* (Kaltenbach) (Hemiptera: Aphididae); the whiteflies (Hemiptera: Aleyrodidae) *Trialeurodes vaporariorum* (Westwood) and *Bemisia tabaci* (Gennadius), which transmits many viruses such as the tomato yellow leaf curl virus (TYLCV) and the mites *Tetranychus evansi* (Baker & Pritchard), *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and *Aculops lycopersici* (Masse) (Acari: Eriophydae) (Table 1.1) (Velden *et al.* 2012, Pérez-Hedo *et al.* 2017).

The biological control strategies used in protected and open-field tomato crops are augmentative biological control, with commercially available biocontrol agents and conservation biological, control with wild natural enemies (Aviron et al., 2016; Messelink et al., 2014). Currently in Europe, the effective management of the tomato key pests (Table 1.1) relies on IPM, in which, biological control is the cornerstone, and it is integrated with either biological or selective insecticides, when necessary (Pérez-Hedo *et al.* 2017). The most important biocontrol agents used in IPM strategies are two mirids, *Nesidiocoris tenuis* (Reuter), and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), which are released and/or conserved, depending on the type of tomato crop and the region (Velden *et al.* 2012;

Pérez-Hedo *et al.* 2017) (Figure 1.2). These mirids are highly polyphagous predators of whiteflies, thrips, leaf miners, Lepidoptera species, aphids and spider mites. Both are endemic in Europe, often appearing naturally in tomato cultivations and they are commercially available as well, for augmentative releases in crops (Urbaneja et al., 2012). Two strategies, based on the use of the two mirids, are currently employed; inoculative releases in crops, during low pest pressure, and the predator-in-first strategy, where the mirids are established already in the seedling nursery (Urbaneja *et al*., 2012, Pérez-Hedo *et al.* 2017). Other natural enemies used in IPM strategies against the tomato key pests in Europe include parasitoids, predatory mites, predatory flies, two-spotted lady beetles and entomopathogenic microorganisms, as shown on Table 1.1 (Velden *et al.* 2012; Pérez-Hedo *et al.* 2017) (Figure 1.2).

Table 1.1. Tomato pests according to economic importance in Netherlands, France, Spain and Italy and their main commercially available natural enemies. Table adapted from Pérez-Hedo *et al.* (2017).

Pests	Natural enemy
Whitefly (Hemiptera: Aleurodidae)	
Bemisia tabaci (Gennadius)	*Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae)
	Eretmocerus mundus (Mercet) (Hymenoptera: Aphelinidae)
	Eretmocerus eremicus (Rose & Zolnerovich) (Hymenoptera: Aphelinidae)
	Encarsia formosa (Gaham) (Hymenoptera: Aphelinidae)
	Beauveria bassiana
	Verticillium lecanii
Trialeurodes vaporariorum (Westwood)	*Nesidiocoris tenuis*
	Macrolophus pygmaeus
	Eretmocerus eremicus
	Encarsia formosa
	Beauveria bassiana
	Verticillium lecanii

Thrips (Thysanoptera: Thripidae) *Frankliniella occidentalis* (Pergande)	*Nesidiocoris tenuis* *Macrolophus pygmaeus* *Amblyseius cucumeris* (Oudemans) (Acari: Phytoseiidae) *Beauveria bassiana* *Hypoaspis miles* (Berlese) (Acari: Laelapidae) *Hypoaspis aculeifer* (Canestrini) (Acari: Laelapidae)
Leaf miners *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) *L. bryoniae* (Spencer) (Diptera: Agromyzidae) *L. huidobrensis* (Blandchar) (Diptera: Agromyzidae)	*Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae) *Nesidiocoris tenuis* *Macrolophus pygmaeus* *Dacnusa sibirica* (Telenga) (Hymenoptera: Braconidae)
Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae)	*Nesidiocoris tenuis* *Macrolophus pygmaeus* *Bacillus thuringiensis* *Trichogramma achaeae* (Nagaraja and Nagarkatti) (Hymenoptera: Trichogrammatidae)
Caterpillars (Lepidoptera: Noctuidae) *Helicoverpa armigera* (Hübner) *Chrysodeixis chalcites* (Esper) *Autographa gamma L.* *Spodoptera littoralis* (Boisduval) *Spodoptera exigua* (Hübner)	*Nesidiocoris tenuis* *Macrolophus pygmaeus* *Bacillus thuringiensis*
Agrotis segetum (Denis & Schiffermüller)	*Bacillus thuringiensis*
Aphids (Hemiptera: Aphididae) *Myzus persicae* (Sulzer)	*Aphidius colemani* (Haliday) (Hymenoptera: Braconidae) *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) *Aphidius matricariae* (Haliday) (Hymenoptera: Braconidae) *Episyrphus balteatus* (DeGeer) (Diptera: Syrphidae)

	Nesidiocoris tenuis
	Macrolophus pygmaeus
	Adalia bipunctata (L.) (Coleoptera: Coccinellidae)
	Beauveria bassiana
Macrosiphum euphorbiae (Thomas) *Aulacorthum solani* (Kaltenbach)	*Aphelinus abdominalis* (Dalman) (Hymenoptera: Aphelinidae)
	Aphidius ervi (Haliday) (Hymenoptera: Braconidae)
	Aphidoletes aphidimyza
	Episyrphus balteatus
	Nesidiocoris tenuis
	Macrolophus pygmaeus
	Adalia bipunctata
	Beauveria bassiana
Spider mites (Acari: Tetranychidae) *Tetranychus urticae* (Koch)	*Nesidiocoris tenuis*
	Feltiella acarisuga (Vallot) (Diptera: Cecidomyiidae)
	Macrolophus pygmaeus
	Phytoseiulus persimilis (Athias-Henriot) (Acari: Phytoseidae)
	Beauveria bassiana
	Nesidiocoris tenuis
Tetranychus evansi (Baker & Pritchard)	*Nesidiocoris tenuis*
	Feltiella acarisuga (Vallot)
	Macrolophus pygmaeus
	Beauveria bassiana
	Nesidiocoris tenuis
Russet mite (Acari: Eriophyidae) *Aculops lycopersici* (Masse)	

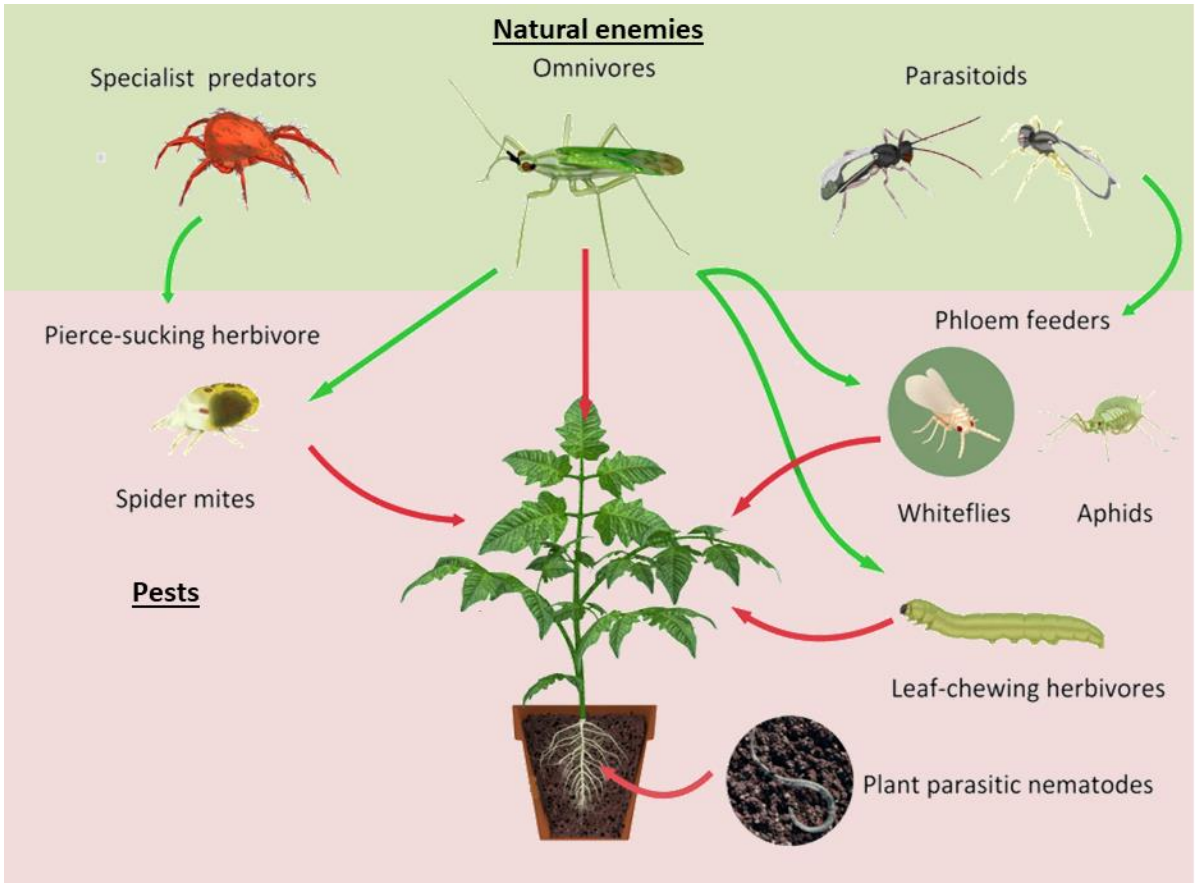


Figure 1.2. Food web of tomato arthropod pests with different modes of plant feeding and their natural enemies. Food chains in green are beneficial for the plant, in red are harmful for the plant. Adapted from Pappas et al. (2017).

In Spain, biological control was applied on protected tomato crops in Catalonia (Northeastern Spain) in the 1970s, and first IPM programmes were based on inoculative releases of the parasitoid *Encarsia formosa* (Hymenoptera: Aphelinidae) (Arno et al., 2009). In southern Spain, where most of tomatoes are cultivated, natural enemies were initially released in protected crops, in the end of 1990s, when Spanish farmers adopted the use of bumblebees for pollination and therefore preferred the application of selective pesticides for the control of pests (van der Blom et al. 2009). However, the main control measure for tomato pests remained the chemical control. When new invasive diseases, such as the TYLCV, transmitted by *B. tabaci*, affected Spanish tomato crops, the biological control of pests was disrupted by

the extensive use of conventional pesticides (Stansly et al., 2004). The use of IPM programs based on mirid predators was widely adopted, after the invasion of the threatening tomato pest *T. absoluta* in 2006 in Europe (Urbaneja et al., 2012). The predatory mirid *N. tenuis* could effectively control *T. absoluta,* as well as the key pests *B. tabaci* and *T. vaporariorum* (Calvo *et al*., 2009; Sánchez *et al*., 2014, Arno et al., 2009). Nowadays, in Spanish tomatoes, conventional pesticides are rarely used and IPM based on the use of mirids is widely applied in tomato crops (Pérez-Hedo *et al.* 2017; Arnó et al., 2009). In Europe, tomato key pests are managed either by *N. tenuis,* or *M. pygmaeus*, because both mirid predators are highly polyphagous. However, the tomato russet mite *A. lycopersici* is not controlled by biocontrol agents, but only by acaricide applications, because of the reported negative effects of the tomato trichomes on their natural enemies, the phytoseiids (van Haren *et al*. 1987; Cedola *et al*. 2001; Trottin-Caudal *et al*. 2003; van Houten *et al*. 2013).

1.3. A hairy problem for biological control: the tomato trichomes

Dense hair like structures called trichomes cover the tomato stems, leaves, petioles and flower buds.

The trichomes of *S. lycopersicum* were first categorized by Luckwill (1943) by their morphology as glandular with types I, VI and VII and non glandular with types III and V (Figure 1.3). Glandular trichomes of tomato have tips called ‘heads’ that release, on contact with arthropods, either herbivores or natural enemies, sticky and/or toxic exudates that entrap and potentially kill them. Non-glandular trichomes pose a barrier to the arthropod movement or to their access to feeding, having as well a negative effect on their fitness.

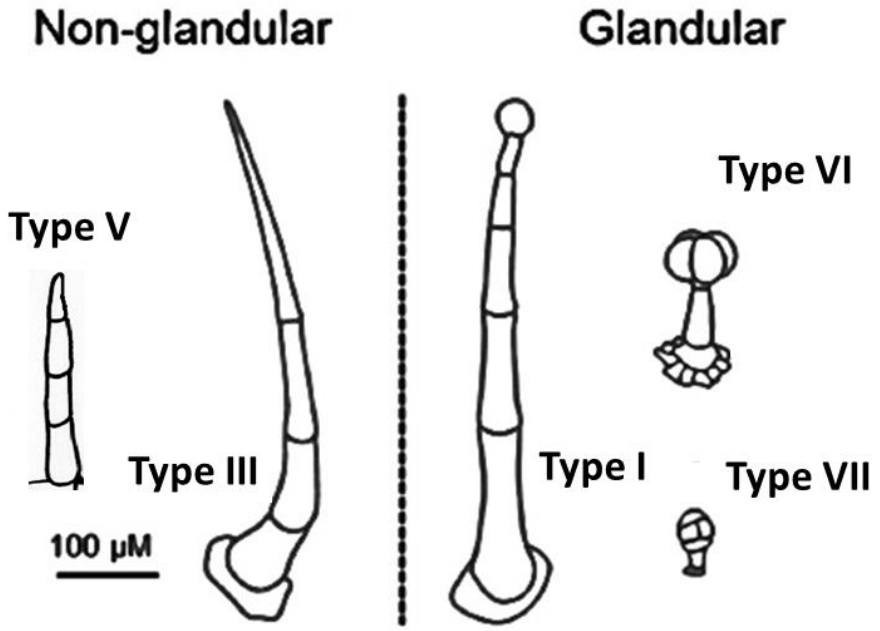


Figure 1.3. Trichome types from *Solanum lycopersicum.* Adapted from Tissier (2012).

Negative effects of trichomes on natural enemies of tomato pests have been reported for both insects and phytoseiids. Parasitism of *Heliothis* spp (Lepidoptera: Noctuidae) by *Trichogramma* spp. was lower on tomato accessions with higher densities of glandular type VI trichomes because these trichomes reduced the searching efficiency, and caused entrapment (Kauffman & Kennedy, 1989). Lifetime fecundity and walking speed of the coleopteran predator of whiteflies *Delphastus (pusillus) catalinae* (LeConte) (Coleoptera: Coccinellidae) were significantly reduced on tomato cultivars with high trichome densities (Heinz & Zalom 1996). The tomato trichomes reduced the searching efficiency and predation of the larvae of another beetle, the predatory *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), as well (Shah 1982). The mobility and the predation rate of *M. pygmaeus* and *Orius niger* (Wolff) (Hemiptera: Anthocoridae) nymphs were reduced on tomato cultivars with higher glandular trichome density (Economou *et al* 2006.*).* Searching efficiency and prey consumption of *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) were reduced on

tomato relative to bean and maize (Coll *et al.* 1997). Moreover the survival of *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) nymphs was lower on tomato plants compared to plastic plants (Lambert 2007) and the searching efficiency and predation rate of *Podisus nigrispinus* (Dallas) (Hemiptera: Pentatomidae) was reduced on the tomato compared to eggplant and pepper plants (De Clercq *et al.* 2000). *Episyrphus balteatus* (DeGeer) (Diptera: Syrphidae) foraging and oviposition rate was lower on tomato compared to broad bean and (Verheggen *et al.* 2009). Type VI trichomes on the stems of *S. lycopersicum* entrapped and killed *P. persimilis*, and higher entrapment rates were associated with high trichome densities (van Haren *et al*. 1987). Increases in temperature appeared to increase entrapment rates because the trichome glandular heads are more sensitive to rupture and may contribute to variation in the effectiveness of mite control by *P. persimilis* (Nihoul 1993, 1994). Tomato trichomes hampered the searching efficiency as well as the prey consumption and oviposition rate of both *Phytoseiulus macropilis* (Banks) and *Phytoseiulus longipes* (Evans) (Acari: Phytoseiidae) (Sato et al., 2011). Tomato cultivars with increased trichome density had direct negative effects on the survival of *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae), as well (Cédola & Sánchez, 2003).

1.4. Phytoseiidae

The family of phytoseiid predatory mites (Acari: Phytoseiidae) has a worldwide distribution and includes 67 different genera and more than 2,000 species (de Moraes *et al.*, 2004). Phytoseiids are considered to be the most effective group in the biological control of the spider mites (Helle et al., 1985). Moreover, phytoseiid predatory mites are especially interesting as mite biological control agents because they have short life cycles, high survival rates, they can thrive at low prey densities better than many predatory insects, some are polyphagous and effective natural enemies of different groups of pests and some are

commercially available (van Lenteren, 2001). Polyphagous phytoseiid species have been used in different crops as biological control agents of phytophagous mites (Helle et al., 1985, van Lenteren, 2000), as well as other pest species, such as thrips, whiteflies and other micro-artropods (Helle et al., 1985; McMurtry and Croft, 1997; Nomikou *et al.*, 2002) with satisfactory results. McMurtry and Croft (1997) categorized the diversity of life styles of phytoseiids, mainly based on their feeding habits, as follows: type I, predators specialized in *Tetranychus* spp. which includes the genus *Phytoseiulus*; type II, predators specialized in tetranichid mites represented by *Galendromus* sp. and some species of the genus *Neoseiulus*; type III generalist predators among which they include the genera *Typhlodromus* spp. and *Amblyseius* spp., and type IV, generalist predators and specialists in pollen, represented by *Euseius* spp., of which some species can survive by feeding only on pollen, in the absence of prey, with a low reduction of its reproduction rate. Because several species of the phytoseid family can develop and reproduce using pollen as food source (Zhimo and McMurtry, 1990, Nomikou *et al.*, 2002, Ragusa *et al.*, 2009), they can persist and even stay at relatively high population densities in the crop, when its main prey is scarse. Therefore, phytoseiids can prevent the reappearance of the pest, without the time lag that is usually associated with the numerical response of other natural enemies (Wiedenmann and Smith, 1997). The ability of these highly polyphagous predators to regulate pest populations has been questioned, but some examples suggest that pollen feeding allows polyphagous predators to control the populations of phytophagous mites and even improve their effectiveness in the field (González-Fernández *et al.*, 2009, Calvo *et al.*, 2015). Currently, mites belonging to the family Phytoseiidae are economically important predators of many phytophagous mites and insects in greenhouses or field crops (van Lenteren *et al.* 2012, 2018). Amongst other biocontrol agents, mass reared phytoseiid mites are commercially available and used particularly in greenhouses against spider mites, thrips and whitefly infestations on plants.

Phytoseiid mites use odors (kairomones) emmited by mite-infested plants, or the spider mite webbing to locate their prey, and are able to distinguish prey eggs from non-prey eggs and plant structures (Nomikou *et al.* 2005).

1.4.1. *Amblyseius swirskii*: description and life cycle

Amblyseius swirskii is a phytoseiid mite described originally in Israel and found in the eastern Mediterranean region (Athias Henriot, 1962). This species is an important biological control agent of mites, thrips and whiteflies in greenhouses and nursery crops (Calvo et al 2015). It can feed and reproduce on a wide range of prey from several orders, including thrips (western flower, onion, melon and chili), whiteflies (greenhouse and silverleaf) (Calvo *et al.* 2015), and plant feeding mites (spider-, broad- and eriophyoid-) (Stansly & Castillo 2009; Onzo *et al.* 2012). For this reason it has been used as a biocontrol agent on various crops including citrus, apples, grapes, apricot, vegetables, cotton and ornamentals. It feeds on the immature stages of thrips, whiteflies and spider mites, although it also consumes the adult stages of phytophagous mites, like spider mites and broad mites (Nomikou et al., 2001, Messelink et al., 2006, Stansly et al., 2010). As other phytoseiids, they search for their prey and then pierce and suck out their body fluids (Juan-Blasco et al., 2012).

Adults have pear-shaped body, 0.5 mm in length without segmentations and four pairs of long legs, and females are slightly larger than males (Figure 1.4). The eggs of *A. swirskii* are round near transparent pale white and measure approximately 0.15 mm in diameter (Figure 1.4). Females lay their eggs on leaf trichomes, in domatia or along the main veins on the underside of leaves. These mites prefer to lay eggs on trichomes on the inner surface of leaves or near plant domatia, maybe as an adaptation to protect the eggs from predators. The eggs hatch in about 2 days at temperature 25 ºC and 80% humidity. Larvae are transparent white in color droplet shaped and only have three short pairs of legs (Figure 1.4). The

protonymph and deutonymph have four pairs of legs and are slightly darker than the larvae, beige in color (Figure 1.4). All stages can be found in domatia, next to the main vein and lateral veins of the underside of leave, and in the flowers of the plant (Messelink *et al.* 2005).

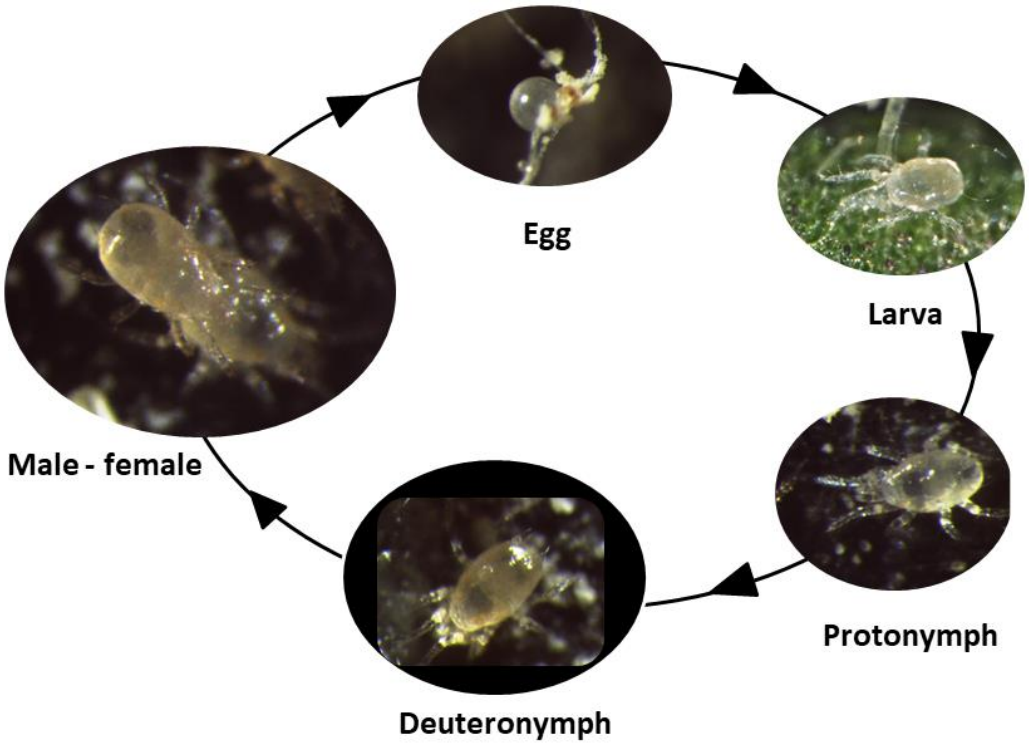


Figure 1.4. Life cycle and developmental stages of *A. swirskii.*

As a type III generalist predator, *A. swirskii* can reproduce on various pollens and plant nectars in addition to arthropod prey, which allow them to persist during periods of low pest density and improve their effectiveness as biocontrol agents (Nomikou *et al.* 2003, Goleva & Zebit. 2013). It can also feed and survive, but cannot reproduce, on honeydew, although with retarded development, suggesting that this diet may serve as a survival diet in the absence of prey or as a nutritional supplement along with prey (Ragusa & Swirskii 1977). Development of *A. swirskii* is influenced by the food source (prey, pollen and nectar), its availability and

the environmental conditions, such as temperature and humidity. Mites develop between 15 and 34 °C and from relative humidity as low as 60% (Lee *et al.* 2011). The egg to adult development period when they feed on prey, at 25 °C is approximately 5 days (Park *et al.* 2010). Mite development is faster feeding on prey and oviposition is higher when compared with feeding on pollen (Momen and Elsaway 1993). *A. swirskii* is commonly used to control whitefly and thrips infestations in greenhouse vegetables (for example cucumber, pepper and eggplant) and some ornamental crops (chrysanthemum, roses), and on citrus and other subtropical crops. This mite can be effectively combined in IPM biological control programs with other biocontrol agents, such as the parasitoid *E. mundus* (Calvo *et al.* 2012) and the predator *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) (Bouagga *et al.* 2018) in order to control whiteflies on sweet pepper crops. Most attention has been focused on *A. swirskii* because it is a very efficient biocontrol agent against the whitefly *B. tabaci*. This species is found worldwide causing severe yield losses in various crops and due to its polyphagy and resistance to insecticides; it rapidly became a serious pest (Cahill *et al.*, 1995). *A. swirskii* has a high reproductive rate and oviposition which is positively correlated to prey consumption during the oviposition period (Momen & El-Saway, 1993). Studies have shown that on sweet pepper and cucumber, *A. swirskii* can generate high population densities and effectively control whiteflies and thrips (Calvo et al., 2011). However, on tomato the population diminishes rapidly (M. Knapp, Koppert Biological Systems, The Netherlands, Personal Communication). This is thought to be a result of the dense trichomes on the leaves of these plants, hampering the mite dispersal and searching efficiency.

1.5. Objectives

The main aims of this thesis are to:

1. Determine the biological parameters of the predatory mite *A. swirskii* which are mostly affected on tomato plants and to quantify this effect. The tomato trichomes and their secondary metabolites which have the highest negative impact on the establishment of this predatory mite were also identified.
2. Estimate the dispersal ability of *A. swirskii* on three monogenic tomato mutants with different trichome phenotypes and their wild-type varieties. On a hairless tomato genotype with distorted trichomes, the efficiency of a biocontrol program, based on the augmentative releases of *A. swirskii* and *P. persimilis* against whiteflies and spider mites, respectively, was investigated.
3. Identify the genes involved in the transcriptomic response of *A. swirskii* to tomato trichome exudates. A further goal was to identify the gene members of three protein superfamilies that are involved in detoxification processes: the cytochromes P450 (CYPs), the glutathione S-transferases (GSTs) and the carboxyl/cholinesterases (CCEs) and compare with the ones present in phytophagous mites such as *T. urticae* and other phytoseiids.
4. Investigate the population genetic variation of field *A. swirskii* from Israel (where the commercial strain was originally collected (Y. Van Houten; Koppert Biological Systems, The Netherlands, Personal Communication)) with the genetic variation in a commercial, long-term reared population of this biocontrol agent. Within this study the potential of applying pooled microsatellite analysis to predatory mites, as a cost-effective solution to low individual DNA yields in minute organisms was explored for first time. Last, we compared the performance of the field populations and the

commercial, long-term reared *A. swirskii*, as biological control agents, by estimating three biological parameters, their predation, oviposition and dispersal ability on a favourable host plant, the sweet pepper plant, and an unfavourable host, the tomato plant.

Chapter 2

The tomato trichomes are deadly hurdles limiting the establishment of *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae)

To be submitted: Angeliki Paspati, José L. Rambla, María Pilar López Gresa, Vicent Arbona, Aurelio Gómez-Cadenas, Antonio Granell, Joel González-Cabrera, Alberto Urbaneja

Abstract

Amblyseius swirskii is a predatory mite widely used in organic farming and in integrated management programs in conventional agriculture as well, for the control of very important pest species such as whiteflies and thrips. However, this species cannot become established on tomato crops, probably due to the negative effect of the plant trichomes and their exudates on its biological parameters. In this work, the effect of tomato plants on *A. swirskii* was evaluated at four different levels: a) the effect of volatile-mediated plant traits on mite preference, b) the effect of plant leaves on the development, predation and oviposition of predatory mites, c) the effect of stem trichomes on the dispersal and survival of mites, and d) the effect of secondary metabolites secreted by tomato trichomes on mite survival. The results showed that *A. swirskii* avoid tomato plants, even if they have been previously in contact with this plant. On the other hand, it was demostrated that survival of *A. swirskii* eggs and juveniles was not affected on tomato leaves but, adult survival was significantly reduced when tested on the whole plant. This is due to the impact of trichomes and their secondary metabolites, present in high concentration on the stems, which affected the mites attempting to disperse on the plant. Finally, it was demonstrated that among the secondary metabolites detected in tomato trichomes, the strongest negative effect was exerted by the acyl sugars. They were highly toxic against the mites and were also detected physically stuck to their bodies after walking on tomato plants. Altogether our results show evidence suggesting why *A. swirskii* is not an efficient biocontrol agent on tomato and set the basis to address new lines of research that would allow the use of this phytoseiid in tomato crops.

2.1. Introduction

Amblyseius swirskii Athias-Henriot (Acari: Phytoseiidae), previously known as *Typhlodromips swirskii*, is a generalist phytoseiid, which feeds and reproduces on various arthropods and it is currently the most widely used biological control agent in augmentative biological control (Knapp et al., 2018). This predatory mite was originally described in 1962 in Israel (Athias Henriot, 1962), where it has been found on various annual and perennial crops, such as citrus, grapes, vegetables and cotton, usually associated with whiteflies (Swirski & Amitai, 1997). Today, it is used to control economically important greenhouse pest species such as whiteflies, thrips and plant feeding mites in vegetables, fruits and ornamentals (Calvo et al., 2015). In addition to its high efficacy, controlling these groups of pests, *A. swirskii* can establish on crops in the absence of prey, using pollen or factitious prey as a food source (Nomikou et al., 2003). Moreover, it can develop under a wide range of temperatures, since it does not enter diapause (Lee & Gillespie, 2011), it can be combined with other biocontrol agents (eg. *Orius* sp and mirid predators) (Doğramaci et al., 2011; Bouagga et al., 2018) and it is easily mass-reared on factious prey (Calvo et al., 2015). All these biological attributes of *A. swirskii* have contributed to its success in augmentative biological control worldwide. The integration of this phytoseiid in Integrated Pest Management (IPM) strategies against whiteflies and thrips in protected sweet pepper crops, in South-eastern Spain, has contributed to the sharp decrease in the use of chemical pesticides and was the first successful showcase of its potential in augmentative biological control of pests in protected crops (Calvo et al., 2011; Calvo et al., 2015; Van Lenteren et al., 2018).

However, *A. swirskii* cannot establish on tomato (*Solanum lycopersicum*), the most important vegetable crop in Europe, with a production of 17,059,000 tons in 2018 (Eurostat, June 2019). On detached tomato leaflets, *A. swirskii* can attack and develop on common tomato

pests, such as the tomato russet mite *Aculops lycopersici* Massee (Acari: Eriophyidae) (Momen and Abdel-Khalek, 2008; Park et al., 2010), the South America tomato pinworm *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) (Momen et al., 2013), the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and the two spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (personal observation). However, on tomato plants the survival and efficacy of phytoseiids are hindered, most likely due to the impact of tomato defenses mediated by the trichomes and their exudates (Kennedy 2003).

Tomato plants are covered by various types of trichomes which are not present on favorable host plants like the sweet pepper. Moreover, it has been shown that high trichomes density on host plants can be detrimental for insect predators (Riddick and Simmons, 2014). Tomato trichomes are diverse in terms of morphology and chemistry and are classified as glandular trichomes with types I, IV, VI and VII and as non-glandular trichomes with types II, III and V (Luckwill, 1934). Non-glandular trichomes are hair-like structures that cover the plant surface and function as physical barriers to arthropod dispersal and herbivory (Baur et al., 1991; Simmons and Gurr, 2005). Glandular trichomes have specialized cells on their tips that form the glandular heads and produce a variety of secondary metabolites with antibiotic and antixenotic effects against herbivores but also affecting natural enemies (Simmons and Gurr, 2005). Among glandular trichomes, the most abundant are type I and VI. They produce a wide array of compounds including high levels of the sticky acyl sugars, but also terpenoids and methyl ketones (Schilmiller et al., 2010). Terpenoids are highly volatile and play an important role in indirect plant defense mostly by attracting predators and parasitoids and repelling herbivores (Dicke et al., 1998; Bleeker et al., 2009). Predatory mites can respond to volatiles and may associate them with positive or negative conditions such as the presence of prey or absence of prey that leads to starvation, respectively (Drukker et al., 2000). Methyl

ketones can be toxic to phytophagous mites, such as the two spotted spider mite, but they are found only at trace levels on cultivated tomato (Chatzivasileiadis & Sabelis, 1997). Tomato glandular trichomes have been previously associated with the entrapment of small arthropods and have a negative impact on both, pests and natural enemies (Cédola and Sánchez, 2003). It has been shown that acyl sugars accumulate on the legs of aphids while walking on the plant, hampering their dispersal (Wagner et al., 2004), and that they can be toxic to mites at very low concentrations (Puterka et al., 2003). Also, these compounds reduce herbivore feeding, development and oviposition of various insect pests such as, the leafminer *Liriomyza trifollii* (Burgess) (Diptera, Agromyzidae), the moths *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), the thrips, *Frankliniella fusca* (Hinds) and *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (Hawthorne et al., 1992; Juvik et al., 1994; Resende et al., 2006; Leckie et al., 2016).

Defensive plant traits against herbivory can be deleterious to natural enemies and subsequently, of great importance for designing effective integrated pest management strategies. In this work, the objective was to unravel the effect of the defensive traits of tomato plants limiting the performance of the generalist predatory mite *A. swirskii* and to identify the secondary metabolites most likely responsible for these effects. To do this, first the behavioral response of *A. swirskii* to the volatiles emitted by tomato plants was evaluated, indicating how stressful the tomato plant is for the mite. Secondly, the mite performance on tomato leaves was measured as predation capacity and oviposition rate. These parameters were compared with those obtained on sweet pepper plants, a favorable host plant for this phytoseiid (Calvo et al., 2011). Thirdly, as the mites usually disperse through the stems to

avoid kin competition and overexploitation, the effect of tomato stem trichomes on the dispersal and survival of *A. swirskii* was assessed. Also, the capacity of trichomes secretions to stick to the mites' body parts was assessed by microscopy. Finally, to move further on the identification of secondary metabolites influencing the deleterious effects of tomato, an extraction of such metabolites with several solvent fractions was performed and their toxicity on mites was characterized.

2.2. Materials and methods

2.2.1 Plants and mites

Tomato plants, *Solanum lycopersicum* cv. Raf Marmande and sweet pepper plants *Capsicum annuum* cv. Lipari were used in the olfactometer experiment and the estimation of life history parameters of *A. swirskii* was performed as described below. Seeds were sown in a mixture of soil and local peat moss. Two weeks after germination seedlings were individually transplanted into pots (8 × 8 × 8 cm). Plants were maintained undisturbed at 25 ± 2 ºC, 65 % ± 5% relative humidity (RH) and 14:10 h (Light: Dark) photoperiod. Pesticide-free plants with 6 fully developed leaves were used for the experiments.

Colonies of *A. swirskii* were initiated from specimens supplied by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). They were maintained in rearing units; a piece of hard black plastic on top of a water saturated sponge, which is placed in a plastic tray with water. The borders of the plastic were covered with water saturated tissue paper to ensure a constant water supply for the phytoseiids, to fix the plastic piece to the sponge, and to prevent phytoseiids from escaping (Abad-Moyano et al., 2009). Cotton threads 2 cm long were provided on the rearing units to serve as oviposition sites. Twice a week, mites were fed *ad libitum* with *Carpobrotus edulis* (L) (Caryophyllales: Aizoaceae) pollen (Ragusa & Swirski,

1975). The colonies were maintained at 25 ± 2 ºC in growth chambers at 14:10 h (Light: Dark) photoperiod and 80 ± 10 % RH.

2.2.2 Olfactory responses to tomato volatiles

The olfactory response to tomato of three different sources of experienced mites was investigated in a Y-tube olfactometer. Experienced mites were obtained by releasing adult female mites for 48 hours on either sweet pepper leaves, tomato leaves or plastic arenas, with *C. edulis* pollen as food source *ad libitum* and left undisturbed at 23 ± 2 °C, 60 ± 10 % RH. Before testing, the mites were collected from the three different arenas and kept for one hour on a clean Petri dish to remove traces of pollen from their bodies. Mites experienced on sweet pepper leaves were tested for their response towards the following experimental treatments: sweet pepper plants (known plant host) vs clean air (no plant host), and sweet pepper plants vs tomato plants (unknown plant host). Female mites experienced on tomato leaves were tested for their response towards the following experimental treatments: tomato plants (known plant host) vs clean air (no plant host), and tomato plants vs sweet pepper plant (unknown plant host). Finally, mites reared on plastic, inexperienced to plants, were tested for their olfactory response towards the following experimental treatments: sweet pepper plant vs tomato plants, sweet pepper plant vs clean air and tomato plant vs clean air.

The Y-tube olfactometer consisted of two glass jars, 5 L volume, connected to a Y-shaped glass tube (2.4 cm diameter), containing a metal string of the same shape (Pérez-Hedo et al., 2015). Unidirectional humidified airflow was pumped at 150 ml/min in each glass jar. Each female mite was individually released on the metal string, at the entrance of the Y-tube and its choice was recorded once the female had walked up one of the Y-tube arms, within 10 minutes. After every 5 valid responses the metal string and the Y-tube was rinsed with water, soap and acetone and the left and right odor source tubes were interchanged in order to

minimize any spatial effect on the olfactory response. All plants used as odor sources were replaced after recording the response of ten mites. A total of 30 valid responses were recorded for each type of mite experience (sweet pepper, tomato, plastic) and each pair of odor sources. The Y-tube experiment was conducted at the following environmental conditions, 23 ± 2 °C, 60 ± 10 % RH. Light was provided by four 60-cm-long fluorescent tubes (OSRAM, L18 W/765, OSRAM GmbH, Germany) positioned 40 cm above the Y-tube and its intensity was measured with a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc., Pullman, WA) at 2,516 lux (Pérez-Hedo et al., 2015).

2.2.3 Immature survival on tomato leaflets *vs* sweet pepper leaves

Approximately one hundred female *A. swirskii* mites from the colony were placed for 24 h on two clean plastic rearing units with cotton threads to allow oviposition (as described in 2.1). Then, with the help of a fine paint brush, the eggs were gently collected from both arenas and transferred evenly to plants. Eggs were individually placed on either one tomato leaflet or on a pepper leaf, whose petioles were covered with tanglefoot odorless glue (The Tanglefoot Company, Grand Rapids, MI, USA) to prevent the escape of the young mites after hatching. In total, twenty eggs were individually placed on the tomato leaflets of two intact tomato plants and another 20 were placed individually on the leaves of four intact sweet pepper plants. Both pepper and tomato plants had 6 fully developed leaves and each fully developed tomato leaf had 5-7 leaflets. Egg hatching and mite survival were evaluated daily for one week. Pollen of *C. edulis* was added every two days on the leaflets and leaves as food source.

2.2.4 Predation and oviposition on tomato leaflets vs sweet pepper leaves

Presumably mated females from the colony were transferred to plastic rearing units with four cotton threads 2 cm long (as described in 2.1) and were allowed to oviposit for 24 hours.

Later, the four cotton threads, with approximately 200 eggs in total (50 eggs on each approximately), were transferred to two rearing units with tomato leaflets and two rearing units with sweet pepper leaves (one cotton thread on each unit). The rearing units consisted of either detached tomato leaflets or sweet pepper leaves, placed on water saturated sponges that were covered with wet cotton, and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs added as food source *ad libitum*. After seven days, 50 females and males were collected from the latter rearing units. Couples of male and female were individually placed, isolated on either tomato or sweet pepper leaf discs with 4 cm diameter (25 pairs per host plant species), with *E. kuehniella* eggs as food source and observed every 24 hours until the first oviposition was detected. After the first oviposition, males were removed, and the number of *E. kuehniella* eggs preyed by *A. swirskii* females and the number of eggs laid per female was counted every 24 hours, during six days. The first day of oviposition was excluded from the analysis because the mites were stressed from the change to a new environment (personal observation). A total of 25 females (replicates) per host plant (tomato or sweet pepper) were tested. The leaf discs were maintained fresh on 1 % agar (w/v) gel, inside plastic cups (5 cm on diameter) with 2 × 2 cm screens on the lid covered with a fine mesh for ventilation but preventing mite escaping. All rearing units and experimental set ups were maintained in growth chambers at 25 ± 2 ºC, 14:10 h (Light: Dark) photoperiod and 80 ± 10 % RH.

2.2.5 Dispersal on tomato stems

Adult female mites from the colony were used to investigate their dispersal on tomato plants with intact stem trichomes and tomato plants with removed stem trichomes. The trichomes of the stem were removed by mechanical pressure, after rubbing softly the stems with a paper tissue and verifying the removal of the trichomes under the stereoscope. The central part of the stem with three successive leaves was used for the observations and it was delimited by

Tanglefoot ® (The Tanglefoot Company, Michigan, USA) glue barriers. On the part with the three successive leaves, the middle leaf was removed and pollen of *C. edulis* was added on the other two leaves. One day later, one female mite was released on the scar left at the stalk base and it was assessed whether the mite had reached the successive leaf or not after two hours (adapted from Van Haren et al., 1987). If the mite reached the successive leaf, the dispersal was recorded as successful. If the mite was stuck to the exudate of the trichomes, the survival was registered after 24 hours. Mites were considered alive if they moved after a gentle probe with a fine paint brush. Thirty-five female mites were tested on each type of tomato plant, with and without trichomes, at 22 ± 3 °C and 50 ± 10 % relative humidity.

2.2.6 Isolation, purification and characterization of trichome secondary metabolites

Tomato plants cv Muchamiel were grown in a greenhouse at Instituto de Biología Molecular y Celular de Plantas at the Universitat Politécnica de Valencia under standard growing conditions. To isolate the tomato trichomes from tomato stems, leaves were removed, and the petioles and the stems were submerged in liquid nitrogen. Then, the frozen stems and petioles were softly rubbed with a fine brush inside a mortar with liquid nitrogen in order to remove and collect the frozen trichomes. Three grams of isolated trichomes were used to extract non-volatile compounds by adding 12 ml of isopropanol:acetonitrile:water (3:3:2 v/v/v). The extract was vortexed vigorously, sonicated for 10 min (Selecta 300683) and then centrifuged at 12,000 rpm for 15 min (Allegra 64R, Beckman Coulter, USA). The supernatant containing trichome secondary metabolites was concentrated by evaporation under vacuum. Then, the concentrated secondary metabolites were combined with Bondesil-C18 40 μM Silica gel (Varian), at a weight to weight ratio of 2:3, secondary metabolites to C18 Silica gel and were purified using a dry column vacuum chromatography protocol. Varian solid phase extraction columns were placed on a filter flask attached to a vacuum and the dried residue was then

loaded on top. Columns were washed under vacuum pressure four times using 5 ml of water. Secondary metabolites were then eluted from the column with two 5 ml methanol washes of decreasing polarity (water containing 25, 50, 75, and 100 % methanol) and were named FII, FIII, FIV and FV, respectively (adapted by Leckie et al., 2016).

For chromatographic analyses, samples were diluted in 80 % methanol (LC/MS grade, supplemented with biochanin A as an internal standard for relative quantitation purposes) to reach a concentration of 1 mg/ml (respect of the dry residue amount).

UPLC/MS analyses were performed using an Acquity SDS LC system (Waters Corp., MA, USA) coupled to a Q-TOF Mass Spectrometer (Micromass Ltd., UK), similarly as described in Ghosh et al., (2014). Ten microliters of each trichome extract fraction (1 mg/ml) were injected. Separation was performed using a C18 Analytical HPLC column (Luna Omega 1.6 µm Polar C18, 2.1 × 100 mm,). The mobile phase consisted of aqueous 10 mM ammonium formate, pH 2.64 (Solvent A) and acetonitrile (Solvent B) using a linear gradient elution of 1 % B at 0–1 min, 1–80 % B at 1–100 min, 80–100 % B at 100–101 min, 100 % B at 101–105 min and 1 % B at 105–106 min. A 4 min re-equilibration time was used between analyses. During analyses, the solvent flow rate was 0.3 ml/min and the column temperature was 40 °C. Analyses were performed in positive and negative ion modes. Source parameters were as follows: capillary voltage 2500 V, sample cone voltage 30 V, desolvation temperature 350 °C, source temperature 120 °C, cone gas flow 40 l/h and desolvation gas flow 350 l/h for the negative ion mode. For positive ion mode, the capillary voltage was set to 3500 V and sample cone voltage was set at 30 V. Mass spectra acquisition was performed within the *m/z* 50 to 1,500 a.m.u. range in both positive and negative ion modes with a scan time of 0.2 s. The fragment ions were obtained by setting a second acquisition function within the same m/z range but including a collision-induced

dissociation step (collision cell energy ramp was set between 5 and 60 eV). Accurate mass values were obtained by co-injecting Leu-enkephalin as a lockmass standard compound ($[M+H]^+$ 556.2771, $[M-H]^-$ 554.2615).

Tentative identification of acyl sugars was performed by comparison of both, mass spectra and retention time with those in the *Solanum* trichome metabolite database version 2015.MSU.004P (Jones, 2015). Identification of all other metabolites was performed by means of comparison of both mass spectra and retention time with those of authentic standards, when available, or by matching precursor and MS/MS mass spectrum with those available in literature or public databases (METLIN, HMDB or KNApSAcK).

2.2.7 Toxicity of trichome extracts

The fractions were concentrated by evaporation and their dry weight was estimated. Then they were diluted again in 75 % methanol to reach a concentration of 20 mg/ml. These solutions were then used to make working dilutions of 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml, for fractions FII – FV. Moreover, even aliquots of fractions FII and FIII were combined to form the more polar fraction (F-MP) while the same was done with fractions IV and V to form the less polar fraction (F-LP). A 1:1 mixture of two fractions was created by combining equal parts of both fraction solutions at 20 mg/ml, thereby creating a mixture with an overall concentration of 20 mg/ml, wherein each fraction was represented at 10 mg/ml upon application. F-MP and F-LP dilutions of 10 mg/ml and 5 mg/ml were tested for toxicity against *A. swirskii,* as well (adapted by Leckie et al., 2016).

To test the toxicity of dilutions of each fraction and the F-MP, F-LP mixtures, a 2 µl droplet was applied, with an automatic micropipette, on a single female mite for 1 minute inside a glass Petri dish. Afterwards, the droplet was dried with a paper tissue and the mortality was

recorded after 30 minutes. For each fraction, 20 mites were tested at room temperature (23 ± 2 ºC) and 50 ± 5 % RH. Also, a solution of 75 % methanol in water was tested on 30 mites as a blank control to test if the buffer solution caused mortality to the mites.

2.2.8 Detection of acyl sugars on mites

To stain the acyl sugars present in the trichomes, the middle part of tomato stems (10 cm long) was cut and the leaves removed. The stem sections were soaked in 0.2 % Rhodamine B (Sigma-Aldrich, St. Luis, Missouri, USA) for 60 minutes and then washed 4 times with distilled water to remove unbound stain (Lin and Wagner 1994). The stems were left to dry for 24 hours, and then adult female mites were released individually for approximately 12 hours on the stems. Then the mites were killed by freezing and they were subsequently observed under the fluorescence microscope (excitation 550 nm / emission 582 nm) (Wagner et al., 2004). All manipulations and solutions were performed at room temperature (22 ± 3 °C).

2.2.9 Statistical analysis

The olfactory responses to tomato and sweet pepper volatiles was analyzed using the Exact Binomial Test (R package), with Clopper-Pearson 95 % confidence interval, to compare the number of mites attracted to volatiles from sweet pepper, tomato or to clean air, against the null hypothesis that the probability of mites choosing any odour is equal. Immature survival probabilities on tomato and pepper plants were compared with a Fisher's Exact Test (R package), where p-values are obtained using the hypergeometric distribution, to test the null hypothesis that these probabilities are similar. The data on predation and oviposition on tomato leaflets *vs* sweet pepper leaves were fitted to a Generalized Linnear Model with quasipoisson distribution and the F-Test was applied to compare the variances. A Fisher's

Exact Test was used as well, to test the null hypothesis that the probabilities of dispersal and survival on tomato plants with trichomes were similar to the probabilities on the tomato plants without the trichomes. Toxicity data were fitted to a Generalized Linnear Model with binomial distribution and the χ^2 test was applied to compare the variances. Dose response curves were fitted to a model and the 50 % lethal concetration (LC_{50}) was estimated using the R package drc version 3.0. To test for effects of mixture of fractions and their interaction on the mite mortality, data were analyzed by a generalized linear model including the rate of each fraction in F-MP and F-LP respectively, and the interaction of these rates. Synergism or antagonism between the two components of each mixture would be indicated by a significant interaction term in the model fit. All the statistical analysis was performed on the software R version 3.5.1.

2.3 Results

2.3.1 Olfactory responses to tomato volatiles

Amblyseius swirskii mites, experienced on tomato leaves, preferred either the unknown host plant, sweet pepper (P = 0.04, N = 30, binom. test) or the absence of plant (clean air) (P = 0.005, N = 30, binom. test) showing a clear avoidance of the known environment, the tomato plant (Figure 2.1). On the contrary, mites experienced on sweet pepper plants preferred to move towards the known host plant, the sweet pepper, instead of the unknown host (tomato plant) (P < 0.001, N = 30, binom. test) or the absence of plant (P = 0.001, N = 30, binom. test) (Figure 2.1). Finally, when mites that did not have a previous experience on any host plant, were tested for their preference between two unknown host plants, the tomato and the sweet pepper, or between one host plant (either tomato or sweet pepper) and the absence of plant, no statistically significant preference was identified, since the mites were choosing

equally any of the two environments offered in each comparison ($P > 0.05$, $N = 30$, binom. test) (Figure 2.1).

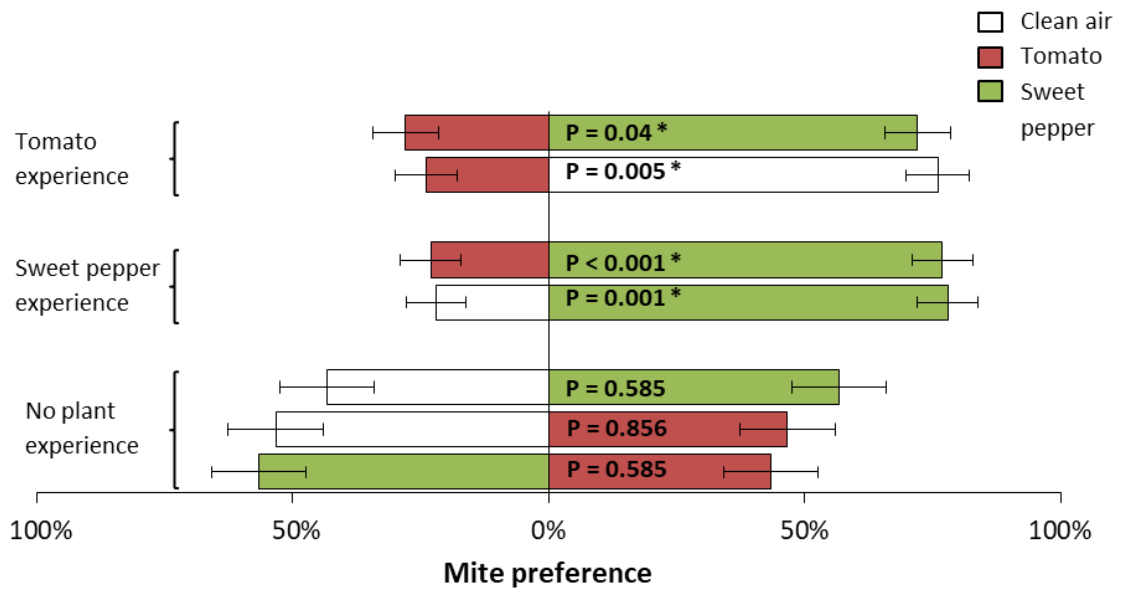


Figure 2.1 Olfactory response rate of *Amblyseius swirskii* females, inexperienced with plants, experienced on sweet pepper leaves and experienced on tomato leaves, towards the volatiles of sweet pepper, tomato or versus clean air. Red color indicates response to tomato volatiles, green color indicates the response to sweet pepper volatiles and white indicates the mite response to clean air. The true responses of 30 mites were collected from each two-side choice experiment. Significant differences, based on the Exact Binomial Test with Clopper-Pearson 95 % confidence interval, are marked with (*) (P<0.05).

2.3.2 Immature survival and adult performance on tomato leaflets

The egg hatching rate was 100 % on both, tomato and sweet pepper leaves and the pre-adult survival rate from egg to adult was 95 % and 100 % for mites reared on tomato and mites reared on sweet pepper leaves, respectively ($P = 1$, $N = 30$, Fisher's Exact Test). The oviposition rate of adult female mites on tomato and sweet pepper leaf discs was estimated for the first five days after oviposition started and was found to be 2.05 ± 0.12 eggs/female/day and 1.8 ± 0.08 eggs/female/day, respectively, which were not significantly different between them ($F = 2.80$; df = 1, 156; $P = 0.09$) (Figure 2.2). The predation rate of *E.*

kuehniella eggs by females of *A. swirskii* was similar in both cases, reaching 4.65 ± 0.22 eggs/female/day on sweet pepper and 4.22 ± 0.17 eggs/female/day on tomato leaf discs (F = 2.47; df = 1, 158; P = 0.11) (Figure 2.2).

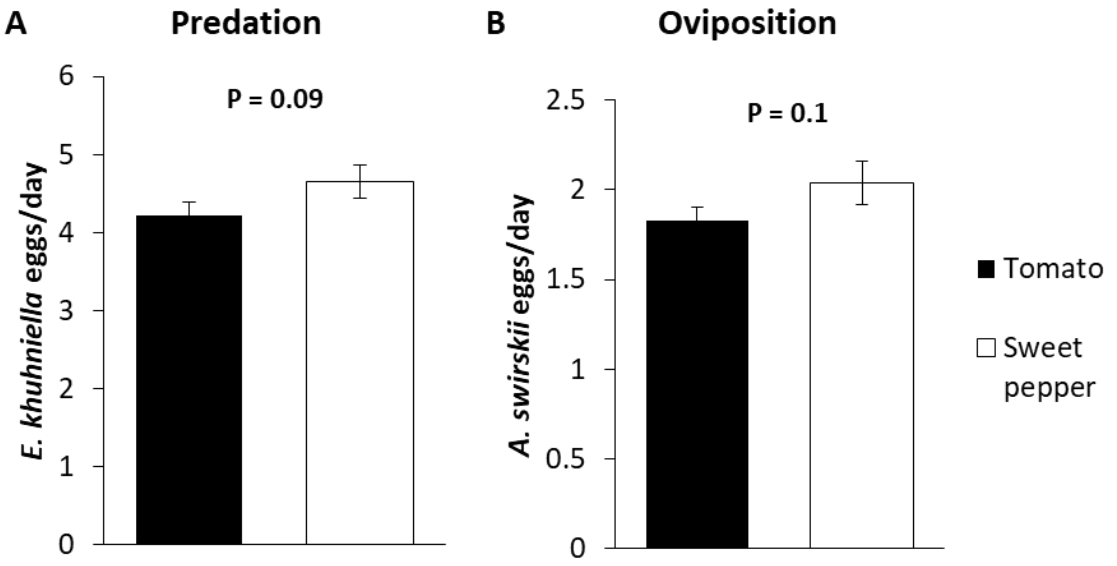


Figure 2.2. A: Mean predation of *A. swirskii* female mites and standard error (SE) on tomato and sweet pepper leaves for five days. B: Mean oviposition rate of *A. swirskii* female mites and SE on tomato and sweet pepper leaves for five days. The data for both parameters were collected from 25 mites for each plant, they were fitted to a generalized linear model and an F-test was applied to the model variances.

2.3.3 Dispersal on tomato stems

The dispersal rate of *A. swirski* adult female was tested on tomato stems with trichomes and it was found to be only 25 ± 9 %, in contrast to the 100 % dispersal rate recorded on tomato plants with removed trichomes (P < 0.001, N = 35, Fisher's Exact Test) (Figure 2.3). On tomato plants without trichomes, all the mites survived for 24 hours, however only 38 ± 10 % of them survived on plants with trichomes (P <0.001 N = 35, Fisher's Exact Test) (Figure 2.3). In particular, on tomato plants with trichomes, the fraction of mites that survived for 24 hours includes the mites that successfully dispersed to an adjacent leaf (25 ± 9 %) and those

that were entrapped on the trichomes but alive, being able to move at least one limb after 24 hours (13 %).

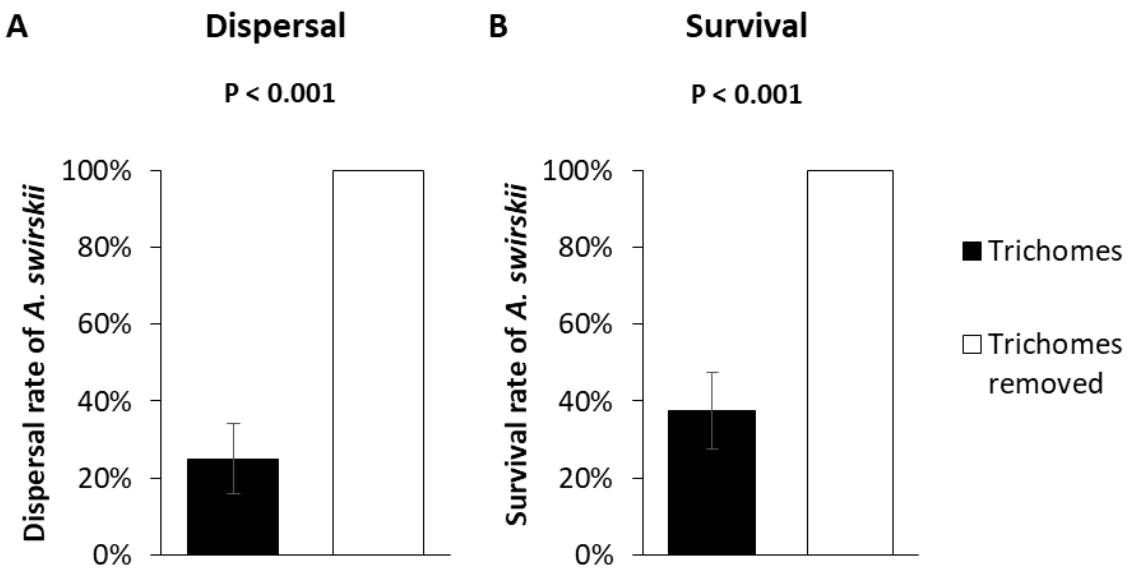


Figure 2.3. A: Mean dispersal and standard error (SE) on tomato stems with trichomes (black bars) and without trichomes (white bars) 2 hours after release. B: Mean survival rate of *A. swirskii* and SE on tomato stems with trichomes (black bars) and without trichomes (white bars) 24 hours after release. The data were analyzed with a Fisher´s Exact Test.

2.3.4 Toxicity of trichome extracts

Trichome extracts of tomato plants were separated by dry column vacuum chromatography into four fractions, based on polarity, with the most polar fraction FII, followed by FIII, FIV and last the least polar fraction FV (Table 2.1). These fractions were tested for their toxicity on adult mites in a range of concentrations from 1.25 to 20 mg/ml (Figure 2.4). The mortality rates were fitted to a model with binomial distribution and it was found that some fractions were significantly toxic to the mites (χ^2; df = 3, 16; $P < 0.001$). The highest toxicity was recorded with fraction FV at 20 mg/ml where 100 % of the mites were killed. In addition, this

fraction caused 95 % mortality at a concentration as low as 5 mg/ml (Figure 2.4). Control testing on 30 mites, with blank 75 % methanol in water, did show any mortality.

Table 2.1. Estimated concentrations, in mg/ml, required to cause 50 % mortality (LC_{50}) and their 95% delta confidence intervals (CI) for four fractions of trichome extracts. Trichome secondary metabolites were extracted and fractioned according to decreasing polarity (water containing 25, 50, 75, and 100 % methanol) and were named FII, FIII, FIV and FV, respectively. Even aliquots of fractions FII and FIII were combined to form the more polar fraction (F-MP) while the same was done with fractions IV and V to form the less polar fraction (F-LP).

Fraction	LC_{50}	Std. Error	Lower CI	Upper CI
FII	26.72	47.74	0.00	120.28
FIII	28.52	11.07	6.83	50.21
FIV	15.23	3.62	8.13	22.34
FV	2.28	0.32	1.66	2.91
F-MP	29.37	134.47	0	292.91
F-LP	6.51	0.74	5.06	7.97

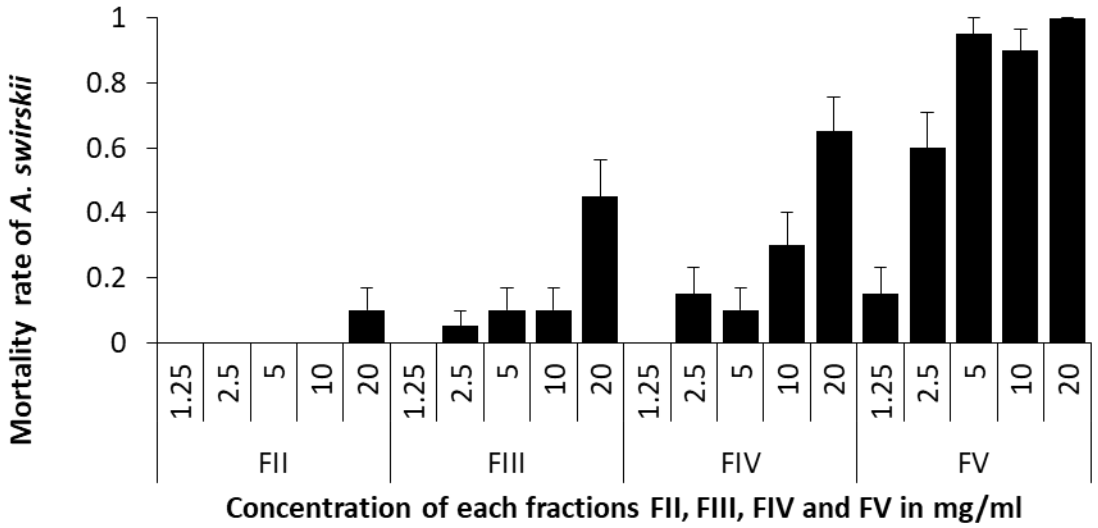


Figure 2.4. Mortality rate of *A. swirskii* mites exposed to four fractions of trichome extracts with different polarity (FII, FIII, FIV, FV). Each fraction was tested at five concentrations (1.25, 2.5, 5, 10, 20 mg/ml) on 20 mites.

Dose response curves were fit for each fraction, and the lethal concentration required to kill 50 % of the mites (LC_{50}), with 95% delta confidence intervals, were estimated (Figure 2.5, Table 2.1). The lowest LC_{50} were observed with fraction FV at 2.28 ± 0.32 mg/ml, followed by FIV at 15.23 ± 3.62 mg/ml, whereas the LC_{50} for the fractions FII and FIII was not in the tested range of concentrations and was estimated by the model approximately two times higher (Table 2.1).

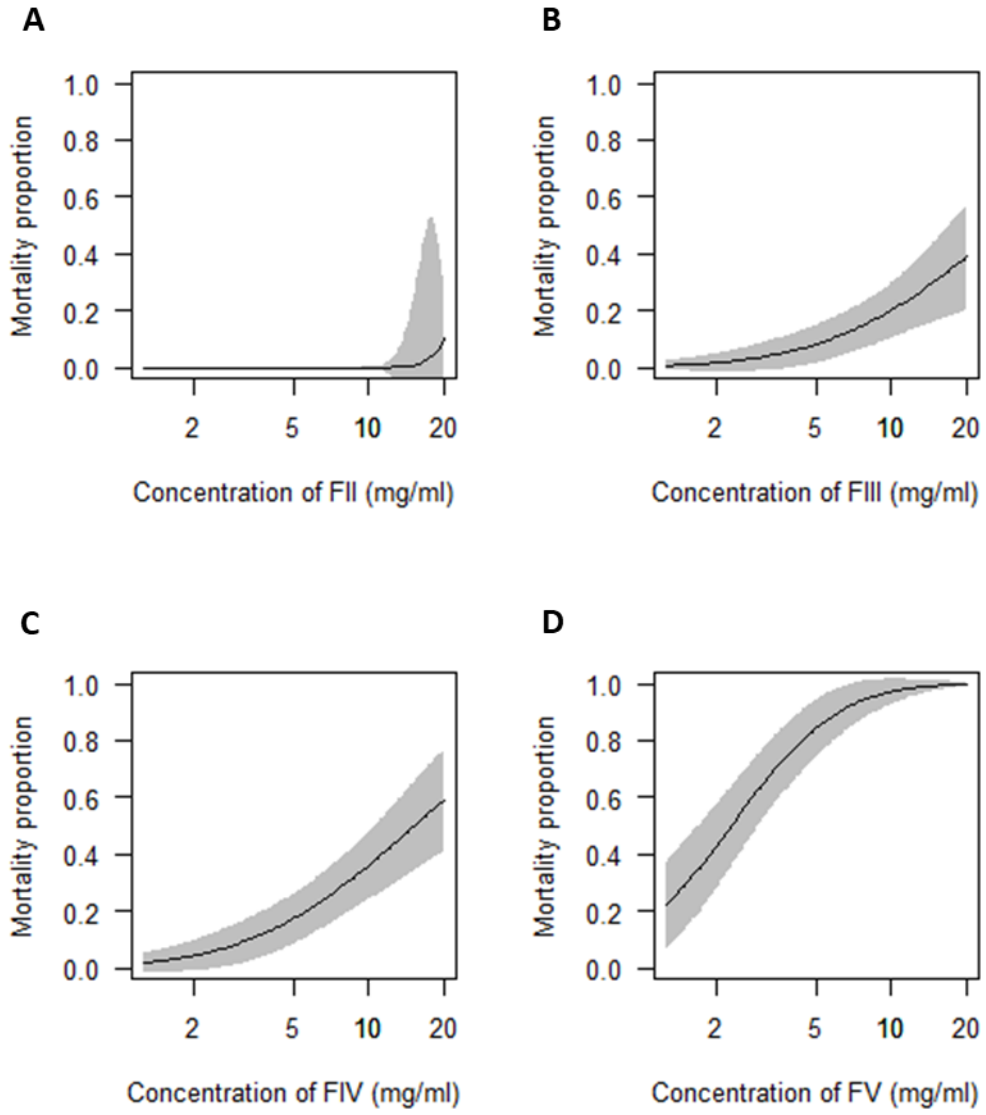


Figure 2.5. The dose response curve and the 95 % delta confidence interval of *A. swirskii* mortality to A) fraction FII, B) fraction FIII, C) fraction FIV and D) fraction FV of trichomes extract.

To investigate possible synergistic or antagonistic effects between fractions, the mixtures F-MP and F-LP were derived from the combination of FII - FIII and FIV-FV, respectively. In the presence of either synergy or antagonism, the mixture should cause a different response than that expected in case of no interactions between the components of the mixture. At a

concentration of 20 mg/ml, fraction F-MP, the 1:1 mixture of fractions FII and FIII, caused 5 % mite mortality, while FII and FIII caused 0 % and 10 %, respectively. Hence, the effect of the mixture F-MP was not stronger than that for the sum of the mixture's components in the toxicity test, neither the interaction effect of FII × FIII rates ($P = 0.19$, χ^2 test). Fraction F-LP, the 1:1 mixture of the fractions FIV and FV, caused 95 % mortality at a concentration of 20 mg/ml, consistent with the combination of the effects of FIV and FV (65% and 100%, respectively). The interaction effect of FIV × FV rates in Fr-LP was not significant ($P = 0.08$, χ^2 test) as well, and only the rate of FV in the mixture had a significant effect on mite mortality ($P < 0.001$, χ^2 test). Hence, there was no synergism or antagonism detected with fraction combinations. This finding is also depicted on the LC_{50} values for F-MP and F-LP, which are not very different from the values found for their individual components (Table 2.1).

2.3.5 Isolation, purification and characterization of trichome secondary metabolites

LC-MS characterization of each fraction revealed that fractions FIV and FV contained several acyl sugars and that the concentration of these compounds was higher in FV (most toxic) by one order of magnitude (Table 2.2). In total, 16 acyl sugars of sucrose were identified in the trichome extract fractions as described on Table 2.2. On the other hand, acyl sugars were not detected in the least toxic fractions, FII and FIII. Some other compounds, such as rutin, kaempferol, tomatine, were detected at similar levels in all fractions.

Table 1.2. The list of acyl sugars identified and characterized by mass spectroscopy with negative electrospray ionization. For each identified acyl sugar, tentative identity, according to the Michigan State University database and its nomenclature, empiric formula, molecular mass, retention time (RT) and its abundance in each of the four fractions (FII, FIII, FIV and FV), measured as peak height of the chromatogram.

Identity	Empiric Formula	Molecular Mass	RT (min)	Abundance (peak height)			
				F V	F IV	F III	F II
S4:16	$C_{28}H_{46}O_{15}$	622.2837	50.75	39	nd	nd	nd
S4:17	$C_{29}H_{48}O_{15}$	636.2993	54.27	750	29	nd	nd
S4:17	$C_{29}H_{48}O_{15}$	636.2993	53.95	104	nd	nd	nd
S3:20	$C_{32}H_{56}O_{14}$	664.3306	68.57	149	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	73.35	92	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	71.66	32	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	58.08	57	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	59.42	45	nd	nd	nd
S3:22	$C_{34}H_{60}O_{14}$	692.3619	74.58	65	nd	nd	nd
S3:22	$C_{34}H_{60}O_{14}$	692.3619	76.26	1990	nd	nd	nd
S3:22	$C_{34}H_{60}O_{14}$	692.3619	76.48	1420	nd	nd	nd
S4:22	$C_{34}H_{58}O_{15}$	706.3776	73.88	59	nd	nd	nd
S4:22	$C_{34}H_{58}O_{15}$	706.3776	79.22	43	nd	nd	nd
S4:23	$C_{35}H_{60}O_{15}$	720.3932	78.68	tr	nd	nd	nd
S4:24	$C_{36}H_{62}O_{15}$	734.4088	75.68	66	nd	nd	nd
S4:24	$C_{36}H_{62}O_{15}$	734.4088	81.75	337	nd	nd	nd

* In Identity: letter = sugar type (S=sucrose), number = number of esters with acyl groups : number = total number of carbons. In Abundance: nd= not detected, tr= found at trace levels.

2.3.6 Detection of acyl sugars on mites

The fluorescent microscopy of the type VI trichomes indicated the presence of sugar esters in the glandular heads, most likely the acyl sugars produced and stored by the glandular cells (Figure 2.6). Moreover, the images of mites that walked on the stems with the stained trichomes revealed that the acyl sugars are released from the trichomes and accumulate on the mite cuticle and in the limb joints and mouth parts from where it is likely to penetrate under the cuticle (Figure 2.6).

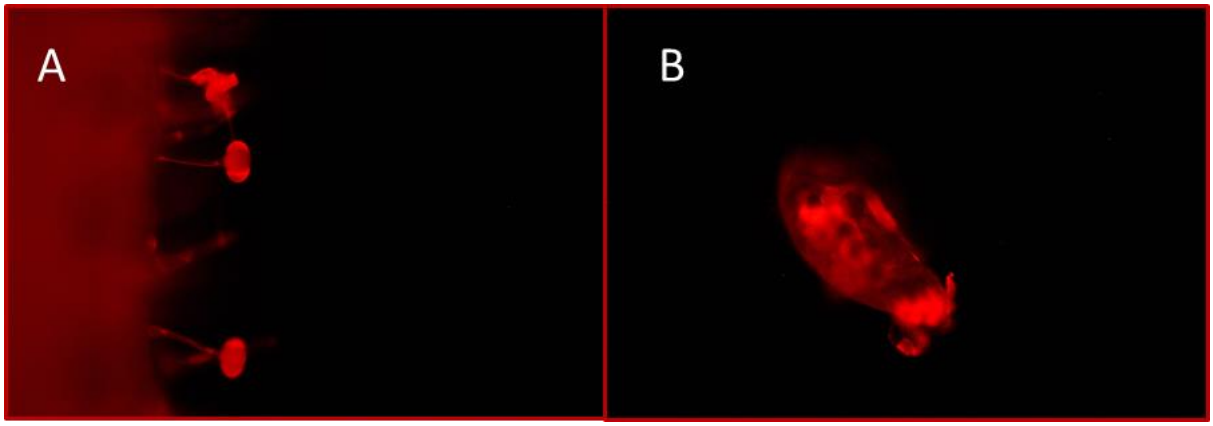


Figure 2.6. Fluorescent microscopy of (A) type VI tomato trichomes and (B) *A. swirskii* mites which were exposed to stained tomato stems for 12 hours.

2.4 Discussion and conclusions

Predatory mites can associate volatile plant cues with positive or negative conditions on the host plant, a process called associative learning (Drukker et al., 2000). In predatory mites, negative associative learning using volatile plant cues has been previously associated with stressful conditions such as starvation, but never with the plant trait itself (Drukker et al., 2000). Here, it was demonstrated that predatory mites experienced with tomato leaves, preferred a new, unknown environment with or without plant, instead of the tomato plant. Therefore, the avoidance of this host indicates a negative, stressful experience of the mites, associated with tomato plants regardless the presence of prey.

The egg hatching, juvenile survival, oviposition and predation rates of predatory mites were not significantly different on tomato leaves compared to sweet pepper leaves under the given experimental conditions. The oviposition rate of *A. swirskii* fed on *E. kuehniella* eggs estimated in this study was higher than the lifelong oviposition rate with the same food source (1.48 eggs/female/day at 23 ºC) (Nguye et al., 2013). This is because oviposition was recorded during the first five days where the peak rate occurs, a methodology widely used to

evaluate this parameter in predatory mites, since these estimates are closed to those obtained from full lifetable analysis (Abad-Moyano, 2009, Argolo et al. 2013, Sabelis and Janssen 1992). A negative effect of the tomato host plant on various biological parameters of other predators, including phytoseiids, has been previously found. *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) survival, adult longevity and predation rate on *T. absoluta* were negatively impacted on tomato plants with high densities of glandular trichomes (Benites Bottega et al., 2017). High density of tomato trichomes was also correlated to lower walking speed and fecundity rate of the predator *Delphastus (Pusillus) catalinae* Horn (Coleoptera: Coccinellidae) (Heinz and Zalom 1996). The movement and predation rate of the predatory larvae *Episyrphus balteatus* De Geer (Diptera: Syrphidae) and *Adalia bipuncata* Linnaeus (Coleoptera: Coccinellidae) was drastically reduced by the tomato trichomes (Shah et al., 1982; Verheggen et al., 2009) and *Podisus maculiventris* Say (Hemiptera: Pentatomidae) experienced high nymphal mortality on tomato plants (Lambert 2007). *Neoseiulus californicus* developmental time and sex ratio were similar on tomato and strawberry, but juvenile survival and oviposition were lower on tomato (Castagnoli et al., 1999). Oviposition rate of *N. californicus* on tomato leaves was negatively affected on tomato leaves, both directly and indirectly through the prey, when compared to bean leaves (Koller et al., 2007). *Phytoseiulus macropilis* and *P. longipes* walking, predation and oviposition rates were reduced on tomato leaves, when compared to strawberry (Sato et al., 2011). *Amblydromalus limonicus* Garman & McGregor (Acari: Phytoseiidae) mites preyed fewer *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) psyllid nymphs per day on tomato than on sweet pepper, but the mite survival was similar on the leaves of both plants (Davidson et al., 2016). *Amblyseius swirskii* walked slower on plant species with increasing trichome density and on tomato leaves their walking speed was lower when compared to rose plants (Buitenhous et al., 2014). On tomato leaves, the trichomes and their exudates seem to affect the phytoseiid

predation rate and their oviposition rate. In this study, an effect of leaf pubescence of different host plants, on predation and oviposition rates of *A. swirskii* was not found. It is possible that offering food *add libitum* and the limited leaf surface used in the experiment could have masked any small differences on predation rate between different host plants.

The detrimental effect of tomato plants on *A. swirskii* dispersal and survival was observed on the stems. This effect was in direct correlation with the presence of trichomes. The profound impact of the glandular trichome density on mite dispersal and survival on tomato stems has been previously shown for *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) as well, with entrapment and mortality rates estimated at 61 % and 73 %, respectively (Van Haren et al., 1987). Also, mite entrapment rate has been positively correlated to the size of glandular of trichome heads, which is influenced by light intensity (Nihoul, 1993). The rates of entrapment and mortality of *A. swirskii* and *P. persimilis* are rather similar and the small differences observed might be explained either by differences in the trichome densities of different tomato varieties used in the experiments or by differences in the mite morphologies, such as the length of legs. Adults are more affected by the glandular trichomes present on the stems than juvenile developmental stages, because young adults disperse in order to mate and avoid prey overexploitation, whereas the juveniles usually stay at the natal patch until molting is completed (Pels and Sabelis, 1999).

Staining of the epicuticular sugar esters of the tomato stems revealed a high concentration of sugar esters in the glandular trichomes type VI. The most abundant secondary metabolite produced by those trichomes, the acyl sugars, are polyesters of glucose or sucrose (Schilmiller et al., 2010). After walking on the stained tomato trichomes the predators, the acyl sugars were released, and accumulated on their cuticle and mostly on their mouth parts and limb joints. Similarly, the staining of the tobacco acyl sugars has shown their

accumulation on the body of aphids after walking on the plant surface (Wagner et al., 2004). This study is the first to demonstrate the attachment and accumulation of acyl sugars on the body of phytoseiids and pinpoints which secondary metabolites are most likely hindering the establishment of predatory mites on tomato plants. Moreover, two main mechanisms of insecticidal action for acyl sugars have been proposed; first, insects suffocate when acyl sugars cover the openings on their cuticle; second, the insects become desiccated when their cellular membranes under their cuticle are disrupted by the fatty acid moiety of acyl sugars (Puterka et al., 2003.).

An additional fact supporting a role of acyl sugars in mite mortality is that acyl sugars were identified in the trichome extract fraction FV that was the most toxic for the predatory mites, and less in the FIV, that was accordingly less toxic for the mites. Other trichome secondary metabolites were identified in similar levels in several of the fractions, hence they were not considered responsible for the observed toxicity. High toxicity of acyl sugars of *Nicotiana gossei* to pear psylla, *Cacopsylla pyricola* (Foerster) (Homoptera: Psyllidae), adults and nymphs has been observed as well (Puterka & Severson, 1995).

High densities of glandular trichomes on the host plant stem are clearly detrimental to the life history of the predatory mites, interfering with the biological control of the pests (Castagnoli et al., 1999, Cédola et al., 2001). Tomato pests on the other hand, have the morphological and behavioral adaptations to avoid the trichomes or the glandular exudates and so, can reproduce on a predator free environment. It is necessary to investigate how plant characters that render resistance against arthropods have fitness tradeoffs, because they provide enemy-free space to herbivores that are adapted to these defenses. Hence, more research is required to understand the influence of the tomato acyl sugars on herbivores and their key predators. Tomato plants with minimal levels of acyl sugars could be used in future studies to understand the effect of

those secondary metabolites on the plant fitness in the presence of biological control. Last, the effect of the plant physiology on natural enemies is important for the application of biocontrol programs on the crops and it should be taken into account by plant-breeding programs.

Chapter 3

Performance of the predatory mites *Amblyseius swirskii* Athias-Henriot and *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) on tomato genotypes with different trichome phenotypes.

To be submitted: Angeliki Paspati, Joel González-Cabrera, Alberto Urbaneja

Abstract

Tomato plants are covered in trichomes that upon touch by pests and natural enemies release toxic or sticky exudates. Currently, cultivars used for tomato production are often selected for high density of trichomes, since they show increased tolerance to herbivore attacks; however, they can be harmful to predatory mites. In this study, we estimated the dispersal ability of the generalist predatory mite *Amblyseius swirskii* on a tomato cultivar and 3 monogenic mutants with different trichome phenotypes: the hairs absent (type I trichomes absent), the hairless (all trichomes distorted), and the wooly (excess of type I trichomes). Moreover, the success of a biocontrol program based on the augmentative release of *A. swirskii* and *Phytoseiulus persimilis* against tomato key pests on the hairless tomato genotype as compared to the wild-type genotype was investigated. The findings indicate that the effect of the plant physiology on natural enemies is important and should be taken into account by plant-breeding programs.

3.1 Introduction

Host plant resistance can be an important component of a pest management strategy. Plant cultivars with high defenses against pests can keep infestation levels even below the economic injury levels and are environmentally safe (Smith, 2005). However, traits that defend plants against herbivory may also be deleterious to natural enemies and they may eventually provide a favoring for the pest, natural enemy-free environment. Therefore, a thorough investigation on the plant-pest-natural enemy system is required to understand whether the effects of resistance traits in plants are beneficial, adverse, or neutral to the natural enemies. To achieve optimal pest control, the tritrophic interactions among the plant-

pest-natural enemy species must be understood and taken into account during crop selection (Bottrell et al., 1998). A careful combination of crop selection for host plant resistance and biological control can result in an effective pest management (Price et al. 1980; Schmidt et al. 2014).

Plant traits are morphological, such as domatia and trichomes, or chemical such as volatile and toxic compounds, which directly affect a natural enemy's fitness, by influencing positively or negatively their efficiency in colonizing a plant, in searching and preying or, parasitizing the pest (Messina and Hanks, 1998; Cortesero et al., 2000). The tomato plants *Solanum lycopersicum* (L) are characterized by a variety of trichome types (types I, III, V, VI, VII), some of which are glandular and secrete a wide range of chemical compounds (I, VI, VII) (Luckwill, 1943, Simmons and Gurr, 2005). The nonglandular trichomes (types III, V) inhibit the movement of predators, increase the searching time and reduce the prey-predator encounter rate (Kennedy, 2003; Simmons and Gurr, 2005). The tomato glandular trichomes release sticky exudates called acyl sugars upon contact with predators, that may get trapped in the exudate (Van Haren et al., 1987 Maluf et al., 2010;). In the case of predatory mites (Acari: Phytoseiidae), it has been found that mite entrapment rate is related to the glandular head size of the trichomes, which is positively influenced by the light intensity (Nihoul, 1993). Furthermore, other glandular trichome exudates such as methyl ketones and zingiberene are toxic to the phytoseiids and their prey, the spider mites (Acari: Tetranychidae) (Chatzivasileiadis & Sabelis, 1997, Antonious & Snyder, 2016).

The negative effects of trichomes on the fitness of predatory mites have been reported for *Phytoseiulus persimilis* (Athias-Henriot), (Krips et al. 1999, Van Haren et al., 1987, Nihoul, 1993), *Neoseiulus californicus* (McGregor) (Castagnoli et al., 1999; Cedola et al., 2001; Cedola & Sánchez, 2003; Koller et al., 2007) and *Phytoseiulus macropilis* (Banks) (Sato et

al., 2011). Tomato cultivars with increased trichome density have direct negative effects on *N. californicus* (Cédola & Sánchez, 2003). Van Haren et al., 1987 found high entrapment and mortality rate of the phytoseiid *P. persimilis* on a *S. lycopersicum* cultivar with high density of trichomes compared to an accession of *Lycopersicon peruvianum* (Mill) almost free of glandular trichomes, where no mite entrapment was observed.

Currently, cultivars used for tomato production are often selected for high density of trichomes, since they show increased tolerance to herbivore attacks. However, tomato trichomes are harmful to natural enemies, important in augmentative biological control, the predatory mites. These mites usually exhibit greater mobility than their prey, thus increasing the likelihood of getting entrapped. Glandular trichomes, which are responsible for the excretion of the sticky acylsugars, are present at low densities on tomato leaves; however, density is higher on stems. Therefore, predatory mites might die during their dispersal from leaf-to-leaf *via* the stems, when trapped in the high concentration of acyl sugars present in the stems (Van Haren et al., 1987). In this study, we estimated the dispersal ability of the generalist predatory mite *Amblyseius swirskii* (Athias-Henriot) on three monogenic tomato mutants with different trichome phenotypes, the hairs absent (type I trichomes absent), the hairless (all trichome types distorted), and the wooly (excess of type I trichomes) and their wild-type varieties. Moreover, we investigated the efficiency of a biocontrol program based on the augmentative release of *A. swirskii* and *P. persimilis* against their prey, the whiteflies and spider mites respectively, on the hairless tomato genotype with distorted trichomes.

3.2. Materials and Methods

3.2.1. Plants and arthropods

Seeds of the tomato plants, *S. lycopersicum* monogenic mutants, hairless (hl, accession number LA3556), hairs absent (h, accession number LA3172), their wild-type cv. Alisa Craig (AC, accession number LA2838), the monogenic mutant wooly (wo, accession number LA0258) and its wild-type cv. Rutgers (RU, accession number LA1090) were originally obtained from Tomato Genetics Resource Center (University of California, Davis, CA, USA). The hairless (hl) tomato plants are characterized by a distorted trichome phenotype, with highly twisted, folded and swollen trichome stems but intact trichome glandular heads (Kang et al., 2010). The hairs absent (h) tomato plants are characterized by the absence of the glandular type I trichomes, caused by the deletion of the entire coding region of the Hair gene (Chang et al. 2018). The typical phenotype of the wolly (wo) tomato plants is a much higher density of trichomes type I, compared to the wild-type plants (Yang et al., 2011). Seeds were sown in a mixture of soil and local peat moss. Two weeks after germination seedlings were individually transplanted into pots (8 × 8 × 8 cm). Plants were maintained undisturbed at 25 ± 2 ºC, 65 ± 5 % Relative Humidity (RH) and 14:10 h (Light: Dark) photoperiod. Pesticide-free plants with 6 fully-developed leaves (approximately 20 cm in height) were used for the experiments at four weeks of age.

Amblyseius swirskii and *P. persimilis* were obtained from commercial products (Swirski-Mite® and Spidex®; Koppert B.V., The Netherlands). *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) were obtained from a laboratory stock colony maintained at IVIA on lemon fruit and tomato plants (*S. lycopersicum* cv. Raf Marmande), respectively. The herbivore pests, *T. urticae* were initially

collected in citrus fields and *B. tabaci*, in tomato fields, both located in the Valencia province (Spain).

3.2.2. Predatory mite dispersal

Tomato plants cv. hairless, hairs absent, Alisa Craig, wooly and Rutgers were used to determine the dispersal ability of *A. swirskii* on the plant stems. Adult female mites from the colony were used to investigate their dispersal on the tomato plants. The central part of the stem with three successive leaves was used for the observations and it was delimited by Tanglefoot ® (The Tanglefoot Company, Michigan, USA) glue barriers. On the part with the three successive leaves, the middle leaf was removed and pollen of *C. edulis* was added on the other two leaves. One day later, one female mite was released on the scar left at the stalk base and it was assessed whether the mite had reached the successive leaf or not after two hours (adapted from Van Haren et al., 1987). If the mite reached the successive leaf, the dispersal was recorded as successful. If the mite was stuck to the exudate of the trichomes, the survival was registered after 24 hours. Mites were considered alive if they moved after a gentle probe with a fine paint brush. Twenty female mites and twenty tomato plants with 6 fully developed leaves were used at temperature 22 ± 3 °C and 50 ± 10 % RH.

3.2.3. Augmentative biological control

A semi field trial was conducted from the beginning of May until the end of June 2018. Tomato plants of the two cultivars, the hairless and Alisa Craig, were placed separately in plastic, meshed cages (BugDorm-2, 60 x 60 x 60-cm, MegaView Science Co., Ltd., Taichung, Taiwan), inside plastic trays that were contained inside larger plastic trays, filled with water and soap to prevent the predatory mite dispersal among plants. The greenhouse was equipped with a fan cooling system, controlling the temperature and the relative

humidity. The mean daily temperature throughout the experimental period was 24.3 °C (maximum and minimum 33.3 C and 14.9 C, respectively). The mean relative humidity inside the cages was 82% and ranged from 91% to 73%. Twelve tomato plants were placed in cages individually, and five pairs of *B. tabaci* were released on each plant twice, with one week time interval. One week after the last *B. tabaci* release, 10 female *A. swirskii* were released, also twice and with one week time of interval between releases, in six of these cages, while the other six remained untreated (6 replicates per treatment). To assess the *A. swirskii* population, three consecutive leaves in the middle of each plant were chosen and observed weekly for 8 weeks and the mobile stages of *A. swirskii* were counted. To assess the *B. tabaci* population, all adult whiteflies were counted on the entire plant weekly.

Similarly, in the other 12 of the cages, 15 female *Tetranychus urticae* were released on each plant twice, with one week time interval. One week after the last *T. urticae* release, 10 female *P. persimilis* were released, and then, one week later, another 10 female *P. persimilis* were released, in six of these cages, while the other 6 remained untreated (6 replicates per treatment). To assess the *P. persimilis* and the *T. urticae* populations, three consecutive leaves in the middle of each plant were chosen and observed weekly for 8 weeks and all the mobile stages of *P. persimilis* and *T. urticae* were counted.

Adult *B. tabaci* were collected from the laboratory stock colony and cooled briefly in a cold room at 8 ^{o}C for counting and sexing and then released in pairs in the cages. The mites, both the pest, *T. urticae*, and the predatory mites, *A. swirskii*, *P. persimilis*, were observed under the stereoscope to identify the sex and females were selected and released on the plant leaves by transferring with a fine humid brash.

3.2.4. Statistical analysis

For the statistical analysis of the adult whitefly population we analyzed the data from week 5 until week 8 and we excluded the data of the first 4 weeks, because we wanted to include the newly emerged generation. For the same reason, the spider mite population data were analysed from week 3 onwards and the data of the first two weeks were excluded. The data of the predatory mite populations of *A. swirskii* and *P. persimilis* from week 4 and week 3 respectively, which correspond to the first week after their release and until the end of the experiment, were used in the analysis.

Dispersal and survival data were fitted to a Generalized Linnear Model with binomial distribution using R version 3.5.1 and the χ^2 test was applied to compare the variances. Pest population data (whiteflies and spider mites) were fitted by maximum likelihood to a linear, generalized, mixed effects model with poisson distribution and penalized quasilikelihood. In this model, we considered the repetitions as a random effect and the tomato plant, hairless (hl) and Alisa Craig (AC), the treatment, biological control (bc) and control (ctl), the time in weeks and their interactions as fixed effects. Predatory mite population data were fitted by maximum likelihood to a linear, generalized, mixed effects model with poisson distribution and Laplace Approximation. In this model, we considered the repetitions as a random effect and the tomato plant, hairless (hl) and Alisa Craig (AC), the time in weeks and their interaction as fixed effects.

3.3. Results

We tested the dispersal ability of female *A. swirskii* mites on the hairless tomato plants with distorted trichomes, on the hairs-absent tomato plants with lack of type I trichomes, and on their wild-type Alisa Craig (Figure 3.1). Moreover, we estimated the dispersal ability of *A.*

swirskii on the wooly tomato mutant with high density of type I trichomes and its wild-type tomato Rutgers (Figure 3.1). We found that the tomato genotype affects significantly the dispersal of *A. swirskii* (χ^2 test, df = 4, P<0.01). *A. swirskii* mites had a significantly higher dispersal rate from one leaf to the neighboring, when released on the hairless plants compared to the wild type Alisa Craig (χ^2 test, df = 15, P = 0.014) (Figure 3.1). On the hairs-absent and wooly plants the mites could not disperse at all (Figure 3.1). No significant difference in the dispersal of the predatory mites on the two wild-type tomato plants, Alisa Craig and Rutgers was found (χ^2 test, *df* = 15, *P*= 0.994).

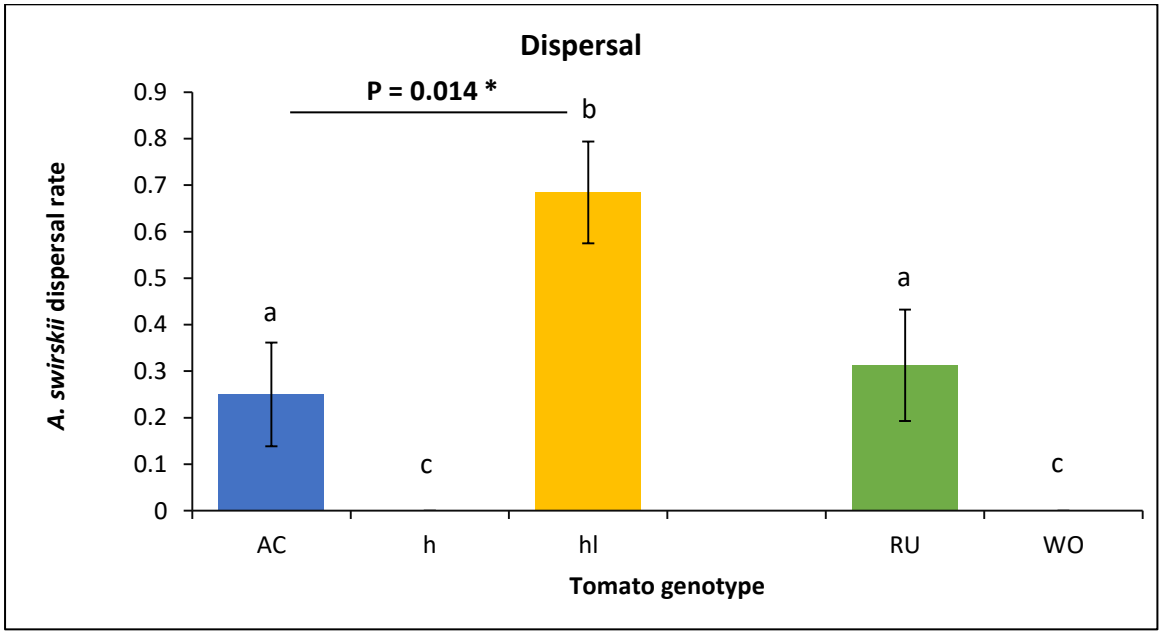


Figure 3.1. Mean dispersal rate of *A. swirskii* and SE on tomato stems of five tomato genotypes with different trichome phenotypes 2 hours after release. Average of 16 mites on tomato genotype. AC (wild-type Alisa Craig), 13 mites on h (monogenic mutant Hairs absent), 19 on hl (monogenic mutant Hairless), 16 on RU (wild-type Rutgers) and 16 mites on Wo (monogenic mutant Woolly). Dispersal data were fitted to a Generalized Linnear Model with binomial distribution and the χ^2 test was applied to compare the variances. Significant differences are marked with (*) (*P*<0.05).

In the semi field experiment, the population density of whitefly adults was similar among treatments and tomato genotypes during the first two weeks after they were released, as it is expected, since the second generation of whiteflies had not emerged yet (Figure 3.2). Thereafter, the number of adults per leaf increased exponentially in the hairless mutant (hl), both treated with *A. swirskii* and the untreated control (Figure 3.2). On the hairless tomato mutant the population density of whiteflies was more than two times higher than in the wild-type Alisa Craig tomato variety (121±16 and 43±7whiteflies/plant respectively), by the end of the experiment on week 8 in the untreated plants (Figure 3.2). In the treated plants, the abundance of whitefly adults was significantly different among the two tomato genotypes, hairless and wild-type Alisa Craig (124±15 and 41±10 whiteflies/plant respectively) on week 8, as well (Figure 3.2). Our analysis suggest that the tomato plant genotype, time and their interaction have a significant effect on the number of whiteflies/plant, but no effect of the biological control by *A. swirskii* was found (Figure 3.2). This finding suggests that the whitefly population is significantly affected by the tomato plant genotype and that *A. swirskii* did not have an effect on suppressing the pest on any of the two tomato genotypes. The population of *A. swirskii* decreased within one week from the time of release on both tomato genotypes, the mutant hairless and the wild-type Alisa Craig and during the following weeks numbers continued to be low on both plant genotypes (Figure 3.3). Overall, significantly more *A. swirskii* were found on the hairless tomato plants compared to the wild-type Alisa Craig (3± 1 mites/leaf and 0.3±0.3 mites/leaf respectively) at the end of the experiment at week 8 and the the tomato genotype affected significantly the population of the predatory mites (Figure 3.3). On the hairless plants, the number of predators/leaf was higher most likely, due to higher prey density on their leaves compared to the prey density on the Alisa Craig tomato plants.

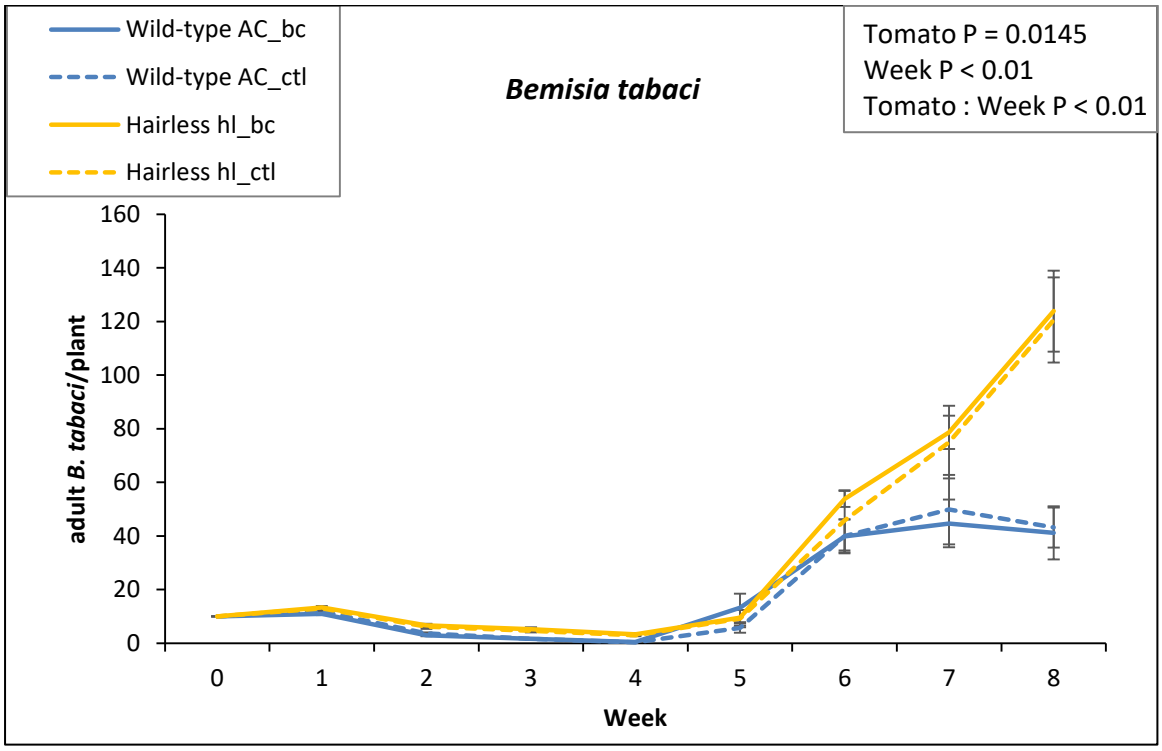


Figure 3.2. Mean number of adult *B. tabaci* per plant. Average of six plants per treatment and per tomato genotype. Blue line; wild-type Alisa Craig tomato plants treated with *A. swirskii* (biological control : bc). Blue dotted line; wild-type Alisa Craig tomato plants untreated control (control : ctl). Yellow line; Hairless tomato plants treated with *A. swirskii* (biological control : bc) Yellow dotted line; Hairless tomato plants untreated control (control : ctl).

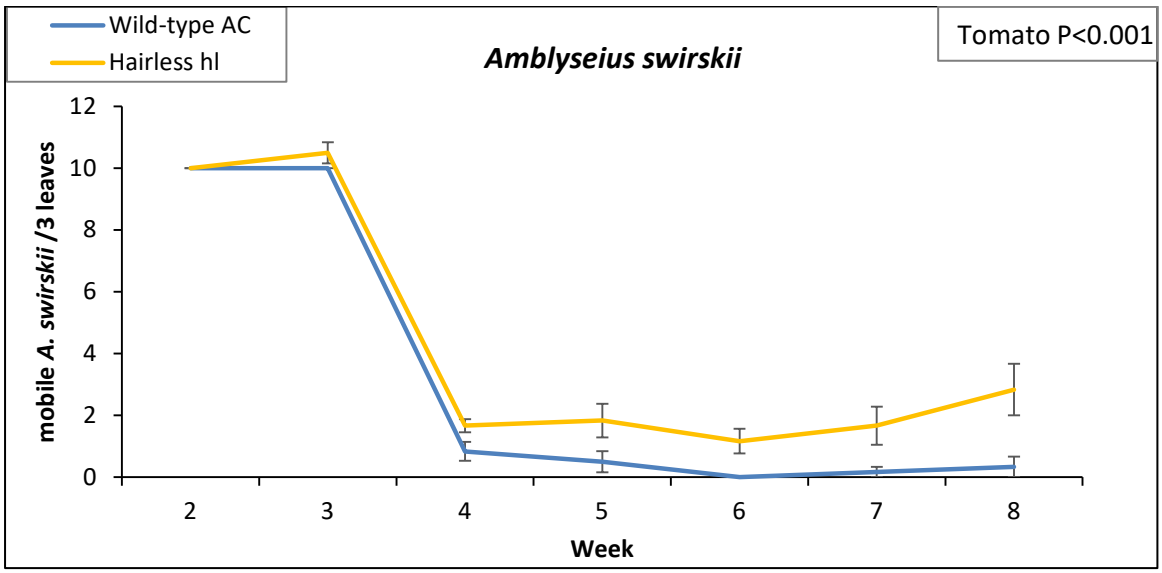


Figure 3.3. Mean number of mobile *A. swirskii* per 3 leaves/plant. Average of six plants per tomato genotype. Blue line; wild-type Alisa Craig tomato plants. Yellow line; Hairless tomato plants.

In the second semi-field experiment, the population density of mobile spider mites increased two weeks after they were released because of the stressed caused by the host plant transfer from citrus to tomato (Figure 3.4). Thereafter, the number of adults per leaf increased in both the hairless mutant (hl) and Alisa Craig plants and mostly on the untreated controls (Figure 3.4). On the untreated plants, the number of spider mites was higher on the hairless tomato plants than on the wild-type Alisa Craig tomato plants, from week 3 and until week 6 (Figure 3.4). On the treated plants, the abundance of spider mites was not significantly different among the two tomato genotypes, hairless and the wild-type Alisa Craig, until week 5, but it was higher on the hairless plants during the last week of the experiment (22±12 and 2±1spider mites/plant respectively) (Figure 3.4). The tomato plant genotype, the biocontrol treatment, the time and the interaction between the tomato plant genotype had a significant effect on the number of spider mites (Figure 3.4). This result indicates that the spider mite population is significantly affected by the tomato plant genotype and by *P. persimilis* which

suppressed the pest on both tomato genotypes (Figure 3.4). The population of *P. persimilis* decreased after their release (week 3) on both tomato plants, the mutant hairless and the wild-type Alisa Craig and during the following weeks the population of the predatory mite did not show an increase on any plant genotype (Figure 3.5). At the end of the experiment at week 6, the number of *P. persimilis* found on the hairless tomato genotype and on the wild-type Alisa Craig was not significantly different (Figure 3.5). The population of the predatory mites was significantly affected by the type tomato plants and was reduced during the time course (Figure 3.5).

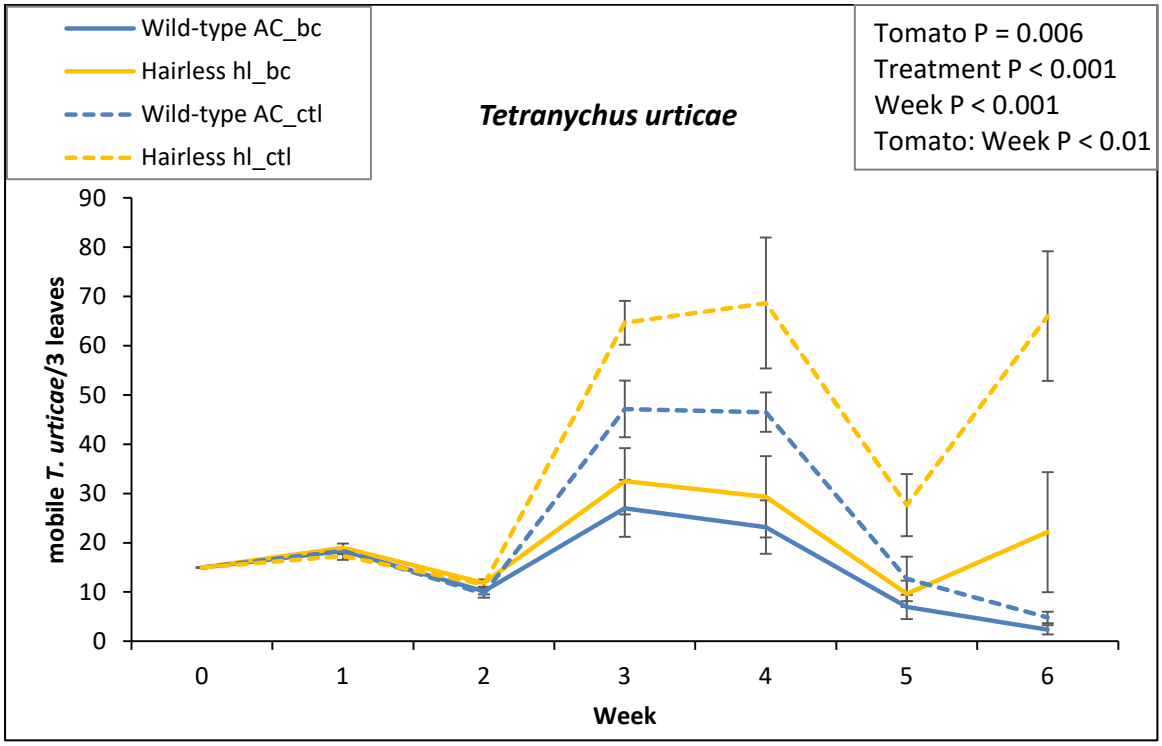


Figure 3.4. Mean number of mobile *T. urticae* per 3 leaves/plant. Average of six plants per treatment and per tomato genotype. Blue line; wild-type Alisa Craig tomato plants treated with *P. persimilis* (biological control : bc). Blue dotted line; wild-type Alisa Craig tomato plants untreated control (control : ctl). Yellow line; Hairless tomato plants treated with *P. persimilis* (biological control : bc) Yellow dotted line; Hairless tomato plants untreated control (control : ctl).

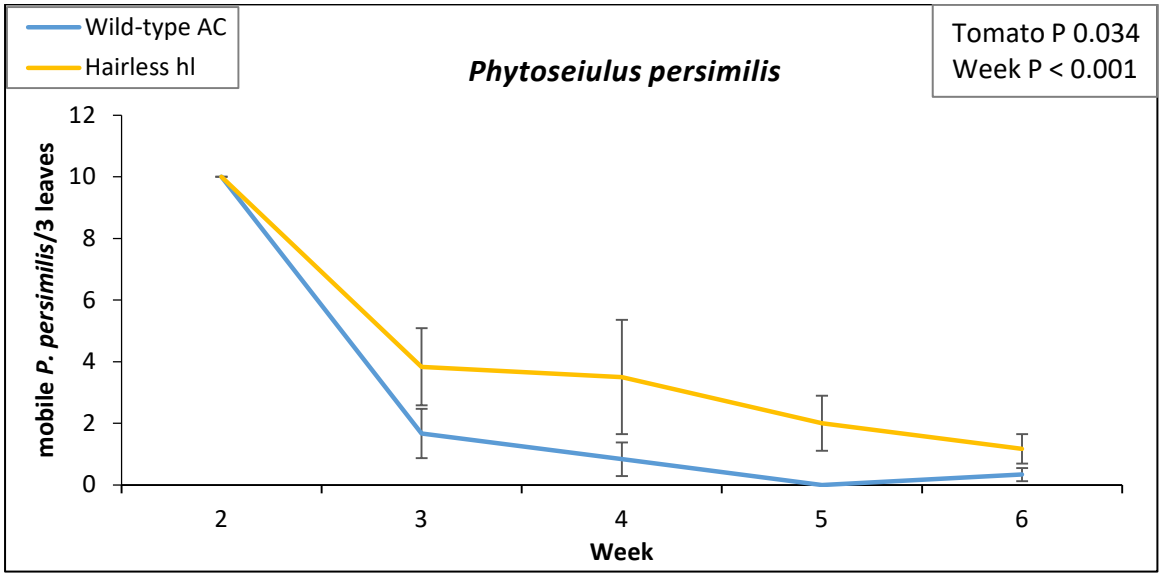


Figure 3.5. Mean number of mobile *P. persimilis* per 3 leaves/plant. Average of six plants per tomato genotype. Blue line; wild-type Alisa Craig tomato plants. Yellow line; Hairless tomato plants.

3.4 .Discussion

High densities of trichomes on tomato plants are detrimental for phytoseiid mites. *P. persimilis* was shown to be entrapped on the tomato trichomes of the stems where their density is much higher compared to the leaves, resulting in high mortality (Van Haren et al., 1987). Also, predatory mite entrapment was higher on tomato plants, grown under higher light intensity and lower temperatures, because of larger heads of the type VI glandular trichomes, than on plants grown under adverse conditions (Nihoul, 1993). On the hairless tomato genotype the dispersal ability of *A. swirskii* was higher than on the wild type genotype Alisa Graig and Rutgers. The hairless tomato plants are characterized by distorted trichome morphology which results in folded trichomes, without affecting however the morphology of the glandular heads (Kang et al., 2010). On the hairless tomato plants, the type VI trichomes, which are short glandular trichomes that produce sticky acyl sugars responsible for the entrapment of small arthropods, are present in similar densities to the wild type plants and

their glandular heads lie on the plant surface. The higher probability of successful dispersal of the predatory mites from one leaf to the next on those plants can be explained by the lying position of the glandular heads, which make it less likely to be touched by the predatory mites while dispersing. However, once upon touch, these glandular heads release their content entrapping the mites, similar to the wild type trichomes. Hence, the negative effect of the tomato plant on the mite dispersal is lower however, still present on the hairless plants.

On this study we found that on the hairless tomato plant the whitefly and spider mite pests performed better than on the wild-type. Our results are consistent with other studies that observed improved performance of the lepidopteran herbivores (Insecta: Lepidoptera), *Manduca sexta* (Linneus) and *Helicoverpa zea* (Boddie) (Kang et al., 2010; Tian et al., 2012). The hairless mutation affects the trichome secondary metabolite composition and the hairless plants have wild-type levels of the monoterpenes, glycoalkaloids, and acyl sugars; however they lack the sesquiterpene and polyphenolic compounds implicated in pest resistance (Kang et al., 2010). Tomato sesquiterpenes have been implicated with tomato resistance against lepidopteran and spider mite herbivores (Eigenbrode et al., 1994; Antonious & Snyder, 2006) and the tomato trichome phenolics in the defense against lepidopteran as well (Duffey & Isman, 1981). The increased performance of pests on hairless plants may results from other factors, such as the changes in the trichome morphology. The glandular trichomes influence negatively the whitefly oviposition and positively the number of trapped whiteflies (Oriani & Vendramim, 2010); hence their distortion on the hairless tomato plants may result in reduced contact between the pest and the glandular exudates.

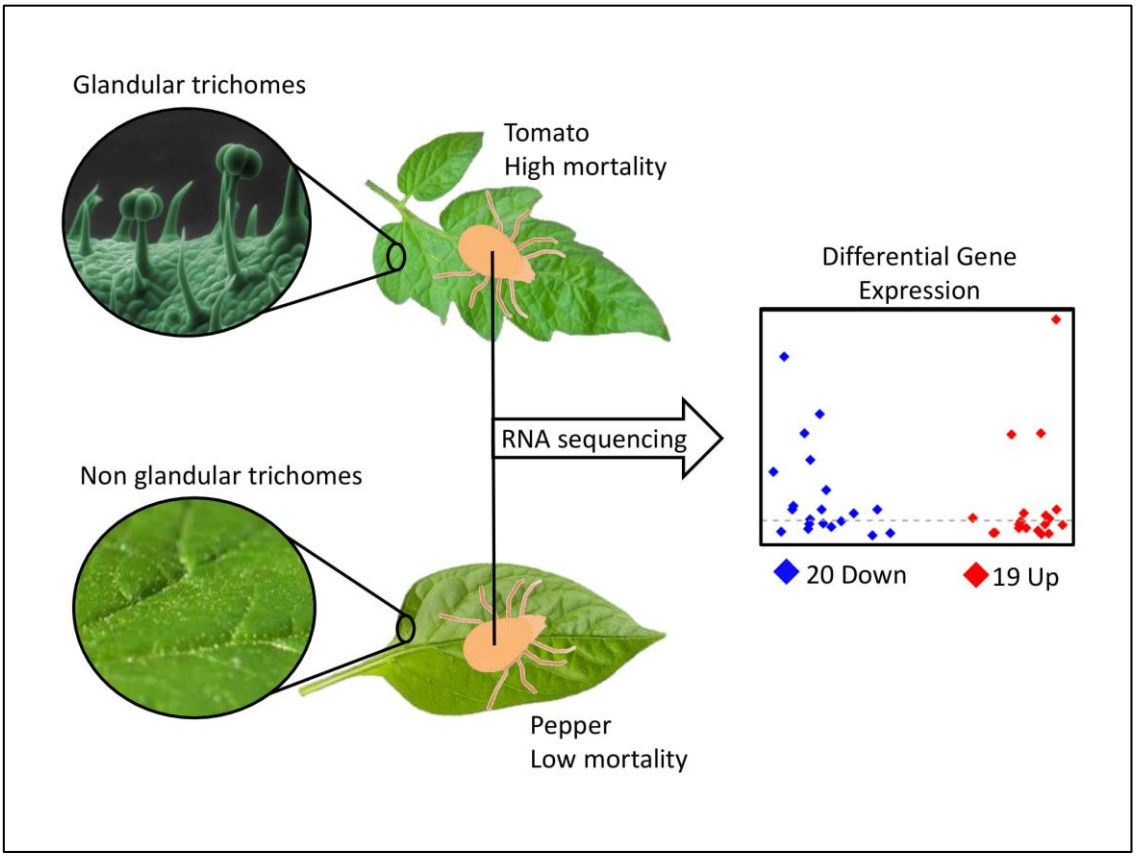
The predatory mites, *A. swirskii* and *P. persimilis,* performed poorly on both the hairless and the wild-type tomato plants and they were not able to suppress the pest. Most likely the predators were negatively affected on both tomato genotypes by the sticky trichome exudates,

which cause their entrapment and do not permit their efficient dispersal in order to exploit new patches of pest, when prey density is reduced. From our findings we conclude that the hairless mutation causing trichome distortion does not favor the predatory mite establishment and efficiency in pest control. Tomato trichomes and their exudates affect negatively the performance of other phytoseiids, as well. For example, *Phytoseiulus longipes* (Evans) (Acari: Phytoseiidae) cannot control *T. urticae* and *Tetranychus evansi* (Baker & Pritchard) (Acari: Tetranychidae) populations at a low predator/prey ratio on tomato plants (Ferrero et al., 2011). The *N. californicus* functional response was much lower on tomato plants compared to other host plants and their fitness did not increase over many generations when reared on tomato (Cédola et al., 2001).

High densities of trichomes on host plants are detrimental to the life history of biocontrol agents, phytoseiids, as well as insects (Kennedy, 2003; Simmons & Gurr, 2005; Riddick & Simmons, 2014). Natural enemies are a cornerstone of integrated pest management and trichome-based plant resistance can be incompatible with their use because of the negative effects of trichomes on them. Crop breeding programmes should take into account whether trichome-based plant resistance can suppress pest densities below the economic injury level and if not, whether it reduces the performance of natural enemies. Hence, future studies should investigate if plant characters that render resistance against arthropods have fitness tradeoffs because they provide enemy-free space to herbivores that are adapted to these defenses. Currently there are no tomato cultivars available where phytoseiid biocontrol agents can be used to control pest infestation. Therefore developing tomato cultivars with lower glandular trichome density and testing them for their compatibility with the effective role of biocontrol agents which are capable of suppressing pests should be investigated more thoroughly.

Chapter 4

The transcriptomic response of the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) to tomato exudates



Submitted to: Insect Science (August 2019)

Angeliki Paspati, Alberto Urbaneja, Joel González-Cabrera

Abstract

Amblyseius swirskii Athias-Henriot (Acari: Phytoseiidae) is a predatory mite, effective at controlling whiteflies and thrips in protected crops. However, on tomato its efficacy as a biocontrol agent is hindered, most probably by the plant trichomes and their exudates. Our aim was to characterize the response of *A. swirskii* to the tomato trichome exudates and identify three major detoxification gene sets in this species, the cytochromes P450 (CYPs), the glutathione S-transferases (GSTs) and the carboxyl/cholinesterases (CCEs). Mites were exposed separately to tomato and pepper, a favourable host plant for *A. swirskii*, after which their transcriptional responses were analysed and compared. The *de novo* transcriptome assembly resulted in 71,336 unigenes with 66.1% of them annotated. Thirty-nine of *A. swirskii* genes were differentially expressed after transfer to tomato leaves when compared to pepper leaves; some of the expressed genes were associated with the metabolism of tomato exudates. Our results illustrate that the detoxification gene sets CYPs, GSTs and CCEs are abundant in *A. swirskii*, but do not play a significant role when in contact with the tomato exudates.

4.1. Introduction

Since its introduction from the Americas, tomato (*Solanum lycopersicum*) has become a very important human food source worldwide. Tomato is one of the most important vegetables in Europe; 17.5 million tonnes were produced in the region in 2017. Spain and Italy are the most important producers accounting for over 2/3 of this production (Eurostat, February 2019). However, high yield losses are reported annually due to damage from various pests including the specialists of Solanaceae: the red tomato spider mite *Tetranychus evansi* (Baker &

Pritchard) (Acari: Tetranychidae), the tomato russet mite *Aculops lycopersici* (Massee) (Acari: Eriophyidae) and the South American tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). In addition to the specialists, other generalist herbivores such as the two spotted spider mite *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and the cotton whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) are listed as major tomato pests. In the majority of protected horticultural crops integrated pest management (IPM) relies on biological control approaches including the use of predatory mites (van Lenteren et al., 2018). However, in tomato, predatory mites, an important group of biological control agents, are not effective due to the challenges involved in their establishment in this crop (e.g. Castagnoli et al., 1999; Cédola and Sánchez, 2003).

In tomato the survival and efficacy of small predators is hindered by plant defences mediated by trichomes and their exudates (Van Haren et al., 1987; Nihoul, 1994; Cédola et al., 2001; Cédola and Sánchez, 2003). Tomato trichomes have diverse morphology and chemistry, placing them in seven categories (types I–VII): types I, VI and VII are glandular and II, III and V are non-glandular (Luckwill, 1943). Non-glandular trichomes are thought to mainly function as a physical barrier to arthropod feeding and movement; obstructing herbivore dispersal throughout the plant surface (Baur et al., 1991; Simmons and Gurr, 2005). Glandular trichomes are characterized by the heads present on their tips, producing sticky, toxic, compounds that may cause lethal entrapment, acute toxicity, or repellence of arthropods (Simmons and Gurr, 2005; Glas et al., 2012; Antonious and Snyder, 2016). Moreover, tomato glandular trichomes and their exudates may have profound effects on herbivore performance (i.e., growth, survival and fecundity) (Duffey and Isman, 1981; Cédola and Sánchez, 2003; Kennedy, 2003; Leckie et al., 2016). A variety of secondary metabolites are secreted by the tomato glandular trichomes, including the sticky acyl sugars

which are the most abundant chemical group, followed by terpenoids, phenols, methyl ketones and others (Schilmiller et al., 2010). The antibiotic and antixenotic effects of these trichome exude metabolites render resistance against herbivores but also negatively affect natural enemies (Simmons and Gurr, 2005).

Phytophagous mites, including the specialists *T. evansi*, *A. lycopersici* and the generalist *T. urticae* have adapted to tomato defences and in some cases evolved mechanisms that suppress them. For example, *A. lycopersici* triggers the degradation of the glandular trichomes (van Houten et al., 2013), whereas *T. evansi* suppresses the signalling pathways of the phytohormones jasmonic acid and salicylic acid (Alba et al., 2015). The polyphagous mite *T. urticae* suppresses the defence downstream of these phytohormones and also expresses various families of detoxification genes (Alba et al., 2015; Grbić et al., 2011). Interestingly, the genome of *T. urticae* is characterized by striking species-specific expansions of gene families associated with detoxification (Grbić et al., 2011). Furthermore, genes acquired by lateral gene transfer from fungi and bacteria, associated with digestion and detoxification, have been identified in *T. urticae* (Grbić et al., 2011). *Tetranychus urticae* changes the regulation of these gene sets and is able to adapt to a poor-quality host, such as tomato (Grbić et al., 2011; Dermauw et al., 2013).

Amblyseius swirskii (Athias-Henriot) (Acari: Phytoseiidae) is a polyphagous predatory mite, which is used in augmentative biological control of whiteflies (greenhouse whiteflies, silverleaf whiteflies), thrips (western flower, onion, chilli thrips), and plant feeding mites (spider mite, broad mite) in many vegetable, fruit, and flower greenhouse crops (Wimmer and Hoffmann, 2008; Stansly and Castillo, 2010; Doğramaci et al., 2011; Hoogerbrugge et al., 2011). There are two main characteristics of *A. swirskii* that have granted its success in biological control. First, this phytoseiid can reproduce on pollen and plant nectar, in addition

to arthropod prey, enabling it to establish and persist on plants when its prey, the pest, is scarce (Ragusa and Swirski, 1975; Nomikou et al., 2003). Secondly, it can develop effectively under a wide range of temperatures (Lee and Gillespie, 2011). On detached tomato leaflets *A. swirskii* can attack and reproduce using common tomato pests, such as *A. lycopersici* (Momen and Abdel-Khalek, 2008; Park et al., 2011), *T. absoluta* (Desneux et al., 2010; Momen et al., 2013), *B. tabaci* and *T. urticae* (personal observation). However, the density of glandular trichomes is much higher on tomato stems where the predatory mites often get trapped and die (Van Haren et al., 1987).

Natural enemies of tomato mite pests, either specialists such as *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), or generalists such as *A. swirskii*, have not been able to establish in tomato. Their ability to detoxify tomato allelochemicals is currently unknown. Our aim is to characterize the transcriptomic response of *A. swirskii* to tomato exudates and identify the genes of three protein superfamilies that are involved in detoxification processes: the cytochromes P450 (CYPs), the glutathione S-transferases (GSTs) and the carboxyl/cholinesterases (CCEs). The number of detoxification genes present in the generalist predator *A. swirskii*, compared to that of *T. urticae*, may explain its difficulty to cope with an unfavourable environment, such as the tomato plant.

4.2. Material and methods

4.2.1. Plant and mites

Tomato plants, *Solanum lycopersicum* cv. Raf Marmande and pepper plants, *Capsicum annuum* cv. Lipari were used to determine the metabolic responses of *A. swirskii* to both host plants. Seeds were sown in a mixture of soil and local peat moss. Two weeks after germination seedlings were individually transplanted into pots (8 × 8 × 8 cm). Plants were

maintained undisturbed at 25 ± 2 ºC, 65 % humidity ± 5% and 14:10 h (Light: Dark) photoperiod. Pesticide-free plants with 6 fully-developed leaves (approximately 20 cm in height) were used for the experiments at four weeks of age.

Colonies of *A. swirskii* were initiated from specimens supplied by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). *Amblyseius swirskii* colonies were maintained in rearing units which consisted of a piece of hard black plastic placed on a water saturated sponge (based on Overmeer, 1985). The sponge with the plastic piece was introduced in a plastic tray with water. The plastic borders were covered with thin sheets of tissue paper to ensure a constant water supply for the phytoseiids, to fix the plastic piece to the sponge, and to prevent phytoseiids from escaping. Twice a week, mites were fed *ad libitum* with *Carpobrotus edulis* (L) (Caryophyllales: Aizoaceae) pollen (Ragusa and Swirski, 1975). The colonies were maintained at 25 ±2 ºC in growth chambers under a 16:8 (Light: Dark) photoperiod and 80 % relative humidity.

4.2.2 Exposure to tomato and pepper leaves and sampling

Thirty *Amblyseius swirskii* females were placed on five tomato or pepper leaf discs (5cm diameter) and left undisturbed for 48 hours with no food source. Fourteen female mites were collected from each leaf disc with a fine paint brush. Thus, seventy female mites per crop were collected in microcentrifuge tubes. They were snap frozen in liquid nitrogen and stored at -80 ºC until use. This process was replicated four times (four biological replicates per crop).

4.2.3. RNA extraction, quantification and qualification

Total RNA from the eight biological replicates was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer recommendations. RNA

degradation and contamination were monitored by electrophoresis in 1 % agar gels. RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

4.2.4. Library preparation and sequencing

Library preparation and sequencing was performed at Novogene (HK) (Wan Chai, Hong Kong). A total amount of 1.5 μg RNA per sample was used as input material for library preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Briefly, mRNA was separated from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer. After second strand cDNA synthesis, remaining overhangs were blunted and NEBNext Adaptors with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150 ~ 200 bp in length, the library of fragments was purified with AMPure XP system (Beckman Coulter, Beverly, USA). Adapter-ligated fragments were amplified by PCR and the amplicons purified with the AMPure XP system. Library quality was assessed on the Agilent Bioanalyzer 2100 system on an Illumina Hiseq 2500 platform to generate paired-end reads.

4.2.5 Data analysis

4.2.5.1. Quality control

Clean reads were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads from the raw data. At the same time, Q20, Q30, GC-content and

sequence duplication level of the clean data were calculated. All downstream analyses were based on clean, high-quality data.

4.2.5.2. Transcriptome assembly

The transcriptome was assembled 'de novo' using Trinity (v. r20140413p1) (Grabherr et al., 2011) with min_kmer_cov set to 2 and all other parameters were set at their default values. A hierarchical clustering of the contigs was performed with Corset (v. 1.05) (Davidson and Oshlack, 2014).

4.2.5.3. Gene functional annotation

The gene function of the *A. swirskii* unigenes resulting from the assembly was annotated using the software NCBI blast (v.2.2.28+) based on the following databases: Nr ("NCBI Protein", 2019), Nt ("NCBI Nucleotide", 2019), KOG (EuKaryotic Orthologous Groups (Koonin et al., 2004)) and UniProt/Swiss-Prot (The UniProt Consortium, 2019), and the description with the highest score calculated from the E-value was accepted. Also, functional annotation was performed using the software KAAS (v. r14 0224) (Moriya et al., 2007) based on the KO database (KEGG Ortholog (Kanehisa et al., 2016)) and the software hmmscan ("HMMER 3," 2019) against the Pfam database (Protein family (Finn et al., 2014)) was run for the longest translated open reading frame of each unigene. Finally, the GO (Gene Ontology (The Gene Ontology Consortium, 2019)) annotation for each top BLAST hit was performed using BLAST2GO (v. b2g4pipe_v2.5) (Gotz et al., 2008).

4.2.5.4. Differential expression analysis

Gene expression levels were estimated by RSEM (v1.2.26) (Li and Dewey, 2011) for each sample:

1. Clean reads were mapped back onto the ‘de novo’ assembled transcriptome

2. Read count for each gene was obtained from the mapping results

Differential expression analysis of the two conditions, exposure to tomato leaves or to pepper leaves, was performed using the DESeq R package (v.1.10.1) (Anders and Huber, 2010). The resulting P values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed.

4.2.5.5. Manual curation and phylogenetic analysis of GSTs, P450s and CCEs

Sequences containing annotations referring to GST, P450 and CCE proteins were manually curated to better characterize the detoxification profile of the mite in comparison with other related species. Unigenes with the same BLAST results and >95 % identity were considered redundant and only the largest contig referring to individual genes were included in further analyses. All possible open reading frames were found using ORF finder (www.ncbi.nlm.nih.gov/orffinder/), then double-checked using BLASTP against the Nr database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the hmmer (v. HMMER 3) to identify the Pfam homologous regions of each unigene. The nucleotide sequences were translated and the amino acid sequences aligned with Clustal W (v. 2) (Larkin et al., 2007). *Tetranychus urticae, Metaseiulus occidentalis, Neoseiulus barkeri, Apis mellifera* and *Drosophila melanogaster* protein sequences were collected from the available proteomes on NCBI (Appendix Tables S4, S5 and S6).

The multiple sequence alignment of CCEs was trimmed at both ends according to the parameters previously set by Claudianos et al., (2006). The best substitution model for the protein alignment was found using the software MEGA v4 (Tamura et al., 2007). According

to the Akaike information criterion, the WAG + I + G + F model was selected for the phylogenetic analyses of CCEs. Phylogenetic trees of the CYP and GST protein families were determined by a maximum likelihood approach (RAxML v8.2.10) and bootstrapping with 1000 replicates. The CCE family Bayesian inference using Mr. Bayes (v3.2.6) was employed with the following parameters: Metropolis-coupled Markov chain Monte Carlo sampling was performed with four chains and the heating parameter, 0.2. Starting trees were random and the analyses were performed for two runs of three million generations. Samplings were performed every 100 generations and the initial 25 % of trees represented burn-in. The analyses were stopped when the average standard deviation of split frequencies dropped below 0.01.

4.3. Results

4.3.1. High-throughput sequencing, assembly and annotation

Eight paired-end cDNA libraries were constructed from RNA samples isolated from adult female mites exposed to either tomato or pepper leaves for 48 hours. A total of 893,330,940 clean reads of 150 base pair (bp) long and 134.01 Giga base pairs (Gbps) were obtained from the RNA sequencing after the removal of adapters, reads containing poly-N, and low-quality reads from the raw reads (Table 4.1). This dataset was uploaded to the National Centre for Biotechnology Information (NCBI) with Sequence Read Archive (SRA) accession number PRJNA484730. The sequencing quality of the clean reads based on the base-calling quality scores of Illumina was at the Q20 level for more than 96.6 % of the clean reads and was higher than Q30 for more than 91 % of them (Table 4.1). The average GC content across the eight samples was 48.25 %. The clean reads were then assembled into 71,345 transcripts, and 71,336 unigenes (Table 4.2). The length distributions demonstrated that 55,316 of the transcripts and 55,313 of the unigenes (77.5 %) were between 500 and 2000 bp and that

17,090 transcripts and unigenes were longer than 1000 bp (Figure 4.1). The mean length of transcripts and unigenes was 1441 bp (Table 4.2).

Table 4.1. Summary of the sequence reads obtained from eight transcriptome libraries of *A. swirskii*. Four transcriptome libraries are from female adults reared on tomato leaves (Treat_T1-Treat_T4) and four from females reared on pepper leaves (Treat_P1-Treat_P4).

Sample	Raw	Clean	Clean	Error(%)	Q20(%)	Q30(%)	GC
Treat_T1	104344962	98272578	14.74G	0.02	96.61	91.64	47.66
Treat_T2	113731580	106805200	16.02G	0.02	96.66	91.74	47.96
Treat_T3	125843276	119195208	17.88G	0.02	96.68	91.84	48.53
Treat_T4	117805522	111478562	16.72G	0.02	97.22	92.88	50.2
Treat_P1	124524702	118665004	17.8G	0.02	96.73	91.95	48.23
Treat_P2	117971816	112701946	16.91G	0.02	96.78	92.03	48.3
Treat_P3	128821170	123369064	18.51G	0.02	96.56	91.71	46.86
Treat_P4	107798420	102843378	15.43G	0.02	96.72	92.04	48.27
Total	940841448	893330940	134.01	0.02	96.75	91.98	48.25

Table 4.2. Summary of the assembled transcripts and unigenes obtained from the combined reads of eight transcriptome libraries of *A. swirskii.*

	Transcripts	Unigenes
Total number	71345	71336
Min length	201	201
Mean length	1441	1441
Median length	914	914
Max length	21101	21101
Total length	102802715	102797693
N50	2239	2239
N90	620	620

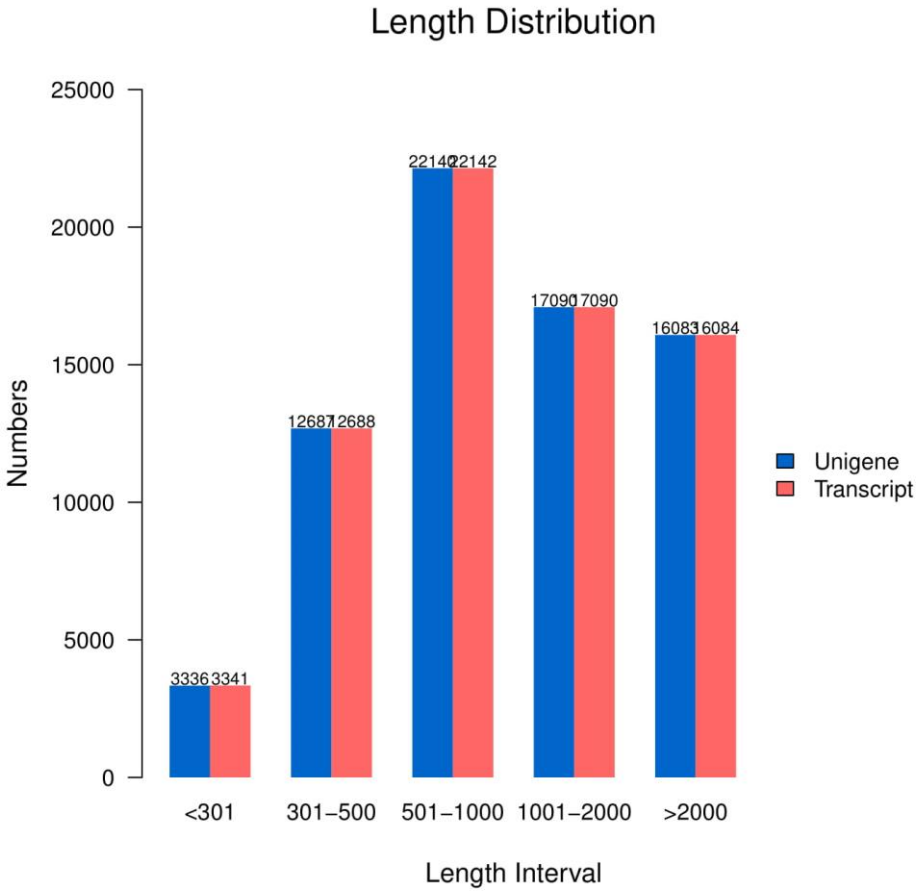


Figure 4.1. Length distribution of the assembled transcripts and unigenes obtained from the combined reads of eight transcriptome libraries of *A. swirskii.*

After annotation, 47,159 (66.1 %) of all unigenes successfully matched known genes in at least one of the databases used: 39,574 in Nr, 18,233 in Nt, 15,927 in KO, 29,384 in Swiss-Prot, 34,165 in Pfam, 34,270 in the GO, 23,070 in KOG. Overall, 10,573 unigenes were annotated in five of the databases (Table 4.3, Figure 4.2). The E-value distribution of the annotated unigenes suggested that 68.2 % of the mapped unigenes had very significant homology with the top hit (E-value $<E^{-45}$); for the other 31.8 % the values were between E^{-45} and E^{-5}, lower but still significant (Figure 4.3). The sequence identity with the top hit was at

least 80 % for more than half of the total annotated unigenes (58.6 %) (Figure 4.4). As expected, *A. swirskii* is more similar to other arachnids than to species from other arthropod classes; 12,033 unigenes (81.8 %) had their best hits with sequences from *M. occidentalis* (a predatory mite), followed by those from *Ixodes scapularis* (1.5 %), *Stegodyphus mimosarum* (0.9 %) and *Acyrthosiphon pisum* (0.5 %) (Figure 4.5).

Table 4.3. Summary of integrated annotations of unigenes

	Number of Unigenes	**Percentage (%)**
Annotated in NR	39574	55.47
Annotated in NT	18233	25.55
Annotated in KO	15927	22.32
Annotated in SwissProt	29384	41.19
Annotated in PFAM	34165	47.89
Annotated in GO	34270	48.04
Annotated in KOG	23070	32.33
Annotated in all Databases	7218	10.11
Annotated in at least one Database	47159	66.1
Annotated in at least five Databases	71336	100

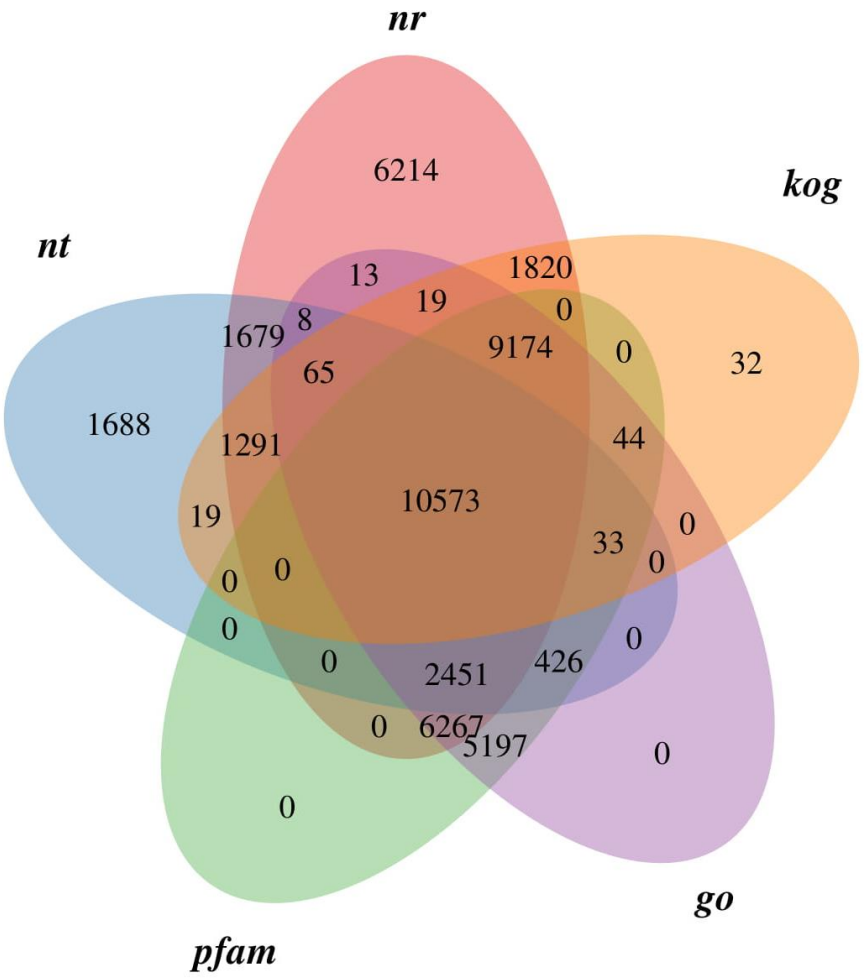


Figure 4.2. Venn diagram of unigenes of *A. swirskii* annotated using various databases. The Venn diagram shows the overlapping unigenes annotated in the Nr, Nt, pfam, GO and KOG databases.

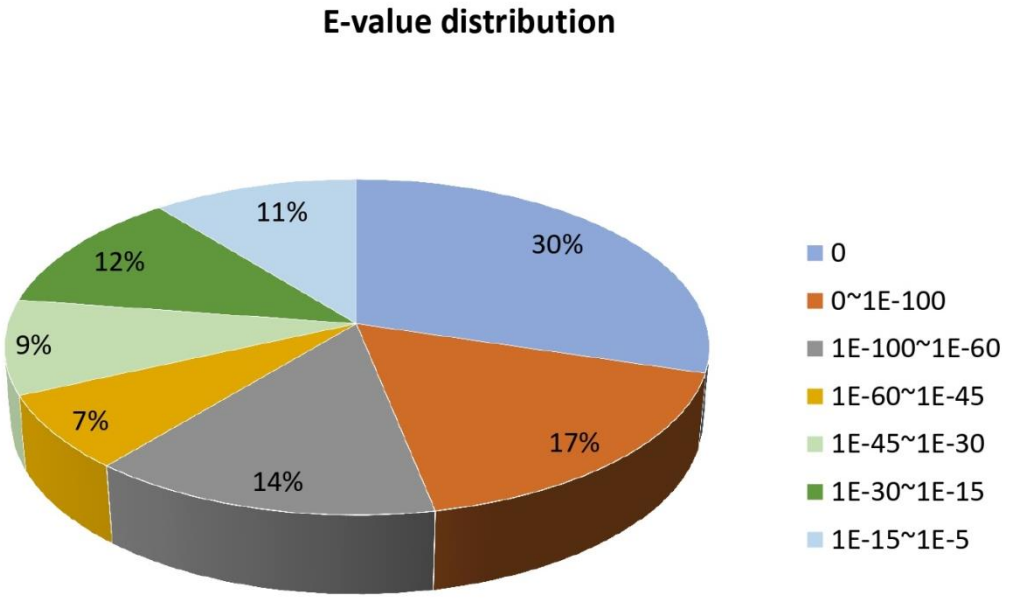


Figure 4.3. E-value distribution of the blast hits for each unigene with E-value $\leq 10^{-5}$ of *A. swirskii* transcriptome data.

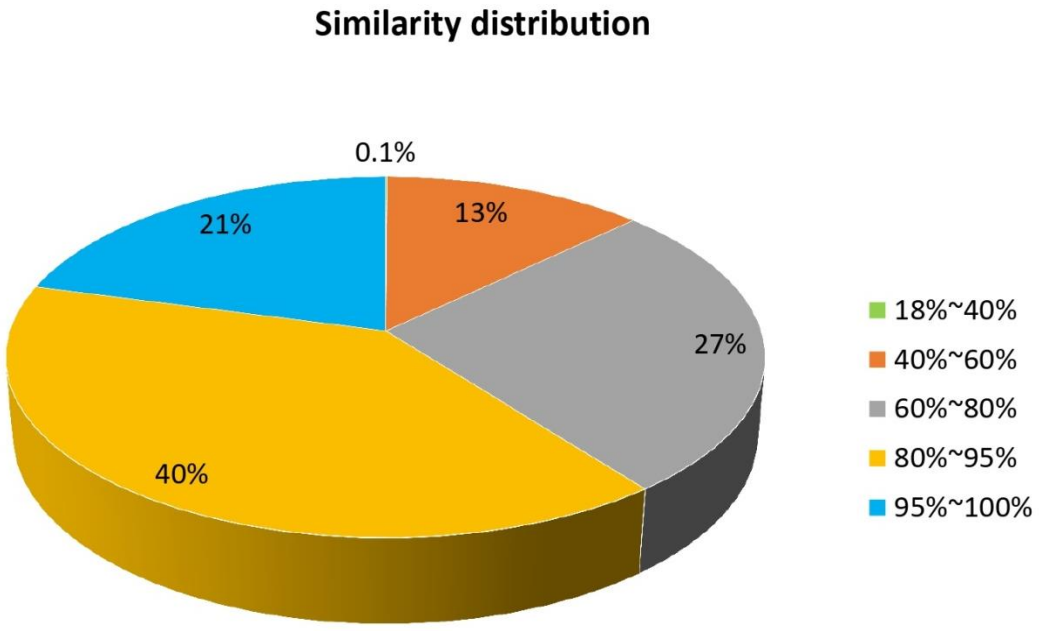


Figure 4.4. Similarity distribution of the top blast hits for each unigene sequence of *A. swirskii* transcriptome

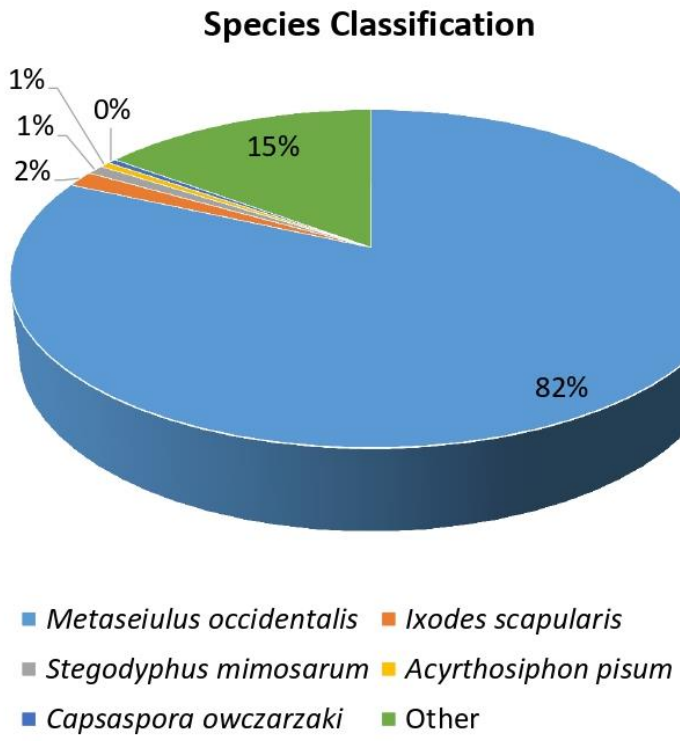


Figure 4.5. Species distribution of the total homologous sequences with E value $\leq 10^{-5}$. The first hit of each sequence was used for statistical analysis.

According to the BLAST2GO results, 34,270 unigenes were classified into 196,539 GO terms and 56 subcategories (Figure 4.6). Also, in total, 23,070 unigenes were mapped and sorted into 26 KOG categories (Table 4.3, Figure 4.7). Among them, "general function prediction" (4,658; 20%) and "Signal transduction mechanisms" (3,491; 15%) were most represented. (Figure 4.7). A total of 15,927 unigenes (22.3%) were mapped to 228 metabolic pathways by the KEGG analysis (Table 4.3). The two most abundant KEGG pathways were: "Signal transduction" (2,084; 13 %) and "Translation" (1,428; 8.9%) (Figure 4.8).

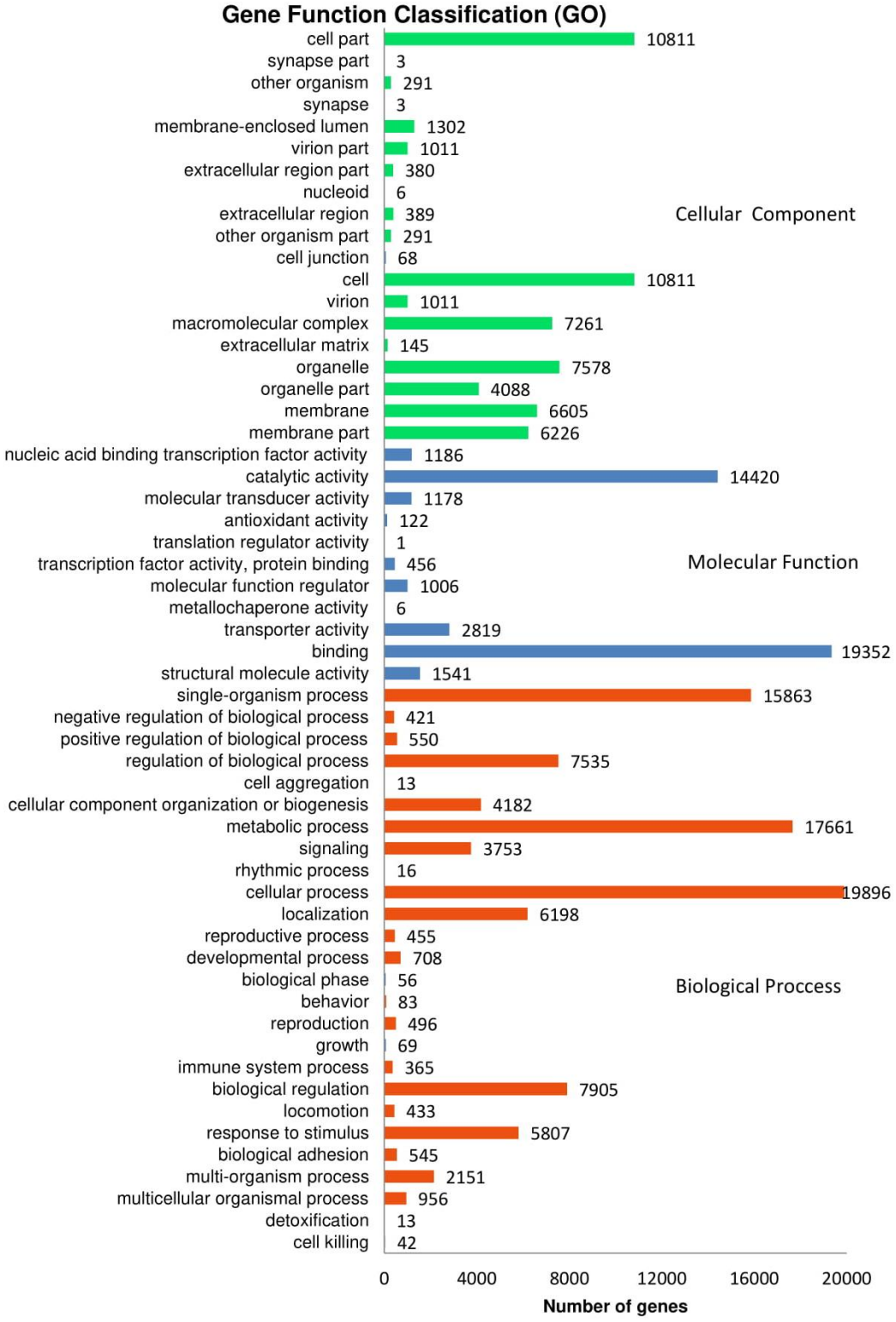


Figure 4.6. GO annotation of the overall unigene dataset. The total transcriptome dataset of *A. swirskii* was classified into biological process, cellular component, and molecular function subcategories. X-axis is the names of the 56 subcategories; Y-axis is the number of unigenes annotated under this subcategory.

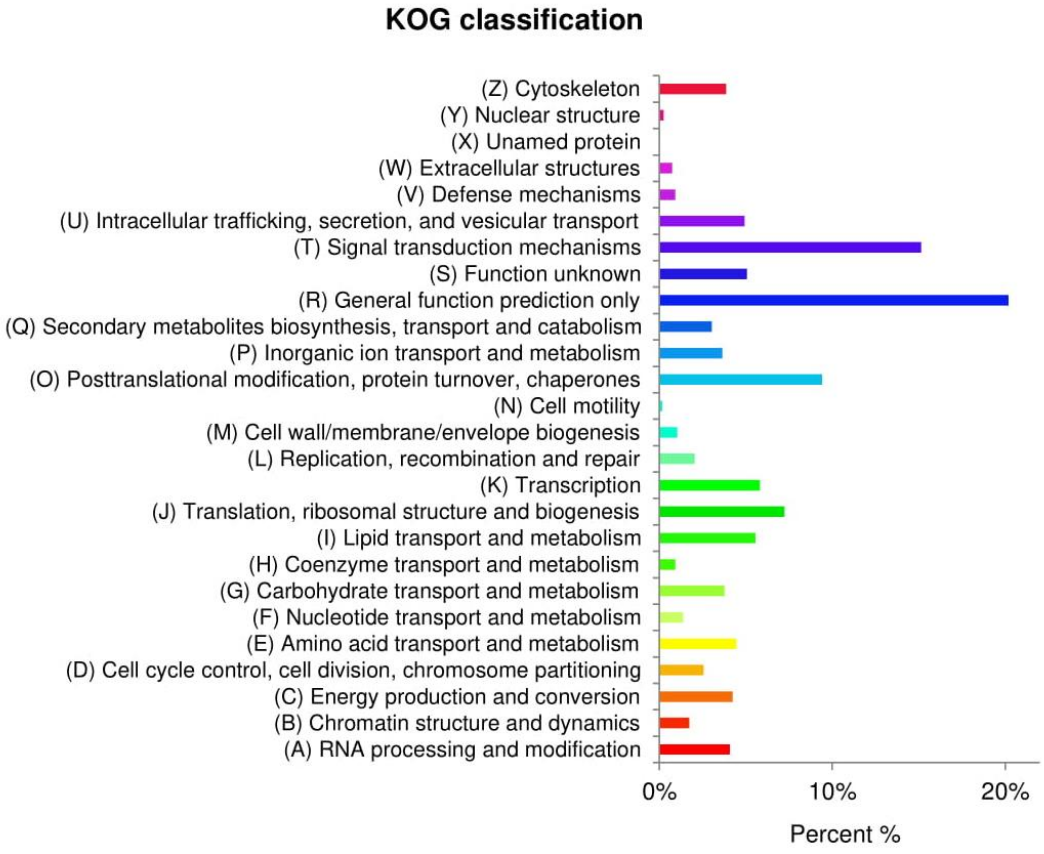


Figure 4.7. Classification of the euKaryotic Orthologous Groups (KOG) analysis of the *A. swirskii* transcriptome. X-axis is the names of the 26 KOG group; Y-axis is the percentage of genes annotated under this group in the total annotated genes.

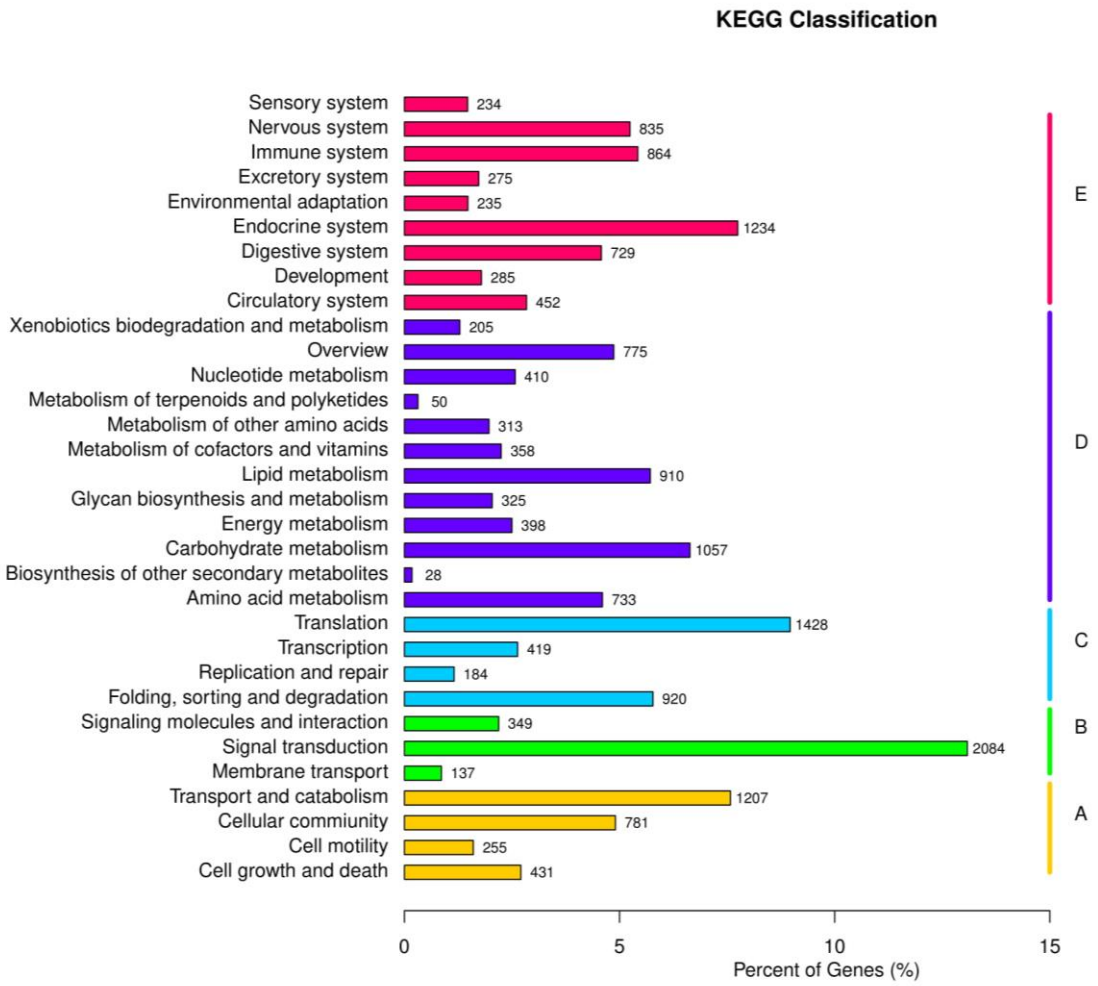


Figure 4.8. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of the *A. swirskii* transcriptome. The top 15 pathways are shown. Y-axis is the names of KEGG pathways; X-axis is the number of the unigenes annotated in the pathway and the ratio between the number in this pathway and the total number of annotated unigenes. The KEGG metabolic pathways are divided into 5 branches: A: Cellular Processes, B: Environmental Information Processing, C: Genetic Information Processing, D: Metabolism, E: Organismal Systems.

4.3.2. Differential expression analysis

In order to gain insight into the differential gene expression of *A. swirskii* when exposed to tomato and pepper, datasets of all samples were mapped against the newly assembled transcriptome. The expression level was quantified estimating the number of Fragments Per

Kilobase of transcript per Million base mapped reads (FPKM) (Figure 4.9). The differential expression analysis was obtained by comparing the read count values of the samples from the two treatments. This analysis elucidated 39 genes to be significantly differentially expressed (DEGs) in the two conditions (padj < 0.05) (Figure 4.10). Among these DEGs, 19 were up-regulated and 20 down-regulated in mites exposed to tomato leaves compared to mites exposed to pepper leaves. Overall, 14 DEGs were found to be exclusively expressed in mites released on tomato leaves and 14 DEGs were expressed only in mites released on pepper leaves. All DEGs had expression differences greater than two-fold between mites exposed to tomato and those exposed to pepper leaves in four biological replicates (Figure 4.10). The expression patterns of DEGs between the two conditions are depicted in Figure 4.11.

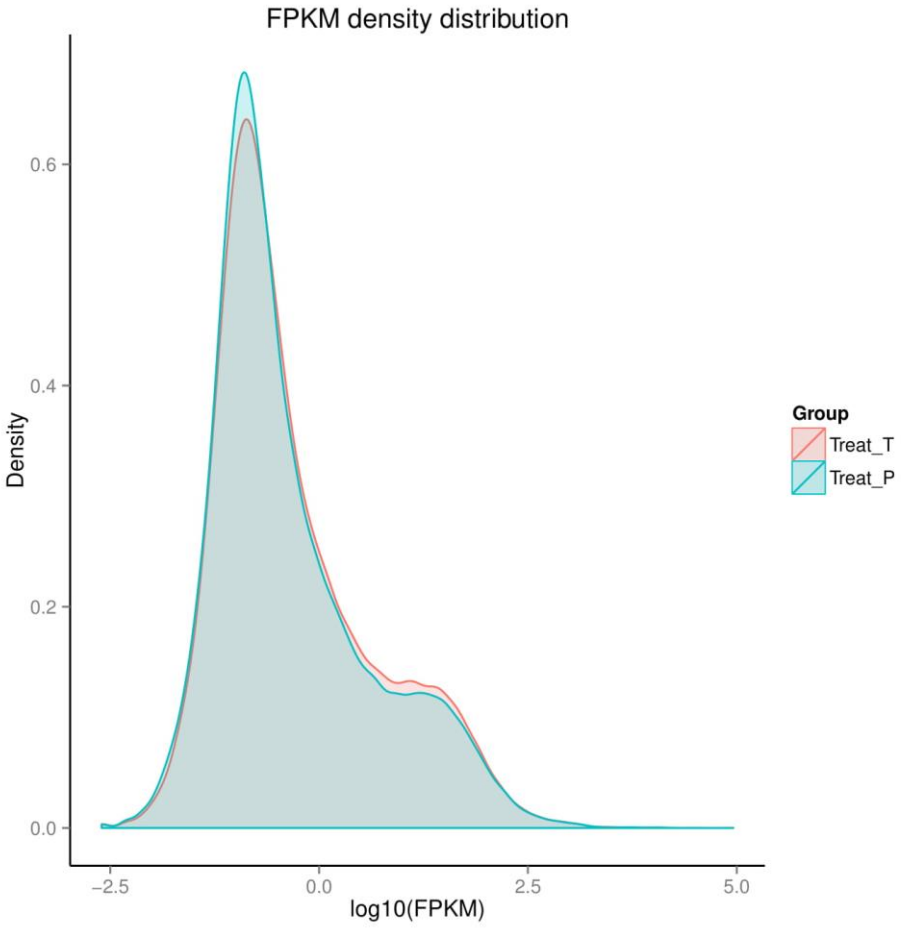


Figure 4.9. Plot of fragments per kilobase of transcript per million mapped reads (FPKM) density of eight transcriptome libraries of *A. swirskii*. Mites exposed to tomato leaves : Treat_T; mites exposed to pepper leaves: Treat_P.

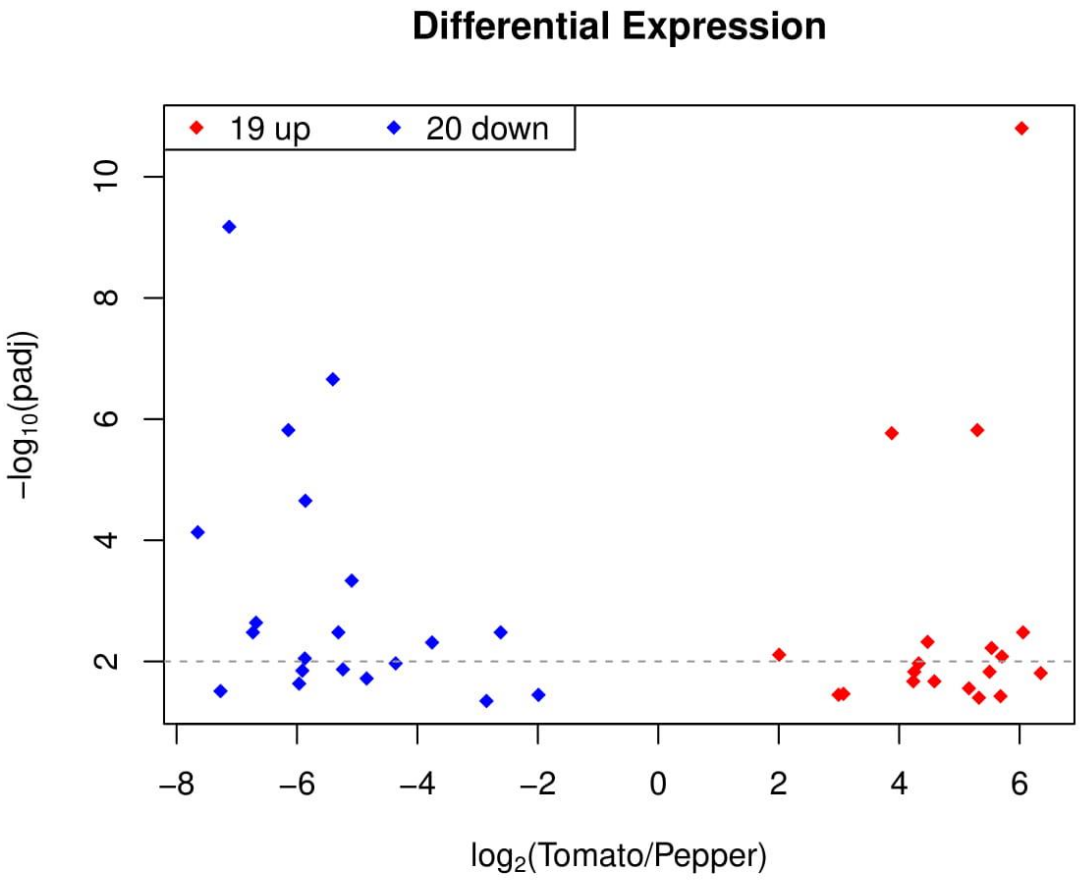


Figure 4.10. Volcano plot showing differentially expressed *A. swirskii* genes when exposed to tomato leaves compared to those when exposed to sweet pepper leaves (P adjusted values < 0.05). Red dots represent upregulated genes, blue dots represent downregulated genes and dashed line represents P adjusted values < 0.01.

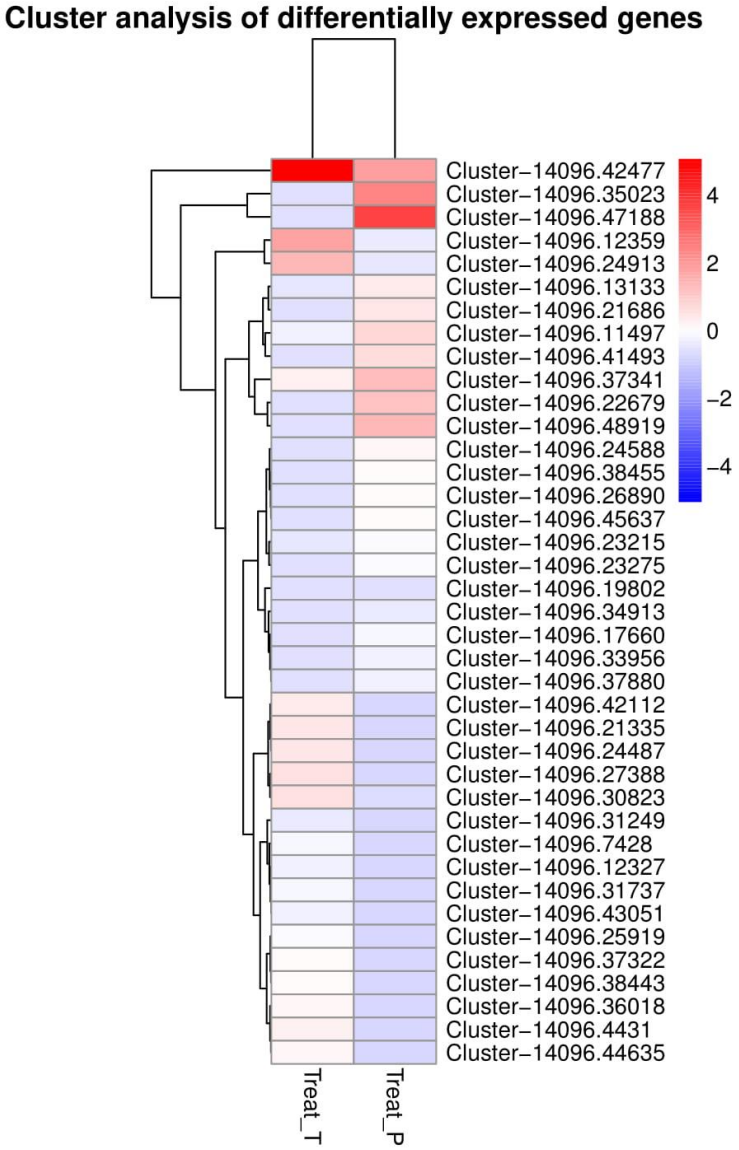


Figure 4.11. Hierarchical clustering heat map of 39 differentially expressed unigenes of *A. swirskii* mites exposed to tomato leaves (Treat_T) compared to mites exposed to pepper leaves (Treat_P) (p adjusted < 0.05). Colours from red to blue represent the fold change in gene expression from positive to negative, respectively.

After functional annotation, 25 out of the 39 DEGs were significantly homologous to previously annotated genes and nine DEGs were similar to uncharacterized protein sequences of *M. occidentalis* (Table 4.4). For the remaining five DEGs, it was not possible to find any significant homology to published sequences (Table 4.4).

Among the 19 DEGs up-regulated in mites exposed to tomato leaves, 11 had annotated homologues in public databases (Table 4.4). Of those, two DEGs showed higher expression levels in mites exposed to tomato leaves. The two DEGs were significantly similar to a *M. occidentalis* lipase member K; one being a protein involved in lipid degradation and metabolism and the other a sodium-coupled monocarboxylate transporter 2 (Table 4.4). The other nine annotated DEGs were expressed in mites exposed to tomato leaves but not expressed in mites exposed to pepper leaves; they were homologous to the *M. occidentalis* proteins as described in Table 4.4.

Moreover, 14 of the 20 down-regulated DEGs in mites exposed to tomato leaves had significant homologies to previously annotated proteins (Table 4.4). Three of those had lower expression levels in mites exposed to tomato compared to mites exposed to pepper and were homologous to the following *M. occidentalis* proteins: ATP-dependent RNA helicase DDX5, sodium-dependent glucose transporter 1 and histone-lysine N-methyltransferase (Table 4.4). The other 11 DEGs were expressed in mites exposed to pepper leaves and not in mites exposed to tomato leaves; they had a significant homology to previously described *M. occidentalis* proteins (Table 4.4).

Table 4.4. Summary of the annotated DEGs. Up or down regulation of gene expression (up/down) in mites exposed to tomato leaves (Treat_T) compared to mites exposed to pepper leaves (Treat_P). Exclusive (E) indicates exclusive gene expression in mites exposed to either tomato or pepper leaves.

Unigene_id	NR Description	Expression -Exclusive
Cluster-14096.42112	solute carrier family 22 member 7	up - E
Cluster-14096.4431	retinol dehydrogenase 12	up - E
Cluster-14096.30823	lipase member K	up
Cluster-14096.25919	mucolipin-3	up - E
Cluster-14096.36018	uncharacterized protein LOC100899406	up

Cluster-14096.44635	No hit	up
Cluster-14096.42477	No hit	up
Cluster-14096.38443	uncharacterized protein LOC100906407	up
Cluster-14096.7428	uncharacterized protein LOC100907771	up
Cluster-14096.12327	dual oxidase	up - E
Cluster-14096.21335	sodium-coupled monocarboxylate transporter 2	up
Cluster-14096.27388	Neuropilin and tolloid protein 2, partial	up - E
Cluster-14096.24487	No hit	up
Cluster-14096.43051	clavesin-2	up - E
Cluster-14096.31249	small G protein signaling modulator 1	up - E
Cluster-14096.24913	uncharacterized protein LOC100901357	up
Cluster-14096.12359	uncharacterized protein LOC100906988	up
Cluster-14096.37322	paired amphipathic helix protein Sin3a	up - E
Cluster-14096.31737	N-acetylated-alpha-linked acidic dipeptidase 2	up - E
Cluster-14096.41493	lipase member K	down - E
Cluster-14096.24588	E3 ubiquitin-protein ligase MIB1	down - E
Cluster-14096.45637	solute carrier family 22 member 7	down - E
Cluster-14096.23275	protein kinase C-binding protein 1	down - E
Cluster-14096.22679	uncharacterized protein LOC100901481	down
Cluster-14096.33956	protein PRRC1	down - E
Cluster-14096.35023	DNAJ homolog subfamily C member 8	down - E
Cluster-14096.11497	uncharacterized protein LOC100902619	down
Cluster-14096.17660	methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	down - E
Cluster-14096.21686	trifunctional purine biosynthetic protein adenosine-3	down - E
Cluster-14096.13133	sodium-dependent glucose transporter 1	down
Cluster-14096.26890	glycosyltransferase protein LARGE2	down - E
Cluster-14096.34913	ATP-dependent RNA helicase DDX5	down
Cluster-14096.48919	No hit	down
Cluster-14096.38455	mitochondrial carrier homolog 2	down - E
Cluster-14096.19802	adenylate cyclase type 6	down - E
Cluster-14096.37880	uncharacterized protein LOC100901512	down
Cluster-14096.47188	No hit	down
Cluster-14096.37341	uncharacterized protein LOC100897466	down
Cluster-14096.23215	histone-lysine N-methyltransferase, H3 lysine-79 specific	down

4.3.3 Detoxification superfamilies CYPs, GSTs and CCEs

In the *A. swirskii* transcriptome, 162 unigenes coding for P450s from the Nr annotation were identified. After manually removing those with short open reading frames (ORFs) (lengths <400 bp), or with more than 95 % identity, along with the published P450s of *T. urticae* and

M. occidentalis (Grbic et al., 2011, Wu & Hoy 2016) the remaining 77 P450 unigenes were used to construct a phylogenetic tree, (Figure 4.12, Appendix Table S1). Cytochrome P450s were classified into four major families; as indicated by the closest hits on the NCBI Nr database and the phylogenetic tree (Figure 4.12, Table 4.5) our analysis illustrated 24 unigenes from *A. swirskii* belong to CYP2 family, 31 to CYP3, 20 to CYP4 and 2 to mitochondrial P450s.

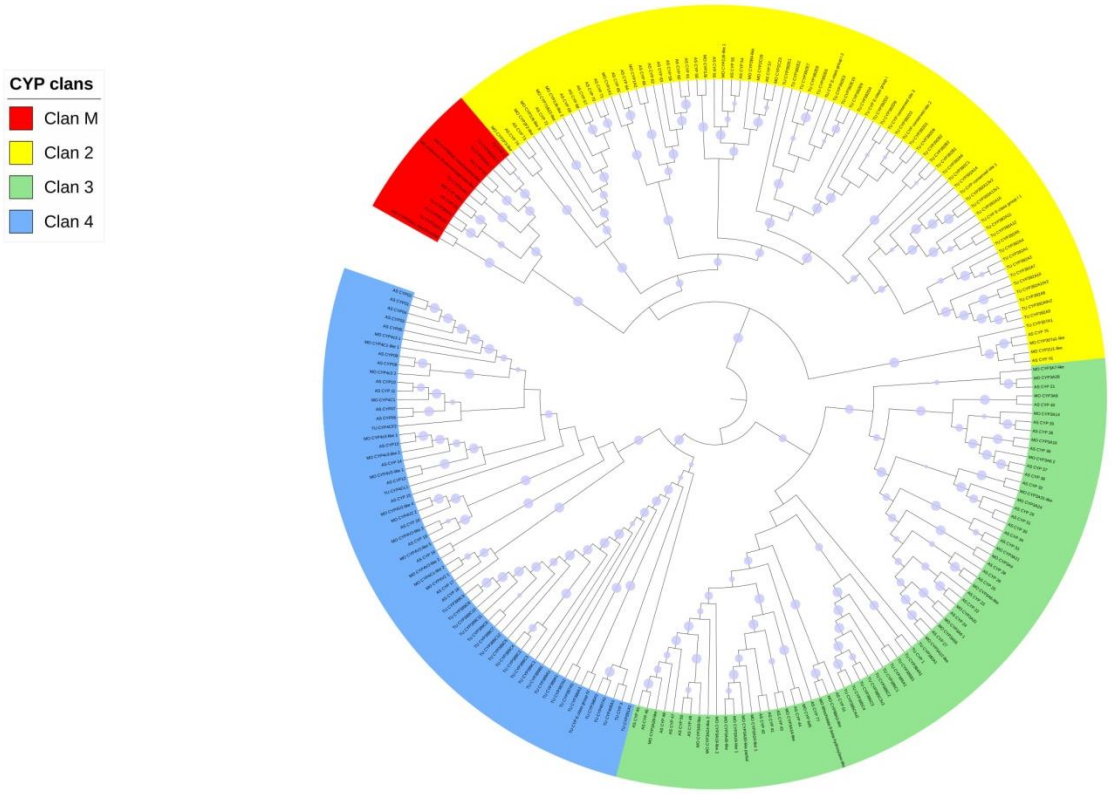


Figure 4.12. Phylogenetic analysis of 77 *A. swirskii* CYP proteins represented with those of *T. urticae* and *M. occidentalis*. The CYP protein clans are represented as follows: in red are the mitochondrial CYP, in yellow the clan 2, in green the clan 3 and in blue the clan 4. The midpoint-rooted tree was generated using a maximum likelihood approach (RAxML v8.2.10) and bootstrapping with 1000 replicates. Blue circles on the nodes represent bootstrap likelihood > 0.5; their size represents their relative value.

In addition, 81 GST genes were identified in the *A. swirskii* transcriptome and, after filtering for short open reading frames (length < 200bp) and high sequence similarity (>95%), 28 putative GST genes were selected to be used in the phylogenetic analysis along with previously published *T. urticae* and *M. occidentalis* GST proteins (Figure 4.13). Based on the phylogenetic analysis and on the annotations from the NCBI Nr database (Figure 4.13, Appendix Table S2), these GST-related unigenes were assigned to the GST classes as follows; 11 to the delta/epsilon, seven to the mu, one to the zeta, five to the omega, two to the kappa, and three to other classes of GSTs (Table 4.5).

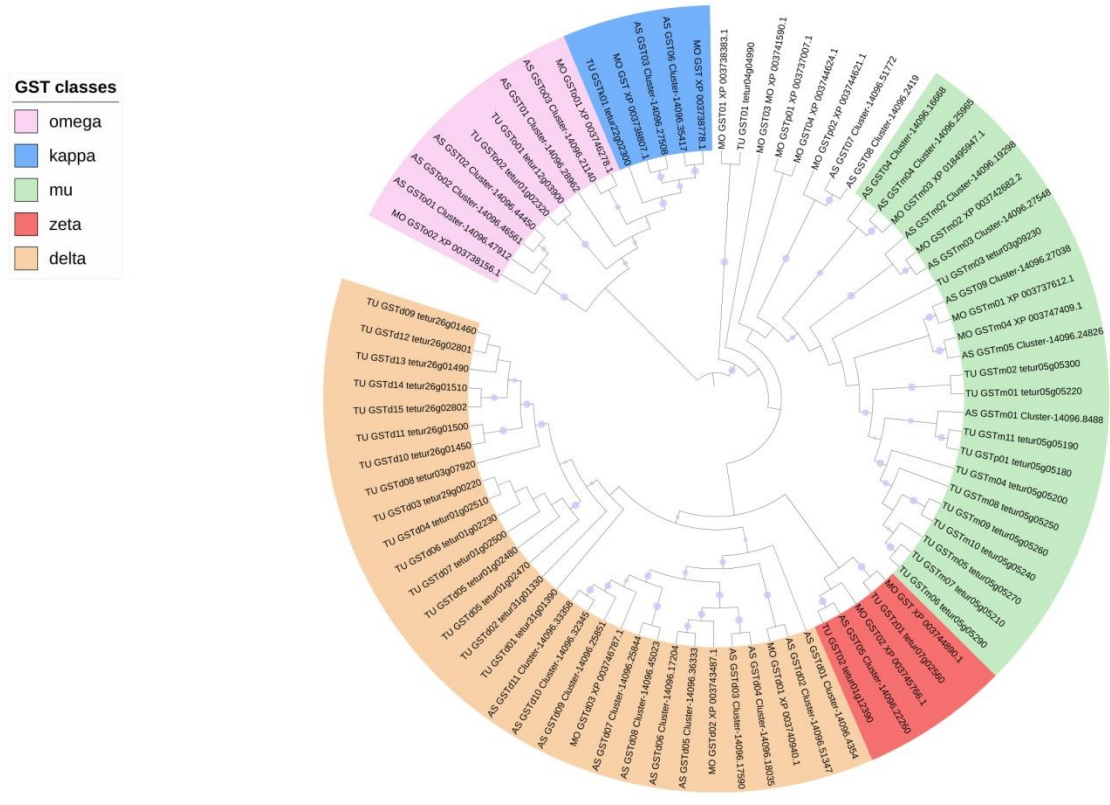


Figure 4.13. Phylogenetic analysis of 28 cytosolic *A. swirskii* GST proteins represented with those of *T. urticae* and *M. occidentalis*. The GST protein classes are represented as follows: in pink are the Omega, in blue the Kappa, in green the Mu, in red the Zeta and in orange the Delta class. Non- coloured proteins belong to uncharacterized clades. The midpoint-rooted tree was generated using a maximum likelihood approach (RAxML v8.2.10) and

bootstrapping with 1000 replicates. Blue circles on the nodes represent bootstrap likelihood > 0.5; their size represents their relative value.

After manual identification of 96 annotated CCEs in the transcriptome of *A. swirskii* 32 nonredundant, putative full-length open reading frames (ORFs) were selected. The translated CCE protein sequences were assigned to the following clades; 13 to the J′, 16 to the J′′, 1 to the K, and two to uncharacterized clades, according to the phylogenetic analysis with known for *T urticae* and *M. occidentalis* CCEs (Figure 4.14, Appendix Table S3). The majority of *A. swirskii* CCEs fall into clades J′ and J′′ which are not present in insects; they are described as Acari-specific clades (Wu and Hoy, 2016) (Figure 4.14, Table 4.5).

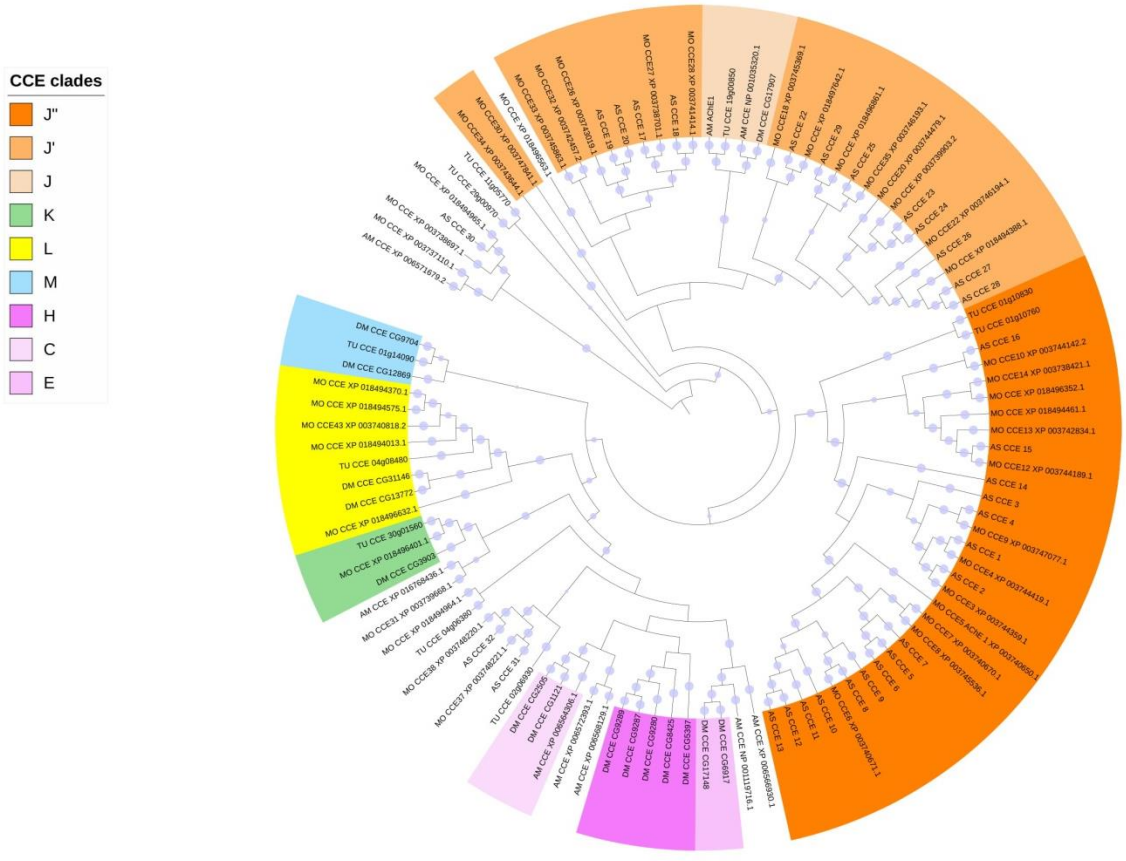


Figure 4.14. Phylogenetic analysis of 32 *A. swirskii* amino acid sequences of

carboxyl/cholinesterase (CCE) genes, represented with those of *T. urticae* and *M. occidentalis*. The different CCE classes/clades are represented as follows: in dark orange the J´´, in light orange the J´, in beige the J, in green the K, in yellow the L, in blue the M, in purple the H, in light pink the C and in pink the E clade. The alignment was trimmed at the N- and C-terminal [ref Claudianos et al. 2006]. The midpoint-rooted tree was generated using Mr. Bayes XSEDE. Blue circles on the nodes represent posterior probabilities > 0.5; their size represents their relative value.

Table 4.5. A comparison of CYP, GST and CCE gene numbers in the genomes of six acarine species*.

	I. scapularis	***T. urticae***	***P. citri***	***M. occidentalis***	***N. barkeri***	***A. swirskii***
CYP						
CYP2		48	12	16	9	24
CYP3		10	9	23	19	31
CYP4		23	26	19	2	20
M. CYP		5	7	5	2	2
Other		25				
Total		86	79	63	32	77
GST						
Delta/Epsilon	12	16	7	3	3	11
Mu	14	12	10	5	4	7
Omega	3	2	1	3	1	5
Sigma	0	0	2	0	0	0
Theta	0	0	1	0	0	0
Zeta	3	1	1	1		1
kappa			2		2	2
Unknown	0	0		1		3
Total	32	31	24	13	10	29
CCE						
Dietary/detoxification class						
Clade A, B and C		0	7	0		0
Hormone/semiochemical class						
Clade D (integument esterases)		0	1	0		0
Clade E (secreted Ξ² esterases)		0		0		0
Clade F (dipteran JhE)		0		0		0
Clade G (lepidopteran JhE)		0		0		0
Clade Fβ€™ (crustacean/Acari JhE)		2		0		0

Neuro/developmental class				0
Clade H (glutactin)	2		0	0
Clade I (uncharacterized clade)	0		0	0
Clade J (AChEs)	1		1	0
Clade K (gliotactin)	1		1	1
Clade L (neuroligins)	5		5	0
Clade M (neurotactins)	1		0	0
Acari-specific class				
Clade J′	34		19	13
Clade J′′	22		15	16
Undetermined	3	5	3	2
Total	71	13	44	32

* Data derived from: *I. scapularis,* Reddy et al. 2011; *T. urticae,* Grbic et al. 2011; *P. citri,* Niu et al 2012; *M. occidentalis,* Wu and Hoy 2016; *N. barkeri,* Cong et al. 2016 and *A. swirskii* from the current study.

4.4. Discussion and conclusions

The implementation of Integrated Pest Management (IPM) has become a priority for the EU since it is a scientifically-based approach to pest control with a strong drive to protect the environment from the negative impacts of conventional pesticides. The EU is not the only zone promoting IPM; this strategy is extensively used with success in many countries since it entails the wise selection of control measures to maximize yields, while limiting chemical residues in the process (Abrol, 2013). Biological control agents (BCAs) are key for the success of IPM; they are major components of this strategy and contribute effectively to pest control, sometimes without a single pesticide input throughout the season (Abrol, 2013). In this context, the predatory mite *A. swirskii* is a valuable player. It is able to feed on several developmental stages of economically important pest species, such as whiteflies, thrips, and plant feeding mites (Wimmer and Hoffmann, 2008; Stansly and Castillo, 2010; Doğramaci et al., 2011; Hoogerbrugge et al., 2011); it can survive and reproduce in the crop, even when the level of prey is low. Moreover, it has been proven to be very effective in several protected

crops, for example strawberries, cucumbers, and peppers (Hoogerbrugge et al., 2011; Calvo et al., 2011; Calvo et al., 2012), but not in tomato, where it has not been able to establish stable populations. Thus, we performed a deep analysis of the transcriptomic response of the mites to the toxic compounds present in tomato leaves, in order to identify key elements of their response, in comparison with that in pepper, a favourable host plant, where the mites thrive and perform very well.

Given the lack of available annotated genome of this species, we used the sequence data generated in our RNAseq approach to assemble and annotate a transcriptome that allowed the characterization of the mite response to tomato. A significant amount of sequencing data was generated (134.01 Gbp), and the transcriptome was *de novo* assembled using a combination of Trinity (Grabherr et al., 2011) and a hierarchical clustering of the contigs using Corset (Davidson and Oshlack, 2014). In the annotation process, 66.1 % of genes were annotated by at least one database, with a total of 47,159 unigenes annotated. This great depth of data and the large transcriptome is a significant genomic resource, now available to the scientific community, from a species with very limited public genomic information.

The number of unigenes annotated here is higher than that in other published transcriptomes of related species e.g. 32,535 from *Panonychus citri* (Niu et al., 2012), 25,888 from *Metaseiulus occidentalis* (Hoy et al., 2013), 20,486 from *Ixodes scapularis* (Gulia-Nuss et al., 2016), 15,866 from *Neoseiulus barkeri* (Cong et al., 2016) and 11,433 from *Tetranychus urticae* (Grbić et al., 2011). As stated above, there is no annotated genome of this species available, thus, a more precise manual curation of the transcriptome could not be performed. Different variants in the population, alternative splicing, or errors in the alignments of the transcripts can be counted as different unigenes erroneously if the transcriptome complexity is high (Chang et al., 2014). On the other hand, given the nature of the experiment and the

biology of the mite, the raw data generated contained significant contamination from tomato, pepper and the *C. edulis* pollen food source. For tomato and pepper there is enough genomic information in the databases that allowed filtering and cleaning the transcriptome from these sources of contamination. However, in the case of contamination with *C. edulis* transcripts, there are no genomic resources available to properly remove them, so it is possible that they contribute to the high the number of unigenes in the transcriptome. As previously suggested, an increase in the starvation period previous to the experiment might reduce the level of contamination from food sources (Hoy et al., 2013). Nevertheless, our experimental design guaranteed the conditions of the mites to be identical before the treatments. Therefore the differential expression measured was related to the response of the mite to tomato and to pepper.

As previously illustrated in *T. urticae* (Dermauw et al., 2013), our analyses elucidated a differential response of *A. swirskii* in tomato compared to pepper. However, a lower number of genes were differentially expressed in *A. swirskii* transferred to tomato for 48 hours (39 genes:19 up-regulated, 20 down-regulated) compared to *T. urticae* transferred on tomato for 12 hours (416 genes: 211 up-regulated, 205 down-regulated) and for five generations of *T. urticae* in another study (1206 genes: 571 up-regulated, 635 down-regulated) (Dermauw et al., 2013). Five transcripts encoding transmembrane transporters (GO ID: 0055085), three of which belong to the Major Facilitator Superfamily (PFAM ID: PF07690), were found. High representation of members of this gene superfamily were found to be differentially expressed in *T. urticae* transferred from bean plants to tomato plants, as well (Dermauw et al., 2013). Furthermore, Dermauw et al., (2013), found that 16 members of a Major Facilitator Superfamily gene cluster (orthoMCL cluster 10032) were up-regulated in mites transferred from bean to tomato for 12 hours and 24 genes of the same gene cluster were differentially

expressed, mostly upregulated (87.5%) in tomato after long term rearing for five generations.

Moreover, we found two annotated transcripts: one alcohol dehydrogenase and one carboxyl transferase possibly related to the metabolism of the most abundant tomato exudates, the acyl sugars. Cultivated tomato acyl sugars are polyols with multiple hydroxyl groups and consist of aliphatic acyl groups esterified to sucrose (Schilmiller et al., 2010); hence, they are putative substrates for arthropod alcohol dehydrogenases (McKechnie and Geer, 1984). The sticky acyl sugars can cover the cuticle and either block the mite respiratory spiracles causing their suffocation or penetrate and degrade the underlying cellular membranes causing their desiccation (Puterka et al., 2003). A chitin synthesiser (Cluster-14096.7428) which synthesizes the major component of the cuticle was found to be differentially expressed. This chitin synthesiser is of great interest because it might be involved in the response against the tomato acyl sugars. It has also been suggested to play an important role in mite resistance to acaricide compounds in the phytophagous mite *Panonychus citri* (Niu et al., 2012). Two other transcripts (Cluster-14096.12359, Cluster-14096.37322) involved in oxidations reduction (GO: 0055114) might be expressed as a response to trichome oxidases such as the polyphenol oxidases, which are involved in the production of quinones; substances harmful to arthropods (Glas et al., 2012).

Intriguingly, none of the 39 differentially expressed genes detected in this study belong to known families of detoxification enzymes such as the cytochrome P450s, the GSTs and the CCEs. In the herbivorous polyphagous mite *T. urticae*, the adaptation to tomato involved a strong transcriptional response that included the differential expression of genes from these families, among others (Dermauw et al., 2013). The mild response to the exposure to tomato plants and the absence of detoxification enzymes among the differentially expressed genes recorded in this study, suggest that the toxic effect of the tomato plants on predatory mites is

different from the toxic effect on herbivory mites. Predatory mite mortality on tomato plants might be caused by suffocation after the physical occlusions of the respiratory openings by the sticky acyl sugars, or the desiccation by the caustic properties of the acyl sugars on the mite cellular membranes (Puterka et al., 2003). Nevertheless, previous findings report that the fitness of another predatory mite, *Neoseiulus californicus* (McGregor) does not increase over many generations when reared on new 'unfavourable' hosts like tomato (Cédola et al., 2001). In contrast, the fitness of *T. urticae* increases rapidly in just a few generations in tomato, which might be the result of a fine tuned evolutionary adaptation for consuming plant material from many plant species (Dermauw et al., 2013).

In this study, we have described a significant number of genes belonging to families of detoxification enzymes that were in the same range found in other related species. Among monooxygenases cytochrome P450 (CYP), 77 genes were identified (Figure 4.12, Appendix Table S1). This number is similar to that recently described in other acarine species like *M. occidentalis* (n = 63) (Wu and Hoy, 2016) or *T. urticae* (n = 86) (Grbić et al., 2011). In other arthropods the number of CYPs ranged from 43 in the predatory mite *N. barkeri* (Cong et al., 2013) to 121 in *P. citri* (Niu et al., 2012), with a significant variation among arthropod species (Zimmer et al., 2014). This family of enzymes plays a crucial role in the detoxification pathways of xenobiotic compounds such as plant toxins, secondary metabolites and pesticides (Scott et al., 1998; Schuler, 2011) and they have been frequently associated with the evolutionary adaptation of several arthropod species to host plant metabolites and or the development of resistance to pesticides (Yang et al., 2011; Dermauw et al., 2013; Niu et al., 2012). In arthropods, genes of this family fall into four major clades, CYP2, CYP3, CYP4 and the mitochondrial clade (Feyereisen, 2012). Our analysis showed that *A. swirskii* has 24 genes belonging to clade CYP2, more than that reported in *M. occidentalis* (16) (Wu and

Hoy, 2016), *N. barkeri* (9) (Cong et al., 2016), and *P. citri* (Niu et al., 2012). However, this is the most abundant clade in *T. urticae* that includes enzymes directly involved in the synthesis of the molting hormone and or in breaking down pesticides which has been associated with the development of resistance in populations (Grbić et al., 2011). In *A. swirskii,* clade CYP3 is the most abundant with 31 genes, very similar to that in the other two phytoseiids with genomic information available, *M. occidentalis* (23) (Wu and Hoy, 2016) and *N. barkeri* (19) (Cong et al., 2016). This distribution of CYP genes in phytoseiid mites resembles that found in some insect species, where CYP3 often forms large clusters (Feyereisen, 2012). Differences in the expression of many enzymes from this clade have been associated with the resistance to a broad range of pesticides and also in the detoxification of host plant secondary metabolites (Dermauw et al., 2013). Clade CYP4 is also abundant in *A. swirskii* with 20 genes, similar to those described in *T. urticae* (23), *P. citri* (26) and *M. occidentalis* (19) (Grbić et al., 2011; Niu et al., 2012; Wu and Hoy, 2016). However, in *N. barkeri* there are only two genes in this clade (Cong et al., 2013). Finally, the mitochondrial clade is the least abundant in *A. swirskii* similar to other closely related phytoseiids like *M. occidentalis* and *N. barkeri* or the herbivores, *T. urticae* and *P. citri* (Grbić et al., 2011; Niu et al., 2012; Cong et al., 2016; Wu and Hoy, 2016).

Glutathione-S-transferases, which also play important roles in detoxification of xenobiotics, were identified as well. GSTs in insects are classified into Delta, Epsilon, Omega, Sigma, Zeta, and Theta classes and among these classes, Epsilon and Delta are the largest in gene numbers in insects (Fang, 2012). However, the classification of GSTs in mites is quite different from that of insects. Five classes of GSTs were found in *A. swirskii*, including Delta/Epsilon, Mu, Omega, Zeta and Kappa (Figure 4.13, Appendix Table S2). The Delta/Epsilon class genes in *A. swirskii*, are the most numerous (11); homologous of this

class are also found in the Acari, *M. occidentalis* (Wu and Hoy, 2016) and *T. urticae* (Grbić et al., 2011). In arthropods, both insects and mites, the Delta/Epsilon class is involved in pesticide resistance (Chen et al., 2003; Pavlidi et al., 2015). The Mu class of GST genes, are homologous to mammalian Mu GSTs and have been identified in *A. swirskii* (7) and in other acarine species, like *M. occidentalis* (Wu and Hoy, 2016), *N. barkeri* (Cong et al., 2016), *I. scapularis* (Niranjan Reddy et al., 2011), *T. urticae* (Grbić et al., 2011) and *P. citri* (Niu et al., 2012). In general, the numbers of the Mu class GSTs in all acarine species are similar to those of the Delta/Epsilon class. The Mu class GSTs have been suggested to participate in pesticide resistance in *T. urticae* mites (Pavlidi et al., 2015). Five Omega class GSTs were found in *A. swirskii*, more than those found in *M. occidentalis* and other acarine species (Wu and Hoy, 2016). Omega class GSTs are involved in the removal of S-thiol groups from proteins and like Zeta class GSTs may also be involved in oxidative stress response (Board et al., 2000; Meng et al., 2014). A single gene of the Zeta class GSTs was identified in *A. swirskii* which is similar to other acarine species with the exception of *I. scapularis* which has three (Wu and Hoy, 2016). These enzymes are involved in the catabolism of amino acids and possibly, in pesticide resistance (Board et al., 1997b). Two Kappa GSTs were identified in the *A. swirskii* transcriptome which is similar to *N. barkeri* (Cong et al., 2016). However, no Sigma, nor Theta GST class genes were found in this species, as in *N. barkeri, M. occidentalis, I. scapularis* and *T. urticae* (Grbić et al., 2011; Niranjan Reddy et al., 2011; Cong et al., 2016; Wu and Hoy, 2016), suggesting a differential feature between mite and insects, even though they have a significant number of enzymes from these two classes.

Carboxyl/cholinesterases are also key enzymes associated with resistance to insecticides/acaricides and plant toxins in Arthropods (Liang et al., 2007; Dermauw et al., 2013; Zhang et al., 2013). Insect CCEs fall into three main phylogenetic classes with distinct

functions: the dietary/detoxification class (A, B, C clades), the pheromone/hormone processing class (D, E, F, G clades) and the neuro/developmental class (H, I, J, K, L, M clades) according to Claudianos et al., (2006). In the transcriptome of *A. swirskii*, 32 CCEs and their phylogenetic relationships with those from other Acari species were investigated (Figure 4.14, Appendix Table S3). The distribution of *A. swirskii* CCEs among different CCE classes/ clades was similar to that of *M. occidentalis* and *T. urticae*. First, *A. swirskii* CCEs, similar to *M. occidentalis* and *T. urticae*, lack homologs to insect CCEs in the dietary/detoxification and hormone/semiochemical classes (clades A, B, C, D, E, F and G) (Grbić et al., 2011; Wu and Hoy, 2016). Secondly, the majority of *A. swirskii* are homologous to the *M. occidentalis* CCEs which fall into two clades, the J′ clade and the J′′ clade (13 and 16 respectively) (Wu and Hoy, 2016). This distribution pattern is similar to that of *M occidentalis* and *T. urticae* CCE superfamily, which have 19 and 34 CCEs falling into the clade J′ respectively and 15 and 22 falling into the clades J′′ (Grbić et al., 2011; Wu and Hoy, 2016). These results add support to the hypothesis that the J′ and J′′ clades represent Acari-specific clades (Wu and Hoy, 2016). Some of the numerous mite CCEs in the J′ and J′′ clades are most likely involved in the detoxification of xenobiotics (e.g. insecticides and plant toxins), since genes of those classes were upregulated in acaricide resistant strains of *T. urticae* and *P. citri* (Niu et al., 2012; Dermauw et al., 2013). In the neuro/developmental class, *A. swirskii* contains one ortholog of the clade K gliotactin, however no *A. swirskii* CCEs were found in the clade L of neuroligins. Furthermore, two *A. swirskii* CCEs do not cluster with known mite CCE clades.

Polyphagous, herbivorous, mites within the Tetranychidae family such as *T. urticae* and *P. citri* thrive on host plants despite the presence of toxic secondary metabolites (Grbić et al., 2011; Niu et al., 2012; Dermauw et al., 2013). However, as evidenced here and elsewhere

(Cédola et al., 2001) predatory mite species do not show the same level of plasticity to adapt to plant defences as their prey, the herbivorous mites. We have created a transcriptome database of a generalist phytoseiid mite, *A. swirskii* and have identified the genes differentially expressed after exposure to an unfavourable host plant, tomato. These identified genes, of both known and unknown function, are most likely associated with *A. swirskii´s* response to excreted plant toxins. In addition, we have illustrated how phytoseiid mites have an evolved detoxification system similar to that found in their prey. Overall, our data will facilitate research on adaptive evolution of predatory mites to plant toxins and will also serve as invaluable public data for other gene analysis of an important biological control agent, *A. swirskii*.

Chapter 5

Effect of mass rearing on the genetic diversity and biological parameters of the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae)

Entomologia Experimentalis et Applicata (2019), 167(7), pp. 670-681.

doi.org/10.1111/eea.12811

Angeliki Paspati, Kim B. Ferguson, Eveline C. Verhulst, Alberto Urbaneja, Joel González-Cabrera & Bart A. Pannebakker

Abstract

Amblyseius swirskii Athias-Henriot (Acari: Phytoseiidae) is a predatory mite used to control whiteflies and thrips in protected crops. This biocontrol agent, originating from the Eastern Mediterranean region, has been mass-reared for commercial use since 2005 and is widely used in augmentative biocontrol programs. As a polyphagous predator, it has to cope with different biotic and abiotic factors. However, possible adaptation to mass rearing for production might be hindering its resilience and capacity for optimum performance in the field. In this study, we investigated the effect of long-term mass rearing on the genetic diversity of *A. swirskii*. We identified six microsatellite loci from whole-genome nanopore sequencing of *A. swirskii* and used these in a comparative analysis of the genetic diversity and differentiation in eight wild populations collected from Israel in 2017 and a commercially available population. Our results indicate that the commercial population is 2.5× less heterozygous than the wild *A. swirskii*. Furthermore, the commercial population has the highest genetic differentiation from all the natural populations, as indicated by higher pairwise F_{st} values. Overall, we show that commercially reared *A. swirskii* have reduced genetic variation compared to their wild counterparts, which may reduce their performance when released to control pests in an integrated pest management (IPM) context.

5.1 Introduction

Food security and safety should be the cornerstone of modern agriculture and the overreliance on chemicals to control pests is jeopardising this goal. The accumulation of residues in the environment and the food chain, their negative impact on human health, biodiversity, soil and water quality and the reduced efficacy due to resistance evolution have

put the spotlight on biological control as the environmentally sound alternative for pest control. In augmentative biological control, mass reared natural enemies are released in large numbers to protect the crops from the negative impact of certain pests (Eilenberg et al., 2001). Today, this approach is key for the success of Integrated Pest Management (IPM) programs for many fruit and vegetable crops, such as cereals, maize, cotton, sugarcane, soybean, grapes and many greenhouse crops on more than 30 million hectares worldwide (Van Lenteren et al., 2018).

The most widely used predatory mite in augmentative biological control is the polyphagous predator *Amblyseius swirskii* (Athias-Henriot) (Acari: Phytoseiidae), followed by two other phytoseiids *Phytoseiulus persimilis* (Athias-Henriot) and *Neoseiulus californicus* (McGregor) (Knapp et al., 2018). *Amblyseius swirskii* was originally described in 1962 on almond trees (*Prunus amygdalus*) in Israel (Athias Henriot, 1962), where it naturally occurs on various other annual and perennial crops, such as citrus, grapes, vegetables and cotton (Swirski & Amitai, 1997). When whitefly resistance to pesticides caused outbreaks in greenhouses at the beginning of this century (e.g. Stansly et al., 2004), attention was drawn on *A. swirskii* because of its fast reproduction and high performance against the key pest *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Nomikou et al., 2001, Calvo et al, 2015). Experiments showed that *A. swirskii* was able to suppress *B. tabaci* populations and also provided good control of thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) on cucumber plants (Nomikou et al., 2002; Van Houten et al., 2005). Today, this species is used to control whiteflies and thrips in greenhouse vegetables, fruits and ornamentals in different parts of the world (Calvo et al., 2015). The importance of *A. swirskii* in current agriculture is showcased in protected sweet pepper crops in South-eastern Spain, where the successful integration of this predatory mite in the IPM strategy against whiteflies

and thrips has led to a sharp decrease in the use of chemical pesticides (Calvo et al., 2011; Van Lenteren et al., 2018). In addition to its high efficacy in managing whitefly and thrips infestations, the success of *A. swirskii* has been further encouraged by its early establishment on crops before the target pests arrive, using pollen or factitious prey as a food source, and by its mass rearing on a plantless system using the stored-product mite *Carpoglyphus lactis* (L.) (Acari: Carpoglyphidae) as factitious prey (Calvo et al., 2015; Bolckmans and Van Houten, 2006).

The effects of long-term mass rearing on the quality and performance of commercial biocontrol agents such as *A. swirskii* in natural or semi-natural environments are largely unknown (e.g. Guzmán-Larralde et al., 2014, Rasmussen et al., 2018, Tayeh et al., 2012). Selection, inbreeding and random genetic drift may lead to loss of genetic variability, loss of fitness and reduced field performance of the mass reared biological control agents (Mackauer, 1976). The constant and regular commercial mass rearing conditions, which are optimized to obtain the maximum number of individuals in the shortest possible time, differ considerably from the greenhouse biotic and abiotic conditions. Furthermore, under these artificial conditions, searching time for preying, mating or dispersal is intentionally restricted to accelerate and synchronize the breeding batches as much as possible. Therefore, long-term laboratory rearing eventually leads to selection for the facility conditions (domestication), which in turn affects the reproductive or behavioural traits of the arthropods (Hoffmann & Ross, 2018). For example, the reproductive fitness of *Drosophila melanogaster* populations captive for 50 generations, was reduced in small populations because of inbreeding depression and in large populations due to genetic adaptation (Woodworth et al., 2002). While domestication is likely to play a key role in the rearing and success of biocontrol agents, only few studies on natural enemies have addressed this process. For instance,

fecundity and emergence of the egg parasitoid *Trichogramma galloi* (Zucchi) (Hymenoptera: Trichogrammatidae) was reduced on the target pest, when the parasitoids were maintained on factitious host compared to natural host (Bertin et al., 2017). Long-term laboratory rearing of the codling moth parasitoid, *Mastrus ridens* (Horstmann) (Hymenoptera: Ichneumonidae), resulted in lower genetic diversity and higher occurrence of diploid males, most likely as an effect of the reduced allelic diversity on a complementary sex determination locus (Retamal et al., 2016). In inbred lines of this parasitoid, the sex ratio of males to females and the proportion of diploid males was higher, and less daughters were produced compared to outbred lines (Zaviezo et al., 2018). A severe lack of genetic diversity and phenotypic differentiation in wing size and abdomen colour were found for one inbred laboratory colony of the crop pollinator *Eristalis tenax* (Linnaeus) (Diptera: Syrphidae) compared with natural populations (Francuski et al., 2014).

The performance of biocontrol agents under variable field conditions depends on its genetic variability, which is defined by the initial size, origin and degree of inbreeding of the founder colony of a biocontrol agent (Mackauer, 1976). If the commercial population is established from a limited number of individuals, then the genetic variation is expected to be low and further reduced due to inbreeding. The genetic variation of commercial populations may be reduced by random genetic drift during rearing as well, such as splitting the colony for commercial distribution, or the occurrence of bottlenecks (Nunney, 2003). One example is the aphid parasitoid *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Braconidae), which experienced a significant founder effect and a strong reduction in the genetic diversity upon its introduction in Australia, compared to the populations from the Old World (Baker et al., 2003). Finally, six biofactory populations of the cereal stem borer parasitoid *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) in Brazil showed genetic differentiation among each

other, resulting in new guidelines for the mixture of populations in the mass rearing strategy to enhance the genetic variability (Freitas et al., 2018).

The commercial *A. swirskii* was originally collected in Israel and is being reared in large scale without its natural prey since 2005 (Y. Van Houten; Koppert Biological Systems, The Netherlands, Personal Communication). The aim of this study was to investigate the population genetic structure of field *A. swirskii* from Israel, as well as the genetic variation in a commercial, long-term reared population of this biocontrol agent. For this, we developed microsatellite markers for *A. swirskii*, using a cost-effective, in-house sequencing process, the MinION Nanopore sequencer for whole genome sequencing, which was applied and evaluated for the first time in sequencing predatory mites. Also, in our study we wanted to explore for the first time the potential of applying pooled microsatellite analysis to predatory mites, thereby offering a cost-effective solution to low individual DNA yields in these minute organisms (Skalski et al., 2006). Last, we wanted to compare the performance of the commercial, long-term reared *A. swirskii* and the field populations, as biological control agents, by estimating three life history parameters, predation, oviposition and dispersal ability on a favourable and an unfavourable host plant.

5.2 Materials and methods

5.2.1 Mites and plants

One colony of *A. swirskii* was initiated from specimens supplied by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain), referred to as the commercial *A. swirskii* population KBS-9. Moreover, a total of eight populations were sampled, from five locations across Israel (see Figure 5.1) and five different host crops (citrus, kiwi, sweet pepper (Sw pep), Jerusalem artichoke (Jer art), cotton). Mites were collected from the field and transferred to the

laboratory where isolated colonies were established and named based on the plant and location found.

Amblyseius swirskii colonies were maintained in rearing units which consisted of a piece of hard black plastic placed on a water saturated sponge. The sponge with the plastic piece was introduced in a plastic tray with water. The plastic borders were covered with thin sheets of tissue paper to ensure a constant water supply for the phytoseiids, to fix the plastic piece to the sponge, and to prevent phytoseiids from escaping. Twice a week, mites were fed *ad libitum* with *Carpobrotus edulis* (L) (Caryophyllales: Aizoaceae) pollen (Ragusa and Swirski, 1975). The colonies were maintained at 25 ±2 ºC in growth chambers under a 16:8 (Light: Dark) photoperiod and 80 % relative humidity.

Pepper plants, *Capsicum annuum* cv. Lipari were used to determine the predation and oviposition rate of commercial and field *A. swirskii* strains and tomato plants, *Solanum lycopersicum* cv. Raf Marmande were used to determine the dispersal rate. Seeds were sown in a mixture of soil and local peat moss. Two weeks after germination seedlings were individually transplanted into pots (8 × 8 × 8 cm). Plants were maintained undisturbed at 25 ± 2 ºC, 65 % humidity ± 5% and 14:10 h (Light: Dark) photoperiod. Pesticide-free plants with 6 fully-developed leaves (approximately 20 cm in height) were used for the experiments at four weeks of age.

5.2.2 Populations and DNA preparation

For the whole-genome sequencing, an inbred population from the commercially available SWIRSKI-MITE® (Koppert Biological Systems, The Netherlands) was established. Five females and five males from the general population were allowed to reproduce for approximately 10 days in a rearing system with cattail *Typha latifolia* pollen as food source,

where eggs were collected and isolated on a new rearing setup. Subsequently five F1 females and F1 males were used to start a new generation. This was repeated for a total of 10 generations. Over 200 individuals from the 10th generation of the inbred population were collected in 96 % ethanol. DNA extraction was performed using the QIAGEN MagAttract HMW DNA Kit (QIAGEN, Hilden, Germany) according to the manual, while the final DNA product was eluted with nuclease free water.

For the microsatellite analysis, the eight field populations, as listed in Table 2 were used. Approximately 18 generations (6 months) after the establishment of the populations in the laboratory, 100 female mites were collected from each colony and placed in 96 % ethanol at -20 °C for DNA extraction. A single batch of 100 *A. swirskii* individuals from the commercially available SWIRSKI-MITE® bottle (Koppert Biological Systems, The Netherlands) was collected directly and placed in 96 % ethanol at -20 °C. The commercial population was not reared in the laboratory before sampling, because this could cause a reduction of its genetic variation. Due to the small size of the mites (0.5 mm) and to reduce genotyping costs (Skalski et al., 2006), we performed pooled DNA extractions for individuals from the same population. Tissue homogenization was achieved by placing dry snap-frozen samples with beads in a shaker for 20 s. Finally, DNA was extracted using a high salt extraction protocol (Maniatis et al., 1982).

5.2.3 Genomic sequencing, markers and experimental procedures

The inbred *A. swirskii* population was sequenced using Oxford Nanopore Technologies (ONT) MinION® sequencer (Ligation Sequencing Kit version 108 (SQK-LSK108), SpotON Flow Cell FLO-MIN107 R9.5) (Oxford Nanopore Technologies, Oxford, UK). Approximately 30,000 reads were generated through direct base calling with the MinKNOW platform (version 1.10.16) or offline base calling using Albacore (version 2.1.3) (ONT). All

raw and uncorrected read files (both FAST5 and FASTQ formats) are available for download (Ferguson, 2018). The basecalled reads, in FASTQ format, were initially trimmed in CLC Genomics Workbench 11.0 (Qiagen, Hilden, Germany) for a minimum read length of 100bp with low quality sequences removed (limit = 0.05), resulting in 11,511 trimmed reads (QIAGEN). These trimmed FASTQ reads were then corrected using CANU version 1.4 pipeline (parameters: genomeSize=100m, correctedErrorRate=0.120, stopOnReadQuality=false) (Koren et al., 2017). This resulted in a total of 574 corrected reads, all longer than 1000bp. These corrected, raw reads are deposited in the NCBI Sequence Read Archive, PRJNA433466. The corrected sequences were used for mining di-, tri-, tetra-, penta-, and hexanucleotide repeats using MSATCOMMANDER v.0.8.2 with the default settings, except for the number of repeats that was set to a minimum of 3 repeats for tri-, tetra-, penta-, and hexanucleotides (Faircloth, 2008). The MSATCOMMANDER program uses Primer3 v1.1.1 to design locus-specific, flanking primers. The settings used for Primer3 were: no perfect repeats; product size 75-500; primer size: min 16, opt 20, max 24 bases; primer annealing temperature: 56 °C - 64 °C. Poor primers were removed based on the following criteria: duplicate, positive for hairpins, high complementarity, and selfing (Rozen & Skaletsky, 2000). The markers with their corresponding primer pairs were sorted by the count of repeats in descending order, as markers with less repeats can be less polymorphic and the first 24 primer pairs of microsatellite loci were selected to be tested for their efficiency using PCR.

A total of six microsatellite loci primers pairs amplified efficiently only the desired microsatellite marker and were selected for analysis, while the rest were rejected for either low efficiency or low specificity. Primer specificity was considered low when the PCR resulted in products different from the expected size. The sequence data of the loci were

deposited in the NCBI's GenBank with accession numbers MK267176 - MK267181 (Table 5.1). The six primer pairs used for the microsatellite amplification and analysis were fluorescently labeled as shown on Table 5.1. The microsatellite markers were amplified for the nine populations of *A. swirskii* according to the following protocol. The PCR reactions were performed in a total volume of 50 µl containing 60 ng of genomic DNA, 1× GoTaq® polymerase buffer, 200 µM dNTP, 1.25 U GoTaq® polymerase (Promega, Madison, Wisconsin, USA) and 0.4 µM of each primer. The PCR protocol was as follows: 5 min denaturation at 95 °C; 35 cycles of [95 °C for 30 s, 50-60 °C for 30 s, 72 °C for 60 s], finally followed by 10 minute at 72 °C. Primer sequences and annealing temperatures are available in Table 1. For analysis, microsatellite amplicons were diluted 250× and pooled into sets of two to three in such a way that overlapping of alleles was avoided even if markers differed for the fluorescent dye (Table 5.1). The samples were denatured at 95 °C and loaded with an internal size standard (GeneScan™ 500 LIZ®) onto an ABI3730 capillary automated sequencer (Thermo Fisher Scientific Inc, Waltham, MA).

Table 5.1. Primer pairs for the mitochondrial and microsatellite markers with corresponding GenBank accession numbers, melting temperature (Tm), PCR product sizes, number of repeats, motifs and fluorescent dyes.

Marker	Accession no.		Primer sequence (5'-3')	T_m (°C)	Product size (bp)	No. of repeats	Motif	Dye
Asw1	MK267176	F	TCT CG GT GG GTT CA AG GA TG	55.5	203-225	13-24	AG	ATTO
		R	AA CGT CG GA AA TTG AG CTG G	56				
Asw2	MK267177	F	TA AC CTC TTG CA CCC TCC TG	55.6	237-253	4-8	AAAG	HEX
		R	TCC TTC ACT CTG TCT CG AC G	56.9				
Asw3	MK267178	F	CTA AG AG GT GC CA AG CC G	58.3	440-443	5-6	AGC	ATTO
		R	TCG CC AT GTT TGC TGT GT AC	55.2				
Asw4	MK267179	F	GA GG GA GC GA GC GT ATC TTC	58.4	425-457	3-9	ACCG	HEX
		R	GT GG GT AC GA TGT TTG GC AC	54				
Asw5	MK267180	F	GC CTG CTC TTC GTC TTT GA C	56.7	419-423	5-6	AGAT	6FAM
		R	GG TCG CGT TAC TAC TTG GCT TAC	55.8				
Asw6	MK267181	F	CAT CAG ACA GCG ATG CGA TC	58.1	277-286	5-8	AGC	ATTO
		R	CAA GAT GAC GGC GGA AAC TC	56.5				

5.2.4 Microsatellite analysis

Genotyping of the pooled DNA samples was performed using the GeneMapper® software (Thermo Fisher Scientific Inc, Waltham, MA). Instead of allele frequencies, peak frequencies were calculated based on the area under the peaks, which is more reliable than to correct for

stutter bands in pooled microsatellite samples (Crooijmans et al., 1996). Microsatellite peak scoring was used to estimate allele frequencies as explained in Hillel et al., 2003. Peak frequencies lower than 0.05 were discarded and peak frequencies higher than 0.05 were subsequently re-calculated to add up to 1 to correct for stutter bands that resulted from incomplete PCR cycles (Megens et al., 2008).

Neighbour Joining clustering was performed for the microsatellite dataset, using Nei's D_A genetic distance in POPTREEW with 1000 bootstraps (Takezaki et al., 2014). Gene diversity was measured as heterozygosity for all loci and for all populations by using peak frequencies instead of allele frequencies according to Hillel et al. (2003). An average heterozygosity was calculated for each population across markers (H) and for each marker across populations (H_m). Similarly, the average allelic richness for each population across markers (A) and for each marker across populations (A_m) and the number of private alleles (N_p) for each population were calculated, as well. Three genetic distance measures for different evolutionary models were calculated based on peak frequencies: the Nei´s genetic distance (D_A), Cavalli-Sforza chord measure (D_{CS}) and Reynolds genetic distance (D_R) (Cavalli-Sforza & Edwards, 1967; Reynolds et al., 1983; Nei et al., 1983). Pairwise distances between each pair of the nine populations (81 estimates) were estimated for each measure. D_A was chosen to be used in the downstream analysis as more appropriate, because this measure assumes that genetic differences are caused by mutations and genetic drift, contrary to the other measures, which do not take into account mutation (Cavalli-Sforza & Edwards, 1967; Reynolds et al., 1983; Nei et al., 1983). The distance matrices were used for Multidimensional Scaling (Gower, 1966) in R 3.4.3. (R Core Team, 2018). Isolation By Distance analysis with a Mantel correlation test (Mantel, 1967) based on 9999 replicates was

also performed using the *ade4* package (Dray et al., 2007) in R 3.4.3. Last, F_{st} measures of genetic differentiation were calculated manually according to Wright (1984).

5.2.5 Predation and oviposition

A panmictic field population was established by placing together 10 mated females from each of the eight field populations from Israel (shown on Table 5.2) for 15 generations (5 months). Then presumably mated females from the panmictic field population and the commercial population KBS-9 were transferred on clean plastic arenas and were allowed to oviposit for 24 hours in climate room at 25C. The eggs were collected and transferred on four pepper leaves, where *Ephestia kuehniella* eggs was added as food source *ad libitum*. After seven days, couples of females and males were collected from the leaves and placed isolated on pepper leaf disks with *E. kuehniella* eggs as food source and observed every 24 hours until first oviposition. Afterwards, the males were removed and every 24 hours during five days, the number of *E. kuehniella* eggs preyed by *A. swirskii* females per female/day and also, the number of eggs laid per female/day was counted. The leaf disks were maintained fresh on 1% agar gel, inside plastic cups with lid of diameter 5 cm and with a 2 cm opening for ventilation, covered with fine mesh to prevent mite escape. Twenty five replicates per population were considered and predation and oviposition data were fitted to a Generalized Linnear Model with quasipoisson distribution using R version 3.5.1.

5.2.6 Dispersal on tomato stems

Ten mated female mites from the field populations EY-3, Had-6, Has-7, collected from citrus trees in Israel (Table 5.2), were pooled together and let reproduce for approximately 15 generations (5 months), in order to establish a citrus population. Adult female mites from the commercial KBS-9 and the field populations from five host plants (kiwi, cotton, sweet

pepper, Jerusalem artichoke, and citrus) (Table 2.5) were used to investigate mite dispersal on tomato plants. The central part of the stem with 3 successive leaves was used for the observations and it was delimited by tanglefoot barriers. On the part with the three successive leaves, the middle leaf was removed and pollen *C. edulis* was added on the other two leaves. One day later, the scar left at the stalk base served as the release point, where one female mite was released. Two hours later we assessed whether the mite had reached a successive leaf and when the mites reached the successive leaf within 2 hours since their release, the dispersal was recorded as successful. Twenty female mites and twenty tomato plants with 6 fully developed leaves were used at temperature 22°C and Relative Humidity 50-60%. Dispersal data were fitted to a Generalized Linnear Model with binomial distribution using R version 3.5.1.

5.3 Results

5.3.1 Microsattelites and genetic diversity

In this study, we investigated the genetic diversity of a commercial *A. swirskii* population, mass reared for long-term and compared to the genetic diversity found in wild populations. Eight wild populations were established from mites collected from citrus, kiwi, sweet pepper, Jerusalem artichoke and cotton crops from Hula Valley, Beit HaEmek, Ezor Yokne'am, Hadera and Hof Ashkelon in Israel as described on Table 5.2 and Figure 5.1 and they were named after the host plant and the source location number. The genetic diversity of the wild populations was compared to one population from a commercial supplier.

Table 5.2. Source (host plant in the field or commercial product), sampling site locations (see map in Figure 1) and number of mites collected (in the field or from the commercial product) for all the *A. swirskii* populations.

Population	**Source**	**Location**	**Sample size (*N*)**
HV-1	Citrus	1 Hula Valley (HV)	100
BHE-2	Kiwi	2 Beit HaEmek (BHE)	30
EY-3	Citrus	3 Ezor Yokne'am (EY)	100
Had-4	Sweet pepper	4 Hadera (Had)	20
Had-5	Jerusalem artichoke	4 Hadera (Had)	10
Had-6	Citrus	4 Hadera (Had)	15
HAs-7	Cotton	5 Hof Ashkelon (HAs)	40
HAs-8	Citrus	5 Hof Ashkelon (HAs)	8
KBS-9	Koppert Biological	-	100

Figure 5.1. Map of showing sampling locations across Israel.

A. swirskii genomic sequencing with the MinION generated 256 Mb total sequence data after basecalling, or approximately 30,000 reads. After sequence trimming and correction, 574 corrected reads of at least 1000 bp long where retained (5.2 Mb) (Table 5.3). Microsatellite mining of the genome sequences identified 2423 microsatellite loci on 532 reads (Table 5.3). The density of microsatellites in the genome is 466 microsatellites per Mb and 92.6 % of our reads contain microsatellite loci. The most common type of microsatellites are dinucleotide repeats (72.10 %), followed by trinucleotide repeats (23.65 %), whereas the least common microsatellites found were the hexanucleotide repeats (0.12 %) (Table 5.3). Primers designed using Primer3 were filtered for quality, resulting in 622 valid primer pairs. Of those, 24 primer pairs of the markers with the largest repeat count were selected and then tested for their specificity and efficiency. Finally, a set of six microsatellite markers was found to be specific and efficient for using in the microsatellite analysis of population pools. The nucleotide motifs of these six microsatellite markers are: di- (Asw1), tri- (Asw3, Asw6) and tetranucleotide repeats (Asw2, Asw4, Asw5) as shown on Table 5.1.

Table 5.3. The composition of the microsatellite repeats found in the genome of *A. swirskii.*

Motif type	**Number**	**Frequency**
Dinucleotide	1747	72.10%
Trinucleotide	573	23.65%
Tetranucleotide	94	3.88%
Pentanucleotide	6	0.25%
Hexanucleotide	3	0.12%
Total	2423	100.00%

In total, 32 different alleles were scored across the six microsatellite loci for the nine populations, with four of those alleles being private alleles, exclusively found in equal number of wild populations as indicated in Table 5.4. All markers were polymorphic in at least four of the nine populations and all of the populations had at least three markers polymorphic (Table 5.4). The average gene diversity H_m for the six markers analysed was 0.42 and the mean allelic richness per locus A_m was 5.3 across populations and 2.9 within populations (Table 5.5). The most polymorphic marker was Asw1 with 12 alleles across populations and a mean allelic richness of A_m = 5.8 per population (Table 5.5). The gene diversity H_m of marker Asw1 was 0.66 and all of the populations were polymorphic for this marker (Table 5.5). On the other hand, the least polymorphic marker was Asw5, with two alleles over all populations, with average A_m of 1.6 alleles per population, gene diversity H_m of 0.19 and it was polymorphic in five of the nine populations (Table 5.5). No bias of field sample size on the allelic richness was found (R^2: 0.01266, p-value: 0.79) (Figure 5.2).

Table 5.4. Frequency of polymorphic markers (P), average heterozygosity (H), average allelic richness (A), the mean genetic distance estimates (MGD) of Nei (DA), Reynolds (DR), Cavalli-Sforza (DCS) and number of private alleles (Np) for nine populations of *A. swirskii* (eight wild and one commercial (KBS-9)).

Population	***P***	***H***	***A***	MGD_A	MGD_{CS}	MGD_R	N_p
BHE-2	0.83	0.34	2.5	0.499	0.333	0.386	1
EY-3	1	0.50	3.7	0.254	0.176	0.210	0
HV-1	1	0.48	2.7	0.323	0.209	0.252	0
HAs-7	0.83	0.39	3	0.430	0.293	0.336	0
HAs-8	1	0.52	3.7	0.423	0.274	0.287	1
Had-4	1	0.53	2.5	0.418	0.250	0.279	0

Had-5	0.83	0.42	3.3	0.362	0.244	0.295	1
Had-6	0.83	0.41	2.7	0.401	0.315	0.318	1
KBS-9	0.50	0.21	1.8	0.430	0.300	0.418	0

Table 5.5. Heterozygosity and allelic richness for all the microsatellite markers per locus (H_m/A_m), and within populations (H/A) across nine populations of *A. swirskii* (eight wild and one commercial (KBS-9)).

Population	Asw1	Asw2	Asw3	Asw4	Asw5	Asw6	Average
BHE-2	0.69/5	0.37/3	0.50/2	0.31/2	0.19/2	0.00/1	0.34/2.5
EY-3	0.63/8	0.50/2	0.50/2	0.63/5	0.13/2	0.63/3	0.50/3.7
HV-1	0.48/4	0.50/2	0.40/2	0.51/3	0.40/2	0.57/3	0.48/2.7
HAs-7	0.74/7	0.51/3	0.46/2	0.33/3	0.00/1	0.27/2	0.39/3.0
HAs-8	0.79/8	0.47/2	0.25/2	0.59/5	0.50/2	0.52/3	0.52/3.7
Had-4	0.55/3	0.38/2	0.50/2	0.65/3	0.48/2	0.62/3	0.53/2.5
Had-5	0.73/6	0.29/2	0.25/2	0.65/6	0.00/1	0.62/3	0.42/3.3
Had-6	0.71/7	0.49/2	0.50/2	0.32/2	0.00/1	0.43/2	0.41/2.7
KBS-9	0.65/4	0.00/1	0.00/1	0.28/2	0.00/1	0.35/2	0.21/1.8
Average	0.66/5.8	0.39/2.1	0.37/1.9	0.47/3.4	0.19/1.6	0.45/2.4	0.42/2.9

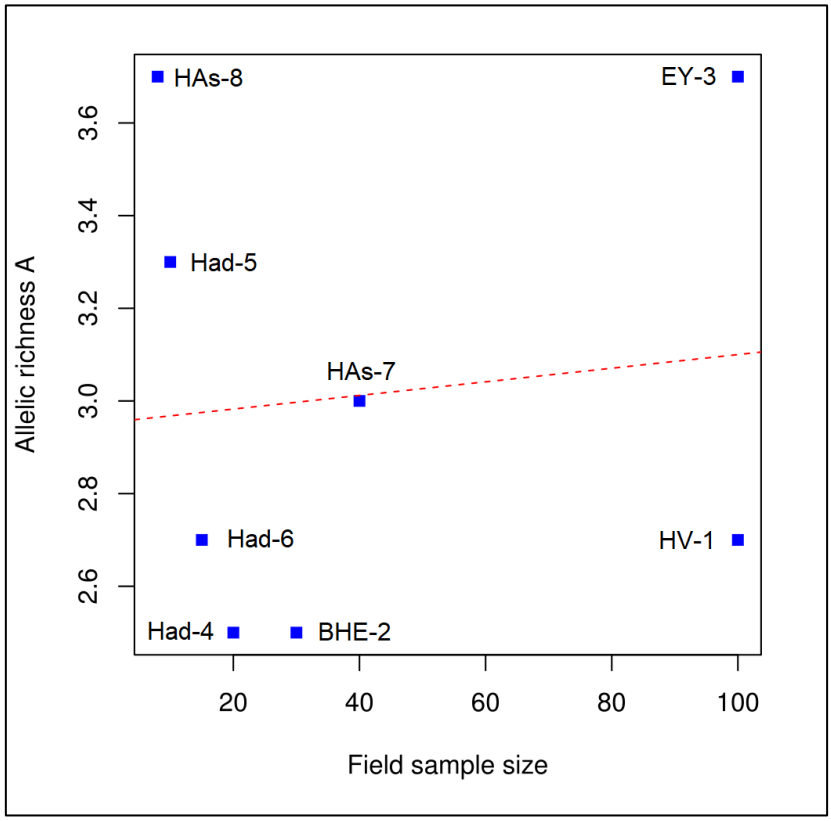


Figure 5.2. Allelic richness A of each population vs field sample size for eight wild *A. swirskii* populations. Dashed red line represents the linear regression fit (R^2: 0.01266, *P-value*: 0.79).

When comparing populations, the lowest frequency of polymorphic markers *P*, average heterozygosity *H* and allelic richness *A* across loci, were found in the commercial population KBS-9 (*P*=0.5, *H*=0.21 and *A*=1.8, respectively). The average heterozygosity *H* in KBS-9 was from 1.6 to 2.5 times lower than the average heterozygosity present in wild *A. swirskii* (*H* ranged from 0.34 to 0.53, *P* ranged from 0.83 to 1 and *A* ranged from 2.5 to 3.7), as indicated in Table 5.4 and Figure 5.3.

Figure 5.3. Heterozygosity (*H*) (mean± standard error) across six microsatellite markers for eight wild and one commercial population of *A. swirskii* (KBS-9).

The heterozygosity H_m of the commercial population KBS-9 ranged from H_m=0.65 and A_m=4 alleles for marker Asw1, to 0 for markers Asw2, Asw3, and Asw5 (Table 5.5). The highest average heterozygosity *H* across loci was found in *A. swirskii* population Had-4 (*H*=0.53), collected from sweet pepper crops in Israel, and ranged from H_m=0.65 and A_m=3 alleles for marker Asw4, to H_m=0.38 and A_m=2 alleles for marker Asw2 (Table 5.5). Moreover, the commercial population KBS-9 has the highest genetic differentiation from all the natural populations, as indicated by the pairwise F_{st} values (Table 5.6).

Table 5.6. Pairwise F_{st} (lower triangle) and Nei´s D_A pairwise genetic distances (upper triangle) among nine populations of *A. swirskii* (eight wild and one commercial (KBS-9)).

Populatio	BHE-2	EY-3	HV-1	HAs-7	HAs-8	Had-4	Had-5	Had-6	KBS-9
BHE-2		0.376	0.507	0.581	0.560	0.471	0.403	0.506	0.586
EY-3	0.0013		0.039	0.303	0.337	0.247	0.222	0.271	0.236
HV-1	0.0312	0		0.410	0.404	0.253	0.278	0.363	0.330
HAs-7	0.1387	0	0		0.301	0.641	0.404	0.309	0.488
HAs-8	0	0	0	0		0.466	0.446	0.443	0.429
Had-4	0	0	0	0	0		0.369	0.431	0.466
Had-5	0.0928	0	0	0.0417	0	0		0.376	0.397
Had-6	0.1117	0	0	0.0605	0	0	0.0147		0.506
KBS-9	0.3428	0.1542	0.1841	0.2916	0.1332	0.1187	0.2457	0.2646	

The genetic distance measures Nei´s D_A, Reynolds D_R, and Cavalli-Sforza chord D_{CS} were calculated between populations and the highest average distances were found for the commercial population and for the population collected on kiwi (Table 5.4). Multidimensional scaling and Neighbour joining clustering of the Nei´s D_A genetic distances derived from the microsatellite genotyping of the wild *A. swirskii* populations suggest three possible clusters, one is the population BHE-2-Kiwi separated from the other populations, a second including the populations Had-6-Citrus, HAs-7-Cotton, HAs-8-Citrus, and last a cluster of the populations Had-5-Jerusalem artichoke, Had-4-Sweet pepper, HV-1-Citrus and EY-3-Citrus (population names indicate the sampling location and the host plant) (Figure 5.4, Figure 5.5). The commercial population KBS-9 falls in the latter cluster of wild populations (Figure 5.4, Figure 5.5). The Neighbour Joining analysis yielded results very similar to the

Multidimensional scaling; however, moderate bootstrap values (<70%) were computed, because the number of microsatellite loci employed in the analysis was lower than the number of populations analysed (Figure 5.5). No evidence for isolation by distance was found among the wild *A. swirskii* populations, according to the Mantel correlation test (simulated p-value: 0.3026) and the linear regression fit (R^2= 0.01653, *P*-value=0.514) (Figure 5.6).

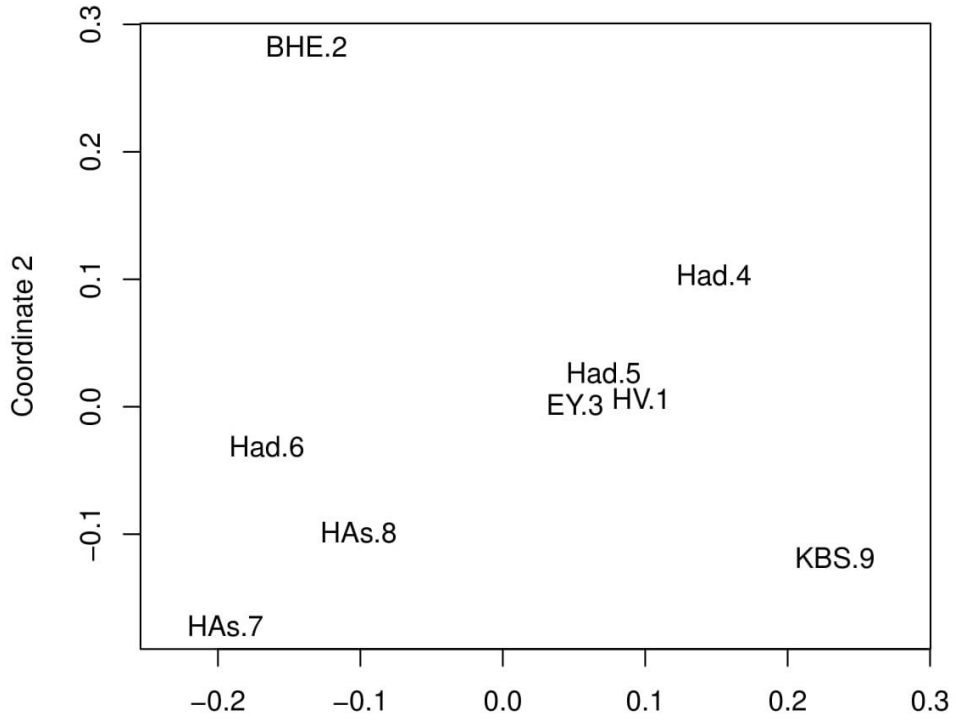


Figure 5.4. Multidimensional scaling using the Nei´s D_A genetic distance matrix for nine populations of *A. swirskii* (eight wild, one commercial (KBS.9)).

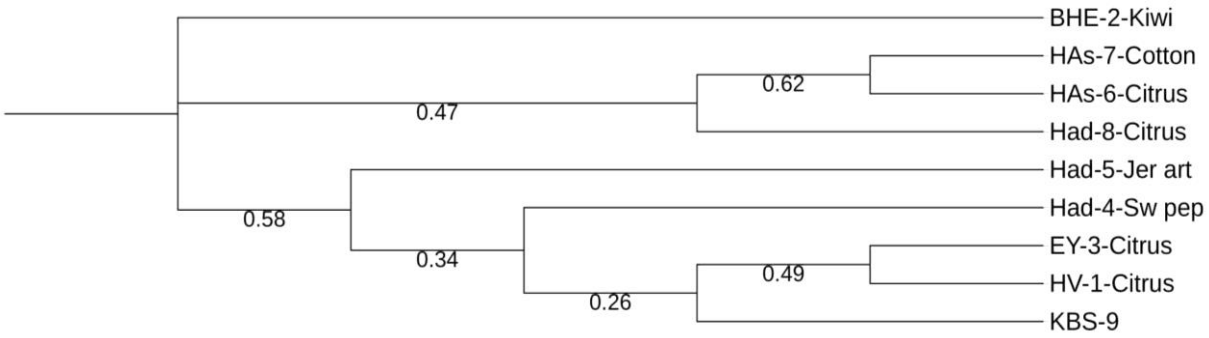


Figure 5.5. Neighbour-Joining clustering of six microsatellite markers with 1000 bootstrap using Nei´s D_A genetic distance matrix for nine populations of *A. swirskii* (eight wild, one commercial (KBS.9)).

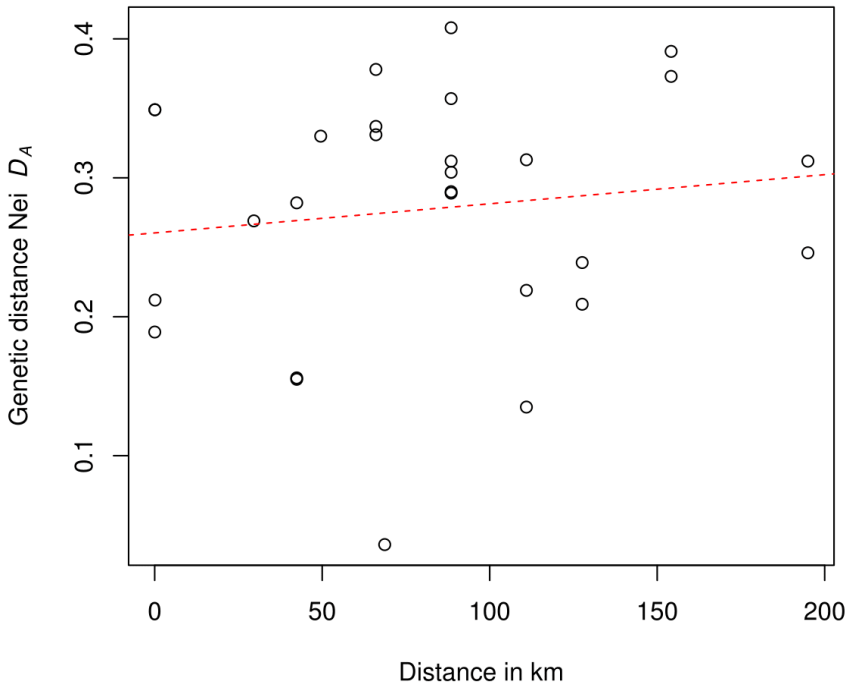


Figure 5.6. Isolation by distance using pairwise Nei´s DA genetic distances versus geographic distances between eight wild populations of *A. swirskii* collected in Israel. Dashed red line represents the linear regression fit (R2= 0.01653, P-value=0.514).

5.3.2 Biological parameters: predation, oviposition and dispersal

The predation rate of *E. khuniella* eggs by female *A. swirskii* mites on pepper leaf discs was estimated and found to be statistically lower for the commercial population KBS-9 (4.65 ± 0.22 SE eggs/female/day) compared to the general field population (5.82 ± 0.24 SE eggs/female/day) (model: Predation ~ mite, family = quasipoisson, F test, P<0.001) (Table 5.7). The oviposition rate of adult female mites on pepper leaf discs was estimated for the four first days of oviposition and it was found to be 2.05 (± 0.12 SE) eggs/female/day for the commercial population KBS-9 and 2.14 (± 0.09 SE) eggs/female/day for the field population respectively, which was not significantly different (model: Oviposition ~ mite, family = quasipoisson, F test, P>0.05) (Table 5.7). The dispersal rate of *A. swirski* on tomato stems was estimated for a commercial population and it was compared to the dispersal rate of field *A. swirskii* collected from five plant species (kiwi, cotton, sweet pepper, Jerusalem artichoke, citrus). The rate of dispersal to the following leaf of field collected mites ranged between 14% and 26%, and was similar to the dispersal rate of commercial *A. swirskii* (25%) (Figure 5.7).

Table 5.7. Mean predation and oviposition rate of *A. swirskii* field and commercial (KBS-9) female mites on sweet pepper leaves for five days, with sample size (N) and standard error (SE). The data for both parameters were fitted to a generalized linear model and an F-test was applied to the model variances.

	KBS-9			Field			F-test
Parameter	**N**	**Mean**	**SE**	**N**	**Mean**	**SE**	**Pr(>F)**
Predation	63	4.65	0.22	76	5.82	0.24	0.0005051
Oviposition	63	2.05	0.12	76	2.14	0.09	0.516

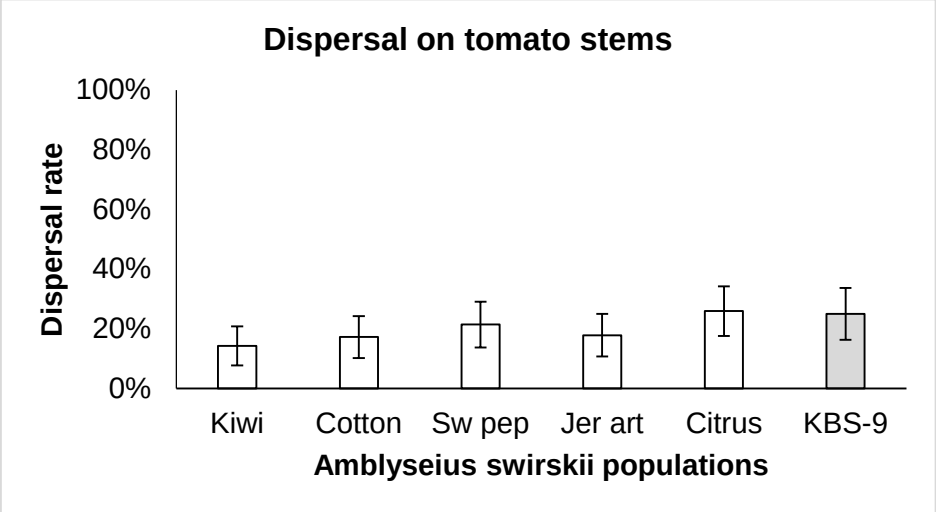


Figure 5.7. Mean dispersal ± SE of eight wild and one commercial (KBS.9, grey bar) populations of *A. swirskii* on tomato stems, 2 hours after release. Twenty female mites were used from each population to collect the data, which were fitted to a Generalized Linnear Model with binomial distribution.

5.4 Discussion

The objective of our study was to estimate and compare the genetic diversity in natural populations and a mass-reared, commercial population of the biocontrol agent *A. swirskii* by analyzing microsatellite markers. In absence of a published genome of *A. swirskii*, the identification of suitable markers for the genetic population inference required the *de novo* sequencing of the genome. However, next-generation sequencing approaches, such as Whole Genome Shotgun (WGS) sequencing need high DNA yield from a highly inbred population, which is intricate to obtain for this minute predatory mite because of its size. In this study, we selected the MinION sequencing platform because it allows small DNA input, and generates long sequence reads (Goodwin et al., 2015). Furthermore, the entire sequencing process, including library preparation can be done in-house and is cost-effective.

Despite the benefits of Nanopore sequencing in generating genomic sequences from the inbred *A. swirskii* strain, the 30,000 sequences (256 Mb) were not enough to generate a

genome assembly, because these sequences did not provide an adequate coverage of the whole genome. We therefore refrained from further assembling the reads into scaffolds, and used the raw reads as input for our microsatellite analysis. However, these reads were beneficial for mining and developing microsatellites for population genetic studies, and add to the low amount of publicly available sequence data for *A. swirskii*, and to the microsatellite panels developed for phytoseiids of economic importance (e.g. Sabater-Muñoz et al., 2012).

Microsatellite mining of the raw reads, primer design and filtering yielded 2,423 microsatellite markers and 622 candidate primer pairs for population genetic analysis. However, when 24 of those primers were tested for efficiency and specificity, only a small fraction (25%) complied with both criteria. One explanation for the low success rate of our microsatellites could be the high error-rate of the nanopore sequencing platform with 1/10 bases called wrongly (Laver et al., 2015) combined with insufficient read depth to properly correct for these errors. Another issue is the likely contamination of the sequencing starting material by the food source cattail (*Typha latifolia*) pollen. Contamination by the food source may have affected the outcome of the genome sequencing of *A. swirskii* providing sequence reads of the contaminant organism (the food source *Typha latifolia* in this case) as well, and has been outlined as a putative hazard for predatory mite studies before (Hoy et al. 2013). Predatory mites are tiny organisms whose gut occupies most of their total volume and it is very difficult to be dissected and excluded, as it is the common technique in larger arthropod species studies. Also, the detection window of prey DNA in the guts of arthropod predators (analyzed by PCR) can range from a few hours to various days post-feeding (King et al., 2008). Unfortunately, the genome of *Typha latifolia* is not known yet and its sequences cannot be filtered out from the consensus genomic sequences. Hence, in order to improve the methodology, the food source provided to the predatory mites should be a species with a

genome sequence available, as those sequences can be filtered out and removed from the sequencing reads. Furthermore, the coverage of the genome should be improved in further genome studies.

Individual genotyping of mites for population genetic inferences is a costly and elaborate method, mainly because of their tiny size and the low amount of DNA available for extraction, which limits the number of loci analysed per individual. However, DNA pooling for assessing relative differences of allele frequency among populations can overcome these limitations and provide a cost effective alternative (Skalski et al., 2006). Although effective number of peaks obtained from DNA pools are systematically overestimated compared to the actual number of alleles obtained from individual typing, those estimates can still detect relative differences in allele frequencies among DNA pools (Skalski et al., 2006). Microsatellite based genetic diversity estimates obtained from DNA pools were found to strongly correlate with those obtained from individually typing in previous studies in chickens and pigs (Hillel et al., 2003; Megens et al., 2008). In those studies, the effective number of peaks correlated strongly with the effective number of alleles, as did the respective heterozygosity estimates. Hence, the use of DNA pools provide reliable estimates for the population's biodiversity (Hillel et al., 2003; Skalski et al., 2006; Megens et al., 2008). Also, the three genetic distance measures D_A, D_{CS} and D_R for different evolutionary models correlate significantly between them, similarly to what was found in chicken and pig lines for pooled samples, suggesting that the downstream inference based on D_A is robust under alternative evolutionary scenarios as well (Hillel et al., 2003; Megens et al., 2008).

A small number of microsatellite loci (n=2,423) was identified in the genomic sequences of *A. swirskii*, similar to the low number of loci found in *Tetranychus urticae* (Koch) (Acari: Tetranychidae) genome (Grbić et al., 2011). However, the actual number of microsatellite

loci is expected to be higher, because the genome of *A. swirskii* was partially covered, yet our estimate is useful for the calculation of the microsatellite density on the genome. For two other mite species: the phytoseiid *Amblyseius fallacis* (Garman) and the tick *Ixodes scapularis* (Say) (Acari: Ixodidae), a low abundance of microsatellites has been proposed as well, following the generation of plasmid libraries enriched for microsatellites (Fagerberg et al., 2001; Navajas et al., 1998), although this method does not provide absolute estimates of microsatellite density to compare. In *A. swirskii*, dinucleotide repeats are the most abundant type of microsatellite, trinucleotides are less than half frequent and longer repeats, such as tetra-, penta- and hexanucleotides, are found markedly less, similar to most arthropod species (Pannebakker et al., 2010) and different from *T. urticae*, in which trinucleotides are the most abundant repeat motif (Grbić et al., 2011). It is unclear why mites have a low microsatellite density compared to other arthropod species (e.g. Abe & Pannebakker, 2017; Pannebakker et al., 2010), but this does not need to reflect an overall low genomic diversity. For instance, despite the low microsatellite abundance, Van Zee et al. (2013) found a very high SNP density in the genome of the tick *I. scapularis*, suggesting SNPs to be a more suitable marker for population genetic analysis. Nevertheless, we did observe high levels of polymorphism in the six microsatellite markers employed in *A. swirskii* in the current study, as reflected by the finding that the least polymorphic marker was polymorphic in 50% of the wild populations and six out of eight markers were polymorphic in all of the wild populations. Our finding is in accordance with the elevated molecular evolution found for the phytoseiid mite *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae) (Hoy et al., 2016).

High genetic variation is observed in natural populations of *A. swirskii*, with the mean observed heterozygosity ranging from H=0.34 to H=0.53, when compared to the commercial population (H=0.21), despite the fact that some natural populations were established by a low

number of individuals. Heterozygosity estimates based on pooled microsatellite analysis found in our study are comparable to the average observed heterozygosity found in a field population of the predatory mite *Neoseiulus womersleyi* (Schicha) (Acari: Phytoseiidae) (Hinomoto et al., 2011). Considerable genetic variation was found also for tetranychid mites; *Tetranychus urticae* field populations from Europe had a spatial genetic structure along a latitudinal gradient (Carbonnelle et al., 2007). *Tetranychus turkestani* (Ugarov & Nikolskii) collected on crops and weeds in southern France had comparable average heterozygosity and mites living on different host plants did not demonstrate clear evidence for genetic differentiation, similar to our results (Bailly et al., 2004). Our results did not show a correlation between host plant and genetic diversity in the wild populations; however, the BHE-2 population collected on kiwi plants did show a lower genetic diversity, which might be due to the presence of high trichome density on this plant.

Natural populations are often characterized by higher levels of genetic variation compared to captive-bred organisms because genetic diversity is crucial for their survival under fluctuating environmental conditions and diverse ecosystems (Barrett & Schluter, 2008). For example, population genetic analysis of chicken using microsatellites showed that wild populations of the Red Jungle Fowl (*Gallus gallus gallus*) had three times higher average heterozygote frequencies than a European, domesticated chicken population (Granevitze et al., 2007). Commercial *A. swirskii* were polymorphic only in 50 % of the microsatellite markers and had lower average allelic richness and lower heterozygosity estimates compared to their wild counterparts, indicating a very low genetic diversity. Low genetic variation of a commercial biocontrol agent may affect their resilience when unexpected and even minor changes or fluctuations of the environmental conditions take place (Lommen et al., 2017; Wright & Bennett, 2018). For instance, in *Drosophila melanogaster* it has been demonstrated

that for higher inbreeding levels, the impact of environmental stress becomes significantly greater and can even lead to extinction (Bijlsma et al., 2000).

The decline in the genetic variation in the mass-reared population of this biocontrol agent may affect traits that are important in their performance as biological control agents, such as reproductive parameters, prey preference, or predation rate. Our laboratory colonies of wild *A. swirskii* populations were founded by low numbers of mites in some cases (8-100); however, still in all cases their genetic diversity was higher than in the commercial strain. Despite the very high numbers at which commercial *A. swirskii* populations are reared, genetic diversity can be low when the initial founding population is low (Mackauer, 1976; Bartlett, 2018). Furthermore, even when a large founding population was used, it may have included a few genotypes that are favoured under mass-rearing conditions, which can result in a bottleneck that reduces the genetic diversity (Nunney, 2003). As the field environment is different from the mass-rearing conditions, adaptation to the rearing environment can have a negative impact on the fitness of mass-reared biocontrol agents in the field (Sørensen et al., 2012). In this study, it was demonstrated that commercially reared *A. swirskii* has lower predation rate compared to a natural population, but oviposition and dispersal rates were similar between populations. Genetic change in mass-reared populations has been described in many arthropods (Nunney, 2003), including experimental populations of *Drosophila melanogaster* (Woodworth et al., 2002), but also in parasitoids reared for biological control; (Guzmán-Larralde et al., 2014; Retamal et al., 2016; Bertin et al., 2017) and various insects reared for Sterile Insect Technique programmes since the 80's (Rössler, 1975; Bush & Neck, 1976; Wong & Nakahara, 1978; Vargas & Carey, 1989). When egg parasitoids *Trichogramma galloi* (Zucchi) were reared on factitious hosts, fecundity and emergence were reduced on the target pest, compared to parasitoids reared on natural hosts (Bertin et al.,

2017). In another case, inbred populations of the mass-reared parasitoid *Trichogramma pretiosum* (Riley) produced fewer offspring than genetically variable populations (Guzmán-Larralde et al., 2014). Also, the entomopathogenic nematode *Steinernema glaseri* (Steiner) had reduced reproduction parameters and virulence, after laboratory adaptation took place (Stuart & Gaugler, 1996).

To further improve the predatory mite *A. swirskii* for augmentative biological control and prevent the decrease in traits related to its fitness, we would suggest enhancing the low genetic diversity present in the mass reared commercial *A. swirskii* by adding genetic variation from natural populations (Nunney, 2003). However, a better understanding of the levels of genetic variation in mass-reared *A. swirskii* populations should be obtained first, before new genetic material is added to mass-rearing. The present study only tested a single commercial population limiting the scope of the conclusions. Additional genetic analysis of commercial populations can further assess the impact of genetic diversity on the performance of *A. swirskii* as a biocontrol agent, which remains one of the most successful natural enemies in augmentative biocontrol of whiteflies and thrips in greenhouse vegetables, fruits and ornamentals in several parts of the world (Calvo et al., 2015; Knapp et al., 2018).

Augmentative biological control of agricultural pests is the environmentally sustainable alternative to synthetic pesticides for safe food production and healthy ecosystems. We found that the genetic diversity of a widely used commercial biocontrol agent is limited compared to the natural conspecifics and this could have implications when the biocontrol agents need to adapt to adverse or novel conditions such as when being released to the field or greenhouse. Also, we showed that pooled microsatellite analysis provides a cost-effective method to determine the genetic diversity of minute biocontrol agents. Hence, adding to our

knowledge approaches to determine the existing genetic variance of commercial biocontrol agents is imperative for the long-term success of IPM strategies and control programmes.

Chapter 6

Discussion and Conclusions

6.1 Discussion

The consumers demand for safe, pesticide free vegetables and the reduction of authorized pesticides products in the EU, have resulted in a shift from insecticide use to the implementation of IPM programs, based on the use of biocontrol agents (Lenteren et al., 2018). The implementation of IPM has become a priority for the EU, since it is a scientifically-based approach to pest control, with a strong drive to protect the environment from the negative impacts of conventional pesticides (Abrol 2013). Biocontrol agents are major components of IPM and contribute effectively to pest control, sometimes without a single pesticide input throughout the season (Abrol, 2013). On tomato crops, the most important biocontrol agents used in IPM strategies are the mirids, *N. tenuis* and *M. pygmaeus* (Velden *et al.* 2012; Pérez-Hedo *et al.* 2017). These mirids are highly polyphagous predators of various pests (whiteflies, thrips, leafminers, Lepidoptera species, aphids, spider mites); however, they are not effective against the tomato rust mite *A. lycopersici*, which is a key pest of tomato crops. Natural enemies of herbivorous mites, such as the predatory mite *A. swirskii*, cannot become established on tomato crops.

Predatory mite populations are not established on plants with high pubescence, because plant trichomes can affect them negatively (Riddick & Simmons, 2014). Tomato trichomes provide excellent shelter for tomato russet mites and are obstacles for its natural enemies. Glandular trichomes present on the stems of *S. lycopersicum* entrap and kill the predatory mite *P. persimilis*, and higher entrapment rates are associated with high trichome densities (van Haren *et al*. 1987). Indeed, it has previously demonstrated that tomato cultivars with increased trichome density have direct negative effects on the survival of *N. californicus* (McGregor) (Acari: Phytoseiidae) (Cédola & Sánchez, 2003) and that tomato trichomes hampered searching efficiency, prey consumption and oviposition rate of the phytoseiids *P.*

macropilis and *P. longipes*. In **chapter 2** it was demonstrated that **there is a detrimental effect of tomato plants on *A. swirskii* survival, during the phytoseiid dispersal through the tomato stems**, where the glandular trichome density is higher than on the leaves. Other life history traits of the predatory mites were not found to be significantly affected on tomato plants. Since juvenile phytoseiids usually stay at the natal patch until molting is completed, it was shown that dispersing adult mites are predominantly affected by tomato trichomes.

In **chapter 2** of this study it was also demonstrated that **the tomato trichome secondary metabolites most likely involved in mite mortality are the acyl sugars**, since they were identified in high concentrations in the most toxic for the predatory mites trichome extract. A high concentration of acyl sugars in the glandular trichomes type VI was demonstrated, confirming the finding of Schilmiller et al. (2010), that they are the most abundant secondary metabolite of the tomato trichomes. When the predators walked on the stained tomato trichomes, the acyl sugars were released, and accumulated on their cuticle and mostly on their mouth parts and limb joints. This study is the first to demonstrate **the toxic impact of the acyl sugars on phytoseiids and morevover, show their attachment and allocation on the body of the mites**. High toxicity of acyl sugars of *Nicotiana gossei* to pear psylla adults and nymphs has been observed as well (Puterka & Severson, 1995). Similarly, the staining of the tobacco acyl sugars has shown their accumulation on the body of aphids, after walking on the plant surface (Wagner et al., 2004). The accumulation of the acyl sugars on the arthropod joints and mouthparts, may allow direct contact with the cellular membranes under the cuticle, which could lead to membrane disruption by the fatty acids of the acyl sugars, causing the mite desiccation, or otherwise, they may block the respiratory pores such as the stigmata and cause asphyxiation of the mite, according to Puterka et al. (2003).

Trichome-based plant resistance can be incompatible with phytoseiid biocontrol agents and can interfere with the biological control of the pests, because the performance of phytoseiids is hindered by the tomato trichomes and their exudates. Tomato pests, on the other hand, have adapted to the defenses mediated by the trichomes and their exudates and so, can reproduce on a predator-free environment. For example, the predatory mite *P. longipes* cannot control *T. urticae* and *T. evansi* populations at a low predator/prey ratio on tomato plants (Ferrero et al., 2011) and the *N. californicus* functional response is much lower on tomato plants compared to other host plants (Cédola et al., 2001). In **chapter 3**, the results suggest that **the distorted trichome phenotype, called hairless, does not favor the predatory mite establishment and performance** in pest control. Both ***A. swirskii* and *P. persimilis,* are not able to suppress the pest on the hairless plants, similar to the wild-type tomatoes**. Furthermore, **whiteflies and spider mites perform better on the hairless tomato plants, than on wild-type plants**. Similarly, other studies have observed improved performance of the herbivorous *M. sexta* and *H. zea* on hairless tomato plants (Kang et al., 2010; Tian et al., 2012). The hairless mutation affects the trichome secondary metabolite composition and the hairless plants lack the sesquiterpene and polyphenolic compounds (Kang et al., 2010), implicated in resistance against herbivorous insects and mites (Duffey & Isman, 1981; Eigenbrode et al., 1994; Antonious & Snyder, 2006). The increased performance of whiteflies on tomato plants with distorted trichomes may be the result of reduced contact with the glandular trichomes, which influence negatively their oviposition and positively the rate of entrapment (Oriani & Vendramim, 2010). Currently, there are no tomato cultivars available where phytoseiids can be used to control pest infestation. Tomato plants with minimal levels of acyl sugars could be used in future studies to understand the effect of those secondary metabolites on the plant fitness, in the presence of biological control.

Herbivorous arthropods have adapted to plant secondary metabolites, however, this might not be the case for their natural enemies. When the herbivorous *T. urticae* was transferred to tomato a large number of genes, including detoxification enzymes, were differentially expressed, as shown by Dermauw et al. (2013). The fitness of *T. urticae* increased rapidly in just a few generations on tomato (Dermauw et al. 2013; Wybouw et al. 2015); however, the fitness of the phytoseiid mite, *N. californicus* did not increase over many generations when reared on tomato (Cédola et al. 2001). In **chapter 4**, **transcriptional gene responses of *A. swirskii* to tomato plants were demonstrated; a small geneset, that did not include known detoxification genes, was differentially expressed in *A. swirskii* transferred on tomato**, very different from what was found for *T. urticae* (Dermauw et al. 2013).

The evolutionary adaptation of several herbivores to host plant toxins and the development of resistance to pesticides have been frequently associated with the expansions of families of enzymes that carry out the detoxification of xenobiotic compounds, such as secondary plant metabolites and pesticides (Yang et al. 2011; Dermauw et al. 2013; Niu et al. 2012). Enzymes involved in the detoxification of plant allelochemicals and pesticides often belong to the CYP, GST and CCE gene superfamilies (Scott et al. 1998; Liang et al. 2007; Schuler 2011; Dermauw et al. 2013; Zhang et al. 2013; Pavlidi et al. 2015). This study provided insights into **the potential gene components of the detoxification superfamilies CYP, GST and CCE of *A. swirskii* and showed that they are comparable in numbers to other phytoseiids, however, less genes are present when compared to herbivorous mites**. The results of the current study support the notion that the detoxification system of predatory arthropods maybe less evolved than the system of their prey, hence they could be more affected by plant allelochemicals or pesticides.

Another fundamental aspect in the performance of a natural enemy is its ability to adapt to different crops and environmental conditions, which is modulated by its standing genetic diversity. Genetic diversity is crucial for the survival of organisms under fluctuating environmental conditions and diverse ecosystems (Barrett & Schluter, 2008). Usually, captive-bred organisms are characterized by lower levels of genetic variation compared to natural populations (Barrett & Schluter, 2008). In the case of *A. swirskii*, the commercial populations that are currently being released come from the same population that has been mass-rearing for more than 15 years and the genetic variation present of the mass-reared population is not known. Despite the very high numbers at which commercial populations are reared, genetic diversity can be low, if the initial founding population is low (Mackauer, 1976; Bartlett, 1993). Furthermore, even if a large founding population is used, it may have included a few genotypes that are favoured under mass-rearing conditions, which can result in a bottleneck that reduces the genetic diversity (Nunney, 2003). As the mass-rearing conditions are different from the field environment, adaptation to the rearing environment can have a negative impact on the fitness of mass-reared biocontrol agents in the field (Sørensen et al., 2012). When environmental conditions change, even to a small degree, low genetic variation of an arthropod may affect their resilience (Wright and Bennett 2018). For instance, in *D. melanogaster* it has been demonstrated that for higher inbreeding levels, the impact of environmental stress becomes significantly greater and can even lead to extinction (Bijlsma et al., 2000). Genetic change in mass-reared arthropod populations used in biological control strategies has been described in various insects reared for 'sterile insect technique' programmes (Rössler, 1975; Bush & Neck, 1976; Wong & Nakahara, 1978; Vargas & Carey, 1989) and also, in parasitoids used as biocontrol agents (Guzmán-Larralde et al., 2014; Retamal et al., 2016; Bertin et al., 2017). **In chapter 5, it was demonstrated that the genetic diversity of commercially reared *A. swirskii* is limited compared to the natural**

conspecifics and this could have implications, when the biocontrol agents need to adapt to adverse or novel conditions, such as when being released to the field or greenhouse.

The decline in the genetic variation in mass-reared populations of biocontrol agents may affect traits that are important in their performance, such as reproductive parameters, prey preference, or predation rate. For example, there was a reduction in the fecundity and emergence of the parasitoid *T. galloi* on the natural host, when the parasitoids were maintained on factitious compared to natural hosts (Bertin et al., 2017). Also, lower fecundity was observed in inbred populations of the mass-reared parasitoid *T. pretiosum* than in genetically variable populations (Guzmán-Larralde et al., 2014). In another case, laboratory adaptation led to reduced reproduction and virulence of the populations of the entomopathogenic nematode *S. glaseri* (Stuart & Gaugler, 1996). In this study, **it was demonstrated that commercially reared *A. swirskii* has lower predation rate compared to a natural population,** indicating an effect of long-term rearing in the performance of biocontrol agents.

Despite its lack of adaptation in tomato, it is unquestionably that the predatory mite *A. swirskii* is one of the most successful natural enemies in augmentative biocontrol in greenhouse vegetables, fruits, and ornamentals in several parts of the world and key player in IPM strategies in these crops (Calvo et al., 2015; Knapp et al., 2018). Here, **the *de novo* transcriptome of *A. swirskii,* a species with very limited public genomic information, was generated**, as a significant genomic resource, now available to the scientific community, which will facilitate research on adaptive evolution of predatory mites to plant toxins. Moreover, **new microsatellite markers were developed for population genetic analysis**, adding to the microsatellite panels available for phytoseiids of economic importance (e.g., Sabater-Muñoz et al., 2012). Overall, our data will serve as invaluable public data for future

population genetic studies, essential for preventing the decrease in traits related to the fitness of the commercial strains for augmentative biological control and improving their resilience (Nunney, 2003). Also, **the current study showed that pooled microsatellite analysis provides a cost-effective method** to determine the genetic diversity of minute biocontrol agents. Hence, adding to our knowledge approaches to determine the existing genetic variance of commercial biocontrol agents is imperative for the long-term success of IPM strategies and control programmes.

6.2 Conclusions

According to the results presented in this PhD thesis, it can be concluded that the tomato acyl sugars produced by the trichomes type VI are detrimental for the phytoseiid *A. swirskii,* independently of the trichome morphology. Also, the current study concluded that the rearing conditions of a commercial biocontrol agent lead to decline of their genetic variation and affect their performance. The information generated in this regard can serve as the basis for future research in plant-breeding programs, which aim to the successful application of natural enemies for crop protection. Overall, the results of this thesis support the following conclusions:

i) Predatory mite mortality on the tomato plants is caused by the acyl sugars, produced by trichomes type VI.

ii) Distortion of the tomato trichomes does not favor phytoseiid performance, but favors the performance of pests.

iii) The detoxification system of phytoseiid mites is not effective against the tomato allelochemicals.

iv) Long-term commercial rearing of biocontrol agents can reduce their genetic diversity and

affect their performance.

Conclusiones

De acuerdo con los resultados presentados en esta tesis doctoral, se puede concluir que los acil azúcares de tomate producidos por los tricomas tipo VI son perjudiciales para el fitoseido *A. swirskii*, independientemente de la morfología del tallo del tricoma. Además, el estudio actual concluyó que las condiciones de crianza de un agente de control biológico comercial causan la disminución de su variación genética y afectan su rendimiento. La información generada a este respecto puede servir como base para futuras investigaciones en programas de fitomejoramiento, cuyo objetivo es la aplicación exitosa de enemigos naturales para la protección de cultivos. En general, los resultados de esta tesis respaldan las siguientes conclusiones:

i) La mortalidad de ácaros depredadores en las plantas de tomate es causada por los acil azúcares, producidos por tricomas tipo VI.

ii) La distorsión de los tricomas del tomate no favorece el rendimiento de los fitoseidos, pero favorece el rendimiento de las plagas.

iii) El sistema de desintoxicación de los ácaros fitoseidos no es efectivo contra los aleloquímicos del tomate.

iv) A largo plazo, la cría comercial de agentes de biocontrol puede reducir su diversidad genética y afectar su rendimiento.

Appendix

Table S1. Classification of the CYP proteins of *A. swirskii.* used in the phylogenetic analysis. Columns are: Protein – assigned protein name; Accession No – the Accession number in SRA; Class – classification based on the results of the phylogenetic analysis shown in Figure 4.12; NR Description, Hit Name, Organism, % Identity; and E-value – the results from the blastp against the NR database.

Protein	Accession No Cluster	Class	NR Description	Hit Name	Organism	% Identity	E Value
AS_CYP_01	12473	4	cytochrome P450 4c3	XP_003744153	Metaseiulus occidentalis	72.40 %	0
AS_CYP_02	14096.329	4	cytochrome P450 4c3	XP_003744153	Metaseiulus occidentalis	72.40 %	0
AS_CYP_03	14096.47136	4	cytochrome P450 4c3	XP_003744153	Metaseiulus occidentalis	74.20 %	0
AS_CYP_04	14096.47566	4	cytochrome P450 4c3	XP_003744153	Metaseiulus occidentalis	73.90 %	0
AS_CYP_05	14096.14832	4	cytochrome P450 4c3	XP_003744153	Metaseiulus occidentalis	76.90 %	0
AS_CYP_06	14096.38673	4	uncharacterized protein	XP_018497071	Metaseiulus occidentalis	85.90 %	0
AS_CYP_07	14096.39632	4	uncharacterized protein	XP_018497071	Metaseiulus occidentalis	83.90 %	1.50E-95
AS_CYP_08	14096.42635	4	cytochrome P450 4c3	XP_003741915	Metaseiulus occidentalis	79.00 %	9.30E-142
AS_CYP_09	14096.46827	4	cytochrome P450 4c3	XP_003741915	Metaseiulus occidentalis	77.80 %	0
AS_CYP_10	14096.29671	4	cytochrome P450 4C1	XP_003740735	Metaseiulus occidentalis	94.10 %	0
AS_CYP_11	14096.32881	4	cytochrome P450 4C1	XP_003740735	Metaseiulus occidentalis	92.20 %	2.39E-68
AS_CYP_12	14096.23635	4	cytochrome P450 4V2-like	XP_003746392	Metaseiulus occidentalis	79.00 %	0
AS_CYP_13	14096.32675	4	cytochrome P450 4V2-like	XP_022657330	Varroa destructor	56.60 %	8.54E-131
AS_CYP_14	14096.24998	4	cytochrome P450 4V2-like	OQR78459	Tropilaelaps mercedesae	45.70 %	1.37E-164
AS_CYP_15	14096.16794	4	cytochrome P450 4V2-like	OQR73969	Tropilaelaps mercedesae	70.10 %	0
AS_CYP_16	14096.17167	4	cytochrome P450 4V2-like	XP_003744207	Metaseiulus occidentalis	67.20 %	0
AS_CYP_17	14096.14981	4	cytochrome P450 4V2	XP_018493906	Metaseiulus occidentalis	88.20 %	0
AS_CYP_18	14096.23655	4	cytochrome P450 4V2	XP_018493906	Metaseiulus occidentalis	67.30 %	0
AS_CYP_19	14096.35067	4	cytochrome P450 4V2-like	XP_018495014	Metaseiulus occidentalis	74.90 %	0
AS_CYP_20	14096.39974	4	uncharacterized protein	XP_003747976	Metaseiulus occidentalis	72.30 %	0
AS_CYP_21	14096.28681	3	cytochrome P450 3A28	XP_003740908	Metaseiulus occidentalis	74.80 %	8.76E-137
AS_CYP_22	14096.14914	3	cytochrome P450 3A31	XP_003744549	Metaseiulus occidentalis	80.90 %	0
AS_CYP_23	14096.11337	3	cytochrome P450 3A31	XP_003744549	Metaseiulus occidentalis	62.20 %	7.04E-154
AS_CYP_24	14096.22859	3	cytochrome P450 3A6	XP_018497017	Metaseiulus occidentalis	68.60 %	0
AS_CYP_25	14096.12758	3	cytochrome P450 3A6-like	XP_003745699	Metaseiulus occidentalis	65.10 %	0
AS_CYP_26	14096.7201	3	cytochrome P450 3A6-like	XP_003745699	Metaseiulus occidentalis	55.10 %	6.81E-130
AS_CYP_27	14096.45017	3	cytochrome P450 3A56	XP_003744550	Metaseiulus occidentalis	54.80 %	0

AS_CYP_28	14096.38234	3	cytochrome P450 3A4	XP_003743686	Metaseiulus occidentalis	78.00 %	0
AS_CYP_29	14096.183	3	cytochrome P450 3A24	XP_003741765	Metaseiulus occidentalis	82.30 %	0
AS_CYP_30	14096.23029	3	cytochrome P450 3A24	XP_003741765	Metaseiulus occidentalis	85.70 %	4.13E-137
AS_CYP_31	14096.14727	3	cytochrome P450 3A24	XP_003741765	Metaseiulus occidentalis	83.80 %	3.41E-138
AS_CYP_32	14096.37721	3	cytochrome P450 3A31-like	XP_003744236	Metaseiulus occidentalis	72.50 %	0
AS_CYP_33	14096.1855	3	cytochrome P450 3A21	XP_003741251	Metaseiulus occidentalis	81.90 %	2.52E-127
AS_CYP_34	14096.2221	3	cytochrome P450 3A21	XP_003741251	Metaseiulus occidentalis	83.50 %	1.85E-112
AS_CYP_35	14096.1389	3	cytochrome P450 3A16-like	OQR73197	Tropilaelaps mercedesae	46.70 %	5.16E-71
AS_CYP_36	14096.38954	3	cytochrome P450 3A16-like	OQR73197	Tropilaelaps mercedesae	50.20 %	0
AS_CYP_37	14096.18325	3	cytochrome P450 3A16	XP_003744817	Metaseiulus occidentalis	51.30 %	1.42E-30
AS_CYP_38	14096.37108	3	cytochrome P450 3A16	XP_003744817	Metaseiulus occidentalis	51.20 %	2.13E-93
AS_CYP_39	14096.8059	3	cytochrome P450 3A16	XP_003744817	Metaseiulus occidentalis	62.70 %	0
AS_CYP_40	14096.17663	3	cytochrome P450 3A9	XP_003739531	Metaseiulus occidentalis	67.90 %	0
AS_CYP_41	14096.39315	3	cytochrome P450 3A24-like	XP_003746429	Metaseiulus occidentalis	70.60 %	0
AS_CYP_42	14096.34995	3	cytochrome P450 3A24-like	XP_003746429	Metaseiulus occidentalis	65.30 %	0
AS_CYP_43	14096.16575	3	cytochrome P450 3A24-like	XP_003746429	Metaseiulus occidentalis	63.20 %	3.89E-165
AS_CYP_44	14096.40867	3	probable cytochrome P450 6d5	XP_003743386	Metaseiulus occidentalis	78.90 %	0
AS_CYP_45	14096.18025	3	cytochrome P450 3A29-like	XP_003737485	Metaseiulus occidentalis	72.50 %	0
AS_CYP_46	14096.23742	3	cytochrome P450 3A29-like	XP_003737485	Metaseiulus occidentalis	74.40 %	0
AS_CYP_47	14096.22491	3	cytochrome P450 3A29-like	XP_003737485	Metaseiulus occidentalis	64.80 %	0
AS_CYP_48	14096.41971	3	cytochrome P450 3A29-like	XP_003737485	Metaseiulus occidentalis	69.00 %	0
AS_CYP_49	14096.19522	3	cytochrome P450 3A9-like	XP_003738548	Metaseiulus occidentalis	59.40 %	0
AS_CYP_50	14096.23153	3	cytochrome P450 3A9-like	XP_003738548	Metaseiulus occidentalis	58.40 %	3.46E-167
AS_CYP_51	14096.16757	3	probable cytochrome P450 6a13	XP_014094156	Bactrocera oleae	36.70 %	6.86E-17
AS_CYP_52	14096.111	M	cytochrome P450 302a1, mitochondrial-like	XP_003745070	Metaseiulus occidentalis	75.50 %	0
AS_CYP_53	14096.16995	M	probable cytochrome P450 49a1	XP_003741296	Metaseiulus occidentalis	92.10 %	0
AS_CYP_54	14096.41654	2	cytochrome P450 2J6-like	XP_018494714	Metaseiulus occidentalis	66.90 %	0
AS_CYP_55	14096.36233	2	cytochrome P450 2J6-like	XP_018494714	Metaseiulus occidentalis	66.10 %	0
AS_CYP_56	14096.22426	2	cytochrome P450 2J6-like	XP_018494714	Metaseiulus occidentalis	64.60 %	0
AS_CYP_57	14096.29831	2	cytochrome P450 2C23	XP_003747006	Metaseiulus occidentalis	76.30 %	0
AS_CYP_58	14096.2349	2	cytochrome P450 2J6	XP_018495238	Metaseiulus occidentalis	91.40 %	0
AS_CYP_59	14096.15944	2	uncharacterized protein	XP_018494895	Metaseiulus occidentalis	57.70 %	0
AS_CYP_60	14096.34069	2	uncharacterized protein	XP_018494895	Metaseiulus occidentalis	59.70 %	0
AS_CYP_61	14096.20597	2	uncharacterized protein	XP_018494895	Metaseiulus occidentalis	69.70 %	2.20E-53
AS_CYP_62	14096.33973	2	uncharacterized protein	XP_018494895	Metaseiulus occidentalis	73.70 %	1.47E-179
AS_CYP_63	14096.12347	2	uncharacterized protein	XP_018494895	Metaseiulus occidentalis	63.20 %	1.34E-158
AS_CYP_64	14096.19055	2	cytochrome P450 1A1	XP_003745229	Metaseiulus occidentalis	80.90 %	4.05E-165

AS_CYP_65	14096.19304	2	cytochrome P450 1A1	XP_003745229	Metaseiulus occidentalis	81.70 %	0
AS_CYP_66	14096.31074	2	cytochrome P450 1A2	XP_003741017	Metaseiulus occidentalis	64.80 %	5.33E-116
AS_CYP_67	14096.24315	2	cytochrome P450 2F3-like, partial	XP_003738046	Metaseiulus occidentalis	53.20 %	4.79E-162
AS_CYP_68	14096.42315	2	cytochrome P450 2J6-like	XP_003748194	Metaseiulus occidentalis	54.10 %	0
AS_CYP_69	14096.16396	2	cytochrome P450 2F3-like, partial	XP_003738046	Metaseiulus occidentalis	56.50 %	0
AS_CYP_70	14096.44766	2	cytochrome P450 2F3-like, partial	XP_003738046	Metaseiulus occidentalis	57.80 %	0
AS_CYP_71	14096.16315	2	cytochrome P450 2F3-like, partial	XP_003738046	Metaseiulus occidentalis	52.50 %	1.38E-180
AS_CYP_72	14096.23357	2	cytochrome P450 71B22-like	XP_003748195	Metaseiulus occidentalis	70.00 %	0
AS_CYP_73	14096.20063	2	cytochrome P450 2F3-like	XP_018493962	Metaseiulus occidentalis	54.90 %	0
AS_CYP_74	14096.25695	2	cytochrome P450 2F3-like	XP_018493962	Metaseiulus occidentalis	59.10 %	0
AS_CYP_75	14096.16263	2	cytochrome P450 307a1-like	XP_003747195	Metaseiulus occidentalis	83.00 %	0
AS_CYP_76	14096.20839	2	cytochrome P450 307a1-like	XP_003747195	Metaseiulus occidentalis	72.70 %	0

Table S2. Classification of the GST proteins of *A. swirskii.* used in the phylogenetic analysis. Columns are: Protein – assigned protein name; Accession No – the Accession number in SRA; Class – classification based on the results of the phylogenetic analysis shown in Figure 4.13; NR Description, Hit Name, Organism, % Identity; and E-value – the results from the blastp against the NR database.

Protein	Accession No Cluster	Class	NR Description	Hit Name	Organism	% Identity	E Value
AS_GST01	14096.28962	omega	pyrimidodiazepine synthase	XP_018496135	Metaseiulus occidentalis	82.30%	6.95E-140
AS_GST02	14096.4445	omega	glutathione S-transferase omega-1	XP_003738156	Metaseiulus occidentalis	79.30%	1.08E-90
AS_GST03	14096.27508	kappa	glutathione S-transferase kappa 1	XP_003738807	Metaseiulus occidentalis	83.60%	1.50E-139
AS_GST04	14096.16668	mu	glutathione S-transferase Mu 1-like	OQR71874	Tropilaelaps mercedesae	62.70%	2.64E-50
AS_GST05	14096.2226	delta	glutathione S-transferase C-terminal domain-containing	XP_003737347	Metaseiulus occidentalis	69.70%	0
AS_GST06	14096.35417	kappa	glutathione S-transferase kappa 1	XP_003738778	Metaseiulus occidentalis	88.80%	2.16E-151
AS_GST07	14096.51772	uncharacterized	glutathione S-transferase 4-like	XP_022669924	Varroa destructor	90.50%	3.29E-125
AS_GST08	14096.2419	uncharacterized	glutathione S-transferase 4-like	XP_022669924	Varroa destructor	81.80%	7.80E-121
AS_GST09	14096.27038	mu	glutathione S-transferase class-mu 26 kDa isozyme 47	XP_003737612	Metaseiulus occidentalis	79.10%	5.60E-60
AS_GSTd01	14096.4354	delta	glutathione S-transferase	EFX81633	Daphnia pulex	58.60%	9.09E-69
AS_GSTd02	14096.51347	delta	glutathione S-transferase 1-1	XP_003740940	Metaseiulus occidentalis	89.50%	1.12E-137
AS_GSTd03	14096.1759	delta	glutathione S-transferase 1	XP_003743487	Metaseiulus occidentalis	64.60%	2.29E-55
AS_GSTd04	14096.18035	delta	glutathione S-transferase 1	XP_003743487	Metaseiulus occidentalis	68.30%	2.27E-103
AS_GSTd05	14096.36333	delta	glutathione S-transferase 1	XP_003743487	Metaseiulus occidentalis	84.10%	2.15E-102
AS_GSTd06	14096.17204	delta	glutathione S-transferase 1	XP_003743487	Metaseiulus occidentalis	87.00%	5.59E-136
AS_GSTd07	14096.25844	delta	glutathione S-transferase 1	XP_003746787	Metaseiulus occidentalis	62.90%	3.76E-78
AS_GSTd08	14096.45023	delta	glutathione S-transferase 1	XP_003746787	Metaseiulus occidentalis	65.70%	2.21E-105
AS_GSTd09	14096.25851	delta	glutathione S-transferase 1	XP_003746787	Metaseiulus occidentalis	84.20%	5.11E-138
AS_GSTd10	14096.32345	delta	glutathione S-transferase 1-like isoform X1	XP_022705046	Varroa jacobsoni	68.40%	9.71E-108
AS_GSTd11	14096.33358	delta	glutathione S-transferase 1	XP_003746787	Metaseiulus occidentalis	78.70%	4.24E-107
AS_GSTm01	14096.8488	mu	hypothetical protein PENSTE	OQE20378	Penicillium steckii	90.50%	9.64E-133
AS_GSTm02	14096.19298	mu	glutathione S-transferase Mu 1	XP_003742682	Metaseiulus occidentalis	68.80%	1.08E-111
AS_GSTm03	14096.27548	mu	glutathione S-transferase Mu 1	XP_003742682	Metaseiulus occidentalis	89.20%	3.63E-146
AS_GSTm04	14096.25965	mu	glutathione S-transferase Mu 1	XP_018495947	Metaseiulus occidentalis	89.10%	4.05E-159
AS_GSTm05	14096.24826	mu	glutathione S-transferases-1	AKO22175	Dermanyssus gallinae	57.40%	1.07E-96
AS_GSTo01	14096.47912	omega	glutathione S-transferase omega-1	XP_003738156	Metaseiulus occidentalis	82.50%	2.77E-146
AS_GSTo02	14096.46561	omega	glutathione S-transferase omega-1	XP_003738156	Metaseiulus occidentalis	80.80%	9.58E-56
AS_GSTo03	14096.2114	omega	glutathione S-transferase omega-1	XP_003746278	Metaseiulus occidentalis	77.70%	1.64E-128

Table S3. Classification of the CCE proteins of *A. swirskii.* used in the phylogenetic analysis. Columns are: Protein – assigned protein name; Accession No – the Accession number in SRA; Class – classification based on the results of the phylogenetic analysis shown in Figure 4.14; NR Description, Hit Name, Organism, % Identity; and E-value – the results from the blastp against the NR database.

Protein	Accession No Cluster	class	NR Description	Hit Name	Organism	% Identity	E Value
AS_CCE_01	14096.17155	J′′	cholinesterase 1-like	XP_003744419	Metaseiulus occidentalis	72.90%	0
AS_CCE_02	14096.11228	J′′	cholinesterase 1-like	XP_003744359	Metaseiulus occidentalis	71.50%	2.98E-154
AS_CCE_03	14096.18193	J′′	liver carboxylesterase 1-like	OQR66681	Tropilaelaps mercedesae	53.10%	4.09E-168
AS_CCE_04	14096.38789	J′′	acetylcholinesterase-like	XP_003747077	Metaseiulus occidentalis	68.30%	0
AS_CCE_05	14096.7546	J′′	cholinesterase-like	XP_003740670	Metaseiulus occidentalis	42.00%	8.67E-45
AS_CCE_06	14096.854	J′′	acetylcholinesterase-like	XP_003745536	Metaseiulus occidentalis	52.30%	1.31E-102
AS_CCE_07	14096.26752	J′′	acetylcholinesterase-like	XP_003745536	Metaseiulus occidentalis	59.40%	4.18E-141
AS_CCE_08	14096.22721	J′′	cholinesterase 1-like	XP_003740671	Metaseiulus occidentalis	69.80%	0
AS_CCE_09	14096.48773	J′′	cholinesterase 1-like	XP_003740671	Metaseiulus occidentalis	56.70%	5.96E-113
AS_CCE_10	14096.6682	J′′	cholinesterase 1-like	XP_003740671	Metaseiulus occidentalis	62.10%	0
AS_CCE_11	14096.23959	J′′	cholinesterase 1-like	XP_003740671	Metaseiulus occidentalis	57.20%	1.95E-130
AS_CCE_12	14096.15138	J′′	cholinesterase 1-like	XP_003740671	Metaseiulus occidentalis	62.90%	1.58E-99
AS_CCE_13	14096.23398	J′′	cholinesterase 1-like	XP_003740671	Metaseiulus occidentalis	56.60%	0
AS_CCE_14	14096.32914	J′′	acetylcholinesterase-like	XP_003742289	Metaseiulus occidentalis	52.30%	4.85E-102
AS_CCE_15	14096.14165	J′′	carboxylesterase 5A-like	XP_003744189	Metaseiulus occidentalis	73.50%	0
AS_CCE_16	14096.6576	J′′	cholinesterase 2-like	XP_003744142	Metaseiulus occidentalis	74.10%	0
AS_CCE_17	14096.49601	J′	cholinesterase 1	XP_003738701	Metaseiulus occidentalis	93.40%	0
AS_CCE_18	14096.44875	J′	acetylcholinesterase-1	XP_003741414	Metaseiulus occidentalis	93.20%	0
AS_CCE_19	14096.42729	J′	acetylcholinesterase-1-like	XP_003743019	Metaseiulus occidentalis	89.70%	0
AS_CCE_20	14096.23198	J′	acetylcholinesterase-1-like	XP_003743019	Metaseiulus occidentalis	89.60%	0
AS_CCE_22	14096.17565	J′	acetylcholinesterase-like	XP_018497642	Metaseiulus occidentalis	91.30%	0
AS_CCE_23	14096.44036	J′	cholinesterase 1	XP_003739903	Metaseiulus occidentalis	82.30%	0
AS_CCE_24	14096.6939	J′	cholinesterase 1	XP_003739903	Metaseiulus occidentalis	84.80%	0
AS_CCE_25	14096.30297	J′	acetylcholinesterase-like	XP_018494386	Metaseiulus occidentalis	80.40%	2.91E-78
AS_CCE_26	14096.19664	J′	acetylcholinesterase-like	XP_018494387	Metaseiulus occidentalis	64.90%	8.92E-112
AS_CCE_27	14096.35506	J′	acetylcholinesterase-1-like	XP_018494388	Metaseiulus occidentalis	55.10%	0
AS_CCE_28	14096.38254	J′	acetylcholinesterase-1-like	XP_018494388	Metaseiulus occidentalis	55.10%	0
AS_CCE_29	14096.11036	J′	acetylcholinesterase	XP_018496861	Metaseiulus occidentalis	87.50%	0
AS_CCE_30	14096.13369	J′	carboxylesterase 1C-like	XP_018494965	Metaseiulus occidentalis	92.00%	0
AS_CCE_31	14096.31091	uncharacterized	cholinesterase-like	XP_003748221	Metaseiulus occidentalis	73.00%	0
AS_CCE_32	14096.22084	uncharacterized	bile salt-activated lipase-like	XP_003748220	Metaseiulus occidentalis	54.80%	1.92E-176

Table S4. *M. occidentalis* CYP, GST and CCE gene accession numbers in GenBank, of the sequences used in the phylogenetic analyses.

Gene CYP	Name in tree	Accession N. GenBank XP		Name in tree	Accession N. GenBank XP
1	MO_CYP1A1	003745229.1	31	MO_CYP3A29 like	003737485.1
2	MO_CYP1A2	003741017.1	32	MO_CYP3A30 like_partial	018497550.1
3	MO_CYP2B4 like	018496574.1	33	MO_CYP3A31	003744549.2
4	MO_CYP2C23	003747006.1	34	MO_CYP3A31 like	003744236.1
5	MO_CYP2C29	003747005.1	35	MO_CYP3A40 like	003743371.2
6	MO_CYP2F2 like	003738704.1	36	MO_CYP3A56_	003744550.1
7	MO_CYP2F3 like_	018493962.1	37	MO_CYP4C1	003740735.2
8	MO_CYP2J6	018495238.1	38	MO_CYP4C1 like_1	018495350.1
9	MO_CYP2J6 like_1	018494714.1	39	MO_CYP4C1 like_2	003737352.2
10	MO_CYP2J6 like_2	003748194.2	40	MO_CYP4c3_1	003744153.1
11	MO_CYP2J6 like_3	003748197.1	41	MO_CYP4c3_2	003741915.1
12	MO_CYP2U1 like	003747196.2	42	MO_CYP4c3 like_1	018497197.1
13	MO_CYP3A4	003743686.1	43	MO_CYP4c3 like_2	003739201.2
14	MO_CYP3A6_1	018497017.1	44	MO_CYP4V2_1	018493906.1
15	MO_CYP3A6_2	003743392.2	45	MO_CYP4V2_2	018496722.1
16	MO_CYP3A6 like	003745699.1	46	MO_CYP4V2 like_1	003746392.1
17	MO_CYP3A7 like	018497741.1	47	MO_CYP4V2 like_2	003744207.1
18	MO_CYP3A9	003739531.2	48	MO_CYP4V2 like_3	018495014.1
19	MO_CYP3A9 like_	003738548.2	49	MO_CYP4V2 like_4	003747974.1
20	MO_CYP3A12 like	003744583.1	50	MO_CYP4V2 like_5	018494969.1
21	MO_CYP3A14	003745878.1	51	MO_CYP71B22 like	003748195.1
22	MO_CYP3A14 like	003743440.1	52	MO_CYP98A3 like_	018495925.1
23	MO_CYP3A16	003744817.1	53	MO_CYP302a1 mitochondrial like	003745070.1
24	MO_CYP3A19 like_1	003742033.2	54	MO_CYP307a1 like	003747195.1
25	MO_CYP3A19 like_2	018496532.1	55	MO_CYP315a1 mitochondrial	003743235.1
26	MO_CYP3A21	003741251.1	56	MO_CYP_6d5_	003743386.2
27	MO_CYP3A24	003741765.1	57	MO_CYP_49a1_	003741296.1
28	MO_CYP3A24 like_1	003746429.1	58	MO_ecdysone 20-monooxygenase like	003748577.1
29	MO_CYP3A24 like_2	003738840.2	59	MO_lithocholate 6-beta-hydroxylase-like	003745726.1
30	MO_CYP3A28	003740908.1			
GST					
1	MO_XP_003738	003738807.1	10	MO_GST04	003744624.1

	807.1				
2	MO_XP_003738778.1	003738778.1	11	MO_GST03	003741590.1
3	MO_GSTp01	003737007.1	12	MO_GST01	003738383.1
4	MO_GSTo01	003746278.1	13	MO_GSTp02	003744621.1
5	MO_GSTo02	003738156.1	14	MO_GSTm01	003737612.1
6	MO_GSTd01	003740940.1	15	MO_GSTm02	003742682.2
7	MO_GSTd02	003743487.1	16	MO_GSTm03	018495947.1
8	MO_GSTd03	003746787.1	17	MO_GSTm04	003747409.1
9	MO_GST02	003745766.1			
CCE					
1	MO CCE3	003744359.1	22	MO CCE34	003743644.1
2	MO CCE4	003744419.1	23	MO CCE35	003746193.1
3	MO CCE5	003740650.1	24	MO CCE37	003748221.1
4	MO CCE6	003740671.2	25	MO CCE38	003748220.1
5	MO CCE7	003740670.1	26	MO CCE43	003740818.2
6	MO CCE8	003745536.1	27	MO CCE	003737110.1
7	MO CCE9	003747077.1	28	MO CCE	003738697.1
8	MO CCE10	003744142.2	29	MO CCE	003739903.2
9	MO CCE12	003744189.1	30	MO CCE	018494013.1
10	MO CCE13	003742834.1	31	MO CCE	018494370.1
11	MO CCE14	003738421.1	32	MO CCE	018494388.1
12	MO CCE18	003745369.1	33	MO CCE	018494461.1
13	MO CCE20	003744479.1	34	MO CCE	018494575.1
14	MO CCE22	003746194.2	35	MO CCE	018494964.1
15	MO CCE26	003743019.1	36	MO CCE	018494965.1
16	MO CCE27	003738701.1	37	MO CCE	018496352.1
17	MO CCE28	003741414.1	38	MO CCE	018496401.1
18	MO CCE30	003747841.1	39	MO CCE	018496563.1
19	MO CCE31	003739668.2	40	MO CCE	018496632.1
20	MO CCE32	003742457.2	41	MO CCE	018496861.1
21	MO CCE33	003745863.1	42	MO CCE	018497642.1

Table S5. *T. urticae* CYP, GST and CCE gene accession numbers in OrcAE, of the sequences used in the phylogenetic analyses.

Gene CYP	Name in tree	Accession N. OrcAE		Name in tree	Accession N. OrcAE
1	TU_CYP4CF2	tetur09g03800	44	TU_CYP392A6	tetur11g00530
2	TU_CYP4CL1	tetur01g00650	45	TU_CYP392A7	tetur16g03500
3	TU_CYP302A1_1	tetur05g02550	46	TU_CYP392A8	tetur02g14020
4	TU_CYP302A1_2	tetur05g02670	47	TU_CYP392A9	tetur47g00090
5	TU_CYP307A1	tetur10g03900	48	TU_CYP392A9v2	tetur02g14330
6	TU_CYP314A1	tetur03g03020	49	TU_CYP392A10	tetur16g03790
7	TU_CYP315A1	tetur06g05620	50	TU_CYP392A10v2	tetur02g14400
8	TU_CYP381A1	tetur13g02850	51	TU_CYP392A11	tetur03g00970
9	TU_CYP381A2	tetur13g02840	52	TU_CYP392A12	tetur03g00830
10	TU_CYP382A1	tetur03g01560	53	TU_CYP392A13v1	tetur08g08050
11	TU_CYP384A1	tetur38g00650	54	TU_CYP392A13v2	tetur03g00020
12	TU_CYP385A1	tetur07g05500	55	TU_CYP392A14	tetur08g07950
13	TU_CYP385B1	tetur05g04000	56	TU_CYP392A16	tetur06g04520
14	TU_CYP385C1	tetur26g01470	57	TU_CYP392B1	tetur20g03200
15	TU_CYP385C2	tetur11g05000	58	TU_CYP392B2	tetur02g06650
16	TU_CYP385C3	tetur11g05540	59	TU_CYP392B3	tetur20g00290
17	TU_CYP385C3v2	tetur46g00170	60	TU_CYP392C1	tetur03g03950
18	TU_CYP385C4	tetur11g05520	61	TU_CYP392D1	tetur23g00260
19	TU_CYP385C4v2	tetur46g00150	62	TU_CYP392D2	tetur03g04990
20	TU_CYP386A1	tetur11g06070	63	TU_CYP392D3	tetur03g05000
21	TU_CYP387A1	tetur08g06170	64	TU_CYP392D4	tetur03g05010
22	TU_CYP387A2	tetur01g06120	65	TU_CYP392D6	tetur03g05030
23	TU_CYP388A1	tetur03g05190	66	TU_CYP392D8	tetur03g05070
24	TU_CYP389A1	tetur25g02050	67	TU_CYP392E1	tetur03g05540
25	TU_CYP389B1	tetur25g02060	68	TU_CYP392E2	tetur06g02400
26	TU_CYP389C1	tetur34g00510	69	TU_CYP392E3	tetur06g02820
27	TU_CYP389C2	tetur05g02950	70	TU_CYP392E6	tetur27g00330
28	TU_CYP389C3	tetur05g02960	71	TU_CYP392E7	tetur27g00340
29	TU_CYP389C4	tetur05g02970	72	TU_CYP392E8	tetur27g00350
30	TU_CYP389C5	tetur05g08390	73	TU_CYP392E9	tetur27g01020
31	TU_CYP389C6	tetur05g06630	74	TU_CYP392E10	tetur27g01030
32	TU_CYP389C7	tetur05g06620	75	TU_CYP406A1	tetur01g04440
33	TU_CYP389C8	tetur05g06610	76	TU_CYP407A1	tetur20g00830
34	TU_CYP389C9	tetur05g06600	77	TU_CYP_1	tetur14g01350
35	TU_CYP389C10	tetur05g06580	78	TU_CYP_2	tetur05g02670
36	TU_CYP389C11	tetur05g06570	79	TU_CYP_3	tetur01g13730
37	TU_CYP389C12	tetur05g06560	80	TU_CYP_conserved_site_1	tetur03g09941
38	TU_CYP390A1	tetur03g00910	81	TU_CYP_conserved_site_2	tetur03g09961
39	TU_CYP391A1	tetur36g00920	82	TU_CYP_conserved_site_3	tetur03g05100
40	TU_CYP392A1	tetur07g06410	83	TU_CYP_E-class_group_I	tetur06g02650
41	TU_CYP392A3	tetur07g06460	84	TU_CYP_E-class_group_I_1	tetur03g00970
42	TU_CYP392A4	tetur07g06480	85	TU_CYP_E-class_group_I_2	tetur27g02598

43	TU_CYP392A5	tetur11g04390	86	TU_CYP_E-class_group_IV	tetur07g08087
GST					
1	TU GSTp01	tetur05g05180	18	TU GSTd13	tetur26g01490
2	TU GSTd15	tetur26g02802	19	TU GSTd05	tetur01g02470
3	TU GSTd12	tetur26g02801	20	TU GSTo02	tetur01g02320
4	TU GSTm05	tetur05g05270	21	TU GSTd03	tetur29g00220
5	TU GSTm07	tetur05g05210	22	TU GSTz01	tetur07g02560
6	TU GSTd08	tetur03g07920	23	TU GSTd07	tetur01g02500
7	TU GSTd10	tetur26g01450	24	TU GSTm10	tetur05g05240
8	TU GSTm03	tetur03g09230	25	TU GSTd05	tetur01g02480
9	TU GSTd01	tetur31g01390	26	TU GSTm11	tetur05g05190
10	TU GST02	tetur01g12390	27	TU GSTd14	tetur26g01510
11	TU GST01	tetur04g04990	28	TU GSTk01	tetur22g02300
12	TU GSTm09	tetur05g05260	29	TU GSTm01	tetur05g05220
13	TU GSTd02	tetur31g01330	30	TU GSTm04	tetur05g05200
14	TU GSTd06	tetur01g02230	31	TU GSTo01	tetur12g03900
15	TU GSTm08	tetur05g05250	32	TU GSTm02	tetur05g05300
16	TU GSTm06	tetur05g05290	33	TU GSTd04	tetur01g02510
17	TU GSTd11	tetur26g01500	34	TU GSTd09	tetur26g01460
CCE					
1	TU_CCE_01g10760	tetur01g10760	6	TU_CCE_04g08480	tetur04g08480
2	TU_CCE_01g10830	tetur01g10830	7	TU_CCE_11g05770	tetur11g05770
3	TU_CCE_01g14090	tetur01g14090	8	TU_CCE_19g00850	tetur19g00850
4	TU_CCE_02g06930	tetur02g08440	9	TU_CCE_29g00970	tetur29g00970
5	TU_CCE_04g06380	tetur04g06380	10	TU_CCE_30g01560	tetur30g01560

Table S6. *A. meliphera* (left columns) and *D. melanogaster* (rignt columns) CCE gene accession numbers in GenBank and FlyBase respectively, of the sequences used in the phylogenetic analyses.

Gene CCE	**Name in tree**	**Accession N. GenBank**		**Name in tree**	**Accession N. FlyBase**
1	AM CCE	NP 001119716.1	1	DM CCE	CG2505
2	AM AChE1	XP_393751.2	2	DM CCE	CG17148
3	AM CCE	XP 006572393.1	3	DM CCE	CG31146
4	AM CCE	XP 006568129.1	4	DM CCE	CG1121
5	AM CCE	XP 006564306.1	5	DM CCE	CG9704
6	AM CCE	NP 001035320.1	6	DM CCE	CG9280
7	AM CCE	XP 016768436.1	7	DM CCE	CG12869
8	AM CCE	XP 006566930.1	8	DM CCE	CG6917
9	AM CCE	XP 006571679.2	9	DM CCE	CG9289
			10	DM CCE	CG9287
			11	DM CCE	CG17907
			12	DM CCE	CG3903
			13	DM CCE	CG8425
			14	DM CCE	CG13772
			15	DM CCE	CG5397

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