ESCOLA SUPERIOR DE TECNOLOGIA I CIÈNCIES EXPERIMENTALS
DEPARTAMENT DE CIÈNCIES AGRARIES I DEL MEDI NATURAL

Citrus defense responses against *Tetranychus urticae*

Tesis

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PhD Thesis

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HACEN CONSTAR QUE:

La presente memoria de Tesis Doctoral, presentada por Blas Agut Capdevila, titulada “Citrus defense responses against *Tetranychus urticae*” y realizada en las Áreas de Fisiología Vegetal y de Producción Vegetal de la Universitat Jaume I de Castellón, reúne las condiciones necesarias para su defensa

Fdo. Dr. Víctor Flors Herrero **Fdo. Dr. Josep A. Jaques Miret**

Agradecimientos

Bien, por donde empezar... Después de tres años esto se va acabando, así que tocará agradecer a toda la gente que ha hecho posible que un joven de Vall d'Alba vaya a ser un futuro doctor!!

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Contents

Introducción

Introducción

España es el mayor productor de cítricos de la Unión Europea y del mundo para consumo en fresco, con una producción anual superior a los 5 millones de toneladas durante los últimos años. Por tratarse de un producto consumido preferentemente en fresco, cualquier daño producido en el fruto lo devalúa económicamente, siendo muy importante el control de las plagas directas. La araña roja *Tetranychus urticae* Koch (Acari: Tetranychidae) es un ácaro plaga que se considera clave, causando el mayor daño cuando ataca a los frutos (Pascual-Ruiz et al., 2014). En la actualidad el principal método de control contra *T. urticae* se basa en la aplicación de acaricidas (Marcic, 2012). Sin embargo, el control químico no siempre es eficaz, debido a la gran capacidad que presenta *T. urticae* para desarrollar resistencia a diferentes tipos de materias activas (Van Leeuwen et al., 2008). Recientemente, se han desarrollado diferentes métodos de control alternativos, como puede ser el uso de cubiertas vegetales para mejorar su control biológico mediante ácaros fitoseidos, los principales depredadores de *T. urticae* (Aguilar-Fenollosa et al., 2012.).

El estudio de los mecánismos de defensa de las plantas puede ayudar a desarrollar nuevas técnicas y herramientas para mejorar el control de agentes bióticos. Las plantas son resistentes a la mayoría de artrópodos herbívoros y hongos fitopatógenos debido a las barreras mecánicas que interponen a su ataque, como la cutícula, tricomas, espinas, etc.. Sin embargo, esto no siempre es suficiente. Algunos artrópodos son capaces de superar esas defensas y ocasionar daño a la planta. En el caso de *T. urticae* el daño que causa a la planta se traduce enuna reducción de la tasa de fotosíntesis y un aumento de la transpiración, debido su alimentación de las células del mesófilo de las hojas a través de sus estiletes, extrayendo su contenido celular (Johnson and Lyon,

1991). Además, en casos como el de los cítricos, el daño aparece también en los frutos, donde el ataque se traduce en la aparición de manchas negruzcas que pueden depreciar enormemente el valor comercial del fruto (Ansaloni et al., 2008).

Para iniciar la respuesta de defensa, la planta debe reconocer eficazmente al atacante. Para ello, la planta presenta unos receptores que reconocen estructuras moleculares asociadas a herbívoros. La activación de estos receptores hace que se inicie una cascada de señalización hasta la producción de compuestos tóxicos para el atacante. Diversos autores han demostrado que la principal ruta de señalización para una respuesta de defensa eficaz contra *T. urticae* es la ruta del ácido jasmónico (Kant et al., 2004; Zhurov et al., 2014). Sin embargo, la naturaleza química de los compuestos con efectos perjudiciales sobre la araña roja es diferente según la especie vegetal de que se trate (Agut et al., 2014; Zhurov et al., 2014), incluyendo inhibidores de proteinasas, glucosinolatos o acil-azucares, para los que se ha demostrado un efecto negativo sobre el desarrollo de *T. urticae*.

En determinadas ocasiones, este tipo de defensa química directa no es del todo eficaz.La planta también puede responder liberando una mezcla de compuestos volátiles que pueden presentar diferentes funciones: atraer a enemigos naturales que se alimentan del fitófago, repeler al fitófago, y avisar a las plantas vecinas o a partes distales de la misma planta de un posible futuro ataque (Dicke and Baldwin, 2010). En el caso de la araña roja, dos compuestos volátiles, ocimeno y metilsalicilato, parece que son los responsables de la atracción de ácaros fitoseidos, el grupo de depredadores considerado más eficaz contra *T. urticae* (Zhang et al., 2009; Ament et al., 2010).

Las variedades comerciales de cítricos se multiplican vegetativamente injertandolas en un patrón. Esto puede proporcionar a la planta resistencia a

diferentes factores ambientales tanto bióticos como abióticos. Bruessow y colaboradores (2011) demostraron que la respuesta de defensa de los cítricos frente a esta plaga estaba mediada por el patrón. Injertando el mismo cultivar sobre diferentes patrones observaron diferentes desarrollo de la araña, siendo la variedad injertada sobre naranjo amargo la que mostró mayor nivel de resistencia mientras que el mandarino Cleopatra fue el que indujo mayor susceptibilidad, sugiriendo la existencia de algún tipo de señal sistémica que viaja desde la raíz a las hojas.

En esta investigación, se pretende dilucidar los principales mecanismos de defensa directa en cítricos mediante el uso de cromatografía líquida acoplada a un cuadrupolo-tiempo de vuelo (QTOF) para la detección de compuestos con capacidad acaricida. Asimismo, se ha determinado el papel que juegan los volátiles liberados en respuesta al ataque, usando la cromatografía de gases para la detección de estos compuestos . Por último, se ha abordado el estudio de las señales sistémicas de las hojas a la raíz y viceversa para determinar la señal móvil que envía el patrón hacia la variedad en respuesta al ataque de *T. urticae*.

Esta investigación ha generado el conocimiento de nuevos compuestos naturales con potencial actividad acaricida y repelente de la propia plaga que combinados adecuadamente pueden conducir en un futuro a nuevos métodos para el control de la araña roja que permitan reducir el uso de plaguicidas químicos de síntesis y desarrollar programas de protección integrada de cultivos con un uso mínimo de plaguicidas en cítricos.

(Todas las referencias se encuentran en las referencias de los capítulos posteriores)

Objetivos

El objetivo general de la investigación es dilucidar los principales mecanismos de defensa de los cítricos frente al ataque de *T. urticae.*

Los objetivos concretos son:

1. Determinar rutas metabólicas y compuestos responsables de la defensa mediante la realización de bioensayos de resistencia directa con patrones de cítricos con diferente nivel de susceptibilidad a la araña roja. Desarrollado en el Capítulo 2.
2. Estudiar la comunicación química planta-planta en respuesta a la infestación por *T. urticae*. Para ello, se determinarán los compuestos volátiles liberados por los patrones en presencia de *T. urticae*, y se analizarán el papel que juegan estos volátiles en la promoción de respuestas en cítricos adyacentes no infestados, así como en la elección del huésped por *T. urticae.* Desarrollado en el Capítulo 3.
3. Identificar las señales sistémicas transmitidas desde el patrón a la variedad en respuesta a la infestación, utilizando el cultivar Clemenules injertado sobre patrones de cítricos con diferente nivel de susceptibilidad a la araña roja. Desarrollado en el Capítulo 4.

Metodología general

- Experimentos planta-artrópodo

Para profundizar sobre los mecanismos de resistencia de cítricos frente a la araña roja se han realizado estudios tanto con patrones de dos meses de edad como con plantas del cultivar Clemenules injertado sobre diferentes patrones.

Se utilizaron dos patrones con diferente nivel de respuesta al ataque de *T. urticae*, naranjo amargo y mandarino Cleopatra. Con estos dos fenotipos distintos se analizaron las posibles rutas metabólicas y compuestos responsables de la resistencia a la araña roja.

- Bioensayos de elección

Para observar el efecto de los volátiles liberados por los patrones en presencia de la plaga se realizaron ensayos de elección con un olfactómetro. Los ensayos de elección se llevaron a cabo mediante la introducción de aire filtrado en el sistema. Los volátiles liberados por las plantas en respuesta a la infestación se hicieron pasar por un tubo en Y para forzar la elección de las arañas en función de la diferente composición de gases emitidos por los patrones.

- RT-qPCR

La expresión génica de los genes de interés se llevó a cabo mediante la técnica de cuantificación de mRNA tras su conversión a cDNA y amplificación mediante cebadores específicos de los genes estudiados, comparando su expresión con los genes constitutivos GAPDH y EF1. Mediante el análisis de la curva de melting se comprobó la pureza del producto amplificado.

- Cromatografía líquida y gaseosa

Las muestras vegetales obtenidas de los diferentes experimentos se prepararon para el análisis por cromatografía líquida mediante homogeneización y posteriormente se centrifugaron y se purificaron mediante partición en fase orgánica, desecado, resuspensión en el disolvente apropiado y posterior filtrado. El desarrollo de todas las técnicas de cromatografía líquida se realizó combinando dos equipos por un lado un Waters Acquity LC (UPLC-TQD; Waters, http://www.waters.com) para las búsquedas dirigidas, y por otro lado,

Waters Acquity acoplado a un detector de masas/masas cuadrupolo-tiempo de vuelo (QTOF MS) para las busquedas no dirigidas.

En cuanto a la cromatografía gaseosa el instrumental utilizado fue un Agilent 7683 acoplado a un espectometro de masas de tiempo de vuelo GCT (Waters Corporation).

Resumen

En la presente tesis doctoral se han estudiado los mecanismos de defensa de cítricos frente al ataque de *T. urticae* a diferentes niveles. En primer lugar se analizaron los mecanismos de defensa directa utilizando dos patrones con diferente nivel de susceptibilidad. Se llegó a la conclusión de que la ruta de defensa del ácido jasmónico (JA) es la efectiva frente a la araña roja. Mediante análisis metabolómicos se observó una gran acumulación de diferentes flavonoides como naringenina o hesperetina en el patrón más resistente. Estos flavonoides con posible actividad acaricida posiblemente estén regulados por la ruta JA, fenómeno que se ha confirmado en otras especies vegetales. A continuación se estudió si los volátiles liberados por los cítricos tenían algún efecto sobre las plantas vecinas o sobre el propio fitófago. Los experimentos demostraron que los volátiles del patrón resistente naranjo amargo infestado con *T. urticae* eran capaces de inducir resistencia en el patrón susceptible mandarino Cleopatra y además que estos volátiles creaban un efecto de repelencia en el fitófago. Se observó que *T. urticae* evitaba las plantas de naranjo amargo previamente infestadas por conspecíficos. La identificación y cuantificación de los volátiles indicó que estos efectos eran causados por limoneno, ocimeno y 4-hidroxi-4-metil-2-pentanona. La última parte del trabajo se basó en estudios sistémicos para tratar de dilucidar las señales que eran transmitidas desde el patrón a la variedad. Se muestreó el eflujo de la raíz en

plantas de la variedad Clemenules injertadas sobre los dos patrones ya citados. Posteriormente el análisis metabolómico de estas muestras indicó que el ácido glutámico parecía ser la señal que se transporta desde el patrón hasta la variedad.

Aportaciones originales

Publicaciones:

1. **Agut B**, Gamir J, Jacas JA, Hurtado M, Flors V. 2014. Different metabolic and genetic responses in citrus may explain relative susceptibility against *Tetranychus urticae*. Pest management science, 2014, 70(11):1728-1741

2. **Agut B**, Gamir J, Jacas JA, Flors V. 2015. *Tetranychus urticae*-triggered responses in citrus promote genotype-dependent conspecific repellence or attractiveness. New phytologist. Accepted article.

3. **Agut B**, Gamir J, Jacas JA, Flors V. 2015. Systemic resistance to Tetranychus urticae in citrus is induced by conspecifics and transmitted by grafting and mediated by mobile aminoacid. In preparation.

4. **Agut B**, Flors V, Jacas JA. *Tetranychus urticae*: current management and new tools based in plant defense mechanisms. In preparation

Aportaciones en congresos:

- Posters:

1. Spider mite response in citrus rootstocks is mediated by the oxylipin pathway. **Agut B**, Jacas J.A, Hurtado M, Flors V.
SEB annual meeting 2012, Salzburg.

2. Relevance of the oxilipin pathway and SA-JA crosstalk in citrus against spider mite infestation. **Agut B**, Jacas J.A, Hurtado M, Flors V.

Environment workshop UIA 2012; Plant-microbe-insect interaction: from molecular mechanisms to ecological implications. Baeza.

3. Rootstock resistance against *T. urticae* in citrus is transmitted to grafted varieties probably activating JA-dependent signalling. **Agut B**, Jacas J.A, Hurtado M, Flors V.

IOBC-WPRS Working Group "Induced resistance in plants against insects and diseases" 2013, Avignon.

- Participaciones orales

1. La respuesta de patrones de citricos frente a *Tetranychus urticae* koch (Acari:Tetranychidae) esta mediada por la ruta del ácido jasmónico. **Agut B.**, Gamir J., Hurtado M., Jacas J., Flors V.

Congreso nacional de Entomología, Mataró. 2013.

2. Sour orange HIPVs repel spider mites and induce defense priming in neighbouring citrus plants. **Agut B.**, Gamir J., Jacas J., Flors V.

SEB annual meeting 2014, Manchester.

3. *Tetranyhus urticae* and Citrus: hijaking or effective defense. **Agut B.**, Gamir J., Hurtado M., Jacas J., Flors V.

XIVth International Congress of Acarology. Kyoto.

CHAPTER 1

Introduction. *Tetranychus urticae*: current management and new tools based in plant defense mechanisms

Abstract

Tetranychus urticae Koch is a cosmopolitan and polyphagous mite, causing economic losses in some agricultural and ornamental plants. There are some traits of *T. urticae* that can explain the difficult control in the field of this pest. Such as a short life cycle, the reproduction and the high ability to adapt to different environmental conditions. Management factors carried out by the farmers such as over fertilization or overuse of pesticides also favor the mite's performance. Nowadays chemical control together with cultural practices are the major control methods. In the recent years some studies have focused their interest in plant defense mechanisms against herbivores. It has been described different families of plant compounds (as flavonoids, glucosinolates or acylsugars) show pesticide activity. Another relevant plant defense against herbivores is the release of volatile compounds that attract natural enemies, prime neighboring plants or repel the conspecifics pest. This new knowledge can be used in the future to reduce the damage caused by *T. urticae* in the field by implementing new crop management strategies.

Introduction

Spider mites (Tetranychidae) are the most important family of plant feeding mites of agricultural relevance (Jepsson el al., 1975). The minute members of this family (200 to 900 µm long) receive this name due the ability of some of them to produce silk threads with different functions, including protection and dispersal from overexploited habitats to new ones (Fleschner et al., 1956). The colonies of several webbing species live within the web that may become a real nest, discouraging natural enemies from entering and mitigating adverse climatic conditions including the interception of risky UV light (Saito, 2010). More than 1200 spider mite species have been described and about 10 % of them can turn into agricultural pests (Helle and Sabelis, 1985; Bolland et al., 1998; Migeon and Dorkeld, 2007). Among these species, the two-spotted spider mite *Tetranychus urticae* Koch is considered as the most serious one. This species is cosmopolitan and polyphagous. Around 4000 host plant species have been described worldwide (Migeon and Dorkeld, 2007) and many of them are important crops, including fruit trees, vegetables and ornamentals, where it is frequently considered as a key pest (Grbić et al., 2011; Attia et al., 2013). *T. urticae* regularly feeds on the mesophyll cells of the underside of the leaves where it is protected from UV light (Ohtsuka and Osakabe, 2009). As a consequence, this mite produces a mechanical damage consisting of empty cells that result in a dull color of the affected organ which may later become blackish as the number of necrotic cells increases. Furthermore, the feeding activity alters cell contents, resulting in lower concentrations of nitrogen, phosphorous or proteins, and cell physiology, reducing photosynthesis and injecting phytotoxic compounds that decrease yields (Johnson and Lyon, 1991). The economic status of *T. urticae* was achieved after World War II (Hoy, 2011) and a plausible explanation of this change is based on the disruption by pesticide

use of existing natural biological control, mostly by Phytoseiidae predatory mites (Huffaker et al., 1969). However, additional causes cannot be neglected and in the following pages we will discuss both intrinsic and extrinsic factors that may have contributed to this increasingly important pest status. Only a thorough knowledge of them may allow us to make educated decisions leading to a better control of this mite. Particularly, studies focused on *T. urticae*-plant interactions may pave the way for novel approaches to improve existing IPM programs for crops where this mite is a key pest.

1. Origin of the problem

As mentioned earlier, there are intrinsic factors, as life cycle or adaptation abilities, that make the two spotted spider mite a relevant agricultural pest. However, management factors have no doubt contributed to the present prevalence of this phytophagous mite. We will first discuss these intrinsic factors, then will focus on current management practices, and finally we will see how, in accordance with the general philosophy of IPM, natural mortality factors acting on *T. urticae*, both top-down and bottom-up regulation mechanisms, could be exploited to increase the resilience of our cropping systems.

1.1 Intrinsic factors

1.1.1 Life cycle and reproduction

Tetranychus urticae is an *r*-strategist (MacArthur and Wilson, 1967). Among the traits that characterize *r*-selected species as small size, short life cycle, early

sexual maturity, high offspring production, or short life time expectancy, *T. urticae* complies with most of them. During their life *T. urticae* females can produce over 100 eggs at 25ºC; they measure 490-515 micrometers long as adults (Helle et al., 1970), in optimal conditions their development time is less than a week. Furthermore, *T. urticae* reproduces through arrhenotoky (Helle et al., 1970), a form of parthenogenesis in which unfertilized eggs develop into males. Males are produced parthenogenetically, while diploid females are usually produced biparentally from fertilized eggs. Reproduction is further altered by the endosymbiont *Wolbachia* spp. (Werren, 1997). This endosymbiont shows different functions when present in *T. urticae*, from manipulation of host reproduction to interactions in nutritional and metabolic pathways, interferences in development and life span, and protection from pathogens and parasites (Moran et al., 2008; Brownlie and Johnson, 2009; Gross et al., 2009; Cook and McGraw, 2010). Because in species with arrhenotokous parthenogenesis, as *T. urticae*, haploid embryos resulting from paternal genome elimination in fertilized eggs can develop into viable males (Breeuwer and Werren, 1990), incompatible *T. urticae* crosses caused by *Wolbachia*, result in fertilized eggs either developing as haploid males or aborting.

1.1.2. High ability to adapt to environmental harsh conditions

Tetranychus urticae shows different traits that let it overcome adverse conditions, such as diapause. It can be defined as a genetically determined state of suppressed development that is controlled by environmental factors (Hunter and McNeil, 1997). Many changes occur when *T. urticae* is in diapause: it stops feeding and searches for hibernation sites (Veerman, 1985), it may change

color, become positively geotactic and negatively phototactic to find protected sites (Foott, 1965). Recently, some authors showed the biochemical changes in the diapause. A genome-wide microarray used by Bryon et al. (2013) revealed changes in pathways implicated with digestion, detoxification, pryoprotection, carotenoid biosynthesis and organization of the cytoskeleton. Also in 2013, Khodayari et al., using metabolomic approaches, found high levels of glucose and gluconolactone in diapausing females. These two metabolites could be implicated the synthesis of polyols, relevant compounds in tolerance to low temperatures. This knowledge may help refining control of *T. urticae*, since winter survival in temperate climates hasimportant consequences for crop protection (Bryon et al., 2013).

The two spotted spider mites have a wide range of hosts and can feed on many plant species (Migeon and Dorkeld, 2007). To defend against herbivore attacks, plants produce a range of compounds with antixenotic effects such as metabolites with insecticidal activity. *Tetranychus urticae* has the ability to detoxify many of these toxic compounds. Dermauw et al. (2012) found that host plant adaptation was due to some proteins families with detoxification properties. like P450 monooxigenases or ring-splitting dioxygenase. When spider mites were moved to a different host plant, the genes implicated were up-regulated.

Dispersal is a key point in the emergence of the pest. *Tetranychus urticae* colonization begins with a mated female followed by aquick population growth and finalizes when the host plant is over-exploited. When the food source becomes scarce spider mites need to disperse. This species has some adaptations related to dispersal. When the host plant deteriorates, a wide range of plant host increases the probability to find a new suitable host. During this

stage males are smaller and less abundant than females while the number of female eggs and male mortality increase (Helle and Sabelis, 1985). Mated females can disperse by crawling (Hussey and Parr, 1963), this kind of behavior normally occurs in parts of the same host plant or in dense aggregations of host plants. However, dispersal from overexploited hosts is usually aerial (Li and Margolies, 1993; Osakabe et al., 2008) . In this case spider mites affix a thread to the substrate, hanging from the thread in the air, and are carried off by the wind. Likewise, when resources become scarce, individuals can disperse together by the formation of silk-balls (Clotuche et al., 2011) that can contain thousands of mites. While individual modes of dispersal (crawling and aerial dispersal) are restricted to mated females, the silk-balls mainly contain immature individuals.

1.2 Management factors

Up to now we have discussed about biological parameters that favor the prevalence of the two spotted spider mite as a pest in the field. However in natural ecosystems spider mite colonies consist of small groups of individuals in equilibrium with their predators and hardly ever cause important damage in their host plant (Helle and Sabelis, 1985). Thus the question arises: why *T. urticae* becomes uncontrolled in the agroecosystems?, and why does it cause severe economically losses to the farmers? To answer these questions we must take into account that there is no single reason contributing to increase the potential damage of *T. urticae* but several.

1.2.1 Pesticides

1.2.1.1 Resistance

An important trait that difficults the control of *T. urticae* is its ability to develop resistance against acaricides in a short time (Knowles et al., 1997; Van Leeuwen et al., 2008). There are several ways by which herbivorous arthropods can become resistant to pesticides. Pests can exhibit more than one resistance mechanism at the same time. They can be classified in four categories (Knowles, 1997):

1) Metabolic resistance, when a resistant population may detoxify the toxin faster than a susceptible population. Resistant pests may possess higher levels or more efficient forms of the enzyme(s) that break down pesticides to nontoxic compounds;

2) Altered target-site resistance, when the site where the toxin usually binds to the enzyme has been modified reducing the pesticide effects;

3) Behavioral resistance, when resistant pests may avoid the toxin by a change from their normal activity, and

4) Penetration resistance, when the resistant population may absorb the toxin slowly than susceptible populations. This resistance occurs when the cuticle develops barriers or avoids the absorption of the chemicals into their bodies.

Tetranycus urticae has been reported to develop resistance to 94 active ingredients with different modes of action (Table 1).

Table 1. Active substances for which resistance in *T. urticae* has been reported categorized according to their mode of action (MoA) (source: IRAC 2014)

MoA (IRAC)		**Active substances**
Group	**Description**	
1A	Acetylcholinesterase (AChE) inhibitors: carbamates	Aldicarb, Formetanate, Methomyl
1B	AChE inhibitors: organophosphates	Acephate, Amidithion, Azinphos-methyl, Bromophos, Carbophenothion, Chlorpirifos, Demeton, Demeton-S-methyl, Diazinon, Dicrotophos, Dimefox, Dimethoate, Disulfoton, EPN, Ethion, Ethoate-methyl, Famphur,, Formothion, Malathion, Mephosfolan, Methamidophos, Methidathion, Mevinphos, Monocrotophos, Naled, Omethoate, Parathion, Parathion-methyl, Phenkapton, Phentoate, Phorate, Phosalone, Phosmet, Phosphamidon, Pirimiphos-methyl, Profenofos, Prothoate, Sulfotep, TEPP, Thiometon, Trichlorfom, Vamidothion
2A	GABA-gated chloride channel antagonist	BHC/cyclodienes, Dienochlor
3A	Na^+ channel modulators	Acrinathrin, Bifenthrin, Deltamethrin, Fenpropathrin, Permethrin
3B		DDT
6	Cl^- channel activators	Abamectin, Milbemectin
10A	Compounds of unknown MoA or non-specific MoA (mite growth inhibitors)	Clofentezine, Hexythiazox, Hexythiazox+Organotin
10B		Etoxazole
12B	Inhibitors of ATP synthase	Azocyclotin, Cyhexatin, Fenbutatin oxide
12C		Propargite
12D		Tetradifon
13	Uncoupler of oxidative phosphorilation via disruption of proton gradient	Binapacryl, Chlorfenapyr,

15	Inhibitors of chitin biosynthesis	Flucycloxuron
19	Octomaminergic agonist	Amitraz, Chlordimeform,
20B	Mitochondrial complex III electron transport inhibitor	Acequinocyl
21A	Mitochondrial complex I electron transport inhibitors	Fenazaquin, Fenpyroximate, Fluacrypyrim, Pyridaben, Tebufenpyrad
23	Inhibitors of lipid synthesis	Spirodiclofen, Spiromesifen, Spirotetramat
UN	Unknown MoA	Azobenzene, Bifenazate Bromopropylate, Chlorbenside, Chlorfenson, Chlorfensulfide, Chlorobenzilate, Chloropropylate, Dicofol, Quinomethionate

1.2.1.2. Secondary pest outbreaks

Secondary pest outbreaks can be caused by broad-spectrum insecticides that disrupt natural pest control in different ways. Direct effects due to the toxicity of the insecticide on non-target natural enemies (in this case, mostly Phytoseiidae mites) are expected. However, insecticides can also cause indirect impacts on the natural enemies as their food resource is reduced or the prey is contaminated (Croft and Brown, 1975; Stark et el., 2007). Moreover, in many cases insecticides affect more natural enemies than the pestiferous species they feed on (Tang et al., 2010). In addition, insecticides may also cause sub-lethal effects on the natural enemies including reduced fecundity, longevity and changes in sex ratios and altered behavior, such as impaired movement and problems to localize their prey (Croft and Brown, 1975; Stark and Banks, 2003; Desneux et al., 2007; Cloyd, 2012; Longley and Jepson, 1996; Desneux et al., 2004). The overuse of insecticides in crops can transform a harmless arthropod into a serious economic problem. The european red mite *Panonychus ulmi*

(Kock) is one of these examples. Its natural control by *Amblyseius fallacis* (Garman) was disrupted by pyrethroids used to control the moth pests in apple. Thereafter *P. ulmi* became a serious problem in apple orchards (Costa-Comelles et al., 1997; Stanyard et al.,1998).

Finally, an additional factor that may prevent correct control of mites in agricultural ecosystems is the stimulatiing effect of some insecticides on diverse biological parameters, the so called hormoligosis phenomenon (Guedes et al., 2009). For instance, James and Pryce (2002) showed that sub-lethal doses of imidacloprid increased in 20% the number of eggs produced by *T. urticae* compared with a water treatment. Likewise, Marcic (2003) measured the intrinsic rate of increase of *T. urticae* after sub-lethal dosis of clofentezine and observed a significant increase compared to water treated females.

1.2.2. Growing highly susceptible cultivars

Plant breeders have been selecting for a long time crops with desirable commercial traits. Unusual or extreme phenotypes, such as large fruit or seed size, intense color, sweet flavor, or pleasing aroma are normally selected and maintained in their cultivars for esthetic reasons, while synchronous ripening or inhibition of seed shattering are selected to facilitate harvest. With the “Green Revolution”, in the 1960s new agricultural practices developed by the industry as fertilizers, pesticides or more productive new cultivars were selected to increase the yield (Brummer et al., 2011). However, high productivity is rarely linked to resistance to pests and diseases. As a consequence, highly productive cultivars selected during the Green Revolution were increasingly susceptible to pests. In the case of *T. urticae*, water stress, heat, or fertilization have been related to increased damages (Aucejo et al., 2004; Ansaloni et al., 2008). A

reasonable explanation for this phenomenon is related to the leaf nutritional status. Some authors have related the levels of nitrogen, phosphorous, potassium and beta-carotene as key compounds that increase damage caused by *T. urticae*. So, the over-fetilization or the use of new cultivars with high productions could alter the spider mite biology and hinder the control of this mite. (Huffaker et al., 1969).

2. Current management of *T. urticae*

According to Rabbinge (1985), the crops suffering most from spider mite damage in 1985 were fruit orchards, as well as many ornamental and horticultural crops both under glasshouse and in open field conditions. This situation has not significantly changed since then and damage should be mostly attributed to *T. urticae* (Attia et al., 2013). Additionally, *T. urticae* is also considered as a key pest in a few more crops as citrus (Pascual-Ruiz et al., 2014) and cotton (Agostini et al., 2014). Management of *T. urticae* in these systems has been for a long time primarily based on the use of pesticides (Marcic, 2012; Van Leeuwen et al., 2013). However, the different side-effects caused by these biocides (see 1.2.1 above) have made the inclusion of alternative more sustainable methods (basically cultural and biological) a requisite of any sound modern approach to manage this mite. Within the context of Integrated Pest Management (IPM), which is considered the key approach to modern crop protection (Hoy, 2011), effective chemical control methods should be not only effective against the target pest but compatible with biological control agents and safe for other non-target organisms (including humans) and the environment.

2.1. Chemical control

Severe widespread outbreaks of *T. urticae* populations occurred during the 1950s (Marcic, 2012). This occurrence coincided with the generalization of chemical control as the primary means to suppress pest populations in increasingly productive crops which became high quality food sources for spider mites. At that moment, the same broad-spectrum neurotoxic insecticide families used against insect pests (organochlorines, and progressively, organophosphates and carbamates) were targeted to spider mites (Attia et al., 2013). Most of these wide-range long-persistent pesticides are currently either banned or not recommended under IPM labels in developed countries mainly due to toxicological and environmental issues. For instance, in the European Union (EU) from the 103 active substances included in the EU database as acaricides that were once authorized in the EU member states, only 32 (Table 2) are presently approved (EU 2014). These products can be arranged in 10 groups according to their mode of action (MoA) (IRAC 2014) including one carbamate and four organophosphates. However, in Spain for instance, none of these broad-spectrum neurotoxic pesticides is currently authorized against *T. urticae* (MAGRAMA, 2014). Specific more selective modes of action centered on the inhibition of mite basic functions as growth, mitochondrial respiration, or lipid biosynthesis have been gaining importance. The sustainable use of these acaricides in combination with additional control methods can be achieved by treatments based on the use of sampling plans and economic injury levels (EIL) when possible (e.g., Pascual-Ruiz et al., 2014), and the rotation of compounds from different MoA groups to delay as much as possible the selection of resistance to any one type of acaricide (IRAC 2014).

2.2. Biological control (entomopathogens, predators, biopesticides).

Spider mites have a relatively large complex of natural enemies, including insects, mites and entomopathogens. Although species may vary locally, taxonomic groups often coincide even at genus level worldwide (Garcia-Marí and Gónzalez-Zamora, 1999). The most effective group of *T. urticae* natural enemies, as well as for all tetranychids, belongs to the Phytoseiidae mite family. Indeed, many IPM prgrams include the sampling and the manipulation, through augmentative releases and conservation, of their populations (Abad-Moyano et al., 2009). Additional less frequent predators can be found in the mite families Anystidae, Bdellidae, Cheyletidae, Erythraeidae, and Stigmaeidae. There is more than 2000 spp. of phytoseiids in 67 different genera (Moraes et al., 2004). These predatory mites have a similar body size to *T. urticae* and they are usually shiny whitish. However, color may change to reddish after ingestion of their prey. Phytoseiidae are usually very active and therefore, they can be easily distinguished from *T. urticae*, which usually moves very slowly. Some Phytoseiidae are almost specialists on *T. urticae* (e.g., *Phytoseiulus persimilus* (Athias-Henriot)), others can feed on other Tetranychidae as well (e.g. *Neoseiulus californicus*), and some phytoseiids are generalist predators that may include *T. urticae* in their diet (e.g. *Euseius* spp.) (McMurtry et al., 2013) Our group has recently developed a multiplex PCR that will allow a quantitative assessment of the impact of these species on *T. urticae* under real field conditions (Perez-Sayas et al., submitted). In addition to mites, some

Table 2. Acaricidal active substances approved in the EU (EU 2014) categorized according to their mode of action (MoA) (IRAC 2014)

MoA (IRAC)		**Active substances**
Group	**Description**	
1A	Acetylcholinesterase (AChE) inhibitors: carbamates	Formetanate
1B	AChE inhibitors: organophosphates	Chlorpyrifos, Chlorpyrifos-methyl, Malathion, Dimethoate
3A	Sodium channel modulator: Pyrethrins, pyrethroids	Acrinathrin, Bifenthrin, Cypermethrin
6	Chloride channel activators: Avermectins, Milbemycins	Abamectin
10A	Mite growth inhibitors	Clofentezine, Hexythiazox
20B	Mitochondrial complex III electron transport inhibitors	Acequinocyl
21A	Mitochondrial complex I electron transport inhibitors	Fenazaquin, Fenpyroximate, Pyridaben, Tebufenpyrad
23	Inhibitors of lipid synthesis	Spirodiclofen, Spiromesifen
25	Mitochondrial complex II electron transport inhibitors	Cyflumetofen
	Acaricides for which the mode of action is unknown	Bifenazate
	Acaricides for which MoA has not been reported by IRAC (2014)	Capric acid (CAS 334-48-5), Caprylic acid (CAS 124-07-2), Fatty acids C7 to C20, Fatty acids C7-C18 and C18 unsaturated potassium salts (CAS 67701-09-1), Fatty acids C8-C10 methyl esters (CAS 85566-26-3), Lauric acid (CAS 143-07-7), Lime sulphur (calcium polysulphid), Methyl decanoate (CAS 110-42-9), Methyl octaonate (CAS 111-11-5) Oleic acid (CAS 112-80-1), Pelargonic acid (CAS 112-05-0), Sulphur

insect predators from different orders can also effectively feed on *T. urticae*. The most important ones, which in some cases are commercially available, include the Coccinellidae *Sthetorus punctillum* Weise, the Neuropterans *Chrysoperla* spp. (Chrysopidae), *Conwentzia* spp. and *Semidalis* spp. (Coniopterygidae), predatory thrips such as *Scolothrips longicornis* Priesner (Thysanoptera: Thripidae) and the midge *Feltiella acarisuga* (Vallot) (Diptera: Cecidomyiidae).

2.3. Cultural control (including plant resistance/tolerance)

It has long been known that high relative humidity may enhance the activity of predatory mites and reduce the reproductive potential of tetranychids whose optimal environment is provided by hot dry conditions (Sabelis, 1986; Nihoul, 1993; Duso et al., 2004). A report in 1969 demonstrated by varying the fertilizer regime applied to the crops that host plant nutrition may affect mite population (Markkula and Tiittanen, 1969). Furthermore, large quantities of nitrogen or a deficiency of potassium can increase the amount of soluble nitrogen available in the plant so that sharp increases in the populations of *T. urticae* may follow such fertilizer regimes (Sabelis, 1986). However, the plant response to such extreme feeding regimes is not economically viable, so variations in host nutrition have not been used for pest control (Helle and Sabelis, 1985).

3. Plant-mediated regulation mechanisms that may contribute for a more sustainable control of *T. urticae*.

Among the methods to control *T. urticae* such as the development of new insecticides with no secondary effect in non-target organism or agronomic practices to improve pest control by natural enemies, the potential of plant defense mechanisms against this herbivore can be used in IPM. However, the knowledge of plant responses to this pest is still limited and further research is needed.

Upon herbivore arthropod attack, plants present a battery of defenses that reduce pest abundance or increase plant tolerance to damage. Plants resistance against arthropods is divided into three different mechanisms:

1) Antibiosis, the resistance affects the biology of the insect (reducing oviposition, longevity or survival). Subsequently damage is reduced compared to plants or cultivars lacking such mechanism (Carmona and Lajeunesse, 2011).

2) Antixenosis, this resistance affects the behavior of an insect pest. The insect shows a non-preference effect when it lands on a resistant plant compared with a susceptible one (Carmona and Lajeunesse, 2011). And

3) Tolerance, in which a plant is able to recover from damage caused by herbivore pest abundance equal to that damaging a plant without resistance characters (susceptible). Tolerance is a plant response to an herbivore pest (Stowe et al., 2000).

3.1. Bottom up mechanisms. Direct defenses

Direct defenses promote directly a detrimental effect on the herbivore. Some examples of this kind of defenses are morphological traits such as thorns, prickles, or high levels of lignification. Another kind or direct defenses are the secondary metabolites, which can be toxic or antidigestive.

3.1.1. Physical plant defenses

Direct defenses include physical barriers such as trichomes and leaf toughness and chemical defenses such as secondary compounds and toxins. It is commonly accepted that physical barriers can be classified as constitutive defenses. Physical barriers are present in all plant species. These barriers are the first defense that faces the herbivore arthropod . They are also the most effective since physical barriers can protect the plant from a variety of herbivore pests, only a little percentage of the herbivore attackers succeed. Different anatomical structures such as trichomes or cuticle constitute the main physical barriers.

The trichomes are hair-like appendages that develop from cells of the aerial epidermis and are produced by most plant species. The trichome structure can be unicellular or multi-cellular. However the most classical division is glandular or non-glandular trichomes. Glandular trichomes present a role in chemical defenses (Wittstock and Gershenzon, 2002). The non-glandular trichome function is to reduce the movement of small arthropods in the leaf surface and to hinder the access the epidermis for feeding (Tian et al., 2012).

Another relevant physical barrier is the cuticle. It is formed by polymeric lipids and soluble waxes that cover the leaf tissues. Normally the cuticle does not

show any adaptations to a specific plant surface with the exception of fruits. Plants with glossy surfaces and reduced wax blooms are usually resistant to smaller arthropods (Eigenbrode, 2004).

3.1.2. Chemical plant defense

Plant secondary metabolites and toxic proteins are another important direct defenses, contrary to physical barriers chemical defenses can be classified as inducible defenses. They are only produced or released in the presence of the herbivore arthropod. Some chemicals can be stored in cell compartments and released once the insect wounds the leave and therefore the cell membranes are disrupted. Such is the case of mustard oil (Stauber et al., 2012; Miresmailli and Isman, 2014).

3.1.2.1 Hormone-mediated plant response

Plants can activate diverse pathways controlled by phytohormones such as abscisic (ABA), salicylic acid (SA), oxilypines (Jasmonic acid, JA derivatives) and ethylene (ET) in response to biotic attack. This fact can induce the production of secondary metabolites and defensive proteins are chemical defenses that cause a detrimental effect in some biological parameter of the herbivore. To activate their biosynthesis the plant first needs to recognize the spider mite attack. However, the knowledge of these first stages of the spider mite infestation is limited. Damage caused by herbivore attack leads a change in the plasma membrane potential, subsequently cytosolic free Ca^{2+} changes, leading a signal that activates a cascade of events. Arimura et al. (2002) demonstrated that a chelator of extracellular Ca^{2+} blocks the defense responses

of lime bean plants after spider mite infestation. Reactive oxigen species (ROS) are a key point in the first steps in plant-pathogen interactions, however the role of H_2O_2 in the signal transduction against spider mites is not clear. Leitner et al. (2005) showed that the production of H_2O_2 appears only at late stages of the attack.

A major regulatory signal against herbivores is the jasmonic acid, it plays an active role in the defense against arthropods. Different authors reported the activation of JA pathway of many plant species: tomato (Kant et al., 2004, Kawazu et al., 2012), arabidopsis (Zhurov et al., 2014) and citrus (Maserti et al., 2011 and Agut et al., 2014). In these species the JA activation occurs only one or two days after intestation, whereas in *Medicago truncatula* the increase JA levels takes place only in yellowing leaves (Leitner et al., 2005). These differences may be caused by the ability of the plant to recognize the spider mite attack. Together JA, SA signaling is also activated in some plant-herbivore interactions, however its role may cover different aspects of plant defense (Kant et al., 2004; Kawazu et al., 2012; Agut et al., 2014). These authors showed in different plant species the activation of both JA and SA against *T. urticae*, but they play different roles in plant-arthropod interaction. Agut et al. (2014) demonstrated that the effective defense pathway against the two spotted spider mite is the JA signaling pathway. MeJA applications to susceptible citrus rootstock reestablish resistance the phenotype. Whereas SA applications did not promote any change in resistance. Zhurov et al. (2014) also showed the relevance of the oxilypin pathway as a major defense against mites. Arabidopsis *aos* (impaired in JA biosynthesis) and *myc2,3,4* (impaired in JA response) mutants showed an increased damage, a faster development from larvae to nymph and reduced larval mortality.

The activation of JA and SA pathway at the same time contrasts the accepted cross-talk JA-SA showed by many authors (Pieterse et al., 2009; Robert-Seilaniantz et al., 2011; Sarmento et al., 2011; Zhang et al., 2009). The negative cross-talk JA-SA may not be active in all rank of concentrations. But more interestingly, the manipulation by effectors of the mite is a tempting hypothesis since it was described for some insects and many pathogens (Hogenhout and Bos, 2011; Sugio et al., 2011). Kant et al. (2008) described a specific line of *T. urticae* that is able to manipulate tomato plants inactivating the JA pathway. *Tetranychus evansi*, another mite species also inhibits the JA pathway by stimulating the SA-dependent responses (Sarmento et al., 2011). Thus, the JA and SA activation could be the consequence of an attempt to manipulate the host by the mite. Simultaneous responses of the plant to the attack and to arthropod effectors are a consequence of a balance between the concentration of the effector and the magnitude of the plant response to the mite detection.

3.1.2.2. Plant secondary metabolites

Terpenes

Terpenes and terpenoids are a large family of plant secondary metabolites. They are characterized by the presence of repeating carbon skeleton of isoprene. Both are regulated by the JA pathway (Zhang et al., 2009; Bleeker et al., 2011). They can act in different manners as toxic compounds, repellents and attractants. The role as repellents/attractants of natural enemies will be covered in the VOCs mediated defenses section. In 2010, Cavalcanti et al. published the negative effect of the essential oils from *Lippia sidoides* in *T. urticae.* An analysis of the essential oil revealed that many terpenes were present at high concentrations.

Among them, thymol and carvacrol showed potent acaricidal activity. Bleeker et al. (2011) reported that tomato plants overexpressing the terpenoid sesquiterpene 7-epizingiberene originating from wild tomatoes, resulted toxic compound to the spider mite reducing its fecundity and population. These authors also showed that the synthesis of this compound was regulated by JA pathway since exogenous JA applications to wild tomato accumulated higher levels of 7-epizingiberene compared with mock-treated plants.

Glucosinolates

The glucosinolates are natural components of the Brassicacea family, they tipically consist in a β-D-glucopyranose residue linked via a sulfur atom to a (Z)-N-hydroximinosulfate ester, plus a variable radical group. Plants containing elevated levels of mustard oils are higly toxic to insects, these oils contain sugar-glucosinolates that are cleaved by mirosinases when the plant leaf is chewed, cut or otherwise damaged (Badenes-Perez et al., 2013). These natural chemicals most likely contribute to plant defense against pests and diseases (Ahmad et al., 2010; Schlaeppi et al., 2010). Gou et al. (2013) described a synergistic regulation between jasmonic acid and glucose in the accumulation of glucosinolates in Arabidopsis thaliana. Lately, Zhurov et al. (2014) reported for the first time the role of JA and glucosinolates in Arabidopsis-*T. urticae* interaction. They observed that mites performed better in Arabidopsis mutants that were impaired in JA. However they also observed increases of gene expression in genes related with tryptophan catabolism and indoleacetic acid biosynthesis which give rise to indolic glucosinolates (IG). Using metabolomic approaches, it was demonstrated that some IG such as I3M (indol-3-ylmethyl glucosinolate), 1-MeO-I3M (1-methoxy-indol-3- ylmethyl

glucosinolates, neoglucobrassicins) and 4-OH-I3M (4-hydroxyindol-3-ylmethyl glucosinolate) levels were higher following spider mite infestation. Acordingly, IGs relevance in defense was assesed using IG mutants lines in which the development of *T. urticae* was increased. The microarray analisys of the spider mite rearing on IG mutants or wildtype genotype showed that increased expression of detoxification genes such as P450 monooxygenases, glycosyltransferases and lipocalins, correlated with IG levels.

Flavonoids

Flavonoids are low molecular weight polyphenolic substances based on the flavan nucleus. Recently, interesting information related non model citrus plants-*T. urticae* interactions was showed by Agut et al. (2014). This herbivore arthropod is a key pest in mandarin orchards. In citrus, flavonoids were found to play a relevant role in defense. Metabolomic analisys showed high levels of flavonoids such as naringenin, hesperetin and the p-coumaric acid (a precursor) in a resistant citrus compared with a very susceptible genotype. The expression of the chalcone synthase, a key gene in the synthesis of flavonoids was also determined, and the result correlated with the metabolomic data. The chalcone synthase was over expressed in the resistant citrus. Reasonably, in the last years the regulation of flavonoid biosynthesis was proposed to be under JA pathway control (Pourcel et al., 2013; Onkokesung et al., 2014).

Acylsugars

Acylsugars are a group of plant secondary metabolites. They typically consist of aliphatic acyl groups of low to medium chain lengths estherified to the hydroxyl groups of glucose or sucrose. These compounds are produced and

secreted from glandular trichomes on the plant leaf and stem surface. High amounts of these acylsugars give a sticky feel to the plant tissue. It is believed that acylsugars provide physical and/or chemical defense to the plant. Acysugars has been extensively studied in the Solanaceae family. Some publications reported their role as defensive compounds (Alba et al., 2009). The density of the glandular trichome type IV and the production of acylsucrose by this kind of trichome was correlated an increased mortality and repellence, and reduced oviposition of *T. urticae*. Hare and Walling (2006) demonstrated that the synthesis of acysugars was controlled be the JA pathway. MeJA applications in *Datura wrightii* (Solanaceae) increased the production of these compounds by 44%.

3.1.2.3. Defensive proteins

In addition to secondary metabolites, plants can also synthesize defensive proteins with different structures and functions. Proteinase inhibitors (PIs), a family of digestive proteases that act in the insect midgut, were widely studied as components of plant defense (Green and Ryan, 1972; Ryan, 1990). They act in the herbivore gut inactivating the gut proteases, disruption of digestive processes causes amino acid deficiencies that negatively affect the performance of the herbivore (Lison et al., 2006; Zavala et al., 2004). The diversity of many PI gene families could reflect the evolution of insect counter-adaptations that have led to the chemical arms race between plants and herbivores (Talyzina and Ingvarsson, 2006). In order to determine the real effect of these PIs Carrillo et al. (2011) characterized a barley cistanin gene that codified a phytocystatin (inhibitor of cysteine-protease). Transgenic maize plants overexpressing the barley cystatin lcy6 gene showed reduced the reproductive performance and

increased days to reach full adult development of *T. urticae*. Santamaria et al. (2012) analyzed the role of a trypsin inhibitor. Arabidopsis plants carrying both the cystatin Icy6 and the trypsin inhibitor Itr1 genes showed reduced damaged leaf area compared with wild type plants. *T. urticae* feeding on these transformant lines presented less activity of cathepsin B, this protein acts in the protein catabolism, therefore a reduction in its this activity could promote aminoacid deficiencies with detrimental effect in the performance of the spider mite.

3.1.2.4. Induced response

Systemic signals

Herbivore induced resistance (Pieterse et al., 2014) in plant arthopod interactions have been poorly studied till now. Previous research showed the effect on aboveground defense against herbivores folllowing an interaction with belowground herbivores. Erb et al. (2008) reported the hormonal signaling involved in the systemic movement to aerial parts after the attack of the root feeder *Diabrotica virgifera virgifer*a Leconte (Coleoptera: Chrysomelidae). A previous attack of *D. virgifera* in maize roots increase the levels of ABA in the leaves, this increase reduced the water content and the nutricious value, and these plants were less attacked by *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae). In the plant-mite interaction Karban and Carey in 1984 showed that a previous infestation with *T. urticae* in cotton seedlings reduced the mite populations compared with plants that had never been exposed to the mite, thus this may be one of the first reports of herbivore induced resistance (HIR) triggered by mites, although there are no molecular studies that provide an explanation for such observation. In 2013, Mousavi et al. showed the electrical

signal as a relevant process in HIR. The authors described that the damage by the herbivore produced a membrane depolarization and this electric signal is transmitted to systemic undamaged leaves. The receptors of this electrical signal in the systemic leaves is the glutamate receptor GLR. Following recognition there is an activation of the JA pathway.

Herbivore induced plant volatiles (HIPVs), as repellents of herbivores

The role of HIPVs as repellents of herbivores is not fully understood. The plant protects itself from further attacks, however from the mite's point of view, these blend may warn other mites of a poor source of nutrites or even a toxic host. Particularly in the case of *T. urticae* the data are still controversial. Dicke (1986) showed that HIPVs emitted by lima bean plants repelled *T. urticae*. Whereas Pallini et al. (1997) showed that the mite was attracted to cucumber plants previoulsy infested with the spider mite. Looking closely to both experiments a plausible explanation can be obtained from such apparent opposite results. Both plant species (lima bean and cucumber) present different levels of resistance that could be associated with differences in the intensity and composition of volatiles released. Another reason could be the level of infestation of the plants. Spider mite may detect a wide infestation, when the host is over-exploted and the mite seems to prefer clean plants. In plants that present a low population, other mites can still be attracted. However the volatiles implicated in this attraction/repellence remain mostly unknown. In Arabidopsis, a transgenic line that overproduce the terpenoids linalool, nerolidol and 4,8-dimethylnona-l,3,7-triene ((E)-DMNT) were less attractive to the aphid Brevicoryne brassicae (L.) (Hemiptera: Aphididae) (Kos et al., 2013). However, sometimes the volatiles released by the plant attract the pest, as the

aromatic compound benzaldehyde, that attract the moth *Cydia molesta* Busck. (Lepidoptera: Tortricidae) in peach orchands (Piñero and Dorn, 2007).

Role of HIPVs as priming agents

Another function previously described for HIPVs is their role as priming agents. Priming is a physiological phenomenon by which a plant responds more quickly or aggressively to future biotic or abiotic stress (Frost et al., 2008). The volatiles released by infested plants can warn distal plant parts as well as neighboring plants. The plants that receive the signal can be prepared for a second attack. In the case of plant-pathogen interactions the priming phenomenon has been extensively studied, while in plant-herbivore interactions knowledge in this field is limited. In 1983, Baldwin and Schultz, showed the first evidence that HIPVs could be involved in defense activation or priming in nearby plants. Engelberth et al, (2004) described the first molecular mechanism about how it works airborne priming. Maize plants after (Z)-3-hexen-1-ol (GLV) exposure showed an increase levels of JA acid. Muroi et al. (2011) showed that lima bean plants after exposure of volatiles from tobaco transgenic lines that overproduced β-ocimene were more resistant to *T. urticae* and more attractive to predatory mites.

3.2. Top-down mechanisms (indirect defense)

Sometimes direct defenses produced by the plant are not effective enough to control the pest. In this point appears the top-down mechanisms, to improve the

pest control through the enhancement of the third trophic level. The main methods to increase the number of natural enemies in the field are the release enhancement of attractants of natural enemies, the improvement of food and shelter provisioning.

3.2.1. HIPVs (attractants of natural enemies)

It is largely known the role of Methyl Salicylate (MeSA) as attractant of predatory mites. De Boer and Dicke showed in 2004 that *Phytoseiulus persimilis* (specialized predator of Tetranychus species) can not distinguish between HIPVs that contains increasing amounts of MeSA and plants infested with *T. urticae*. However, it is needed the presence of MeSA for a positive choice of *P. persimilis*. The absence of choice in MeSA free blends can be complemented by adding synthetic MeSA, the presence of this chemical significantly increases the mite preference for this odor, suggesting an important role for MeSA. Ament et al. (2010) silenced the salicylic acid methyl transferase (SAMT) gene in tomato that catalyzes the conversion of SA to MeSA. Interestingly, this enzyme is under regulation of JA pathway (Ament et al., 2004). *P. persimilis* was unable to distinguish between SAMT silenced uninfested tomato plants and *T. urticae* infested plants. Another important predatory mite of the two spotted spider mite is *Neoseiulus californicus* (specialized predator of tetranychid mites). Shimoda et al. (2010) worked with the tritrophic interaction lima bean-*T. urticae-N. californicus*. The authors identified several volatiles promoting the attraction of *N. californicus* such as methyl salicylate, linalool and three green-leaf volatiles (GLVs) of (Z)-3-hexen-1-ol, (Z)-3- hexenyl acetate and (E)-2-hexenal. Mixtures of this compounds without MeSA were not attractive to the predatory mite. However

when MeSA was added to this mixture, *N. californicus* clearly preferred this odor.

In other plant species the role of the MeSA as attractant of predatory mites remains unclear. Kappers et al. (2011) showed that differences in volatile production by different varieties of cucumber had an impact in the preference of *P. persimilis*. The levels of (E)-β-ocimene and (E,E)- TMTT (homoterpene (E,E)- 4,8,12-trimethyltrideca-1,3,7,11-tetraene) correlated positively with the attraction of predatory mites. In contrast some compounds such as MeSA showed a negative correlation contrasting with other studies that described this compound as an attractant. Probably these contrasting results could be due to the different plant species used in the experiments. It cannot be discarded a coordinated action with other unknown compounds with negative effect in the attraction of predatory mites that therefore could be masking the attractiveness of methyl salicylate.

Terpenoids were also related with the attraction of natural enemies. Previously we discussed the role of terpens in direct defenses. They trigger detrimental effects in the biology of *T. urticae*. Additionally, they have a relevant role as attractants of predatory mites. Several studies reported this dual effect of β-ocimene. Zhang et al. (2009) reported that infestations with the whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) in lima bean that were previously infested with *T. urticae* stopped the release of some volatiles that affected the recognitions by *P. persimilis*. When β-ocimene was added to the plants with *T. urticae* and *B. tabaci*, the predatory mite was attracted. These results are consequence of the activation of multiple pathways. The attack of *B. tabaci* increases the levels of SA in lima beans, this increase blocks the JA pathway suppressing the β-ocimene synthase that is regulated by JA levels, and

therefore the production of β-ocimene is reduced.

Another terpenoid with a role in tritrophic interactions is the homoterpene (E,E)- TMTT. Brillada et al. (2012) produced a transgenic line of *Lotus japonica* that overproduces the (E,E)-TMTT. This line altered the behavior of two predatory mites. *N. californicus* was clearly attracted to uninfested transgenic line of *Lotus japonica* compared to uninfested wildtype plants. In contrast *P. persimilis* did not show any preference in a choice test. However this predatory mite showed a significant attraction to infested transgenic lines compared to infested wild-type plants. These contrasting observations may be due to the different phytoseids employed in the assays. Whereas *P. persimilis* is a voracious and specialized predator of *T. urticae t*his mite seems to respond mainly to severely infested plants since they need a high amount of *T. urticae* to survive. Contrastingly, *N. californicus* is a generalist predator that can feed on polen and other tetranychid mites. For this reason this mite may be also attracted by uninfested transgenic lines.

Mycorrhizal fungi colonizes the host plant roots to form a mutualistic symbiosis (Pozo et al., 2013). It is known their role inducing disease and drough resistance, but the relevance of mycorrhiza in plant-herbivore interactions was recently proposed. Schausberger et al. (2012) described that the colonization of the mycorrhizal fungus *Funneliformis mosseae* (formerly *Glomus mosseae*) in lima bean plants altered the volatile production following spider mite attack. Two main terpenoids were overproduced β-ocimene and β-caryophyllene, and the consequence was a significant attraction compared to infested lima bean plants without mycorrhiza.

In order to determine the real effect of these volatiles as attractants of natural enemies field assays with different synthetic volatiles dispensers have been

performed (Mérey et al., 2011; Uefune et al., 2012; Sun et al., 2012; Simpson et al., 2011). Rodriguez-Saona et al. (2011) revised several studies related with MeSA in field assays. Forty one out of 91 observations showed a significant attraction. There are evidences that natural enemies are broadly attracted to MeSA in the field, however to export this knowledge to field treatments further investigations are needed.

Trying to improve the biological control of *T. urticae* in the field, van Wijk et al. (2008) offerred to predatory mites a single volatile compound related with the attraction of natural enemies, surprisingly most of the choices showed an opossite result. These experiments suggested that predatory mites do not respond to one compound but to a a mixture of volatiles. Probably using dispensers with many volatiles may be a better strategy. Achieving a good attraction of predatory mites with the dispensers is just a first step since the predators once established in the field would need to feed on alternative food if there is not enough prey, particularly for specialist such as *P. persimilis*. Another possibility to the use of dispensers is growing selected varieties with a huge production of HIPVs when they are attacked by the two spotted spider mite. These genotypes would be more efficient attracting natural enemies

3.2.1. Food and shelter provision (nectar or pollen and alternative prey)

Another method to increase the abundance of the third level trophic could be through the food provision in times of the year when the principal food resource (the pest) is not present or in low densities. Normally the ground covers are used to increased the food provision to natural enemies, but at the same time the cover can be used to provision of refuges, hibernation or aestivating sites, which are required for predatory and parasitic arthropods to successfully

develop to adulthood and reproduce.

The use of the ground covers to increase the food provision is not new, however this cultural practice is not used by mayor part of the farmers. Citrus farmers in China planted *Agerantum conyzoides* L. (Asteraceae), since this plant produce big amounts of pollen that is used by Amblyseius spp. As alternative food when the densities of *P. ulmi* are low (Liang and Huang, 1994). Particularly two phytoseids species, *P. persimilis* and *N. californicus* are the key in the control of *T. urticae*. *Festuca arundinaceae* Schreb. (Poaceae) was described by Aguilar-Fenollosa et al. (2011) as an effective cover crop to increase the densities of these two phytoseids. In this cover crop there was a specific strain of *T. urticae* and Poaceae-specific thrips species that could be used by the two phytoseids as alternative prey (Aguilar-Fenollosa et al., 2012; Aguilar-Fenollosa and Jacas, 2013). Another relevant parameter to improve the biological control is the presence of pollen. Nevertheless not all the pollen shows the same effectiveness. Pina el al. (2012) showed in semi-field experiments, that the pollen from *Carpobrotus edulis* (L.) L. Bolus increased the proportion of *Euseius stipulatus* and this effect could impair the levels of phytoseiid species suffering intraguild predation by *E. stipulatus*, while this fact did not occur in *F. arundinacea* pollen.

4. Concluding remarks

T. urticae a worldwide pest is a serious problem in many crops and ornamental plants due to its own characteristics as a quick reproduction, dispersal abilities or ability to detoxify toxic compounds. However there are other factors caused by farmers that difficult *T. urticae* control, as the overuse of pesticides or the over-fertilization of the field. Figure 1 shows a summary of different plant

compounds that have been described as keys elements to control mites with different function: with toxic activity or attractants of natural enemies.

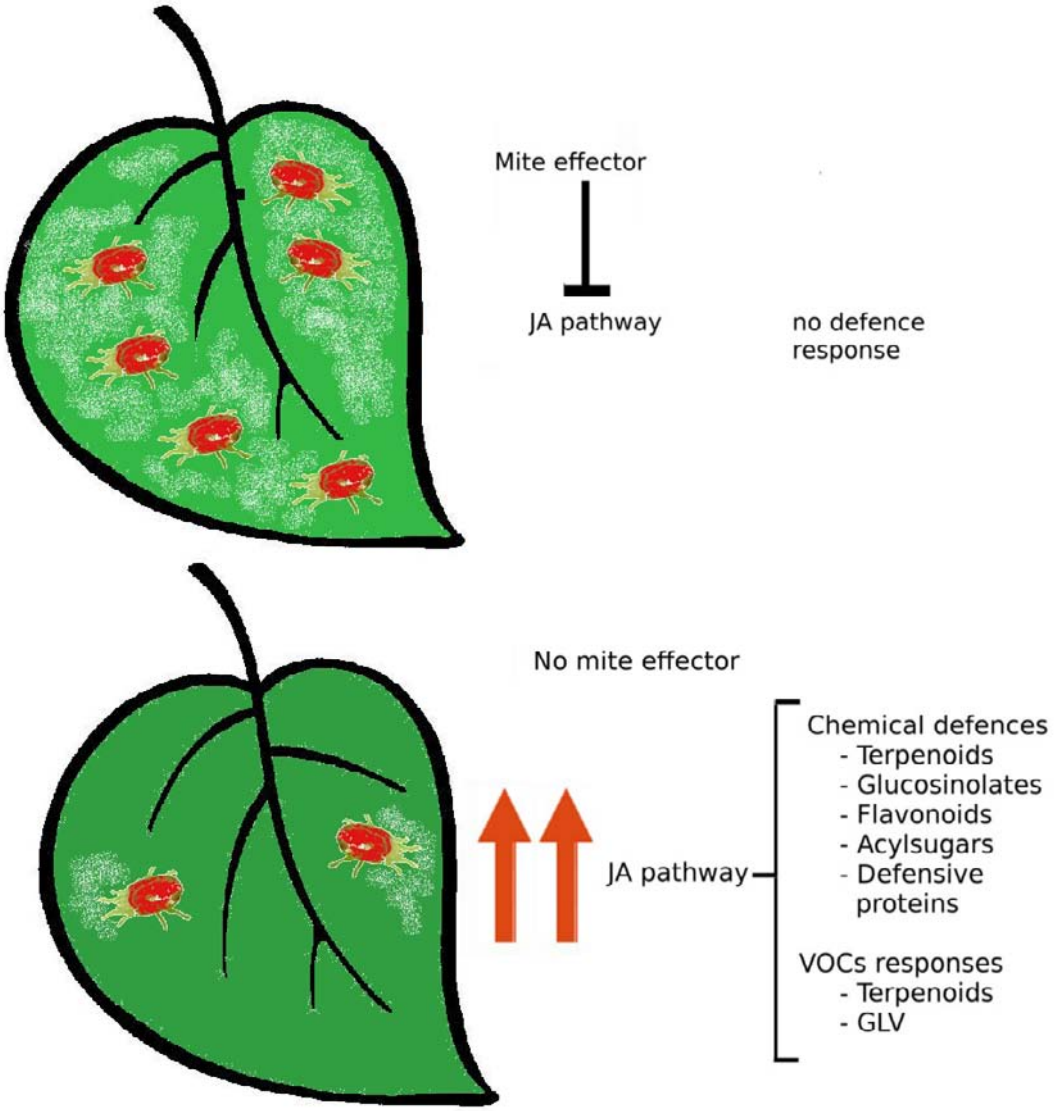

Figure 1. New control strategies based in plant-arthropod studies. Different lines of *T. urticae* can be found in the field, some lines can modify the defense mechanisms of the plant by injecting effectors. These compounds show the ability to interfere the activation of defense pathways. When the effector is absent in the mite the plant

respond to the infestation activating the JA pathway. This JA activation give rise the production and accumulation of many compunds with detrimental effect on the mite, as terpenoids, glucosinolates, flavonoids, acylsugars or defensive compounds. Other compounds as the volatiles GLV and terpenoids can play a different roles in VOCs responses.

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CHAPTER 2

Different metabolic and genetic responses in citrus may explain relative susceptibility against *Tetranychus urticae*

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Abstract

BACKGROUND: Life history parameters of the phytophagous spider mite *Tetranychus urticae* in citrus depend on the rootstock where the cultivar is grafted. To unveil the mechanisms responsible of this effect, we have carried out comparative experiments of *T. urticae* performance on two citrus rootstocks, the highly *T. urticae*-sensitive Cleopatra mandarin and the more resistant sour orange.

RESULTS: Sour orange showed reduced leaf damage symptoms, supported lower mite populations and reduced oviposition rates compared to Cleopatra mandarin. Hormonal, metabolomic and gene expression analyses of the main defense pathways suggest a relevant role of the oxylipin and the flavonoid pathways in the response against *T. urticae*. Sour orange showed an increased activity of the JA pathway which was hardly active in the most susceptible rootstock. Moreover, treatments with the LOX inhibitor Phenidone abolished the enhanced resistance of sour orange. Therefore, oxylipin-dependent defense seems to be rootstock-dependent. The metabolome analysis showed the importance of the flavonoid pathway, which is implicated in the interaction between plants and their environment.

CONCLUSION: Our findings suggest that sour orange enhanced resistance to spider mites can be sustained by a combination of pre-existing and induced responses standing on high levels of flavonoids and a fast and effective activation of the oxylipin pathway.

1. Introduction

Understanding how plant respond and coordinate defense activation upon biotic attack remains a major challenge in plant-arthropod/microbe interactions. This information could pave the way for novel approaches for pest control. Plants can activate diverse pathways controlled by phytohormones such as abscisic, salicylic and jasmonic acids (ABA, SA and JA, respectively) and ethylene (ET) (Ton et al., 2009; Glacebrook, 2005; Pozo et al., 2005) in response to attack by primary consumers as pests and pathogens. Plant responses against arthropods are generally triggered by receptor complexes that recognize Herbivore Associated Elicitors (HAEs) and Fatty Acid-Aminoacid Conjugates (FACs) (Bonaventure et al., 2011). Once the plant has identified the attack, it can respond through synthesis activation of diverse proteins. These proteins include Lipoxygenase-2 (LOX-2) and other antibiotic proteins as PR (Pathogenesis-Related proteins), miraculine-like and lectin-like proteins. LOX proteins are a family of enzymes involved in the synthesis of JA that play important roles in the metabolic responses to wounding (Howe and Jander, 2008). Miraculines are highly glycosylated proteins that belong to a family of protease inhibitors, whereas lectin-like proteins belong to a heterogeneous group of carbohydrate-binding proteins that play a role as insecticides in plant tissue (Van Damme, 2008). These plant-derived proteins, when ingested, remain active in the arthropod gut, and are detrimental to phytophagous insects through the degradation of essential nutrients (Chen et al., 2008).

Among many other defense mechanisms, those presented above play essential roles in direct plant defense. However, depending on the biology of the primary consumer, they can result too slowly to effectively protect the host. Plants can also release volatiles that play several functions in defense; one is to prime

faster responses of defense mechanisms in distal parts of the plant or neighbouring plants (Ton et al., 2007; Ton and Heil, 2008), another one is an indirect defense mechanism by attracting beneficial secondary consumers such as parasitoids and predators and finally volatiles can also act as repellents for phytophagous species. Cell content feeders presenting short stylets, such as the spider mite *Tetranychus urticae* Koch (Acari: Prostigmata) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), usually stimulate JA-inducible genes (known as the oxylipin pathway) upon attack (De Vos et al., 2007; Kawazu et al., 2012). However, there are reports that confirm the activation of both SA and JA-inducible genes in tomato and Arabidopsis upon attack by *T. urticae* and *F. occidentalis*, respectively (Kant et al., 2004; Kawazu et al., 2012). No clear answer for these controversial findings has been reported till now. However, it is hypothesized that phloem-feeding insects with long stylets as aphids have evolved the technique of suppressing or weakening the JA-inducible plant defenses by stimulating the SA-inducible pathway (Pieterse and Dicke, 2007; Walling, 2008; Kawazu et al., 2012). Spider mites can also suppress plant responses by reducing both volatile emission and the oxylipin pathway. This suppression affects spider mite performance (Kant et al., 2008). There are also reports of other mite species as *Tetranychus evansi* Baker & Pritchard, which can improve plant quality for herbivores by eliminating plant responses that act as deterrents of spider mite feeding (Sarmento et al., 2011). The tomato mutant *def-1*, deficient in the accumulation of JA and defense proteins, displays enhanced susceptibility to *T. urticae* which performs better on this mutant producing higher damage than wild type plants (Ament et al., 2004). In addition, *def-1* has low methyl salicylate (MeSA) rates that fail to attract predatory phytoseid mites (Ament et al., 2010). In another study, Maserti et al. (2011) analyzed proteome changes in citrus leaves (*Citrus*

clementina Hort. ex Tanaka grafted on trifoliate orange, *Poncirus trifoliata* (L) Rafinesque (cv Kryder) rootstocks) induced either by *T. urticae* or treatments of methyl jasmonate (MeJA), a compound that plays a key role in defense mechanisms of plants against arthropods (Howe and Jander, 2008). High levels of LOX, chitinase, miraculine- and lectin-like proteins were found after mite attack as well as after MeJA treatment.

T. urticae, is an important pest of clementine mandarins. This species causes damage to plant cells resulting in chlorotic spots on leaves. Sudden defoliations can occur when high infestations coincide with water stress. However, the most important damage caused by this mite is a characteristic fruit scarring which downgrades the fruit severely affecting market price (Jacas et al., 2010; Aguilar-Fenollosa et al., 2011; Pascual-Ruiz et al., 2013). Commercial citrus cultivars are always propagated vegetatively by grafting onto a rootstock. For a long time, sour orange (*Citrus aurantium* L.) was the most widely used rootstock worldwide (Wutscher, 1979). Despite its good agricultural properties, it has been progressively displaced from the orchards because it is highly sensitive to the Citrus Tristeza closterovirus (CTV) (Cambra et al., 2000). Nowadays, most citrus species are grafted on citrange Carrizo (*P. trifoliata* X *Citrus sinensis* (L.) Osbeck), which is CTV-tolerant (Pina et al., 2000). Although this replacement has effectively solved the problem of CTV, new problems as salinization are forcing growers to use salt-tolerant rootstocks like Cleopatra mandarin (*Citrus reshni* Hort. ex Tanaka) or Alemow (*Citrus macrophylla* Wester). Interestingly, Bruessow et al. (2010) compared the fitness of *T. urticae* when reared on satsuma mandarin plants (*Citrus unshiu* Marcovitch) grafted on different commercial rootstocks. These authors showed that *T. urticae* performed the worst (lowest intrinsic rate of increase, r_m) on satsuma grafted on sour orange, closely followed by Troyer citrange (*C.*

sinensis × *P. trifoliata*) and trifoliate orange, then by Alemow, Volkamer lemon (*Citrus volkameriana* Tanaka & Pasquale) and finally Cleopatra mandarin which yielded an intrinsic rate of increase (r_m) 89.1 % higher than sour orange. For a species with a very short generation time as *T. urticae*, the enormous increase in r_m observed when comparing sour orange with Cleopatra mandarin as a rootstock could result in dramatic increases of its population numbers in the field and therefore its increasing prevalence as a citrus pest of economic importance. These results have triggered our interest in this system as a means to disentangle the response of citrus to *T. urticae.*

In the present research, we take advantage of the aforementioned genetically-based variation in the response to *T. urticae* of the citrus genotypes sour orange and Cleopatra mandarin, commonly used as rootstocks, to elucidate the molecular mechanisms implicated. We aim to shed light on how citrus plants orchestrate responses to mite attack using transcriptomic, metabolomic and hormonal analyses of the major defense pathways induced.

2. Materials and methods

2.1 Plant material

Two different genotypes used commonly as rootstocks were used in our assays: sour orange and Cleopatra mandarin. Twelve week-old citrus seedlings were maintained in a climatic chamber at 25° C and 50–70 % RH under a 16 h photoperiod. These plants were grown on vermiculite and peat (1:3; vol:vol) in 320 ml pots. All the experiments performed in the experiments described below were carried out using non-grafted plants (= rootstocks). No insecticides or

acaricides were applied to these plants, which were fertilized using a modified Hoagland's solution(Bañuls et al., 1997) every 3 days.

2.2 Spider mite stock colony

The *T. urticae* colony used in our assays was initiated with specimens collected in clementine orchards in the region of La Plana (Castelló, Spain) (Aucejo-Romero et al., 2004). The colony was maintained on detached leaves of young mandarin plants, *C. clementina* cvar Clementina de Nules (INIASEL 22) in a climatic chamber at 25 °C, 70–80% RH and 12:12 h (L:D) photoperiod. The rearing took place on detached leaf units consisting of a single Clemenules leaf placed upside down on moistened cotton, placed on top of a water-saturated foam cube (3–4 cm thick) in an open plastic box (35 × 20 × 7 cm) half-filled with water. Moist cotton was folded over the edges of the leaves to prevent mites from escaping. When necessary, cohorts of the same age were produced by transferring gravid females from the stock colony to freshly set detached leaf units for a controlled period of time. Afterwards, females were removed and the eggs kept undisturbed until reaching the target stage and age. These cohorts were maintained under the same environmental conditions as the stock colony.

2.3 *T. urticae* performance and damage on selected citrus rootstocks

Assays were conducted in the climatic chamber described above for plants. Ten plants of each rootstock were used per assay which was repeated three times. Prior to infestation, the lower part of the trunk of each plant was painted with

Tangle-Trap Insect Trap Coating (Tanglefoot Company, Bozeman, MT, USA) to prevent ambulatory mite dispersal from plant to plant. To further prevent dispersal, plants were set in a tray (55 × 40 × 10 cm) half filled with water. Five plants per rootstock were artificially infested with five 48-h-old *T. urticae*, which were randomly transferred to each plant with a fine camel paintbrush. Fourteen days after infestation, leaves were inspected visually to count the number of adult *T. urticae* females per leaf, as this is a good estimator of total *T. urticae* population (Gonzalez-Zamora et al., 1993; Martinez-Ferrer et al., 2006). Subsequently, these trees were defoliated and leaves were scanned to estimate damaged (chlorotic) upper leaf area (DULA). The GIMP software (GNU Image Manipulation Program v 2.6) was used for area determinations. Based on these values, percentage damaged area per leaf was determined. According to these values, leaves were further classed into four damage levels (Castagnoli et al., 2003): I, no visible spider mite symptoms; II, DULA ≤ 3%; III 3 %< DULA ≤ 10%; and IV, DULA > 10 %. The remaining five plants per rootstock received three 48-h-old *T. urticae* females onto three leaves. In this case, plants were left undisturbed for 3 days. At that time, the three infested leaves were detached and observed under binocular microscope to determine the number of eggs deposited. All these measurements were compared by *t*-test analysis at $P < 0.05$.

2.4 *T. urticae* oviposition on hormone treated rootstocks

Twenty plants per rootstock were used. These plants were divided into 4 groups per rootstock. One group was sprayed with an aqueous solution of 0.01% (v/v) glycerol (controls); the other three groups received the same treatment supplemented with either 100 μM methyl jasmonate (MeJA), 1mM salicylic

acid (SA) or 4mM phenidone (Sigma–Aldrich, Barcelona, Spain), an inhibitor of LOX, until run-off. After 24 h at 22–24°C, 50–70% RH and a 16:8-h L:D photoperiod, these plants were used to estimate oviposition as above. Likewise, this experiment was repeated three times. Results were subjected to analysis of variance (ANOVA) and significance was tested with an *LSD* test at $P < 0.05$.

2.5 Plant defense mechanisms against *T. urticae* in selected citrus rootstocks

A total of 36 plants per rootstock were used in this assay (18 infested and 18 uninfested). To prevent mite dispersal during the assay, all plants received Tangle-Trap Insect Trap Coating and were set in trays as previously described. Eighteen plants per rootstock received five 48-h-old *T. urticae* as described above. Plants were defoliated and their leaves pooled in one single sample and frozen at -80º C for further processing. A time course analysis was performed at 0, 1, 2, 3, 7 and 14 days after infestation (dpi). For each time and rootstock, quantitative real-time PCR analyses and hormone determinations were carried out, whereas polyamine, aminoacid and metabolome analyses were performed for 3 dpi and control samples only. As in previous assays, the gene expression experiment and those for polyamine and aminoacid quantification were repeated three times. The experiments for hormonal determination were repeated four times.

2.6 Quantitative real-time PCR analysis

mRNA was extracted using the Plant RNA Kit (Omega Bio-Tek Inc, Doraville, Georgia, USA). For RT-qPCR experiments, 1,5 µg of total RNA was digested

with 1 unit of DNase (RQ1 RNase-Free DNase) in 1 µl of DNase buffer and Milli-Q water up to 10 µl (Promega Corporation, Madison, Wisconsin, USA) and incubated for 30 min at 37°C. After incubation, 1 µl of RQ1 DNase stop buffer was added and incubated again at 65°C for 10 min to inactivate DNase. The RT reaction was performed by adding 2 µl of RT buffer, 2 µl of 5 mM dNTP, 2 µl of 10 µM Oligo(dT) 15 primer (Promega, Oligo(dT)15 Primer), 1 µl of 10 U/µl RNase inhibitor (Promega Rna- sin RNase inhibitor) and 1 µl of Omniscript reverse transcriptase (Qiagen, Barcelona, Spain). The reaction mixture was incubated at 37°C, for 60 min. Complementary DNA from the RT reaction, 10 x diluted, was used for qPCR. Forward and reverse primers (0.3 µM) were added to 12,5 µl of PCR SYBR reaction buffer, 2 µl of cDNA and Milli-Q sterile water up to 25 µl of the total reaction volume (Takara Bio, Kyoto, Japan). Quantitative PCR was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA USA) sequence detector with standard PCR conditions. Differences in cycle numbers during the linear amplification phase between samples. The data were transformed with the formula $2^{\Delta Ct}$ (Yuan et al., 2006). RT-qPCR analysis were performed at least three times using sets of cDNA samples of independent experiments. Gene expression levels between both infested rootstocks were compared using a *t*-test.

Primers of marker genes of defense signalling pathways such as *PR-5*, *LOX-2*, *PR-3*, *PI TYPE1* and *ABA-4* were used. *EF 1 α (elongation factor 1-alpha)* and *GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)* were used as housekeeping genes to normalize the results.

Primers of *PR-3, LOX-2* and *ABA-4*, were designed using the technical documentation of Citrus Genome Array of Affimetrix (Affimetrix Inc, Santa Clara, CA) and Genebank (Nucleic acids research. URL

http://www.nbci.nlm.nih.gov/genbank). *PI TYPE 1* and *EF 1 α* sequences were taken from Mozoruk et al. (2006). *PR-5 and CHS* sequences were obtained from the citrus EST database (Version 1.20 of HarvEST:citrus, *Florida Citrus Production Research Advisory Council. http://harvest.ucr.edu*). All primers were designed using the software Primer 3. The sequences employed for the RT-Q-PCR amplifications are described in Supplementary table 1.

2.7 Hormone analysis

The hormones 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), JA-isoleucine (JA–Ile), abscisic acid (ABA) and salycilic acid (SA) were analysed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) as described by Flors et al. (2008) and Forcat et al. (2008). Hormone levels between both infested rootstocks were compared using a *t*-test.

2.8 Polyamine analysis.

50 mg of freeze-dried samples were used. The tissue was homogenized in 2.5 ml of ultra-pure water and centrifuged at 5000 g for 40 min. A mixture of internal standard containing 100 ng 1,4-diaminobutane $^{13}C_4$ (ISOTEC, Miamisburg, OH, USA) was added to each sample prior to extraction. Three technical and three independent biological replicates per sample processed by High-performance liquid chromatography using a Waters (Milford, MA, USA) Alliance 2690 system, which consists of an auto-sampler and a quaternary pump. Aliquots (20 µl) were injected in a Pack ODS-A reversed-phase C18 column (100x2 mm, 5 µm, 2nm; YMC, Europe GmbH). Polyamines were

eluted according to Sánchez-López et al. (2009). Briefly, a gradient of methanol containing 1% propionic acid and water containing HFBA 1 mM and 1% propionic acid was used. The gradient started with 10:90 (v/v) and linearly reached 90:10 (v/v) in 9 min. In the following 1.5 min, the gradient was kept in isocratic conditions and then returned into initial conditions in 3 min. The column was allowed to equilibrate for 2.5 min, giving a total time of 16 min per sample. The solvent flow rate was 0.3 ml min1 with working pressures around 70–110 bar. Polyamine amounts were analysed on the basis of a calibration curve of polyamine standards (Sigma–Aldrich, Barcelona, Spain).

2.9 Aminoacid analysis

50 mg of freeze-dried samples were used. The tissue was homogenized in 2.5 ml of ultra-pure water and centrifuged at 5,000 g for 40 min. A mixture of internal standards containing 100 ng of Thr-$^{13}C^{15}N$(Aldrich), 100 ng of Phe-$^{3}C^{15}N$ (Aldrich) and 100 ng of d^{2}IAA (Isotec; Sigma) was added to each sample prior to extraction. Three technical and three independent biological replicates per sample processed by High-performance liquid chromatography using a Waters (Milford, MA, USA) Alliance 2690 system, which consists of an auto-sampler and a quaternary pump. Aliquots (20 µl) were injected in a Pack ODS-A reversed-phase C18 column (100 × 2 mm, 5 µm, 2 nm; YMC Europe GmbH, Dinslaken Germany). Solvent gradient and additional chromatographic conditions were performed according to Gu et al. (2007).

2.10 Metabolome analysis. LC-ESI full scan mass spectrometry Q-TOF

50 mg of frozen dried plant samples were extracted with MeOH:H2O (10:90) containing 0.01% of HCOOH. After polytron homogenization on ice, the samples were centrifuged for 15 min at 15000 g and the supernatant was filtered and an aliquot was used for subsequent analysis. Metabolome analysis was performed using an Acquity UPLC system (Waters, Mildford, MA, USA) interfaced to hybrid quadrupole time-of-flight (QTOF Premier). Three technical and three independent biological replicates per sample were randomly injected. The LC separation was performed by HPLC SunFire C18 analytical column, 5 µm particle size, 2,1 × 100 mm (Waters). Analytes were eluted with a gradient of methanol and water containing 0.01 %HCOOH. The gradient started with aqueous mobile solvent 90 % and linearly reached 10% in 12 min. In the following 3 min, the gradient was kept in isocratic conditions and then returned to initial conditions in 4 min. The column was allowed to equilibrate for 3 min, giving a total time of 22 min per sample. The solvent flow rate was 0.3 ml min^{-1}. The injection volume was 20 µl. The drying gas and the nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 600 L/h, and the cone gas flow was set to 60 L/h. A cone voltage of 20 V and a capillary voltage of 3.3 kV were used in the negative ionization mode. The nitrogen desolvation temperature was set at 350°C, and the source temperature was set at 120°C. The instrument was calibrated in the m/z 50-1000 range with a 1/1 mixture of 0.01 M NaOH /1% HCOOH ten-fold diluted with acetonitrile/water (80/20, v/v). A solution of Leucine enkephalin at a concentration of 2 ppm in acetonitrile/water (50/50, v/v) with 0.1 % of formic acid was simultaneously introduced into the Q-TOF instrument via the lock-spray needle for accurate m/z determinations. The $[M - H]^-$ ion of leucine enkephalin at m/z 554.2615 was used for recalibrating the m/z axis. Metabolite amounts were analysed on

the basis of normalized peak area units relative to the dry weight. Kruskal-Wallis test ($p < 0.05$) was applied to test the metabolomic differences between rootstocks vs infestation, significance was assessed by the absence of overlapping in a box-and-plot graphical method.

2.11 Full scan data analysis

Three technical and three independent biological replicates injected randomly. Centroid aquired raw data were transformed into .cdf files using the Databridge from Masslynx 4.1 software (Masslynx 4.1, Waters) and subsequently subjected to analysis using the software R for Statistical Purposes. Signals from positive and negative electro-spray analysis (ESI^+; ESI^-) were processed separately. Peak peaking, grouping and signal corrections were developed applying the algorithm XCMX. This statistical package can be used to preprocess full scan LC/MS data for relative quantification and statistical analysis (Smith et al., 2006). The statistics and the heat map analysis were carried out with the Mar-Vis Suit software, a tool for clustering and visualization of metabolic biomarkers (Kaever et al., 2012). Mar-Vis was used to process exported files from the XCMS and perform statistical analysis, adduct and isotope correction and clustering and colour heatmap visualization. To determine a global behaviour of the signals Principal Component Analyses (PCA) was used.

2.12 Statistical analyses

All statistical analyses (ANOVAS, post hoc and *t*-test) were conducted using Statgraphics Plus 3.1. (Rockville, MD), the software ‘‘R’’ version 2.9.2 (R

Development Core Team) and the package XCMX (Kaever et al., 2012). All the experiments were repeated at least three times except otherwise explained (see above).

3. Results

3.1 Sour orange displays reduced susceptibility to spider mite infestation compared with Cleopatra mandarin.

Fourteen days after mite infestation, sour orange rootstock sustained significantly lower mite populations than Cleopatra mandarin (Fig 1A). Female counts on this rootstock were 80 % higher than on sour orange. Likewise, oviposition on sour orange was significantly reduced by more than 17 % compared with Cleopatra mandarin (Fig 1 B). As a result of the lower mite population in sour orange, damage was lower as well (Fig 1 C).

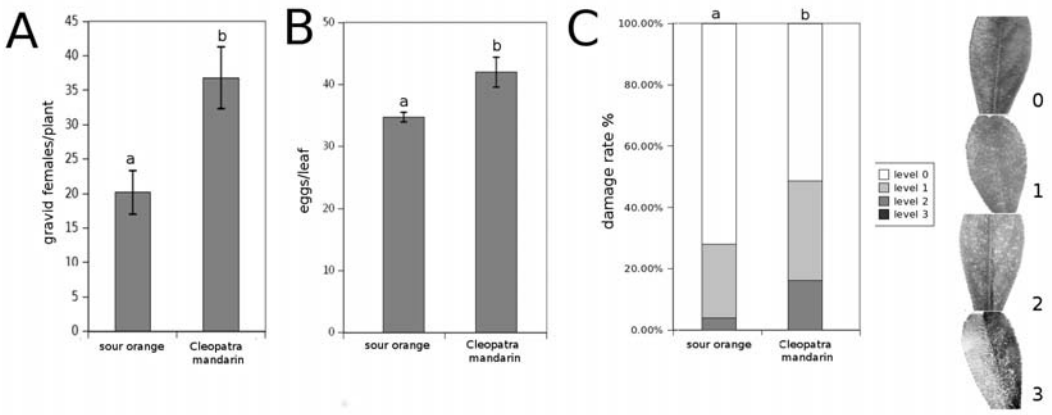

Figure 1. Spider mite performance on two citrus rootstocks Cleopatra mandarin and sour orange. A) # Spider mite in different rootstocks. The mites were counted 14 days after infestation with 5 mites per plant on 12 week-old citrus plants. Different letters indicate significant differences ($P < 0.05$; *t*-test) between treatments. B) Spider mite

oviposition in sour orange and Cleopatra mandarin. Plants were infested with 2-d-old *T. urticae* females (3 specimens per leaf) and 3 d later the number of eggs was determined. Asterisks indicate significant differences between treatments ($P < 0.05$; *t*-test). C) Damage rate 14 d after infestation. Symptom severity is expressed based on damaged (chlorotic) upper leaf areas (DULA). According to these values, leaves were classed into four damage levels: I, no visible spider mite symptoms; II, DULA ≤ 3%; III 3 %< DULA ≤ 10%; and IV, DULA > 10 %. Different letters indicate significant differences between treatments ($P < 0.05$; *t*-test).

3.2 Rootstock response to spider mites is associated with a concomitant enhancement of SA and JA- dependent signalling.

To understand citrus responses to spider mite attack, several hormonal and gene expression analysis were undertaken. The JA pathway marker genes *LOX-2* and *PR-3* homologous (*Cit1727.1.S1* and *BA/63287.1* respectively) were analysed. Sour orange responded more efficiently than Cleopatra mandarin by activating *LOX-2* gene expression as fast as 1 dpi (Fig 2A). Furthermore, *LOX-2* transcripts remained at high levels along all the experiment. Contrarily, Cleopatra mandarin hardly responded to the infestation. *LOX-2* expression in this rootstock was lower than in sour orange. Accordingly, the JA marker gene *PR-3* dramatically increased in sour orange compared with the more sensitive rootstock (Fig 2B). Hence sour orange genotype activated JA-dependent genes faster in response to *T. urticae* compared with Cleopatra mandarin. The latest needed more than 3 days to show a detectable expression of *PR-3* that remained at very low levels even at later stages of infestation (Fig 2B). Transcript levels of the SA marker gene *PR-5* citrus homologous Cit6759.1.S1 were rapidly up-regulated at 1 dpi in sour orange whereas this gene was hardly altered in Cleopatra mandarin (Fig 2C). An interesting observation is that major changes in gene expression of SA- and JA-signalling associated to citrus response

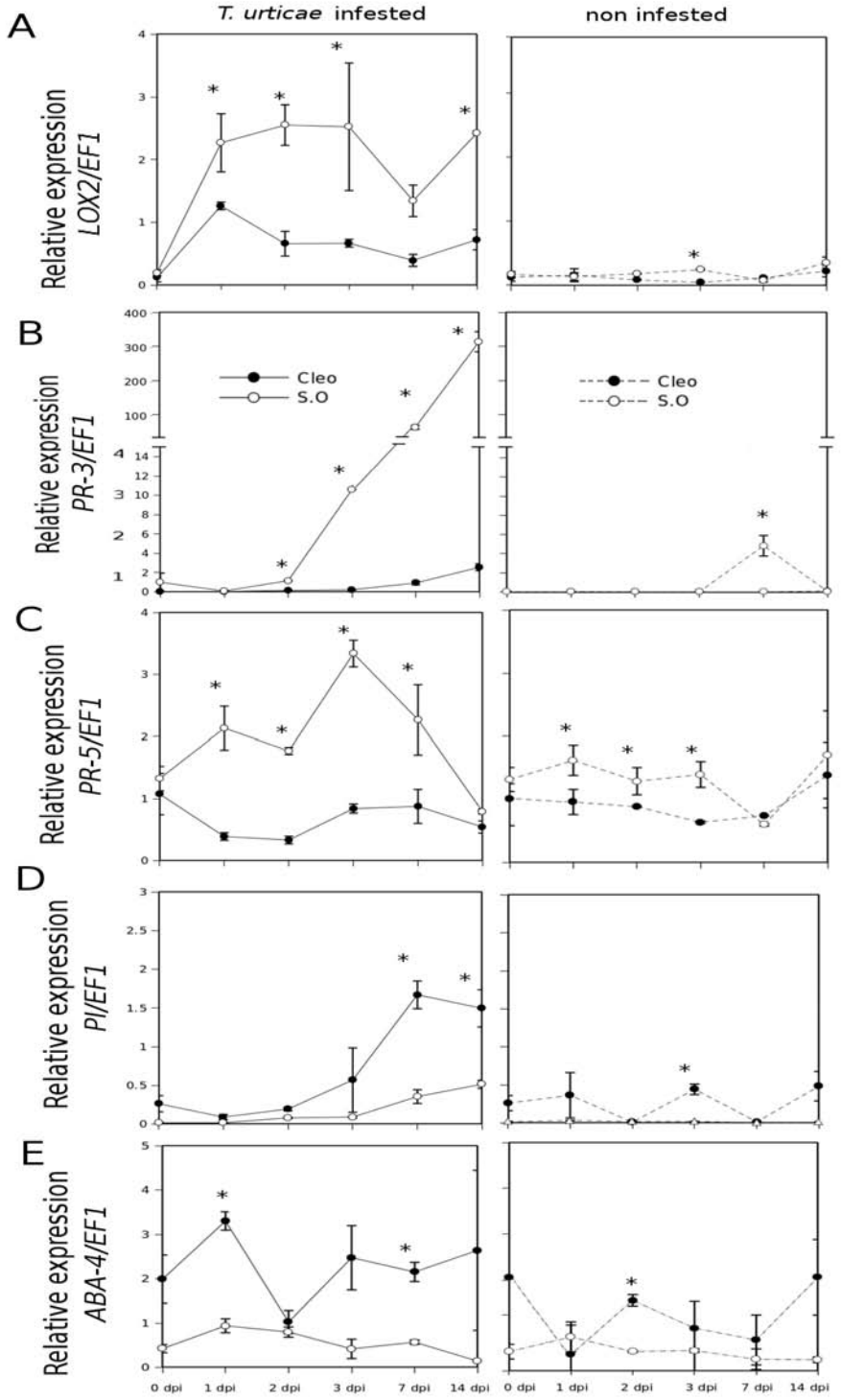

Figure 2. Impact of *T. urticae* infestation on defense gene expression in sour orange

(s.o.) and Cleopatra mandarin (Cleo.). Total RNA was extracted from the leaves of three plants at 0, 1, 2, 3, 7, and 14 dpi converted to cDNA, and subjected to quantitative RT-PCR analysis. The *LOX-2* (cit16759.1S1; A), *PR-3* (Cit.17271.S1; B), *PR-5* (BAI63287.1; C), *PI TYPE 1* (DR908940; D), *ABA-4* (318.1.S1; E) transcript levels in uninfested and *T. urticae*-infested plants were normalized to the expression of EF1α measured in the same sample. Data are presented as a mean of three independent analysis of transcript expression relative to the housekeeping gene plants ± SD (n = 3). Significant differences in relative transcript levels between both rootstocks were estimated performing the t-test on both rootstocks time-points with ΔCt values as mentioned by Yuang et al. (2006). The experiment was repeated three times with the same results.

against spider mites take place between 2 and 3 dpi. The citrus *PI TYPE 1 (DR908940)* mRNA accumulation was quantified. The expression of this gene responded clearly to the spider mite infestation but was more active in Cleopatra mandarin than in sour orange, where it did not change (Fig 2D). This gene profiling, suggests that this *PI* in citrus may act as an infestation marker rather than playing any direct function in defense against mites. Another major defense pathway involved in plant resistance such as ABA was also studied. *ABA-4* transcripts of the citrus homologue (*318.1S1*) showed higher levels in Cleopatra mandarin than in sour orange. These elevated levels may be not due to the mite attack since prior to infestation this rootstock already presented higher levels than sour orange (Fig. 2E). Despite the ABA marker gene was not clearly induced upon mite attack, sour orange sowed lower levels of *ABA-4* expression probably as a consequence of the antagonistic effect since SA-dependent marker was strongly induced. In the absence of infestation, expression of all the genes analysed were not different between rootstocks with the exception of PR-5 that showed an increased expression in sour orange compared with Cleopatra mandarin (Fig. 2). Taken together the genetic analysis of the different defense pathways and phenotypes, the results suggests a relevant role of concomitant increases in the SA and JA pathway to confer

lower susceptibility to *T. urticae* in sour orange.

3.3 Impact of *T. urticae* in defense related hormones

To gain more insight into the coordinated increase of SA- and JA-signalling pathways associated to *T. urticae* response in citrus, the impact on hormone homoeostasis was determined. Analysis by UPLC-coupled tandem mass spectrometry (LC-MS/MS) allowed the simultaneous quantification of free and Ile-conjugated JA, the JA precursor OPDA, ABA, and SA from each rootstock. The phytohormone SA showed a response to spider mite attack in the two rootstocks considered. However, the magnitude of these responses differed among them. Little response was observed in Cleopatra mandarin. Contrastingly, sour orange showed a strong early response and higher levels of SA than the other rootstock along the experiment (Fig 3A). Notably, mite attack induced strong changes in the oxylipin pathway in sour orange that showed higher levels of OPDA and JA (Fig 3B and C). Basal levels of SA, JA and OPDA in the resistant cultivar were higher in the absence of infestation, thus this genotype displays constitutively higher levels of these hormones compared with the susceptible rootstock. JA-Ile increased at early time-points in the most resistant rootstock. However, it decayed at the end of the experiment and reached similar levels compared with Cleopatra mandarin (Fig 3D). ABA levels showed an increase during the first two days after infestation and a subsequent decay. No differences in ABA levels between rootstocks were observed in spite of the increased resistance of sour orange. These levels showed an increase during the first two days after infestation and a subsequent decay. Therefore, a minor contribution of ABA to the observed response against *T. urticae* is hypothesised.

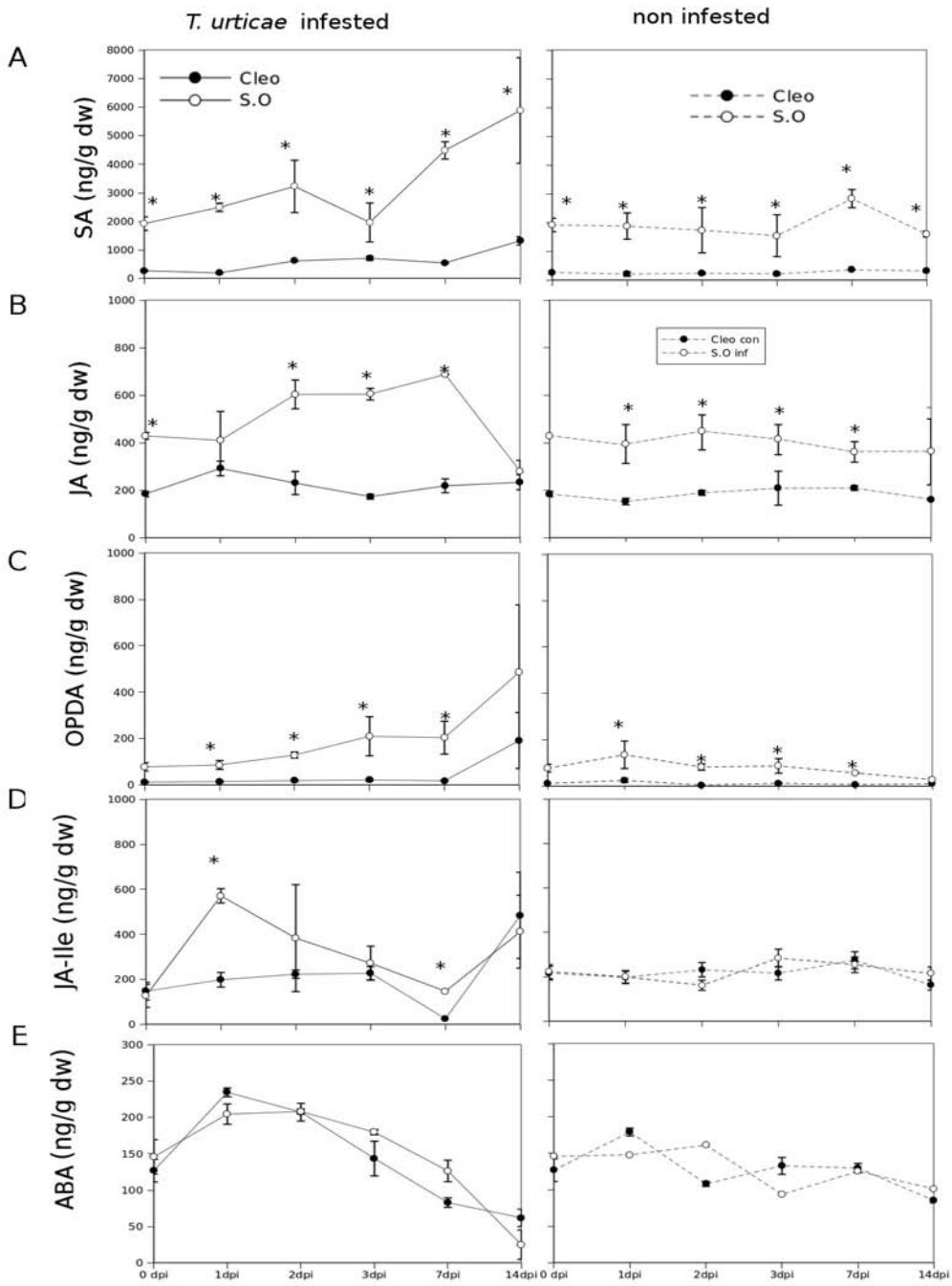

Figure 3. Hormonal content in sour orange and Cleopatra mandarin upon *T. urticae* attack. Infested and uninfested leaves were collected at different time points (0, 1, 2, 3,

7, and 14 dpi), and SA (A), JA (B), OPDA (C), JA-Ile (D), ABA (E) levels were determined in freeze-dried material by HPLC-MS. The results shown are mean hormone levels of four independent analysis of ± SD (n = 4). Asterisks mean significant differences between both rootstocks on individual time-points ($P < 0.05$; *t*-test). The experiment was repeated four times with the same results.

3.4 Inhibition of LOX results in increased susceptibility in sour orange while MeJA protects Cleopatra mandarin

Because of the absence of citrus mutants in the oxylipin pathway, the use of chemicals results very helpful for a closer analysis of resistance. Therefore, to determine the relevance of the oxylipin pathway in the reduced susceptibility of sour orange to *T. urticae*, a series of experiments treating plants with Phenidone, MeJA and SA were performed. The reduced oviposition in sour orange disappeared in Phenidone treated plants (Fig 4), whereas the number of eggs per plant was not significantly different in MeJA- and SA-treated plants relative to the control. By contrast, oviposition in Cleopatra mandarin treated with MeJA was reduced compared with control plants. The reduction or inhibition of JA-signalling by using either the antagonistic phytohormone SA or the LOX inhibitor Phenidone did not alter Cleopatra mandarin basal response against *T. urticae* that already exhibited very low levels of JA. These results confirm that the JA-signalling is a relevant pathway in citrus regulating the defense against this mite.

3.5 Infestation leads to a change in metabolites in sour orange

To analyse the metabolic changes under *T. urticae* intestation in the two selected rootstocks, a metabolome analysis was performed using

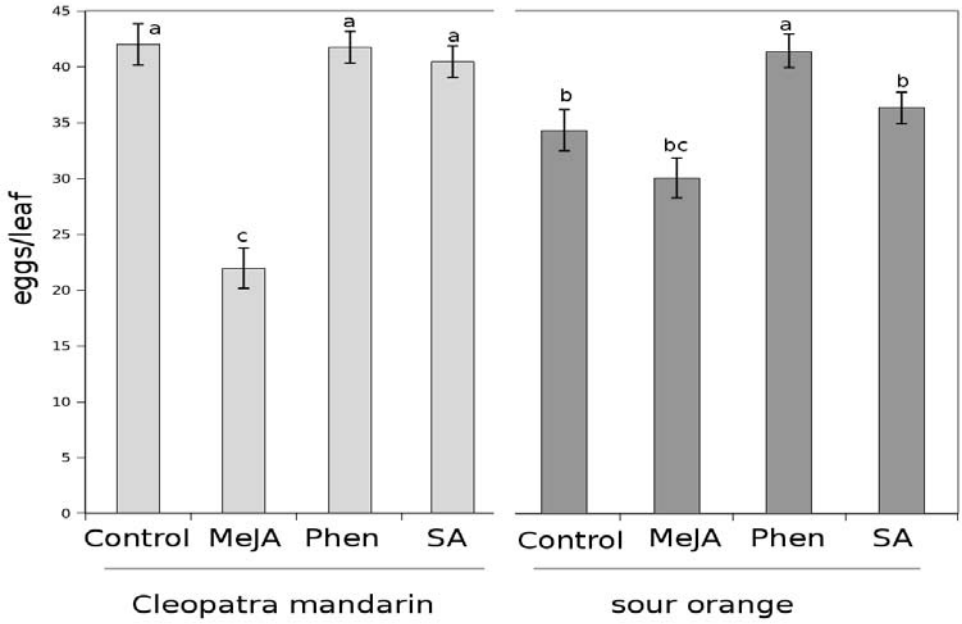

Figure 4. Effect of phenidone, SA and MeJA treatment on oviposition in sour orange and Cleopatra mandarin. Twelve week-old plants were infested 24 h after chemical treatments. Different letters indicate significant differences (One way ANOVA, $P < 0.05$; *LSD*) between treatments. Oviposition was determined at 3 dpi.

UPLC-QTOFMS. Since major changes happen three days after infestation we selected this time-point to perform metabolomic analysis. Results were processed using the package XCMX of ‘‘R’’ version2.9.2.[35] The statistics, the heat-maps and pathway analyses were carried out with the Mar-Vis Suit software.[36] PCA in both electrospray modes showed an almost total overlay in the metabolites from Cleopatra mandarin independently of mite infestation (Fig 5). Interestingly, sour orange in the absence of mite attack, displayed a completely different metabolic profile and behaviour compared with the other rootstock. In addition, the presence of the mite, induced radical changes in the most resistant rootstock, as demonstrated by the absence of overlay in the PCA in any of the ESI modes (Fig 5).

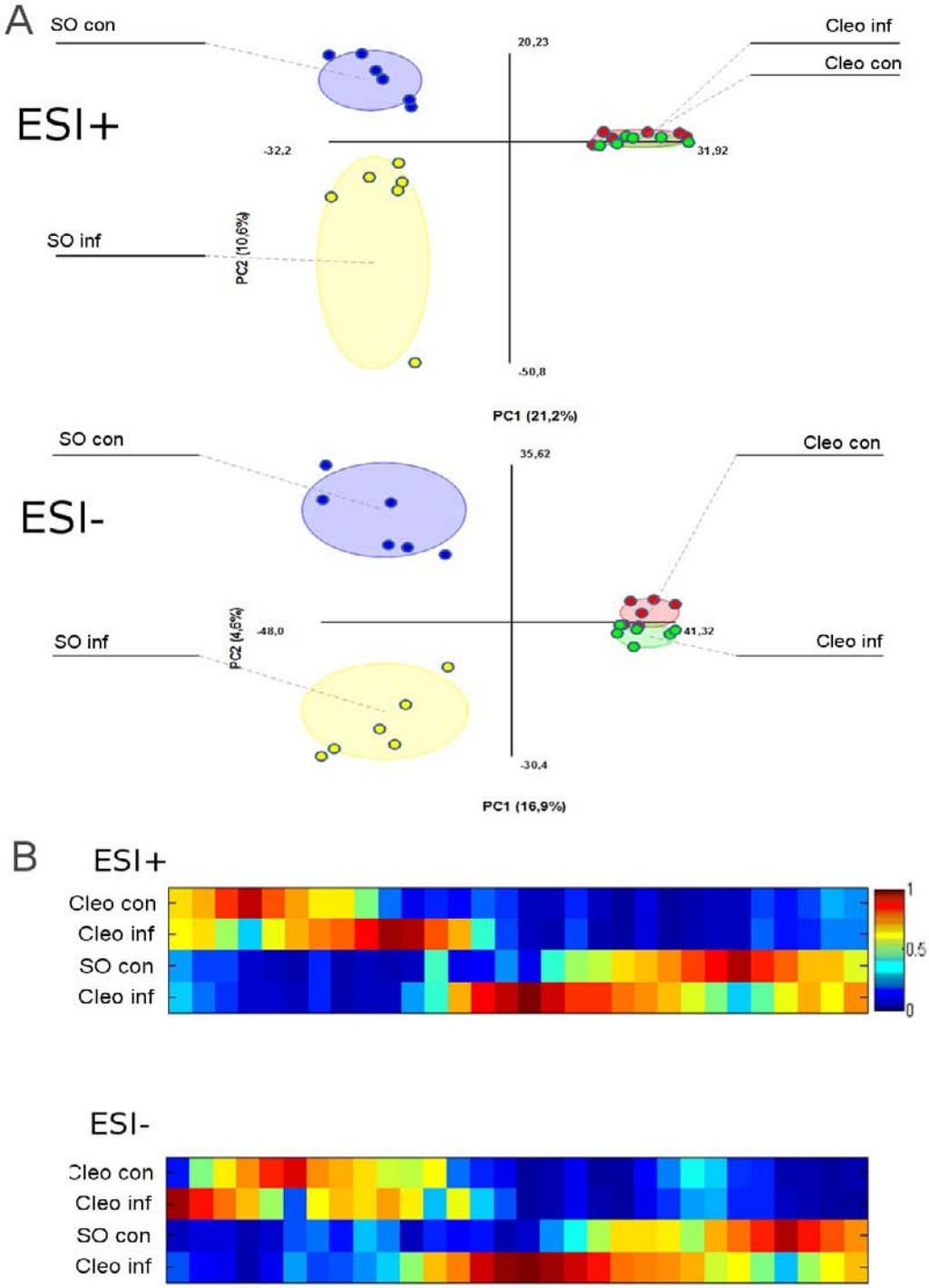

Figure 5. Metabolomic analysis of sour orange and Cleopatra mandarin against *T.*

urticae infestation. A) Non supervised principal component analysis (PCA) representation of major sources of variability of ESI+ and ESI- signals obtained from a non-targeted analysis by HPLC-QTOFMS to monitor metabolomic changes during spider mite infestation. Four different sets of samples were tested: in the first place sour orange non-infested (SO con), sour orange infested (SO inf), Cleopatra mandarin non-infested (Cleo con) and Cleopatra mandarin infested (Cleo inf). 12 week old plants were infested with 5 mites/plant and samples for analysis were collected 3 dpi. Leaf material from 6 individual plants was pooled together for each genotype combination. Three independent biological and two technical replicates were randomly injected and analysed (n = 6). B) Heatmap analysis generated by Mar-Vis Filter and Cluster following a Kruskal-Wallys test ($P < 0.05$). The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Heat map representation is divided in two electrospray ionization modes, positive (ESI+) and negative (ESI-) from HPLC-QTOF analysis.

3.6 Pathway analysis and identification of relevant metabolites mediating the response against *T. urticae*

The QTOF data that showed significant differences ($P < 0.05$) were used to obtain a heat map representation and perform a cluster analysis with the Mar-Vis program (Fig 5, Table S1 and S2). The heatmap representation showed clusters of signals differentially accumulated in sour orange and Cleopatra mandarin in the absence of challenge confirming the previous observations obtained from the PCA analysis. We focused our attention on those clusters of signals that were induced by the mite in the most susceptible genotype as putative host-manipulated metabolites, and also in those clusters induced in the most resistant rootstock, as putative metabolitescontributing to resistance. We found 377 signals in ESI+ and 122 signals in ESI- that were over-accumulated in sour orange when infested. Among all these signals, those up-regulated only in sour orange in presence of *T. urticae* could be responsible of the lower susceptibility of this rootstock (Table 1, Supplementary figure 1). The accurate mass offered by the QTOF analyser was employed to assign putative candidate

compounds and biological pathways in different on-line biological databases such as KEGG, AraCyc and Metlin. Among the compounds up-regulated in sour orange, we found signals related to the flavonoid, phenylpropanoid and anthocyanin biosyntheses and alkaloids derived from shikimate pathway and aminobenzoate degradation (Table 1). Another group of signals of relevance were those up-regulated in the susceptible genotype in the presence of the mite. These metabolites are putative targets for host manipulation. Among signals with this behaviour we isolated 164 in ESI+ and 69 in ESI- in Cleopatra mandarin. For a total identification of compounds over-represented in sour orange upon spider mite attack, we compared the fragmentation spectrum of the signal with the theoretical fragmentation spectra from Mass Bank database (http://metlin.scripps.edu/). We could successfully identify 3 compounds: p-coumaric acid, naringenin and hesperetin. All these compounds are related to the flavonoid pathway (Fig 6). All of them are significantly increased in sour orange in the absence of infestation compared with Cleopatra mandarin. However, these compounds seem to be further induced after spider mite attack (Fig 6A). Both, the theoretical exact masses (1: TOF MS) and the

Table 1. Signal induced in sour orange leaves upon *T. urticae* infestation.

Accurate (Neutral) Mass Determination	**Ion Mode**	**Putative Pathway based on exact mass identification**
204,128	ESI+	Alkaloids derived from shikimate pathway
311,149	ESI+	Alkaloids derived from shikimate pathway

392,109	ESI+	Alkaloids derived from shikimate pathway
270,056	ESI+	Flavonoid biosynthesis
288.066	ESI+	Flavonoid biosynthesis
450,114	ESI+	Flavonoid biosynthesis
448,101	ESI+	Flavonoid biosynthesis
314,080	ESI+	Flavonoid biosynthesis
316,062	ESI+	Flavonoid biosynthesis
346,069	ESI+	Flavonoid biosynthesis
610,190	ESI-	Flavonoid biosynthesis
354,094	ESI+	Phenylpropanoid biosynthesis
216,044	ESI+	Phenylpropanoid biosynthesis
478,110	ESI+	Anthocyanin biosynthesis
578,164	ESI+	Anthocyanin biosynthesis
950,230	ESI+	Anthocyanin biosynthesis
150,034	ESI-	Aminobenzoate degradation
126,034	ESI-	Aminobenzoate degradation
338,064	ESI-	Purine metabolism

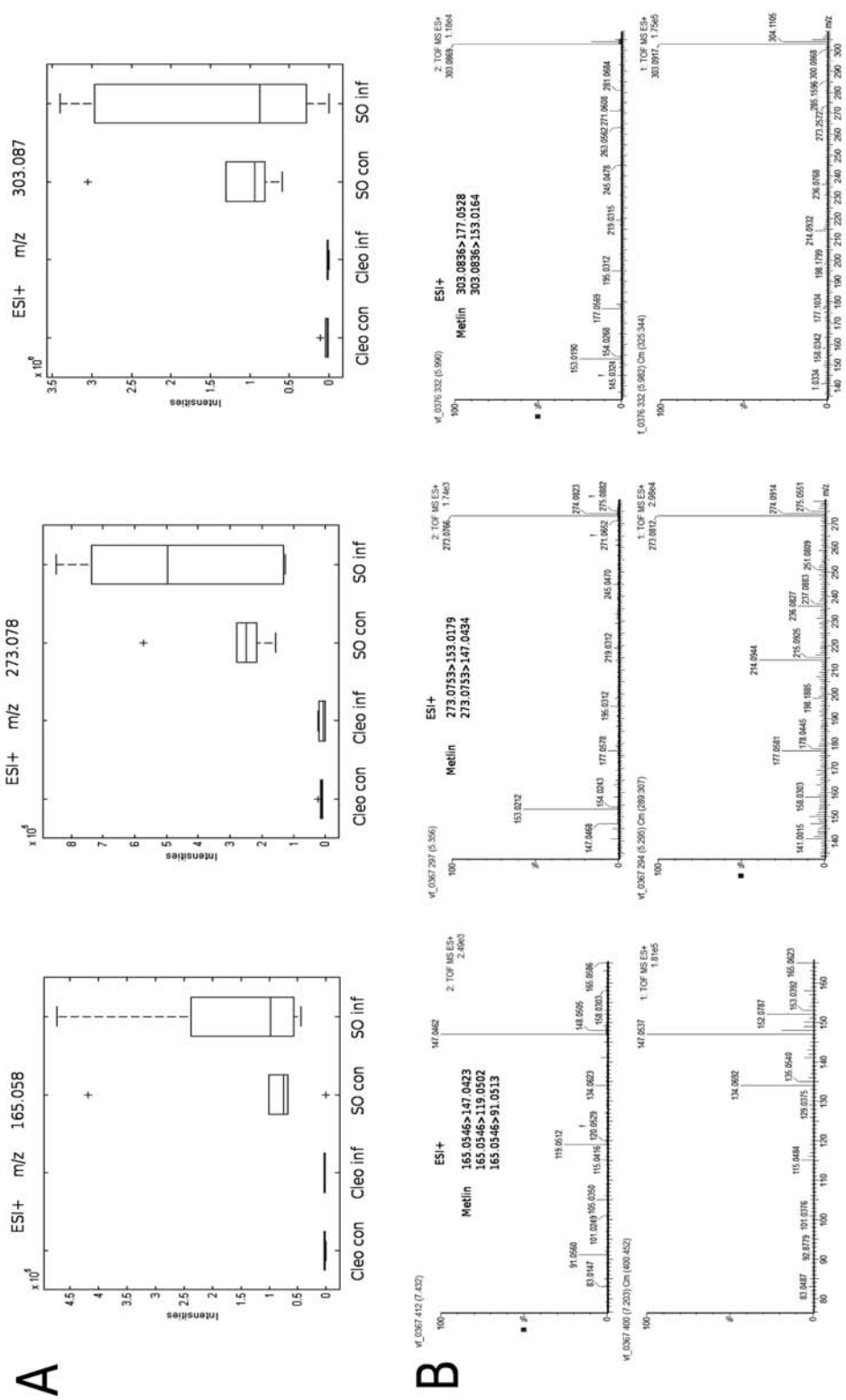

Figure 6. Precise identification of three signals over-accumulated in sour orange:

165.058, 273.078 and 303.087 corresponding to 4-coumaric acid, naringenin and herperetin respectively. A) Boxplot analysis of the relative abundance of the three compounds in sour orange (SO) and Cleopatra mandarin (Cleo) either in the absence (con) or in the presence of spider mites (inf). B) Identification of the compounds. To determine the exact mass of the parental ion, no collision energy is applied (1: TOF MS EI+/-). To obtain fragmentation spectrum a collision energy ramp from 5 eV to 45 eV is applied to the parental ion (2: TOF MS EI+/-). Theoretical transitions were first checked in mass spectra databases (Metlin).

fragmentation spectra (2: TOF MS) matched with the experimental masses and the fragmentation obtained in the Metlin database (Fig 6B). Since chalcone synthase catalyses one of the key steps in the flavonoid pathway, we tested the levels of mRNA of the citrus homologous gene. As expected, the relative CHS (CF40417078) gene expression was 3-fold higher in sour orange compared with Cleopatra mandarin (Fig 7) and this result explains the increased amount of flavonoids found.

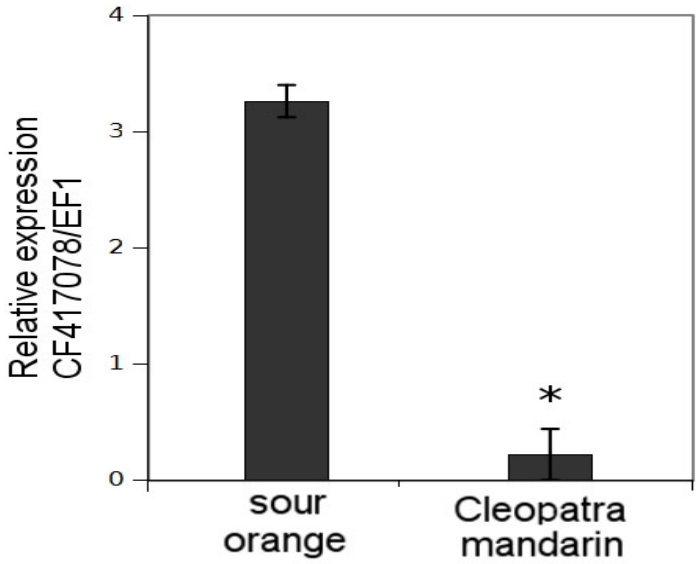

Figure 7. Relevance of *chalcone synthase (CHS)* in citrus defense against *T. urticae*. Plant material was processed as mentioned in figure 2. cDNA was subjected to quantitative RT-PCR analysis. The *CHS* (CF417078) transcript levels in uninfested and *T. urticae* infested plants were normalized to the expression of EF1α measured in the

same sample. Data are presented as a mean of transcript expression relative to uninfested plants ± SD (n = 3). Significant differences in relative transcript levels between infested rootstocks were estimated performing a *t*-test. Asterisks mean statistical significant differences ($P < 0.05$; $n = 3$).

To gain more insight into the citrus responses to spider mites, the levels of aminoacids and polyamines were determined. Ornithine was hardly affected by infestation. However, a contrasting response in the diamine putrescine (Put) compared with spermidine (Spd) and spermine (Spn) was found. The most resistant genotype was associated to increased basal levels of Put that seemed to be further boosted by mite infestation. On the other hand, Spn and Spd levels were not altered by mite presence in sour orange and remained lower compared to Cleopatra mandarin (Figure 8).

Since a major source for nutrients during herbivory are the primary plant metabolites, we also determined the aminoacid profiling in both citrus genotypes when in presence and absence of mite infestation. Basal levels of aminoacids remained very similar in both rootstocks (Fig 9). Additionally, *T. urticae* herbivory did not change significantly the levels of most aminoacids after 3 dpi. Despite these observations, tryptophan, tyrosine, histidine and Leucine+Isoleucine (which could not be separated in our analysis) were induced in sour orange upon mite infestation compared to Cleopatra mandarin. Since these induced aminoacids are precursors of other secondary metabolites involved in plant resistance, this result suggests that sour orange may be adapting its metabolism upon mite attack, whereas this phenomenon is not observed in the more susceptible Cleopatra mandarin.

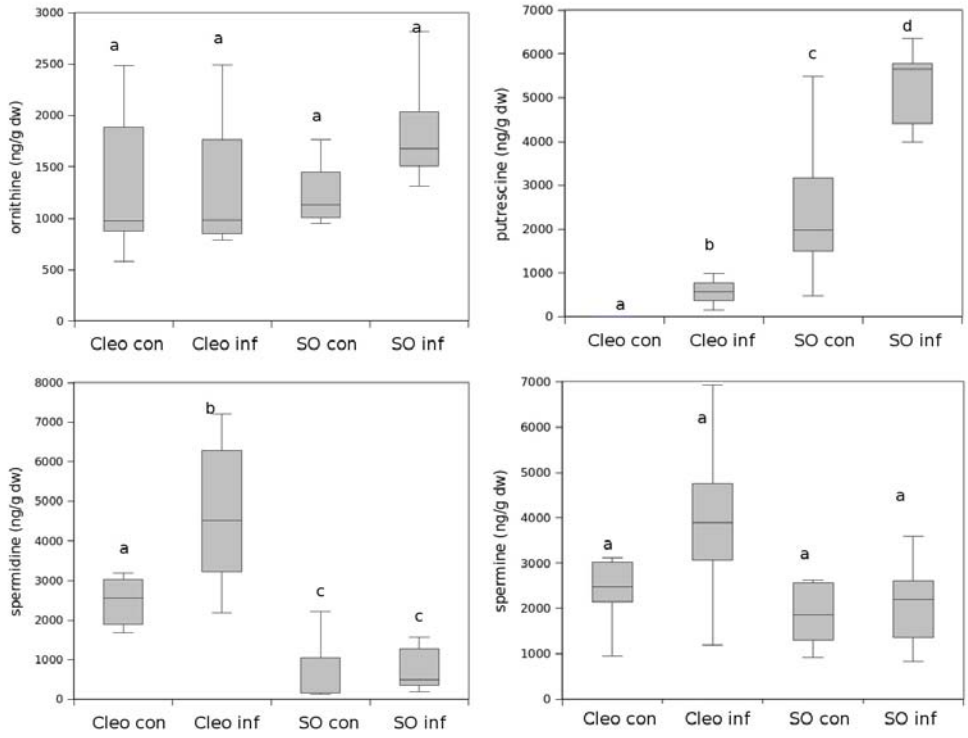

Figure 8. Profile of polyamines in sour orange and Cleopatra mandarin following spider mite infestation. Uninfested sour orange (SO con), infested sour orange (SO inf), uninfested Cleopatra mandarin (Cleo con) and infested Cleopatra mandarin (Cleo inf). 12-week-old plants were infested with 5 mites per plant and samples for analysis were collected 3 dpi. Leaf material from 6 individual plants was pooled together for each genotype combination. Samples were processed for polyamine quantification analysis by HPLC-TQD. Polyamine concentration was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Boxplots represent the average of three independent experiments with two technical replicates (n = 6). Different letters indicate significant differences (One way ANOVA, $P < 0.05$; *LSD*) between treatments.

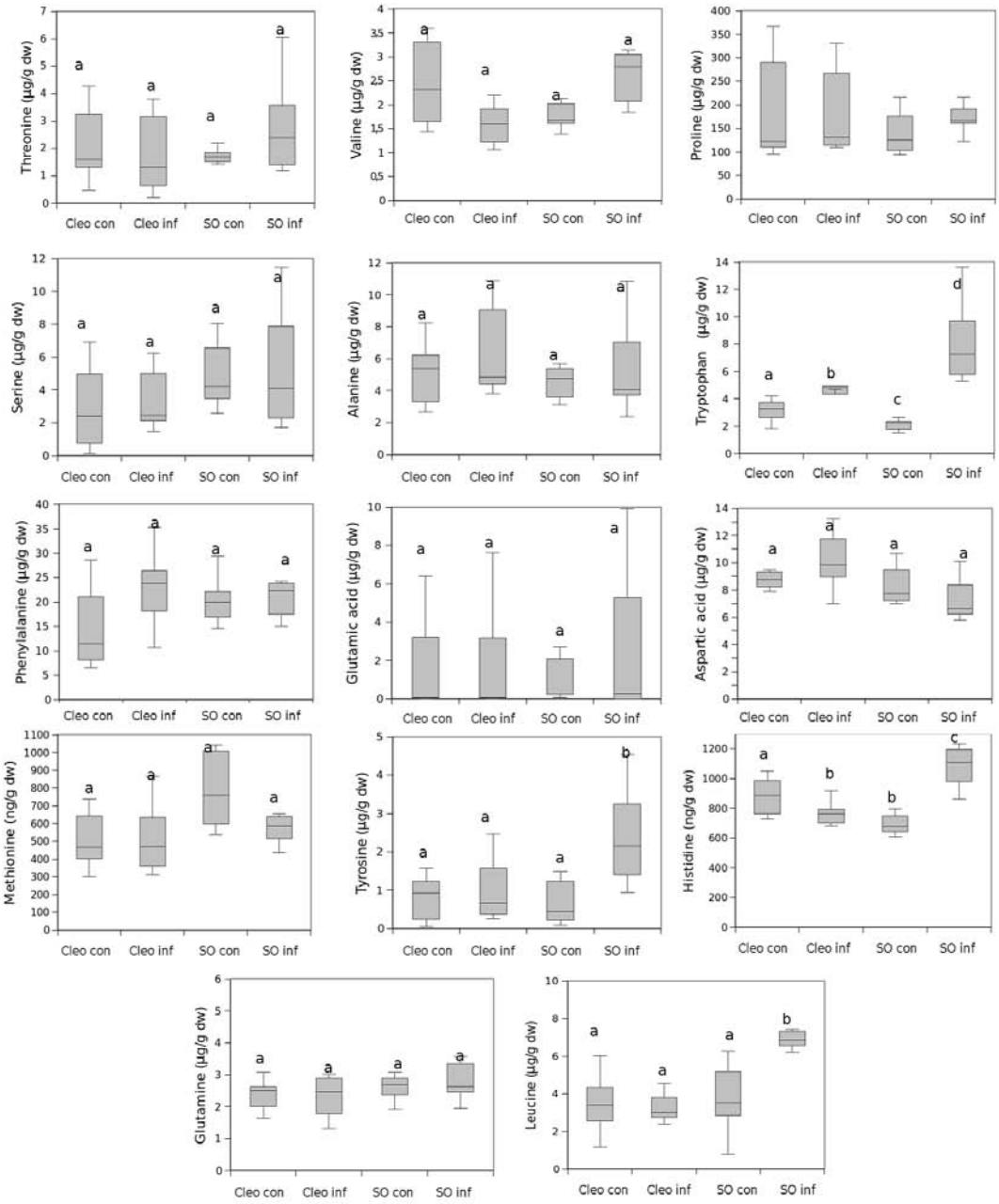

Figure 9. Profile of aminoacids in sour orange and Cleopatra mandarin following spider mite infestation. Uninfested sour orange (SO con), infested sour orange (SO inf), uninfested Cleopatra mandarin (Cleo con) and infested Cleopatra mandarin (Cleo inf). 12-week-old plants were infested with 5 mites/plant and samples for analysis were collected 3 dpi. Leaf material from 6 individual plants was pooled together for each genotype combination. Samples were processed for aminoacid quantification analysis

by HPLC-TQD. Aminoacid concentration was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Boxplots represent the average of three independent experiments with two technical replicates (n = 6). Different letters indicate significant differences (One way ANOVA, $P < 0.05$; *LSD*) between treatments.

4. Discussion

Spider mites are key pests of many economically important crops including *Citrus* sp (Garrido and Ventura, 1993; Jacas and Urbaneja, 2010; Maserti et al., 2011; Pascual-Ruiz et al., 2013). Here, we show a first report of a plausible resistance mechanism of citrus trees against *T. urticae,* as deduced from the different molecular mechanisms observed when comparing rootstocks with genetically-based natural variation in resistance to spider mite attack. Sour orange showed reduced levels of infestation, oviposition and leaf damage compared with Cleopatra mandarin. The less susceptible rootstock (sour orange) showed a fast and strong activation of SA and oxylipin dependent pathways. These results suggest that *T. urticae* may be activating SA and JA dependent defenses in the most resistant cultivar, while slight responses are observed in Cleopatra mandarin resulting in an enhanced susceptibility. In addition some flavonoid precursors and phytoalexins are strongly induced in sour orange upon infestation and this result demonstrates a genetic natural variation of the response against spider mite infestation in citrus. According to the results obtained, the oxylipin pathway plays a key role in the response of citrus against *T. urticae*. This hypothesis is supported by the constitutive high levels of *LOX-2* and the faster and stronger increase in the JA-marker gene *PR-3* in the less susceptible rootstock. The enhanced levels of JA, OPDA and the active hormone JA-Ile that showed increased levels at very early time-points after infestation in sour orange is reflected in the enhanced

expression of the oxylipin-related genes. These results, especially levels of JA-Ile, suggest that sour orange may detect the mite faster than Cleopatra mandarin and consequently respond in a more effective manner. Before the infestation JA was already higher in sour orange, thus basal levels may also contribute to mount effective defensive reactions earlier than in Cleopatra mandarin against *T. urticae*. There are evidences suggesting that oxylipins and JA derivatives contribute to the resistance against spider mites in different crop plants (Howe and Jander, 2008; Kant et al., 2008). In fact, the threshold of oxylipins that are triggered during mite infestation has a direct consequence of the performance of these mites on tomato leaves. There is an inverse correlation between the level of the JA marker *WIPI-II* and population densities and oviposition of the different lines of *T. urticae* according to their adaptation. Since most research links plant resistance against mites to oxylipin-dependent responses, it is surprising the observed enhanced levels of SA and PR-5 gene expression in the most resistant cultivar sour orange. According to these observations it would be also possible that the SA-dependent pathway was contributing to its reduced susceptibility together with the JA pathway. To clarify the relevance of both signals we performed experiments by using SA, MeJA and the LOX inhibitor Phenidone as there are no JA mutants available in citrus species. Phenidone inhibits the conversion of linoleic acid into 13-hydroperoxilinoliec acid, which is the first step of the oxylipin pathway (Bruinsma et al., 2009). Phenidone-treated sour orange plants lost their resistance against *T. urticae*. As a consequence, this mite could increase oviposition relative to untreated control plants. Hence, an intact JA signalling is necessary in sour orange to inhibit mite population growth and consequently decrease damage. It is noteworthy that the well-known negative crosstalk between JA and SA defense pathways (Pieterse et al., 2009; Robert-Seilaniantz

et al., 2011) is not observed in sour orange in response to *T. urticae*. MeJA treatments protected the most susceptible genotype but did not enhance the response of sour orange and this was attributed to its already elevated JA-dependent signals. It is known that there are lines of spider mites that can suppress plant defense responses (Kant et al., 2008). In fact, *T. evansi* can improve plant quality for herbivores by eliminating plant responses that act as deterrents of spider mite feeding (Sarmento et al., 2001). In relation to Cleopatra mandarin, *T. urticae* may be effective in suppressing plant defensive responses through a yet unknown effector machinery, although this possibility is still a matter of future research. The fact that there is no effective suppression of defenses upon Phenidone treatments, leaves open the question whether *T. urticae* can downregulate Cleopatra mandarin defenses or it is due to the inability of this host to activate JA-dependent responses against herbivores. In addition to SA-JA interplay, the participation of proteinase inhibitors and cystein proteases in several plant species upon mite attack has been described (Kant et al., 2004; Santamaria et al., 2012). Proteinase inhibitors have been described as effective defenses against phytophagous arthropods by a direct deterrent activity. The induction of proteinase inhibitors by spider mites in tomato is jasmonate dependent (Li et al., 2002). Reasonably, the proteinase inhibitors such as miraculine-like proteins are also up-regulated in citrus by spider mite attack (Maserti et al., 2011). The pyramiding of two barley proteinase inhibitors expressed in Arabidopsis provided a very effective response against *T. urticae* (Santamaria et al., 2012). However, in our experiments the function of proteinase inhibitors is less clear since the most resistant rootstock showed much lower expression levels than Cleopatra mandarin. In tomato, antisense depletion of *LOX* decreases expression of *PI* genes, but does not down-regulate JA levels (Royo et al., 1999). Other authors

claim alternative regulation of *PI*s unrelated to JA (Mozoruk et al., 2006). It is still unclear whether the citrus *LOX-2* homologue participates in *PI* regulation since the strong up-regulation of citrus *LOX-2* and oxylipin accumulation in sour orange is not triggering elevated *PI* expression levels. This result suggests a JA-independent regulation, at least, of this particular citrus *PI* (DR908940). A similar observation was obtained by Kawazu et al. (2012) who found the up-regulation of the JA-related gene *LapA1* but not *PIN2* after *T. urticae* infestation in tomato. Interestingly, ABA-singalling was reported to positively regulate *PIN2* in tomato (Peña-Cortes et al., 1995). In our results, the more sensitive rootstock, Cleopatra mandarin, showed higher levels of *ABA-4* expression and accordingly there is an up-regulation of *PI*. Despite the expression of *ABA-4* upon infestation suggests a *PI* regulation by ABA-signalling it is still a matter of future research since ABA levels are not significantly different in Cleopatra mandarin and sour orange.

The metabolomic profiling performed on both rootsocks following spider mite infestation allowed exact and tentative assignation of many signals that may contribute to the enhanced resistance of sour orange to *T. urticae* infestation. A pathway analysis of the cluster of signals over-represented in sour orange upon infestation allowed us to determine that most of these compounds are derivatives of the shikimate pathways and subsequent phenolic acid metabolism, specifically biosynthesis of alkaloids derived from shikimate, flavonoids, phenylpropoanoids and anthocyanins. MS/MS analysis and subsequent fragmentation spectra confirmation, allowed us to identify 4-coumaric acid, naringenin and hesperetin. These three substances are derivatives of the shikimate.

Phenolic compounds and flavonoids are long time known to possess antifungal

and insecticidal properties (Lattanzio et al., 2006). One function for flavonoid precursors is the formation of physical barriers as components of lignin (Boerjan et al., 2003). 4-coumaric acid is a key component, because it is the starting point for lignin biosynthesis through the 4-coumarate-CoA ligase. Lignin polymers are a complex protecting network based on 4-coumaryl, coniferyl, and sinapyl alcohols within the plant cell wall (Boerjan et al., 2003; Lattanzio et al., 2006;). However, these compounds can be actively induced in the plant upon biotic stress. There is a negative correlation between biosynthesis of secondary compounds and flavonoids and growth and development of almost all phytophagous insects. Flavonoids and secondary metabolites reduce or eliminate the palatability of the plant in which they are synthesised and act as determinants of host-plant range in phytophagous insects (Burghardt et al., 2001; Lattanzio et al., 2006). In this regard, the up-regulation of citrus CHS in sour orange and the concomitant increase of 4-coumaric acid, naringenin and hesperetin could contribute to the reduced susceptibility of this genotype. Noteworthy, sour orange displays elevated basal levels of all three compounds which boosted upon *T. urticae* infestation. Contrastingly, Cleopatra mandarin had very low levels of 4-coumaric acid, naringenin and hesperetin. An interesting study has pointed to a regulatory role of flavonoid homeostasis in the JA biosynthesis (Pourcel et al., 2013). The excess of flavonoids and naringenin in Arabidopsis display a negative feedback in JA and JA-Ile (Loreti et al., 2008). In our plant-arthropod system, it is very likely that the defensive reactions in sour orange pass through a fast activation of the oxylipin pathway that triggers a strong enhancement of the flavonoids naringenin and hesperetin. These flavonoids had no negative effect in the JA-singalling pathway according to our observations, since shikimic acid biosynthesis is necessary for coumaric acid and flavonoids occurrence, the biosynthetic branch shared with SA needs

to be active. This fact may explain why sour orange displayed elevated SA and PR5 gene expression levels compared with the more susceptible rootstock Cleopatra mandarin.

The influence of polyamine on spider mite performace in plants has been hardly documented. There are some reports establishing a positive link between Spn treatments and hydrogen peroxide (H_2O_2) and JA accumulation contributing to resistance against insects (Ozawa et al., 2009). Noteworthy, the degradation of putrescine generates H_2O_2 by the action of diamineoxidases. We obseved strong increases of putrescine in infested sour orange. This may contribute to sour orange reduced susceptibility. Further, it may play a role in up-regulation of JA singalling (Arimura et al., 2011).

Host quality in terms of sugar and aminoacid contents is important to sustain reproduction of sap feeding insects (Awmack et al., 2002). For instance, the levels of phloem aminoacids are determinant of aphid fecundity. According to our observations, the levels of phloem aminoacids does not change significantly upon infestation. However, we observed some particular increases in tyrosine, tryptophan, isoleucine-leucine and histidine. Among them, the four former are precursors of coumaric acid and therefore of flavonoids and phenolic alkaloids. It is likely that specific changes in aminoacid concentrations in sour orange constitute a metabolic reprogramming in order to increase resistance reactions after mite herbivory.

In conclusion, the results presented here reveal the importance of the oxylipin pathway and flavonoids in response to *T. urticae*. Both higher constitutive levels and increased/earlier response in combination in sour organge had consequences on the performance of this mite, its reproduction and, therefore, plant damage. These observations point out the relevance of rootstock choice

since it could have a relevant impact for pest management. Indeed, since most citrus varieties around the world are grafted, it is of key relevance to study the signals transmission to distal plant parts and also between variety and rootstock. Unfortunately, Cleopatra mandarin, a CTV-tolerant rootstock, is more susceptible to spider mites than sour orange and, as mentioned by Bruessow et al. (2010), replacement of sour orange by CTV-tolerant rootstocks may have triggered the prevalence of *T. urticae* as a key pest of clementine mandarins in Spain. This is a demonstration that enhanced tolerance or resistance to a single pest, disease, or abiotic factor should not be a unique trait to take into account for rootstock breeding. All this knowledge could result extremely helpful to develop new rootstocks with better response to the spider mite attack, thus achieving new approaches for a more sustainable pest management.

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Suporting information

Figure S1. Boxplot analysis of the 19 selected signals from the table 1 and heatmap analysis showing the strongest induction in sour orange infested and uninfested plants compared with Cleopatra mandarin. Four different sets of samples were tested: in the first place uninfested sour orange (SO con), infested sour orange (SO inf), uninfested Cleopatra mandarin (Cleo con) and infested Cleopatra mandarin (Cleo inf). 8-week-old plants were infested with 5 mites per plant and samples for analysis were collected 3 dpi. Leaf material from 6 individual plants was pooled together for each genotype combination. Three independent biological replicates and two technical replicates were randomly injected and analysed (n = 6). Boxplots were generated by using Mar-Vis Filter and Cluster following a Kruskal-Wallis test ($P < 0.05$). The concentration of the

metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample.

Supplementary table1. Primers used for RT-qPCR analysis of gene expression.

Description	Accesion	Forward primer 5' → 3'	Reverse primer 5' → 3'
PR-3	Cit.1727.1.S1_s_at	CTGGACATTTGTGCTACATAGAAG	CACAAGGCAGTCTTAAATGACA
LOX2	Cit.16756.1.S1_s_at	GAACCATATTGCCACTTTCG	CGTCATCAATGACTTGACCA
EIN3	Cit.5616.1.S1_s_at	AGGTGTGAGGCTTCTTCCTT	AAAGCAGTTTTGGACATTGG
ABA4	Cit.318.1.S1_s_at	CACTCATGGTTTTTGCTCCT	AGCTCTGGGAGCCAGTATTT
PR-5	BAI63297.1	CATCAAGCTTCACAGTGCTTAG	CCACAACGTACAGACTGATGAC
PI TYPE1	DR908940	CTTTCTCTTTGCCTTTGCTG	CCGTTTACACCAACCAGTTC
EF 1 α	DR908282	CCGGACATCGTGACTTTATC	TCCATCTTGTTACAGCAGCA
GAPDH	Cit.122.1	GGAAGGTCAAGATCGGAATCAA	CGTCCCTCTGCAAGATGACTCT

Supplementary table 2. Results for metabolomic markers in control and *T. urticae* infected leaves of sour orange (SO) and Cleopatra mandarin (Cleo) (positive electro-spray analysis).

Supplementary table 3. Results for metabolomic markers in control and *T. urticae* infected leaves of sour orange (SO) and Cleopatra mandarin (Cleo)

(negative electro-spray analysis).

CHAPTER 3

Tetranychus urticae-triggered responses in citrus promote genotype-dependent conspecific repellence or attractiveness.

Adapted from:

Agut B, Gamir J, Jacas JA, Flors V. 2015. *Tetranychus urticae*-triggered responses in citrus promote genotype-dependent conspecific repellence or attractiveness. *New phytologist*. Accepted article.

Abstract

- The citrus rootstocks sour orange and Cleopatra mandarin display differential levels of resistance against the two spotted spider mite *Tetranychus urticae*. Sour orange supports reduced oviposition, growth rates and damage levels compared with Cleopatra mandarin. Jasmonic acid (JA) signalling activation and flavonoid accumulation revealed as key mechanisms for the enhanced resistance of sour orange.

- In the present research we observed that herbivore induced plant volatiles (HIPVs) from sour orange have a marked repellent effect on conspecific mites that can be associated with the production of the terpenes α-ocimene, α-farnesene, pinene, and D-limonene, as well as with that of the green leafy volatile 4-hydroxy-4-methyl-2-pentanone. Contrastingly, HIPVs from Cleopatra mandarin promote conspecific mite attraction associated with an increase in (2-Butoxyethoxy) ethanol, Benzaldehyde, and Methyl salicylate levels.

- Sour orange HIPVs released by sour orange following *T. urticae* infestation induce resistance in Cleopatra mandarin reducing oviposition rates and stimulating the oxylipin biosynthetic gene lypoxigenase2 LOX2.

- We conclude that sour orange can promote herbivore induced resistance in Cleopatra mandarin, that despite its weak basal resistance can be primed by components of HIPVs blend such as α-ocimene and D-limonene.

1. Introduction

Plant direct defenses against herbivores comprise diverse reactions that differs depending on the timing and the nature of the plant-arthropod interaction. For example, early stages of plant response are regulated by phytohormones (Pieterse and Dicke, 2007; Pieterse et al., 2009; Erb et al., 2012) whereas late stages usually involve the synthesis of phytoalexins and secondary defensive metabolites (Mithöfer and Boland, 2012). The type of mechanical injury caused by the arthropod also modifies the hormone profiling (Kawazu et al., 2012). Injury directly depends on the mouthparts and feeding habits of the herbivore. Hemipteroid insects with piercing-sucking haustellate mouthparts, such as aphids and whiteflies, pierce plant tissues and ingest phloem through their long stylets. However, orthopteroid and other non hemipterioid insects, such as lepidopteran larvae and grasshoppers, have mandibulate mouthparts that lacerate leaf tissue to be subsequently ingested. Phytophagous mites pierce individual epidermal cells with their short stylets and ingest their celullar contents. From the most relevant plant hormones related to plant defense against biotic stresses (Jasmonic Acid/JA, Salicylic Acid/SA, cytokinins, Abscisic Acid/ABA, Indole-Acetic Acid/IAA, and Ethylene), it is recognized that JA, SA and cytokinins can promote resistance against phloem-feeders (Soler et al., 2013). Nevertheless, plant responses to phytophagous spider mites (Acari: Tetranychidae) do not follow the same pattern.

Spider mite attacks trigger both JA and SA and the role of the latter is not fully clear. It has been proposed that SA may be induced as a strategy to repress plant responses regulated by JA that provide an effective resistance against phloem feeder arthropods (Walling et al., 2008). For instance, JA can itself regulate

other plant responses such as systemin and proteinase inhibitors (PIs) as part of a set of direct defenses (Howe and Jander, 2008). Kant et al. (2008) demonstrated that tomato plants overexpressing the prosystemin gene (35S:PS) support reduced oviposition of JA-sensitive two-spotted spider mite *Tetranychus urticae* Koch. Polyphenol oxydase (PPOX) and lipoxygenase (LOX) modify dietary proteins hampering arthropod development (Johnson and Barbehenn, 2000). PIs can block gut proteases resulting in amino acid deficiencies and arthropod growth reduction (Lison et al., 2006; Zavala et al., 2004).

Following hormonal signalling, defensive proteins and secondary metabolites have emerged as key elements for an effective defense against arthropods. Among them, terpenoids are the most diverse class of secondary metabolites functioning in plant defense (Aharoni et al., 2005). Other tryptophan derivatives such as DIMBOA in maize and wheat and glucosinolates in Arabidopsis can act as toxic compounds for arthropods (Halkier and Gershenzon, 2006; Ahmad et al., 2011). The flavonoids hesperetin and naringenin overaccumulated in citrus with enhanced resistance against *T. urticae* and these compounds are probably responsible for the decreased mite performance observed (Agut et al., 2014).

Plant volatile organic compounds (VOC) can mediate both direct defenses and attraction of beneficial arthropods, so called indirect defenses. VOCs can benefit the plant by deterring conspecific oviposition (De Moraes et al., 2001) but also by attracting entomophagous predators and parasitoids (Kessler et al., 2001; Schnee et al., 2006). In this regard, sesquiterpenoids such as (E)-β-farnesene and (E)-α-bergamotene mediate indirect defenses in these tritrophic interactions. A specific blend of VOCs that are released upon herbivore attack, the herbivore induced plant volatiles (HIPV), can also affect

the behavioural responses of the attacker. However this issue has been poorly investigated. Some studies report that HIPVs can act as herbivore repellents (Dicke, 1986, Bernasconi et al., 1998), whereas other reports show that they can also be attractants (Pallini et al., 1997; Halitschke et al., 2008). Despite priming has been described thoroughly in plant-pathogen interactions, this phenomenon has also been documented with arthropods. Not only conspecific neighbours but also distal plant parts can be primed. However, our knowledge of plant-herbivore interactions is still limited (Frost et al., 2008; Kim and Felton, 2013). Priming by VOCs was observed in maize plants after exposure to the green leaf volatile, GLV, (Z)-3-hexen-1-ol (Engelberth et al., 2004). This GLV induced an increase in the levels of JA acid. VOCs-mediated priming was also found in different plant-arthropod interactions including the accumulation of plant secondary metabolites (Kessler et al., 2006; Hirao et al., 2012), increased PI activity (Kessler et al., 2006), increased transcription of antiherbivore defense genes (Ton et al., 2007; Peng et al., 2011), and secretion of extrafloral nectar (Heil and Kost, 2006), resulting in reduced herbivore performance (Kessler et al., 2006; Ton et al., 2007; Muroi et al., 2011; Peng et al., 2011), and increased attraction of natural enemies (Ton et al., 2007; Muroi et al., 2011; Peng et al., 2011; Rezende et al., 2014).

Despite all efforts in understanding direct and indirect defenses in plant-mite interactions, the response of citrus to *T. urticae* remains poorly understood. In the present research we focus our attention on the HIPVs-induced resistance, so called volatile-induced resistance (VOC-IR) in the citrus-*T. urticae* system. This mite is a key pest of citrus (Jacas et al., 2010; Jacas and Urbaneja, 2010; Pascual-Ruiz et al., 2014). Bruessow et al. (2010) demonstrated that the fitness of *T. urticae* developing on satsuma mandarin plants (*Citrus unshiu* Marcovitch) depended of the rootstock where the cultivar was grafted. *T.*

urticae performed the worst on satsuma grafted on sour orange (*Citrus aurantium* L.) and the best on Cleopatra mandarin (*Citrus reshni* Hort. ex Tanaka). Later on, Agut et al. (2014) determined that sour-orange enhanced resistance to spider mites could be sustained by high levels of flavonoids and a fast and effective activation of the oxylipin pathway upon mite attack. In fact, naringenine and hesperetine accumulation correlated with an increase in chalcone synthase which is a key enzyme in their synthesis (Pourcel et al., 2013).

There is little information about citrus immune system against mites and even less is known about the plant-plant communication in citrus. Here we propose a study to determine the molecular cues of mite triggered HIPVs-induced resistance using a resistant citrus rootstock, sour orange, that can promote resistance in a neighbour susceptible citrus, Cleopatra mandarin. We have performed a comparative analysis of VOCs emission in infested and uninfested citrus plants. We have observed that release of HIPVs upon mite infestation differ between both plant species. Noteworthy, only HIPVs from sour orange can induce resistance in the susceptible species stimulating plant defense responses that result in reduced mite oviposition rates. Interestingly the resistance response triggered in the susceptible rootstock differes from the immune responses observed in the resistant sour orange.

2. Materials and methods

2.1 Plant material

Sour orange (*Citrus aurantium*) and Cleopatra mandarin (*Citrus reshni*), two rootstocks with different response to *T. urticae* (Bruessow et al., 2010; Agut et al., 2014) were used. 12 week-old of both rootstocks were maintained in a climatic chamber at 25º C and 50–70% RH under a 16:8 h L:D photoperiod. These plants were grown on vermiculite and peat (1:3, vol:vol) in 320 mL pots. No insecticides or acaricides were applied to these plants, which were fertilized using a modified Hoagland's solution every 3 days.

2.2 Spider mite stock colony

The *T. urticae* colony used in the present assays was initiated with specimens collected in clementine orchards in the region of La Plana (Castelló, Spain). The colony was maintained on detached leaves of young clementine mandarin plants, *Citrus clementina* Tanaka cv. Clemenules (INIASEL 22), in a climatic chamber at 25º C, 70–80% RH and 12:12 h (L:D) photoperiod. The rearing took place on detached leaf units consisting of a single Clemenules leaf placed upside down on moistened cotton, placed on top of a water-saturated foam cube (3-4 cm thick) in an open plastic box (35 × 20 × 7 cm) half-filled with water. Moist cotton was folded over the edges of the leaves to prevent mites from escaping. When necessary, cohorts of the same age were produced by transferring gravid females from the stock colony to freshly set detached leaf units for a controlled period of time. Afterwards, females were removed and the eggs were kept undisturbed until reaching the immature target stage and age. These cohorts were maintained under the same environmental conditions as the stock colony.

2.3 Collection of Headspace Volatiles.

Volatiles emitted by the two different citrus species including both uninfested and infested plants were collected using a headspace collection system similar to that described by Bruinsma et al.(2010). Plants were individually transferred to 5-L glass vessels (Duran). These vessels were ventilated with carbon-filtered pressure-air at 1.5 L hr^{-1}. The system was purged for 1 h with purified air before attaching a pipeline filled with 240 mg Porapak (Sigma-Aldrich, Spain) to the air outlet in the lid to trap the headspace volatiles. Headspace collections took place in a climatic chamber at 23 ± 1 °C, 60 ± 5% R.H. When necessary, plants were infested with 20 *T. urticae* adult females 1 day before volatile collection. In this case, volatiles were collected for 24 h following infestation.

2.4 Gas Chromatography Instrumentation

Gas Chromatography (GC) instrumentation consisted of an Agilent 6890N GC system (Paloalto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a time-of-flight mass spectrometer, GCT (Waters Corporation, Manchester, UK), operating in electron ionization (EI) mode. The GC separation was performed using a fused silica DB- 5MS capillary column of 30m length, 0.25mm i.d. and a film thickness of 0.25m (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 50 ºC (1min); 5 ºC/min to 210 ºC (1 min); 20 ºC/min to 300 ºC (2 min), resulting in a total analysis run of 40.50 min. Splitless injections were carried out. Helium was used as carrier gas at 1mL/min. The interface and source temperatures were both set to 250º C and a solvent delay of 3min was selected. TOF MS was

operated at 1 spectrum/s acquiring the mass range m/z 50–650 and using a multi-channel plate voltage of 2800 V. TOF MS resolution was about 8500 (FWHM) at m/z 614. Heptacose, used for the daily mass calibration as well as lock mass, was injected via syringe in the reference reservoir at 30 ºC. 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 samples.

Volatile compounds were identified by using the retention time and fragmentation spectrum against the following commercial standards: MeSA and MeJA (Sigma, Barcelona, Spain). Other volatile compounds were identified using GC–MS and matching to the NIST Library, using Match values of at least 850 as a threshold for identification as it has been described previously by Wallis et al. (2008).

2.5 Y-tube olfactory choice assays

Olfactory-choice assays for spider mites were conducted using a Y-tube olfactometer, based on that described by Bruin et al. (1992). It consisted of a Y-shaped glass tube 4 cm in diameter with a 13 cm base and two 13 cm arms containing a Y-shaped 1 mm in diameter metal wire of the same dimensions which occupied 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 (same as described for volatile collection) containing different odor sources. Each vessel was connected to an air pump that produced a unidirectional airflow of 1.5 L hr^{-1} from the arms to the base of the tube. Air was purified with a granulated activated charcoal filter (Sigma-Aldrich, Spain). The light intensity

was maintained at 2,516 lux measured with a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc. Pullman, WA, USA). The environmental conditions of the Y-tube experiments were 23 ± 2 ºC and 60 ± 10 % RH. Adult female *T. urticae* deprived of food for 24 h prior to the bioassay were individually deposited at the beginning of the long arm of the wire with a soft-bristle paintbrush. Individual females were allowed to make a choice within 5 min. As soon as a mite reached the end of one of the two short arms of the tube, it was removed from the set-up and discarded. Mites were scored as 'no choice' when they failed to reach either end of the short arms within the allocated time. A minimum of 45 spider mites per odour combination (see below) were tested. Glass containers were switched after every five females had been tested to reduce the effects of any spatial influence on choice. After every ten females had been used, plants were replaced and the whole system was rinsed with acetone (99 %) and then air-dried. To exclude any bias from the setup, prior to the beginning of the assays, a minimum of 20 mites were offered clean air in both arms.

2.6 Effect of HIPVs on neighbouring plants

To determine the effect of volatiles released by infested sour orange and Cleopatra mandarin plants on untreated plants, an oviposition assay was carried out. First, sour orange plants and Cleopatra mandarin plants were infested with 20 adult females per plant as already described. 24 h later, two infested plants of either sour orange or Cleopatra mandarin were placed in a tray with 10 untreated sour orange and 10 untreated Cleopatra mandarin plants. Subsequently, the tray was covered with a transparent lid. To avoid mite ambulatory dispersal, the tray was filled with water. 72 h later, five plants for

each species were defoliated and leaves frozen at -80ºC for further analysis (mRNA and metabolome analyses, see below). The remaining five plants per citrus species were infested with six 2-day old adult females. 72 h later, the number of eggs per plant was counted. This process was repeated three times with the corresponding control untreated plants (not exposed to any volatile). The semi-field experiments were performed in a 40 m^2 greenhouse by placing the plants randomly. To ensure a better communication in open air conditions, five infested plants were alternated every 10 untreated plants for the HIPVs treatment. Sampling and infestations were performed as described above with the exception of the trays.

2.7 Quantitative real-time PCR analysis

RNA was extracted using the Plant RNA kit (Omega Bio-Tek Inc., Doraville, GA). For RT-qPCR experiments, 1.5 μg of total RNA was digested with 1 unit of DNase (RQ1 RNase-Free DNase) in 1 μL of DNase buffer and Milli-Q water up to 10 μL (Promega Corporation, Madison, WI) and incubated for 30 min at 37º C. After incubation, 1 μL of RQ1 DNase stop buffer was added and incubated again at 65º C for 10 min to inactivate DNase. The RT reaction was performed by adding 2 μL of RT buffer, 2 μL of 5 mM dNTP, 2 μL of 10 μM Oligo(dT) 15 primer (Promega), 1 μL of 10 U μL−1 RNase inhibitor (Promega) and 1 μL of Omniscript reverse transcriptase (Qiagen, Barcelona, Spain). The reaction mixture was incubated at 37º C for 60 min. Complementary DNA from the RT reaction, 10× diluted, was used for qPCR. Forward and reverse primers (0.3 μM) were added to 12.5 μL of PCR SYBR reaction buffer, 2 μL of cDNA and Milli-Q sterile water up to 25 μL of the total reaction volume (Takara Bio, Kyoto, Japan). Quantitative PCR was carried out using the Smart Cycler II

(Cepheid, Sunnyvale, CA) sequence detector with standard PCR conditions. Because there were differences in cycle numbers during the linear amplification phase between samples, the data were transformed with the formula $2^{\Delta\Delta Ct}$. RT-qPCR analysis was performed at least 3 times using sets of cDNA samples from independent experiments. The primers of *LOX2*, *PR3*, *PR5* and *CHI* were used to observe the activation of the JA and SA pathways (Supplementary table 1). Relative expression was compared with the housekeeping genes EF1α and GADPH with the same results.

2.8 Hormone analysis

A quantity of 50 mg of freeze-dried samples was used. The hormones 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), JA-isoleucine (JA–Ile), were analysed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS), as described by Flors et al. (2008) and Forcat et al. (2008).

2.9 Metabolome analysis: LC-ESI full-scan mass spectrometry Q-TOF

50 mg of lyophilised leaf samples were extracted with MeOH:H_2O (10:90) containing 0.01% HCOOH. After polytron homogenisation on ice, the samples were centrifuged for 15 min at 15 000 × g, the supernatant was filtered and an aliquot was used for subsequent analysis. Metabolome analysis was performed using an Acquity UPLC system (Waters) interfaced to hybrid quadrupole time-of-flight (QTOF Premier). Three technical and three independent biological replicates per sample were randomly injected. The LC separation was

performed on an HPLC SunFire C18 analytical column, 5 μm particle size, 2.1 × 100 mm (Waters). Analytes were eluted with a gradient of methanol and water containing 0.01% HCOOH. The gradient started with 90% aqueous mobile solvent and linearly reached 10% in 12 min. In the following 3 min, the gradient was kept in isocratic conditions and then returned to initial conditions in 4 min. The column was allowed to equilibrate for 3 min, giving a total time of 22 min per sample. The solvent flow rate was 0.3 mL min^{-1}. The injection volume was 20 μL. The drying gas and the nebulising gas was nitrogen. The desolvation gas flow was set to approximately 600 L h^{-1}, and the cone gas flow was set to 60 L h^{-1}. A cone voltage of 20 V and a capillary voltage of 3.3 kV were used in the negative ionisation mode. The nitrogen desolvation temperature was set at 350º C, and the source temperature was set at 120º C. The instrument was calibrated in the m/z 50–1000 range with a 1/1 mixture of 0.01 M NaOH/1% HCOOH tenfold diluted with acetonitrile/water (80/20, v/v). A solution of leucine enkephalin at a concentration of 2 ppm in acetonitrile/water (50/50, v/v) with 0.1% formic acid was simultaneously introduced into the Q-TOF instrument via the lock-spray needle for accurate m/z determinations. The [M − H]− ion of leucine enkephalin at m/z 554.2615 was used for recalibrating the m/z axis. Metabolite amounts were analysed on the basis of normalised peak area units relative to the dry weight. Kruskal–Wallis test ($P < 0.05$) was applied to test the metabolomic differences between rootstocks versus infestation.

2.9 Full-scan data analysis.

Centroid acquired raw data were transformed into .cdf files using Databridge from the Masslynx 4.1 software (Waters) and subsequently subjected to analysis

using the software R for statistical purposes. Signals from positive and negative electrospray analysis (ESI+; ESI−) were processed separately. Peak peaking, grouping and signal corrections were developed applying the algorithm XCMX. This statistical package can be used to pre-process full-scan LC/MS data for relative quantification and statistical analysis. The statistics and the heat map analysis were carried out with the Mar-Vis Suit software including MarVis Filter and Mar Vis Cluster (Kaever et al. 2012), a tool for clustering and visualisation of metabolic biomarkers. Mar-Vis was used to process exported CSV. files from the XCMS and perform statistical analysis, adduct and isotope correction and clustering and colour heat map visualisation. To determine a global behaviour of the signals, principal component analysis (PCA) was used.

2.10 Effect of selected synthetic volatiles on plants

To elucidate which of the volatiles identified in infested sour orange plants were the responsible of the induced resistance observed in Cleopatra mandarin plants, transcrip levels of LOX2 were measured after 72 h of exposure to selected synthetic compounds commercially available. For induction, 100 µg of either D-limonene, ocimene, or 4-hydroxy-4-methyl-2-pentanone (dissolved in dichloromethane, 1 µg/µl) (Sigma-Aldrich, Spain) were pipetted onto a cotton ball. This ball was introduced in a covered tray as before with five untreated Cleopatra mandarin plants. Controls received a ball with 100 µl of pure dichloromethane. 72 h later the leaf material was collected for gene expression analysis. Three replicates per volatile were performed.

2.11 Statistical analysis

Statistical analyses of genetic and metabolomic data were conducted using Statgraphics Plus 3.1 (Rockville, MD) and the software R v.2.9.2 (R Development Core Team) and the package XCMX respectively. All experiments were repeated at least 3 times unless specified otherwise. A control consisting of plants exposed to uninfested plants under the same environmental conditions was considered.

3. Results

3.1 Volatiles emitted by *T. urticae*-infested citrus plants affect conspecific mite choice

In a previous research (Agut et al. 2014) we determined that enhanced resistance against *T. urticae* observed in sour orange compared to Cleopatra mandarin can be attributed to antibiosis. However, the existence of antixenosis (or repellence) in this system could be excluded. To elucidate the role of the VOCs released in the citrus-*T. urticae* interaction, an analysis of the volatile metabolome either in the presence or in the absence of infestation was carried out (Fig 1A). In absence of infestation the basal volatile profile of the two citrus species tested showed mainly quantitative differences. On the one hand, Cleopatra mandarin plants released higher amounts of pinene,

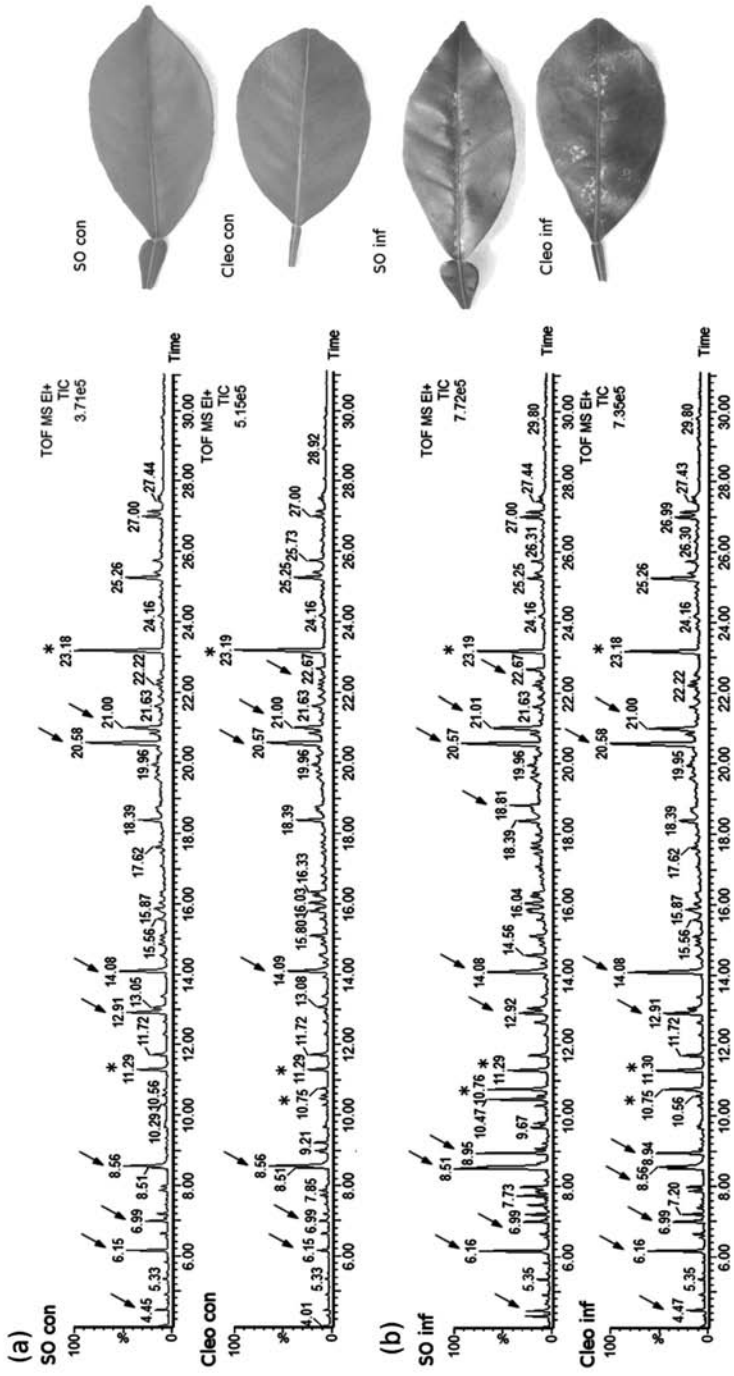

Figure 1. Volatile emission by citrus rootstosck that displays different resistance levels to *T. urticae* changed quantitatively and qualitatively upon infestation. Sour orange (SO) and Cleopatra mandarin (Cleo) plants were either kept uninfested or infested with 20 *Tetranychus urticae* adult females for 24 hours. (a) Volatile emission was determined by gas chromatography and the identification of the volatiles was made by

comparison of their fragmentation spectrum with those reported in the NIST library spectra (for the details of volatile collection and identification, see text in the Materials and Methods section). (b) Representative picture of uninfested leaves and symptoms of *T. urticae* in sour orange and Cleopatra mandarin leaves. An asterisk (*) indicates air contamination and arrows are the compounds identified in Table 1.

4-hydroxy-4-methyl-2-pe*ntanone*, 3,7-dimethylocta-2,6-dienyl benzene and 2(2-butoxyethoxy ethanol compared to sour orange (Fig 2, Fig S1, Table 1). On the other, sour orange showed higher levels of 6,11-dimethyl-2,6,10-dodecatrien-1-ol, α-ocimene, D-limonene, benzoic acid 2-(methylamino)-methyl ester, methyl salycilate (MeSA) and methyl jasmonate (MeJA) compared with Cleopatra mandarin. Despite these differences *T. urticae* did not show any preference for any of these genotypes (Fig 3). Upon spider mite infestation, volatile emission of the two citrus showed both quantitative and qualitative differences (Fig 1B). Several compounds, such as α-farnesene, α-ocimene, D-limonene, 4-hydroxy-4-methyl-2-pentanone,benzoic acid 2-(methylamino)-methyl ester and MeJA showed increased intensity in sour orange (Fig 2). 6,11-dimethyl-2,6,10-dodecatrien-1-ol remained undetectable in Cleopatra mandarin whereas it strongly accumulated in sour orange. Based on this result, we wondered whether these blends may have an antixenotic effect. Therefore a choice test with *T. urticae* infested plants was conducted. Infested sour orange plants were strongly repellent to conspecific mites compared with uninfested plants (Fig 3). Almost 75 % of mites preferred uninfested sour orange plants suggesting a strong antixenotic response that may be mediated by the overaccumulated HIPVs. Interestingly, a strong preference of *T. urticae* for infested Cleopatra mandarin compared with uninfested plants was found. This observation suggests a manipulation of this host by *T. urticae* resulting in enhanced attraction for conspecific mites.

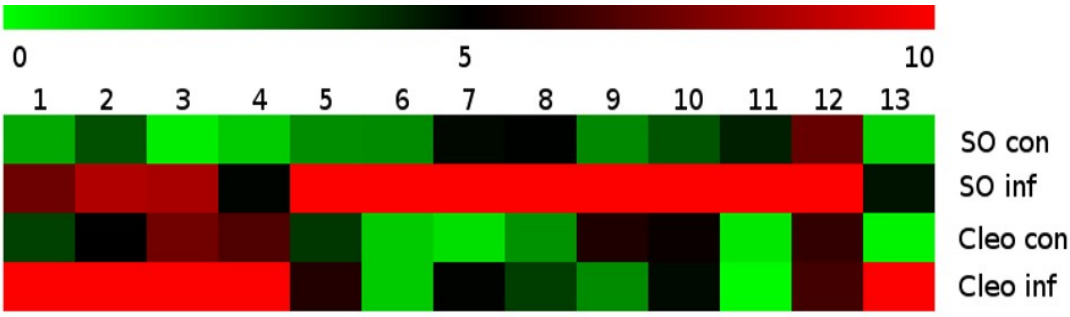

Figure 2. Heatmap analysis of the volatile profiling. Results are shown for sour orange control (SO con), sour orange infested (SO inf), Cleopatra mandarin control (Cleo con) and Cleopatra mandarin (Cleo inf) plants. Quantitative data are shown in Table 1. 1, benzaldehyde; 2, Benzothiazole; 3, (3,7-dimethylocta-2,6-dienyl) benzene; 4, (2-Butoxyethoxy) ethanol; 5, α-Farnesene; 6, 6,11-Dimethyl-2,6,10-dodecatrien-1-ol; 7, α-Ocimene; 8, D-limonene; 9, 4-hydroxy-4-methyl-2-pentanone; 10, Pinene; 11, Benzoic acid, 2-(methylamino)-, methyl ester; 12, MeJA; 13, MeSA.

This attraction could be favoured by both repressed and overaccumulated volatiles triggered by *T. urticae* infestation in Cleopatra mandarin. Emissions of MeJA and 4-hydroxy-4-methyl-2-pentanone were reduced in Cleopatra mandarin following mite attack. Contrastingly, compounds such as 2-butoxyethoxy ethanol, benzaldehyde and MeSA were strongly induced by mite infestation (Fig 2). Thus the release of these compounds may be triggered by *T. urticae* to manipulate/suppress host defenses and increase host attractiveness for conspecific mites. As expected, mites highly preferred infested Cleopatra mandarin compared with infested sour orange (Fig 3).

Table 1. Volatile profiling in the headspace of citrus upon attack of *T. urticae*.

Volatile profiling of sour orange (SO) and Cleopatra mandarin (Cleo) plants in absence of the mite or infested.

Volatile compounds	RT (min)	SO control		SO infested		Cleo control		Cleo infested	
Benzaldehyde	6.99	1.307,33 ± 285,13	a	5.518,67 ± 871,82	b	2.885,33 ± 152,21	a	7.720,33 ± 784,25	c
Benzothiazole	14.11	9.594,67 ± 2.605,01	a	23.988,30 ± 4584,44	b c	14.199,30 ± 1678,73	a b	28.289,00 ± 5.639,73	c
(3,7-dimethyloct a-2,6-dienyl) benzene	20.57	14.18 ± 4922,97	a	34.213,70 ± 4406,85	b	29.777,70 ± 9379,43	a b	41.143,70 ± 2980,72	b
(2-Butoxyethoxy) ethanol	12.92	0 ± 0	a	12.572,00 ± 1513,06	b	16.912,70 ± 2695,89	b	25.809,30 ± 3293,59	c
α-Farnesene	21.03	9.127,33 ± 3739,27	a	41.863,70 ± 11861,30	b	16.325,70 ± 4105,07	a	23.732,70 ± 2426,34	ab
6,11-Dimethyl-2, 6,10-dodecatrien -1-ol	22.68	2.416 ,00 ± 989,50	b	10.598,30 ± 3787,48	c	nd ±	a	nd ±	a
α-Ocimene	8.91	13.606,30 ± 2680,18	b	31.181,00 ± 5341,87	c	1.855,00 ± 263,213	a	13.067,70 ± 1547,03	b
D-limonene	8.51	16.347,00 ± 4392,98	a	33.191,00 ± 1928,56	b	7.068,67 ± 1646,92	a	12.460,70 ± 2263,42	a
4-hydroxy-4-met hyl-2-pentanone	4.48	3.430,33 ± 441,02	a	14.844,00 ± 1228,83	c	8.299,33 ± 1669,88	b	3.335,00 ± 1117,30	a
Pinene	6.16	4.601,33 ± 509,75	a	14.208,30 ± 4625,88	c	7.402,50 ± 510,71	b	6.770,33 ± 1359,75	b
Benzoic acid, 2-(methylamino)- , methyl ester	18.81	6.603,00 ± 2589,49	a	15.199,30 ± 2995,22	b	684,00 ± 684,00	a	110,66 ± 110,67	a
MeJA	13.43	32.805,70 ± 5044,86	a	46.780,00 ± 7531,13	b	28.093,30 ± 2.374,65	a	2.9440,30 ± 2583,68	a
MeSA	9.23	92.595,00 ± 24.877,90	a	488.936,00 ± 150.483,00	c	22.958,30 ± 6933,94	b	1.062.700,00 ± 139.42	d

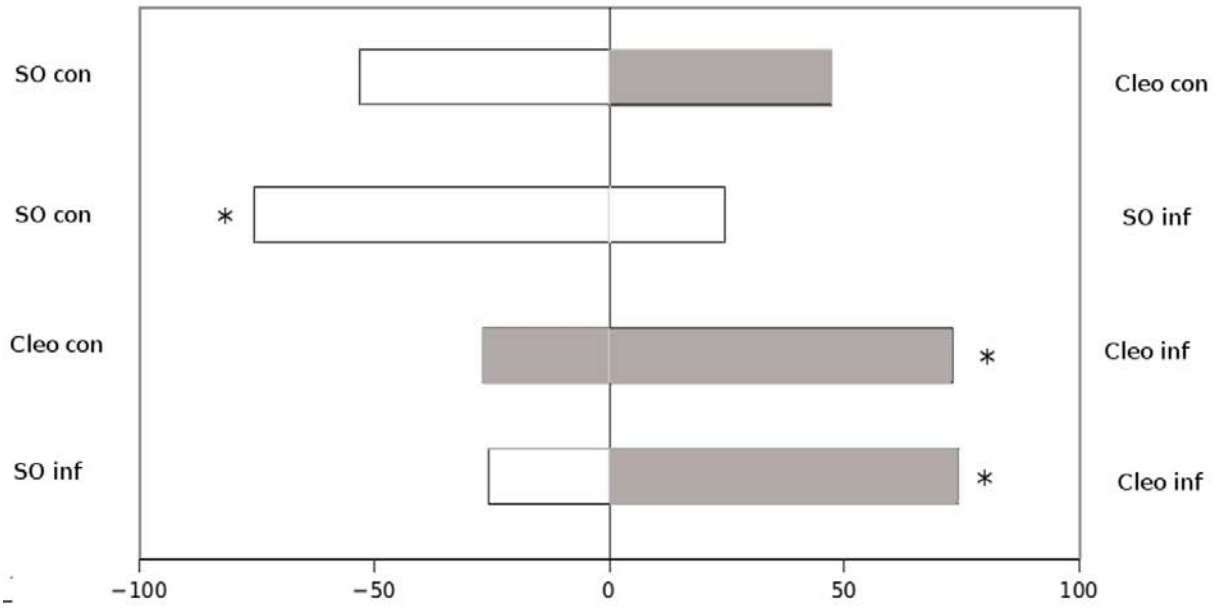

Figure 3. Olfactory response of *Tetranychus urticae* when offered untreatedplants or plants that previously experienced conspecific mite infestation. Four different combinations where *T. urticae* had to choose between two plant odours were carried out. A minimum of 45 mites were tested in each choice combination. First: sour orange untreated plants (SO con) versus Cleopatra mandarin untreated plants (Cleo con); second: sour orange untreated plants (SO con) versus sour orange infested plants (SO inf); third: Cleopatra mandarin untreated plants (Cleo con) versus Cleopatra mandarin infested plants (Cleo inf) and fourth: sour orange infested plants (SO inf) versus Cleopatra mandarin infested plants (Cleo inf). Plants were infested with 20 adult females 24 hours before the onset of the assay. Asterisks indicate significant differences from a 50:50 distribution (binomial test; Asterisks indicate significant differences ($P < 0.05$) between treatments).

HIPVs released by infested sour orange induce resistance and prime defense responses in Cleopatra mandarin

To determine whether citrus volatiles have an impact on the resistance of neighbouring plants against mites, we assessed the response of both rootstocks to different air blends. In agreement with results previously obtained by our group (Agut et al 2014), sour orange supported lower oviposition rates compared with Cleopatra mandarin when both were exposed to clean air (Fig 4A). Subsequently, both sour orange and Cleopatra mandarin were exposed

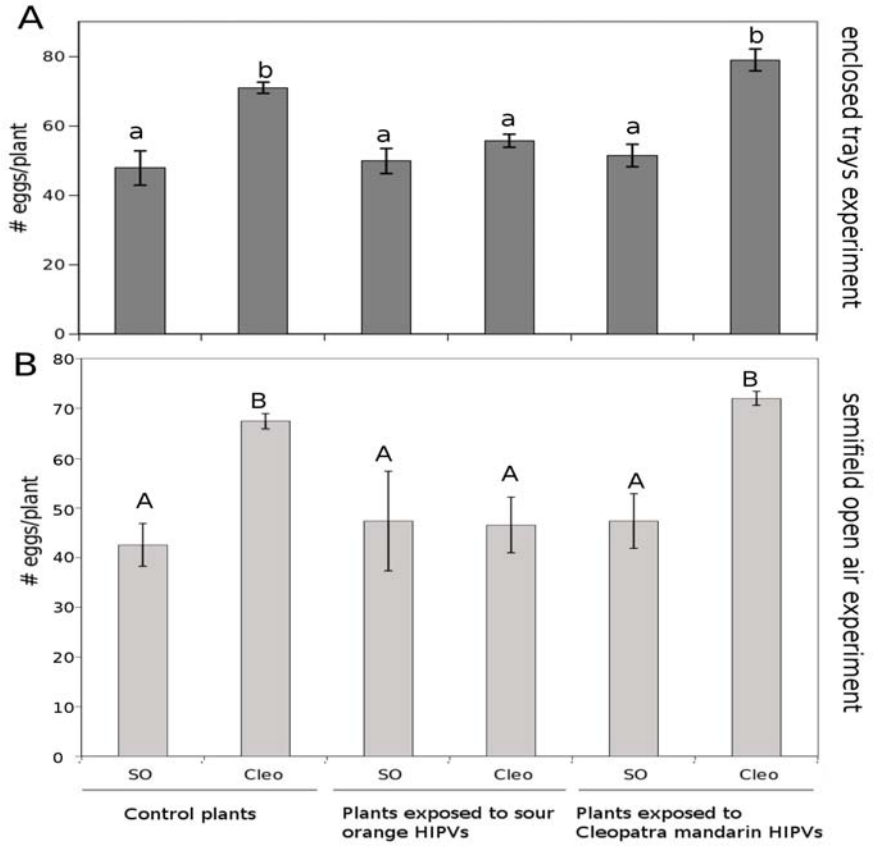

Figure 4. Spider mite oviposition on the rootstocks sour orange and Cleopatra mandarin following exposure to different HIPVs. Plants were exposed to different airborne blends during 3 days, subsequently plants were infested with 2-day-old *T. urticae* adult females (six specimens per plants), and 3 days later the number of eggs was determined. Plants were exposed to clean air, to infested sour orange HIPVs and to

infested Cleopatra mandarin HIPVs. The experiment was performed in enclosed trays (A) and open air semi-field conditions (B). Different letters mean statistical differences between treatments (ANOVA; LSD $p<0.05$; n=10 in A and B).

to the HIPVs of infested sour orange. Cleopatra mandarin supported reduced *T. urticae* egg densities similar to those observed in the resistant sour orange, while sour orange did not improve its resistance response. Neither Cleopatra mandarin nor sour orange exposed to infested Cleopatra mandarin HIPVs altered the ovipossition rates of *T. urticae* relative to those observed when exposed to clean air (Fig 4A). Additionally, control plants exposed to VOCs from uninfested Cleopatra mandarin and sour orange showed the same oviposition rates as clean air-exposed ones (data not shown). In a different set of experiments, we reproduced the previous assays in open air conditions in two semi-field trials. Although in a slightly different extent, oviposition rates in the receiver Cleopatra mandarin were also reduced by sour orange HIPVs (Fig 4B). These results demonstrate that infested sour orange HIPVs can induce functional resistance against *T. urticae* in Cleopatra Mandarin. Interestingly, only sour orange HIPVs induced resistance in the susceptible genotype Cleopatra mandarin.

HIPVs released from sour orange induce *LOX2* in Cleopatra mandarin

Previous results demonstrated that sour orange antibiosis to *T. urticae* was mainly mediated by the oxylipin and the flavonoid pathways (Agut et al 2014). *LOX2* was strongly induced in sour orange upon mite infestation whereas it remained at basal levels in infested Cleopatra mandarin. Following the observation that HIPVs-IR in Cleopatra mandarin was only functional after

exposure to sour orange HIPVs, we wondered whether the oxylipin pathway could be activated in Cleopatra mandarin upon appropriate stimulation. First, Cleopatra mandarin plants were exposed for three days to HIVPs from *T. urticae*-infested sour orange plants. The expression of *LOX2* significantly increased compared with unexposed Cleopatra mandarin controls (Fig. 5a). To determine candidate compounds that may trigger LOX2, we selected three doses of two terpenoids (D-limonene and ocimene) and the green leaf volatile (GLV*)* 4-hydroxy-4-methyl-2-pentanone among the HIPVs released by infested sour orange. These compounds fulfilled the criterion of being highly increased in infested sour orange compared with infested Cleopatra mandarin. LOX2 did not responded to the volatiles at early timepoints (24hpt) (Fig 5B). After 72 hours of exposure of the plants to the volatiles, a clear LOX2 induction was observed. In the case of the 4-hydroxy-4-methyl-2-pentanone, it was only effective inducing LOX2 at the highest concentration (1000 µg). Limonene was the most effective activating LOX2 gene expression since it was significantly induced at 10 µg, although the induction was stronger at the highest doses. Finally, ocimene stimulated LOX2 expression at 10 and 100 µg, but surprisingly, when plants were treated with the highest concentration, the LOX2 expression remained at the same levels as in control treaments. Despite 6,11-dimethyl-2,6,10-dodecatrien-1-ol and benzoic acid, 2-(methylamino)-, methyl ester compounds also matched the selection criterion mentioned above, we were unable to find commercial standards to test them individually.

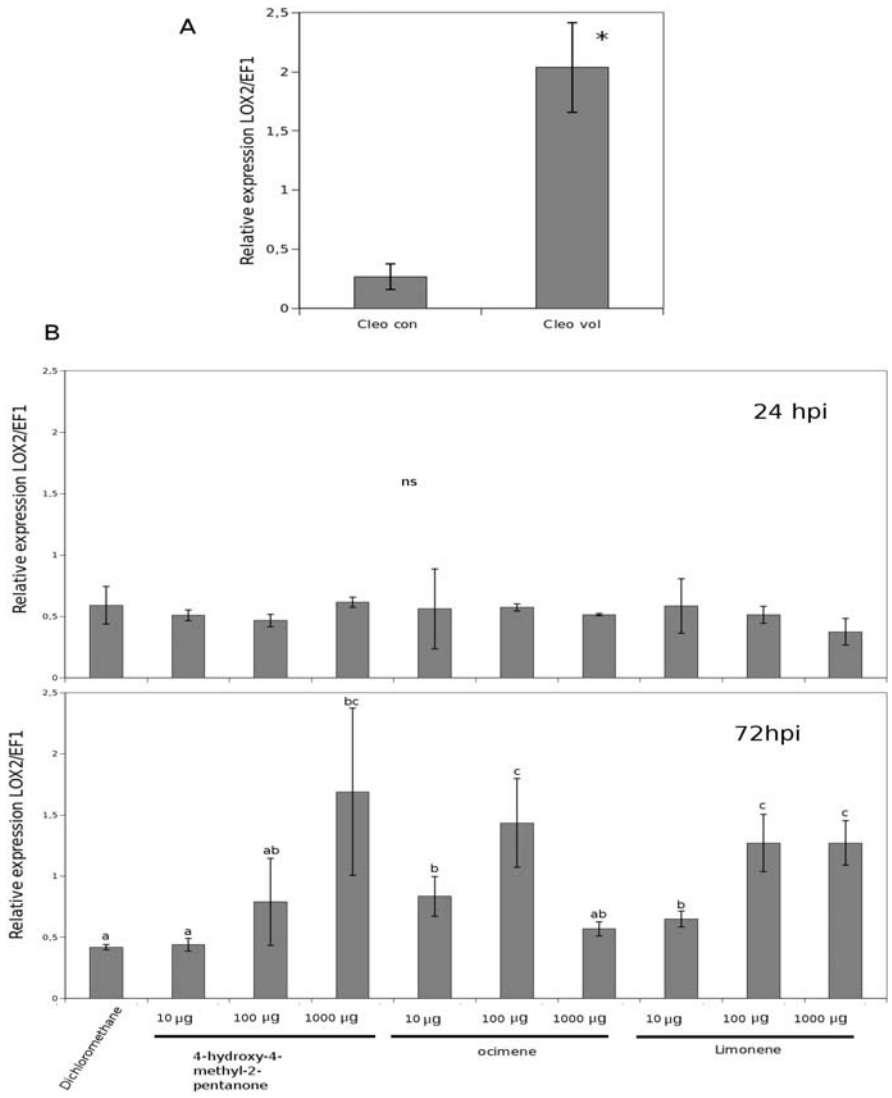

Figure 5. *LOX2* induction following exposure to different volatile blends. (a) *LOX2* expression in untreated Cleopatra mandarin after 24 or 72 hours of exposure to sour orange HIVPs. (b) Effects of 4-hydroxy-4-methyl-pentanone, ocimene, limonene at three different concentrations (10, 100 and 1000 µg) in 12-liter plastic container on *LOX2* expression in Cleopatra mandarin. The *LOX2* transcript levels were normalised to the expression of EF1α measured in the same sample. Data are presented as a mean of three independent experiments of transcript expression ± SD (n = 3). Significant differences in relative transcript levels between different treatments were estimated, performing the t-test with $2^{\Delta\Delta Ct}$ values as described by Yuan et al 2006 in (a). The experiment was repeated 3 times with dose of 100 µg and two times with the other two doses (10 and 1000 µg) with the same results. In (b), different letters mean statistical

differences between treatments (ANOVA; LSD $p<0.05$; n=3; n.s. means non significant).

HIPVs from sour orange alters metabolomic responses of Cleopatra mandarin.

Previous experiments demonstrate that Cleopatra mandarin retains the ability to respond to mite attack and, upon appropriated stimulation, efficient defenses can be activated. To determine the mechanisms behind VOCs-IR in Cleopatra mandarin, leaf material from HIPVs-primed Cleopatra mandarin was collected. *A* non-targeted metabolomic analysis was performed using UPLC coupled to QTOF MS (quadrupole-time of flight mass spectrometer). Metabolomic analysis and data reporting were performed in accordance with the methods described by Pitzschke and Hirt (2010) and Kaever et al., (2012). To determine how exposure to sour orange HIPVs modifies the response of Cleopatra mandarin plants, a Principal Component Analysis (PCA) was used to compare Cleopatra mandarin either exposed to clean air (control) or to sour orange HIPVs (Fig. 6a). Unexpectedly, the metabolomic response of both treatments in Cleopatra mandarin plants was not significantly different either in positive or negative electrospray ionization modes (ESI+ and ESI-). Although Cleopatra mandarin plants exposed to sour orange HIPVs showed an increased resistance against *T. urticae*, the metabolic changes affecting this resistance response were rather subtle as it was confirmed following a heatmap analysis (Fig 6b). Clusters containing compounds with similar behavio*ur* (low colour distance) contained many more signals compared with clusters overaccumulated in sour orange HIPVs-treated Cleopatra mandarin. This result confirms that changes imposed by exposure to sour orange HIPVs are restricted to a few metabolites that may be the responsible for the induced resistance to spider mite.

Reasonably, those metabolites should correspond with those exhibiting higher abundance in the heatmap of sour orange HIVPs-exposed Cleopatra mandarin plants. The accurate masses provided by the QTOF detector were contrasted with online biological databases such as Metlin for mass identity and KEGG and AraCyc for pathway search. The compounds that showed a strong response were related to the flavonoids, nucleotides and IAA conjugated biosynthesis, and alkaloids derived from the shikimate pathway (Table 2, Fig S2). Interestingly the signal 392.109 m/z assigned to the alkaloid derived from

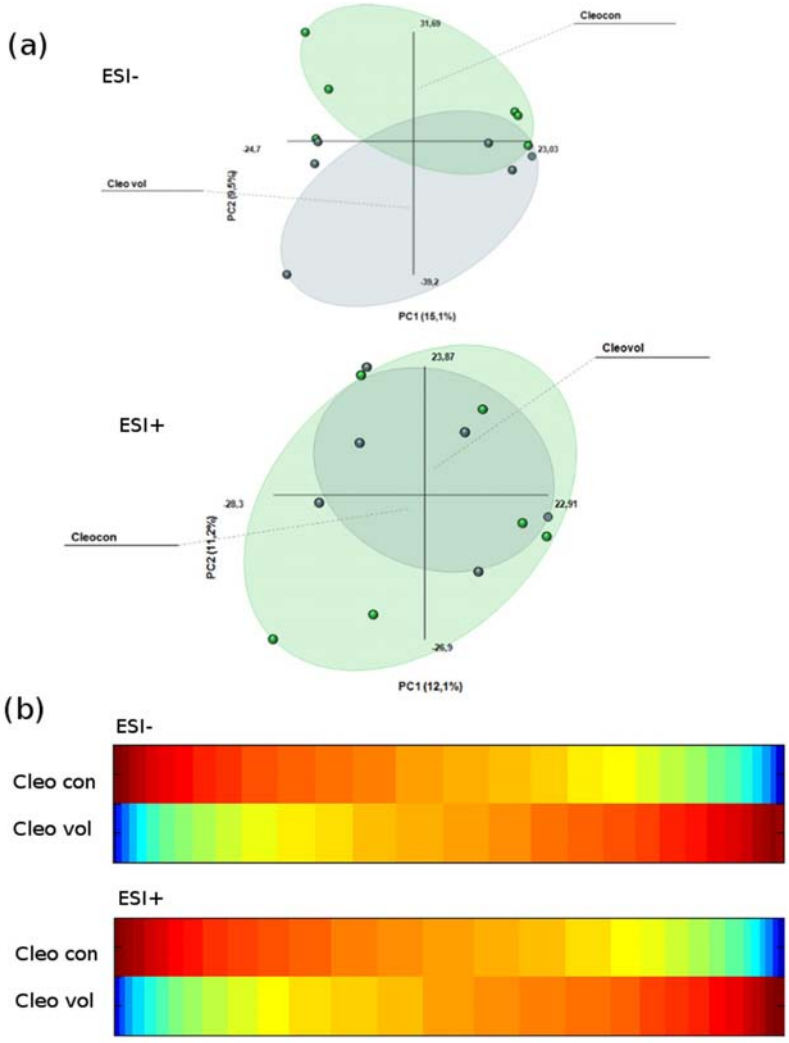

Figure 6. Metabolomic profilling of Cleopatra mandarin during VOCs-IR. (a) Non-supervised principal component analysis (PCA) representation of major sources of variability of ESI+ and ESI− signals obtained from a non-targeted analysis by HPLC-QTOFMS to monitor metabolomic changes. Two different sets of samples were tested: Twelve-week old Cleopatra mandarin exposed to clean air (Cleo con) and Cleopatra mandarin exposed to sour orange HIVPs (Cleo vol). After 3 d of exposure samples for analysis were collected. Leaf material from six individual plants was pooled together. Three independent biological and two technical replicates were randomly injected and analysed (n = 6). (b) Heat map analysis generated by Mar-Vis Filter and Cluster following a Kruskal–Wallis test ($P < 0.05$). The concentration of the metabolites was determined in all the samples by normalising the chromatographic area for each compound with the dry weight of the corresponding sample. Heat map representation is divided into two electrospray ionisation modes, positive (ESI+) and

negative (ESI−), from HPLC-QTOF analysis.

Table 2. Cleopartra mandarin metabolites induced following sour orange HIPVs-treatments.

Accurate (Neutral) Mass Determination	Ion Mode	Putative Pathway based on exact mass identification
331.075	ESI+	Alkaloids derived from shikimate pathway
392.109	ESI+	Alkaloids derived from shikimate pathway
358.088	ESI+	Flavonoid biosynthesis
390.093	ESI+	Flavonoid biosynthesis
400.107	ESI+	Flavonoid biosynthesis
596.164	ESI+	Flavonoid biosynthesis
480.108	ESI+	Flavonoid biosynthesis
464.095	ESI+	Flavonoid biosynthesis
964.283	ESI-	Flavonoid biosynthesis
652.181	ESI-	Flavonoid biosynthesis
283.092	ESI+	Nucleotide biosynthesis
323.055	ESI+	Nucleotide biosynthesis
337,118	ESI+	IAA conjugate biosynthesis
880.431	ESI+	Unknown
390.211	ESI+	Unknown
830.271	ESI+	Unknown
168.117	ESI+	Unknown
192.197	ESI-	Unknown

shikimate pathway was previously found by our group in the resistant sour orange after infestation with *T. urticae* (Agut et al., 2014). According to these previous observations in which enhanced resistance of sour orange was determined, we performed a Venn diagram analysis of metabolites trying to

identify metabolites in HIPVs-treated Cleopatra mandarin plants overlapping with basal defense metabolites found in sour orange (Figure 7). We could determine nine metabolites shared in the profiling of sour orange basal resistance and Cleopatra mandarin VOCs-IR (Table 3). Three of these compounds were tentatively identified based on their accurate mass as methyl benzoate and two flavones, while a fourth compound was fully identified as macarpine (329.109 m/z; Figure S3), a shikimate derivative alkaloid.

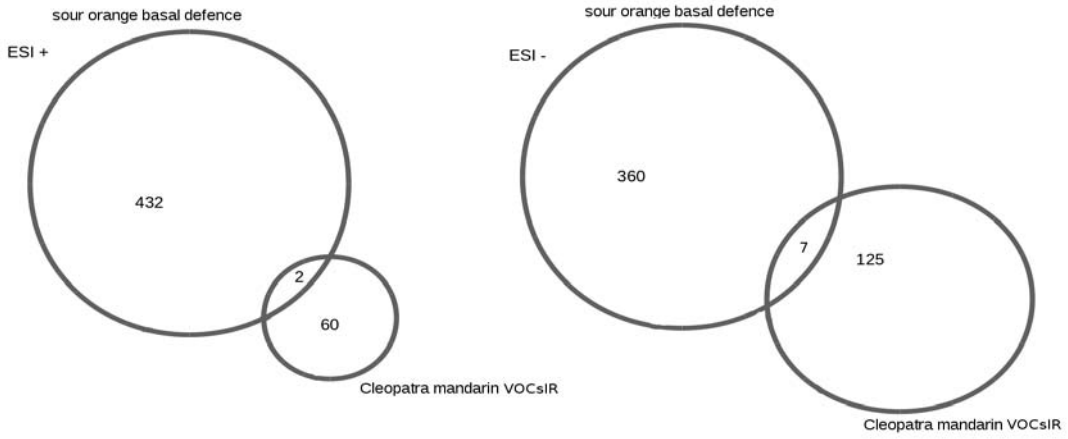

Figure 7. Venn diagrams representation; electrospray ionization positive (ESI+) and electrospray ionization negative (ESI-) from HPLC-QTOF analysis built up from selected clusters in Cleopatra mandarin exposed to volatiles (Volatile-Induced resistance; VOCs-IR) and augmented signals in sour orange infested plants. Numbers inside of the each region represent number of metabolites overaccumulated compared with their respective control uninfested and not primed plants.

In addition to metabolic responses we also tested whether HIPVs could induce marker genes of the main hormone-regulated plant defense pathways. Surprisingly the oxylipin pathway marker Pathogenesis Related protein-3 (PR-3) was not upregulated by the HIPVs treatment, while the Chalcone Synthase (CHS) involved in flavonoid and flavone synthesis and the SA marker

Pathogenesis Related protein-5 (PR-5) were both upregulated following volatile exposure (Figure 8). These experimental evidences are very likely to be linked to the flanovonoids identified among the HIPVs-induced metabolites in Cleopatra mandarin that were either absent or present at very low levels in control plants.

Table 3. Compounds found in the Venn Diagram analysis shared between primed Cleopatra mandarin and infested sour orange.

Accurate (Neutral) Mass Determination	Ion Mode	Putative Pathway based on exact mass identification
400,102	ESI-	3,5,3'-Trimethoxy-6,7:4',5'-bis(methylenedioxy) flavone
618,187	ESI-	Unknown
136,054	ESI-	Methyl benzoate
472,194	ESI-	Unknown
960,243	ESI-	Unknown
510,137	ESI-	Unknown
625,176	ESI-	Unknown
430,136	ESI+	3,5,8,3'-Tetramethoxy-6,7:4',5'-bis(methylenedioxy)flavone
392,109	ESI+	Macarpine

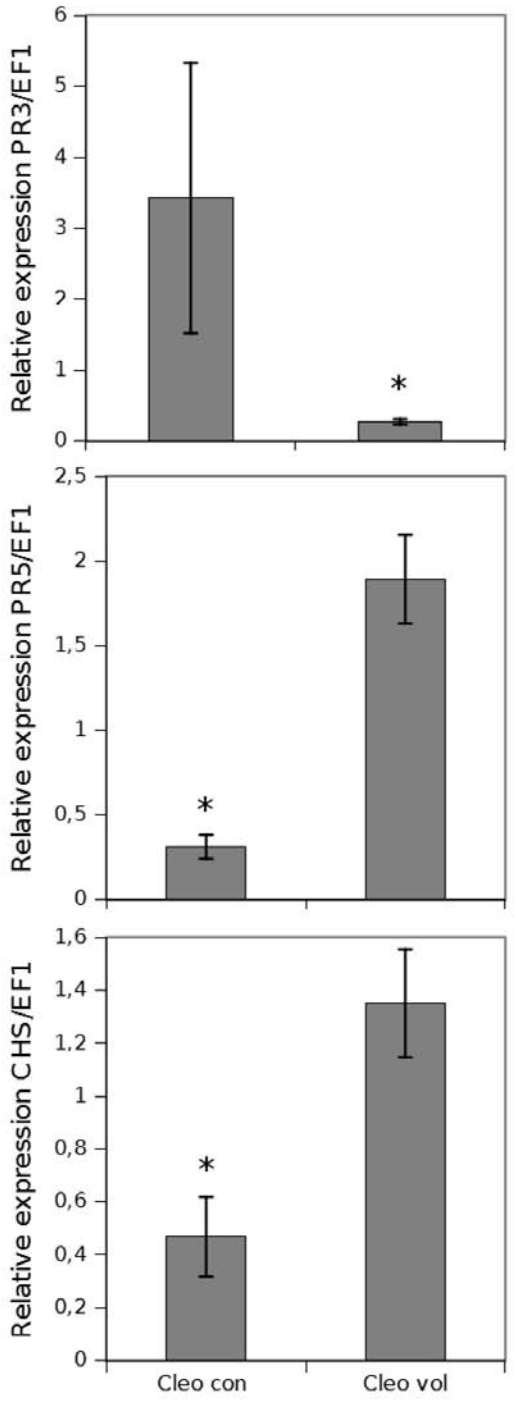

Figure 8. Impact of sour orange HIPVs *on defense gene expression in Cleopatra mandarin (Cleo.).* Total RNA was extracted from the leaves of three plants converted to cDNA and subjected to quantitative RT-PCR analysis. The *PR-3*, *PR-5*, *CHS* transcript levels were normalised to the expression of EF1α measured in the same sample. Data are presented as a mean of three independent analyses of transcript expression relative to the housekeeping gene plants ± SD (n = 3). Significant differences in relative transcript levels between different treatments were estimated, performing the t-test with $2^{\Delta\Delta Ct}$ values as described by Yuan et al (2006). The experiment was repeated 3 times with the same results.

To determine the role of the JA pathway, oxylipin concentrations were assessed by UPLC-MS. JA and JA-Ile (data not shown) were not changed by HIPVs treatments in Cleopatra mandarin whereas OPDA and SA were clearly induced in the Cleopatra mandarin exposed to sour orange HIPVs (Fig 9).

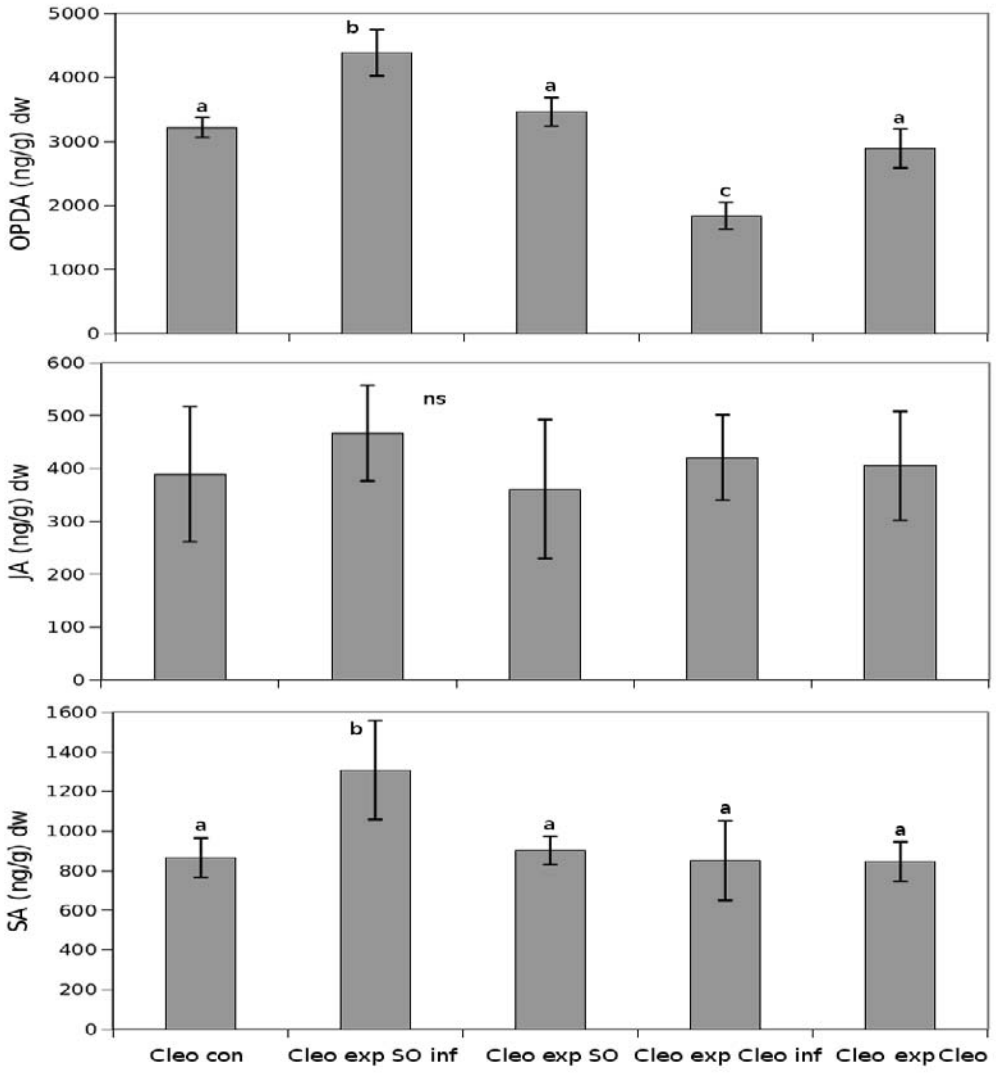

Figure 9. OPDA, SA and JA content in Cleopatra mandarin exposed to volatiles from unifested and infested Cleopatra mandarin and sour orange rootsocks. Leaves were collected after 3 days of volatile exposure. The levels were determined in freeze-dried

material by HPLC-MS. The results shown are mean hormone levels of three independent analyses ± SD (n = 4). Asterisks indicate significant differences between the different treatments ($P < 0.05$; t-test). The experiment was repeated 3 times for treatments with volatiles from infested plants and two times for treatments with uninfested plants. All the experiments showed similar results.

4. Discussion

Tetranychus urticae is a polyphagous mite that feeds on many crops such as tomato, citrus, cotton, and also many ornamentals (Bolland, 1998). In our previous work (Agut et al., 2014) we described the main basal resistance mechanisms of citrus to *T. urticae*. In the present research we focused our attention on the role of citrus HIPVs in the direct and induced resistance against *T. urticae*.

Most attention claimed by HIPVs is linked to indirect defenses attracting insect natural enemies (De Boer et al., 2004; Ton et al., 2007). Although no so widely studied, there are also some reports studying HIPVs implication in direct defenses implicating antixenotic effects on herbivores (Dicke 1986, Bernasconi et al., 2008). Sour orange displays enhanced resistance against *T. urticae* compared to Cleopatra mandarin because the latter supported higher mite densities resulting in increased plant damage (Agut et al., 2014; Bruessow et al., 2010). Whether this resistance could be complemented with additional antixenotic volatile-mediated effects had not been previously addressed. Some earlier reports confirm that HIPVs can act as repellents of arthropod pests (Dicke 1986, Bernasconi et al, 1998), while others demonstrate the opposite (Pallini et al., 1997; Botler et al., 1997; Halitschke et al., 2008). We have observed that both effects can occur in closely related species. In fact, sour orange *T. urticae*-induced HIVPs act as conspecific repellents whereas those from Cleopatra mandarin act as conspecific attractants. Because in the case of

sour orange, which displays enhanced antibiotic mechanisms of defense against *T. urticae*, these warning volatiles are beneficial to both the releaser and the receptor, they constitute a synomone. From the mite's point of view these volatiles may warn conspecifics of the existence of a poor quality host. However, in the case of Cleopatra mandarin, which is highly susceptible to *T. urticae*, probably as a consequence of the ability of this mite to downregulate plant defenses, HIPVs benefit the receptor only and act as a kairomone.

Despite the conspicuous differences in the basal volatile profile of sour orange and Cleopatra mandarin, mite differential response was only observed once the plant had been infested by *T. urticae* and these changes were quite fast as sour orange became less attractive to subsequent mite infestations in less than 24 hours of exposure to mite attack.

Sour orange released higher amounts of volatiles related to the terpenoid family, such as D-limonene, α-ocimene and α-farnesene compared with Cleopatra mandarin. Similarly, Kos et al. (2013) showed that transgenic Arabidopsis overproducing the terpenoids linalool, nerolidol and 4,8-dimethylnona-l,3,7-triene ((*E*)-DMNT) were less attractive to the aphid *Brevicoryne brassicae* L. (Hemiptera: Aphidae). It is known that some enzymes in the terpenoid pathway, such as β-ocimene synthase which catalyses the biosynthesis of β-ocimene, are under control of the JA pathway (Zhang et al., 2009). The more active JA-dependent signalling pathway observed in sour orange (Agut et al., 2014) is probably the responsible for the release of higher levels of terpenoid-related volatiles in response to mite attack. Similarly, lima bean plants exposed to transgenic tobacco plants overexpressing an ocimene synthase gene were better protected against *T. urtica*e (Muroi et al., 2011). Therefore, the production of the GLV 4-hydroxy-4-methyl-2-pentanone, which

increased in infested sour orange plants could contribute to the repellent effect observed.

Contrary to what we observed in sour orange, Cleopatra mandarin presented higher amounts of (2-Butoxyethoxy) ethanol, MeSA, and the aromatic compound benzaldehyde. Moayeri et al. (2007) showed that (2-Butoxyethoxy) ethanol was released in bean plants after the attack of the mirid bug *Macrolophus caliginosus* (Wagner). Benzaldehyde has been described as a relevant volatile in the attraction of the peach moth *Cydia molesta* Busck (Lepidoptera: Tortricidae) (Piñero and Dorn, 2007). Accordingly, Cleopatra mandarin HIPVs blend resulted in increased attractiveness to *T. urticae*. Assuming that the negative cross-talk between JA and SA pathways occurs in citrus, the high levels of MeSA could be used by *T. urticae* as indicator of low activation of JA pathway. Taken together, these compounds are very likely to be responsible of the enhanced attraction of infested Cleopatra mandarin plants.

Plant-plant induced resistance, which is a widespread indirect mechanism of defense in plants, had not been previously described in citrus. One of the first studies describing this mechanism was observed in corn plants exposed to several GLVs, which overexpressed JA-dependent genes and released terpenoids (Engelberth et al., 2004). More recently Zakir et al. (2013) showed that cotton plants infested with *Spodoptera litoralis* could inhibit conspecific oviposition in neighbouring plants. In the present research, we show that HIPVs released by sour orange following spider mite infestation induces resistance in susceptible Cleopatra mandarin. Molecular analyses of Cleopatra mandarin plants exposed to sour orange HIPVs showed a strong activation of the *LOX2* gene (Figure 5). This gene is a key element in the resistance response of citrus to *T. uriticae* (Agut et al., 2014). Cleopatra plants infested with *T. urticae* were

not able to induce the expression of *LOX*2 that only increased after exposure to sour orange HIPVs. Based on these results we have demonstrated that Cleopatra mandarin retains the genetic mechanisms to activate an effective defense against *T. urticae*. However, Cleopatra mandarin needs to be appropriately stimulated or primed because its basal defense is rapidly counteracted by the mite, which promotes susceptibility and the release of kairomones which make this genotype more attractive to subsequent infestations. Host manipulations by other mite species such as *T. evansi* have been previously reported in tomato (Kant et al., 2008). The sour orange HIPVs *α*-ocimene and D-limonene triggered *LOX2* in Cleopatra mandarin. In addition to *LOX2*, a comparative metabolomic analysis of Cleopatra mandarin treated with clean air or HIPVs revealed a set of metabolites that were overaccumulated in response to volatiles treatments. A Principal Component Analysis showed an overlap in the metabolite distribution between exposed and non-exposed plants suggesting that HIPVs-IR targeted a small number of metabolites in Cleopatra mandarin in the absence of mite infestation. A subsequent heatmap analysis revealed several clusters of signals overrepresented in Cleopatra mandarin exposed to sour orange HIPVs. The identification of the signals showed that flavonoids, alkaloids derived from shikimate pathway, and an IAA conjugate were overaccumulated following sour orange HIPVs-IR. Flavonoids display a wide variety of functions in plants such as a major role in UV stresses, floral pigmentation, and an inhibitory activity against microorganisms that cause plant diseases (Balmer et al., 2013). Additionally, in the last years an important role of flavonoids in defense against herbivores has been suggested (Lattancio et al., 2006; Pourcel et al., 2013; Agut et al., 2014; Onkokesunget al., 2014).

The Venn diagram analysis was performed to determine whether the metabolomic fingerprint of primed Cleopatra mandarin was similar to the

response of infested sour orange. Interestingly, primed Cleopatra mandarin shared only nine metabolites with infested sour orange, among them three were tentatively identified as two flavonoids and methyl benzoate. Noteworthy, by contrasting exact mass and fragmentation spectra, the macarpine, a benzophenanthridine alkaloid derived from shikimate acid was precisely identified. Either individually or by combination, these compounds are likely to mediate defense priming in Cleopatra mandarin. Macarpine was also previously identified by Agut et al. (2014) as one of the metabolites overaccumulated in sour orange upon *T. urticae* infestation. Benzophenanthridine alkaloids are common compounds in many families as Rutaceae or Papaveraceae. Alkaloids from this biosynthetic branch are relevant as antifungal compounds and also possess insecticidal activity. Remarkably, benzophenanthridine alkaloids are also under oxylipin control (Gundlach et al., 1992; Pauli et al., 1998) and accordingly, HIPVs-IR of Cleopatra mandarin enhanced *LOX2* and also *CHS* expression, which are involved in the JA-signaling and flavonoid biosynthesis, respectively (Richard et al., 2000). Surprisingly, *PR3* and JA were not induced in Cleopatra mandarin. However, we observed an increase in OPDA following sour orange-HIPVs treatment. In addition to OPDA also SA and several other uncharaterized flavonoids and alkaloids derived from shikimate pathway such as macarpine have been also induced by sour orange HIPVs treatments. Despite it is temptim to asume a function of these compounds in the induced resistance, further studies are needed to fully comfirm they have an active role in the resitance against *T. urticae*.

Plants, and citrus are not an exception, have developed direct and indirect mechanisms of resistance against herbivore pests (Heil and Kost, 2006). Breeders have focused genetic improvement programs mostly in crop production traits. As a consequence, many commercial plant cultivars available

depend on chemical control to adequately deal with pests. The present scenario, where agricultural policies are aimed at decreasing the use of pesticides and increasing the effect of natural mortality factors of pests in our crops, makes the identification of the key metabolites and genes involved in natural resistance to herbivores, a pre-requisite for the development of more resilient crops. In this study, we have identified several metabolites mediating direct and induced defenses against *T. urticae* that can be further exploited to increase the resilience of citrus to pest injury.

Acknowledgments

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Supporting information

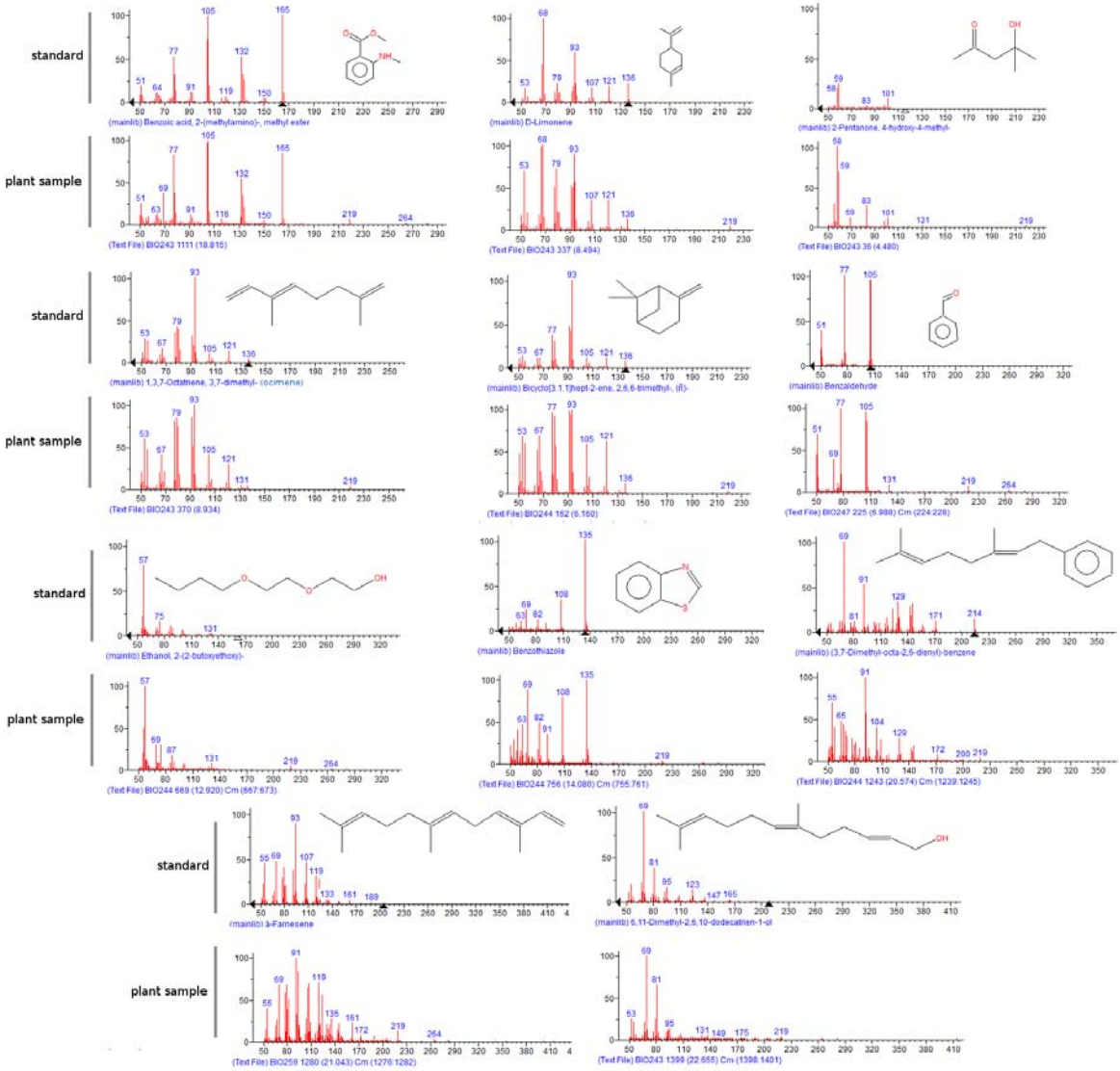

Figure S1. Volatile identification by comparison of their fragment spectrum agaisnt those reported in the library spectra NIST.

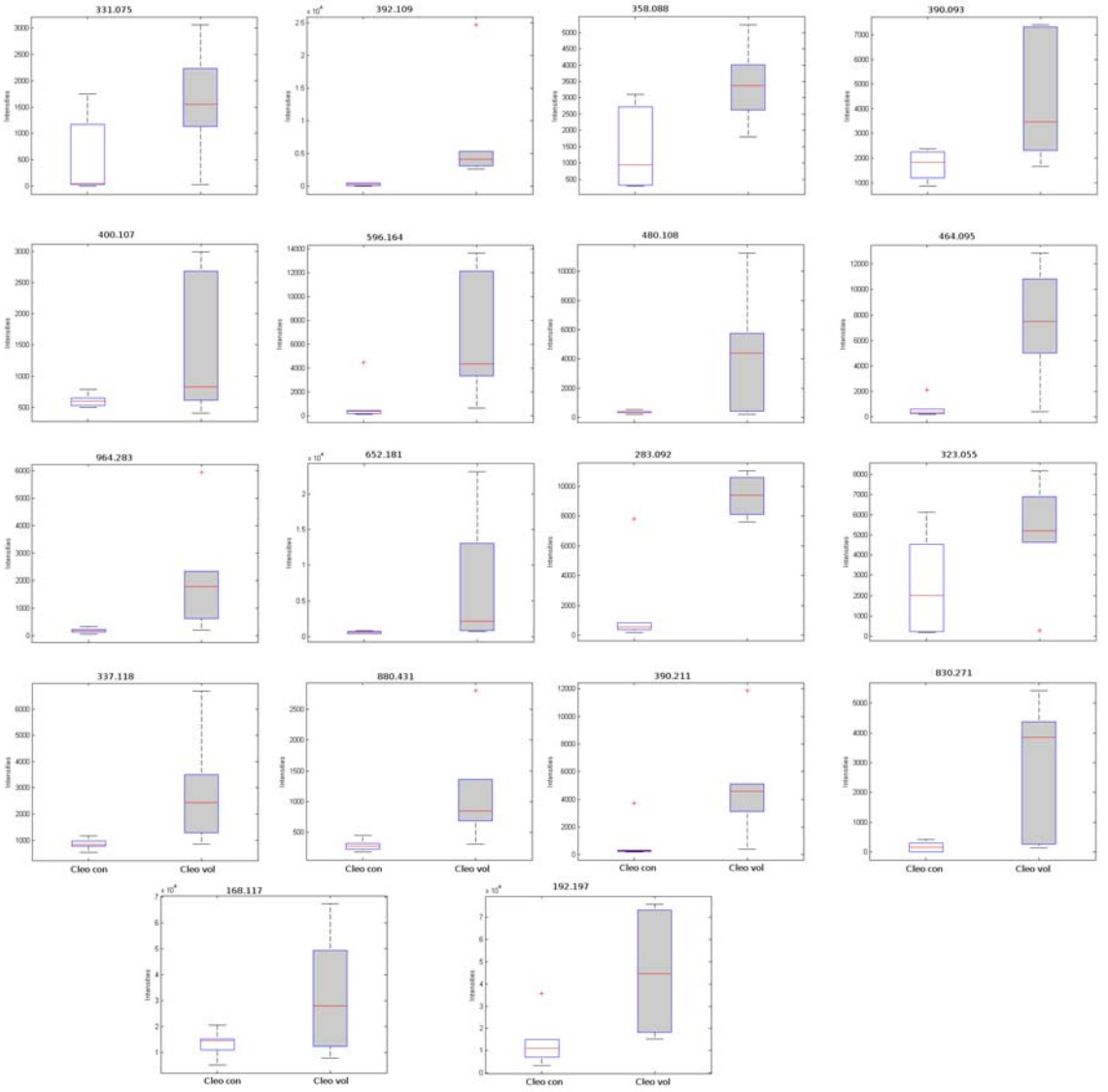

Figure S2. Boxplot analysis of the 18 selected signals from the table 2. Two different sets of samples were tested: in the first place Cleopatra mandarin treated with clean air (Cleo con) and Cleopatra mandarin treated with sour orange HIPVs (Cleo vol). Leaf material from 6 individual plants was pooled together for each genotype combination. Three independent biological replicates and two technical replicates were randomly injected and analysed (n = 6). Boxplots were generated by using Mar-Vis Filter and Cluster following a Kruskal-Wallis test ($P < 0.05$). The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample.

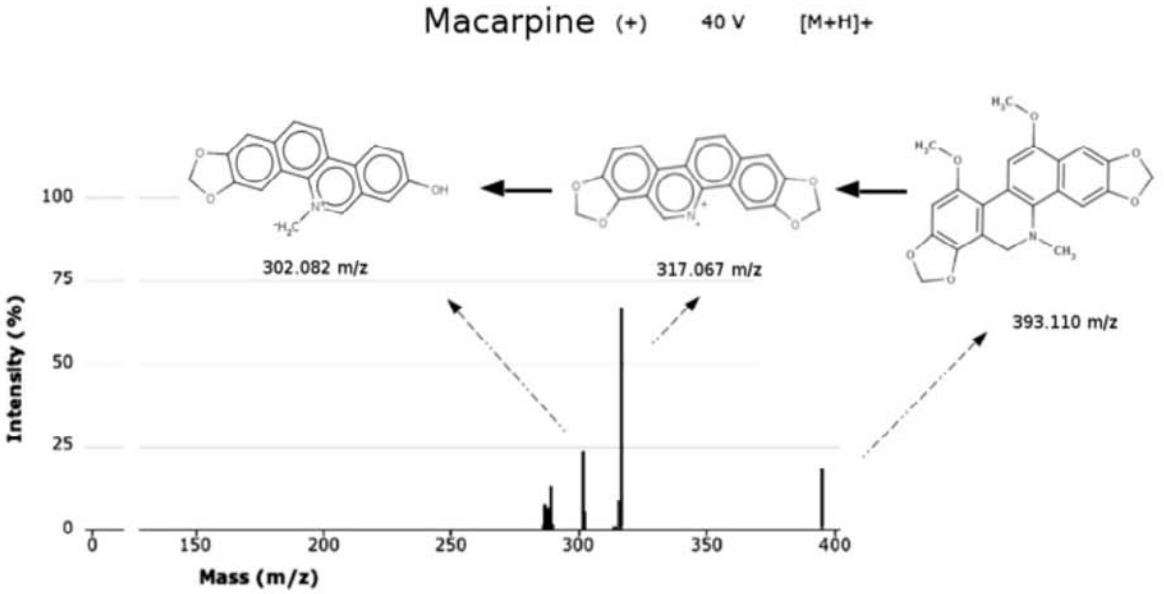

Figure S3. Precise identification of the signal overaccumulated in sour orange infested plants and Cleopatra mandarin exposed to sour orange HIVPs: 392.109, corresponding to macarpine. To determine the exact mass of the parental ion, no collision energy is applied (1 – TOF MS EI+/−). To obtain the fragmentation spectrum, a collision energy ramp from 5 to 45 eV is applied to the parental ion (2 – TOF MS EI+/−). Theoretical transitions were first checked in mass spectrum databases (Metlin).

CHAPTER 4

Systemic resistance to *Tetranychus urticae* in citrus is induced by conspecifics and transmitted by grafting and mediated by mobile amino acids

Abstract

Systemic signalling and plant communication between roots and leaves has been recently recognized to play an important role in plant defense against herbivores. In the present research we show that oviposition in *Citrus aurantium* (sour orange) by *Tetranychus urticae* was reduced by 50% when the plant had been previously infested by conspecifics. Working with the same cultivar grafted onto different rootstocks, as sour orange and *Citrus unshiu* (Cleopatra mandarin), there was a reduction in symptomatic leaves and *T. urticae* populations in sour orange plants. We observed that there is a mobile signal that is transmitted by the rootstock to the leaves. Metabolomic and gene expression analyses revealed the important role of glutamic acid as mobile signal that increased the expression of glutamate receptors. This receptor has been recently described as regulator of the JA pathway. Our results support this hypothesis, sour orange uninfested systemic leaves showed increased expression of glutamate receptors and higher amounts of JA and OPDA in plants that have been previously infested.

1. Introduction

Plant react locally activating defense barriers around penetration sites or woundings against herbivore attack, plants present many different defense strategies/mechanisms. There are receptors responsible for herbivore recognition through herbivore-associated molecular patterns (HAMPs) or damage-associated molecular patterns (DAMPs) in the plant cellular membrane. Once this recognition has taken place in the local damaged tissue, a cascade of events that ends up mainly in the activation of jasmonic acid (JA) signalling and the synthesis of toxic secondary metabolites with detrimental effects to the herbivore begins (Kant et al., 2004; Agut et al., 2014; Zhurov et al., 2014). A later defense mechanism is the activation/synthesis of toxic compounds in the distal undamaged leaves that hamper herbivore development/reproduction. This phenomenon has been largely studied in plant-microbe interactions, and it is so called systemic acquired resistance (SAR). For a long time the systemic signal that is transported to undamaged leaves was elusive. In the last years some compounds such as methyl salycilate, azelaic acid, dehydroabietinal or pipecolic acid have been described as mobile signals that may be responsible for the systemic induction of salycilic acid (SA) (Park et al., 2007; Vlot et al., 2008: Liu et al., 2010; Manosalva et al., 2010; Jung et al., 2009; Chaturvedi et el., 2012; Návarová et al., 2012).

JA has also been proposed as possible mobile signal in SAR. Buhot et al. (2004) showed that spray treatment with JA in tobacco plants enhanced the resistance to pathogenic bacteria. They suggested the formation of JA-protein complex behaving as a long-distance defense signal. In contrast, other authors did not detect any supression of SAR induce by *P. syringae* in Arabidopsis

JA-biosynthesis/signaling mutants (Cui et al., 2005; Attaran et al., 2009).

The role of JA in the systemic resistance in response to herbivore attack is widely accepted (Heil and Ton, 2008). This term resembles SAR in plant-herbivore interactions. Evidence of this plant defense mechanism has been observed in several plant-arthropod systems. The first evidence of herbivore-induced systemic resistance was provided by Karban and Carey (1984). These authors showed that cotton seedlings that had previously experienced an infestation with *Tetranychus urticae* (Koch) (Acari: Tetranychidae) sustained reduced populations compared with newly infested plants. It is known that such a systemic resistance is mainly related with the JA signalling pathway (Heil and Ton, 2008; Soler et al., 2013; Tian et al., 2014). In fact, the induction and expression of proteinase inhibitors in undamaged distal leaves after insect attack are under the control of the JA pathway (Howe et al., 1996); Li et al. (2005). However the mobile signal responsible for the JA activation in the systemic leaves still remains unknown. Recently, in 2013, Mousavi et al. showed the implication of glutamate receptors (GRLs) in the systemic response in *Arabidopsis thaliana*. Besides binding glutamate, these receptors are able to detect electric changes in the cell surface caused by wounding. Interestingly, these receptors must be intact for the activation of JA signals in the systemic undamaged tissues. Roots have an important role producing mobile signals that target receptors in distal leaves. Erb et al. (2008) showed that an attack by the root feeder *Diabrotica virgifera virgifer*a Leconte (Coleoptera: Chrysomelidae) increased the resistance in maize against *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) in aboveground tissues. Leaf hormonal profiling in maize plants infested with *D. virgifera* showed an accumulation of absisic acid that was produced in the roots. The accumulation of this hormone altered the water content affecting the nutritional quality of the

leaves for leaf feeders.

More evidences of the relevance of the roots were observed in *Nicotiana bentamiana* plants. Leaves attacked by herbivores produced an unknown mobile signal that was perceived by the root. Subsequently, this tissue began to produce nicotine, a secondary toxic compound with insecticidal activity. Recently, using *N. bentamiana* plants impaired in JA signaling in the roots it was demosntrated that a proper functioning of the JA pathway in the roots is necessary to sustain a correct defense response in the leaves (Fragoso et al., 2014). In addition, regulation of local production of leaf JA and ABA and some shoot metabolites is regulated by JA from the root was evidenced.

The relevance of the oxylipin pathway in the resistance against *T. urticae* was recently demonstrated in citrus (Agut et al., 2014). Despite its rapid adaptation to synthetic pesticides, there are families of natural secondary metabolites that remain toxic to *T. urticae* such as flavonoids and glucosinolates (Agut et al., 2014; Zhurov et al., 2014). JA seems to influence in the accumulation of phenylpropanoid phytoalexins such as naringenin and hesperetin, in citrus. In addition, the release of volatiles with repellent effect to spider mite and induced resistance properties is probably related to JA and oxylipin pathway (Agut et al., submitted). Citrus rootstocks provide resistance/tolerance to many abiotic stresses as salinity, drought, and diseases as Phytophthora or Citrus Tristeza Virus. Nevertheless rootstock choice in citrus has never been based on resistance/tolerance to arthropod pests. In 2010, Bruessow et al. showed how rootstock altered the intrinsic rate of increase of *T. urticae*. The same cultivar grafted onto different rootstocks modified the population densities of the mite. Despite all the new advances, citrus resistance against spider mites remains largely unknown. Particularly, those events regulated by the belowground

tissues and the rootstock are still elusive.

In the present research we have studied the systemically transmitted resistance in upper parts of citrus plants when bottom leaves are infested by spider mites. We also investigate how different rootstock-cultivar combinations determine the resistance in the grafted cultivar and the nature of the mobile signals responsible for the systemic resistance that are released by the rootstocks through the vascular vessels.

Material and methods

2.1 Plant material

In this study we used two different kind of plants: rootstocks (sour orange and Cleopatra mandarin plants) and grafted plant (Clemenules cvar grafted onto sour orange and Cleopatra mandarin rootstocks). 12 week-old citrus rootstock of both species and 2 year-old grafted plants were maintained in a climatic chamber at 25º C and 50–70% RH under a 16:8 h L:D photoperiod. These plants were grown on vermiculite and peat (1:3, vol:vol). The leaves for maintaining spider mite colony were obtained from *Citrus clementina* Tanaka cv. Clemenules (INIASEL 22). No insecticides or acaricides were applied to these plants, which were fertilized using a modified Hoagland's solution every 3 days.

2.2 Spider mite stock colony

The *T. urticae* colony used in the present assays was initiated with specimens

collected in clementine orchards in the region of La Plana (Castelló, Spain). The colony was maintained on detached leaves of young clementine mandarin plants, The rearing took place on detached leaf units consisting of a single Clemenules leaf placed upside down on moistened cotton, placed on top of a water-saturated foam cube (3–4 cm thick) in an open plastic box (35 × 20 × 7 cm) half-filled with water. Moist cotton was folded over the edges of the leaves to prevent mites from escaping. When necessary, cohorts of the same age were produced by transferring gravid females from the stock colony to freshly set detached leaf units for a controlled period of time. Afterwards, females were removed and the eggs were kept undisturbed until reaching the immature target stage and age. These cohorts were maintained under the same environmental conditions as the stock colony.

2.3 Systemic resistance in rootstocks

Cleopatra mandarin and sour orange plants were used in these assays. We infested plants of each rootstock with 10 adults females (with their respective uninfested control plants). To prevent mite dispersal to distal parts of the plants, a ring of the trunk right above the infested leaves was painted with Tangle-Trap insect trap coating (Tanglefoot Company, Bozeman, MT). Three days later, clean distal part of the rootstocks (infested and uninfested control plants) were infested with 6 two day-old *T. urticae* females.Three days later the number of eggs per plant was assessed. This experiment was repeated a minimum three times.

2.4 Hormonal analyses in systemic resistance experiments

Same as before control and infested plants per rootstock were considered. Three days later, the uninfested distal leaves were collected and the hormonal content was analyzed. The hormones 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), JA-isoleucine (JA–Ile), abscisic acid (ABA) and salycilic acid (SA) were analysed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS), as described by Flors et al. (2008) and Forcat et al. (2008).

2.5 Collection of leaf efflux

Same as before control and infested plants per rootstock were considered. Three days later, mature leaves were excised by cutting the petioles from the leaf blade under the surface of 5 mM 2-Na-ethylenediaminetetra- acetic acid, disodium salt (EDTA), pH 7.0. The petioles of excised leaves were inserted into 1.5 ml Eppendorf tubes with 1.0 ml of EDTA solution. Tubes were then placed in a closed chamber under low light conditions and close to 100% humidity to reduce transpiration. After 8 hours we removed the leaf and kept the liquid solution at -20 ºC for metabolomic analysis.

2.6 Response of clemenules mandarin plants grafted on selected citrus rootstocks to mite attack

Two year-old plants of the Clemenules cultivar grafted on either sour orange or Cleopatra mandarin were used. These plants were maintained in a greenhouse located at UJI at 22 ± 5 °C and 50–70% RH under natural photoperiod. Plants

were grown on a substrate consisting of sand and peat (1:1) in 6 L-cylindrical containers. The base of the trunk of each plant was painted with tanglefood to prevent ambulatory mite dispersal between plants. Six plants (= replicates) per rootstock-cultivar combination were infested with 20 females directly taken from the stock colony and randomly transferred to each plant with a fine camel paintbrush. For the following two weeks, spider mite numbers were scored in three leaves randomly selected per plant. During this 2-week period, symptomatic leaves per plant (those exhibiting chlorotic spots) were also scored. These parameters were checked daily until mite induced plant defoliation started.

2.7 Collection of root efflux

Clemenules variety grafted onto sour orange (SO) and Clemenules variety grafted onto Cleopatra mandarin (Cleo) plants uninfested (con) or infested (inf). Two year-old grafted plants were infested with 20 mites per plant. Three days later root sap samples were collected by cutting plant stems near the base (5–10 mm above the ground) and inserting them (upside down) in a Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA). The cut end protruded by about 5–8 mm through the air-tight rubber compression gland. Pressure was applied by filling the chamber with compressed air. The resultant excreted root sap was immediately collected with a micropipette and stored in a 1.5 ml Eppendorf tube. In most cases, between 50 and 100 µl of sap was collected for each replicate, thus samples from two or three plants were pooled. The collected samples were weighed and diluted with double distilled water (typically, 10–20 times), frozen and kept at −18°C until measured. On average, six collective samples for each treatment were collected.

2.8 Quantitative real-time PCR analysis

GLR expression analysis of Cleopatra mandarin and sour orange, and Clemenules cultivar grafted either on sour orange and Cleopatra mandarin was extracted using the Plant RNA kit (Omega Bio-Tek Inc., Doraville, GA). For RT-qPCR experiments, 1.5 μg of total RNA was digested with 1 unit of DNase (RQ1 RNase-Free DNase) in 1 μL of DNase buffer and Milli-Q water up to 10 μL (Promega Corporation, Madison, WI) and incubated for 30 min at 37º C. After incubation, 1 μL of RQ1 DNase stop buffer was added and incubated again at 65º C for 10 min to inactivate DNase. The RT reaction was performed by adding 2 μL of RT buffer, 2 μL of 5 mM dNTP, 2 μL of 10 μM Oligo(dT) 15 primer (Promega), 1 μL of 10 U μL−1 RNase inhibitor (Promega) and 1 μL of Omniscript reverse transcriptase (Qiagen, Barcelona, Spain). The reaction mixture was incubated at 37º C for 60 min. Complementary DNA from the RT reaction, 10× diluted, was used for qPCR. Forward and reverse primers (0.3 μM) were added to 12.5 μL of PCR SYBR reaction buffer, 2 μL of cDNA and Milli-Q sterile water up to 25 μL of the total reaction volume (Takara Bio, Kyoto, Japan). Quantitative PCR was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA) sequence detector with standard PCR conditions. Because there were differences in cycle numbers during the linear amplification phase between samples, the data were transformed with the formula $2^{\Delta\Delta Ct}$. RT-qPCR analysis was performed at least 3 times using sets of cDNA samples from independent experiments. The primers of GLR and the housekeeping gene EF1 (Agut et al. 2014) were used. Sequence of GLR primers: left primer GGGGCGGGACATTAAATCTT; right primer CTGCGGATACCCATGTTCAA.

2.9 Metabolome analysis: LC-ESI full-scan mass spectrometry Q-TOF.

Metabolome samples from leaf and root efflux were used. Metabolome analysis was performed using an Acquity UPLC system (Waters) interfaced to hybrid quadrupole time-of-flight (QTOF Premier). Three technical and three independent biological replicates per sample were randomly injected. The LC separation was performed on an HPLC SunFire C18 analytical column, 5 µm particle size, 2.1 × 100 mm (Waters). Analytes were eluted with a gradient of methanol and water containing 0.01% HCOOH. The gradient started with 90% aqueous mobile solvent and linearly reached 10% in 12 min. In the following 3 min, the gradient was kept in isocratic conditions and then returned to initial conditions in 4 min. The column was allowed to equilibrate for 3 min, giving a total time of 22 min per sample. The solvent flow rate was 0.3 mL min^{-1}. The injection volume was 20 µL. The drying gas and the nebulising gas was nitrogen. The desolvation gas flow was set to approximately 600 L h^{-1}, and the cone gas flow was set to 60 L h^{-1}. A cone voltage of 20 V and a capillary voltage of 3.3 kV were used in the negative ionisation mode. The nitrogen desolvation temperature was set at 350º C, and the source temperature was set at 120º C. The instrument was calibrated in the m/z 50–1000 range with a 1/1 mixture of 0.01 M NaOH/1% HCOOH tenfold diluted with acetonitrile/water (80/20, v/v). A solution of leucine enkephalin at a concentration of 2 ppm in acetonitrile/water (50/50, v/v) with 0.1% formic acid was simultaneously introduced into the Q-TOF instrument via the lock-spray needle for accurate m/z determinations. The [M − H]− ion of leucine enkephalin at m/z 554.2615 was used for recalibrating the m/z axis. Metabolite amounts were analysed on the basis of normalised peak area units relative to the dry weight. Kruskal–Wallis test ($P < 0.05$) was applied to test the metabolomic differences between rootstocks versus infestation.

2.10 Full-scan data analysis.

Centroid acquired raw data were transformed into .cdf files using Databridge from the Masslynx 4.1 software (Waters) and subsequently subjected to analysis using the software R for statistical purposes. Signals from positive and negative electrospray analysis (ESI+; ESI−) were processed separately. Peak peaking, grouping and signal corrections were developed applying the algorithm XCMX. This statistical package can be used to preprocess full-scan LC/MS data for relative quantification and statistical analysis. The statistics and the heat map analysis were carried out with the Mar-Vis Suit software including MarVis Filter and Mar Vis Cluster (Kaever et al. 2012), a tool for clustering and visualisation of metabolic biomarkers. Mar-Vis was used to process exported CSV. files from the XCMS and perform statistical analysis, adduct and isotope correction and clustering and colour heat map visualisation. To determine a global behaviour of the signals, principal component analysis (PCA) was used.

2.11 Statistical analysis

Statistical analyses of genetic and metabolomic data were conducted using Statgraphics Plus 3.1 (Rockville, MD) and the software R v.2.9.2 (R Development Core Team) and the package XCMX respectively. All experiments were repeated at least 3 times unless specified otherwise.

Mean mite densities and symptomatic leaves counts in the Clemenules cultivar grafted on each rootstock along time in the third assay were compared using a repeated measures Generalized Linear Mixed Model (the fixed factor was rootstock and the sampling date was the random factor).

When required, data were square root transformed to fulfill the assumption of

normality. Our first approach in the variable symptomatic leaves was to use a normal distribution, which resulted adequate based on results of Akaike's information criterion and the distribution of residuals compared to negative binomial and Poisson distributions. In contrast for the variable mite densities we used a gamma distribution, as it best adapted to the requirements already mentioned. When significant differences were found, pairwise comparisons of the fixed factor levels were performed with the least significant difference (LSD) post hoc test ($P < 0.05$).

3. Results

Both rootstocks Celopatra mandarin and sour orange can perceive systemic induced induced resistance in a different extent.

The phenomenon of systemic resistance following infestation by *T. urticae* was previously showed by Carey and Karban (1984). In a previous research we demonstrated that the rootstock Cleopatra mandarin is highly susceptible to this mite and contrastingly, sour orange shows elevated levels of resistance (Agut et al 2014). Furthermore, HIPVs released by sour orange are able to stimulate induced resistance in Cleopatra mandarin, therefore both roostocks retain genetic mechanisms to express induced resistance against the mite. We wondered whether both rootstocks could also express systemic resistance against *T. urticae* if they had experienced a first contact with the mite in distal plant tissues. To solve this question we infested bottom leaves with 10 mites per plant. After three days, a second infestation with females was performed. Both rootstocks, showed a significant reduction of egg oviposition compared with their respective controls that had not been previously infested. Both rootstocks displayed systemic resistance in distal leaves (Fig. 1). However, the level of

protection was not the same. Sour orange plants previously infested showed a reduction of 50% in the number of eggs, whereas Cleopatra mandarin plants showed around 30% of reduction. In conclusion, both genotypes can express systemic resistance although this resistance is more efficient in sour orange than in Cleopatra mandarin.

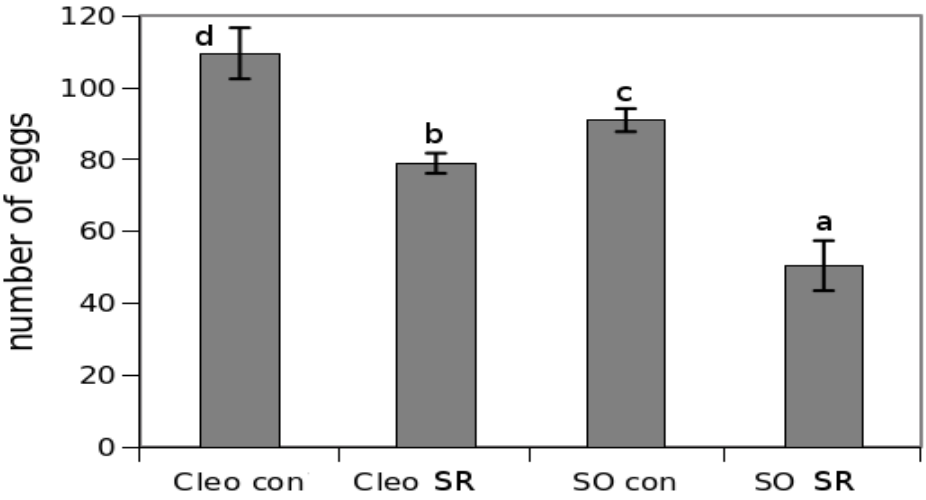

Figure 1. Effect of systemic resistance (SR) treatments of Cleopatra mandarin and sour orange on spider mite oviposition rates. Half of the plants were previously infested (SR treatments) with 10 *T. urticae* adult females. Three days later all the plants were infested with two day-old *T. urticae* adult females in distal clean leaves. Egg number was determined 3 days after the second infestation. Different letters indicate significant differences ($P < 0.05$; ANOVA) between treatments.

Metabolomic analyses revealed candidates responsible for systemic induced resistance.

To determine candidate signals that are transmitted by the infested leaves to distal clean leaves, we collected the sap efflux from bottom infested leaves and an analysis by LC-ESI-Q-TOF was performed. The data from the Q-TOF was processed by a principal component analysis (PCA). The Q-TOF detected around 800 signals in each treatment. In the absence of infestation the

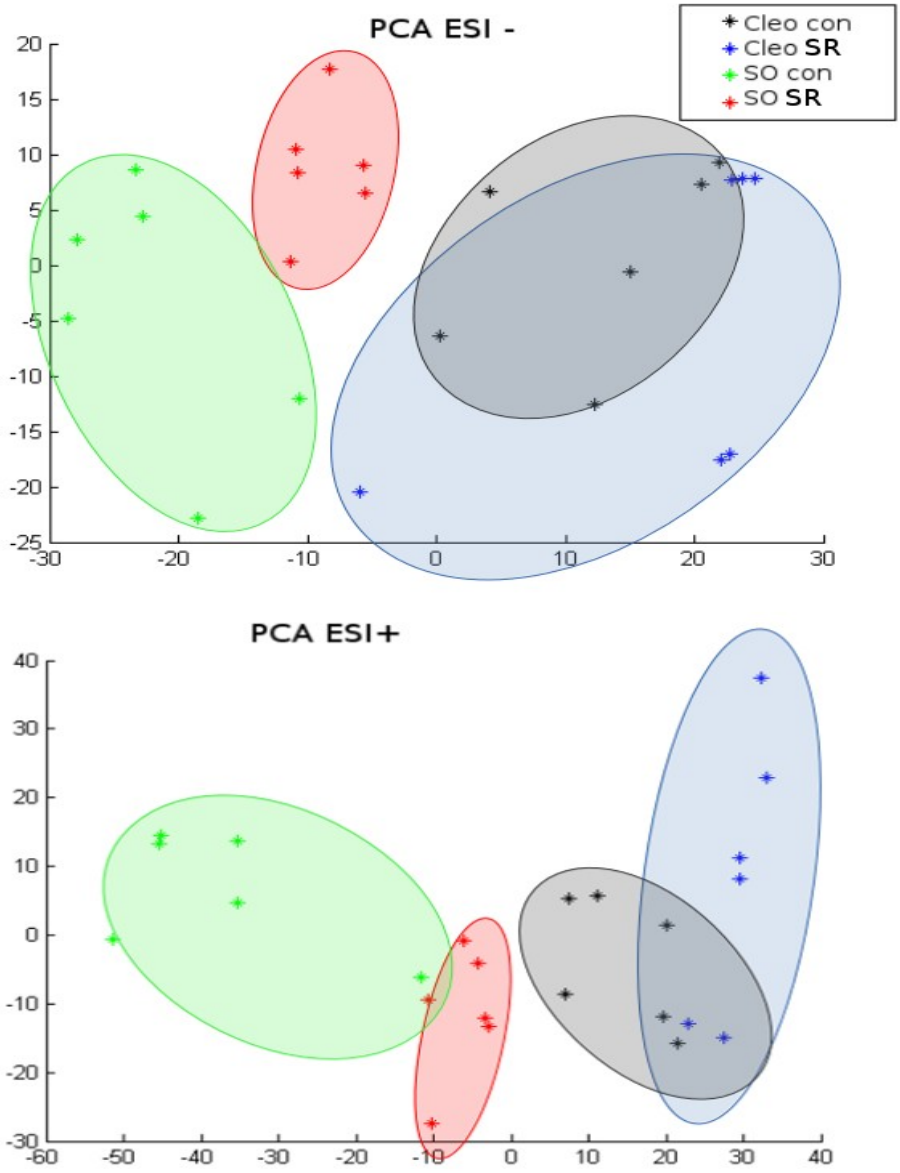

Figure 2. Metabolomic analysis of sour orange and Cleopatra mandarin following SR treatments against *T. urticae infestation*. Non-supervised principal component analysis (PCA) representation of major sources of variability of ESI + and ESI − signals obtained from a non-targeted analysis by HPLC-QTOFMS to monitor metabolomic changes during spider mite infestation. Four different sets of samples were tested: sour orange non-infested (SO con), sour orange previously infested (SO SR), Cleopatra mandarin non-infested (Cleo con) and Cleopatra mandarin previously infested (Cleo SR). Twelve-week old plants were infested with 10 mites per plant. Three days later, infested leaves were cut and the petiole was submerged in an EDTA solution for eight hours to collect the leaf eflux. Three independent biological and two technical

replicates were randomly injected and analysed (n = 6).

metabolic contents of the leave exudate between the two rootstock was rather different revealing strong basal differences in sap composition (Fig. 2). When we compared both rootstocks previously infested, a significant separation of the metabolites in SIR sour orange was observed, while there was a small overlapping in the pool of compounds from Cleopatra mandarin. Therefore infestation strongly modified the composition of metabolites secreted by the leaves of sour orange. In contrast, although Cleopatra mandarin also showed systemic resistance to , changes in the global behaviour of the metabolites was ore subtle compared with control plants. To determine the global behaviour of the data, a principal component analysis was used to allow comparison among data classes. The comparative unsupervised PCA showed that infestation explained 18,2 and 16,1% of the total variation for ESI+ and ESI− signals, respectively.

The accurate masses provided by the QTOF detector were contrasted with online biological databases such as Metlin for mass identity and KEGG and AraCyc for pathway search. Several compounds that were up-regulated in both Cleopatra mandarin and sour orange rootstocks previously infested were determined (Fig 3). One of them was identified as mio-inositol, its identity was confirmed by matching the exact mass and the fragment spectrum with commercial standard. Three aditional masses were identified in a tentative level, 112.01, 124.28 and 469.39 were also found in higher concentrations in plants previously infested. Although the two rootstocks showed a functional systemic resistance , the induction of this phenomenon was stronger in sour orange, so we were focused our attention on those metabolites that may be

involved sour orange systemic resistance as possible warning signals. Three masses matched the criteria of large accumulation in sour orange plants upon infestation. We were able to fully identify the citric acid, and two fatty acids, octadecanoid acid (256.24) and hexadecanoid acid (282.25) (Fig 4).

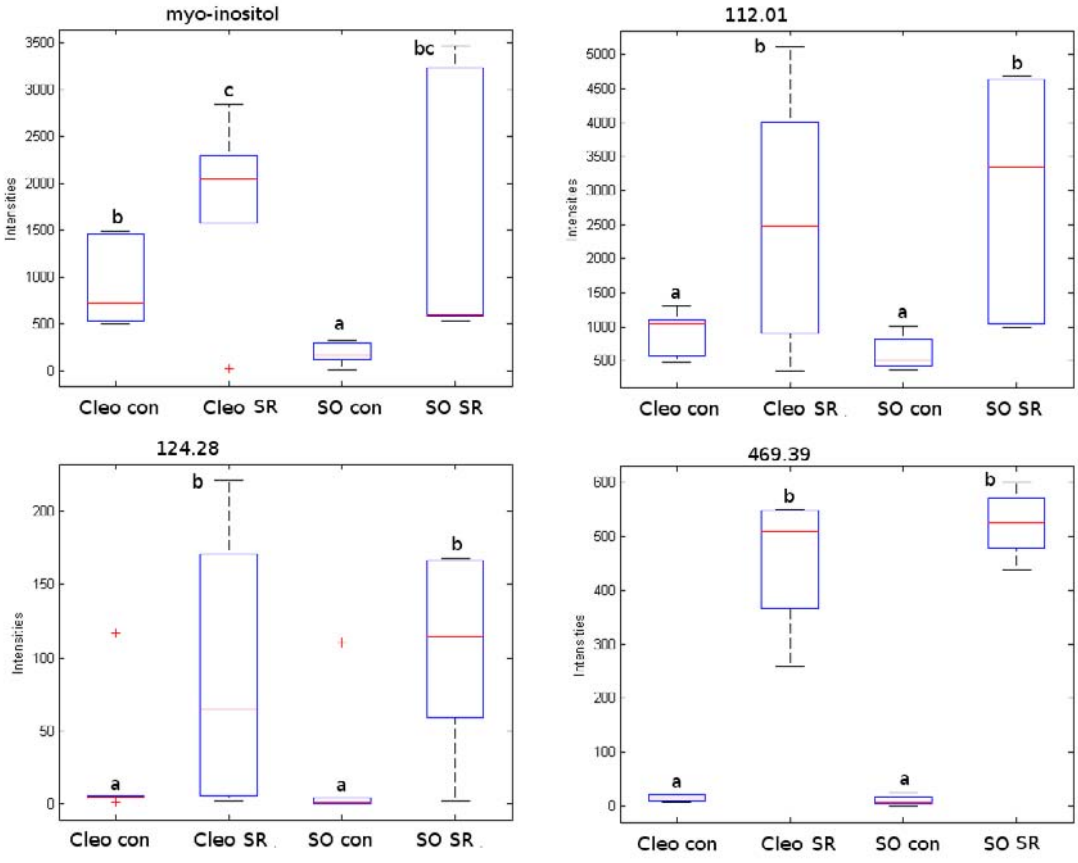

Figure 3. Four signals overaccumulated in the leaf eflux of sour orange and Cleapatra mandarin SR plants: one of them were fully identified as myo-inositol and the other remain ed unknown (112.01, 124.28 and 469.39 m/z). Boxplot analysis of the relative abundance of the four compounds in sour orange (SO) and Cleopatra mandarin (Cleo) either in the absence (con) or previously infested with spider mites (SR). Different letters indicate significant differences ($P < 0.05$; ANOVA) between treatments.

Since amino-acids are small and mobile compounds that could be transported systemically, we target-analysed their relative quantities in the leaf exudate. All amino acids were determined an quantified using a library of commercial standards injected in the same set of samples. Retention time, exact mass and fragment spectrum were used for aminoacid- determination. As it can be observed in the figure 5, the amino-acid profiling in the exudate is clearly influenced by the infestation by *T. urticae*. Leucine, methionine, phenylalanine and threonine were found in higher concentrations in Cleopatra mandarin plants previously infested than in their respective uninfested control plants. Interestingly, the set of amioacids that increase their concentration in sour orange was radically different. Glutamic acid, tyrosine and proline were

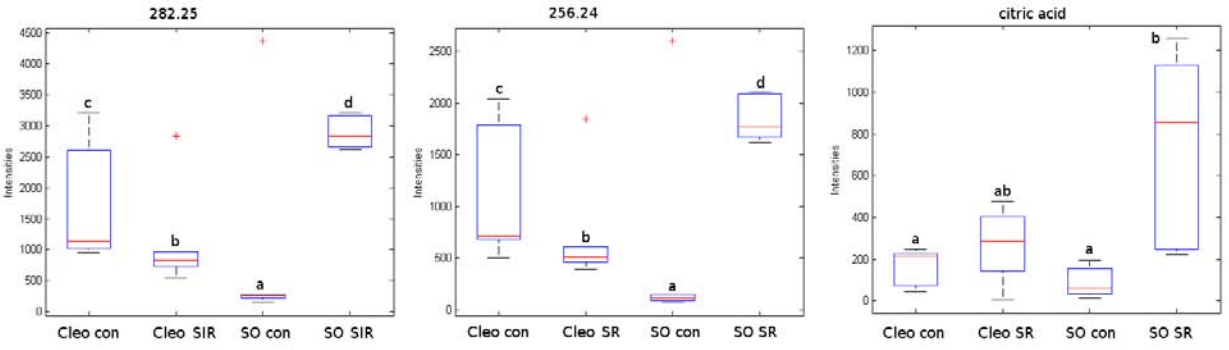

Figure 4. Three signals overaccumulated in the leaf efflux of SR-treated sour orange, that showed a 50% of egg reduction. One of them were fully identified as citric acid and the other two were identified by exact mass as: 282.25 (hexadecanoic acid) and 256.24 (octadecanoic acid). Boxplot analysis of the relative abundance of the four compounds in sour orange (SO) and Cleopatra mandarin (Cleo) either in the absence (con) or previously infested with spider mites (SR). Different letters indicate significant differences ($P < 0.05$; ANOVA) between treatments.

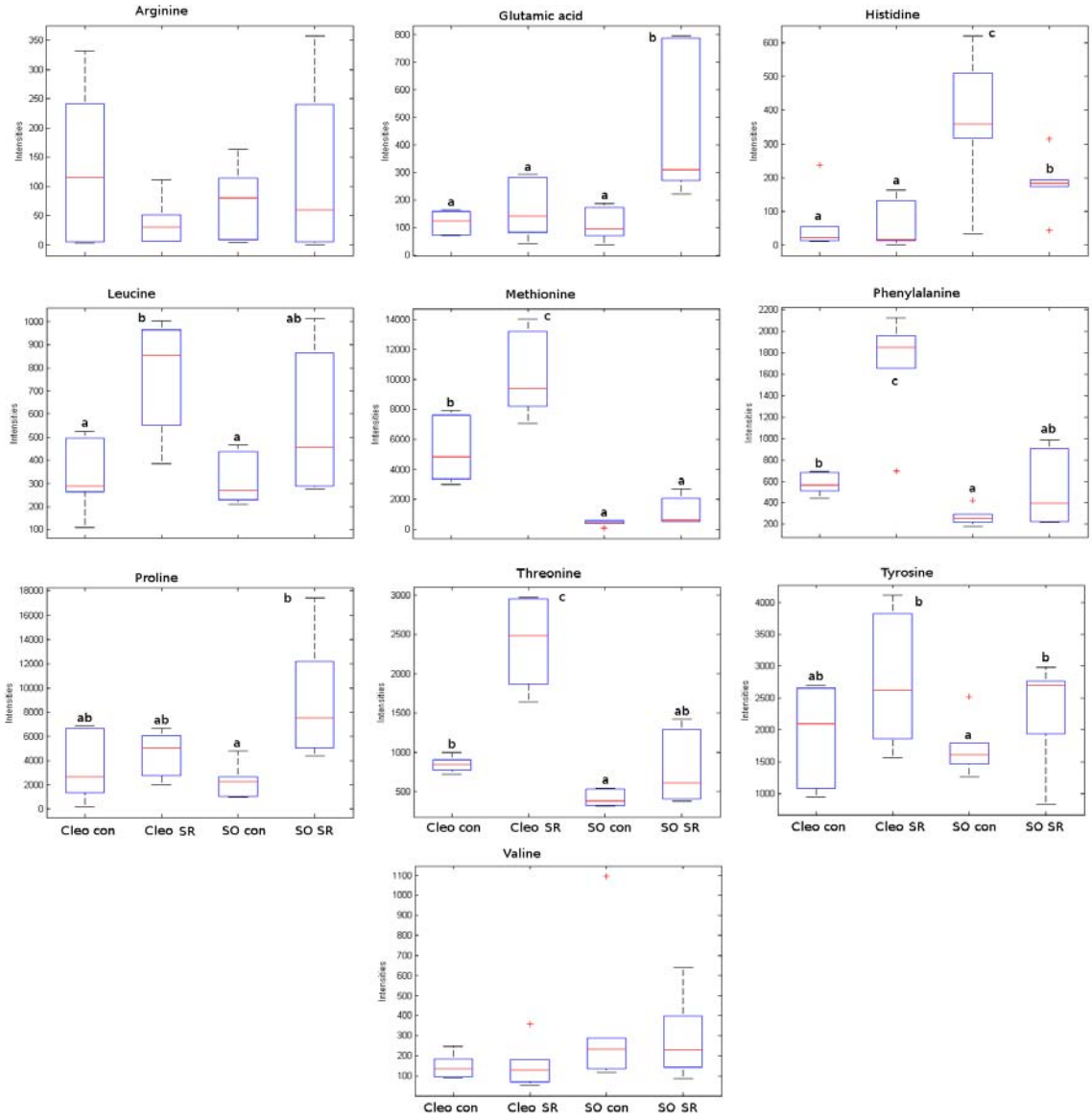

Figure 5. Amino acid profile in the leaf efflux in sour orange and Cleopatra mandarin following spider mite infestation. Uninfested sour orange (SO con), sour orange previously infested (SO SR), uninfested Cleopatra mandarin (Cleo con) and Cleopatra mandarin previously infested (Cleo SR). Twelve-week-old plants were infested with 10 mites per plant. Three days later in infested leaves were cut and during eight hours we collected the leaf efluxe in a EDTA solution. The samples were quantified by HPLC-QTOFMS and were processed using a amino acid library. Boxplots represent the average of three independent experiments with two technical replicates (n=6). Different letters indicate significant differences (one-way ANOVA, $P < 0.05$; LSD) between treatments.

transported in higher amounts when sour orange plants were infested. Therefore these aminoacids from Cleopatra mandarin and sour orange are likely to be the systemically transported following *T. urticae* infestation.

Glutamate receptor-like gene expression confirms the key role of the glutamic acid.

Glutamate receptor-like proteins (GLRs) are involved in leaf-to-leaf transport communicating signals that provide information about the neighbouring leaf status following wounding (Mousavi et al. 2013). Furthermore these receptors seem to promote JA-dependent signals in distal undamaged leaves. In fact, GLR Arabidopsis mutants are not able to activate the JA pathway in distal leaves in response to insect attack. As we showed previously, glutamic acid accumulated in the exudate of leaves from sour orange upon mite infestation, although this accumulation was more remarkable in sour orange than in Cleopatra mandarin. We measured the orthologous GLR citrus gene expression in clean distal plants (Fig 6). Interestingly, the GLR gene expression was strongly induced in uninfested leaves of SIR-treated sour orange while it remained hardly detectable in Cleopatra mandarin. This observation suggest a very likely implication of Glutamic acid and citrus GLRs in sour orange SIR whereas it still remains unclear in Cleopatra mandarin.

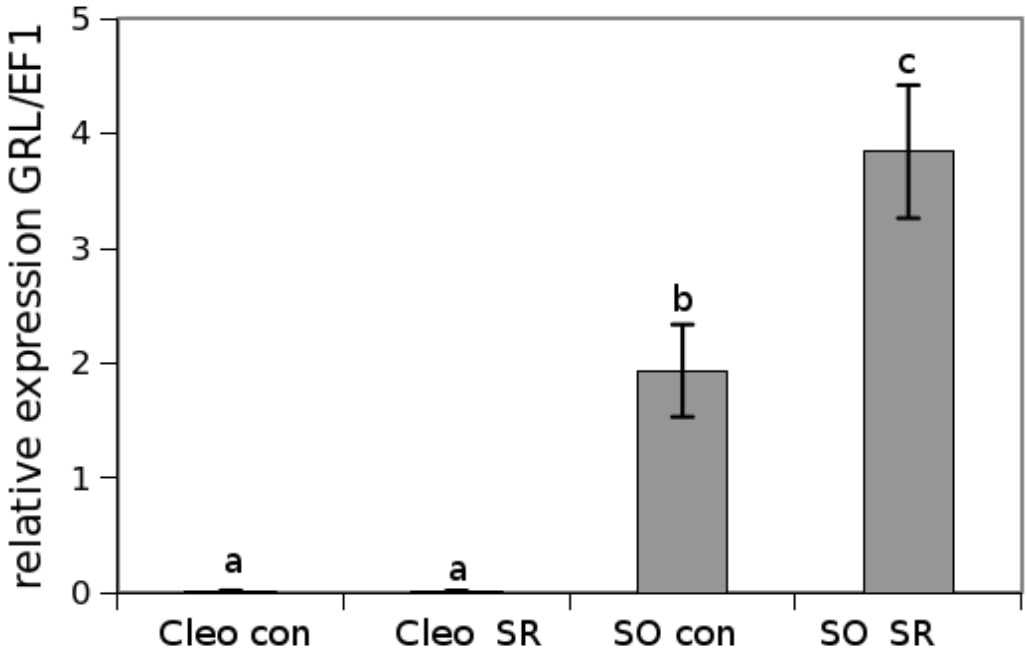

Figure 6. Relevance of glutamate receptor-like genes (GLR) in systemic herbivore induced resistance in citrus rootstocks against *T. urticae*. Citrus rootstocks were previously infested with 10 *T. urticae* adult females. Three days later distal uninfested leaves of these plants were collected for RT-PCR analisys. Data are presented as a mean of three independent analyses of transcript expression relative to the housekeeping gene plants ± SD (n = 3). Different letters indicate significant differences (one-way ANOVA, $P < 0.05$; LSD) between treatments with C t values as described by Yuan et al.

The activation of oxylipin pathway in distal leaves is linked to systemic resistance in sour orange while it may be related to ABA siganling in Cleopatra mandarin

In order to determine the influence on the main defense pathways regulated by hormones, the hormonal content in distal leaves of SIR-treated plants was determined (Fig 7). In SR-treated sour orange plants the oxylipin hormones

OPDA and JA showed a high accumulation in distal clean leaves compared to sour orange control plants. Contrastingly, Cleopatra mandarin plants did not show any significant change either in OPDA or JA. Despite the absence of oxylipin increase in Cleopata mandarin, it was still observed a reduction in oviposition induced by SR. Looking at the hormonal levels, this observation may be linked to ABA since it was significantly induced in distal leaves of SIR-treated Cleopatra mandarin while it remained at control levels in sour orange. Finally, SA was not altered by SR treatments in any of the two genotypes studied.

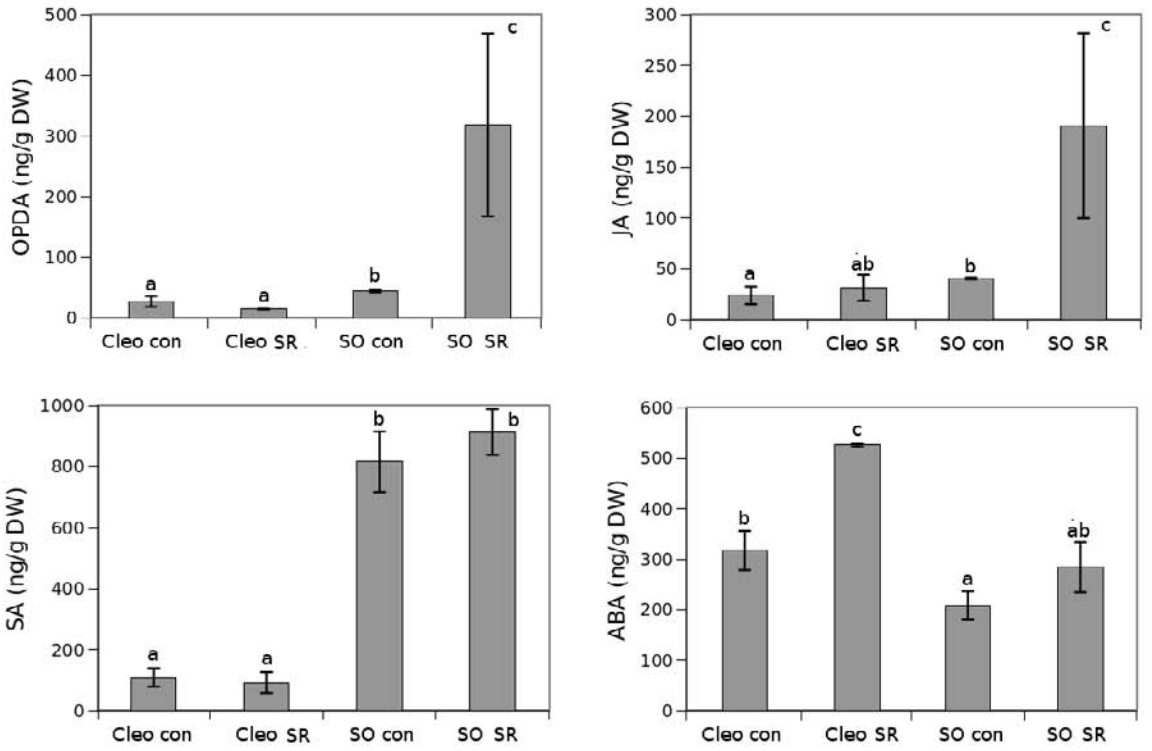

Figure 7. Hormonal content in sour orange and Cleopatra mandarin upon SR treatment. Citrus rootstocks were previously infested with 10 *T. urticae* adult females. Three days later distal uninfested leaves of these plants were collected for hormonal

measures. JA, OPDA ,SA and ABA levels were determined in freeze-dried material by HPLC-MS. The results shown are mean hormone levels of three independent analyses ± SD (n =3). Different letters indicate significant differences (one-way ANOVA, $P < 0.05$; LSD) between treatments.

The systemic induced resistance in sour orange is transmitted by grafting

Rootstocks are commonly used in agriculture as they provide the grafted commercial scions a better performance in terms of tolerance to different stresses and agronomic characteristics. Previously we showed that sour orange displays enhanced resistance against *T. urticae* compared with Cleoaptra mandarin (Agut et al. 2014). In the present research we also observed that sour orange transmited more efficiently the systemic resistance to uninfested distal leaves and this phenomenon is very like to be regulated by the oxylipin pathway. Despite this phenomenon is also present in Cleopatra mandarin it seems to be regulated by different mechanisms. In both cases, the transmitted signals seem to be generated in the bottom part of the plant that has been exposed to *T. urticae*. In the following experiments we wondered whether the resistance observed in the different rootsocks could be transmitted to grafted plants. We used a common scion, Clemenules on both rootocks, sour orange and Cleopatra mandarin.

Two year old plants containing both the combinations of scion-rootstock were infested with adult 20 females of T. urticae. The number of chlorotic leaves and the number of adult females per leaf was assessed for two weeks. Both parameters determined showed same trend. The combination Clemenules scion-sour orange displayed enhanced resistance showing lover population and reduced levels chlorotic leaves compared with Clemenules scion grafted onto Cleopatra mandarin (Fig 8).

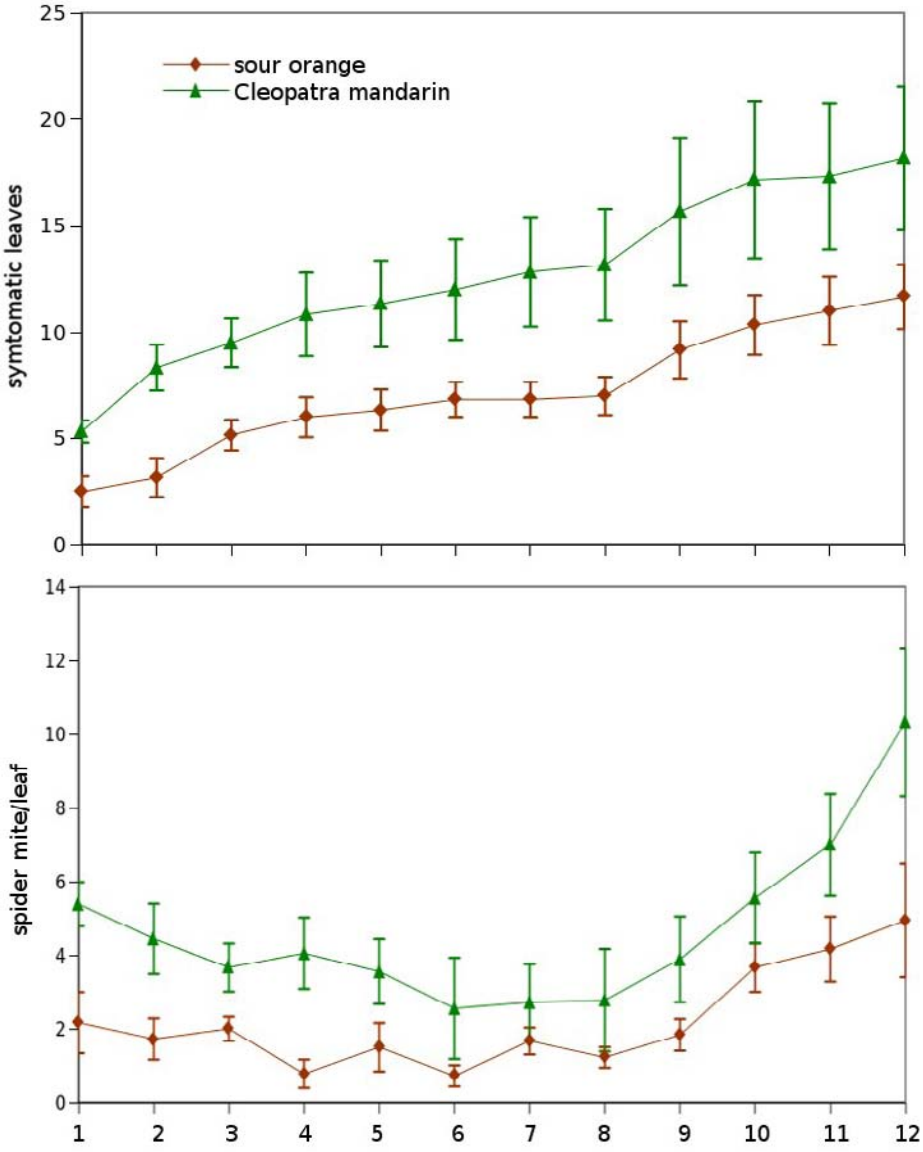

Figure 8. Symptomatic leaves and spider mite performance in Clemenules variety grafted onto sour orange or Cleopatra mandarin rootstocks. Two year-old plants were infested with 20 *T. urticae* adult females. The samplings were conducted since the emergence of the first chlorotic leaves until the first symptoms of defoliation. The statistical analysis were conducted by Generalized Linear Mixed Model.

Metabolic changes in the scion are rootstock-dependent

In order to determine the mobile signal that is transmitted from the roots to the leaves, the root efflux from infested plants was collected and analysed by LC-Q-TOF. We did not observe any relevant change in JA, OPDA or IAA measured in the efflux of infested plants compared with their respective controls (Fig 9). However, the JA was reduced in sour orange follwing infestation. Subsequently, we identified signals corresponding to different amino-acids (Fig 10). None of the amino acids identified in Cleopatra mandarin was significantly changed.

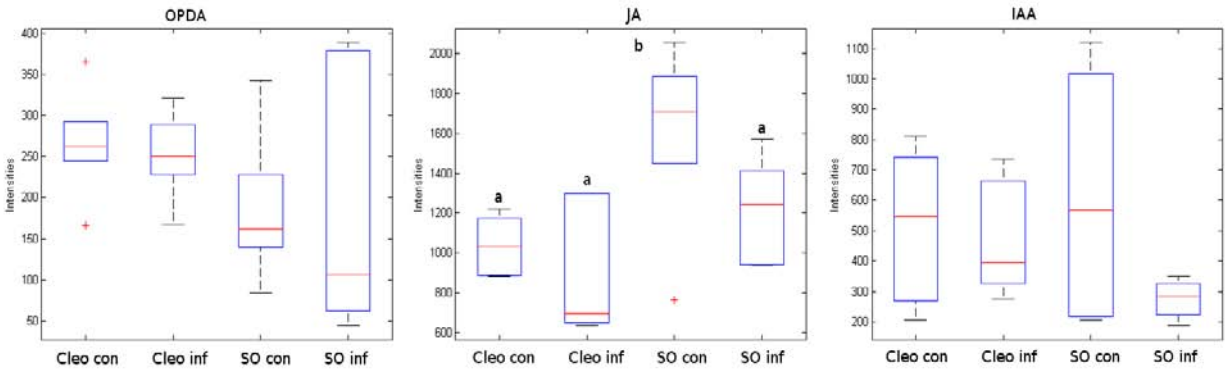

Figure 9. Hormonal profile in the root efluxe in grafted plants following spider mite infestation. Clemenules variety grafted onto sour orange (SO) and Clemenules variety grafted onto Cleopatra mandarin (Cleo) plants uninfested (con) or infested (inf). Two year-old grafted plants were infested with 20 mites per plant. Three days later the stem was cut and the root efluxe was collected using a Scholander-type pressure chamber. The samples were quantified by HPLC-QTOFMS and were processed using a hormonal library. Boxplots represent the average of three independent experiments with two technical replicates (n=6). Different letters indicate significant differences (one-way ANOVA, $P < 0.05$; LSD) between treatments.

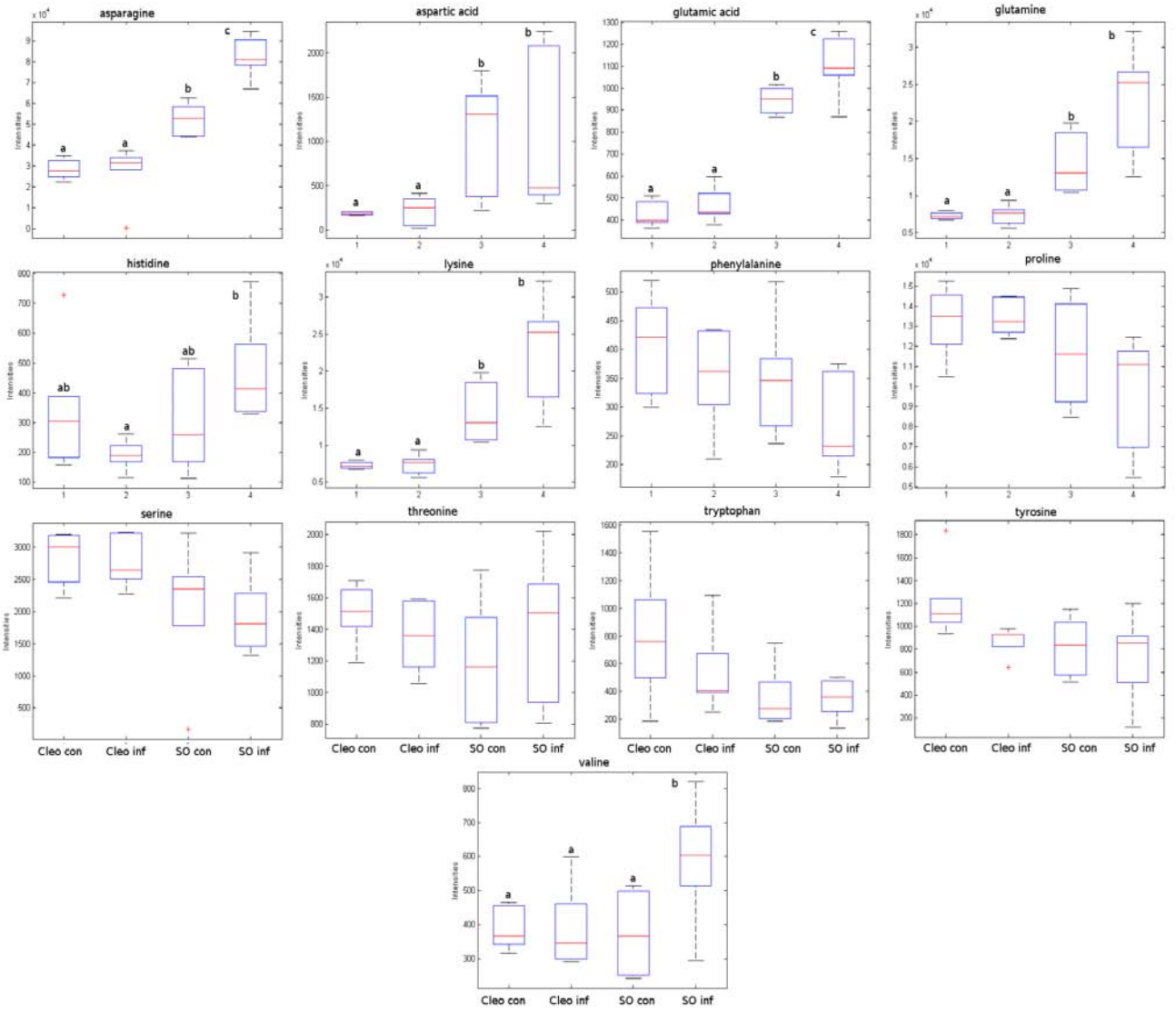

Figure 10. Amino acid profile in the root efflux in grafted plants following spider mite infestation. Clemenules variety grafted onto sour orange (SO) and Clemenules variety grafted onto Cleopatra mandarin (Cleo) plants uninfested (con) or infested (inf). Two year-old grafted plants were infested with 20 mites per plant. Three days later the stem was cut and the root efluxe was collected using a Scholander pressure chamber. The samples were quantified by HPLC-QTOFMS and were processed using a amin oacid library. Boxplots represent the average of three independent experiments with two technical replicates (n=6). Different letters indicate significant differences (one-way ANOVA, $P < 0.05$; LSD) between treatments.

Contrastingly, sour orange responded to the infestation by increasing the levels of asparagine, aspartic acid, valine, glutamine and glutamic acid. Again glutamic acid behaved as a key metabolite in sour orange resistance and a very likely transported signal. We collected leaf material from Clemenules grafted onto sour orange and Cleopatra mandarin after infestation for futher analysis. Despite the leaves belonged to the same genotype, the expression of GLR varied strongly depending on the rootstock (Fig 11). Clemenules variety grafted onto sour orange showed a GLR increased expression, probably due the elevated concentrations of glutamic acid in the root current that may trigger more efficiently the glutamate receptor GLR. Nevertheless a contribution of the other amino acids cannot be fully discarded.

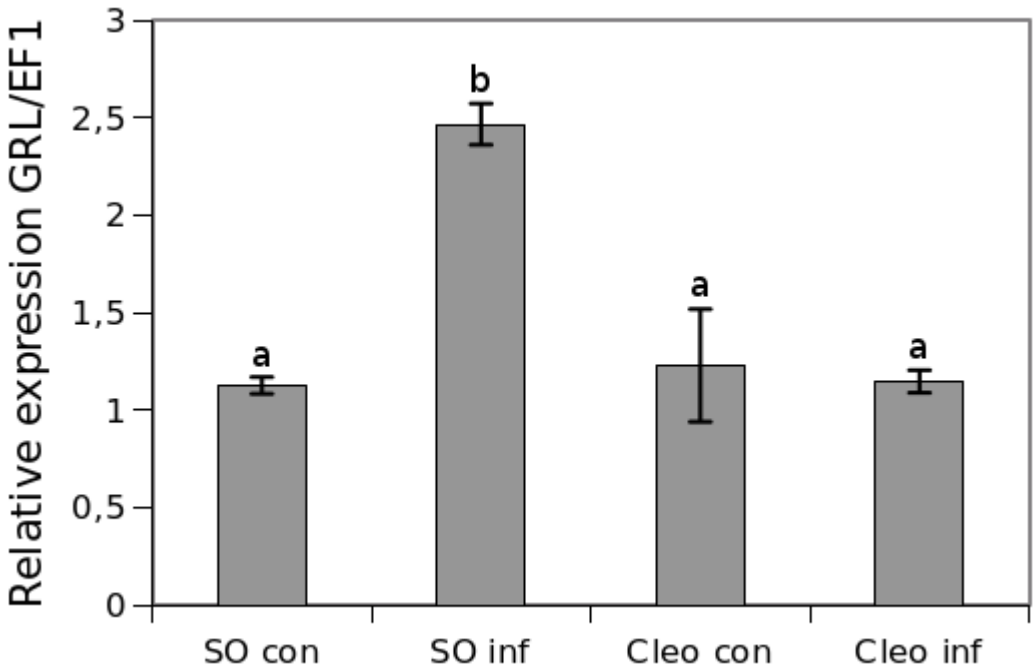

Figure 11. GLR expression in grafted plants affected by the spider mite. Clemenules variety grafted onto sour orange (SO) and Clemenules variety grafted onto Cleopatra mandarin (Cleo) plants uninfested (con) or infested (inf). Two year-old grafted plants were infested with 20 mites per plant. Three days later, leaves were collected for mRNA analisys. Data are presented as a mean of three independent analyses of

transcript expression relative to the housekeeping gene plants ± SD (n = 3). Different letters indicate significant differences (one-way ANOVA, P < 0.05; LSD) between treatments with C t values as described by Yuan et al.

4. Discussion

Following biotic attack, the locally damaged tissue can send a warn signal to undamaged distal leaves to prepare defense to subsequent attacks. Several mobile signals that trigger the defense response in undamaged tissue after pathogen infection were recently described. MeSA, azelaic acid, pipecolic acid, dehydroabietinal or the lipid transfer protein DIR1 (Park et al., 2007; Vlot et al., 2008: Liu et al., 2010; Manosalva et al., 2010; Jung et al., 2009). These compounds play a co-operative role to activate SAR. In 1970, Green and Ryan described the accumulation of proteinase inhibitors in distal leaves after wounding or herbivory in tomato leaves. Some authors suggested the systemin as the mobile signal (McGurl et al., 1992), however Li et al. (2005) using grafting techniques and JA mutant plants determined that systemin was not the mobile signal and was not required to induce SIR. In 2013, Mousavi et al., showed that membrane depolarization is a critical step for signal transmission. The glutamate receptor-like is sensitive to this depolarisation and activates downstream expression of JA marker genes, that ends up in the synthesis of toxic compounds with insecticidal activity. The relationship between GLR and JA had been reported before. Kang et al. (2006) showed that Arabidopsis plants overexpressing a radish GLR had enhanced resistance against a fungal pathogen due to a up-regulation of jasmonic acid-responsive and jasmonic acid-biosynthetic genes.

Our results show that both rootstocks, Cleopatra mandarin and sour orange, are

able to express SR although to a different extent. We could fully identify the myo-inositol from the leaf efflux that was accumulated in both rootstocks when infested. Myo-inositol and its derivatives are crucial compounds for development and signalling in plants. They act as mediate signalling in several pathways in response to stress, hormones, and nutrients, contributing to stress tolerance in plants. Nevertheless the resistant rootstock sour orange showed a stronger SR compared with the susceptible rootstock Cleopatra mandarin. Accordingly, we found high levels of citric acid in infested sour orange whitch may contribute to the stronger SR response observed in this rootstock. Silva et al. (2013) speculated that citric acid increased in soya bean could be used to enhance the production of energy for biosynthesis of secondary metabolites.

Kuc (2001) already proposed glutamic acid as a likely mobile signal that can trigger SR. The free glutamic acid in the plant can be perceived by the GLR in undamaged distal leaves and start the signal transduction to activate the JA-dependent pathway as it was observed in sour orange. Surprisingly, the susceptible rootstock still retained the ability to express SR but we did not observe any change in glutamic acid levels in the efflux and any JA activation in distal leaves. It is likely that the first infestation triggers alternative defenses in distal parts that can prime Cleopatra mandarin for subsequent attacks. However the signals that may be transmitted from below-ground remain to be elucidated. The susceptible rootstock Cleopatra mandarin is not able to respond to mite attack by activation of the JA pathway (Agut et al., 2014) probably due the manipulation of the host defenses by the mite. However, the undamaged distal leaves of Cleopatra mandarin previously infested showed a strong accumulation of ABA. Previously, Erb et al, (2008) described the negative effect of ABA accumulation on the growth of *Spodoptera littoralis* . The increased ABA content contributed to reduce the nutritional quality of the leaf

resulting in a reduced insect growth. Given the poor activation of the JA signaling in Cleopatra mandarin, an alternative activation of ABA-dependent responses is a likely strategy for this rootstock to improve its defenses against T. urticae.

Roots were usually considered as a support tissue and their main function the water and nutrient uptake from the soil. However, in the last years this oversimplified vision has changed. The roots are able to send and deliver signals to the shoots to enhance and coordinate the defensive response against insects such as *S. littoralis* (Erb et al., 2008). Wu and Baldwin (2010) proposed the roots as a manufacturer of toxic metabolites. After wounding by lepidopteran larvae , nicotiana plants sent to the roots an undetermined signal that induced the biosynthesis of the toxic secondary metabolite nicotine. Recently, Fragoso et al. (2014) showed the necessary proper function of the JA pathway in the roots. The JA produced in the roots controls aboveground JA levels. When wild type tomato plants were grafted JA mutants showed an increased damage caused by *Empoasca* spp leafhoppers and *Tupiocoris notatus* Distant (Hemiptera: Miridae).

Our work and the data published by Bruessow et al. (2010), show the evidence of a systemic signal travelling from the roots to the shoots, since the same variety grafted onto different rootstocks modified the performance of *T. urticae*. Taken together, we previously hypothesized that either JA or a conjugate may be the mobile signal. Surprisingly the JA levels released by the roots after *T. urticae* infestation in sour orange were lower compared sour orange control plants making this hypothesis unlikely. In absence of infestation, sour orange showed more root JA compared with Cleopatra mandarin, supporting our previous data of higher basal levelsof this hormone (Agut et al., 2014).

Following the observation of amino-acid profiling from the root exudate of sour orange, we hypothesize that glutamic acid as the candidate mobile signal inducing SR. Accordingly, increased GLR expression in the Clemenules cultivar grafted onto sour orangesupported lower densities of *T. urticae* and presented less symptomatic leaves than the same cultivar grafted onto Cleopatra mandarin.

Finally, we collected the the efflux of rootstocks from plants exposed to *T. urticae* infestation in order to find candidates transmiting a mobile signal to the grafted cultivar triggering enhanced resistance. For such propose, we used Clementine plants grafted onto the sensitive Cleopatra mandarin and the resistant sour orange. Interestingly, amino-acid profiling was substantially modified in the below-ground efflux of sour orange with the strongest changes in the levels of glutamic acid. Since nitrogen is one of the nutrients that pathogens and herbivores try to obtain grom their host plants, the glutamic reallocation observed in sour orange previously infested by *T. urticae* can be a strategy to reduce the free ammonium in the damaged tissue and decrease the nutritional quality of the leaf as it was proposed previously (Seifi and Hofte, 2013).

Concluding, citrus responses to spider mites is spread systemically through leave and root eflux (Fig 12). In addition, citrus responses vaires in a rootstock-dependent manner. While leaf efflux from Cleopatra mandarin contains high amounts of myo-inositol, sour orange eflux also contains citric acid, glutamic acid and two fatty acids. These signals are perceived by the root and then the root delivers compounds move to distal leaves orchestrating a defense response. Accordingly, sour orange increase the expression of GLR that activates the JA pathway (high levels of OPDA and JA) reducing the

oviposition of *T. urticae* by 50%. The reduction of *T. urticae* oviposition in Cleopatra mandarin is 30%, however the mechanisms for such reduction is still elusive depite ABA is a likely candidate further experimentation is needed to confirm its participation in citrus resistance against arthropods.

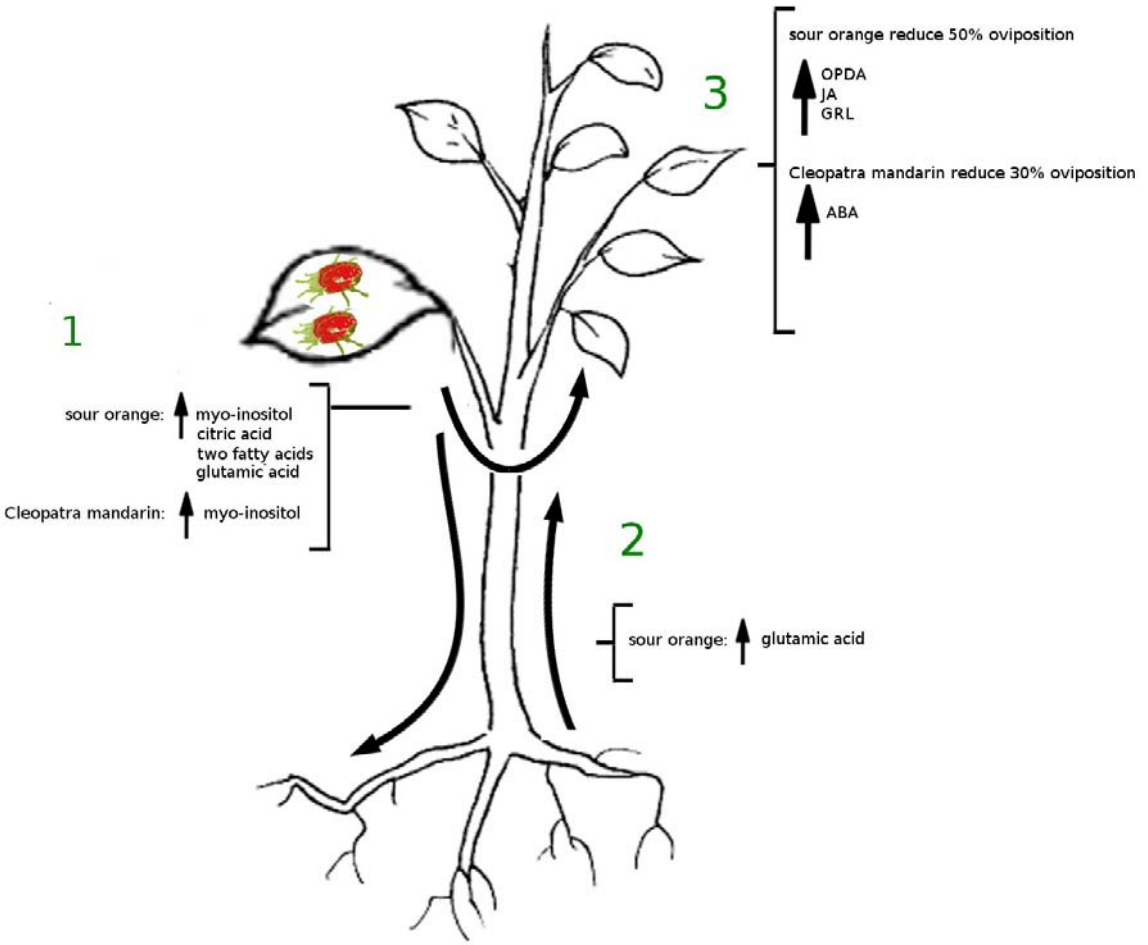

Figure 12. Model for systemic resistance in citrus against *T. urticae.* Spider mite attact rapidly induces changes in the leaf efflux. The two rootstocks respond differently to mite infestation, leaf efflux from Cleopatra mandarin contains high amounts of myo-inositol, while sour orange in addition also releases citric acid, glutamic acid and two fatty acids. These compounds can move to distal leaves or the roots. Once the roots detect the signals from the infested leaves, the resistant rootstock sour orange increases the transport of glutamic acid to the shoots. The distal leaves receive the signals from the roots and/or from the infested leaves and respond to a future attack. In

consequence, sour orange increases the expression of GLR that active the JA pathway (high levels of OPDA and JA) and reduce the oviposition of *T. urticae* by 50%. The reduction of *T. urticae* oviposition in Cleopatra mandarin is 30% probably due to an increase of ABA levels.

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Conclusions

1. Resistance bioassays showed different resistance of two citrus rootstocks against *Tetranychus urticae*. Sour orange behaved as a resistant rootstock whereas Cleopatra mandarin as a susceptible one. Gene expression and hormone analyses correlated the resistance of sour orange with the activation of JA signaling pathway.

2. Metabolomic data analized by Acquity UPLC system interfaced to hybrid quadrupole time-of-flight suggest the key role of flavonoids as toxic compounds against *T. urticae*, since these compounds over-accumulated in the resistant rootstock in response to infestation.

3. Volatiles released by infested sour orange plants repelled conspecific mites, whereas infested Cleopatra mandarin plants were more attractive. These different responses could be attributed to different occurrence of some terpenoids.

4. Volatiles released by *T. urticae* infested sour orange plants induced resistance in the susceptible rootstock Cleopatra mandarin. The exposure to a blend of compounds from *T. urticae* infested sour orange plants increased the expression of the JA biosynthesis gene *LOX2*.

5. Exposure of Cleopatra mandarin plants to different synthetic volatiles confirmed the key role of terpenoids and Green Leaf Volatiles in the defense reaction against *T. urticae*. Only exposure to ocimene, limonene and 4-hydroxy-4-methyl-2-pentanone increased *LOX2* expression, a key gene in the defense response against *T. urticae*.

6. Although *T. urticae* may manipulate the defense of Cleopatra mandarin, this

rootstock retains an effective response against the pest when appropriately stimulated.

7. Prior *T. urticae* infestation in sour orange induced a strong systemic resistance. An accumulation of OPDA and JA in undamaged distal leaves was observed.

8. The resistance in sour orange is retained in the grafted scion cultivar. This is indicative that there are some mobile signals between the rootstock and the cultivar. Metabolomic analysis of leaf, root efflux and *GLR* gene expression suggest that glutamic acid is the mobile signal transmitting the distal defense responses.