UNIVERSITAT JAUME I

Àcars en cítrics:
defensa de les plantes i interacció del microbioma

TESI DOCTORAL

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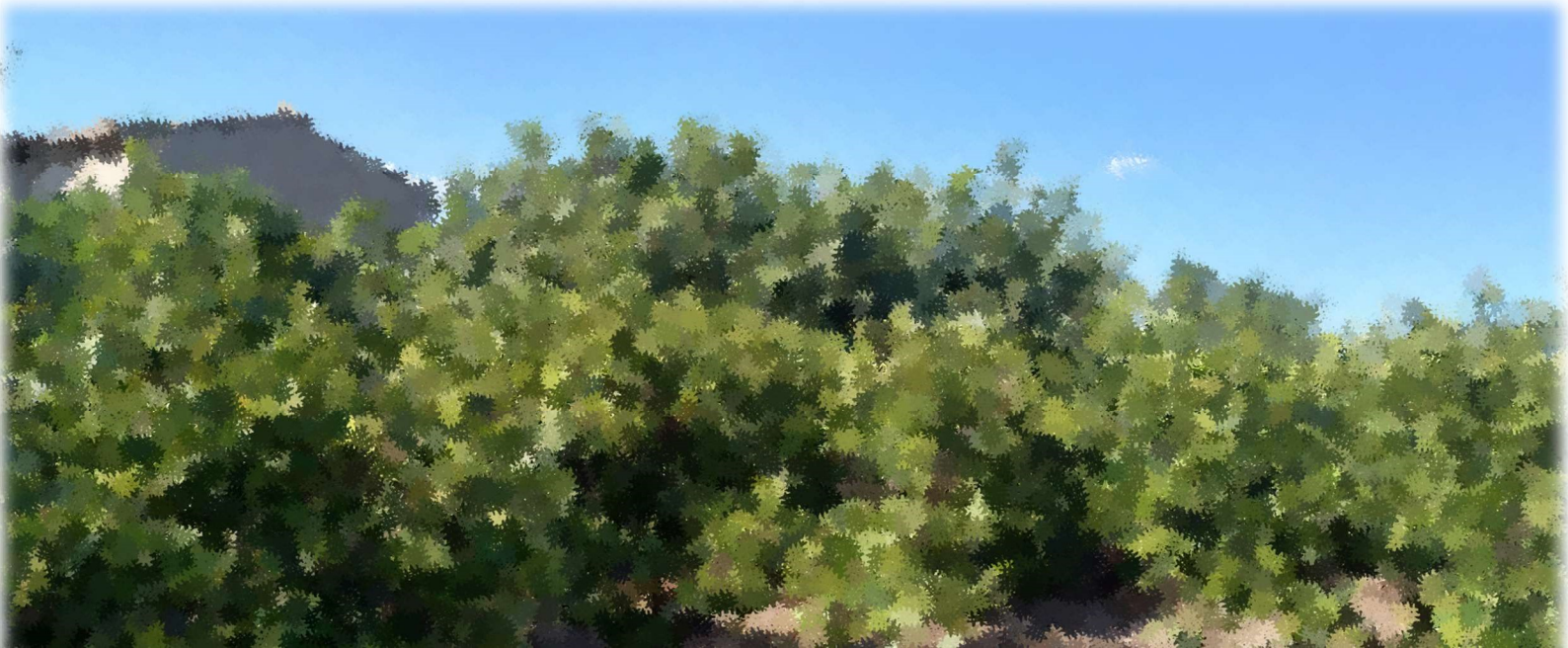
Castelló de la Plana, Octubre 2019



Programa de Doctorat en Ciències

Escola de Doctorat de la Universitat Jaume I

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defensa de les plantes i interacció del microbioma

Memòria presentada per Marc Cabedo López per a optar al grau de doctor per la Universitat Jaume I

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Agraïments

Es diu que el temps passa molt ràpidament i, tirant la vista enrere, queda clar que és així. Quatre anys han passat des que vaig arribar a la Universitat Jaume I, amb grans expectatives i un poc de temor al nou repte al qual, sense esperar-ho, m'hauria d'enfrontar. Havia estudiat, treballat a l'estranger i viscut moltes experiències, però fer una tesi doctoral eren paraules majors. A poc a poc vaig anar entrant en aquest fabulós (però molt maltractat) món de la investigació i em va enganxar, tant per la responsabilitat i el món universitari com pel repte d'aconseguir fer un món millor.

Durant aquests quatre anys he conegut molta gent, he fet una estada a Amsterdam, he anat i participat en molts congressos i reunions i m'he adonat que les universitats, els científics i les investigacions són un pilar fonamental per a la societat i que cal cuidar.

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Mil gràcies!

Resum

En la present tesi doctoral s'ha estudiat la resposta defensiva dels cítrics a dos espècies d'àcars fitòfags, l'aranya roja *Tetranychus urticae* i l'àcar blanc *Polyphagotarsonemus latus*, així com la interacció amb els principals enemics naturals de l'aranya roja. A més a més, s'ha realitzat l'estudi del microbioma d'aquest fitòfag per tal d'esbrinar quins són i quin paper poden tenir els bacteris simbionts.

En primer lloc, es van analitzar els mecanismes de defensa indirecta de dos patrons de cítrics amb diferent nivell de susceptibilitat per a l'herbívor *T. urticae* (el més resistent, el taronger bord *Citrus aurantium*, i el més susceptible, el mandariner Cleòpatra *C. reshni*). S'han realitzat una sèrie d'assajos de comportament amb els tres depredadors fitoseids de l'aranya amb diferents nivells d'especialització en la seua dieta per determinar el paper dels volàtils de planta induïts per herbivorisme (HIPVs) en la defensa indirecta dels cítrics. També, s'han caracteritzat els volàtils associats a l'aranya roja i al mandariner Cleòpatra induït pels HIPVs de taronger bord infestat amb *T. urticae*. Encara que la preferència per plantes menys defensades amb presumiblement major densitat de presa era esperable, les respostes dels tres àcars fitoseids estudiats depenen del genotip de la planta, la presència de la presa i els seus hàbits alimentaris. En segon lloc, s'ha aprofundit en l'estudi de la plaga clau *T. urticae*, concretament estudiant el seu microbioma, ja que s'ha demostrat que els bacteris simbionts poden influir en els paràmetres biològics dels artròpodes. Una de les espècies bacterianes més importants és *Wolbachia* spp., que forma part d'un grup de proteobacteris intracel·lulars capaços de manipular la reproducció dels seus hostes. La caracterització de la microbiota de *T. urticae* podria portar a noves estratègies de control biològic d'aquest fitòfag. Per açò, s'ha analitzat la comunitat de bacteris per seqüenciació massiva del 16S ADNr població de dues subpoblacions de *T. urticae* (tractada i no tractada amb l'antibiòtic tetraciclina) derivades d'un hort de clementí de la comarca de La Plana que es mantenen en laboratori, i s'han realitzat experiments de creuaments entre aquestes dues línies per determinar l'efecte de la microbiota en la reproducció de l'herbívor. Els resultats mostren com l'espècie *Wolbachia* és la més abundant i pot ser eliminada per l'antibiòtic i com aquest bacteri indueix incompatibilitat citoplasmàtica en les femelles tractades, afectant als paràmetres reproductius . Finalment, s'ha caracteritzat la resposta dels dos genotips de cítrics a la infestació de *P. latus* per dilucidar les defenses, el perfil volàtil i els mecanismes moleculars implicats en aquesta interacció. Els resultats demostren que la

resposta dels cítrics a *P. latus* és depenent del genotip de la planta i afecta, almenys, l'expressió dels gens de les vies defensives principals, és a dir, àcid jasmòmic (JA) i àcid salicílic (SA) i el perfil de volàtils alliberats. Curiosament i contrari al que ocorre amb *T. urticae*, en *C. aurantium* es va obtenir una major taxa de creixement d'aquest àcar que en *C. reshni*. La caracterització d'aquests efectes pot ajudar a millorar les practiques de control actuals front a esta plaga.

Abstract

In the present doctoral thesis, the defensive response of citrus to two phytophagousmite species, the two-spotted spider mite *Tetranychus urticae* and the broad mite, *Polyphagotarsonemus latus*, has been studied, as well as the interaction of the main natural enemies of the two-spotted spider mite. In addition, the study of the microbiome of this phytophagous has been carried out to determine its composition as well as the role of symbiont bacteria. First of all, the mechanisms of indirect defense of two citrus rootstocks with different levels of susceptibility to the herbivore *T. urticae* (the most resistant, sour orange *Citrus aurantium*, and the most susceptible, Cleopatra mandarin *C. reshni*) were analyzed. To do this, a series of behavioral tests were conducted with three phytoseiid predator species of the spider mite with different diet levels of specialization to determine the role of herbivory-induced volatiles plant (HIPVs) in the citrus indirect defense. Moreover, volatiles associated with the spider mite and Cleopatra mandarin induced by *T. urticae*-infested sour orange have been characterized. Although the preference for less defended plants with presumably more prey density was expected, the response of the three phytoseiid mites studied depends on the plant genotype, the presence of the prey and their feeding habits. Secondly, the study of the key pest *T. urticae* has been deepened, specifically studying its microbiome, since it has been shown that symbiont bacteria can influence the biological parameters of arthropods. One of the key species is *Wolbachia*, which is part of a group of intracellular proteobacteria capable of manipulating the reproduction of their hosts. The microbiota characterization of *T. urticae* could provide new biological control strategies. For doing this, the bacterial community has been analyzed by massive sequencing of the 16S rDNA of two related sub-populations of *T. urticae* (treated and not treated with tetracycline antibiotic) from a clementine orchard in the region of La Plana maintained as lab strain. Mating assays have been performed between these two lines to determine the effect of the microbiota on the reproduction of the herbivore. The results show how the *Wolbachia* specie is the most abundant, it can be removed by the antibiotic and this bacterium induces cytoplasmic incompatibility affecting the reproduction of the mite. Finally, the response of the two citrus genotypes has been characterized when they are infested by *P. latus* to elucidate the defenses, the volatile profile and the molecular mechanisms involved in this interaction. The results demonstrate that the response of citrus to *P. latus* depends on the plant genotype and affects, at least, the expression of the main defensive pathways genes,

that are, jasmonic (JA) and salicylic acid (SA) and the profile of released volatiles. Curiously and contrary to what happens with *T. urticae*, Cleopatra mandarin sustains lower *P. latus* populations compared with sour orange. The characterization of these effects can help improve current control practices against this pest.

Resumen

En la presente tesis doctoral se ha estudiado la respuesta defensiva de los cítricos a dos especies de ácaros fitófagos, la araña roja *Tetranychus urticae* y el ácaro blanco, *Polyphagotarsonemus latus*, así como la interacción con los principales enemigos naturales de la araña roja. Además, se ha realizado el estudio del microbioma de este fitófago para determinar quiénes son y qué papel juegan. En primer lugar, se analizaron los mecanismos de defensa indirecta de dos patrones de cítricos con diferente nivel de susceptibilidad para el herbívoro *T. urticae* (el más resistente, el naranjo amargo *Citrus aurantium* y el más susceptible, el mandarino Cleopatra *C. reshni*). Para ello, se realizaron una serie de ensayos de comportamiento con los tres fitoseidos depredadores de la araña con distintos niveles de especialización en su dieta para determinar el papel de los volátiles inducidos por herbivoría (HIPVs) en la defensa indirecta de los cítricos. También, se han caracterizado los volátiles asociados a la araña roja y al mandarino Cleopatra inducido por los HIPVs de naranjo amargo infestado con *T. urticae*. Aunque la preferencia por las plantas menos defendidas con presumiblemente mayor densidad de presa era de esperar, la respuesta de los tres ácaros fitoseidos estudiados depende del genotipo de la planta, la presencia de la presa y sus hábitos alimentarios. En segundo lugar, se ha profundizado en el estudio de la plaga clave *T. urticae*, concretamente estudiando su microbioma, ya que se ha demostrado que las bacterias simbiontes pueden influir en los parámetros biológicos de los artrópodos. Una de las especies bacterianas más importantes es *Wolbachia*, la cual forma parte de un grupo de proteobacterias intracelulares capaces de manipular la reproducción de sus hospedadores. La caracterización de la microbiota de *T. urticae* podría llevar a nuevas estrategias de control biológico de este fitófago. Para ello, se ha analizado la comunidad de bacterias por secuenciación masiva del 16S ADNr de dos subpoblaciones de *T. urticae* (tratada y no tratada con antibiótico tetraciclina) procedente de un huerto de clementinos de la comarca de La Plana que se mantienen en laboratorio, y se han realizado experimentos de cruzamientos entre estas dos líneas para determinar el efecto de la microbiota en la reproducción del herbívoro. Los resultados muestran como la bacteria *Wolbachia* es la más abundante y puede ser eliminada por el antibiótico y como esta bacteria induce incompatibilidad citoplasmática en las hembras tratadas, afectando a sus parámetros reproductivos. Finalmente, se ha caracterizado la respuesta de los dos genotipos de cítricos cuando son infestados por *P. latus* para dilucidar las defensas, el perfil volátil y

los mecanismos moleculares implicados en esta interacción. Los resultados demuestran que la respuesta de los cítricos a *P. latus* depende del genotipo de la planta y afecta, al menos, la expresión de los genes de las principales vías defensivas, es decir, ácido jasmónico (JA) y ácido salicílico (SA) y el perfil de volátiles liberados. Curiosamente y contrario a lo que ocurre con *T. urticae*, en *C. aurantium* se obtuvo una mayor tasa de crecimiento de este ácaro que en *C. reshni*. La caracterización de estos efectos puede ayudar a mejorar las prácticas de control actuales frente a esta plaga.

ÍNDEX

INTRODUCCIÓ

Àcars plaga en cítrics

Alguns àcars fitòfags poden esdevenir plagues importants que afecten un ampli rang de cultius (Simon et al. 2011; Agut et al. 2018), provocant greus danys si les seues densitats no es mantenen per baix dels llindars econòmics de danys. Destaquen les famílies dels *Tetranychidae*, *Tenuipalpidae*, *Tarsonemidae* i *Eriophyidae*, que poden causar perjudicis ja siga per alimentació directa o per transmissió de patògens (van Leeuwen et al. 2015).

Els cítrics són un dels principals cultius de l'àrea mediterrània (MAPAMA 2018) i la seua resistència front a plagues és clau. Existeixen nombrosos fitòfags que afecten el cultiu, però sols 15 espècies són considerades plagues clau en el nostre país (Jacas i Urbaneja 2008). Destaquen la mosca de la fruita *Ceratits capitata* (Wiedemann) (Diptera: Tephritidae), els pugons *Aphis spiraecola* Patch i *A. gossypii* Glover (Hemiptera: Aphididae), el poll roig de Califòrnia *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), i l'aranya roja *Tetranychus urticae* Koch (Prostigmata: Tetranychidae). A més d'aquestes plagues clau, ocasionalment poden aparèixer plagues secundàries, normalment per l'ús inapropiat dels tractaments químics, com és el cas, entre d'altres, de l'àcar blanc *Polyphagotarsonemus latus* (Trombidiforme: Tarsonemidae) (Gerson 1992), sent aquest junt amb l'aranya roja les espècies en què es centra aquesta tesi doctoral.

Gestió integrada i control biològic en cítrics

L'últim terç del segle XX va ser clau per a iniciar un canvi en les polítiques europees respecte l'ús sostenible de pesticides en agricultura per a reduir l'impacte mediambiental i protegir la salut humana, que van culminar establint els principis bàsics de la Gestió Integrada de Plagues (GIP) com a política europea (OJEU 2009). Aquest canvi de paradigma s'havia estat forjant fa dècades als països desenvolupats implementant pràctiques agrícoles sostenibles per a respectar el medi ambient i la biodiversitat (Wijnands i Kroonen-Backbier 1993). Per complir amb la sostenibilitat en l'agricultura es necessita redirigir la gestió de plagues des del control químic cap a altres tipus de control, especialment al control biològic. Tot açò queda englobat en la GIP que es defineix com la combinació de tots els mètodes de control disponibles tant biològics, biotecnològics, químics i culturals, inclosa la no intervenció, per a reduir al mínim imprescindible l'ús de productes fitosanitaris d'una manera sostenible. En l'actualitat, i després de l'entrada en vigor l'any 2014 de la Directiva 2009/128/CE del Parlament Europeu, els principis de la GIP són obligatoris per als productors europeus.

Tetranychus urticae

L'aranya roja *T. urticae* (Figura I.1) és una espècie polífaga i cosmopolita amb capacitat per desenvolupar-se sobre més de 1000 hostes distints, dels quals 150 tenen una gran importància econòmica (Attia et al. 2013). Un d'aquests cultius són els cítrics, afectant principalment al mandariner clementí [*Citrus clementina* Hort. Ex Tan. (Rutaceae)] i la llimera [*Citrus lemon* (L.) Burm f. (Rutaceae)] (Abad-Moyano et al. 2008), sent una plaga molt important a Espanya a causa dels danys que ocasiona en fruits (Aucejo-Romero et al. 2004; Ansaloni et al. 2007). En clementiner, aquest herbívor ocasiona taques cloròtiques sobre les fulles sent l'estiu el període quan es poden produir greus defoliacions de l'arbre (Aucejo i Jacas 2005). Però el dany més important a nivell comercial són les taques als fruits que redueixen el seu valor comercial (Pascual-Ruiz et al. 2014a).

Figura I.1. Colònia de *T. urticae* sobre fulla de *Phaseolus vulgaris* L. (Fabaceae).

Tetranychus urticae és una espècie que segueix una estratègia de desenvolupament tipus "r" (en referència al model logístic de creixement poblacional), amb un gran potencial biòtic, cicle de vida curt i una ràpida taxa de desenvolupament i dispersió (Speight et al. 2008). Aquest fitòfag desenvolupa les seves colònies en el revers de les fulles dels cítrics, la teranyina que produeix, pot retenir la humitat de la transpiració de la planta, creant un microclima favorable que el permet sobreviure en climes secs (Garcia-Marí et al. 1991; Aucejo i Jacas 2005), a més de protegir-les d'alguns dels seus depredadors. El cicle de vida consta de quatre etapes de desenvolupament: ou, larva, 2 estats nimfals (protonimfa i deutonimfa) i adult. En condicions òptimes, aproximadament 30°C, completa el seu cicle en 9-10 dies (Garcia-Marí et al. 1991; Aucejo i Jacas 2005). Aquest àcar es caracteritza per tenir un sistema reproductiu haplodiploide, en el qual els mascles es desenvolupen a partir d'ous no fertilitzats i són haploides i les femelles d'ous fertilitzats i són diploides (King et al. 2006).

Interacció planta-herbívor

Les plantes han desenvolupat mecanismes de defensa directa per fer front a l'herbivorisme, els quals poden ser constitutius (presents a les plantes independentment de l'ambient) o induïts mitjançant la seua activació quan se sotmeten a un estrès biòtic o abiòtic. La defensa, a més, es divideix en directa i indirecta (Aljbory i Chen 2018). El primer cas inclou l'activació o producció de compostos tòxics que afecten negativament el creixement o la supervivència dels herbívors (Howe i Jander 2008), que estan lligats a la síntesi de fitohomornes com l'àcid jasmònic (JA) i l'àcid salicílic (SA) (Pieterse et al. 2009). Contràriament, la defensa indirecta tracta d'atraure els enemics naturals dels herbívors, com depredadors i parasitoides (Sabelis et al. 2001; Flórez et al. 2015), gràcies a l'alliberació de compostos orgànics volàtils (VOCs) coneguts com a volàtils de plantes induïts per herbívors (HIPVs) i que també s'han relacionat amb el JA i el SA (Wei et al. 2014; Helms et al. 2019). Aquestes mescles de volàtils difereixen quantitativament i qualitativament en funció de l'espècie de planta (van den Boom et al. 2004), de l'herbívor (Birkett et al. 2003), a més d'altres factors biòtics i abiòtics (Dicke i van Loon 2000). El coneixement sobre aquests compostos en les interaccions planta-herbívor és limitat (Frost et al. 2008; Kim i Felton 2013) però pot obrir les portes a noves maneres més sostenibles de gestionar les poblacions d'espècies plaga.

Interacció herbívor-microbioma

L'estudi i la caracterització de la microbiota d'aquests artròpodes és essencial per aprofundir en el seu coneixement i desenvolupar noves ferramentes biotecnològiques per a l'estudi i control sostenible de la plaga (Berasategui et al. 2016). Les principals espècies d'endosimbionts presents en artròpodes són bacteris amb la capacitat de manipular la reproducció del seu hoste com *Wolbachia* (Rickettsiales) (Breeuwer i Jacobs 1996), *Cardinium* (Cytophagales), *Spiroplasma* (Entomoplasmatales) i *Rickettsia* (Rickettsiales) (Zhang et al. 2016).

La biologia molecular s'ha desenvolupat de manera exponencial durant els últims anys i ha estat incorporada als estudis de biodiversitat, permetent la seua aplicació directa al camp de l'entomologia i l'entorn de protecció de cultius. Les tècniques moleculars són més ràpides, específiques, sensibles i precises que les tècniques tradicionals i poden identificar microorganismes no cultivables, facilitant les decisions en el control de plagues. Les noves plataformes de seqüenciació massiva (New Generation Sequencing:

NGS) permeten la detecció múltiple de les espècies de bacteris presents en la microbiota dels artròpodes en poc temps i a un preu cada vegada més reduït. Com és ben sabut, només es pot cultivar un xicotet percentatge de microorganismes, limitant els coneixements sobre la comunitat present en una mostra (Hugenholtz 2002; Alves et al. 2018).

Per a la caracterització del microbioma s'utilitza la regió del 16S d'ADN ribosomal bacterià (16S rADN) com a principal marcador filogenètic, ja que presenta regions altament conservades i altres de molt variables, la qual cosa permet establir diferències entre comunitats bacterianes (Snel et al. 1999). Les 9 regions altament variables (V1-V9) oscil·len entre 30 i 100 pb i estan implicades en l'estructura secundària de la subunitat ribosòmica menuda (Figura I.2). Els encebadors més comuns - 27F i 1492R - han estat dissenyats per Weisburg et al. (1991). La mida de tota aquesta regió del 16s (~ 1500 pb), també és molt adequada per a les anàlisis genètiques de seqüències (Janda i Abbott 2007). La seqüenciació massiva d'aquesta regió es coneix com a seqüenciació profunda del 16S rDNA o Super16S.

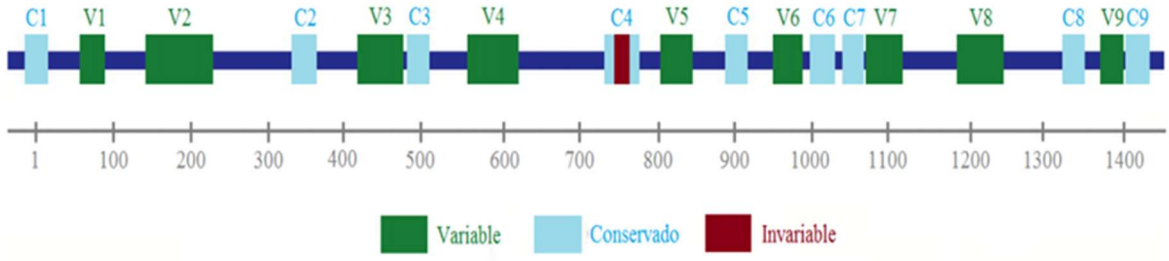

Figura I.2: Representació gràfica de la regió 16S rDNA utilitzada en la caracterització de bacteris (Risinger et al. 2015).

Principals àcars enemics naturals de *Tetranychus urticae*

Els principals enemics naturals de *T. urticae* són àcars depredadors de la família Phytoseiidae (Acari: Mesostigmata). *Euseius stipulatus* (Athias-Henriot), *Neoseiulus californicus* (McGregor) i *Phytoseiulus persimilis* Athias-Henriot són els fitosèids més comuns associats a *T. urticae* en els horts de cítrics espanyols (Abad-Moyano et al. 2009b; Aguilar-Fenollosa et al. 2011b).

Euseius stipulatus és un depredador generalista, omnívor i pal·linòfag (fitosèid de tipus IV) (McMurtry i Croft 1997), sent el més abundant en els horts de cítrics espanyols (70% del total dels fitosèids aproximadament) tant als arbres com a la flora adventícia associada (Abad-Moyano et al. 2009b; Aguilar-Fenollosa et al. 2011b; Pérez-Sayas et al. 2015).

S'alimenta de pol·len, àcars fitòfags, insectes menuts i melassa (Pina et al. 2012). Les poblacions d'*E. stipulatus* són elevades des d'octubre fins a juliol en els horts de cítrics, amb màxims poblacionals durant l'hivern. A l'estiu es produeix un fort descens de la densitat, que és degut a les altes temperatures i la baixa humitat relativa. El descens afecta a tots els estats de desenvolupament del fitosèid per igual, i té lloc quan la temperatura supera els 30-35°C i la humitat relativa descendeix per sota del 40% . El cicle biològic consta de les fases d'ou, nimfa (protonimfa i deutonimfa) i adult sent la seua durada entre 5 a 12 dies aproximadament depenent de la temperatura (Ferragut et al. 1986, 1987, 1988).

Aquesta superioritat numèrica el converteix en un depredador molt important de *T. urticae*, però no és el més eficaç. Un estudi on s'analitzava el contingut del tracte intestinal usant tècniques moleculars va mostrar que el fitosèid més eficaç era *P. persimilis*, el qual era capaç de depredar aranya roja fins a cinc vegades més que *E. stipulatus* (Pérez-Sayas et al. 2015). D'altra banda, s'ha descrit que el gènere *Euseius* De Leon presenta els quelícers adaptats per a xuclar de la planta hoste i així complementar la seua nutrició (Adar et al. 2012, 2015; Gómez-Martínez et al. 2019). Estudis recents del nostre grup han demostrat que és capaç d'activar la resposta defensiva en patrons de cítrics (Cruz-Miralles et al. 2019) i de perforar membranes artificials per a ingerir líquid tenyit (Gómez-Martínez et al. 2019).

Neoseiulus californicus és un depredador polífag que s'alimenta de tetraníquids, pol·len i insectes menuts (fitosèid de tipus II) (Escudero i Ferragut 1996; McMurtry i Croft 1997; Easterbrook et al. 2001). El cicle biològic d'aquest àcar consta de diferents fases: ou, nimfa (protonimfa i deutonimfa) i adult sent la seva durada entre 4 a 12 dies depenent de la temperatura. Les femelles ponen 2 ous al dia de mitjana sent el màxim 4 ous al dia. Els adults poden viure fins a 20 dies. El seu desenvolupament és més ràpid quan s'alimenta d'aranya roja en lloc d'altres fonts d'alimentació. Les condicions òptimes per al seu desenvolupament són amb temperatures entre 20 - 30ºC i entre 40 - 80% d'humitat relativa (Rhodes i Liburd 2015).

Aquest àcar depredador no aconsegueix augmentar les seues poblacions quan s'utilitza pol·len com a únic recurs (Pina et al. 2012) i a més, per estudis de morfologia dels seus quelícers, no està adaptat a alimentar-se de les plantes (Adar et al. 2012). Tot i que s'utilitza en agricultura com agent de control biològic de l'aranya roja, el control natural

que exerceix en horts de clementiners s'ha considerat insuficient (Abad-Moyano et al. 2009b).

Phytoseiulus persimilis és considerat el principal depredador de *T. urticae* (Aguilar-Fenollosa et al. 2011b; Pérez-Sayas et al. 2015), donat que és un àcar entomòfag estricte especialitzat en aquesta espècie (fitosèid de tipus I) (Badii i McMurtry, 1984). El seu cicle biològic consta de 5 fases: ou, larva, 2 estadis nimfals (protonimfa i deutonimfa), i adult. La seva durada depèn, entre altres factors, de la temperatura. A 20°C, els ous eclosionen als 3 dies, i completen el seu desenvolupament en 10 dies, mentre que la seva presa *T. urticae* necessita 17 dies a la mateixa temperatura. A 30°C, el temps de desenvolupament total d'aquest fitosèid es redueix a 5 dies, i el de l'aranya roja a més de 7 dies. Les femelles poden pondre fins a 50-60 ous durant tota la seva vida (Hoque et al. 2008).

Un dels factors essencials que afavoreix o limita la dinàmica poblacional de *P. persimilis*, és la humitat relativa. Aquest paràmetre incideix directament sobre la fecunditat i la longevitat de les femelles, així com sobre el desenvolupament dels ous i dels estats immadurs. Una humitat baixa, per baix del 60%, té un efecte negatiu. Per tant, aquest àcar fitosèid manté un control efectiu amb temperatures entre 15 i 25°C en intervals de 60-90% d'humitat relativa. Per sobre de 30°C, la seva activitat decreix (Badii i McMurtry 1984).

Per les seues característiques, *P. persimilis,* és considerat l'àcar millor adaptat per al control biològic tant augmentatiu com de conservació de *T. urticae* (Jacas i Urbaneja 2010; Aguilar-Fenollosa et al. 2011a,b,c). El principal problema és que no es reprodueix amb aliments alternatius, de manera que no es manté al camp quan la seua presa desapareix (Abad-Moyano et al. 2009a).

Figura I.3. Femelles adultes d'*Euseius stipulatus*, *Neoseiulus californicus* i *Phytoseiulus persimilis*.

El cas de *Polyphagotarsonemus latus*

Polyphagotarsonemus latus és una de les plagues d'àcars més comunes en àrees tropicals i subtropicals. És capaç d'atacar un ampli rang d'hostes, incloent més de 50 espècies de dicotiledònies entre les quals destaquen els cítrics i moltes ornamentals (Gerson 1992). Amb uns 0,2 mm de llargada, resulta impossible la seua observació a simple vista provocant que no siguen detectats inicialment en els cultius, sinó que es detecten quan les plantes presenten símptomes (Venzon et al. 2008) (Figura I.4).

El seu cicle de vida és d'una a dues setmanes aproximadament. Les femelles poden pondre entre 2 a 5 ous al dia a la part inferior de les fulles joves, a les flors i als fruits. Sense l'aparellament, les femelles produiran només mascles; altrament, la proporció de sexe és d'un mascle a 3 - 4 femelles. Les condicions òptimes per al seu desenvolupament són entre 21 – 27ºC i una elevada humitat relativa. *Polyphagotarsonemus latus* es dispersa pel vent des dels teixits vegetals infestats, així com per contacte del fullatge i a través d'insectes (Rodríguez et al. 2017), és a dir, posseeix una relació forètica amb les mosques blanques, especialment *Bemisia* sp. i *Dialeurodes* sp. (Hemiptera: Aleyrodidae), utilitzant-los com a portadors per arribar a altres plantes (Luypaert 2015).

Aquest àcar ataca principalment les parts més joves de la planta i fa la seua posta a la part inferior de les fulles (van Maanen et al. 2010). El dany es caracteritza per la malformació dels teixits en creixement (fulles, brots i flors), provocant l'arrugament, l'enrotllament i la caiguda de les fulles (Gerson 1992). El dany al fruit es produeix a les zones d'ombra a l'interior dels arbres i pot passar desapercebut però si l'atac és sever, pot provocar la decoloració i la caiguda del fruit (Rodríguez et al. 2017).

Figura I.4. Femella de *Polyphagotarsonemus latus* i dany causat per l'àcar en cítrics en laboratori.

Sistema d'estudi

El control biològic de plagues implica la interacció de diferents actors dins d'un agroecosistema incloent les relacions microbiota-planta-artròpode-atròpode. En aquesta tesi doctoral es pretén abordar l'estudi de l'agroecosistema dels cítrics incloent tots quatre actors.

En estudis anteriors es van dilucidar els mecanismes de defensa dels cítrics front l'atac de l'aranya roja utilitzant dos patrons de cítric amb diferent nivell de susceptibilitat front a aquest àcar, com són el taronger bord *Citrus aurantium* L. (Sapindales: Rutaceae) i el mandariner Cleòpatra *Citrus reshni* Hort. ex Tan. (Sapindales: Rutaceae). A més a més, de la inducció de les respostes defensives dels cítrics pels principals enemics naturals d'aquesta plaga i les interaccions que es produeixen en el sistema planta-presa-depredador (Agut et al. 2014, 2015, 2016;Cruz-Miralles et al. 2019). En la present tesi doctoral es continuen aquests treballs incloent l'espècie *P. latus*.

El primer capítol tracta sobre la defensa indirecta dels cítrics analitzant la resposta dels principals enemics naturals de *T. urticae* als volàtils de planta induïts per herbivorisme (HIPVs).

El segon capítol se centra en l'estudi de la microbiota de *T. urticae* dels horts de cítrics i el possible paper en la defensa de la planta dels principals bacteris endosimbionts, els quals podrien jugar un paper fonamental en el desenvolupament de l'àcar alterant principalment la reproducció i per tant afectant al seu desenvolupament.

Per últim, al tercer capítol s'analitza la resposta dels mateixos patrons de cítrics a l'herbívor *P. latus*, seguint el mateix procediment que es va realitzar per a l'aranya anys enrere (Agut et al. 2014, 2015). Es determinarà si la resposta és depenent del genotip de la planta i si aquest àcar és capaç de desencadenar una resposta defensiva com *T. urticae* estudiant els principals gens defensius i els HIPVs.

OBJECTIUS

L'objectiu principal de la present tesi doctoral és incrementar el coneixement sobre les possibilitats de control biològic de dos plagues importants dels cítrics (*P. latus* i *T. urticae*). Per això, es pretén estudiar la interacció d'aquestes plagues en l'agroecosistema dels cítrics atenent a les relacions microbiota-planta-artròpode. D'aquesta manera es plantegen els següents objectius específics:

1. Determinar el paper dels volàtils de planta induïts per herbivorisme en la defensa indirecta del taronger bord i el mandariner Cleòpatra analitzant la resposta dels principals enemics naturals de l'aranya roja, *T. urticae*. Aquest objectiu es desenvolupa en el capítol 1.
2. Caracteritzar el microbioma d'una població de *T. urticae* procedent d'un hort de clementiners de la comarca de La Plana, per determinar quins endosimbionts dels descrits com a possibles manipuladors de la reproducció de l'àcar en són responsables. Aquest objectiu es tracta al capítol 2.
3. Dilucidar l'efecte dels endosimbionts en els paràmetres biològics d'una població de *T. urticae* procedent d'un hort de clementiners de La Plana. Aquest objectiu es tracta al capítol 2.
4. Caracteritzar la resposta de dos genotips de cítric a la infestació de *P. latus* per determinar les defenses de la planta, el seu perfil volàtil i els mecanismes moleculars implicats en aquesta interacció. Aquest objectiu es desenvolupa al capítol 3.

CAPÍTOL 1

The olfactive responses of *Tetranychus urticae* natural enemies in citrus depend on plant genotype, prey presence, and their diet specialization

Adapted from:

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ABSTRACT

Sour orange, *Citrus aurantium*, displays higher constitutive and earlier inducible direct defenses against the two-spotted spider mite, *Tetranychus urticae*, than Cleopatra mandarin, *Citrus reshni*. Moreover, herbivore induced plant volatiles (HIPVs) produced by sour orange upon infestation can induce resistance in Cleopatra mandarin but not vice-versa. Because the role of these HIPVs in indirect resistance remains ignored, we have carried out a series of behavioral assays with three predatory mites with different levels of specialization on this herbivore, from strict entomophagy to omnivory. We have further characterized the volatile blend associated with *T. urticae*, which interestingly includes the HIPV methyl salycilate (MeSA), as well as that produced by induced Cleopatra mandarin plants. Although a preference for less defended plants with presumably higher prey densities (i.e., *C. reshni*) was expected, this was not always the case. Because predators' responses changed with diet width, with omnivore predators responding to both HIPVs and prey-related odors and specialized ones mostly to prey, our results reveal that these responses depend on plant genotype, prey presence, and predator diet specialization. As the different volatile blends produced by infested sour orange, induced Cleopatra mandarin and *T. urticae* itself are attractive to *T. urticae* natural enemies but not to the herbivore, they may provide clues to develop new more sustainable tools to manipulate these agriculturally relevant species.

1.1 INTRODUCTION

Spider mites (Acari: Tetranychidae) comprise more than one thousand plant-feeding species worldwide (Migeon and Dorkeld 2006-2017). One of these species is the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), a highly polyphagous and cosmopolitan species (Migeon and Dorkeld 2006-2017). The pest status of this herbivore changed from minor to key pest of many food and ornamental crops after World War II (Hoy 2011; Pérez-Sayas et al. 2015). The disruption of existing top-down regulation mechanisms (i.e., natural enemies) by pesticide abuse during the second half of the XX century is recognized as one of the main causes for that change (Huffaker et al. 1970). More recently, the implication of bottom-up regulation mechanisms by replacement of traditional resistant crops by more susceptible genotypes has been also highlighted (Bruessow et al. 2010; Agut et al. 2014). These studies focused on citrus, one of the many crops where *T. urticae* is considered a pest (Jacas and Urbaneja 2010). Indeed, in the case of clementine mandarins [*Citrus clementina* Hort. ex Tan. (Sapindales: Rutaceae)], *T. urticae* can achieve the status of key pest (Pascual-Ruiz et al. 2014a; Gómez-Martínez et al. 2018).

Commercial citrus plants are regularly propagated vegetatively by bud-grafting onto a seedling rootstock. Sour orange, *Citrus aurantium* L. (Sapindales: Rutaceae), was the most widespread rootstock until the 1950s, when the emergence of the citrus quick decline disease caused by the Citrus Tristeza Virus (CTV, *Closteroviridae*) proved lethal for this rootstock. This triggered its massive replacement around the world (Cambra et al. 2000). Sour orange, though, is highly resistant to *T. urticae*, while one of the alternative CTV-tolerant rootstocks, Cleopatra mandarin, *Citrus reshni* Hort. ex Tan. (Sapindales: Rutaceae), is highly susceptible to this mite (Bruessow et al. 2010). Agut et al. (2016) provided evidence that resistance in sour orange was systemically transmitted from the roots to the shoots of the grafted cultivar. Both the jasmonic acid (JA) and the salicylic acid (SA) pathways were upregulated in sour orange plants upon mite attack, while these pathways remained unchanged in infested Cleopatra mandarin. However, the SA pathway proved irrelevant for the enhanced direct defense of sour orange (Agut et al. 2014). Further studies (Agut et al. 2015) showed that the release of *T. urticae* HIPVs (herbivore induced plant volatiles) from sour orange [namely, the terpenes α-ocimene, α-farnesene, pinene and D-limonene, and the green leaf volatile (GLV) 4-hydroxy-4-methyl-2-pentanone] had a marked repellent effect on conspecific mites and induced resistance in

Cleopatra mandarin plants. Oviposition rates decreased while both the JA and the SA pathways were stimulated in this rootstock. Contrarily, Cleopatra mandarin HIPVs [namely, (2-butoxyethoxy) ethanol, benzaldehyde, and methyl salicylate, MeSA] had a marked attractant effect on conspecific mites and did not induce any resistant response in uninfested Cleopatra mandarins. However, the potential role of these induced volatiles in indirect defense, i.e., the attraction of the natural enemies of the herbivore (Aljbory and Chen 2018; Cortés et al. 2016), remains unknown. Therefore, this system offers a good opportunity to study the possible effect of plant genotype on the behavior of *T. urticae* natural enemies. Because for a predator, directing its food search toward HIPVs emitted by well-defended plants may reduce its fitness, as its chances of finding abundant and well-nourished prey are lower, we would expect a higher attraction of clean Cleopatra mandarin relative to induced Cleopatra plants and clean sour orange.

The main natural enemies of *T. urticae* are predatory mites of the family Phytoseiidae (Acari: Mesostigmata). *Euseius stipulatus* (Athias-Henriot), *Neoseiulus californicus* (McGregor) and *Phytoseiulus persimilis* Athias-Henriot are the most common phytoseiids naturally associated with *T. urticae* in the canopy of Spanish citrus orchards (Abad-Moyano et al. 2009b; Aguilar-Fenollosa et al. 2011a,b). These predators have different diet specializations, ranging from selective predators of *Tetranychus* spp., as *P. persimilis*, to extreme diet generalists, omnivores feeding on both animal and plant derived food, as *E. stipulatus*, for which plant cell-sap feeding is suspected (Adar et al. 2012). The Tetranychidae specialist *N. californicus* would occupy an intermediate position feeding on both prey and plant derived food (i.e., pollen) (McMurtry and Croft 1997; McMurtry et al. 2013). However, same as *P. persimilis*, *N. californicus* is not considered a plant cell-sap feeding phytoseiid (Adar et al. 2012). These diet specializations may also have consequences on the behavior of predators and affect their choices. Although, as pointed out earlier, predators would benefit from choosing less defended plants, plant cell-sap-feeding, which would allow this type of omnivorous predators to switch to plant feeding when prey is scarce could result in a stronger attraction for these plants, which could be missing in strict entomophagous predators (i.e., *P. persimilis*).

Here, we present a study of the effects of plant genotype and predator diet specialization on the indirect plant defense responses triggered by *T. urticae* in citrus. To achieve this goal, we have carried out a series of Y-tube olfactory choice assays (Bruin et al. 1992)

using the two extreme citrus genotypes partly characterized in terms of their response to *T. urticae* herbivory (defensive pathways and HIPV profiles): sour orange and Cleopatra mandarin (Agut et al. 2014, 2015, 2016). We have also characterized the volatile blends produced by induced Cleopatra mandarin and *T. urticae*.

1.2 MATERIALS AND METHODS

1.2.1 Plant material

Sour orange, Cleopatra mandarin, clementine mandarin (*C. clementina* cv. Clementina de Nules grafted on citrange Carrizo rootstock) and bean (*Phaseolus vulgaris* L. (Fabaceae) cv. Buenos Aires roja) plants were used in our assays. These plants were grown on vermiculite and peat (1:3, v:v). No pesticides were applied to these plants, which were watered every 3 days with approximately 30 ml of a 1:100 (v:v) modified Hoagland's solution (Bañuls et al. 1997). Bean plants were used for rearing purposes only (see below).

Three-month-old plants of sour orange and Cleopatra mandarin were used in the behavioral assays (see below). They were maintained in a climatic chamber at 22 ± 2.5°C and 60 ± 10% relative humidity (RH) under a 16:8 h L:D (Light:Dark) photoperiod. Two-year-old clementine mandarin plants maintained in a greenhouse at 25 ± 10 °C, 75 ± 30% RH, under natural photoperiod and lemon (*Citrus limon* (L.) Burm f. (Sapindales: Rutaceae)) fruit obtained from a pesticide-free orchard at Universitat Jaume I Riu Sec Campus (UJI; 30º59'38''N; 0º03'59''W, 30 m alt.), the same location, were used to maintain *T. urticae* stock colonies. Finally, pesticide-free bean leaves obtained from plants grown at UJI greenhouses were used to maintain *E. stipulatus* and *P. persimilis* colonies.

1.2.2 Spider mite stock colony

The colony of *T. urticae* used in the assays was initiated with specimens collected in clementine mandarin orchards in the region of La Plana (Castelló, Spain) in 2011. Mites were maintained on lemons kept in a climatic chamber (22 ± 2.5°C and 75 ± 5% RH and 16:8 h L:D photoperiod). Colonies consisted of 8–10 lemons, which were replaced weekly in groups of four. Adult females (5-6 day-old) obtained from these stock colonies were used in the behavioral assays (see below), either directly to infest citrus plants (48h

before the assays) or to use as an odor source (25 adult females were either deposited on different leaves of the same plant using a soft-bristle paintbrush or moved to a meshed bag (10 × 5 cm) closed with a magnet), or subjected to a previous 24-h starvation period, before measuring their preferences. For the characterization of *T. urticae* associated volatiles, we used individuals from these colonies but also from an additional colony maintained on detached clementine mandarin leaves. These leaves were placed upside down on top of sponges (14 × 14 × 4 cm) covered with cotton in water-containing trays (35 × 20 × 7 cm) that served both as a water source for leaves and mites and as a barrier against mite dispersal.

1.2.3 Phytoseiidae mite stock colony

Three different phytoseiid mite species were used in our studies: *E. stipulatus*, *N. californicus* and *P. persimilis*. Colonies of *P. persimilis* and *E. stipulatus* were initiated with specimens collected in clementine mandarin orchards in the region of La Plana (Castelló, Spain) whereas *N. californicus* was obtained from Koppert Biological Systems (SPICAL®) and these specimens were directly used in our choice tests. The colonies of *P. persimilis* and *E. stipulatus* were maintained on detached leaves of bean plants in a climatic chamber at the same conditions as above. The rearing took place on units consisting of a single bean leaf placed upside down on moistened cotton, placed on top of a water-saturated sponge in water-containing trays as before. Moist cotton was folded over the edges of the leaves to prevent mites from escaping. A mix of different stages of *T. urticae* was provided twice a week to *P. persimilis*, whereas *E. stipulatus* was supplied *Typha* L. spp. (Typhaceae) pollen, only. 5-6 day-old phytoseiid adult females obtained from these stock colonies were used in the behavioral assays (see below).

1.2.4 Y-tube olfactory choice assays

Olfactory choice assays were conducted using a Y-tube olfactometer according to Bruin et al. (1992). This assay involves the use of a 4-cm-diameter Y-shaped glass tube with a 13 cm base and two 13 cm arms containing a Y-shaped 1-mm diameter metal wire of the same dimensions, which occupies the core of the olfactometer. The two short arms were directly connected via a plastic pipeline to the outlets of two identical 5-l glass vessels (Duran, Mainz, Germany) containing different odor sources (mite odors, plant odors or a combination of both, see Figure 1.1-1.4). Each vessel was connected to an air pump that

produced a unidirectional airflow of 1.5 l h^{-1} (measured with a flowmeter) from the arms to the base of the tube. The air was purified with a granular activated charcoal filter (Sigma-Aldrich). The environmental conditions inside the Y-tube were 23 ± 2°C and 60 ± 10% RH. Adult females either of *T. urticae* or its predators offered water only during the 24 h before the assay, were individually deposited at the beginning of the basal arm of the wire using a soft-bristle paintbrush. Females were allowed to make a choice within 10 min. As soon as a mite reached the end of one of the two arms of the Y-tube, the mite was removed from the set-up and discarded. Mites failing to reach either end of the two arms within the allocated time were scored as 'no choice'. Each combination was evaluated four times at different dates (i.e., four replicates). Each replicate included 10 responding mites which meant that up to 13 mites per combination per date were tested as the non-choice rate ranged from 0 to 3. The glass vessels were switched after five females had been tested. After every 10 females had been tested, the plants were replaced and the whole system was rinsed with ethanol (70%), followed by air drying. The glass vessels were switched to reduce the effects of spatial influence on choice. To exclude any bias from the set-up, before the beginning of the assays, 10 mites were exposed to clean air in both arms.

1.2.5 Effect of HIPVs on neighboring plants

To determine the effect of the volatiles released by Cleopatra mandarin plants previously exposed to *T. urticae*-infested sour orange on mite behavior, an olfactory choice assay was performed. First, sour orange plants were infested with 25 adult *T. urticae* females per plant. After 24 h, one infested sour orange plant was placed in a tray (65 × 50 × 30 cm) containing five untreated Cleopatra mandarin plants. Subsequently, the tray was covered with a transparent lid. To avoid mite ambulatory dispersal, the tray was filled with water. After 72 h, one Cleopatra mandarin and one sour orange plants were defoliated. Detached leaves were immediately frozen at -80°C for further analysis (mRNA expression, see below). The remaining four presumably-induced Cleopatra mandarin plants were used in an olfactory choice assay together with control plants where the preferences of *T. urticae, E. stipulatus, N. californicus* and *P. persimilis* were studied following the procedure explained above.

1.2.6 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

RNA was extracted using a plant RNA protocol with trizol (Kiefer et al. 2000). For qRT-PCR experiments, 1 µg of total RNA was digested with 0.7 µg of DNase (RNase-free DNase I) in 0.7 µl of DNase buffer and Milli-Q water up to 4.9 µl and incubated for 30 min at 37°C. After incubation, 0.7 µl of EDTA was added and incubated again at 65°C for 10 min to inactivate DNase (Thermofisher Scientific Inc.). The RT reaction was performed by adding 7 µl of DNase reaction, 2 µl of PrimeScript buffer and 0.5 µl of PrimeScript RT and Oligo-dT respectively (PrimeScript RT Reagent Kit, Takara Bio Inc.). The reaction mixture was incubated at 37°C for 15 min. Complementary DNA from the RT reaction, 10X diluted, was used for qPCR. Forward and reverse primers (0.3 µM) were added to 5 µl of Maxima SYBR Green qPCR Master Mix, 1 µl of cDNA and 3 µl Milli-Q sterile water (Maxima SYBR Green/ROX qPCR, Thermofisher Scientific Inc.). qPCR was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA, USA) sequence detector with standard PCR conditions (95ºC-10 min; 40×(95ºC-10 sec; 55ºC-10 sec; 72ºC-20 sec); 60ºC-10 sec; 95ºC-15 sec). qRT-PCR analysis was replicated three times. The primer for lipoxygenase2 (*LOX2*) and pathogenesis-related protein 5 (*PR5*) genes were used to determine their expression. Relative expression was compared with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (primer sequences in Table 1.1 Suppl.).

1.2.7 Characterization of Cleopatra-mandarin volatiles induced by exposure to sour orange HIPVs

Volatiles emitted by Cleopatra mandarin plants previously exposed to *T. urticae*-infested sour orange (see above) and Cleopatra mandarin control plants were collected using a headspace collection system similar to that described by Bruinsma et al. (2010). Open glass vials containing 300 mg of Porapak (Sigma-Aldrich, Barcelona, Spain) were used as volatile retention filters. They were connected to the air outlet hole at the top of 5-l glass vessels described above. This system was ventilated with carbon-filtered pressure-air at 1.5 l/h. The system (glass vessels and Porapak filters) was cleaned with acetone and dried in an oven 1 hour prior to the assay. Plants were set individually inside these glass vessels. Volatile compounds were collected in 1 ml of ethyl acetate. This collection took place in a climatic chamber at 22 ± 2.5°C and 60 ± 10% relative humidity (RH) under a

16:8 h L:D photoperiod during 24 hours. An Agilent 6890N GC system (Palo-Alto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a time-of-flight mass spectrometer (TOF-MS), GCT (Waters Corp., Manchester, UK), operating in electron ionization (EI) mode was used to characterize the volatiles. A fused silica DB-5MS capillary column of 30 m length, 0.25 mm internal diameter and a film thickness of 0.25 µm (JandW Scientific, Folson, CA, USA) was used to the GC separation. The temperature program for this process was the following; 50°C (1 min); 5°C min^{-1} to 210°C (1 min); 20°C min^{-1} to 300°C (2 min); this resulted in a total analysis run of 40.50 min. Splitless injections were carried out. Helium was used as carrier gas at 1 ml min^{-1}. The interface and source temperatures were both set to 250°C and a solvent delay of 3 min was selected. The TOF-MS was operated at 1 spectrum s^{-1} acquiring the mass range m/z 50–650 and using a multi-channel plate voltage of 2800 V. The TOF-MS resolution was c. 8500 (full width at half-maximum, FWHM) at m/z 614. Heptacose, used for the daily mass calibration as well as lock mass, was injected via syringe into the reference reservoir at 30°C. The m/z ion monitored was 218.9856. The application manager ChromaLynx, a module of MassLynx software, was used to investigate the presence of non-target compounds in the samples. Volatiles were identified by matching to the National Institute of Standards and Technology library (NIST\EPA\NIH Mass Spectral Library, version 2.0, build 4/2005) using match values of at least > 80% as a threshold for identification, as described by Wallis et al. (2008). Finally, for each volatile identified the TOF-MS-derived peak areas were calculated.

1.2.8 Characterization of *Tetranychus urticae* associated volatiles

Groups of 1000-2000 spider mite individuals (mixed instars and sexes, see above) were placed in 20-ml closed screw-cap headspace vials by carefully brushing the rearing substrate. Volatiles were collected in static conditions by solid-phase microextraction (SPME) using Supelco SPME holders equipped with a polydimethylsiloxane/divinylbenzene fiber (PDMS/ DVB), film thickness = 100 µm (Supelco Inc., Bellefonte, PA, USA). SPME fibers were conditioned before volatile sampling in a GC injector at 250°C for 10 min under a 20 ml min^{-1} helium flow rate. SPME needles were inserted through the polytetrafluoroethylene (PTFE)-silicone septa, and fibers were exposed to each sample for 24 h at 23 ± 2°C, under a 16:8 h L:D photoperiod. This sampling period was chosen in order to achieve maximum sensitivity

(Alfaro et al. 2011). Then, fibers were removed and inserted into the GC injection port to desorb volatiles. Nine replicates were carried out with different groups of *T. urticae* individuals, six of them obtained from the colony maintained on lemons, and three from the colony on clementine mandarin leaves. SPME fibers were thermally desorbed into the GC injection port, set at 250°C for 1 min, and operated in the splitless mode. The extracted volatiles were analyzed by GC-MS using a Clarus 600 GC-MS (PerkinElmer Inc., Wellesley, MA, USA). The column used was a 30 m × 0.25 mm i.d., 0.25 μm film thickness, ZB-5MS fused silica capillary column (Phenomenex Inc., Torrance, CA, USA). The oven was held at 40°C for 2 min and then programmed at 5°C min^{-1} to 180°C; when reached, temperature was raised to 280°C at 10°C min^{-1} and maintained at 280°C for 1 min (total analysis run of 41 min). Helium was used as the carrier gas with a flow rate of 1.2 ml min^{-1}. Detection was performed in the EI mode (ionization energy, 70 eV; source temperature, 180 °C), and spectra acquisition was done in the scanning mode (mass range m/z 35−400). Chromatograms and spectra were recorded with GC-MS Turbomass software version 5.4 (PerkinElmer Inc.). Volatiles were identified by either comparing their retention times and mass spectra with those of pure standards (Sigma-Aldrich) or, same as before, by matching to the National Institute of Standards and Technology library (NIST\EPA\NIH Mass Spectral Library, version 2.0, build 4/2005) using match values of at least > 80% as a threshold for identification, as described by Wallis et al. (2008). For each rearing substrate, the different peak areas in the chromatogram corresponding to these compounds were calculated and used to estimate their relative abundance in the blend.

1.2.9 Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 23. The results of the two-choice assays were initially subjected to logistic regression to check for the effect of replicate (n = 4) on mite preference. Depending on whether this effect was significant or not ($P > 0.05$), either each single replicate or the combination of the four, respectively, were subjected to chi-square analysis to test whether they departed from a 1:1 distribution. Student *t*-tests were used to compare the results of genetic expression results. The TOF-MS-derived peak areas were compared using a Generalized Linear Model (GLM) with a normal distribution of the error and identity function (i.e., linear regression). Treatment and replicate were used as fixed effects ($P < 0.05$).

1.3 RESULTS

In order to understand the role of HIPVs in direct and indirect defense we first confirmed that sour orange strongly reacts to *T. urticae* infestation by triggering expression of both *LOX2* and *PR5* marker genes of the JA and the SA-signaling pathways, respectively (Figures 1.1A and 1.2A Suppl.). Likewise, Cleopatra mandarin could be stimulated by sour orange HIPVs that triggered an upregulation of *LOX2* and *PR5* gene expression (Figures 1.1B and 1.2B Suppl.).

As the effect of the factor 'replicate' was not significant in any case, for each 2-choice experiment, the results were pooled and subjected to chi-square test. Preferences of adult *T. urticae* females when exposed to the odors of clean and infested plants, which had already been recorded in our previous work (Agut et al. 2015), were studied again. In addition, we also checked the responses to conspecific mites alone, and to induced Cleopatra mandarin. These preferences are shown in Figure 1.1. Without plant, adult females did not respond to the blend of volatiles associated to conspecifics. However, when plants were considered, Cleopatra mandarin was always preferred to sour orange, irrespective of the infestation status. Moreover, when comparing the same genotype, clean versus infested plants, infested sour orange became repellent, whereas infested Cleopatra mandarin became attractive, which correlates the level of direct response with the infestation observed in both genotypes (Agut et al. 2015; Figure 1.1 Suppl.),. Remarkably, Cleopatra mandarin plants induced by sour orange HIPVs became repellent as well. This result correlates not only with the enhanced expression of SA and JA markers in induced Cleopatra (Figure 1.1 and 1.2 Suppl.) but also with a specific volatile profile. From the eight volatiles reported in Table 1.1, the production of the GLV 2-ethyl-1-hexanol increased in induced Cleopatra, whereas that of two aromatic derivatives and two additional GLVs decreased. These results confirm that Cleopatra mandarin is sensitive to the volatile organic compounds (VOCs)-induced direct resistance producing an antixenotic response, which is likely based on the production of a specific blend of volatiles.

The preferences of the three phytoseiids when exposed to the odors of *T. urticae*, plants, and the combination of these two are shown in Figures 1.2, 1.3 and 1.4. Contrary to what was observed for *T. urticae*, the three predators always preferred the odor of its prey, *T. urticae*, to clean air. This clearly suggests that these predators can effectively smell the herbivore. The characterization of *T. urticae* volatile profile allowed the identification of

twelve compounds that were consistently detected regardless of the mite rearing substrate (Table 1.2). Seven of them were confirmed with commercial standards and include six GLVs: three simple isoprenoid alcohols, two short-chain aldehydes, and hexanoic acid. The last confirmed volatile in the blend is the HIPV MeSA. Four additional volatiles were tentatively identified as the structurally related lilac ketone and lilac aldehyde isomers. In the experiments where both clean genotypes (no previous mite infestation) were contrasted, all three predators preferred sour orange independently of their degree of specialization (Figures 1.2 to 1.4). This behavior changed when the phytoseiids had to choose between *T. urticae*-infested plants. The generalist *E. stipulatus*, same as its prey, preferred Cleopatra mandarin whereas the other two phytoseiids showed no preference for any of them. When comparing the same plant genotype, either infested or not, predators always preferred infested plants. Despite these interesting observations, in the experiments where we studied the VOCs-induced indirect defense, we observed that both *E. stipulatus* and *N. californicus* preferred Cleopatra mandarin-induced plants while *P. persimilis* remained neutral. These diverging results may be related predator diet specialization.

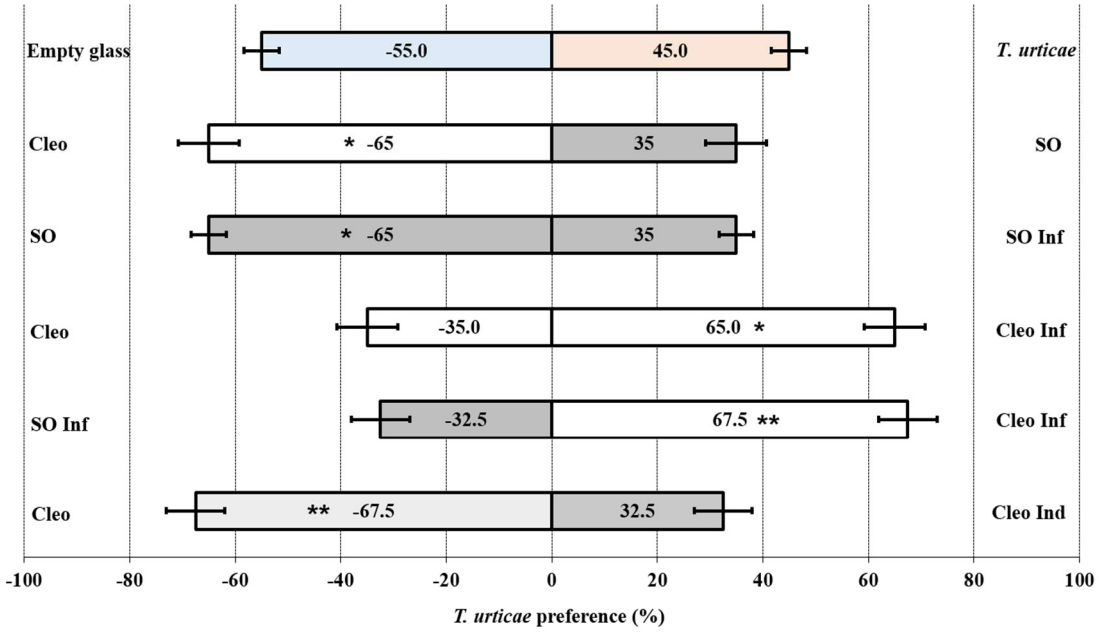

Figure 1.1. Olfactory response of *T. urticae* to conspecific mites either with or without plant substrate. Six different combinations, in which *T. urticae* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus conspecifics, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

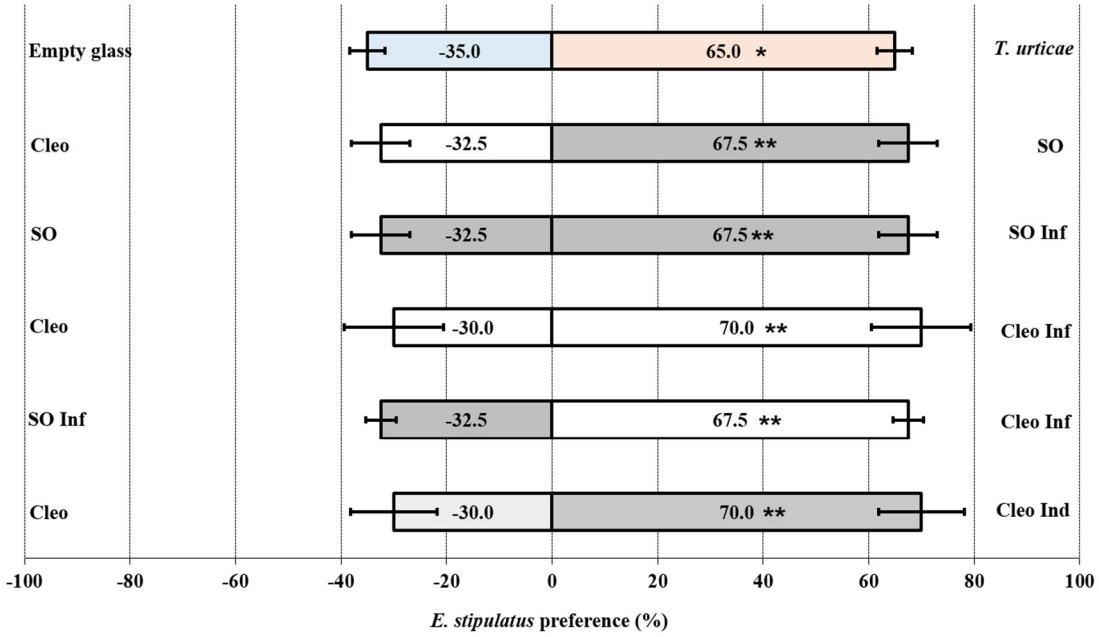

Figure 1.2. Olfactory response of *E. stipulatus* to *T. urticae* either with or without plant substrate. Six different combinations, in which *E. stipulatus* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus*T. urticae*, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

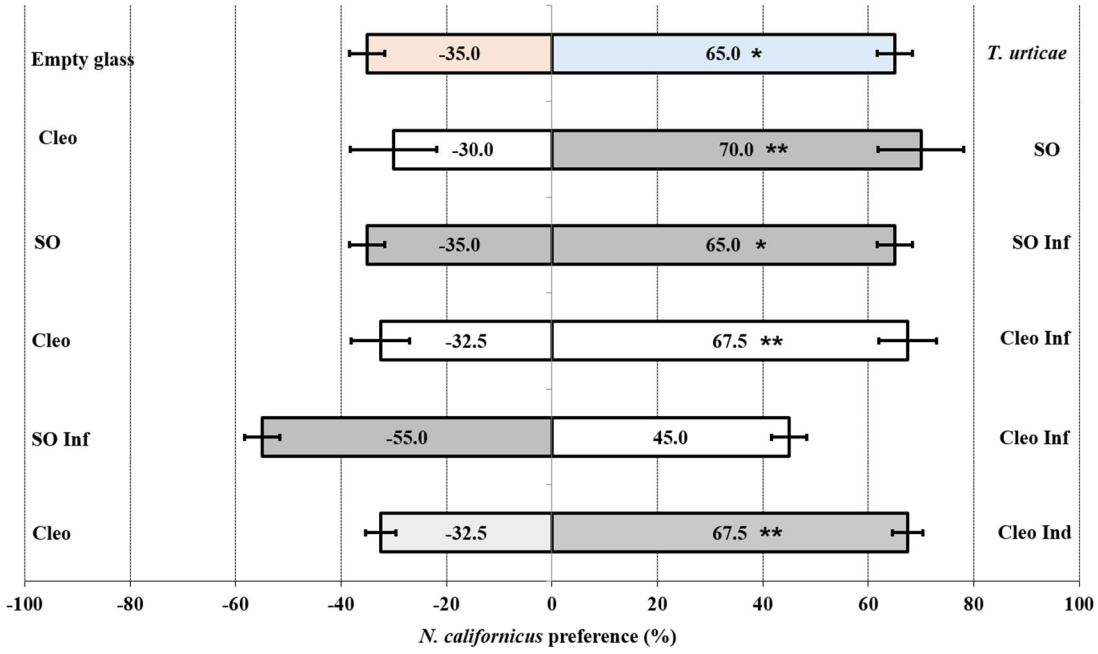

Figure 1.3. Olfactory response of *N. californicus* to *T. urticae* either with or without plant substrate. Six different combinations, in which *N. californicus* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus *T. urticae*, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

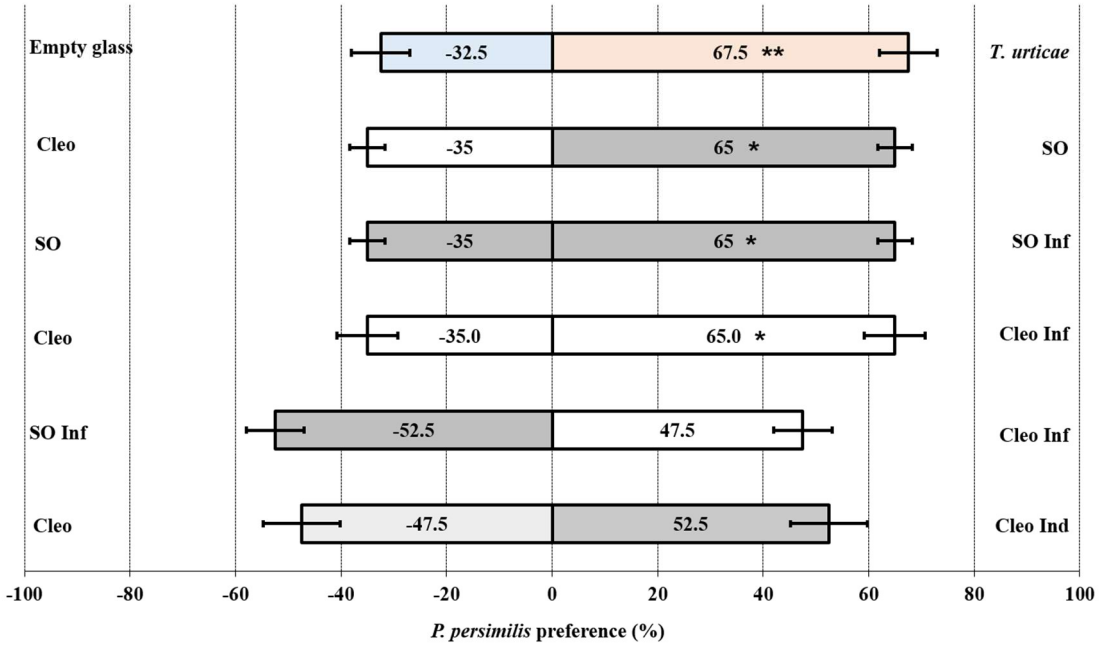

Figure 1.4. Olfactory response of *P. persimilis* to *T. urticae* either with or without plant substrate. Six different combinations, in which *P. persimilis* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus *T. urticae*, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo Ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

Table 1.1. Tentative identification[1] of the compounds detected in the headspace of Cleopatra mandarin (Cleo) plants without treatment (Cleo control) or induced by the HIPVs from *T. urticae* infested sour orange plants (Cleo induced) (mean TOF-MS-derived peak areas ± standard error). For each volatile, TOF-MS-derived peaks areas were compared using a Generalized Linear Model where treatment and replicated were used as fixed effects. Replicated was significant ($P < 0.05$) in the second, third, fifth and seventh compound, nevertheless as the relative differences observed for treatment were consistent in all replicates, results were interpreted in a qualitative manner. Therefore, different letters represent significant differences between treatments ($P < 0.05$).

Volatile Compounds	Cleo control	Cleo induced
(1-methylethyl)-Benzene	8,413.0 ± 455.9 b	15,407.5 ± 1,485.6 a
1-ethyl-2-methyl-Benzene	30,487.5 ± 6,152.8 b	43,507.5 ± 3,093.2 a
2-ethyl-1-Hexanol	15,468.7 ± 3,909.6 b	50,200.3 ± 9,780.5 a
3-ethyl-3-methyl-Pentane	88,573.0 ± 8,009.3 a	44,584.7 ± 870.6 b
2-butoxyethyl Acetate	20,543.8 ± 7,199.3 b	38,083.7 ± 3,746.1 a
3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester Benzenepropanoic acid	2,550.8 ± 289.9 a	1,717.7 ± 513.9 a
4-hydroxy-4-methyl-2-Pentanone,	28,166.5 ± 4,526.2 a	24,584.8 ± 1,477.6 a
1R-α-Pinene	60,245.0 ± 21,100.1 a	47,417.2 ± 6,888.6 a

[1]Tentative identification of the compounds with spectra and high probability matches (> 80%) according to NIST mass spectral database (Wallis et al. 2008).

Table 1.2. Compounds detected in volatile collections of *T. urticae* (relative mean ± standard error[1] percentage considering the total chromatogram area of the detected compounds) reared on either lemon fruits or clementine mandarin leaves.

Compound	id.[4]	Rearing substrate	
		Lemon fruits	Clementine leaves
2-methyl-3-buten-2-ol	C	18.34 ± 5.05	0.51 ± 0.37
3-methyl-3-buten-1-ol	C	6.44 ± 2.00	6.31 ± 4.06
3-methyl-2-buten-1-ol	C	18.08 ± 9.43	2.22 ± 1.00
Hexanal	C	3.07 ± 1.13	10.21 ± 8.92
Hexanoic acid	C	10.73 ± 4.41	50.91 ± 20.81
5-ethenyldihydro-5-methyl-2(3H)-furanone[2]	T	3.07 ± 1.17	5.29 ± 2.33
Nonanal	C	28.48 ± 10.04	15.27 ± 6.48
5-ethenyltetrahydro-α,5-dimethyl-2-Furanacetaldehyde[3] isomer	T	4.33 ± 2.54	2.28 ± 0.48
Lilac aldehyde isomer	T	7.44 ± 4.11	3.23 ± 0.87
Lilac aldehyde isomer	T	2.39 ± 1.39	0.73 ± 0.26
Methyl salicylate	C	5.23 ± 3.26	3.20 ± 2.62

[1]Means of six replicates for volatile samplings of individuals of the stock colony maintained on lemons and three replicates for samplings of individuals from a colony maintained on clementine mandarin leaves

[2] lilac lactone

[3] lilac aldehyde

[4] Identification of the compound: C, confirmed with commercial standard; T, tentative with spectra and high probability matches (> 80%) according to NIST mass spectral database (Wallis et al. 2008).

1.4 DISCUSSION

Predators are not always attracted to less defended plants

Sour orange plants display higher constitutive and faster inducible direct defense against *T. urticae* compared with Cleopatra mandarins, which eventually results in the latter supporting higher *T. urticae* densities and increased plant damage (Bruessow et al. 2010; Agut et al. 2014, 2015). Therefore, according to our initial hypothesis, infested Cleopatra mandarins were expected to be more attractive for phytoseiids than infested and well-defended sour orange plants. However, in our experimental conditions only the omnivorous predator *E. stipulatus*, same as the herbivore, preferred Cleopatra mandarin when the two infested genotypes were simultaneously offered (Figures 1.1 and 1.2). The other two predators showed no preference for these infested genotypes (Figures 1.3 and 1.4). Following the same rationale, induced Cleopatra mandarin plants, which exhibit enhanced expression of *LOX2* and *PR5* genes (Figures 1.1B and 1.2B Suppl.), should not have been chosen by predators when simultaneously offered with clean Cleopatra mandarin plants. Indeed, this is what the herbivore did. However, both *E. stipulatus* and *N. californicus* preferred the better-protected and void-of-prey Cleopatra induced plants, whereas *Tetranychus* spp.-specialist *P. persimilis* did not show any preference. Consequently, these results provide evidence that predator responses depend on plant genotype and diet specialization. Interestingly, predators are not always attracted to the less defended plants. For omnivores, plant defense induction could be a general clue of *T. urticae* presence in the area.

The well-known negative crosstalk between JA- and SA- defense pathways may be missing in citrus

Although some trade-offs between direct and indirect defenses have been suggested in specific plant-arthropod interactions (Koricheva et al. 2004), there are also reports in which both sorts of defense function synergistically (Rasmann et al. 2011; Pellissier et al. 2016). This could be the case for citrus as well, as evidenced by our observations in sour orange and induced Cleopatra mandarin plants (Figures 1.1B and 1.2B Suppl.). Indeed, sour orange appears to be a jack-of-all-trades, as it seems to have maximized different types of defense against this mite. A clear observation in the absence of infestation is that all predators are more attracted to sour orange, contrary to what was observed for the herbivore. Furthermore, the volatile profile of infested sour orange and induced Cleopatra

mandarin changed relative to clean plants. Remarkably, the VOC profiles described in infested sour orange (Agut et al. 2015) and those found in induced Cleopatra mandarin are different and just share the monoterpene pinene. It is very likely that these defense responses in sour orange are responsible for the repellence of *T. urticae* as well as the attractiveness of phytoseiids. Therefore, the three volatile blends identified so far (those corresponding to infested sour orange, induced Cleopatra mandarin, and *T. urticae*) are triggering similar behavioral responses in the four mite species studied: attraction of natural enemies but not of the herbivore. These blends deserve further studies, as they may provide new tools to manage these mites in crops.

Plant feeding by spider mites can activate both JA- and SA-related signaling pathways (Kant et al. 2004; Kawazu et al. 2012). However, the decreased performance of these mites (i.e., direct defense) has been associated with the induction of JA-related defenses and the accumulation of additional secondary metabolites such as glucosinolates (Kant et al. 2007; Agut et al. 2014, 2016; Zhurov et al. 2014). Therefore, the simultaneous upregulation of both defensive pathways in infested sour orange (Figures 1.1A and 1.2A Suppl.; Agut et al. 2014) and in induced Cleopatra mandarin (Figures 1.1B and 1.2B Suppl.) indicates that the well-known negative crosstalk between JA- and SA- defense pathways (i.e., the antagonistic interaction between the SA- and the JA-response pathways) (Pieterse et al. 2009; Robert-Seilaniantz et al. 2011) may be missing in citrus.

***Tetranychus urticae*-associated volatiles include MeSA**

Interestingly, our results have shown that *T. urticae* associated odors include MeSA (Table 1.2), a volatile that had been previously identified in Cleopatra mandarin and sour orange HIPVs (Agut et al. 2015). However, we suspect that the amount of MeSA produced by the mite is orders of magnitude below what plants can produce, as we have been unable to detect this compound in infested lemons using the method described above for induced Cleopatra mandarin HIPVs. MeSA had been also found in the blend of volatiles produced by *T. urticae* female teliochrysalis and adult males (both stages were likely present in the mixed pool of mites used to characterize *T. urticae* associated volatiles (Table 1.2)) together with three additional volatiles, including methyl *cis*-dihydrojasmonate (Oku et al. 2015). In their study, this blend was shown to mediate male discrimination between male-guarded and solitary female teliochrysalis. Although different butterfly species of the genus *Pieris* Schrank (Lepidoptera: Pieridae) can use the amino acid phenylalanine as a precursor to MeSA (Andersson et al. 2000, 2003), *T.*

urticae most probably obtains this volatile from its host plants (Oku et al. 2015). Because SA has been widely recognized as a key factor for predator recruitment by infested plants (i.e., indirect defense) (Mallinger et al. 2011; Rodríguez-Saona et al. 2011; Kaplan 2012; Rowen et al. 2017; Salamanca et al. 2017), the question of why a plant volatile exploited by natural enemies as a kairomone is not immobilized/degraded by its potential prey, deserves further investigations.

Blends rather than single compounds matter

Importantly, it is often the whole blend rather than single volatiles what predatory mites exploit to communicate (McCormick et al. 2012). Indeed, in their study Oku et al. (2015) could not attribute the behavioral differences observed in male *T. urticae* to a single compound but to the whole blend. Moreover, van Wijk et al. (2008, 2011), showed that although MeSA alone, which was produced by *T. urticae*-injured lima bean plants, was attractive to *P. persimilis*, attraction increased when MeSA was part of the natural HIPV blend produced by the plant. Interestingly, one of the volatiles in that blend, the GLV (Z)-3-hexenyl acetate, was repellent to *P. persimilis* when tested alone. Likewise, in our case, attraction to the three phytoseiids tested could be attributed to the blend in Table 1.2 rather than to a single volatile. Most of these compounds have been reported as aggregation pheromones in several bark beetles (Bakke et al. 1977; Stoakley et al. 1978; Bowers et al. 1991). Lilac-related compounds have been described as volatile constituents of plant essential oils (Jerković et al. 2017; Peron et al. 2017). Moreover, lilac aldehyde stereoisomers have been identified in the flower scent of many plant species, with an important role for the attraction of pollinators (Dötterl and Jürgens 2005; Dötterl et al. 2006). Although the role of *T. urticae* associated volatiles needs further investigations, their origin, same as MeSA, is likely the host plant (Castro-Vázquez et al. 2009), from where they may have been acquired either directly or as precursors (Reddy and Guerrero 2004).

Diet specialization may partly explain phytoseiid choices

As pointed out earlier, the SA-dependent signaling pathway is considered key for indirect defense. Actually, MeSA has been shown to attract phytoseiid mites (de Boer and Dicke 2004; van Wijk et al. 2008, 2011; Shimoda 2010). Therefore, plants with relatively enhanced activation of the SA signaling pathway were expected to be selected by phytoseiids in our two choice-tests. However, this was not always the case. For most of

these exceptions, an over-ruling of prey-related odors, which interestingly include MeSA (Table 1.2), can explain the results. This is the case of *N. californicus* and *P. persimilis*, which showed no preference when offered the two infested genotypes (when a preference for infested Cleopatra mandarin was anticipated as MeSA levels are higher in this genotype, Agut et al. 2015). Nevertheless, this prey over-ruling hypothesis does not explain the preferences of *E. stipulatus* and *N. californicus* for induced Cleopatra mandarin over clean Cleopatra plants (where no preference was expected as MeSA was not differentially produced in these genotypes; Table 1.1). These differences among predators may be partly due to their different diet specializations (McMurtry and Croft 1997; McMurtry et al. 2013), which may affect the interpretation of the meaning of the different volatile blends.

The high polyphagy of *T. urticae* (Migeon and Dorkeld 2006-2017) results in the induction of quantitatively and qualitatively different HIPVs in different host plants (Van den Boom et al. 2004) and this might hamper prey location by its natural enemies. *Phytoseiulus persimilis* can locate their prey from a distance using volatiles, including MeSA, emitted by plants infested with spider mites (Sabelis and van de Baan 1983; Sabelis et al. 1984; Dicke et al. 1990). However, this phytoseiid selected volatiles from prey-infested leaves, *T. urticae*, rather than leaves infested with a non-prey close relative, *Panonychus ulmi* (Koch) (Acari: Tetranychidae) (Sabelis and van de Baan 1983). For specialist predators (i.e., *P. persimilis*), the density of its main prey on the infested plant has to be enough as a reward as this is their only suitable food for complete development and successful reproduction. Therefore, it is not surprising that in our experiments *P. persimilis* responded mainly to the blend of *T. urticae*-associated volatiles (Figure 1.4). Although it detected and reacted to the upregulation of SA-signaling *PR5* gene in clean sour orange when offered together with clean Cleopatra mandarin, the lower levels in induced Cleopatra mandarin (Figure 1.2B Suppl.) did not trigger the same behavior of *P. persimilis* when the predator had to choose between induced and clean Cleopatra mandarin plants. Indeed, this predator is known to respond to MeSA, which was induced in both sour orange and Cleopatra mandarin by *T. urticae* (Agut et al. 2015), in a dose-dependent manner (de Boer and Dicke 2004). However, for extreme omnivorous predators, including zoophytophagous species, which can obtain their food from different prey species and even from the host plant, both prey-specific chemical cues and HIPVs may be equally important to select patches with enough prey diversity and abundance but

also with minimal plant direct defense. *Euseius stipulatus* is the only predator from the three species included in this study that most probably belongs to the group of phytoseiids that may complement their nutrition requirements by feeding on leaf epidermal cells (Adar et al. 2012; McMurtry et al. 2013). Therefore, *E. stipulatus* may benefit from choosing the plant genotype showing the weakest defense when infested by *T. urticae* (Agut et al. 2014). By preferring Cleopatra mandarin to sour orange when both genotypes were infested (Figure 1.2), *E. stipulatus* also selects the host likely offering higher densities of the prey and this would eventually benefit the plant as well, as this omnivorous predator may choose to feed preferentially on the prey and not on the plant. As MeSA was not differentially produced in the blend of volatiles produced by Cleopatra mandarin upon induction by sour orange HIPVs (Table 1.1), other volatiles must have a more important role in governing *E. stipulatus* choices and this should be partly true for *N. californicus* as it exhibited a behavior in between this generalist and the specialist *P. persimilis*.

1.5 CONCLUSION

To sum up, our results provide evidence that the response of the four mite species included in this study is plant genotype dependent and is modulated by their feeding habits, as well as by the presence of the herbivore on the plant. Some of these behavioral responses in *T. urticae* had already been described by our group (Agut et al. 2015). Interestingly, the discrimination by *T. urticae* between Cleopatra mandarin plants either clean or induced with HIPVs from *T. urticae*-infested sour orange, and the fact that this mite did not show any preference when exposed to volatiles emitted by conspecifics, confirms that this behavior is triggered by plant HIPVs only.

Further research focused on the three volatile blends that have been identified in this study as attractive for *T. urticae* natural enemies but not for the herbivore could provide new more sustainable tools with clear applications in crop protection (i.e., use of volatile dispensers for predator recruitment and plant defense enhancement). Furthermore, the accumulation of MeSA in *T. urticae*, which, on the one hand, may have a direct impact on plant defense (i.e., priming) and, on the other, on recruiting natural enemies, should be also further studied.

Supplementary material

Table 1.1 suppl. Primers used in qRT-PCR reactions.

Description	Accession	Forward primer 5'→3'	Reverse primer 5'→3'
LOX2	Cit.16756.1.S1_s_at	GAACCATATTGCCACTTTCG	CGTCATCAATGACTTGACCA
PR5	BAI63297.1	CATCAAGCTTCACAGTGCTTAG	CCACAACGTACAGACTGATGAC
GAPDH	Cit.122.1	GGAAGGTCAAGATCGGAATCAA	CGTCCCTCTGCAAGATGACTCT

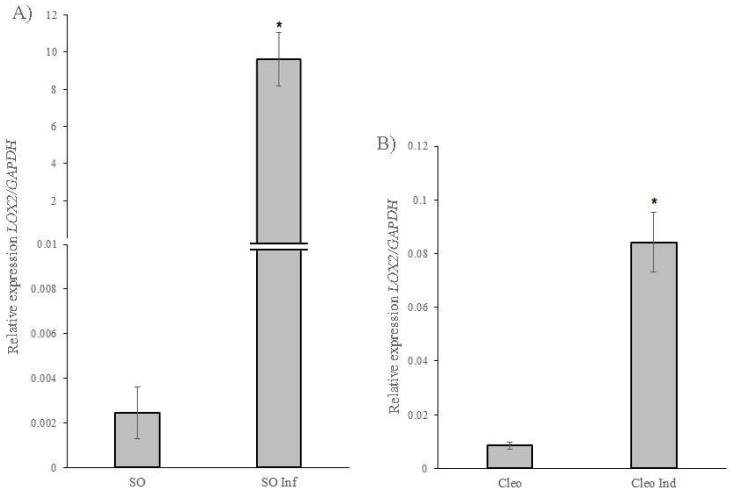

Figure 1.1 suppl. Induction of defensive pathways in Cleopatra mandarin by exposure to HIPVs produced by neighboring sour orange plants infested with *T. urticae*. Lipoxygenase2 gene (*LOX2*) induction following different treatments; A) *LOX2* expression in untreated sour orange plants and 72 h post-infested sour orange plants with *T. urticae*. B) *LOX2* expression in untreated Cleopatra mandarin plants and at 72 h post-exposure to sour orange herbivore-induced plant volatiles (HIPVs). The *LOX2* transcript levels were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured in the same sample. The data are presented with a representative figure for four independent experiments of the analysis behavior through the olfactometer of the mites studied in the present work, in Cleopatra mandarin induced plants. Significant differences in the relative transcript levels between different treatments were estimated using a *t*-test. The asterisk indicates significant difference to different treatments (*t*-test; $P < 0.05$).

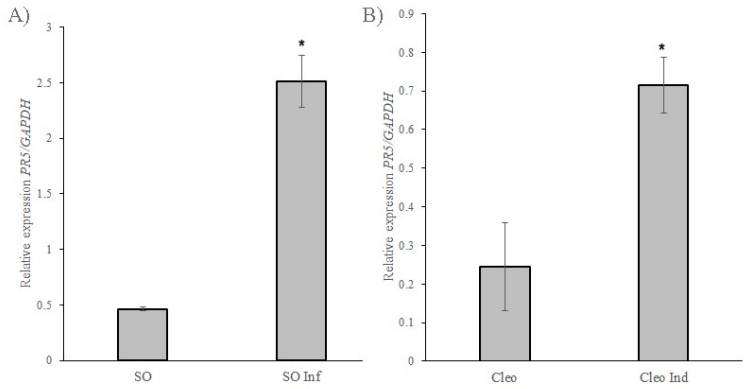

Figure 1.2 suppl. Induction of defensive pathways in Cleopatra mandarin by exposure to HIPVs produced by neighboring sour orange plants infested with *T. urticae*. Pathogenesis-related protein 5 (*PR5*) induction following different treatments; A) *PR5* expression in untreated sour orange plants and 72 h post-infested sour orange plants with *T. urticae*. B) *PR5* expression in untreated Cleopatra mandarin plants and at 72 h post-exposure to sour orange herbivore-induced plant volatiles (HIPVs). The *PR5* transcript levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured in the same sample. The data are presented with a representative figure for the four independent experiments of the analysis behavior through the olfactometer of the mites studied in the present work, in Cleopatra mandarin induced plants. Significant differences in the relative transcript levels between different treatments were estimated using a *t*-test. The asterisk indicates significant difference to different treatments (*t*-test; $P < 0.05$).

CAPÍTOL 2

Tetracycline-treated *Tetranychus urticae* shows a differential mating behavior and microbiota

ABSTRACT

The two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is an important pest of citrus in Spain. Bacteria in the genus *Wolbachia* infect this mite, which can also harbor other endosymbionts. Facultative symbionts as *Wolbachia* spp. are mutualistic in the context of various ecological interactions, and they can have important implications for the management of pest species. The genus *Wolbachia* is a strict intracellular bacteria that cause several types of reproductive distortions in its host like male feminization, thelytokous parthenogenesis, cytoplasmic incompatibility, or male death. The presence of different *Wolbachia* strains can affect mite reproductive parameters. The effect of tetracycline treatment on spider mite reproduction was evaluated through different crosses between treated and untreated mites (T- and U- mites respectively). Four different crosses were tested: 1) U-♀ × U-♂; 2) T-♀ × T-♂; 3) U-♀ × T-♂; 4) T-♀ × U-♂; with two controls: 5) virgin untreated females (U-♀) and 6) virgin treated females (T-♀). To study the effect of antibiotic treatment in mite microbiota, 16S deep sequencing has been done using two different combination of primers. The sequences obtained were compared with two databases for OTUs (Operational Taxonomic Unit) identification: (1) SILVA and (2) a non-redundant nucleotide database.

Tetracycline treatment induced cytoplasmic incompatibility in *T. urticae* by affecting offspring sex ratio, indicating the presence of a mating distorter bacterium in our citrus *T. urticae* population. The antibiotic treatment removes mainly *Wolbachia* spp. Bacteria diversity identification was affected not only by the selected primers, but also by the database used.

2.1 INTRODUCTION

Bacterial symbionts are tremendously abundant among invertebrates, particularly among arthropods (Zchori-Fein and Bourtzis 2011; Zug 2018). On these hosts, these symbionts have been shown to significantly influence diverse processes including nutritional status, reproduction, lifespan, and resistance to insecticides (Bourtzis and Miller 2008). Commonly, the prevalence of these reproductive parasites in a host population is secured by increasing the proportion of infected females through various mechanisms including cytoplasmic incompatibility (CI), feminization, parthenogenesis or male killing (Duron et al. 2008; Werren et al. 2008; Engelstädter and Hurst 2009). These reproductive disorders may have interest in their application in biological control programs (Zabalou et al. 2004).

The two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a citrus key pest in Spain (Aucejo-Romero et al. 2004; Ansaloni et al. 2007; Pascual-Ruiz et al. 2014a). This mite is a polyphagous herbivore with a haplodiploid arrhenotokous reproductive system (unfertilized eggs develop haploid males, and fertilized eggs develop diploid females) (King et al. 2006). The characterization of the microbiota inhabiting this mite has been previously targeted. This species harbors so-called reproductive bacterial-manipulators like *Wolbachia* (Rickettsiales) (Breeuwer and Jacobs 1996), *Cardinium* (Cytophagales), *Spiroplasma* (Entomoplasmatales) and *Rickettsia* (Rickettsiales) (Zhang et al. 2016).

Sequence data on the arthropod symbionts can provide valuable information for elucidating the microbiome arthropod composition. The application of New Generation Sequencing (NGS) technologies allows the detection of every bacterium, fungus or virus present in an organism at low cost and in a short time. It also holds the promise of revealing the genomes of the majority of microorganisms that cannot be readily obtained in pure culture (Hugenholtz 2002). The importance of the microbiome has been widely recognized for a wide range of animals, and for arthropods this topic is gaining considerable importance. Meta-omics techniques revealed useful information in microorganism pests' life and interaction with the environment (Malacrinò 2018).

The genus *Wolbachia* is the most abundant intracellular bacterium in arthropods and nematodes, estimated to infect 52% arthropod species (Weinert et al. 2015). *Wolbachia* has also been found infecting a large number of mites (Zhang et al. 2016) especially

Tetranychidae (Gotoh et al. 2003; Pina et al. 2019). This bacterium has been found as the main endosymbiont in *T. urticae* Spanish citrus populations (Pina et al. 2019). Although the genus *Wolbachia* is the most common endosymbiont in arthropods, other mating distorter bacteria can play an important role in their mating behaviour. Bacteria in the genus *Cardinium* have been found in co-infections with *Wolbachia* (Gotoh et al. 2003, 2007; Liu et al. 2006; Xie et al. 2011; Ros et al. 2012; Suh et al. 2015; Zhang et al. 2016; Zélé et al. 2018a) and occasionally also with *Spiroplasma* (Zhang et al. 2016; Staudacher et al. 2017) and *Rickettsia* (Jeyaprakash and Hoy 2005; Zhang et al. 2016). To determine the microbiota diversity in an organism the 16S region of ribosomal bacterial DNA (16S rDNA) is used as the main phylogenetic marker (Snel et al. 1999). Combining NGS with the amplification of this region provides the possibility to use 16S rDNA deep sequencing methodologies to get the Operational Taxonomic Units (OTUs) of an organism, and can determine all mating disorder bacteria present in an arthropod population. This approach has been used already in arthropods like aphids (Gauthier et al. 2015; Jousselin et al. 2016), ticks (Couper and Swei 2018) and mites (Pekas et al. 2017).

The effects of *Wolbachia* in the life parameters of their arthropod hosts have been intensively studied. The evolutionary success of this α-proteobacterium is due to its ability to manipulate the host reproductive system and promote the spreading of infection throughout the arthropod populations through a variety of strategies including CI, male killing, thelytokous parthenogenesis, and feminization of genetic males (Stouthamer et al. 1999; Dedeine et al. 2001; Stevens et al. 2001; Hosokawa et al. 2010). From those, CI is the most common effect of *Wolbachia* infection. This endosymbiont is present in the female germline stem cells and is vertically transmitted to the next generation with a transmission efficiency close to 100% (Jiggins et al. 2002).

To elucidate the role of *Wolbachia* in the *T. urticae* reproduction Breeuwer (1997) investigated the effect of this symbiont in crosses between an infected and a cured *T. urticae* strain collected from tomato plants, finding CI produced by *Wolbachia*. It has been proven that *Wolbachia* can provide a reproductive isolation mechanism in a sympatric speciation process (Vala et al. 2000). Probably, this endosymbiont also promote the avoidance of the CI process by favoring the mating between individuals in compatible crosses (Vala et al. 2004). It is unclear whether the *Wolbachia* found in *T. urticae* Spanish citrus orchards populations can induce the same reproductive disorders.

The aim of this work is to elucidate the effect of the microbiome in the biological parameters of *T. urticae* populations from Spanish clementine orchards, to find new approaches to improve biological control strategies. To obtain this information, we analyzed the bacterial symbiont community by 16S rDNA deep sequencing in a laboratory reared tetracycline-treated (T-line) and untreated (U-line) *T. urticae* populations. Crossing experiments between these lines could determine the effect on the affected microbiota in *T. urticae* reproduction mode.

2.2 MATERIALS AND METHODS

2.2.1 *Tetranychus urticae* rearing

Tetranychus urticae stock colony used in our assays was initiated in 2001 with specimens collected from clementine mandarin orchards in the region of La Plana (Castelló, Spain). This colony was reared on lemons [*Citrus limon* (L.) Burm f. (Rutaceae)] maintained at room conditions and natural photoperiod (Aucejo-Romero et al. 2004; Pérez-Sayas et al. 2015).

2.2.2 Plant material

Pesticide-free leaves of clementine mandarin (*Citrus clementina* Hort ex Tan. (Rutaceae) cv. Clementina de Nules (INIASEL 22)) grafted on citrange Carrizo rootstock (*Poncirus trifoliata* (L.) Rafinesque-Schmaltz x *Citrus sinensis* (L.) Osbeck (Rutaceae)) were obtained from greenhouse-protected 2-years-old trees grown at University Jaume I (UJI). Leaves were collected as required, washed with chlorinated tap water, rinsed in distilled water and dried prior to use. Bean leaflets (*Phaseolus vulgaris* L. (Fabaceae)) –from UJI specific greenhouse– were handled following the same procedure.

2.2.3 Antibiotic treatment of spider mite cohorts and determination of *Wolbachia* presence or absence

Clean-fresh bean leaflets were treated by dipping into a 0.1% tetracycline hydrochloride solution (Sigma-Aldrich cat no. T3383) and then placed upside down over a cotton piece imbibed on 0.1% tetracycline solution (approx. 1 liter of solution) kept in a 35 × 20 × 7 cm tray. A control batch of bean leaves was set up in the same way, replacing the tetracycline solution by distilled water.

Around 50 adult female mites (from mother colony) were placed on tetracycline treated bean leaflets for four days. At day 5, tetracycline-treated mites (T-mites) were transferred to control leaves to allow the hatching of the treated line (T-line) F1 eggs. Afterwards, T-line F1 larvae were transferred to tetracycline treated-fresh bean leaves for another 12 days (with leaves replacement every 4 days) until adult development. The same treatment was followed with T-line F2 eggs, which constituted the T-line cohort. In a similar way, an untreated control cohort (U-line cohort) was established using control bean leaflets. Afterwards, T and U-line cohorts were maintained isolated in separate climatic chambers (25 ± 2.5°C, 75 ± 5% RH and 16:8 h L:D photoperiod) in a semi-mass rearing environment on clementine mandarin leaves without further antibiotic treatments.

T- and U-lines were checked regularly by polymerase chain reaction (PCR) to ascertain the presence of *Wolbachia*, including the first check at F2 prior line establishment. Mites were surface sterilized with 70% ethanol, air dried, and used individually (at F2), or in a 12 individuals pool (every 10 generations), for DNA extraction following Salting-OUT protocol (Sabater-Muñoz et al. 2012). All DNA extractions were verified by PCR on mite ITS2 region (Pérez-Sayas et al. 2015) prior to *Wolbachia* screening to ascertain the presence of DNA in the sample. *Wolbachia* screening PCR was conducted with primers 99F and 994R (O'Neill et al. 1992) using 1x PCR buffer (FIREPol® without $MgCl_2$), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U. Taq pol (FIREPol®, Solis BioDyne, Tartu, Estonia), in 25 µl reaction volume. Amplification was conducted in a thermal cycler (Bio-Rad C1000™ Thermal Cycler) with one first denaturation step at 94ºC for 5 min, followed by 30 cycles of 95ºC for 1 min, 52ºC for 1 min, 72ºC for 1 min and a final extension at 72ºC for 5 min. Amplifications were verified in 2% agarose D-A low EEO (Pronadisa, Sumilab S.L., Madrid, Spain) gel electrophoresis in 1x TAE stained with GelRed (Biotium,

Hayward, CA), using the 100 bp DNA ladder as size molecular marker (Invitrogen, Carlsbad, CA, USA).

2.2.4 Bacteriome characterization

Bacterial communities inhabiting *T. urticae* T- and U-lines were determined by 16S rDNA NGS sequencing (Illumina). Fifty live individuals (mixed ages and sexes) of each *T. urticae* population (T- and U- lines) were soaked into 70% ethanol: 10% bleach solution (~ 0.5 ml per batch) for 20 seconds, then washed with 70% ethanol for 60 seconds, and allowed to air-dry before DNA extraction. DNA extraction was performed with ZymoBIOMICS® DNA mini kit (cat no. D4301, ZYMO, Irvine (CA), USA) following manufacturer's protocol. DNA samples were quantified with nanodrop. 16S rDNA amplicon targeting V3-V4 region were amplified following the 16S rDNA gene metagenomics sequencing library preparation protocol (cod 1504423 rev. A, Illumina, NY, USA) with two primer pairs: (i) 16SrDNAampliconPCR_forward and 16SrDNAampliconPCR_reverse (corresponding to primers Bakt_341F and Bakt_805R from (Herlemann et al. 2011; Thijs et al. 2017)) named as old lines (primer pair 1) and (ii) 16SrDNAampliconPCR_F2 (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG YCT ACG GRN GGC WGC AG-3') – primer developed after alignment of 16S full sequences from several *Wolbachia, Spiroplasma, Rickettsia* and *Cardinium* species over the same region as 341F, containing at its 5' end the Illumina adapter– and 16SrDNAampliconPCR_reverse named as new lines (primer pair 2). 50 ng of each DNA sample was used as starting material for each primary PCR (performed in triplicate with Kapa HiFi polymerase as indicated in the manual), followed by indexing labelling for multiplexing step using Nextera XT DNA Library preparation kit (cat no. FC-131-1096, Illumina). Libraries were size verified in a DNA 1000 chip (Agilent 2100 Bioanalyzer, Waldbronn, Germany) prior sequencing in a MiSeq (Illumina) at 2x300 pb paired-end run (MiSeq reagent kit v3; cat no. MS-102-3001; Illumina) following manufacturer's instructions with 150 cycles. 16S amplicons, libraries and runs were performed in VALGENETICS, an external services' UJI authorized provider (VALGENETICS SL, València, Spain).

2.2.5 *Tetranychus urticae* mating assays

The effect of tetracycline on spider mite reproduction was evaluated through different crosses between T- and U- mite lines. A total of six different crosses (treatments) were tested: (1) untreated females and males (U-♀ × U-♂); (2) treated females and males (T-♀ × T-♂); (3) untreated females with treated males (U-♀ × T-♂); (4) treated females with untreated males (T-♀ × U-♂); (5) virgin untreated females (U-♀) and; (6) virgin treated females (T-♀).

To perform these crosses, separate cohorts from T- and U-lines were established on clementine leaves with ca. 80 adult females each. Founder females were discarded after 24 h and synchronized eggs were allowed to complete development until the teleiochrysalis stage (the final molting stage previous to adulthood). Female teleiochrysalis from both lines were separated in three batches of 20 individuals with a similar number of newly emerged adult males, except for those females ("treatment") that should remain virgin. Adult males came from T- or U- lines, depending on the crosses tested. After 72 h, males were removed, and at least 12 females per treatment were isolated and transferred daily for 4 consecutive days to new clementine leaves. The number of eggs laid (clutch size), the egg hatching, offspring sex ratio (% females) and number of males was recorded per female and day. On day 5, females were collected and individually tested by PCR as described previously (both for DNA and *Wolbachia* presence). Each cross type (treatment) was repeated twice (2 replicates). All crosses were performed in climatic chambers as described above.

2.2.6 Bacteriome data analysis

Bacteriome raw sequences were pre-processed in using a custom pipeline with cutadapt v1.9.1 (Martin 2011) and uchime v4.2 (Edgar et al. 2011). Sequences were analysed for quality, read length, reads pairing and chimera formation, after removal of library barcode and sequencing primers. Cleaned sequences were mapped against the SILVA NR database v132 and then clustered in OTUs at 97% similarity using the MOTHUR pipeline v1.39.5. Taxonomy of OTUs was assigned using the RDP classifier trainset16_022016 (Wang et al. 2007; Schloss et al. 2009). The OTUs-tables were depleted of non-bacterial sequences (plant-organelles, Eukaryota, Archea) prior assessment of species richness and bacterial composition, by determining Shannon diversity (weight of the number of species

by their relative evenness data, Shannon 1948) and Inverse Simpson indexes ((1/lambda) measure of the effective number of parties, richness, Simpson 1949) (Smith and Wilson 1996) for each data set in MOTHUR (Schloss et al. 2009). Venn diagrams were used to highlight shared and unique bacterial taxa (OTUs) between libraries and mite lines (Schloss et al. 2009). This representation was created using the bioinformatics tool Venny 2.1 (https://bioinfogp.cnb.csic.es/tools). Final representative sequences were aligned using MUSCLE and phylogenetic trees were constructed from filtered alignments using Fast Minimum Evolution model, as implemented in MOLE-BLAST (Altschul et al. 1997; Edgar 2004). In addition, those OTUs that could not be assigned to a bacterial species genus, directly with MOTHUR, were further screened against the whole Genebank 16SrRNA repository with blastN (Altschul et al. 1997).

2.2.7 Crosses data analysis

To study the effects of the different factors (Treatment and Replicated) on the variables: oviposition, mortality, hatching and sex-ratio separately, we used generalized linear models GLM (McCullagh and Nelder 1989), with a Poisson distribution of the error of the counted data. In all cases, the factors “Treatment” (cross 1 to cross 4, as crosses 5 and 6 are controls) and “repetition” (1 or 2), were used as fixed effects. Once tested the signification of both factors, Tukey post-hoc pairwise multiple comparisons tests were performed when necessary to separated means, using the package multcomp for R (Hothorn et al. 2008).

Omnibus test, the absence of over dispersion (deviance/df close to 1) and the relative model quality according to log-likelihood and Akaike information criterion (AIC) were used as criteria for model selection.

2.3 RESULTS

2.3.1 Tetracycline treatment affects *T. urticae* microbiota

The MiSeq runs generated a total of 745,380 and 192,822 raw reads for primer pair 1 and primer pair 2 respectively, with average raw read lengths of 250bp for primer pair 1 and 300bp for primer pair 2. After read processing and quality filtering, a total of 242,828 high quality bacterial reads were retained. The primer pair 1 (see material and methods section) was found to be more specific to proteobacteria than primer pair 2, despite the improvement in their sequence by the new two bubbles included. Nevertheless, the primer pair 2 allowed identifying some bacterial species known to be involved in reproductive manipulation of their hosts.

The 242,828 bacterial sequences that clustered at 97% identity to the SILVA database sequences generated a total of 4,539 OTUs, with 717 OTUs from T-line (being distributed into 359 and 358 from primer pair 1 and 2 respectively) and 3,822 from U-line (with 3,534 from primer pair 1, and only 288 from primer pair 2). The distribution of OTUs is depicted in Figure 2.1. Despite these differences, all libraries achieved at least 94% of coverage, which together with the diversity indexes indicated that all putative bacterial species present in each library were identified to the corresponding known species (Table 2.1). The core microbiome of *T. urticae* is composed of 16 OTUs, with a great difference between the groups in each library (Figure 2.1). At the SILVA database, 1,819 OTUs of the U-line were classified as Proteobacteria unclassified, whereas a posterior blastN alignment against the full 16SrRNA GeneBank indicated that almost all these OTUs corresponded to *Wolbachia* species, with 92-97% homology.

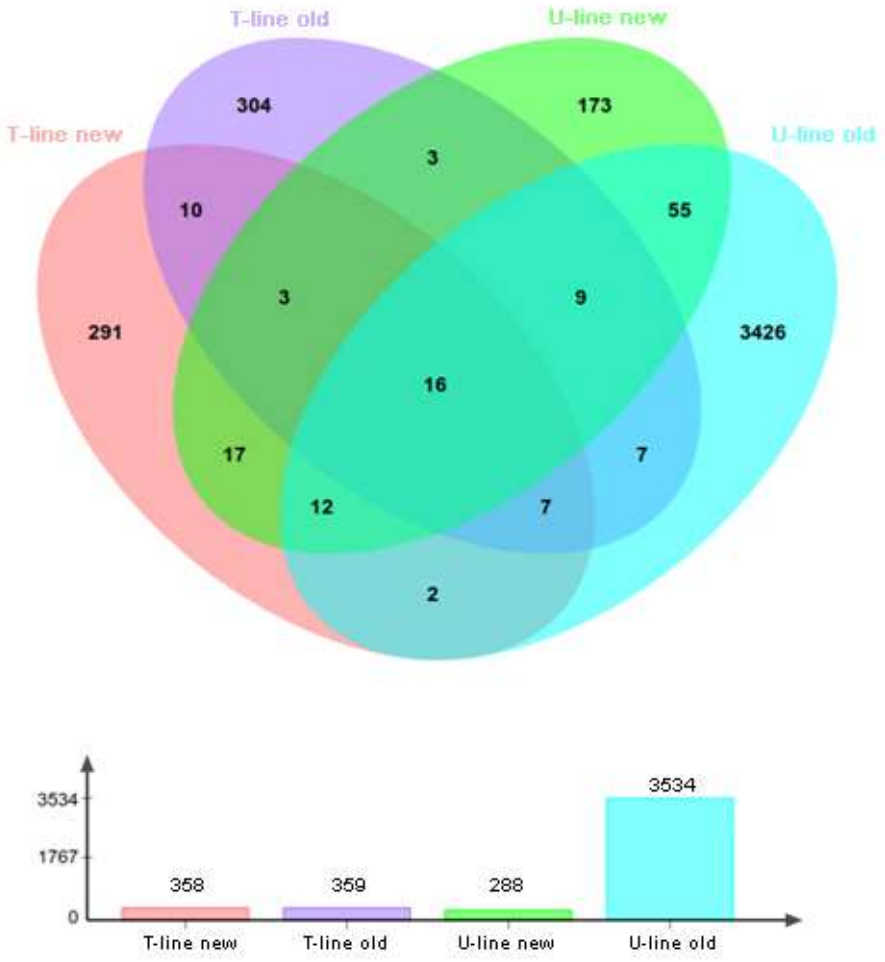

Figure 2.1. A Venn diagram representation of the distribution of the 4,539 OTUs from T and U-lines libraries performed with each primer pair, being primer pair 1 indicated as old and primer pair 2 indicated as new.

Table 2.1. *Tetranychus urticae* microbiome alpha diversity indexes.

Library	Number of reads	Coverage	Observed OTUs	invSimpson	invSimpson lci	invSimpson hci	Shannon	Shannon lci	Shannon hci
T-line old	4,666	0.950922	322	17.795736	16.675632	19.077149	3.797886	3.749236	3.846535
U-line old	159,308	0.983102	3,261	1.105571	1.103128	1.108026	0.518448	0.508794	0.528102
T-line new	41,961	0.995424	298	1.747255	1.728847	1.766059	1.514154	1.492416	1.535892
U-line new	27,072	0.99505	260	1.383033	1.369702	1.396626	0.908627	0.885471	0.931783

lci, lower coefficient interval; hci, higher coefficient interval.

2.3.2 *Wolbachia* alters mating system in *T. urticae*

Table 2.2 shows the effect of tetracycline treatment in the biology parameters: clutch size, egg hatching, immature mortality, offspring sex ratio (as % females from total offspring) and offspring males of *T. urticae* crosses. As indicated at the Methods section, each variable was adjusted to GLM models using cross type and repetition as fixed factors. From the control crosses (5 and 6, untreated and treated virgin females) an only-males F1 was obtained as expected, being removed from the subsequent statistical analysis. Concerning clutch size, the tetracycline treatment itself (♀ T x ♂ T) affected significantly the parameter (Z = -2.301, P = 0.0214) as reported previously, which render the GLM model fit with statistical differences between crosses ($F_{3, 77}$ = 3.0359; P = 0.0341). As described in other unidirectional incompatibility examples, the cross between ♀ T x ♂ U rendered lower egg hatching ($F_{3, 77}$ = 29.94; P = 6.184e-13) and higher immature mortality ($F_{3, 77}$ = 43.05; P = 2.22e-16) when compared with intra-strain crosses (♀ T x ♂ T or ♀ U x ♂ U) or to the reciprocal cross (♀ U x ♂ T). Tetracycline treatment affected the F1 sex ratio of cross between ♀ T x ♂ U (Z = -4.066, P = 4.78e-05) affecting also the GLM fitting model ($F_{3, 75}$ = 5.702; P = 0.0014) despite it did not affect the number of F1 males ($P > 0.05$).

Table 2.2. Effect of tetracycline treatment (mean ± SE) on F1 clutch size, egg hatching, immature mortality, sex ratio (as % females) and offspring males for assayed crosses.

Cross type	N	Clutch size (#)	Egg hatching (%)	Immatures mortality (%)	Sex ratio (% Females)	Offspring males (#)
♀ **U** × ♂ **U**	22	12.6 ± 0.7	87.8 ± 2.5	8.8 ± 2.4	76.1 ± 1.6	2.4 ± 0.2
♀ **T** × ♂ **T**	20	10.2 ± 0.9*	81.1 ± 4.1	7.1 ± 2.7	66.3 ± 4.2	2.5 ± 0.3
♀ **U** × ♂ **T**	22	12.4 ± 1.0	78.7 ± 4.9	12.2 ± 2.9*	71.2 ± 4.2	2.1 ± 0.2
♀ **T** × ♂ **U**	18	13.4 ± 0.7	19.3 ± 2.1*	14.9 ± 6.6*	3.6 ± 2.0*	2.2 ± 0.3
♀ **U**	13	11.3 ± 0.9	78.9 ± 5.7	13.3 ± 4.5	0	7.5 ± 0.8*
♀ **T**	18	10.7 ± 1.1	83.1 ± 4.1	6.6 ± 2.7	0	8.1 ± 0.9*

N = sample size; T = *T. urticae* tetracycline treated; U = *T. urticae* tetracycline untreated. * in the same column indicate significant differences between crosses (Tukey's Test, $P < 0.05$).

2.4 DISCUSSION

Microbiome assessment shows how tetracycline treatment affects *T. urticae* microbiota

Microbiome characterization has been recently highlighted as a key factor in plant protection. Many bacterial symbionts are affecting arthropod host biological parameters. The knowledge of who is in and what is their contribution to the ecology of the species is becoming the target of new pest management strategies, especially considering the urgency of reducing chemical treatments and pesticide resistance development (Kopecky et al. 2014; Lv et al. 2018).

We have determined by the 16S rDNA NGS that the microbiota of our *T. urticae* population is drastically affected by tetracycline with a reduction in the number of OTUs identified, being *Wolbachia* spp. the most frequently found. It affected bacterial diversity. Tetracycline is a broad-spectrum antibiotic (Li et al. 2014) that removes mainly *Wolbachia*. Tetracycline inhibits protein synthesis by binding to the bacterial 30S ribosomal subunit, which means that this molecule does not kill the bacteria, just renders it static, not being able to reproduce neither to be transmitted to the next host generation. This antibiotic is being used to determine the presence of bacterial reproductive manipulator species in many arthropods, which were determined to be infected mainly by *Wolbachia* (Mariño et al. 2017; Bagheri et al. 2019). As in the present work, there are other studies about the use of tetracycline to reduce the relative proportion of this endosymbiont in the microbiota of arthropods. *Wolbachia* was reduced from 0.49% to 0.04% in the microbiota of the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), the most devastating coffee pest worldwide (Mariño et al. 2017). Bagheri et al. (2019) used this antibiotic to study if the parasitoid wasp *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae), an important biological control agent of many lepidopteran larvae, is infected with *Wolbachia*.

While performing this work, Pekas et al. (2017) compared the microbiota of the predatory mite *Neoseiulus cucumeris* (Oudemans) (Acari: Phytoseiidae) and its prey *Tyrophagus putrescentiae* (Schrank) (Acari: Acaridae), and found that the microbiota is also altered depending on the rearing system, similary to the modification that can be induced by the tetracycline treatment.

***Wolbachia* alters mating system in *T. urticae*.**

The 16S rDNA deep sequencing results indicate that the principal bacterial endosymbiont found in the *T. urticae* colony used is *Wolbachia* spp, which is practically removed by the tetracycline treatment. The analysis of crosses performed between treated and untreated isolines (Table 2), indicate that the removed bacterial species induces cytoplasmic incompatibility (CI) in our *T. urticae* colony, indicated by a stadistical significant reduction of egg hatchability. The CI observed could be produced by any of the bacterial mating distorters described for *T. urticae* (Zélé et al. 2018b). Nevertheless, the differences found with the microbiome of this study indicate that the endosymbiont responsible for the mating disorder observed in our *T. urticae* population is *Wolbachia*.

These results are comparable with those obtained by Breeuwer (1997) and Zhao et al. (2013), in which CI produced by *Wolbachia* is expressed by the reduction in egg hatchability and sex ratio. As Zhao et al. (2013), we have obtained a reduction in the number of female descendants in the cross ♀ T × ♂ U, not affecting the number of males, which is not significantly different to the compatible crosses. Vala et al. (2000, 2004) studying *T. urticae* in cucumber and rose showed the same phenomenon describing this reproductive failure as hybrid breakdown. They said that the 'sterilization' of uninfected females by infected males confers a fitness advantage to *Wolbachia* in infected females. Similar results are observed in the study of the parasitoid wasp, *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae), where the presence of *Wolbachia* in the females increased fecundity and female offspring (Bagheri et al. 2019). In *T. urticae* from soybean (*Glycine max* (L) (Fabaceae)), *Wolbachia* induced strong cytoplasmic incompatibility, increasing host fecundity without affecting females' longevity or males mating competitiveness. Most recently, Knegt et al. 2017 investigated genetic causes for hybrid incompatibility between differentiated lineages of the haplodiploid spider mite *Tetranychus evansi* Baker and Pritchard (Acari: Tetranychidae), and showed that strong, but incomplete, hybrid breakdown occurs. The genetic structuration found in Spanish *T. urticae* populations (Aguilar-Fenollosa et al. 2012, 2016; Pascual-Ruiz et al. 2014b) can be linked to the presence of different *Wolbachia* strains as pointed out by several authors (Navajas and Fenton 2000; Vala et al. 2000; Gotoh et al. 2003; Koukou et al. 2006; Li et al. 2009; Xie et al. 2011). Cytoplasmatic incompatibility between *T. urticae* harbouring different *Wolbachia* strains has already been demonstrated in the past (Gotoh et al. 2007; Xie et al. 2011; Suh et al. 2015). Pina et al. (2019) found that the Spanish *T. urticae*

populations are infected by two phylogenetically different *Wolbachia* races, similary to the different *Wolbachia* OTUs identified in this work, whose implication in the mating isolation and the biological control of Spanish *T. urticae* populations deserves further research.

Outstanding remarks

The identification of putative several *Wolbachia* OTUs in our *T. urticae* colony, originally collected in citrus, which can be removed by tetracycline treatment, and that induce directional incompatibility, highlights the importance of microbiome studies in pest ecology (Li et al. 2009; Suh et al. 2015; Sun et al. 2016) and in the applied pest control field. This fact makes possible the use of this bacterial species in biological control programs, by means of para-transgenesis (as developed for the fruit flies in Zabalou et al. 2004) or by means of increasing the most outstanding sex in a natural enemy (like females in insect parasitoids, as reviewed in Saridaki and Bourtzis 2010).

2.5 CONCLUSION

1. Microbiome assessment shows how tetracycline treatment removes mainly *Wolbachia* spp. from our *T. urticae* colony.

2. *Wolbachia* induces cytoplasmic incompatibility in treated *T. urticae* females and affects the mating behavior in *T. urticae* from Spanish citrus crops utilizing these strategies to increase the frequency of infected females.

3. The presence of *Wolbachia* in *T. urticae* females from Spanish citrus orchards increases the fecundity and female offspring sex ratio.

CAPÍTOL 3

The response of citrus plants to the broad mite *Polyphagotarsonemus latus* (Banks) (Acari: Tarsonemidae)

ABSTRACT

Polyphagotarsonemus latus (Banks) (Acari: Tarsonemidae) is a polyphagous mite pest common in tropical and subtropical areas which can become an important citrus pest. To understand the citrus response to *P. latus* infestation, we have characterized the volatile profile and the molecular defense mechanisms of two citrus genotypes, sour orange (*Citrus aurantium*) and Cleopatra mandarin (*Citrus reshni*), to *P. latus* infestation. Our results prove the differential response of these two citrus species, with the activation of both the jasmonic acid- and the salicylic acid-dependent pathways in sour orange, which remained unchanged in Cleopatra mandarin. These differences corresponded to sour orange allowing larger population growth rates than Cleopatra mandarin.

3.1 INTRODUCTION

Mites are important pests in indoor and outdoor agricultural ecosystems usually controlled with synthetic pesticides (Fathipour and Maleknia 2016). Chemicals adversely affect natural enemies and lead to environmental pollution, human health problems, harmful side effects, and pest resistance development (Rajendran and Singh 2016). In recent decades, the focus on crop production has moved from yield to quality, safety, and sustainability. To achieve this goal, the concept of integrated pest management based on the use of biocontrol agents, has gained importance. Understanding the mechanisms that plants have evolved to defend themselves and the identification of the ecological drivers of this evolution have been major challenges during recent time (Kant et al. 2015).

A key aspect in plant defense is distinguishing between constitutive and induced defensive traits. Constitutive defense refers to the basal levels of a defense trait and induced defense to increased levels in response to herbivory damage (Moreira et al. 2018). These responses can be triggered by simple wounding or through arthropod-derived elicitors such as certain enzymes, fatty acid-derived conjugates, other low-molecular-weight aliphatic compounds and peptides generated from degradation of ingested plant material (Mithöfer et al. 2018). Inducible defense is turned off until arthropod attack is detected to minimize the metabolic cost of defense for plants (Dicke and van Poecke 2002). It involves the production of signalling molecules which results in the upregulation of the biosynthesis of specific compounds. Furthermore, constitutive defenses are present all the time and can be physical (e.g., presence of trichomes) or chemical. Inducible defense is often subdivided into direct and indirect defense. Direct defense includes the activation or production of antifeedants, such as toxins and inhibitors of digestion, which negatively affect the growth and/or survival of herbivores (Howe and Jander 2008). It also includes traits that can reinforce the endogenous ability of plants to counter arthropod attacks (Aljbory and Chen 2018). By contrast, indirect defense refers to plant traits that enhance attraction or arrestment of natural enemies of the herbivore, such as predators and parasitoids (Sabelis et al. 2001). Whereas phytohormones such as jasmonic acid (JA) or salicylic acid (SA) play a primary role in regulating defense responses (Li et al. 2019), organic compounds that are released by plants (volatiles) can trigger indirect defenses under natural conditions (Kessler and Baldwin 2001). In particular, plants attacked by herbivores release much greater quantities or produce *de novo* low molecular weight volatiles which are called herbivore-induced plant volatiles (hereafter, HIPVs) that attract

natural enemies of the herbivores (Gebreziher 2016). Many natural enemies can use HIPVs as a cue to find their prey (Aljbory and Chen 2018).

Polyphagotarsonemus latus (Banks) (Acari: Tarsonemidae) is a common mite pest in tropical and subtropical areas. This species has a large host range, comprising plants in more than 60 botanical families including Rutaceae (Gerson 1992). Indeed, *P. latus* is considered as one of the most important citrus pests worldwide (Imbachi et al. 2012; Rodríguez et al. 2017). With about 0.2 mm in length, the naked eye observation of broad mites results impossible. Although these mites are not initially noticed in crops, they are detected when plants show damage symptoms (Venzon et al. 2008). Broad mites attack actively growing plant parts (e.g., leaves, flowers, fruit, buds) and oviposit on the underside of leaves (van Maanen et al. 2010). Common symptoms observed after mite infestation include leaf edge curl and rolling down (Gerson 1992), which are frequantly confused with phytotoxicity from pesticides. In citrus, Rodríguez et al. (2017) observed first leaf symptoms of *P. latus* infestation in Valencia orange plants between 7.0 and 11.8 days after infestation. Plant resistance to *P. latus* has been found in several plant species such as pepper (*Capsicum annuum* L. (Solanaceae)), cucumber (*Cucumis sativus* L. (Cucurbitaceae)), aubergine (*Solanum melongena* (brinjal) (Solanaceae)), tomato (*Solanum lycopersicon* L. (Solanaceae)), or azalea (*Rhododendron simsii* Planch (Ericaceae)) (Luypaert et al. 2014). The presence and density of trichomes has been pointed out as the predominant factor related to resistance/defense to this mite (Thungrabeab and Boonlertnirun 2002; Matos et al. 2009; Luypaert et al. 2014) but differences in plant defense are most probably key. Although literature on induced defense responses upon *P. latus* herbivory is scarce, the activation of the JA- and SA-dependent pathways in cucumber after infestation was demonstrated by Grinberg et al. (2005). Likewise, the JA-pathway was induced and could be related to resistance in tomato (Grinberg-Yaari et al. 2015). Compared to these poorly known mechanisms for *P. latus*, the two-spotted mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is probably the best characterized mite in terms of its interactions with the plant (Agut et al. 2018).

Same as *P. latus*, *T. urticae* is a polyphagous and cosmopiltan mite (Migeon and Dorkeld 2006-2017) which can be an important pest of citrus (Jacas et al. 2010; Imbachi et al. 2012; Pascual-Ruiz et al. 2014a). Several studies have reported activation of the JA pathway after *T. urticae* infestation in citrus. This activation occurs only one or two days after infestation as a consequence of the plant's recognition of the spider mite attack. In addition to JA, SA-mediated signaling is also activated in some plant–herbivore interactions. Although both hormones can be induced following mite attack, they play different roles in the plant–mite interactions (Agut et al. 2018). Metabolic and genetic responses of citrus to *T. urticae* were characterized by Agut et al. (2014, 2015). Two important rootstocks for the citrus industry are sour orange (*Citrus aurantium* L.) and Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) (Sapindales: Rutaceae), which display differential resistance against *T. urticae*. Sour orange, which had been massively used in the past and had to be replaced by other rootstocks because of its susceptibility to the *Citrus Tristeza virus* (*Closteroviridae*) (Berk 2016), showed elevated levels of constitutive resistance associated with the JA pathway compared with Cleopatra mandarin (Agut et al. 2014). Furthermore, the release of HIPVs from sour orange plants induced by *T. urticae* had a marked repellent effect on conspecific mites and triggered resistance in Cleopatra mandarin (Agut et al. 2015). Therefore, both plant species retain different mechanisms to express induced resistance against this mite.

Induced plant direct defenses against herbivores involve diverse reactions that differ depending on the timing and nature of the plant-arthropod interaction. The type of mechanical injury caused by the arthropod can modify hormone profiling (Kawazu et al. 2012) and the appropriate recognition of the attacker is relevant to the survival of the host because depending on the nature of the herbivore, each reaction may be different (Ali and Agrawal 2012; Agut et al. 2016). Because of these differences, in this study we have characterized the response of the two citrus genotypes already characterized in relation to *T. urticae* infestation (*C. aurantium* and *C. reshni*) to *P. latus* infestation. Our aims has been to elucidate the defense, the volatile profile, and the molecular mechanisms implicated. Our initial hypothesis is that *P. latus* will trigger defense responses in citrus similar to those elicited by *T. urticae*.

3.2 MATERIAL AND METHODS

3.2.1 Plant material

Sour orange (*C. aurantii*) and Cleopatra mandarin (*C. reshni*) plants were used in the assays. Three-month-old plants of both species (with about 10 fully developed leaves) were maintained in a climatic chamber at 60 ± 10% relative humidity (RH) and under a 16:8 h L:D (Light:Dark) photoperiod combined with a day/night thermal regime of 25 ± 2º and 20 ± 2ºC, respectively. These plants were grown on vermiculite and peat (1:3; v:v) in 320-ml pots. No pesticides were applied and fertilization consisted of a modified Hoagland's solution applied every 3 days (Bañuls et al. 1997).

Bean plants (*Phaseolus vulgaris* L. (Fabaceae) cv. Buenos Aires roja) grown at UJI greenhouse in pesticide-free conditions were used to maintain *P. latus* colonies.

3.2.2 *Polyphagotarsonemus latus* stock colony

The *P. latus* colony used in the present assays was initiated with specimens collected in Azalea (*Rhododendron* L.) in Merelbeke (Belgium, 50°58′59″N, 3°46′46″W). The colony was maintained on detached bean leaves, in a climatic chamber set at 22 ± 2.5°C and 60 ± 10% RH under a 16:8 h L:D photoperiod. Single bean leaflets were placed upside down on moistened cotton, on top of water-saturated foam cube (3 – 4 cm thick) in an open plastic box (35 × 20 × 7 cm^3) half-filled water. The edge of the leaflet was covered with folded moistened cotton to prevent mites from escaping.

3.2.3 *P. latus* performance on selected citrus rootstocks

Five citrus plants of each rootstock were isolated using Tanglefoot® (Tanglefoot Co., Grand Rapids, MI 49504, USA) around its base as barrier to prevent mites from escaping. Every plant was infested with 5 adult females of *P. latus* and maintained under the same climatic chamber conditions as described above. When first obvious symptoms of infestation were observed (21 days after infestation), mites were recovered from infested plants (see below) and counted. The experiment was repeated three times as true biological replicates.

3.2.4 Extraction of specimens

Infested plants were individually placed in a beaker with 1 liter of the extraction solution proposed by de Lillo (2001), which consists of tap water, 0.2% detergent (dishwashing soap), and 2% sodium hypochlorite. This mixture was stirred for 10 minutes with a magnetic stirring bar. Then, the suspension was poured into stainless steel sieves stacked in the top to bottom sequence 850, 180, 53 and 25 µm (de Lillo 2001). This material was further washed with tap water and eventually the material in the bottom sieve was carefully washed out into a Petri dish using distilled water. Finally, two drops of detergent were added to the recovered solution and mixed gently in order to keep the material in the bottom of the Petri dish for specimen counting under stereomicroscope.

3.2.5 Collection of volatiles

Following the same methods used by our group (Cabedo-López et al. 2019; Cruz-Miralles et al. 2019) for *T. urticae*, volatiles emitted by Cleopatra mandarin and sour orange plants, either uninfested or infested with 25 *P. latus* adult females after 48 h, were collected using a headspace collection system similar to that described by Bruinsma et al. (2010). Open glass vials containing 300 mg of Porapak (Sigma-Aldrich, Barcelona, Spain) were used as volatile retention filters. They were connected to the air outlet hole at the top of 5-l glass vessels described above. This system was ventilated with carbon-filtered pressure-air at 1.5 l/h. The system (glass vessels and Porapak filters) was cleaned with acetone and dried in an oven 1 hour prior to the assay. Plants were set individually inside these glass vessels. Volatile compounds were collected in 1 ml of ethyl acetate. This collection took place in a climatic chamber following the same procedure as in our previous work (Cabedo-López et al. 2019; Cruz-Miralles et al. 2019). An Agilent 6890N GC system (Palo-Alto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a time-of-flight mass spectrometer (TOF-MS), GCT (Waters Corp., Manchester, UK), operating in electron ionization (EI) mode was used to characterize the volatiles. A fused silica DB-5MS capillary column of 30 m length, 0.25 mm internal diameter and a film thickness of 0.25 µm (JandW Scientific, Folson, CA, USA) was used to the GC separation. The temperature program for this process was the following; 50°C (1 min); 5°C min^{-1} to 210°C (1 min); 20°C min^{-1} to 300°C (2 min); this resulted in a total analysis run of 40.50 min. Splitless injections were carried out. Helium was used as carrier gas at 1 ml min^{-1}. The interface and source temperatures were both set to 250°C and a solvent delay of 3 min

was selected. The TOF-MS was operated at 1 spectrum s^{-1} acquiring the mass range m/z 50–650 and using a multi-channel plate voltage of 2800 V. The TOF-MS resolution was c. 8500 (full width at half-maximum, FWHM) at m/z 614. Heptacose, used for the daily mass calibration as well as lock mass, was injected via syringe into the reference reservoir at 30°C. The m/z ion monitored was 218.9856. The application manager ChromaLynx, a module of MassLynx software, was used to investigate the presence of non-target compounds in the samples. Volatiles were identified by matching to the National Institute of Standards and Technology library (NIST\EPA\NIH Mass Spectral Library, version 2.0, build 4/2005) using match values of at least > 80% as a threshold for identification, as described by Wallis et al. (2008). Finally, for each volatile identified the TOF-MS-derived peak areas were calculated and used to estimate their relative concentration.

3.2.6 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Three assays including 3 plants per treatment each were carried out. For each assay, six sour orange and six Cleopatra mandarin plants were used. For each genotype three plants were infested as previously explained (3.2.5), whereas the other three remained uninfested and were used as controls. Forty eight hours after infestation at the same temperature, RH conditions and photoperiod as before, leaves were cut and immediately introduced into 50 ml Falcon™ vials, which were immersed in liquid nitrogen and stored at -80ºC until extraction. Leaves for the same treatment were pulled together in the same vial. RNA was extracted using a plant RNA protocol with trizol (Kiefer et al. 2000). For qRT-PCR experiments, 1 µg of total RNA was digested with 0.7 µg of DNase (RNase-free DNase I) in 0.7 µl of DNase buffer and Milli-Q water up to 4.9 µl and incubated for 30 min at 37°C. After incubation, 0.7 µl of EDTA was added and incubated again at 65°C for 10 min to inactivate DNase (Thermofisher Scientific Inc.). The RT reaction was performed by adding 7 µl of DNase reaction, 2 µl of PrimeScript buffer and 0.5 µl of PrimeScript RT and Oligo-dT respectively (PrimeScript RT Reagent Kit, Takara Bio Inc.). The reaction mixture was incubated at 37°C for 15 min. Complementary DNA from the RT reaction, 10X diluted, was used for qPCR. Forward and reverse primers (0.3 µM) were added to 5 µl of Maxima SYBR Green qPCR Master Mix, 1 µl of cDNA and 3 µl Milli-Q sterile water (Maxima SYBR Green/ROX qPCR, Thermofisher Scientific Inc.). qPCR was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA, USA)

sequence detector with standard PCR conditions (95ºC-10 min; 40× (95ºC-10 sec; 55ºC-10 sec; 72ºC-20 sec); 60ºC-10 sec; 95ºC-15 sec). qRT-PCR analysis was replicated three times. The expression of lipoxygenase 2 (*LOX2*; accession Cit.16756.1.S1_ s_at; forward primer: 5′ GAACCATATTGCCACTTTCG 3′; reverse primer 5′ CGTCATCAATGACTTGACCA 3′) and pathogenesis-related protein 5 (*PR5*; accession BAI63297.1; forward primer: 5′ CATCAAGCTTCACAGTGCTTAG 3′; reverse primer 5′ CCACAACGTACAGACTGATGAC 3′) genes was determined (Agut et al. 2014). Relative expression was compared with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; accession Cit.122.1; forward primer: 5′ GGAAGGTCAAGATCGGAATCAA 3′; reverse primer: 5′ CGTCCCTCTGCAAGATGACTCT 3′).

3.2.7 Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 23. *Polyphagotarsonemus latus* performance on the three rootstocks was compared with one-way ANOVA. Previously, data was checked for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). For each volatile identified in the blends produced by plants, TOF-MS-derived peak areas were compared using a Generalized Linear Model (GLM) with a normal distribution (Kolmogorov-Smirnov test) of the error and identity link function (i.e., linear regression). Plant genotype, infestation status, and replicate (and their interactions) were used as fixed effects. When necessary, we used Bonferroni post-hoc test ($P < 0.05$) for mean separation. Finally, student t-test was used to compare the results of gene expression assays.

3.3 RESULTS

3.3.1 Cleopatra mandarin sustains lower *P. latus* populations compared with sour orange

Twenty one days after infestation, Cleopatra mandarin sustained significant lower mite population than sour orange. The mean number of specimens counted on sour orange was 250 % higher than in Cleopatra mandarin (Figure 3.1).

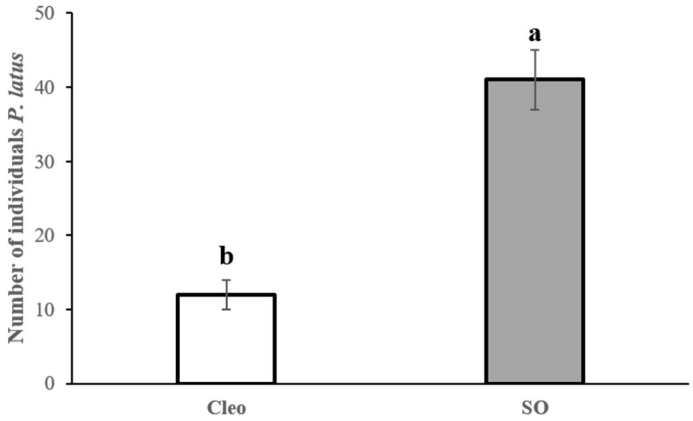

Figure 3.1. Number of *P. latus* in Cleopatra mandarin (Cleo) and sour orange (SO). The mites were counted 21 days after infestation with 5 adult females per plant. Different letters indicate significant differences between treatments ($P < 0.05$; One-way ANOVA).

3.3.2 The mite *P. latus* triggers the production of volatiles (HIPV) in sour orange and Cleopatra mandarin in a different way

To elucidate differences in volatile organic compounds (VOCs) released from different citrus rootstocks–*P. latus* interactions, a series of repeated experiments were performed. The factor 'replicate' and all the 2- and 3-factor interactions were significant. The reason is that for each HIPV identified, the TOF-MS-derived peak areas obtained for each replicate could be up to two orders of magnitude apart. However, as the relative differences observed for the other two factors considered (plant genotype and infestation) for each volatile were consistent (Figure 3.2), results were interpreted in a qualitative manner and according to these two factors only.

In the absence of infestation, the basal volatile profiles of the two citrus species tested showed quantitative differences. When mites were infesting the plants, 5 compounds were detected (Table 3.1). Four of them showed differences depending on either or both the genotype and the infestation status. Two of these four, the aromatic compound

4-ethenyl-1,2-dimethyl-Benzene, and the Green Leaf Volatile (GLV) 1-(3,4-dimethylphenyl)-Ethanone, showed no significant differences between plant genotypes but their concentration was reduced by mite infestation. The aromatic Diphenylmethane was more expressed in Cleopatra mandarin than sour orange but showed no changes with infestation. Finally, the concentration of the aromatic 1,1'-ethylidenebis-Benzene was higher in sour orange than in Cleopatra mandarin and in infested plants. However, the interaction between genotype and infestation was not significant for this compound.

Table 3.1. Volatile profiling in the headspace of sour orange (SO) and Cleopatra mandarin (Cleo) plants either clean or infested (inf). For each volatile, TOF-MS-derived peak areas were compared using a Generalized Linear Model. Plant genotype, infestation status, and replicate were used as fixed effects. Replicate and all the interactions including this factor were significant ($P < 0.05$) and these results are not presented in the table. As the relative differences observed for the other two factors considered were consistent for each volatile, results were interpreted in a qualitative manner and according to these two factors only. Volatiles were tentatively identified by comparing to the National Institute of Standards and Technology (NIST) Library as described by Wallis et al. (2008).

Volatile compounds	GLM results (Wald-χ^2; *P*)		
	Plant genotype (A)	**Infestation status (B)**	**A*B**
Benzene, 4-ethenyl-1,2-dimethyl-	0.04; 1; 0.839	33.60; 1; <0.001	0.04; 1; 0.840
	SO = Cleo	clean > inf	
Ethanone, 1-(3,4-dimethylphenyl)-	0.02; 1; 0.89	238.16; 1; <0.001	10.16; 1; 0.001
	SO = Cleo	clean > inf	Cleo clean > SO clean > SO inf > Cleo inf
1-Dodecene	1.22; 1; 0.27	1.04; 1; 0.31	4.04; 1;0.44
	SO = Cleo	clean = inf	
Diphenylmethane	5.18; 1; 0.023	0.05; 1; 0.829	7.87; 1; 0.005
	SO < Cleo	clean = inf	Cleo inf > SO clean = Cleo clean > SO inf
Benzene, 1,1'-ethylidenebis-	6.19; 1; 0.013	22.57; 1; <0.001	0.28; 1; <0.599
	SO > Cleo	clean < inf	

For volatiles for which the Plant*Infestation interaction is significant, means were separated according to Bonferroni ($P < 0.05$).

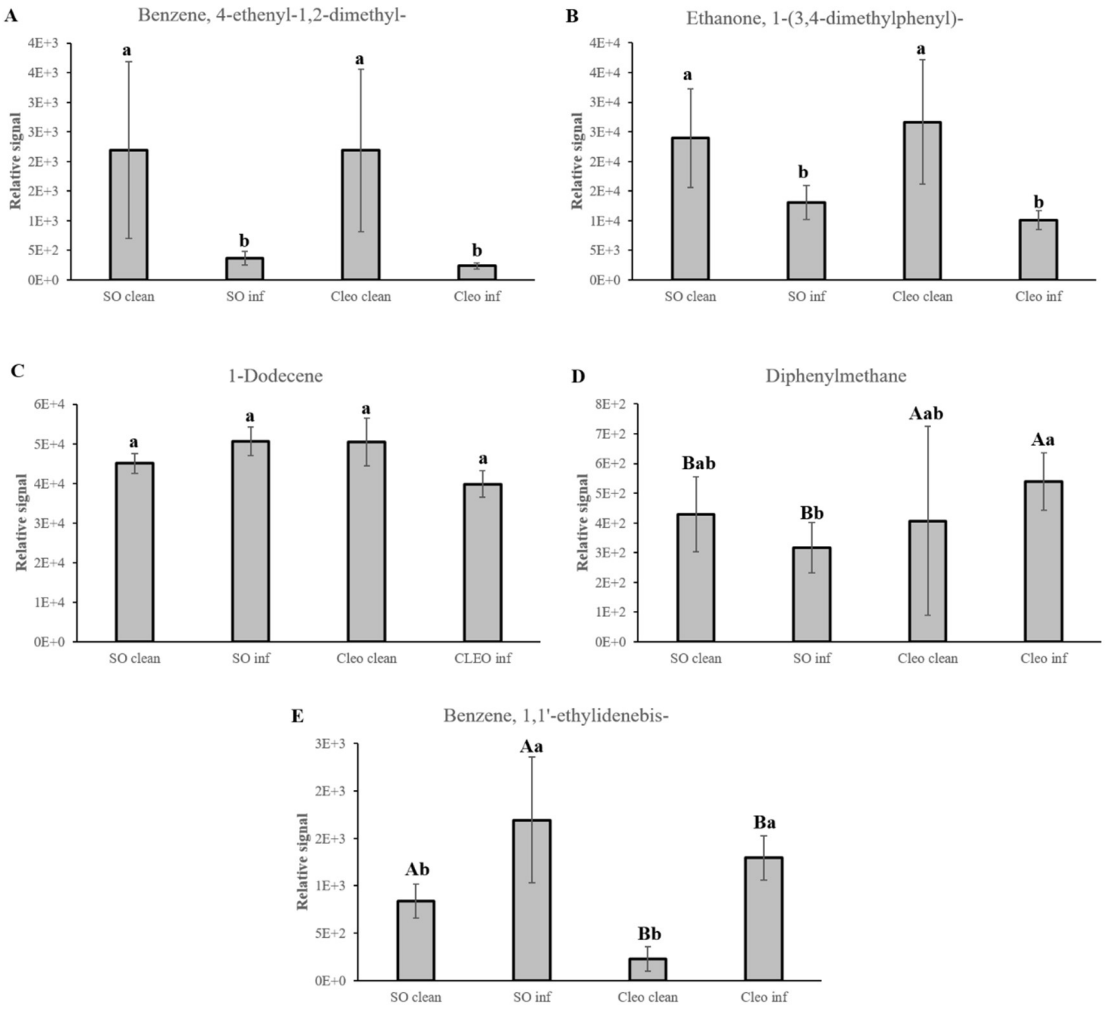

Figure 3.2. Relative signal (TOF-MS-derived peak areas) of the volatiles differentially produced in infested (inf) and clean sour orange (SO) and Cleopatra mandarin (Cleo) plants during the first 24 hours of infestation with 25 *P. latus* adult females. **A**. 4-ethenyl-1,2-dimethyl-Benzene; **B**. 1-(3,4-dimethylphenyl)-Ethanone; **C**. 1-Dodecene; **D**. Diphenylmethane; **E**. 1,1'-ethylidenebis-Benzene. For figures A-D, bars with the same letter are not significantly different ($P < 0.05$). For figures D and E, capital letters refer to the factor plant genotype, whereas lowercase letters to the infestation status.

3.3.3 *P. latus* triggers defensive responses in sour orange and Cleopatra mandarin plants.

The JA and SA signaling pathways homologous marker genes *LOX2* and *PR5* respectively, were analyzed in clean and *P. latus*-infested plants. The same results have been obtained for both genes (Figures 3.3 and 3.4). The presence of *P. latus* in sour orange significantly induced gene expression in *LOX2* and *PR5*. No induction was observed in Cleopatra mandarin.

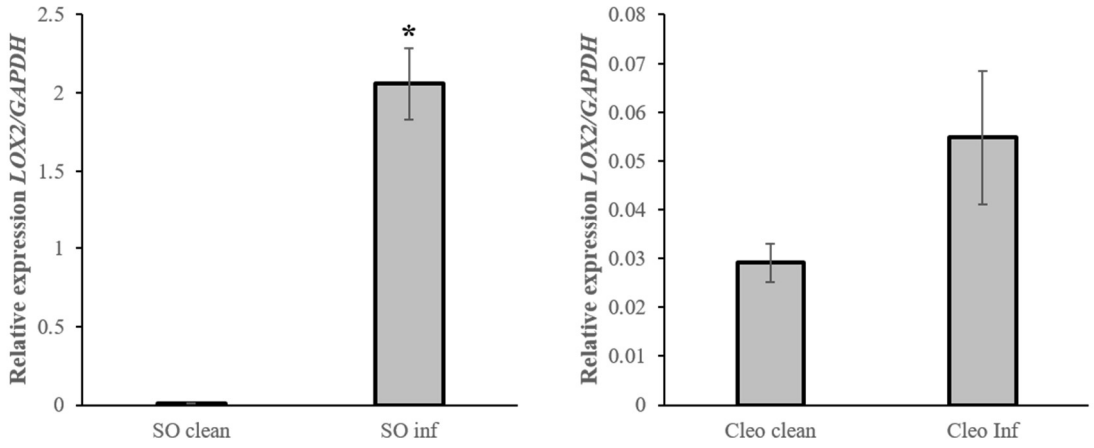

Figure 3.3. Relevance of lipoxygenase 2 (*LOX2*) in citrus defense triggered by *P. latus*. Total RNA was extracted from the leaves of three plants per genotype (sour orange, SO, and Cleopatra mandarin, Cleo) and infestation status (clean and infested with 25 mites, inf) 48 hours after infestation, converted to cDNA and subjected to quantitative RT-PCR analysis. The *LOX2* transcript levels were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured in the same sample. Significant differences between clean and infested plants were estimated performing a t-test for each genotype. Asterisks indicate statistically significant differences ($P < 0.05$).

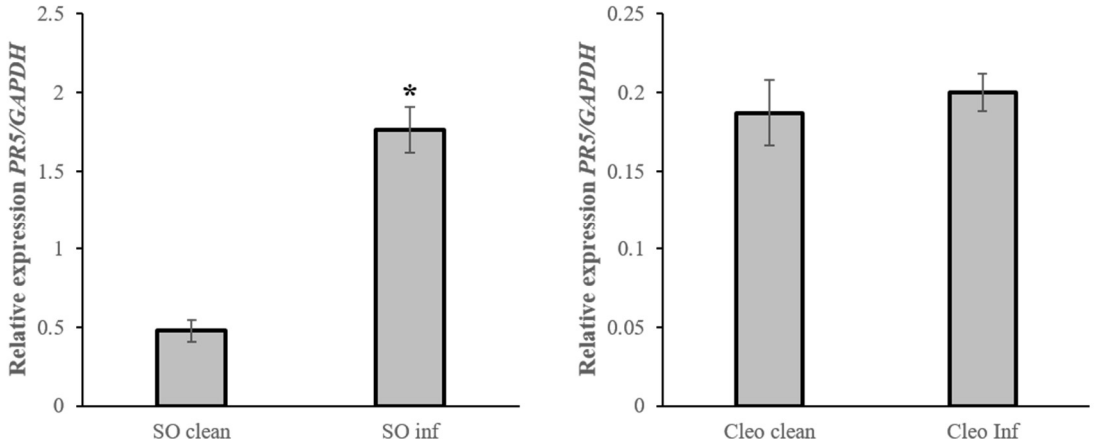

Figure 3.4. Relevance of the pathogenesis-related protein 5 (*PR5*) in citrus defense triggered by *P. latus*. Total RNA was extracted from the leaves of three plants per genotype (sour orange, SO, and Cleopatra mandarin, Cleo) and infestation status (clean and infested with 25 mites, inf) 48 hours after infestation, converted to cDNA and subjected to quantitative RT-PCR analysis. The *PR5* transcript levels were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured in the same sample. Significant differences between clean and infested plants were estimated performing a t-test for each genotype. Asterisks indicate statistically significant differences ($P < 0.05$).

3.4 DISCUSSION

In this study we have tried to shed light into the mechanisms of citrus defense against *P. latus* following the same approach used by our group for *T. urticae*. Although the same gene regulation patterns and similar HIPVs (a mixture of GLV and aromatic compounds) were observed in both cases 48 h after infestation, contrary to *T. urticae,* sour orange appeared as the most susceptible rootstock, as it sustained higher populations of this mite.

Rodríguez et al. (2017) observed first symptoms of leaf damage by *P. latus* in Valencia orange plants between 7.0 and 11.8 days after infestation. However, in our case it took longer. Only 21 days after infestation, clear symptoms (leaf curling, leaf drop, and bud abortion; Figure 3.5) were observed. Mite counting at that date showed that 3- and 8-fold population increases had occurred in Cleopatra mandarin and sour orange, respectively, which confirms the huge biotic potential of *P. latus*.

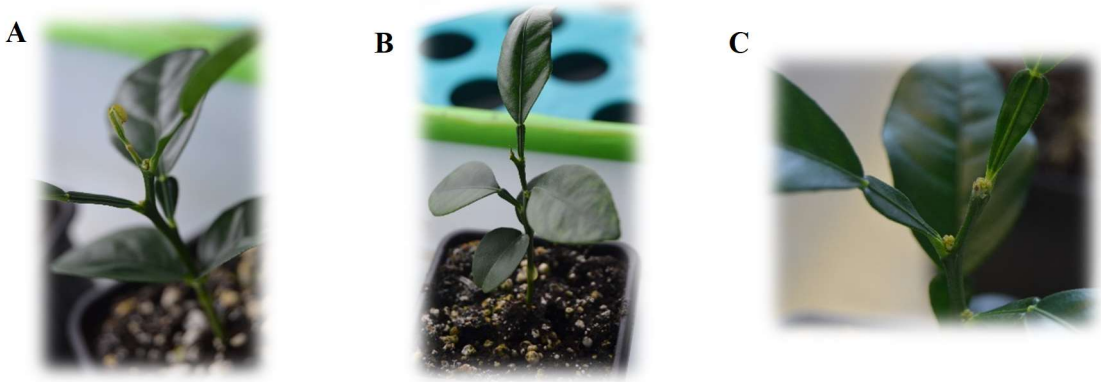

Figure 3.5. Typical symptoms of *P. latus* infestation 21 dpi. **A.** leaf curling in sour orange. **B.** leaf drop in Cleopatra mandarin. **C.** Bud abortion in sour orange.

Although, as pointed out earlier, literature on induced defense responses upon *P. latus* herbivory is scarce, Grinberg et al. (2005) proved the resistance against *P. latus* infestation was related to the activation of the JA- and SA-dependent pathways in cucumber. Likewise Grinberg-Yaari et al. (2015) proved the same for JA in tomato. The activation of both pathways occurred in sour orange (Figure 3.3 and 3.4), whereas no activation was observed in Cleopatra mandarin. These results taken together with the higher basal levels of these hormones in this genotype relative to Cleopatra mandarin would have made us hypothesize that, same as *T. urticae* (Agut et al. 2014), *P. latus* would perform better on less protected Cleopatra mandarin than in sour orange. However, this was not the case. Intriguingly, activation of *LOX2* and *PR5* genes by both mites was

similar (Agut et al. 2014) in spite of important quantitative differences between their piercing-sucking mouthparts and the plant tissues where they feed. While *P. latus* chelicerae are 43 μm long (Gui et al. 2001) and feeds on the epidermal cells (Grinberg et al. 2005), those of *T. urticae* range from 100 to 150 μm in larvae and adult females, respectively (Avery and Briggs 1968; Ekka 1969; Sances et al. 1979), which allows this mite to feed on mesophyll cells (Bensoussan et al. 2016). Indeed, the activation of the same genes by the zoophytophagous phytoseiid mite *Euseius stipulatus* (Athias-Henriot) (Cruz-Miralles et al. 2019), which can feed on the plant cell sap by grasping, is orders of magnitude below that observed for the two tetranychoids. Because gene expression was measured 2 days after infestation by *P. latus* but mite counts were performed 19 days later, additional changes may have occurred along this period that could explain this apparent contradiction. Therefore, further research aimed at monitoring the expression of these defense genes during a longer period, is necessary.

None of the five HIPVs observed in the present study had been previously observed in the same genotypes upon infestation by either *T. urticae* (Agut et al. 2015) or *E. stipulatus* (Cruz-Miralles et al. 2019). However, they belong to the same groups, GLV and aromatic compounds, and differences observed between infestation status and plant genotype are similar. HIPVs are usually exploited by arthropods in plant selection (Bernasconi et al. 1998; Halitschke et al. 2008). Interestingly, *P. latus* is phoretic and can attach themselves to the tibiae and tarsi of adult whiteflies in the genera *Trialeurodes*, *Dialeurodes*, *Bemisia*, and *Aleyrodes* (Palevsky et al. 2001), which are used as a carrier for their dispersal. Therefore, it would be interesting to study the effect of these HIPVs on the behavior of whiteflies within these genera infesting citrus (e.g., *Dialeurodes citri*), as well as on its natural enemies. As the interaction between plant genotype and infestation status was significant for the production of two of these volatiles, namely 1-(3,4-dimethylphenyl)-Ethanone and Diphenylmethane, the response of whiteflies to these compounds could be a first step towards better understanding the chemical ecology of this system. However, we should keep in mind that it is widely recognized the importance of considering the whole blend of volatiles instead of single compounds, when studying this type of behavioral responses (Gregg et al. 2018). Therefore, further studies with more complex mixtures mimicking the natural blend should be also considered.

3.5 CONCLUSION

Our results prove the differential response of two citrus genotypes infested by *P. latus* in terms of expression of the defense genes as of the volatiles profile, which were related to the population growth differences found. The characterization of such effects may help refining current biological control practices.

DISCUSSIÓ GENERAL

Els àcars fitòfags poden esdevenir plagues molt importants que afecten un ampli rang de cultius agrícoles en el món (Simon et al. 2011; Agut et al. 2018), provocant greus danys si les seues densitats no es mantenen per baix dels llindars de danys econòmics. Aquest és el cas dels cítrics, un dels principals cultius de l'àrea mediterrània i per tant, el seu control és molt important. Les plantes han desenvolupat mecanismes de defensa per fer front a atacs per herbivorisme, els quals poden ser constitutius (presents constantment a les plantes) o induïts mitjançant l'activació de les seues defenses quan són estressades. Treball previs del nostre grup (Agut et al. 2014, 2015 i 2016) van analitzar els mecanismes de defensa directa dels cítrics front a l'atac de *T. urticae* utilitzant dos patrons de cítrics amb diferent nivell de susceptibilitat a l'aranya: el taronger bord i el mandariner Cleòpatra els quals mostren, dins de tot el rang de cítrics estudiat, una extrema resistència i susceptibilitat respectivament (Bruessow et al. 2010). A més a més, es va demostrar que la ruta de l'àcid jasmònic (JA) i de l'àcid salicílic (SA) estaven sobre-expressades en el taronger bord quan era atacat, mentre que aquestes rutes no variaven en mandariner Cleòpatra infestat. També van concloure que la ruta del JA és efectiva front a aquest herbívor i es van identificar i quantificar els volàtils alliberats per plantes infestades demostrant que aquests, en el cas del taronger bord, eren capaços d'induir resistència en les plantes veïnes i provocar repel·lència per al fitòfag. El paper d'aquests compostos en defensa directa estava ben definit i el pas següent va ser determinar la rellevància d'aquests en defensa indirecta, és a dir, la manera en què influïen en el comportament dels principals enemics naturals de l'aranya roja en els cítrics, sent aquest l'objectiu principal del capítol 1.

Les respostes olfactives dels enemics naturals de *T. urticae* en cítrics depenen del genotip de la planta, la presencia de la presa i els hàbits alimentaris

El capítol 1 estudia els efectes del genotip de la planta i els hàbits alimentaris específics dels depredadors en la defensa indirecta de les plantes desencadenada per *T. urticae* en cítrics. La hipòtesi inicial estableix que les plantes infestades de mandariner Cleòpatra serien més atraients per als fitoseids que les plantes de taronger bord infestades, les quals tenen una major defensa. No obstant això, sols el depredador omnívor *E. stipulatus*, al igual que l'herbívor, preferien el mandariner Cleòpatra (Figures 1.1 i 1.2). Els altres dos depredadors no van mostrar preferència alguna (Figures 1.3 i 1.4). Seguint amb la mateixa idea, les plantes de mandariner Cleòpatra induïdes pels volàtils alliberats pel taronger bord infestat, deurien ser repel·lents front a plantes control, però una vegada més no va

ser així per al cas dels depredadors: els dos omnívors van preferir la planta induïda més protegida ja que podria ser interpretada com la presència de la presa a prop, mentre que l'especialista d'aranya no va tenir preferència al no haver presa. Per tant, aquests resultats clarifiquen que la resposta dels depredadors és depenent del genotip de la planta i del tipus de dieta del fitosèid.

L'estudi dels volàtils alliberats per la pròpia presa són de gran interès, ja que aquests són els compostos pels quals poden sentir preferència els depredadors. Els nostres resultats mostren que les olors associades a *T. urticae* inclouen metil salicilat (MeSA) (Taula 1.2), el qual va ser identificat en el conjunt de HIPVs alliberats pel taronger bord i el mandariner Cleòpatra (Agut et al. 2015). *T. urticae* probablement obté aquest volàtil de les plantes hoste (Oku et al. 2015). Degut a què el SA ha sigut reconegut com un factor clau per a l'atracció dels depredadors per part de plantes infestades, és a dir, en defensa indirecta (Mallinger et al. 2011; Rodríguez-Saona et al. 2011; Kaplan 2012; Rowen et al. 2017; Salamanca et al. 2017), el perquè de com aquest compost pot estar retés en la presa en perjudici d'ella no queda clar i deuria ser motiu per a continuar indagant. Però a pesar d'açò, l'atracció dels tres fitoseids avaluats seria atribuït a tot el conjunt de volàtils (Taula 1.2) i no a un sols, ja que és tot el conjunt més que volàtils individualitzats els que usen els àcars depredadors per a comunicar-se (McCormick et al. 2012).

Com s'ha explicat abans, la via del SA és clau en la defensa indirecta de les plantes, ja que MeSA provoca l'atracció dels fitoseids (de Boer i Dicke 2004; van Wijk et al. 2008, 2011; Shimoda 2010). Per tant, les plantes amb major expressió d'aquesta ruta deurien ser atraients per als depredadors, però aquest no ha sigut sempre el cas. Per als depredadors *N. californicu*s i *P. persimilis*, la presència de la presa és clau en la seua elecció independentment del genotip de la planta tenint en compte que els olors de l'aranya inclouen MeSA (Taula 1.2). Però aquest fenomen no ocorre quan entren en joc les plantes de Cleòpatra induïdes, on per als depredadors omnívors hi ha una preferència per aquestes plantes sobre plantes control on la no preferència era d'esperar al no haver presa ni haver una diferència significativa en la producció de MeSA (Taula 1.1). Per tant, aquestes diferències entre els depredadors poden ser parcialment explicades pels distints hàbits alimentaris (McMurtry i Croft 1997; McMurtry et al. 2013), els quals afecten a la interpretació dels volàtils.

En els resultats de comportament a l'olfactòmetre, no és sorprenent que *P. persimilis* responga al conjunt de volàtils associats a la presa (Figura 1.4), ja que és un especialista

de tetraníquids. Malgrat això, per a les espècies omnívores que poden obtenir aliment de la presa però també dels recursos que ofereix la planta, els dos conjunts de volàtils (els produïts per la planta i els produïts per la presa) són igual d'importants, provocant diferències en l'elecció (Figura 1.2 i 1.3). *Euseius stipulatus* és l'únic depredador de les tres espècies d'estudi que probablement complementa la seua nutrició xuclant planta (Adar et al. 2012; McMurtry et al. 2013; Gómez-Martínez et al. 2019). Estudis recents del nostre grup (Cruz-Miralles et al. 2019) han demostrat com *E. stipulatus* és capaç de desencadenar una resposta defensiva en els cítrics. Per tant, aquest omnívor es pot beneficiar de la tria de la planta que estiga pitjor defensada quan és atacada per *T. urticae* (Agut et al. 2014). Tal com mostra la figura 1.2, la preferència és el mandariner Cleòpatra que a més a més, tindrà més quantitat de presa i açò li proporciona un avantatge a la pròpia planta ja que el depredador preferirà alimentar-se de presa en lloc de picar planta.

Per tant, els nostres resultats proven que la resposta de les 4 espècies d'àcars d'aquest estudi (*E. stipultaus, N. californicus, P. persimilis* i *T. urticae*) és depenent del genotip de la planta i modulada pels hàbits alimentaris i la presència de l'herbívor. A més a més, la discriminació per part del fitòfag entre el mandariner Cleòpatra induït i el control i el fet de què no mostre preferència quan és exposada als volàtils dels conspecífics, confirma que el seu comportament està determinat pels HIPVs.

El tractament amb tetraciclina de la colònia de *T. urticae* de laboratori procedent de cítrics efecta a la seua microbiota i provoca alteracions reproductives

Una part important de la investigació sobre la relació planta - herbívor és el paper que pot jugar la microbiota del fitòfag. La seua caracterització ha sigut recentment posada en valor com a factor clau en la protecció de cultius. Els bacteris simbionts afecten als paràmetres biològics dels artròpodes i per tant el coneixement de qui són i quina és la seua contribució a l'ecologia de l'espècie s'està convertint en l'objectiu de les noves estratègies per al control de plagues (Kopecky et al. 2014; Lv et al. 2018). El capítol 2 pretén dilucidar l'efecte del microbioma en els paràmetres biològics de les poblacions de *T. urticae* procedents d'horts de clementins, per a tal propòsit s'ha determinat, per la tecnologia de seqüenciació massiva 16S rDNA, que la microbiota de la colònia de *T. urticae* es veu dràsticament afectada pel tractament amb l'antibiòtic tetraciclina provocant una reducció en el nombre de seqüències identificades (però augmentant la diversitat ja que l'eliminació de *Wolbachia*, fa possible la detecció d'altres bacteris), sent la *Wolbachia* spp l'espècie bacteriana més present i afectada pel tractament amb tetraciclina.

Aquest fenomen també ocorre en altres estudis recents com en *Hypothenemus hampei*, una de les principals plagues del cafè (Mariño et al. 2017) i el parasitoide *Habrobracon hebetor* (Bagheri et al. 2019) entre d'altres. Després de l'aplicació del tractament amb l'antibiòtic a les colònies d'aranya de cítrics del laboratori, s'observa en un dels creuaments (♀ T x ♂ U) incompatibilitat citoplasmàtica (CI). Aquests resultats són comparables amb estudis pioners com els de Breeuwer (1997) i Vala et al. (2000, 2004), que descriuen aquest fenomen en *T. urticae* procedent de cultius de tomata i cogombre respectivament. La CI observada es podria produir per qualsevol dels endosimbionts que, al igual que *Wolbachia*, causen alteracions en la reproducció de *T. urticae* i que han sigut prèviament descrits en co-infecció amb aquest àcar (Zélé et al. 2018). No obstant això, les diferències trobades amb l'estudi del microbioma de les línies tractades i no tractades de *T. urticae* indiquen que l'endosimbiont responsable de les alteracions observades en la nostra població de *T. urticae* és *Wolbachia*. La presència de dues races de *Wolbachia* en les poblacions de *T. urticae* en cítrics (Pina et al. 2019) pot explicar l'estructuració poblacional trobada en estudis previs (Aguilar-Fenollosa et al. 2012, 2016; Pascual-Ruiz et al. 2014b) i obre noves possibilitats per a la seua aplicació al control d'aquesta plaga en cítrics.

La resposta de les plantes de cítric a l'àcar *P. latus*

El capítol 3 tracta de dilucidar els mecanismes de defensa dels cítrics front a *P. latus* seguint el mateix enfocament utilitzat pel nostre grup per a l'estudi de *T. urticae*. Tot i que es van observar els mateixos patrons de regulació gènica i similars HIPVs (una barreja de GLV i compostos aromàtics) en ambdós casos, 48 hores després de la infestació, al contrari que amb l'aranya, el taronger bord va ser el patró més susceptible, ja que va mantenir poblacions més elevades d'aquest àcar. A diferència del què va ocórrer amb el treball de Rodríguez et al. (2017), solament després de 21 dies post-infestació dels patrons es van observar símptomes clars com la curvatura i caiguda de les fulles i l'avortament de brots.

Estudis anteriors de Grinberg et al. (2005) en cogombre van demostrar que la resistència front a una infestació de *P. latus* estava relacionada amb l'activació de les vies defensives depenents del JA i SA. Per la seua banda, Grinberg-Yaari et al. (2015) va obtenir l'activació de la via de l'àcid jasmònic en tomata. Aquestes dues vies també s'activen en el taronger bord quan és infestat amb l'àcar, mentre que no hi ha diferències per al mandariner Cleòpatra (Figures 3.3 i 3.4). Aquests resultats obtinguts, juntament amb els

nivells basals més elevats d'aquestes hormones en aquest genotip en relació amb el mandariner Cleòpatra ens feien deduir que, al igual que amb *T. urticae* (Agut et al. 2014), *P. latus* obtindria un millor rendiment en el genotip menys protegit (mandariner Cleòpatra). Però no va ser així. Curiosament, l'activació dels gens defensius d'ambdós àcars va ser similar a l'observada en altres treballs (Agut et al. 2014), malgrat que es tracta de dos àcars que presenten importants diferències en la seua estructura bucal i la manera d'alimentar-se, també cal destacar que aquesta activació va ser superior al depredador fitosèid *E. stipulatus* (Cruz-Miralles et al. 2019). A pesar d'açò, són necessàries futures investigacions que es centren en l'estudi de l'expressió dels gens als 21 dies, què és el moment on es van detectar els símptomes de manera clara la qual cosa podria explicar aquesta contradicció.

Cap dels cinc volàtils detectats en els cítrics infestats amb *P. latus* van ser detectats en els mateixos genotips infestats d'aranya (Agut et al. 2015) i *E. stipulatus* (Cruz-Miralles et al. 2019), malgrat això, pertanyen als mateixos grups (compostos aromàtics i GLV). Els HIPVs són explotats pels artròpodes en la selecció de les plantes (Bernasconi et al. 1998; Halitschke et al. 2008). Cal destacar que *P. latus* és forètic i per tant, seria interessant estudiar l'efecte d'aquests volàtils sobre el comportament de les mosques blanques dins dels gèneres que infesten els cítrics (per exemple, *Dialeurodes citri*), així com sobre els seus enemics naturals. Degut a què la interacció entre el genotip de la planta i l'estat d'infestació va ser significativa per a la producció de dos d'aquests volàtils, l'1-(3,4-dimetilfenil)-Etanona i el difenilmetà, la resposta de les mosques blanques a aquests compostos podria ser un primer pas per a una millor comprensió de l'ecologia química d'aquest sistema. Tanmateix, es reconeix àmpliament la importància de considerar tota la barreja de volàtils en lloc de compostos únics, a l'hora d'estudiar aquest tipus de respostes (Gregg et al. 2018). D'aquesta manera, els nostres resultats demostren la resposta diferencial de dos genotips de cítrics infestats per *P. latus* en termes d'expressió dels gens de defensa i del seu perfil de volàtils, relacionats amb les diferències en el grau de infestació. La caracterització d'aquests efectes pot ajudar a afinar les pràctiques actuals de control biològic.

CONCLUSIONS

1. La resposta dels quatre àcars protagonistes d'aquesta tesi (*E. stipultaus, N. californicus, P. persimilis* i *T. urticae*) depèn del genotip de la planta, la presència de la presa i els hàbits alimentaris específics de cadascun d'ells. Els fitoseids depredadors són atrets per les plantes amb nivells de defensa més elevats, que probablement interpreten com a senyal d'herbivorisme, fins i tot quan la inducció és a través de volàtils, sense presència de la presa.

2. Els tres conjunts de volàtils identificats en el capítol 1 (taronger bord infestat, mandariner Cleòpatra induïda i *T. urticae*), que atrauen els enemics naturals però no l'herbívor, podrien proporcionar noves eines per a manipular aquests àcars i s'haurien de seguir estudiant.

3. Les olors associades a *T. urticae* inclouen el MeSA, un volàtil que s'havia identificat prèviament en els HIPVs del mandariner Cleòpatra i taronger bord. L'acumulació d'aquest compost, per una banda podria tenir un impacte directe en la defensa de la planta (*priming*) i per l'altra, ser clau en l'atracció dels enemics naturals, és a dir, en la defensa indirecta.

4. La identificació majoritària de seqüències de *Wolbachia* en la microbiota de *T. urticae* de cítrics, la qual es pot eliminar mitjançant tractament amb tetraciclina, i que indueix una incompatibilitat citoplasmàtica provocant alteracions en la reproducció del fitòfag, posa de manifest la importància dels estudis de microbiomes en ecologia de plagues i la seua possible aplicació per al control de plagues en els cultius.

5. La resposta dels cítrics a *P. latus* és depenent del genotip de la planta i afecta, almenys, l'expressió dels gens de les vies defensives principals, és a dir, JA i SA i el perfil de volàtils alliberats. La caracterització d'aquests efectes pot ajudar a millorar les pràctiques actuals de control biològic d'aquest àcar.

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