Induced resistance in citrus by hexanoic acid

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Tesi Doctoral

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PhD Thesis

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FEM CONSTAR QUE:

La present memòria de Tesi Doctoral, presentada per Eugenio Llorens Vilarrocha titulada:

"Induced resistance in citrus by hexanoic acid"

i realitzada a l'àrea de Fisiologia Vegetal en la Universitat Jaume I de Castelló, reuneix les condicions per a la seva defensa.

Signat:

Dra. Leonor Lapeña Barrachina Dra. Pilar García-Agustín

Agraïments

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Part dels resultats d'aquesta tesi han estat publicats en les següents aportacions originals

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Resum

Resum

En l'actualitat es considera un tema prioritari el desenvolupament de sistemes alternatius a l'ús de plaguicides en la lluita contra les malalties en cultius d'interès agronòmic. Una de les estratègies plantejades a aquest efecte, es basa en la cerca de nous compostos naturals, com a alternativa a tractaments químics agressius.

Fa uns anys, en el grup de Bioquímica i Biotecnologia del Departament de Ciències Agràries i del Medi Natural, es va iniciar una línia de recerca amb l'objectiu de desenvolupar mètodes alternatius a l'ús de plaguicides, mitjançant el desenvolupament de nous compostos inductors, l'aplicació dels quals afavoreixi l'adaptació de les plantes enfront de situacions d'estrès biòtic i abiòtic. Els inductors són composts que poden actuar en diversos punts de les rutes naturals d'activació de respostes de les plantes, podent imitar en part, o íntegrament, l'activació dels mecanismes biològics de resistència.

Els estudis realitzats fins avui han demostrat que l'àcid hexanoic (Hx), un compost natural present en plantes i fruits, és un inductor de resistència en plantes de tomata enfront de necròtrofs (*Botrytis cinerea*) i biòtrofs (*Pseudomonas syringae*). A més actua com un inductor de "priming" ja que predisposa als sistemes de defensa de la planta perquè responguin de forma més ràpida i eficaç enfront del possible atac de patògens.

Basant-nos en aquests antecedents i amb l'objecte d'estudiar l'eficàcia i el mecanisme d'acció de l'àcid hexanoic en cítrics, un cultiu d'interès agronòmic a la Comunitat Valenciana, plantegem realitzar aquest treball.

Un dels greus problemes als quals s'ha enfrontat en els últims anys l'agricultura d'aquesta zona, ha estat eradicar el fong necròtrof *Alternaria alternata* pv *citri*. Aquest organisme, capaç d'atacar a diverses varietats de cítrics especialment a la varietat 'Fortune', ha arribat a disminuir la producció en més de 120000 t/any. Per això, un dels objectius parcials d'aquest treball ha estat estudiar l'efecte de l'inductor (Hx) en plantes de la varietat 'Fortune' davant de l'atac de *A. alternata*. S'ha demostrat l'efectivitat d'aquest compost en aplicar una concentració de 1mM a les plantes en haver-se reduït la incidència de la malaltia en més d'un 50%. A més, s'ha observat que la deposició de callosa s'incrementa així com l'expressió dels gens de proteïnes inhibidores de poligalacturonases (implicades en la resistència enfront de patògens) en plantes infectades i tractades amb Hx. L'anàlisi hormonal ha revelat que la ruta de l'àcid jasmònic (JA) sembla que també està implicada en el mecanisme d'acció, ja que s'ha observat una major acumulació de compostos implicats en aquesta ruta. Així mateix, els resultats han demostrat un ràpid

increment dels nivells d'àcid abscísic (ABA), que podria actuar com un regulador positiu de la deposició de callosa.

Un altre dels objectius d'aquest treball, ha estat estudiar l'eficàcia de l'inductor (Hx) a llarg termini en plantes de la varietat 'Fortune' i veure com responen enfront de l'atac d'*A. alternata*. Els resultats obtinguts han mostrat que, després de la segona inoculació, es manté i fins i tot s'augmenta la protecció davant del patogen en un 20%, disminuint tant el nombre de fulles infectades com la grandària de la infecció. No obstant això, la resposta hormonal després de la segona inoculació, no ha mostrat diferències significatives entre plantes tractades i no tractades, mentre que s'ha observat un augment dels compostos fenòlics en plantes tractades. Això fa pensar que en el mecanisme de protecció mediat per Hx també estan implicats aquests compostos.

Encara que en l'actualitat a Espanya el patogen *Xanthomonas citri* subsp. *citri* no és un greu problema per a la nostra agricultura, si ho és per a certs països tals com Estats Units o Brasil. Per això, ens plantegem estudiar l'efecte de l'inductor (Hx) enfront d'aquest biòtrof. L'aplicació de l'Hx en taronger dolç va reduir la infecció en més d'un 50% quan es van infectar amb *X. citri*, perllongant-se aquest efecte fins a 50 dies després de l'aplicació de l'inductor. Enfront d'aquest patogen, l'àcid hexanoic actua activant l'expressió dels gens *PR2* i *AOS*, així com la deposició de callosa. Aquests resultats mostren l'eficàcia del inductor davant de diferents patògens (biòtrofs i necròtrofs) sent el seu mecanisme d'acció diferent depenent de l'estratègia de patogenicitat.

A fi d'aprofundir en el mecanisme d'acció de l'inductor en plantes de cítrics davant d'*Alternaria alternata*, s'ha realitzat un estudi del perfil metabolòmic. Les anàlisis han revelat que en totes les condicions estudiades (control, tractades amb Hx, infectades i tractades i infectades) s'observen canvis en aquest perfil.

Així mateix, l'anàlisi fisiològica ha revelat que no només es modifiquen els paràmetres, tals com la fotosíntesis, transpiració i contingut de clorofil·les, sinó que incrementen els seus nivells, la qual cosa ens indica que la planta no està fent una gran despesa energètica, quan s'aplica l'inductor.

Seguint aquesta línia, per conèixer la localització de l'inductor en les plantes de cítrics després del tractament, es va utilitzar àcid hexanoic marcat amb ^{13}C. Els resultats han mostrat que l'inductor no es mobilitza cap a les fulles sinó que s'acumula a nivell radicular.

D'altra banda, s'ha demostrat que l'aplicació de l'àcid hexanoic indueix l'emissió de compostos volàtils en plantes de cítrics infectades amb *A. alternata*. Per primera vegada, s'ha observat que l'inductor Hx promou l'emissió de derivats

del geranil difosfat provinents de la ruta de l'àcid mevalònic i àcid linoleic en plantes infectades i tractades. A més, pels resultats previs obtinguts, determinant les masses exactes dels composts aigües a dalt d'aquests dos metabòlits, ens ha fet proposar la hipòtesi que el precursor de tots dos pot estar prop de l'Acetil CoA. No obstant això, s'han de realitzar més estudis per demostrar aquesta hipòtesi.

De tots aquests resultats podem concloure que l'àcid hexanoic actua com inductor de priming protegint a les plantes de cítrics tant enfront del necròtrof *A. alternata* com al biòtrof *X. citri*. En tots dos casos està activat el mecanisme de protecció intervingut per callosa i la ruta de l'àcid jasmònic. A més, a llarg termini, l'inductor (Hx) activa altres mecanismes d'acció en els quals estan implicats els compostos fenòlics, sent específic pel biòtrof *X. citri* l'activació dels mecanismes de defensa mediats per àcid salicílic. Finalment, es conclou per primera vegada, que en el mecanisme de resistència induïda per àcid hexanoic poden estar implicats compostos volàtils.

Summary

Nowadays it is considered a priority the development of alternative systems to the use of pesticides for combating diseases in crops with agronomic interest. One of the strategies proposed for this purpose is based on the search for new natural compounds as an alternative to harsh chemical treatments.

A few years ago the group of Biochemistry and Biotechnology of the Department of Agricultural Sciences and Natural Environment, started a line of research in order to develop alternative methods to the use of pesticides through the development of new inducer compounds, the application of which improve the adaptation of plants to face biotic or abiotic stresses. Inductors are compounds that may act in diverse steps of the natural pathways of defensive responses, and mimic in part, or in whole, the activation of the resistance.

Studies performed to date have shown that hexanoic acid (Hx), a naturally compound present in plants and fruits, is an inducer of resistance in tomato plants against necrotrophs (*Botrytis cinerea*) and biotrophs *(Pseudomonas syringae*). It also acts as an inducer of "priming" because predisposes the defense systems of the plant to respond more rapidly and effectively against a possible attack by pathogens.

Based on this background, and in order to study the efficacy and mechanism of action of hexanoic acid in a crop with agronomic interest in Valencia Community such as citrus, we proposed this work.

One of the serious problems occurred in recent years in the agriculture in this area has been eradicating necrotrophic fungus *Alternaria alternata* pv citri. This organism, capable of attacking several varieties of citrus, especially the variety 'Fortune', has come to reduce production by more than 120,000 t / year. Therefore, one of the sub-objectives of this work was to study the effect of the inductor (Hx) in plants of the variety 'Fortune' against *A. alternata*. The effectiveness of this compound has been demonstrated by reducing the incidence of the disease by 50% when the compound was applicated to the roots at final concentration of 1mM. Moreover, in treated and infected plants, the deposition of callose was enhanced, as well as, the expression of genes of polygalacturonase inhibiting proteins (involved in resistance against pathogens). Hormonal analysis has revealed that the jasmonic acid pathway (JA) may be also involved in the mechanism of action, since there has been a further accumulation of compounds of this pathway. Also, the results have shown a rapid increase in the levels of abscisic acid (ABA), which ld act as a positive regulator of callose deposition.

Another objective of this work was to study the long lasting effectiveness of the inductor (Hx) in plants of the variety 'Fortune' and how they respond to the attack of *A. alternata*. The results have shown that, after the second inoculation, the protection against the pathogen is maintained and even increased by 20% and reduce both the number of infected leaves and the size of the infection. However, the hormonal response after the second inoculation, showed no significant differences between treated and untreated plants, but there has been an increase in the phenolic compounds in treated plants. This suggests that these compounds are also involved in the mechanism of protection mediated Hx.

Although currently in Spain, *Xanthomonas citri* subsp. *citri* isn´t a serious problem for our agriculture, it is a harmful pest for some countries such as USA and Brazil. Therefore, we propose to study the effect of the inductor (Hx) against this biotrophic pathogen. Hx application in sweet orange plants reduced infection by more than 50% when the plants were infected with *X. citri*, showing a long lasting effect until 50 days after application of the inducer. Against this pathogen, hexanoic acid acts by activating the expression of genes *PR2* and *AOS* and callose deposition. These results show the effectiveness of this inductor against different pathogens (biotrophs and necrotrophs) and its mechanism of action is different depending on the strategy of pathogenicity.

To delve into the mechanism of action of the inductor in citrus plants against *Alternaria alternata*, we made a study of the metabolomic profile. The analysis revealed that changes are observed in the metabolic profile under all studied conditions (control, treated with Hx, infected and treated and infected).

Furthermore, the physiological analysis has revealed that the parameters, such as photosynthesis, transpiration and chlorophyll content are not changed, but also increase their levels, which indicates that the application of Hx not supposes a high energetic cost.

Along this line, for the location of the inductor in citrus plants after treatment, hexanoic acid labeled with ^{13}C was used. The results have shown that the inductor does not move to the leaves but accumulates to the root level.

Furthermore, it has been shown that the application of hexanoic acid induces the release of volatile compounds in citrus plants infected with *A. alternata*. For the first time, it has been observed that the inductor (Hx) promotes the emission of compounds derived from the geranyl diphosphate from mevalonic acid and linoleic acid pathways in infected and treated plants. Moreover, by the previous results, determining the exact masses of compounds upstream of these two metabolites, we have proposed the hypothesis that the precursor of both may be

near the acetyl CoA. However, more studies should be performed to prove this hypothesis.

With all these results we conclude that the hexanoic acid acts as an inducer of priming protecting citrus plants against both necrotrophic *A. alternata* and the biotrophic *X. citri*. In both, the mechanism of protection is related with callose and jasmonic acid pathway. In addition, in the long lasting protection, the inductor (Hx) activates other mechanisms of action related to phenolic compounds, being specific for *X. citri* biotrophic the activation of defense mechanisms dependent of salicylic acid. Finally, it is concluded for the first time, the relation between the induced resistance by hexanoic acid and the emission of volatile compounds.

Resumen

En la actualidad se considera un tema prioritario el desarrollo de sistemas alternativos al uso de plaguicidas en la lucha contra las enfermedades en cultivos agronómicos de interés. Una de las estrategias planteadas con este fin, se basa en la búsqueda de nuevos compuestos naturales, como alternativa a tratamientos químicos agresivos.

Hace unos años en el grupo de Bioquímica y Biotecnología del Departamento de Ciencias Agrarias y del Medio Natural, se inició una línea de investigación, con el objetivo de desarrollar métodos alternativos al uso de plaguicidas, mediante el desarrollo de nuevos compuestos inductores, cuya aplicación favorezca la adaptación de las plantas frente a situaciones de estrés biótico y abiótico. Los inductores son compuestos que parecen actuar en diversos puntos de las rutas naturales de activación de respuestas de las plantas, pudiendo imitar en parte, o en su totalidad, la activación de los mecanismos biológicos de resistencia.

Los estudios realizados hasta la fecha han demostrado que el ácido hexanoico (Hx), un compuesto natural presente en plantas y frutas, es un inductor de resistencia en tomate frente a necrótrofos (*Botrytis cinerea*) y biótrofos (*Pseudomonas syringae*). Además actúa como un inductor de "priming" ya que predispone a los sistemas de defensa de la planta para que respondan de forma más rápida y eficaz frente al posible ataque de patógenos.

Basándonos en estos antecedentes y con el objeto de estudiar la eficacia y el mecanismo de acción del ácido hexanoico en cítricos cultivo de interés agronómico en la Comunidad Valenciana, planteamos realizar este trabajo.

Uno de los graves problemas a los que se ha enfrentado en los últimos años la agricultura de esta zona, ha sido erradicar el hongo necrótrofo *Alternaria alternata* pv *citri*. Este organismo, capaz de atacar a diversas variedades de cítricos en especial a la variedad 'Fortune', ha llegado a disminuir la producción en más de 120000 t/año. Por ello, uno de los objetivos parciales de este trabajo ha sido estudiar el efecto del inductor (Hx) en plantas de la variedad 'Fortune' frente al ataque de *A. alternata.* Se ha demostrado la efectividad de este compuesto al aplicar una concentración de 1mM a las plantas al haberse reducido la incidencia de la enfermedad en más de un 50%. Además, se ha observado que la deposición de callosa se incrementa así como la expresión de los genes de proteínas inhibidoras de poligalacturonasas (implicadas en la resistencia frente a patógenos) en plantas infectadas y tratadas con Hx. El análisis hormonal ha revelado que la ruta del ácido jasmónico (JA) parece que también está implicada en el mecanismo de acción, ya que se ha observado una mayor acumulación de compuestos

implicados en esta ruta. Asímismo, los resultados han demostrado un rápido incremento de los niveles de ácido abscísico (ABA), que podría actuar como un regulador positivo de la deposición de callosa.

Otro de los objetivos de este trabajo, ha sido estudiar la eficacia del inductor (Hx) a largo plazo en plantas de la variedad 'Fortune' y ver cómo responden frente al ataque de *A. alternata*. Los resultados obtenidos han mostrado que, tras la segunda inoculación, se mantiene e incluso se aumenta la protección frente al patógeno en un 20%, disminuyendo tanto el número de hojas infectadas como el tamaño de la infección. Sin embargo, la respuesta hormonal tras la segunda inoculación, no ha mostrado diferencias significativas entre plantas tratadas y no tratadas, mientras que se ha observado un aumento de los compuestos fenólicos en plantas tratadas. Esto hace pensar que en el mecanismo de protección mediado por Hx también están implicados estos compuestos.

Aunque en la actualidad en España el patógeno *Xanthomonas citri* subsp. *citri* no es un grave problema para nuestra agricultura, si lo es para ciertos países tales como Estados Unidos o Brasil. Por ello, nos planteamos estudiar el efecto del inductor (Hx) frente a este biótrofo. La aplicación del Hx en naranjo dulce redujo la infección en más de un 50% cuando se infectaron con *X. citri*, prolongándose este efecto hasta 50 días después de la aplicación del inductor. Frente a este patógeno, el ácido hexanoico actúa activando la expresión de los genes *PR2* y *AOS*, así como la deposición de callosa. Estos resultados muestran la eficacia del inductor frente a diferentes patógenos (biótrofos y necrótrofos) siendo su mecanismo de acción diferente dependiendo de la estrategia de patogenicidad.

Con objeto de profundizar en el mecanismo de acción del inductor en plantas de cítricos frente a *Alternaria alternata*, se ha realizado un estudio del perfil metabolómico. Los análisis han revelado que en todas las condiciones estudiadas (control, tratadas con Hx, infectadas y tratadas e infectadas) se observan cambios en este perfil.

Asimismo, el análisis fisiológico ha revelado que no sólo se modifican los parámetros, tales como fotosíntesis, transpiración y contenido de clorofilas, sino que incrementan sus niveles, lo que nos indica que la planta no está haciendo un gran gasto energético, cuando se aplica el inductor.

Siguiendo esta línea, para conocer la localización del inductor en las plantas de cítricos tras el tratamiento, se utilizó ácido hexanoico marcado con ^{13}C. Los resultados han mostrado que el inductor no se moviliza hacia las hojas sino que se acumula a nivel radicular.

Por otro lado, se ha demostrado que la aplicación del ácido hexanoico induce la emisión de compuestos volátiles en plantas de cítricos infectadas con *A. alternata.* Por primera vez, se ha observado que el inductor Hx promueve la emisión de derivados del geranil difosfato provenientes de la ruta del ácido mevalónico y ácido linoleico en plantas infectadas y tratadas. Además, por los resultados previos encontrados, determinando las masas exactas de los compuestos aguas arriba de estos dos metabolitos, nos ha hecho proponer la hipótesis de que el precursor de ambos puede estar cerca del Acetil CoA. Sin embargo, se deben realizar más estudios para demostrar esta hipótesis.

De todos estos resultados podemos concluir que el ácido hexanoico actúa como inductor de priming protegiendo a las plantas de cítricos tanto frente al necrótrofo *A. alternata* como al biótrofo *X. citri*. En ambos casos está activado el mecanismo de protección mediado por callosa y la ruta del ácido jasmónico. Además a largo plazo el inductor (Hx) activa otros mecanismos de acción en los que están implicados los compuestos fenólicos, siendo específico para el biótrofo *X. citri* la activación de los mecanismos de defensa mediados por ácido salicílico. Por último, se concluye por primera vez, que en el mecanismo de resistencia inducida por ácido hexanoico pueden estar implicados compuestos volátiles.

Abbreviations

Abbreviations

2-DDG, 2-deoxy-D-glucose
ABA, abscisic acid
ABS, alternaria brown spot
ACT, alternaria citri toxin
AM, arbuscular mycorrhiza
AOS, allene oxide synthase
AP, apetala
ASM, acibenzolar-S-methyl
AzA, azelaic acid
BABA, β-aminobutyric acid
BTH, benzo-(1,2,3)-thiadiazole-7-carbotionic acid S-methyl ester
DA, dehydroabietinal
DAB, diaminobenzidine
CalS1, callose synthase 1
Cfu, colony forming units
CHS, chalcone synthase
CTV, citrus tristeza virus
ERF, ethylene response factor
ESI, electrospray ionization
ET, ethylene
ETI, effector-triggered immunity
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GC, gas cromatography
HIR, herbivore-induced resistance
HLB, huanglongbing
HPL, hydroperoxide lyase
HPLC, high performance liquid chromatography
HR, hypersensitive response
Hx, hexanoic acid
Hx-IR, hexanoic acid-induced resistance
I3CA, indol-3-carboxilic acid
ICS, isochorismate synthase
IMID, imidacloprid
INA, 2,6-dichloroisonicotinic acid
IR, induced resistance
ISR, induced systemic resistance
JA-Ile, jasmonic-isoleucine
KCC, kasugamycin, cephalexin, chlorothalonil medium
LB, Luria Bertani broth
LC, liquid chromatography
LOX, lipoxygenase
LSD, fisher´s least significant difference test

MeJA, methyl jasmonate
MeSA, methyl salicilate
MS, mass spectrometry
NADPH, nicotinamide adenine dinucleotide phosphate
NBT, nitroblue tetrazolium
OPDA, 12-oxo-phytodienoic acid
ORA, octadecanoid-responsive arabidopsis
PAL, phenylalanine ammonia lyase
PAMP, pathogen (microbe)-associated molecular patterns
PBS, saline phosphate buffer
PCA, principal component analisys
PDA, potato dextrose agar
PDB, potato dextrose broth
PDBe, pee dee belemite
PDF, plant defensin
PGIP, polygalacturonase-inhibiting protein
PIP, pipecolic acid
PLS, partial least square analisys
PR, pathogenesis-related proteins
PTI, PAMP-triggered immunity
QTOF, quadrupole-time of flight mass spectrometer
RD22, responsive to desiccation 22
ROS, reactive oxygen species
RT-qPCR, quantitative real-time PCR
SA, salicylic acid
SAR, systemic acquired resistance
UPLC, ultra-high performance liquid cromatogarphy
UTC, untreated checks
VSP, vegetative storage protein
WUE, water use efficiency
Xcc, *Xanthomonas citri* subsp. *citri*

General Introduction

Importance of citrus

Citrus is one of the world's major fruit crops whose global availability and popularity contribute to human diets. The most well-known examples of commercially important citrus fruits are oranges, lemons, limes, grapefruits and mandarins. Most grown crops are located around the equator and cover tropical and subtropical areas of the world between 35ºN and 35ºS (Liu et al., 2012). In recent decades, annual global production of citrus fruits has strongly and rapidly grown from approximately 57 million metric tons in 1980 to a total estimate of over 122 million metric tons in 2013 (FAO, 2013).

According to the 2013 data of the Food and Agriculture Organization of the United Nations (FAO), China, Brazil, the USA, India, Mexico, and Spain are the world's leading citrus fruit-producing countries, which represent around 70% of global production. Among the four major varietal groups (oranges, mandarins, lemons/limes and grapefruits), world production is dominated by oranges with 73 million tons (60%), followed by mandarins with 26.5 million tons (21%), lemons/limes with 14.5 million tons (12%) and finally by grapefruits with 8.5 million tons (7%). Although many citrus fruits, such as oranges, tangerines and grapefruit, can be eaten fresh, about one third of the worldwide citrus fruit production is utilised after processing mostly for juice production. The main countries involved in juice production are the USA and Brazil, which produce more than 85% of the world market. More than 90% of world mandarin production is destined to fresh consumption (USDA, 2013). The citrus production of Mediterranean countries is destined for the fresh fruit market, and 3.3 million tons of mandarins were exported in 2011 (FAO, 2012). Clementine mandarins and related fruits from Spain and Morocco dominate the easy-peelers category (Ladanyia, 2007). Spain has a surface of 317,605 ha cultivated with citrus, of which 287,620 are productive. The main crops grown are oranges (48%) and mandarins (38%). Spain exports 57% of citrus fruit production. In the mandarin group, 70% of production is clementines, and 81% of production is navel oranges in the orange group. The Valencia region (east Spain) is the first Spanish citrus producer with 3.5 million tons and a cultivated surface of over 170,000 ha, which represents 56% of total Spanish citrus production (MAGRAMA, 2013).

Current landscape of citrus pests

One of the main problems of this crop is the costs incurred to manage exotic citrus diseases that become endemic or established in the citrus industry. The worldwide orange industry faces more than 100 different pests (García-Marí, 2009; Dreistadt, 2012). Not all diseases and pest are present in all citrus-growing

areas, and quarantine policies avoid their worldwide spread. They can almost all be controlled with chemical, biological or cultural measures to minimise damage to fruits and trees. The appearance of a new disease in a given area can change the entire crop basis. One example is citrus tristeza virus (CTV), which spread to all citrus areas in the 20th century, caused losses of 100 million trees and entailed having to renew the entire crop. This virus disease forced a change in the sour orange rootstock, which is very sensitive to the virus, to tolerant rootstocks of poorer agronomic quality. In this way, the spread of new diseases continuously changes crop management, which implies sharp increases in production costs.

One example of this situation is 'Fortune' mandarin. This variety was one of the most important late maturing cultivars in Spanish citrus regions, with production over 120,000 tons per year. Since the appearance of *Alternaria alternata* in Spain in 1998 (Vicent et al., 2000), this disease has proven to be a limiting factor for the production of this mandarin. Production has declined annually by about 20% and is almost exclusively due to the difficulty to control this fungus. The symptoms that this fungus causes are brown and black spots of various sizes, subsequently surrounded by yellow halos (Figure 1). Currently, fungicides are commonly used to control this disease. In Spain, there are only two compounds used to control this fungus: copper compounds (copper oxychloride, cuprocalcic sulphate, etc.); or dithiocarbamate-based fungicides (Maneb or Mancozeb) (MAGRAMA, 2014).

Figure 1. Detail of the lesions caused by *Alternaria alternata* (Source, the author)

However due to lack of effectiveness, these products must be sprayed several times, sometimes as many as 12 applications or more, per cultivation cycle (de Souza et al., 2009; Vicent et al., 2009). Even after several spray applications, quite often the pathogen cannot be completely controlled, and the use of susceptible cultivars becomes impractical. Concern about the incidence of this fungus is so

serious that new varieties must prove to be resistant before being released (Cuenca et al., 2010).

New diseases have appeared in recent years and have caused huge economic losses all over the world. Two examples are citrus canker, caused by *Xanthomonas citri* subsp *citri* (Xcc), and HLB associated with "*Candidatus* Liberibacter asiaticus". These two pests affect citrus orchards in North and South America and imply having to spend hundreds of millions of dollars on prevention and eradication plans. Xcc is a biotrophic bacterial phytopathogen and is one of the most severe in terms of economic loss (Khalaf et al., 2011). Susceptibility to citrus canker disease varies among citrus types and relatives, but most commercially grown citrus types are susceptible hosts to Xcc. Disease symptoms include canker lesions on green aerial plant parts and fruits; infections can result in both foliar and fruit abscission and, therefore, reduce the productivity of affected trees (Figure 2). Profitability can also lower as a result of blemished fruit that can be harvested, but not sold on the fresh market (Gottwald et al., 2002). At present, the primary disease management approach is to use chemicals like copper-containing bactericides (Das, 2003). Nevertheless, copper reduces bacterial populations on the leaf surface, but correct control demands multiple applications, which not only increase management costs, but also raise the environmental contamination issue (Graham and Myers, 2013). Disadvantages of long-term use of copper bactericides include induced copper resistance in xanthomonad populations (Behlau et al., 2012). Therefore, other preventive measures to be used as an effective supplementation for copper-based disease control are required.

Figure 2. Detail of the lesions caused by *Xanthomonas citri* (Source, the author)

Currently, one of the most severe diseases to affect citrus trees is HLB. This disease is associated with a bacterium that affects all citrus cultivars. From the genus *"Candidatus* Liberibacter asiaticus*"*, this phloem-limiting, gram-negative bacterium inhibits the flow of nutrients throughout the tree, causes decreased fruit

production, and ultimately the tree dies. Hodges and Spreen (2012) estimated that a 23% reduction in orange production during the 2006-2010 period was attributed to the presence of HLB. Symptoms may not be exhibited in infected trees until 2 years afterwards. To date, no compound or cultural method has been identified to control HLB and to stop it from spreading to new citrus production areas (Wang and Trivedi, 2013). Nowadays, citrus canker and HLB are present in the worlds' principal citrus-producing countries, but have not been reported in Europe, where they are considered a quarantine organism (EPPO, 2012). The international strategies adopted by the European and Mediterranean Plant Protection Organization mean that it is possible to avoid the introduction and spread of new pests that damage cultivated and wild plants in natural and agricultural ecosystems. Thanks to such regulations, transporting live material is restricted and incoming sources of pests and diseases are avoided (Brunel et al., 2013).

Induced resistance, a general approach.

The different biotic and abiotic stresses that plants are subjected can lead to alterations in the physiological state which, in the species of agronomic interest, can provoke major reductions in yield and crop quality. Faced with these situations, plants must react specifically thanks to their immobility. For this reason, primary defenses have been developed to face the biotic and abiotic stresses that can appear in the environment. The first of these barriers is passive protection, provided by the waxy cuticle and the accumulation of antimicrobial compounds, such as saponins, piretrines and other secondary metabolites (Osbourn, 1996). However, plants have also developed sophisticated defense mechanisms to perceive pathogen attacks and to translate that perception into an adaptive response.

The immune response of plants starts by recognising the pathogen. The sequence of responses was described by Jones and Dangl (2006) in the 'zigzag' model. The plant's reaction starts by recognising pathogen (microbe)-associated molecular patterns (PAMPs). PAMPs are recognised by pattern-recognition receptors, which leads to PAMP-triggered immunity (PTI) that can delay or stop infection. Some pathogens are able to release effectors that interfere with PTI, which results in effector-triggered susceptibility. However, these effectors can be recognised by plant R proteins, which results in effector-triggered immunity (ETI). ETI is an amplified PTI response that results in disease resistance and, usually, in a hypersensitive cell death response (HR) at the infection site. Afterwards, natural selection allows some pathogens to avoid ETI by shedding or diversifying the recognised effector gene, or by acquiring additional effectors that suppress ETI.

Upon pathogen recognition, the speed and the accuracy with which the plant cell can mobilise its defenses often determine if the plant can resist attack (Pastor et al., 2014). The time elapsed between pathogen recognition and the induction of defense responses might actually be the difference between resistant and susceptible plants (Hammond-Kosack and Parker, 2003).

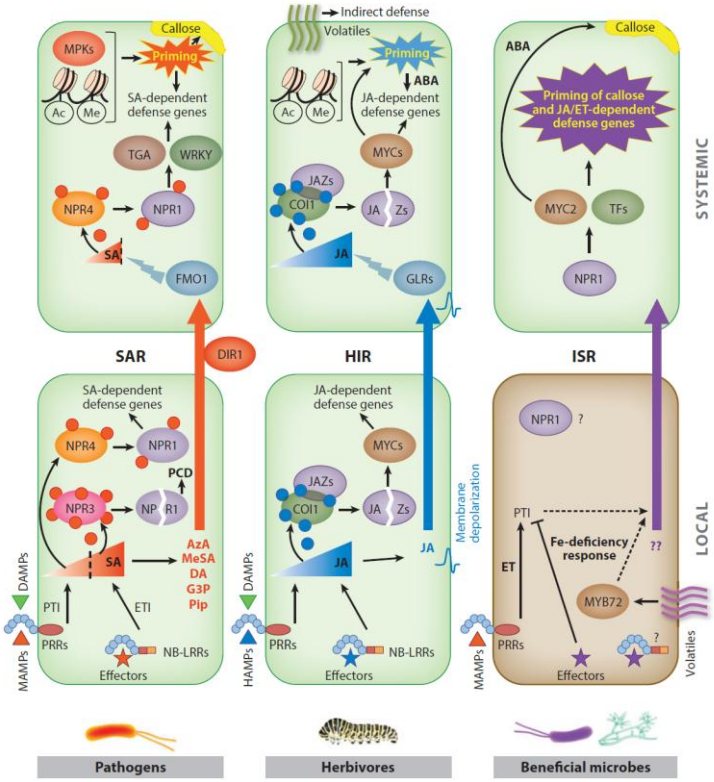

Figure 3. Schematic representation of the molecular components and mechanisms involved in pathogen-induced systemic acquired resistance (SAR), herbivore-induced resistance (HIR), and induced systemic resistance (ISR) triggered by beneficial soil-borne microbes. (Source, Pieterse et al. 2014)

Induced resistance triggered by pathogens can be divided depending on pathogen type: systemic acquired resistance (SAR), herbivore-induced resistance (HIR) and induced systemic resistance (ISR) (Figure 3). The SAR term is often associated with the resistance that uninfected systemic plant parts can acquire in response to localised infection elsewhere in the plant (Ryals et al., 1996), but it also defines the resistance that is dependent of salicylic acid (SA) accumulation

and resistance against biotrophic pathogens. SA is a phytohormone synthesised by either the phenylalanine ammonia lyase (PAL) pathway or isochorismate synthase (ICS) (Wildermuth et al., 2001). This molecule accumulates after pathogen infection, which leads to a hypersensitive response (HR) that inhibits the growth of challenge pathogens (Ryals, et al., 1996; Dempsey et al., 1999). SA is required in SAR to induce the expression of several pathogenesis-related proteins (PR), such as PR1, chitinases (PR3) and glucanases (PR2) (Sticher et al., 1997).

Despite SA accumulating in the phloem of plants, the experiments by Vernooij (1994) with tobacco performed demonstrated that SA is not the systemic SAR signal. However, recent studies have revealed several metabolites that may be involved in long-distance SAR signalling, such as the methyl ester of SA (MeSA), dehydroabietinal (DA), azelaic acid (AzA) and pipecolic acid (Pip) (Dempsey and Klessig, 2012; Shah and Zeier, 2013).

ISR is a defensive response triggered by the action of non-pathogenic microbes (Alstrom, 1991; Vanpeer et al., 1991). It is widely known that this mechanism occurs with no accumulation of PR proteins, the main characteristic of SAR (Pieterse et al., 1996; Ton et al., 2002). Experiments with *Pseudomonas fluorescens,* and *P. putida* have evidenced an SA-independent signalling pathway, which suggests that ISR and SA-dependent SAR are regulated by different signals (VanWees et al., 1997; Pieterse et al., 2000). These differences were the basis to refer to SAR when induced resistance is SA-dependently triggered, and also to ISR when induced resistance is SA-independent (Pieterse et al., 2012; 2014).

The resistance pathway independent of SA is mediated by jasmonic acid (Figure 4). The two major JA signalling branches are the MYC branch and the ERF branch. The MYC branch is controlled by basic helix-loop-helix leucine zipper proteins MYC2, 3 and 4 (Fernández-Calvo et al., 2011; Niu et al., 2011) and a synergistic action of abscisic acid that antagonises the ERF branch (Anderson et al., 2004). This branch is activated in response and defense to wound-provoked insect herbivores (Kazan and Manners, 2012) and includes JA-responsive marker gene vegetative storage protein2 (VSP2). The ERF branch is regulated by members of the apetala2/ethylene response factor (AP2/ERF) family of transcription factors, such as ERF1 and octadecanoid-responsive arabidopsis59 (ORA59) (Dombrecht et al., 2007; Pré et al., 2008), and includes JA-responsive marker gene plant defensin1.2 (PDF1.2). The ERF branch of the JA pathway is associated with enhanced resistance to necrotrophic pathogens (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003), whereas the MYC branch is associated with response and defense against insects. However, it has been demonstrated that MYC2 plays a role in induced resistance against pathogens (Pozo et al., 2008).

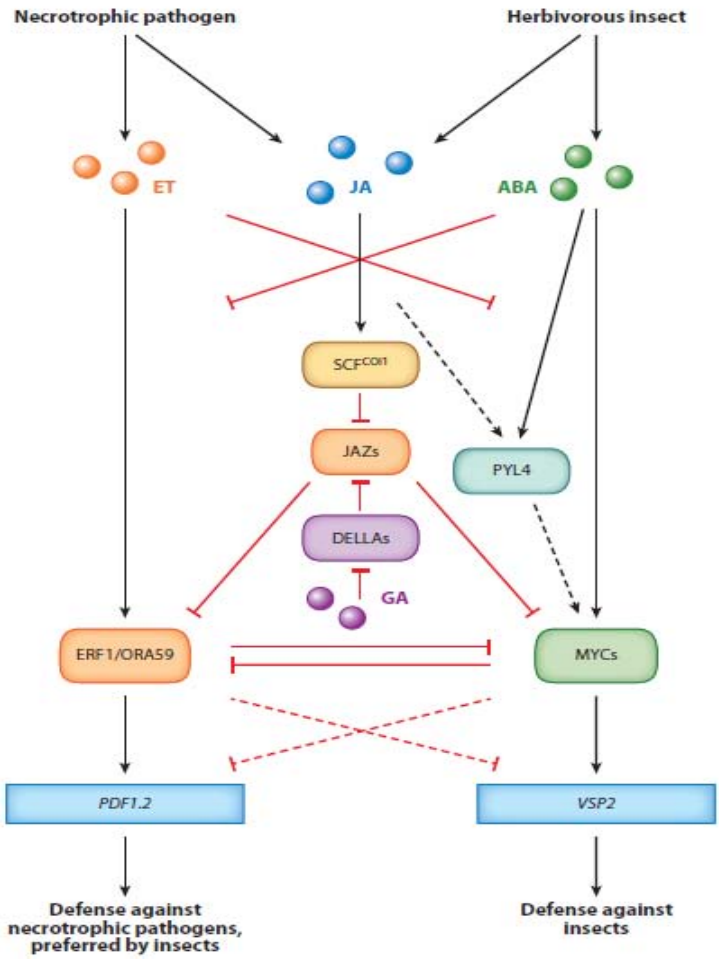

Figure 4. Modulation of the JA signalling pathway by ET, ABA, and GA. (Source, Pieterse et al. 2012)

In response to wounding, in plant leaves the systemic accumulation of proteinase inhibitors occurs which inhibit digestive enzymes in the insect gut, and the long-distance signals produced at the tissue injury site mediate a systemic resistance against herbivorous insects (Howe and Jander, 2008; Wu and Baldwin, 2010). Along with the production of anti-insecticidal toxins and feeding deterrents, the herbivory also triggers the production of volatiles that attract natural predators of the attacking herbivore (Dicke and Baldwin, 2010). These leafy volatiles originate in the hydroperoxide lyase (HPL) branch of the oxylipin pathway (Matsui, 2006). Practically all green plants are able to produce and release volatiles against different stresses, such as abiotic stresses, herbivores or

pathogens (Turlings et al., 1995; Heiden et al., 2003; Halitschke et al., 2004; Brilli et al., 2011).

It is well-known that exposure of plants to some stresses can induce a sensitisation state of the whole plant for enhanced defense, characterised by a faster and stronger activation of cellular defenses upon invasion. This state is known as priming of defense (Goellner and Conrath, 2008; Conrath, 2009; Jung et al., 2009; Pastor et al., 2013).

Application of certain chemicals can deliver a priming response, which is often more consistent and less variable than that produced by natural agents. (Oostendorp et al., 2001). Chemical priming inducers can be divided into xenobiotic or endogenous compounds. Among xenobiotic compounds, β-amino butyric acid (BABA), the Benzothiadiazol (BTH, ASM) and 2,6-Dichloroisonicotinic acid (INA) are the most widely studied. Application of these resistance inducers can protect plants against a broad spectrum of diseases (Jakab et al., 2001; Conrath, 2009), including some crop diseases that are difficult to control by conventional disease management strategies. Unlike INA and SA, ASM is sufficiently tolerated by most crops. Therefore, the compound is appealing for practical agronomic use. In 1996, ASM was introduced as a ‘‘plant activator’’ under trade names Bion®, Actigard® or Boost® (Ruess et al., 1996).

The other group includes endogenous plant compounds, or functional analogues, which are synthesised by the plant in response to biotic stress signals; e.g., SA (Kauss and Jeblick, 1995), JA (Frost et al., 2008) and azelaic acid (Jung, et al., 2009). There are other priming agents produced only by several plants or microorganisms, like hexanoic acid (Hx). This monocarboxylic acid is a natural compound produced by strawberry (Zabetakis et al., 2000) and *Arbutus unedo* (Soufleros et al., 2005). It is also detected in butter and butter oil (Peterson and Reineccius, 2003) and in cheeses (Morales et al., 2006), and contributes to their aroma, thus supporting that the toxicity of this compound at low concentrations is lacking. In the last years, some studies reported that Hx is able to induce resistance in *Solanum lycopersicum* and *Arabidopsis thaliana* plants against *Botrytis cinerea* (Vicedo et al., 2009; Kravchuck et al., 2011). The resistance against this fungus is mediated by callose accumulation and by the activation of the synthesis of OPDA and bioactive signal JA-Ile (Vicedo et al., 2009). On the other side, these authors demonstrated that Hx induces resistance against *P. syringae*. Hx treatment has also been reported to potentiate the SA response and 12-oxo-phytodienoic acid accumulation upon *P. syringae* infection, and to diminish that of JA-Ile (Scalschi et al., 2013).

Although most of the research conducted into the effectiveness of priming agents has been studied in model plants such as *Arabidopsis taliana* (Pieterse, et al., 2000; Jakab et al., 2005; Fernández-Calvo, et al., 2011; Luna et al., 2011; Kravchuk et al., 2011), some studies have been performed in crop plants; e.g. tomato and pepper (Flors et al., 2001; Achuo et al., 2004; Flors et al., 2007; Leyva et al., 2008; Vicedo et al., 2009; Scalschi et al., 2013), apple (Ortega et al., 1998; Brisset et al., 2000; Macarisin et al., 2009; Yi et al., 2012), pear (Faize et al., 2004; Yu et al., 2014), and several reviews about induced resistance in crops have been published (Vallad and Goodman, 2004; Walters et al., 2008; Walters and Fountaine, 2009; Ahmad et al., 2010; Gozzo and Faoro, 2013). In the last few years, some studies about the efficacy of priming agents have been tested in citrus crops. These works have suggested that the application of different types of resistance inducers may be effective as a pest control and can be a realistic alternative to classical means.

Induced resistance in citrus

The majority of studies about resistance inducers in citrus have focused on protection against *Xanthomonas citri* (Francis et al., 2009; Graham and Myers, 2011; Graham and Myers, 2013). In recent years however, the potential of these compounds has been tested against other devastating diseases such as HLB, its vector *Diaphorina citri* or phytophtora.

ASM has been extensively evaluated as a component for plant disease control in the field (Vallad and Goodman, 2004; Walters and Fountaine, 2009). This functional analogue of SA provides a non-insecticidal option to neonicotinoids with a low risk of movement below the root zone into the soil profile. Recently in field studies, Graham et al. (2011) demonstrated that this compound can be effective to alleviate citrus canker effects if combined with copper or other bactericides. The intensive use of foliar-applied ASM resulted in stunted growth and yield when used in tomato or pepper crops (Louws et al., 2001; Romero et al., 2001). This reduction has been attributed to the physiological cost of constitutive induction of plant defense (van Loon et al., 2006; Walters and Fountaine, 2009). In greenhouse trials with citrus, ASM caused mild leaf chlorosis, but symptoms were temporary and no such symptoms were observed in field trials (Francis, et al., 2009). Similar protective results have been obtained with applications of INA and insecticide Imidacloprid. Recent studies have revealed that the expression of the PR protein (β -1,3 glucanase) gene, *PR-2*, in citrus increased in response to ASM and isonicotinic acid (INA). Insecticide imidacloprid (IMID) broke down *in planta* into 6-chloronicotinic acid, an analogue of INA which induced the SAR response

(Ford et al., 2010; Sur and Stork, 2003). In a greenhouse pot trial, Francis et al., (2009) confirmed that soil drenches of IMID, and of INA and ASM, induced a high and persistent up-regulation of the *PR-2* gene expression, which correlated with fewer canker lesions for up to 24 weeks.

Non-protein amino acid, β-aminobutyric acid, is perhaps the most studied resistance inducer. This compound has been reported to be a powerful inducer of resistance against abiotic stress (Jakab, et al., 2005; Zimmerli et al., 2008), nematodes (Oka et al., 1999), insects (Hodge et al., 2005) and microbial pathogens (Jakab, et al., 2001; Conrath, 2009). Applications in citrus have demonstrated that all three developmental stages of HLB vector *D. citri* were negatively impacted by BABA through the induction of host-plant resistance in citrus. Foliar applications significantly reduced the number of eggs, nymphs and adults per plant. Tiwari et al. (2013) demonstrated that the *PR-2* gene was up-regulated by more than 150-fold in the citrus crops treated with BABA in combination with *D. citri* adult feeding compared to the control or citrus crops treated with BABA or *D. citri* feeding alone. This result is an alternative tool for current *D. citri* management programmes based exclusively on insecticides. Other studies have also revealed the efficacy of BABA against *Xanthomonas citri* in key lime, but the implication of PR proteins remains unclear (Beheshti et al., 2011).

Very few of the natural compounds that induce resistance in citrus have been tested in citrus crops. A study investigated a 3-day SA application which resulted in attenuated citrus canker disease in *Citrus sinensis*, a highly susceptible cultivar (Wang and Liu, 2012). These authors observed one effect of SA, which they attributed to the signalling role it plays when activating the key enzymes and *PR* genes involved in the defense response. This activation of defensive responses provides protective barriers against Xcc ingress. SA also promotes stomatal closure by raising the H_2O_2 level, which limits bacterial entry into host tissues.

Arbuscular mycorrhiza (AM) symbiosis usually confers better plant growth, higher nutrient uptake, greater tolerance to abiotic and biotic stresses, and an improved soil structure in the host plant. Several studies have demonstrated that AM-inoculated citrus plants show greater tolerance to drought stress or salt-affected soils (Nadeem et al., 2013; Wu et al., 2013). Abiotic stress strongly restricted both the development of non-AM-citrus and AM-citrus mycorrhizal development, but AM colonisation had a positive effect on plant growth and photosynthesis, even in drought or salinity stress situations. The colonisation of roots with arbuscular mycorrhiza can also induce greater plant resistance to some pathogens. The studies by Watanarojanaporn et al (2011) demonstrated that the use of a certain AM improves both citrus growth and *Phytophtora* tolerance.

However, the recent studies of Graham et al (2012) concluded that the protection of citrus roots against infection by *Phytophthora spp*. by hypovirulent *P. nicotianae* is not related to induced systemic acquired resistance.

Enhanced resistance in plants is not restricted to the soil or foliar applications of inducers. Novel studies about nutrition in citrus plants have demonstrated that ammonium-based fertilisation can also induce resistance in trees. Fernández-Crespo et al (2012) observed that NH_4^+ treatments induced a mild stress condition which primed the citrange Carrizo defense response by stress imprinting and conferred protection against subsequent salt stress.

Current and possible uses in the field

Despite all the research conducted on induced resistance in citrus, applications in orchards is not a common practice. Although the protection conferred by inducers is often incomplete, its use as preventive compounds or its combination with classical pesticides can be a valuable alternative to classical treatments. One of the most important problems of abusing treatments is the appearance of resistant strains. Copper-resistant strains of Xcc have been identified in citrus groves in Argentina (Behlau et al., 2013). In a previous evaluation, ASM proved particularly useful for the management of bacterial speck and bacterial spot where copper-resistant strains predominated (Louws, et al., 2001). Therefore, soil-applied SAR inducers can be employed for copper-resistance management as they lower the rate and reduce the frequency of copper bactericide applications.

The current results also suggest the potential of investigating other commercially available SAR-inducing products, perhaps in combination with other pesticides, to achieve improved citrus against insect pests and pathogens (Edreva, 2004). The use of natural endogenous compounds, such as Hx, implies improved resistance against pests with no chemical residues in fruits. The correct treatment schedule that includes an alternation between these compounds and classical ones can be used to cut both pest damage and chemical residues. Utilising nutritional compounds, like NH_4^+ (Fernández-Crespo et al., 2012) or colonisation with arbuscular mircorhiza (Watanarojanaporn et al., 2011), is also an interesting alternative to preventive and curative treatments. By adopting these strategies, plants can achieve enhanced basal resistance that is permanently primed against possible stresses.

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Objectives

OBJECTIVES

In recent years, worldwide agriculture is claiming for safer pesticides with lower residues and lower impact in the environment. The development of resistance-inducing molecules represents an attractive alternative to protect crops against pathogens. But the largest part of the studies about effectiveness and mode of action of these compounds are performed only in model plants. The effectiveness of these compounds in crops against pests that suppose real problems, is barely tested. For these reasons the goal of this research was to study the mode of action of hexanoic acid in citrus plants and the analysis of the changes produced in the plants. The specific objectives were:

1. To study the mechanisms behind hexanoic acid induced resitance in 'Fortune' mandarin plants against the necrotrophic pathogen *Alternaria alternata* (Chapter 1).

2. To check the long lasting effect of protection and the response of the primed plants against a second challenge inoculation with *Alternaria alternata* (Chapter 2).

3. To study the effect of the hexanoic acid as a resistance inductor against bacterial disease citrus canker, caused by *Xanthomonas citri* subsp. *citri* (Chapter 3).

4. To unravel the metabolic changes involved in the resistance mediated by the hexanoic acid in citrus plants (Chapter 4).

Chapter 1

Enhancement of the citrus immune system provides effective resistance against Alternaria brown spot disease

ABSTRACT

In addition to basal defense mechanisms, plants are able to develop enhanced defense mechanisms such as induced resistance (IR) upon appropriate stimulation. We recently described the means by which several carboxylic acids protect Arabidopsis and tomato plants against fungi. In this work, we demonstrate the effectiveness of hexanoic acid (Hx) in the control of Alternaria brown spot (ABS) disease via enhancement of the immune system of 'Fortune' mandarin.

The application of 1 mM Hx in irrigation water to 2-year-old 'Fortune' plants clearly reduced the incidence of the disease and led to smaller lesions. We observed that several of the most important mechanisms involved in induced resistance were affected by Hx application. Our results demonstrate enhanced callose deposition in infected plants treated with Hx, which suggests an Hx priming mechanism. Plants treated with the callose inhibitor 2-DDG were more susceptible to the fungus. Moreover, *polygalacturonase-inhibiting protein* (*PGIP*) gene expression was rapidly and significantly upregulated in treated plants. However, treatment with Hx decreased the levels of reactive oxygen species (ROS) in infected plants. Hormonal and gene analyses revealed that the jasmonic acid (JA) pathway was activated due to a greater accumulation of 12-oxo-phytodienoic acid (OPDA) and JA along with a rapid accumulation of JA-isoleucine (JA-Ile). Furthermore, we observed a more rapid accumulation of abscisic acid (ABA), which could act as a positive regulator of callose deposition. Hence, our results support the hypothesis that both enhanced physical barriers and the JA signalling pathway are involved in hexanoic acid-induced resistance (Hx-IR) to *A. alternata*.

INTRODUCTION

Alternaria brown spot (ABS) is an important disease in many types of tangerines and their hybrids that is caused by the necrotrophic fungus *Alternaria alternata*. This pathogen can affect leaves, twigs, and immature fruit (Pegg, 1966; Canihos et al., 1999) causing decreased productivity and complete loss of commercial value in infected fruits (Peever et al., 2004). The 'Fortune' mandarin is one of the most important late-maturing cultivars in Spanish citrus regions, and its production has reached over 120 000 tonnes per year. The appearance of *Alternaria alternata* in Spain in 1998 has proven to be a limiting factor in the production of 'Fortune' mandarin (Vicent et al., 2000), and the annual decrease in fruit production is almost exclusively due to the difficulty in controlling this fungus.

Alternaria alternata causes brown spots in various citrus cultivars due to the production of a host-selective ACT toxin (derived from *Alternaria citri* Tangerine toxin) (Kohmoto et al., 1993). This toxin damages the cell membrane and induces electrolyte leakage from citrus cells resulting in necrotic lesions (Wang et al., 2011). Moreover, the ACT toxin is primarily active in "Dancy" mandarin and its hybrids (such as the 'Fortune' mandarin) and in tangerine/grapefruit and tangerine/sweet orange hybrids (Vicent et al., 2009; Wang, et al., 2011).

Disease symptoms in the form of brown and black spots of various sizes appear up to 24 h after the infection begins, and these spots are subsequently surrounded by yellow halos caused by the specific toxin. The host-selective toxin is released by conidia during germination and rapidly affects susceptible tissues (Canihos et al., 1999). This toxin is sometimes translocated through the vascular system producing chlorosis and necrosis, which extends outwardly along the veins. Lesions typically continue to expand, and a large amount of foliar tissue can be destroyed (Vicent et al., 2004; Reis et al., 2007). Currently, fungicides are commonly used to control this disease. In Spain, there are only two compounds used to control this fungus: copper compounds (copper oxychloride, cuprocalcic sulphate, etc.) or dithiocarbamate-based fungicides (maneb or mancozeb) (MAGRAMA, 2012). However, due to lack of effectiveness, these products must be sprayed several times, and the number of applications sometimes exceeds 12 per cultivation cycle (de Souza et al., 2009; Vicent et al., 2009). Even with the increased number of sprays, the pathogen often cannot be completely controlled, and the use of sensitive cultivars becomes impractical. Thus, it is particularly important to promote alternatives to synthetic chemicals to lower the risks and impacts of pesticide use on human health and the environment.

Non-synthetic treatments have been studied in recent years, and certain essential oils have been shown to exhibit antifungal activity *in vitro*. Carvalho et al. (2011) demonstrated that the application of natural extracts of *Anadenanthera colubrina* to inoculated Murcott fruits reduced infection to levels that were similar to those obtained with commercial fungicides. Another study highlighted the inhibitory effect of *Citrus reticulata* extracts on spore germination when it was applied to the culture medium at 2 mL/L (Chutia et al., 2009). However, the efficacy of these compounds in field applications is still unclear.

Plants have a wide variety of chemical and physical defenses against fungal infections such as those caused by *A. alternata*. Plant cells have developed pre-invasive structural defenses including a cuticular layer and various modifications of the cell wall and the compaction of the extracellular matrix, all of which are crucial for the obstruction of pathogens (Mendgen et al., 1996; van Kan, 2006; Hückelhoven, 2007).

In the second barrier of early post-invasive defenses, pathogens face callose formation as well as the rapid accumulation of reactive oxygen species (ROS) and other enzymes that make cell invasion difficult (Asselbergh et al., 2008). If pathogens overcome these physical barriers, plant defenses are finally triggered by transcriptional reprogramming and corresponding changes in metabolic and hormonal profiles (Anderson et al., 2004; Lorenzo et al., 2004; Yadav et al., 2005). Thus, the plant immune system is controlled by a network of signalling pathways, and the cross-talk between these pathways results in variable responses depending on the challenging pathogens (Glazebrook, 2005; Pieterse et al., 2009). Some of these defensive mechanisms can be induced by certain natural (Leyva et al., 2008; Vicedo et al., 2009) or synthetic (Beckers and Conrath, 2007) compounds.

The application of certain chemicals prepares cells to respond to stress more rapidly and robustly upon subsequent pathogen attack; this phenomenon is referred to as priming of defense (Conrath et al., 2006; Pastor et al., 2013). Plants primed by treatments that induce resistance exhibit a faster and/or stronger activation of defense responses. In recent years, priming-induced activity in plants has been reported in response to many compounds; some of the best characterised of these are 2,6-dichloroisonicotinic acid (INA), benzo-(1,2,3)-thiadiazole-7-carbotionic acid S-methyl ester (BTH), and β-aminobutyric acid (BABA) (Oostendorp et al., 2001; Conrath et al., 2002). Our research group has demonstrated the efficacy of carboxylic acids for protecting tomato plants against *Alternaria solani* and *Phytophthora citrophthora* (Flors et al., 2003). Recently, we found that hexanoic acid (Hx) can protect Arabidopsis and tomato plants against

Botrytis cinerea (Leyva et al., 2008; Vicedo et al., 2009; Kravchuk et al., 2011). This natural short-chain monocarboxylic acid displays antimicrobial activity and can also induce plant defense responses when used as a priming agent. Upon infection, the oxylipin 12-oxo-phytodienoic acid (OPDA) and the bioactive molecule jasmonate-isoleucine (JA-Ile) were significantly induced in treated plants. Additionally, callose deposition was primed, and abscisic acid (ABA) acted as a positive regulator of hexanoic acid-induced resistance (Hx-IR) by enhancing callose accumulation (Vicedo et al., 2009).

Induced resistance (IR) is a relatively new tool that is still not widely utilised for crop protection. Very few studies have been performed to examine the efficacy of priming agents in woody plants and their possible applications in the field. Here, we present evidence that Hx improves the resistance of 'Fortune' mandarin to *Alternaria alternata* and ascertain the possible mechanisms of action of the Hx-primed defense response.

MATERIALS AND METHODS

In vitro antimicrobial activity assays

Alternaria alternata was isolated from the infected leaves of 'Fortune' mandarin and germinated on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 24ºC. The assay mixture contained $4 \cdot 10^6$ conidia/mL of potato dextrose broth (PDB) (Difco, Detroit, MI, USA). Hx was added to PDB from stock solutions to achieve various final concentrations (0, 0.1, 0.5, 1.5, 3, 6, 12, 20 and 30 mM) and the pH was adjusted to 4 or 6 using HCl or NaOH, respectively. The percentage of germinated spores and mycelial growth data were obtained as previously described by Vicedo et al. (2006). In both experiments, four replicates were performed for each treatment.

Plant material and pathogen inoculation

For all experiments, we used two-year-old 'Fortune' mandarin plants grafted onto Carrizo citrange plants and grown in a greenhouse in 10-L pots using perlite as a substrate. One month before the commencement of each experiment, the leaves were removed to encourage uniform sprouting.

Spores were collected from 10- to 15-day-old cultures with sterile water containing 0.02% (v/v) Tween-20. The solutions were then filtered, quantified

with a hemocytometer and adjusted to 10^5 spores/mL. Infection was carried out by dispensing 5 µL of the spore solution onto each leaf.

Determination of treatment concentration with the induction effect

An *in vivo* test was performed by applying 0, 0.5, 1 and 3 mM Hx to roots. Concentrations of less than 3 mM were used according to effects observed in the antimicrobial assay. Four days prior to infection, the plants were treated with Hx. Two days prior to infection, the plants were covered with transparent plastic bags to increase humidity and facilitate infection. Four days after infection, we measured the diameter of necrosis and the number of infected leaves.

Analytical measurements

Callose deposition and the inhibitory effects of 2-deoxy-D-glucose (2-DDG) were evaluated as described by Flors et al. (2007). Counting was performed using Photoshop CS4 software (Adobe).

The quantification of peroxide and superoxide was performed by staining sheets with diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) (Thordal-Christensen et al., 1997; Lin et al., 2009).

RT-qPCR analysis

Gene expression by quantitative real-time PCR (RT-qPCR) was performed using RNA samples extracted from leaf tissue using the E.Z.N.A. Total RNA Kit II (Omega Bio-tek; Norcross, GA. U.S.A; http://www.omegabiotek.com). Citrus leaf tissue samples for RNA isolation were collected at 0, 6, 48 and 96 h post-infection (hpi), and tissue was collected from both treated and non-treated plants. We used the RT-qPCR conditions previously described by Flors et al. (2007). The primers used in the RT-qPCR are listed in Table 1 and were previously described by Fernández-Crespo (2012). *GAPDH* gene expression was used as an internal standard.

Table 1. Primers sequences

Primer name	Forward primer	Reverse primer
GAPDH	5′- ggaaggtcaagatcggaatcaa - 3′	5′ cgtccctctgcaagatgactct -3′
AOS	5′- cgaatttcaatccccaagaa-3′	5′- ttggtgggttgttcatcaga-3′
RD22	5′- ttggaaaaggacttgcatcc-3′	5′- atgccagcgtcttcacactc-3′
PGIP	5′- gttgaacaagacgacgcaga-3′	5′- ccaaagctctgcaactttcc-3′

Chromatographic analysis

The extractions and experimental procedures used in hormone analysis were performed as described by Flors et al. (2008). We analysed the levels of salicylic acid (SA), jasmonic acid (JA), JA-Ile, OPDA and ABA using [$^2H_6$]-ABA, prostaglandin B1, dihydrojasmonic acid and propylparaben as internal standards.

Statistical analysis

Statistical analyses were performed using the Statgraphics software support. The data are expressed as the means ± standard error. Mean values were compared by LSD (least significant difference) test. All experiments were repeated at least three times.

RESULTS

Characterisation of the antifungal activity of hexanoic acid against Alternaria alternata

Alternaria alternata conidia were incubated in the presence of various concentrations of Hx at pH 4 or pH 6. After 20 h of treatment, microscopic analyses of the spores showed a pH-independent reduction in germination when the Hx concentration was increased to 30 mM (Fig. 1a). The germination rate was strongly dependent on the Hx concentration in the medium, and a more pronounced effect was observed at Hx concentrations greater than 3 mM. Statistical analyses indicated that there were significant pH-dependent differences in the spore inhibition potential of Hx. For example, the inhibitory effect of Hx on

spores was 50% higher at lower pH levels when concentrations between 6 and 12 mM were used.

The effect of Hx on hyphal growth was also examined. Mycelial growth was inhibited by Hx treatment in a concentration-dependent manner. The results obtained indicate that mycelial growth was decreased by 50% at an Hx concentration of 3 mM, and at higher Hx concentrations, mycelia were unable to develop (Fig. 1b). Surprisingly, the growth patterns of *A. alternata* differed depending on the pH of the medium. When the spores were germinated at pH 4, the hyphae were much more elongated and less branched.

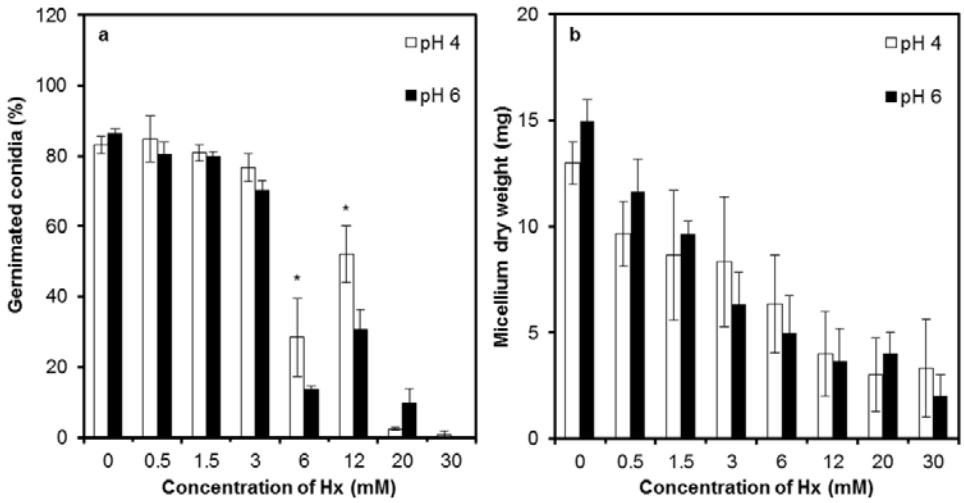

Figure 1. Effect of different Hx concentrations on *A. alternata* germination in a range of Hx values at pH 4 and pH 6. (a) The percentage of germinated spores after 24 h incubation. (b) Mycelial growth. Growth was estimated by measuring mycelia dry weight after 7-day incubation. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. Asterisks indicate a significant difference ($P<0.05$).

Hexanoic acid enhances 'Fortune' resistance to A. alternata infection

Compared with the non-treated control plants, all the Hx treatments resulted in a significantly reduced lesion diameter at day 5 post-challenge inoculation at the three concentrations tested. Compared with the lesions measured in control plants, the 0.5 and 1 mM concentrations reduced the infection diameter by 15% and 55%, respectively. The number of infected leaves was also decreased in treated plants (Table 2), and the ratio of infected leaves to inoculated leaves was 84% at 0.5 mM

and 54% at 1 mM. Additionally, treatment with 3 mM Hx completely inhibited leaf infection. Further analyses of the induction effects of this chemical were performed by treating plants with 1 mM Hx.

Table 2. Effect of different concentrations of hexanoic acid on 'Fortune' plants infected with *Alternaria alternata*. The infection diameter measured on day 4 post-inoculation is expressed in mm. Infected leaves expressed as a percentage. Data show the average of three independent experiments obtained with 10 plants per point ± SE. Different letters in a row represent statistically significant differences ($P < 0.05$).

Concentration	Infection diameter (mm)	Infected leaves (%)
0 mM	8.425 ± 0.287 a	86.36 ± 5.24 a
0.5 mM	7.137 ± 0.316 b	84.54 ± 8.19 a
1 mM	5.168 ± 0.218 c	54.77 ± 10.22 b
3 mM	0 d	0 c

Hexanoic acid treatment primes callose deposition and PGIP response

To assess whether Hx-IR to *A. alternata* was associated with an increase in callose accumulation, cytological observations were performed at the infection sites. We found that Hx significantly increased callose accumulation upon infection. Interestingly, Hx-treated plants showed no callose accumulation in the absence of infection (Fig. 2a). To confirm whether increased callose deposition was related to enhanced resistance to *A. alternata*, we treated plants with 2-deoxy-D-glucose (2-DDG), an inhibitor of callose synthesis (Flors, et al., 2007; Vicedo, et al., 2009). We found that both Hx-treated and non-treated plants showed diminished callose deposition upon treatment with 2-DDG compared with non-inhibited callose plants (Fig. 2b and c).

The infection rate was estimated by measuring the average lesion diameters (Fig. 2d). Treatment with 2-DDG enhanced the susceptibility to *A. alternata* in both Hx-treated and non-treated plants.

To perform an in-depth study regarding the physical barriers involved in Hx-IR the expression level of a polygalacturonase-inhibiting protein (*PGIP*) gene was determined. These ubiquitous plant cell wall proteins counteract the action of fungal polygalacturonase proteins by preventing cell wall degradation and interfering with invasion. We observed that *PGIP* gene expression in infected plants was upregulated more rapidly in Hx-treated vs. control plants (Fig. 3).

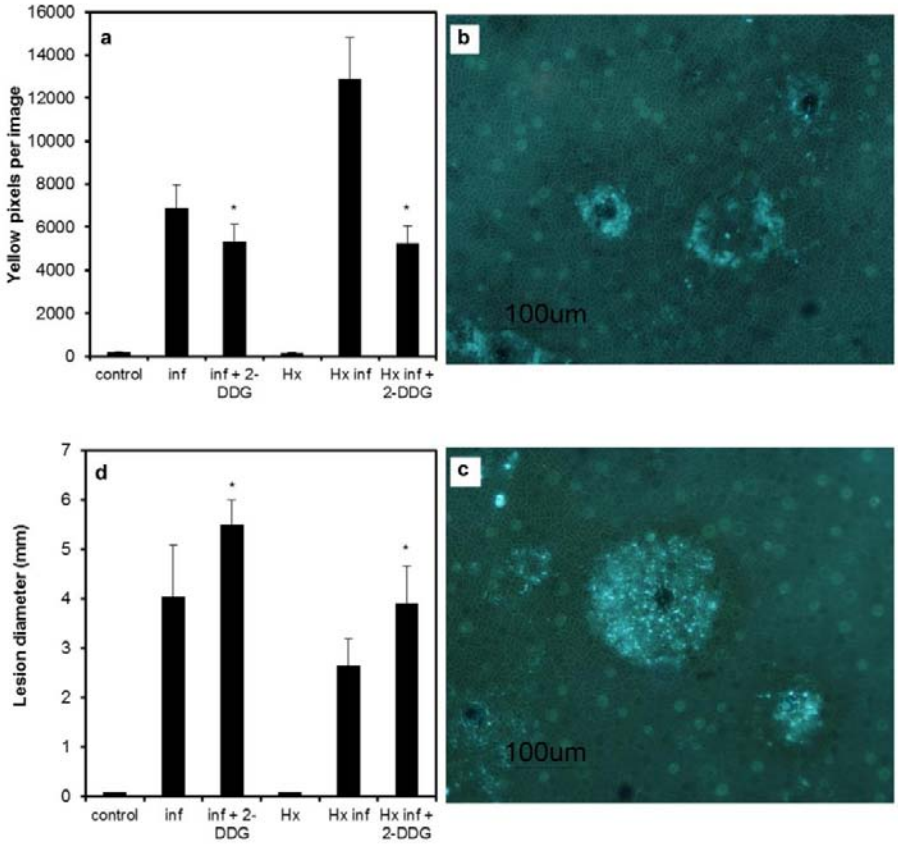

Figure 2. Contribution of callose deposition to hexanoic acid-induced resistance. (a), Callose deposition was measured in the 'Fortune' plants treated with hexanoic acid upon infection with *Alternaria alternata*. Quantification was performed by determining the number of yellow pixels per image corresponding to pathogen-induced callose shown on digital photographs of infected leaves. Data show average values ± standard error (SE) (b), Effect of callose inhibition on hexanoic acid-induced resistance against *A. alternata*. Lesion diameter was measured at 96 h post-inoculation in water- and hexanoic acid-treated plants treated locally with callose inhibitor 2-DDG. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. Asterisks indicate a significant difference ($P<0.05$). Representative images of experiment (c) infected plants (d) treated and infected plants.

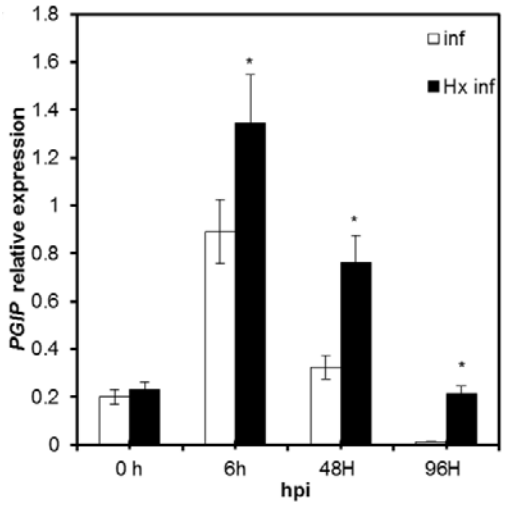

Figure 3. Effect of hexanoic acid treatment on gene expression in 'Fortune' plants upon *A. alternata* infection. Total RNA was isolated from leaves at 0, 6, 48 and 96 h post-inoculation, and was converted into cDNA and subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The relative level of *PGIP* was analyzed in infected, and in treated and infected citrus plants. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. Asterisks indicate a significant difference ($P<0.05$).

Hx reduces oxidative damage and ROS

The accumulation of H_2O_2 in response to *A. alternata* was also determined using 3,3-diaminobenzidine (DAB) as a substrate. The formation of a dark brown insoluble precipitate was observable at 96 h post-inoculation, which was indicative of H_2O_2 accumulation in leaves. Dark brown pigments noticeably surrounded by a distinct brownish ring (also indicative of H_2O_2 accumulation) were observed at all sites inoculated with *A. alternata* after staining with DAB (Fig. 4).

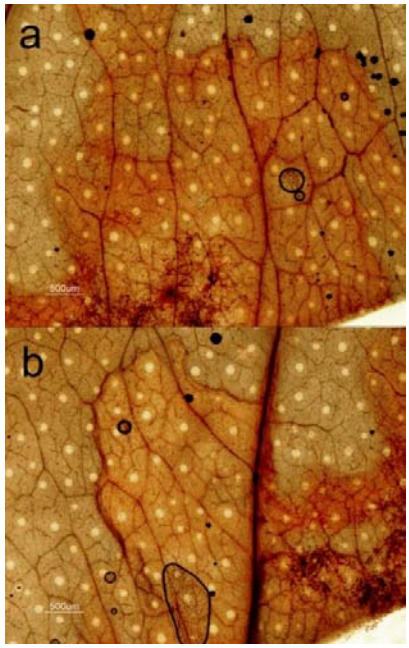

Figure 4. H_2O_2 staining, estimated by using DAB staining in the leaves of the infected and the treated and infected 'Fortune' plants. Images are from a representative experiment that was repeated three times with similar results. (a) infected plants (b) treated and infected plants

Infected plants treated with Hx exhibited fewer dark brown pigments, indicating reduced H_2O_2. As shown in Table 3, the level of peroxide quantified in infected but non-treated plants was 1.8 times higher than that in infected plants treated with Hx. Together with H_2O_2, superoxide ($O_2^{\bullet-}$) radicals are the main ROS generated in cells. Table 3 illustrates the increase in superoxide ions in infected non-treated leaves. This increase was 3-fold higher than that observed in infected plants treated with Hx.

Table 3. Effect of Hx on peroxide and superoxide (the results are expressed in pixels per image). The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. Asterisks indicate a significant difference ($P<0.05$).

	Inf	**Hx inf**	**Sig**
Peroxide	$1.45 \cdot 10^5$	$8.14 \cdot 10^4$	*
Superoxide	$3.65 \cdot 10^4$	$1.30 \cdot 10^4$	*

Hormone levels during hexanoic acid-induced resistance

The potential role of various signalling pathways in Hx-IR was analysed. The results reveal that the ABA response was more rapid at 12 h post-inoculation in treated plants compared with non-treated plants (Fig. 5a). Hx treatment induced a more rapid accumulation of OPDA, JA and JA-Ile at 48 h post-inoculation compared with water-treated plants (Fig. 5b, c and d).

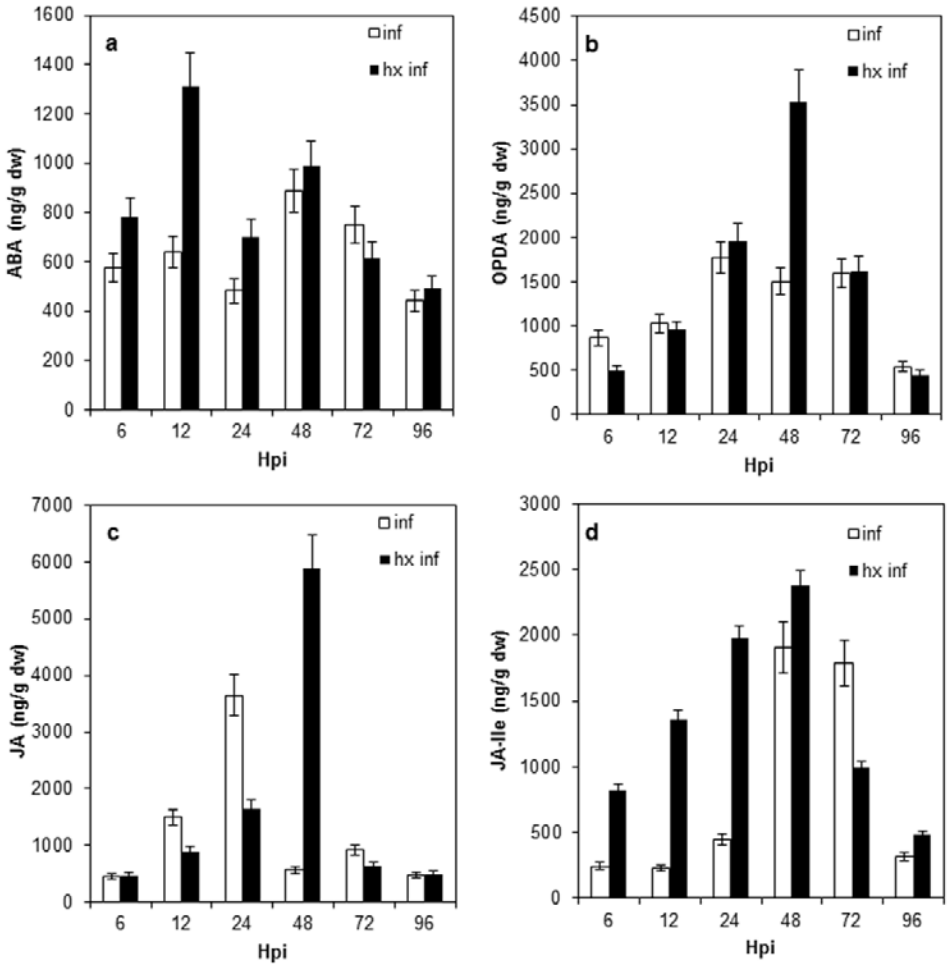

Figure 5. Hormone levels in infected, and treated and infected 'Fortune' plants upon infection. Leaves were collected at 0, 12, 24, 48, 72 and 96 h post-inoculation. (a) The ABA (b) OPDA (c) JA and (d) JA-Ile levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE.

Interestingly, the increase in JA observed in water-treated plants upon infection slightly increased at 24 hpi, but this increase was not observed in Hx-treated plants. Moreover, Hx-treated plants exhibited significant differences compared with controls in the absence of infection, and the results obtained for salicylic acid (SA) content were not significantly different among any of the treatments (data not shown).

The *AOS* and *RD22* genes are markers for jasmonic acid (JA) and abscisic acid, respectively. In non-treated plants, a greater level of *AOS* gene expression was observed at an early stage (24 hpi); this level of expression was not achieved until 48 hpi in treated plants (Fig. 6a). *RD22* expression in infected plants was increased at 6 hpi in treated plants when compared with non-treated plants (Fig. 6b). Moreover, both marker genes were well correlated with the hormone levels observed. The *PR5* gene, a marker for SA, was also analysed; however, we did not uncover any differences in *PR5* expression between treated and non-treated plants (data not shown).

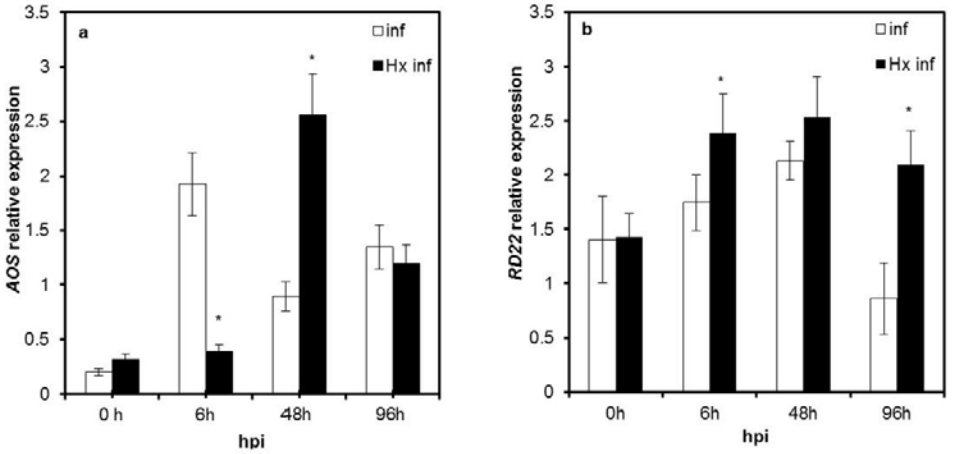

Figure 6. Effect of hexanoic acid treatment on the gene expression in 'Fortune' plants upon *A. alternata* infection. Total RNA was isolated from leaves at 0, 6, 48 and 96 h post-inoculation and was converted into cDNA and subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The relative levels of (a) *AOS* and (b) *RD22* were analyzed in infected, and treated and infected citrus plants. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. Asterisks indicate a significant difference ($P<0.05$).

DISCUSSION

In the present work, we have analysed the effectiveness of Hx for the control of ABS disease. We have demonstrated that Hx presents antifungal activity at a non-phytotoxic concentration. Additionally, Hx prevents germ tube formation *in vitro*, suggesting that it could affect the processes taking place during the early stages of germination, which was demonstrated previously in tomato using *Botrytis cinerea* (Leyva et al., 2008; Vicedo et al., 2009). We have demonstrated that Hx levels exceeding 16 mM are sufficient to inhibit the growth of the fungus. However, in this work, 30 mM Hx was required to achieve 99% inhibition. This result is similar to that of Mondal et al. (2005), who observed that citrus pathogens were generally much less sensitive to Strobilurin fungicides than other fungi. In our particular case, Hx does not inhibit germination at concentrations lower than 3 mM regardless of pH. Combrink et al. (2011) demonstrated that other natural compounds such as Thymol and Eugenol can achieve 100% inhibition at 3.2 mM.

To determine the concentration at which Hx induces resistance, we applied several concentrations (in irrigation water) to 2-year-old plants. Hx concentrations of 0.5 and 1 mM reduced both the infection diameter and the number of infected leaves. Plants treated with the highest concentration tested (3 mM) showed complete lack of infection. This result reinforces the hypothesis that the application of Hx enhances plant resistance because 3 mM Hx has no fungicidal effect *in vitro* at any pH and is a weak mycelial growth inhibitor. For the subsequent experiments, we used a concentration of 1 mM because it caused a significant reduction in infected leaves and infection diameter, and the use of a lower concentration ensures the lack of an antifungal effect. We observed that treating citrus plants with 1 mM Hx 4 days prior to infection clearly reduces the disease incidence and leads to smaller lesions at 96 h post-inoculation. This concentration has no antimicrobial effect on *A. alternata*, which was also the case for other pathogens such as *B. cinerea* (Leyva et al., 2008). The fact that the concentration of Hx required in plants is lower than the concentration required to inhibit germination *in vitro* suggests that the protective effects of Hx might result from specific interactions with plant defense systems.

In this work, we also analysed the main parameters involved in Hx-IR. A characteristic cellular response of early post- invasive defenses that occurs on the inner surface of the epidermal cell wall is papillae accumulation. Papillae are composed mostly of callose, an amorphous high molecular weight β-1,3-glucan, and other constituents (Thordal-Christensen et al., 1997; Hématy et al., 2009). Callose acts as a physical barrier or as a matrix that concentrates antimicrobial compounds at attempted sites of fungal penetration (An et al., 2006). We

demonstrated herein that Hx treatment induces enhanced callose deposition in infected plants, which could act as a physical barrier to decrease the severity of infection. Moreover, plants treated with the callose inhibitor 2-DDG exhibited enhanced susceptibility to *A. alternata,* which correlates directly with lower levels of callose deposition. Additionally, uninfected plants did not present callose accumulation regardless of whether they were treated with Hx. These results support the involvement of this mechanism in inducible defense responses against necrotrophs and suggest that Hx acts as a priming agent. Previous experiments in our group have revealed that Hx-treated tomato plants infected with *B. cinerea* exhibit increased callose accumulation (Vicedo et al., 2009). Related experiments by Kravchuk et al. (2011) indicated no differences in callose accumulation between Hx-treated and non-treated Arabidopsis plants infected with *Botrytis cinerea*. Taken together, these findings suggest that callose deposition induced by Hx treatment is a mechanism that may delay infection in a pathogen- and crop-dependent manner.

It has been demonstrated that, in addition to previous mechanisms, the PGIP–endoPG interaction limits the ability of endoPG to allow pathogens to colonise plants. The importance of PGIPs in plant defense has been elucidated in a series of studies (Ridley et al., 2001). We demonstrated that *PGIP* expression was upregulated more rapidly in infected plants treated with Hx compared with non-treated plants, and these higher levels were maintained throughout the experiment. It has been reported that *PGIP* overexpression mutants of both Arabidopsis (Ferrari et al., 2003) and tomato (Powell et al., 2000; Agüero et al., 2005) have fewer symptoms and exhibit less *B. cinerea* colonization.

When plants are attacked by a pathogen, ROS are produced by cells via the enhanced enzymatic activity of NADPH-oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast (Grant and Loake, 2000; Hammond-Kosack and Jones, 2000). We observed that inoculation of the necrotrophic pathogen *A. alternata* rapidly induced the production of H_2O_2 in 'Fortune' leaves, which was also observed by Lin et al. (2011) in Minneola tangelo. However, in our experiments, we found that infected plants treated with Hx accumulate lower levels of peroxide and superoxide ions when compared with infected control plants.

During the defense response, plants produce more ROS while their ROS scavenging capacities diminish simultaneously. These results in the accumulation of oxidative species that are capable of reacting with a wide variety of biomolecules thus causing irreversible damage, which can lead to tissue necrosis and may even ultimately, kill the plant (Mittler et al., 1999; Delledonne et al.,

2001; Girotti, 2001). The increase in ROS can be explained as a defensive response as opposed to a harmful effect caused by stress. These results support the hypothesis that physical and primary barriers to *A. alternata* could be implicated in the Hx-IR mechanism in citrus.

As hormonal pathways play an important role in induced resistance, we analysed the major molecules implicated in this process. Our results indicate that Hx-treated plants exhibit a more rapid ABA response at 12 h post-inoculation compared with un-treated plants. ABA can act as a positive regulator of Hx-IR by enhancing callose deposition, which was previously reported for BABA-IR in Arabidopsis (Ton and Mauch-Mani, 2004; Flors et al., 2008) and Hx-IR in tomato infected with *B. cinerea* (Vicedo et al., 2009). Based on our results, the elevated expression of *RD22* in treated plants was correlated with the ABA levels observed. It has been demonstrated that various stress conditions in citrus plants could trigger the upregulation of this gene (Souza et al., 2007).

For the first time in citrus plants, we have revealed that the JA signalling pathway also plays a role in the defense against *A. alternata*. The levels of JA, OPDA, and JA-Ile increased after *A. alternata* inoculation in 'Fortune' plants. Additionally, treatment with Hx induced their greater accumulation if compared with untreated plants, indicating that the JA pathway could perform an important role in Hx-IR. Interestingly, the JA-Ile level was higher than the levels of all other hormones examined at earlier time points. This rapid accumulation could be due to the rapid transformation of basal amounts of OPDA (accumulated in chloroplasts), which allows for rapid JA-Ile production after the pathogen is detected. However, the increase in JA noted upon infection in non-treated plants took place earlier than it did in Hx-treated plants.

Gomi et al. (2003) demonstrated that the transcription of the *AOS* gene, which is involved in the first step of the JA pathway in citrus, occurred within 2 h of wounding and led to the production of oxylipin derivatives of linoleic and linolenic acids such as C6-aldehydes, C6-alcohols and MeJA. A number of these volatiles are known to play a role in plant signalling. Our results show that *AOS* is upregulated early in infected plants but not in Hx-treated plants. However, a delayed increase in the expression of this gene in Hx-treated plants was observed. This delay in the expression of *AOS* combined with the lower JA level at early time points supports the hypothesis that the establishment of an infection can be slowed down by Hx prior to the JA-controlled defense responses. Hx may enhance early defenses such as the accumulation of callose and ROS.

In our analysis of SA levels and *PR5* gene expression, we observed no significant differences between treatments. These results support the hypothesis

that the response pathways that target necrotrophic fungi are controlled by JA, while SA is implicated in the response against biotrophic fungi (Pieterse, 2009).

In conclusion, our results demonstrate that treatment with Hx can be an effective means of controlling the pathogenic fungus *Alternaria alternata* in 'Fortune' mandarin. In fact, treatment with Hx enhances plant defenses via callose priming and prevents the establishment of infection by stimulating initial responses such as *PGIP* gene expression and ROS accumulation. Additionally, the levels of metabolites involved in the JA pathway confirm its role in Hx-induced resistance.

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Chapter 2

Hexanoic acid provides long-lasting protection in ‘Fortune’ mandarin against *Alternaria alternata*

ABSTRACT

Alternaria alternata causes Alternaria brown spot disease, for which there are no highly effective disease control measures, in many mandarins and their hybrids worldwide. Only repeated spray applications of copper are employed to protect fruit from this fungal infection, leading to copper phytotoxicity and accumulation in the soil. In recent years, induced plant resistance has been studied as an actual alternative to classical pesticides. Few studies on the effectiveness of these products and their long-lasting effects in woody crops have been performed. Citrus plants were grown under controlled greenhouse conditions and treated with a single dose of hexanoic acid at 1mM. Two infections, with a month between them and without Hx retreatment, were performed using spores of *A. alternata*. Plant defense mechanisms were measured to evaluate the disease resistance in these plants. Overall, the application of hexanoic acid is capable of inducing a long-lasting protection in citrus against Alternaria brown spot disease, providing an alternative to the current chemical treatments.

INTRODUCTION

The necrotrophic fungus *Alternaria alternata* is the cause of *Alternaria* brown spot disease. This fungus can attack many types of mandarins, and their hybrids cause damage to leaves, twigs, and immature fruit, (Canihos et al., 1999). The toxin released by the pathogen is primarily active in "Dancy" mandarin and its hybrids, such as the 'Fortune' mandarin, and in mandarin/grapefruit and mandarin/sweet orange hybrids (Vicent et al., 2009). The severity and lack of control of this fungus makes growing susceptible varieties unprofitable. The field usefulness of any pesticide or protective compound is directly related to the persistence of its effect. There are no curative compounds against this pest; therefore, all the means of control are preventive. Classical means of controlling *A. alternata*, such as copper (Cu) applications, must be sprayed several times, and the number of applications sometimes exceeds 12 per cultivation cycle (de Souza et al., 2009; Vicent, et al., 2009). Recent studies showed that covering at least 50% of leaves with Cu is necessary to achieve disease control, but with a high inoculum pressure a 75% coverage may be required (van Zyl et al., 2013). This represents a residue of 60 and 150 mg Cu per kg of biomass. However, the problem with Cu is that it can easily be rinsed off by water. When rain fell 2 days after Cu treatments, Wilson et al. (2012) observed levels up to 233 µg Cu/L in the runoff water, and additional Cu that remained in the soil. Current recommendations in Spain suggest an application of Cu every 15 days during the high-risk periods and extra applications after each rainfall (IVIA, 2013). In sub-tropical areas in the north hemisphere, recommendations are to spray on a 10- to 14-day schedule from late February to mid-July to control in heavily infested orchards. In tropical areas, where rainfall and high temperatures increase the risk of infection, weekly sprays year-round were required to achieve control (Canihos, et al., 1999). Even with the increased number of sprays, the pathogen often cannot be completely controlled, and the use of sensitive cultivars becomes impractical.

Therefore, it is necessary to find efficient control alternatives to improve natural plants defense mechanisms in response to microbial pathogens and insect herbivores. The variety of responses depends on the nature of the pathogen and its mechanism of pathogenicity. The activation of some disease responses can be detrimental to plant growth. The first means of protection against pathogens is constitutive resistance, which consists of structural defenses, such as waxes or essential oils. When these barriers fail to prevent the entry of pathogens, plants activate a second level of defense responses called pathogen-induced resistance. Generally, these responses are controlled by plant hormones. The two main pathways, controlled by salicylic acid (SA) and jasmonic acid (JA), can activate the defense responses against biotrophic and necrotrophic pathogens, respectively

(Beckers and Spoel, 2006). In addition, other compounds have been shown to play an important role in signal transduction. For example, methyl salicylate or pipecolic acid have recently been identified as a mobile signal for systemic resistance (Park et al., 2007).

Over the past decades, there has been increasing evidence that the more efficient activation of cellular defense responses can be induced with xenobiotic compounds. This induction is associated with enhanced resistance to various biotic or abiotic stresses. This phenomenon, referred to as priming the defense, has been well characterized in a wide number of species (Conrath et al., 2006; Pastor et al., 2013; Scalschi et al., 2013). The majority of studies on xenobiotic compounds and their effects are performed in a laboratory setting using model plants not using crop plants. It has also been demonstrated that some chemical inducers, such as beta-aminobutyric acid, acibenzolar-S-methyl and neonicotinoid-based insecticides, can induce a long-lasting induction of defenses. Studies performed in *Arabidopsis* plants showed that the primed defense state can be maintained long after the initial stimulus, indicating a form of plant immunological memory (Luna et al., 2012).

Recently, we found that hexanoic acid (Hx) can protect *Arabidopsis* and tomato plants against *Botrytis cinerea* (Leyva et al., 2008; Vicedo et al., 2009; Kravchuk et al., 2011) and *Pseudomonas syringae* pv. *tomato* (Scalschi, et al., 2013). This natural short-chain monocarboxylic acid displays antimicrobial activities and can also induce plant defense responses when used as a priming agent. Upon infection, the oxylipin 12-oxo-phytodienoic acid (OPDA) and the bioactive molecule jasmonate-isoleucine were significantly induced in treated plants. Additionally, callose deposition was primed, and abscisic acid (ABA) acted as a positive regulator of hexanoic acid-induced resistance (Hx-IR) by enhancing callose accumulation (Vicedo, et al., 2009).

Hx's effectiveness as a systemic resistance inducer in woody plants has only been tested in citrus against *A. alternata* over short time periods (Llorens et al., 2013), where it was able to reduce the number and size of lesions 5 days after inoculation and stimulate the defense pathways of citrus. The aim of this work is to evaluate the long-lasting effect of Hx in 'Fortune' mandarins against *A. alternata*, which may contribute to minimize the excessive use of harmful chemical pesticides and their effects on the environment.

MATERIALS AND METHODS

Plant material

For all experiments, we used 2-year-old 'Fortune' mandarin plants grafted onto Carrizo citrange plants and grown in a greenhouse in 10-L pots with peat based substrate. One month before the commencement of each experiment, the leaves were removed to encourage uniform sprouting. The leaves with size suitable to inoculation (75% expanded) were labeled and infected.

After the first inoculation, when the samples were collected, all the remaining leaves were removed again to force a new flush. Four weeks later, when the new leaves achieved the correct size; a second inoculation was performed in the same plants but without a new treatment. The second inoculation was performed 6 weeks after the Hx treatment.

Chemicals and inoculation procedures

Compounds were applied in a single application as a soil drench (500ml of solution per pot). The timing of treatments and their rates were chosen based in previous reports (Llorens, et al., 2013) of effective dosages and timing of applications. In brief: hexanoic acid was applied as a soil drench at 1mM 4 days before inoculation. In all the experiments, untreated and inoculated plants were included as controls.

Spores of *Alternaria alternata* were collected from 10- to 15-day-old cultures with sterile water containing 0.02% (v/v) Tween-20. The solutions were then filtered, quantified with a hemocytometer, and adjusted to 10^5 spores/mL. Leaves were infected by dispensing 5 µL of the spore solution onto each leaves' surface. After 48 and 96 h, leaves were sampled.

Gene expression

A gene expression analysis by real-time quantitative PCR (RT-qPCR) was performed with RNA samples extracted from leaf tissue using the E.Z.N.A. total RNA Kit II (Omega Bio-tek; Norcross, GA. USA; http://www.omegabiotek.com). Citrus leaf tissue samples for RNA isolation were collected at 0, 48, and 96 h post-infection (hpi), and tissues were collected from both treated and non-treated plants. We used the RT-qPCR conditions previously described by Flors et al. (2007). The primers used in the RT-qPCR were *CALs1* described by Enrique et al (2011),

PGIP described by Llorens et al (2013) and *AOS* and *GAPDH* (as internal standard) described by Fernandez-Crespo et al. (2012).

Quantification of hormones and phenolic compounds

The extractions and experimental procedures used in the hormone analysis were performed as described by Erb et al. (2009). We analyzed the levels of JA, OPDA, ABA, chlorogenic acid and caffeic acid using prostaglandin B1, dihydrojasmonic acid, [$^2H_6$]-ABA, and propylparaben as internal standards.

Statistical analyses

Treatments were analysed by one-way ANOVA using Statgraphics centurion XVI.I software (Statistical Graphycs Corp.), and means were separated using Fisher's least significant difference (LSD) at 95%. Treatments were 1: non-inoculated untreated plants (data not shown), 2: non-inoculated Hx treated plants (control), 3: inoculated untreated plants (inf), and 4: inoculated Hx treated plants (Hx inf). All experiments were repeated three times with six plants per treatment. Figures show the average of three independent experiments.

RESULTS AND DISCUSSION

Protection mediated by Hx lasts 6 weeks

Treatment with Hx reduces the number and size of lesions produced by *A. alternata*. The results obtained showed that treating citrus plants with 1 mM Hx 4 days prior to infection clearly reduced the disease incidence and led to smaller lesions at 96 hpi. The number of infected leaves was lower in treated plants and this protective effect remained up to the time of the second infection. In both inoculations, the ratio of infected leaves to inoculated leaves was nearly 30% lower in treated plants compared with non-treated plants, achieving a protection rate of more than 50% in inoculated leaves (Table 1). The area of the lesions was also lower in plants infected and treated with Hx. In comparison with non-treated plants, lesions of treated plants were 30% smaller after the first and 25% smaller after the second infection (Table 1). The results obtained from the second inoculation are similar at those obtained from the first, suggesting that only one application of Hx is necessary to protect citrus against this fungus for at least 2 months. These results are in accordance with previous tests performed in 'Fortune'

mandarins inoculated with *A. alternata*. However, the long-lasting effects of natural compounds in other citrus has not been tested yet. In citrus, Francis et al. (2009) demonstrated that some neonicotinoids, such as Imidacloprid, and other resistance inducer chemical compounds, such as Acibenzolar-S-methyl, provided long-lasting protection against citrus canker (Francis, et al., 2009).

Table 1. Lesions and ratio of infection in infected (Inf) and treated and infected (Hx inf) plants. The necrotic area measured at 96 h post-inoculation is expressed in mm^2. The number of infected leaves is expressed as a percentage. Data show the average of three independent experiments obtained with 10 plants per point ± SE. Asterisk (*) in a row represent statistically significant differences ($P < 0.05$).

	First inoculation	
	Necrotic area (mm^2)	**Infected leaves (%)**
Hx inf	3.3+0.2*	45.33+4.27*
Inf	4.8+0.5	77.63+11.33
	Second inoculation	
	Necrotic area (mm^2)	**Infected leaves (%)**
Hx inf	1.4+0.1*	31.72+8.57*
Inf	2.1+0.3	60.83+10.61

Additionally, the long-lasting protective effect of resistance inductors was also described in *Arabidopsis* by Luna et al. (2012), who observed trans-generational effects in the progeny of plants that had repeatedly been infected with the virulent strain *P. syringae* pv. *tomato* DC3000. At the same time, Rasmann et al. (2012) demonstrated that *Arabidopsis* and tomato treated with JA, or exposed to insect herbivory, produce more resistant progeny against caterpillar feeding. In citrus, long lasting antibacterial effects of some natural compounds have been described (Fornes et al., 2005). However, this is the first report of a long-lasting protective effect against a fungal disease induced by a natural priming agent in citrus.

Hx enhances the defensive physical barriers

To perform an in-depth study regarding the physical barriers involved in Hx-IR, the expression level of the polygalacturonase inhibiting protein (*PGIP*) and the callose synthase 1 (CalS1) gene were determined. *PGIP* is a ubiquitous plant cell wall protein that counteracts the action of fungal polygalacturonase proteins by preventing cell wall degradation and interfering with invasion. In this experiment we observed that *PGIP* gene expression in infected plants was up-regulated more rapidly in Hx-treated plants (Fig. 1).

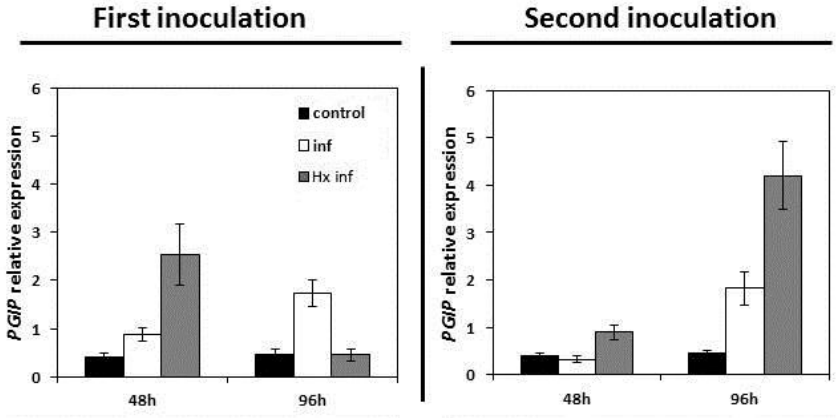

Figure 1. Relative levels of polygalacturonase inhibiting protein (*PGIP*) analyzed in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated. Total RNA was isolated from leaves at 48 and 96 h after each inoculation, converted into cDNA, and subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The data show the average of three independent experiments obtained with a pool of 10 plants per point ± SE.

After the first and second inoculations, higher expression levels of *PGIP* were observed in Hx-treated plants compared with untreated. However, after the first inoculation, the expression level was higher at 48 h, whereas after the second inoculation higher levels were achieved at 96 h. It has been demonstrated that, in addition to other mechanisms, the PGIP-endoPG interaction limits the ability of endoPG to allow pathogen colonization of plants. The importance of PGIPs in plant defense has been demonstrated by Ridley et al. (2001). It has also been reported that *PGIP* overexpression mutants of both *Arabidopsis* (Ferrari et al., 2003) and tomato (Powell et al., 2000; Aguero et al., 2005) have fewer symptoms and exhibit lower levels of *B. cinerea* colonization. Our results suggest that the

enhanced expression of the PGIP gene after both inoculations could be implicated in the protection mediated by Hx against *A. alternata.*

Another characteristic cellular response of early post-invasive defenses that occurs on the inner surface of the epidermal cell wall is papillae accumulation (Fig 2a). Papillae are composed mostly of callose, an amorphous high-molecular-weight 1,3-glucan, with other minor constituents (Hématy et al., 2009). Callose acts as a physical barrier, or as a matrix, that concentrates antimicrobial compounds at attempted sites of fungal penetration (An et al., 2006). In previous experiments, we observed that the callose accumulation is important in the prevention of infection against *A. alternata* (Llorens, et al., 2013). In the present experiment, after the second inoculation is done, we did not observe differences in the accumulation of callose between treated and untreated plants (Fig. 3a, b).

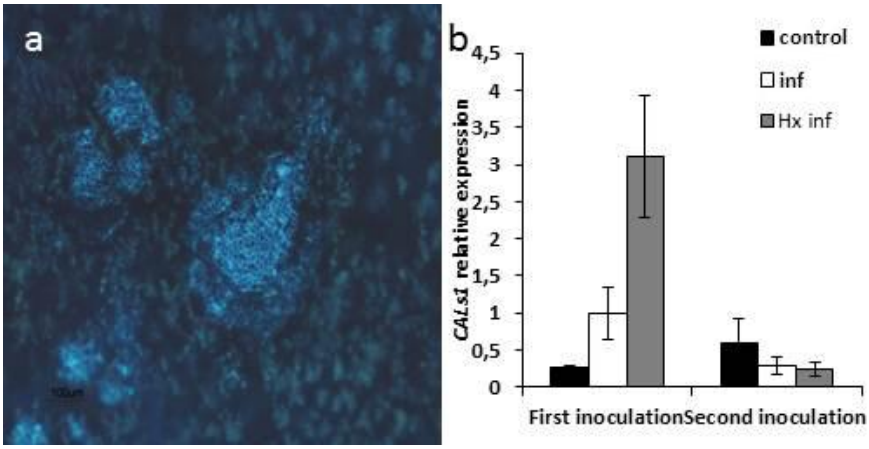

Figure 2. a) Callose deposition detail. b) Relative levels of *CALs1* analyzed in in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated. Total RNA was isolated from leaves at 96 h post-inoculation, converted into cDNA, and subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The data show the average of three independent experiments obtained with a pool of 10 plants per point ± SE.

To confirm the results observed in the enhancement of callose deposition, we also analyzed the expression of the CALS1 gene. Figure 2b shows the higher expression level of this gene only in Hx-treated plants after the first inoculation, whereas after the second inoculation no significant differences were observed. The

non-activation of callose synthesis after the second infection may be related to the promotion of other defense pathways. The accumulation of this polysaccharide appears necessary for the prevention of infection once activated other induced resistance processes.

Application of Hx induces higher accumulation of phenolic compounds

Phenols are known to play an important role in plant defenses. Some occur constitutively, whereas others are formed in response to pathogen ingress and associate as part of an active defense response in the host. The defensive activity of phenolic compounds is due to their direct toxic effects on the pathogen and also their capacity to strengthen the cell wall (Hückelhoven 2007). In relation to induced resistance, Lavania et al. (2006) observed that phenolic compounds induced by plant growth-promoting Rhizobacteria have an important protective role in *Piper betle* against *Phytophthora nicotianae*. Direct evidence for decreases in fungal growth in tomato because of phenolic profile changes in response to inoculation with *Verticillium alboatrum* is available in the literature (Lattanzio et al., 2006). Our results showed that Hx treatment increased the level of caffeic acid at 48 h after the first inoculation, whereas the chlorogenic acid increased only in untreated plants. At 96 h, levels of both phenolic compounds had higher concentrations in Hx-treated plants compared with in control plants, and the level in the Hx-treated plants was higher than in untreated plants, but not significantly (Fig. 3). However, after the second inoculation, the results showed a higher accumulation of caffeic acid in Hx-treated and infected plants compared with infected but not treated plants, and chlorogenic acid showed, in general, an enhancement both in treated and untreated plants compared with control plants. These observations suggest that cell wall reinforcement, mediated by caffeic acid, in the Hx-IR could be implicated in disease resistance.

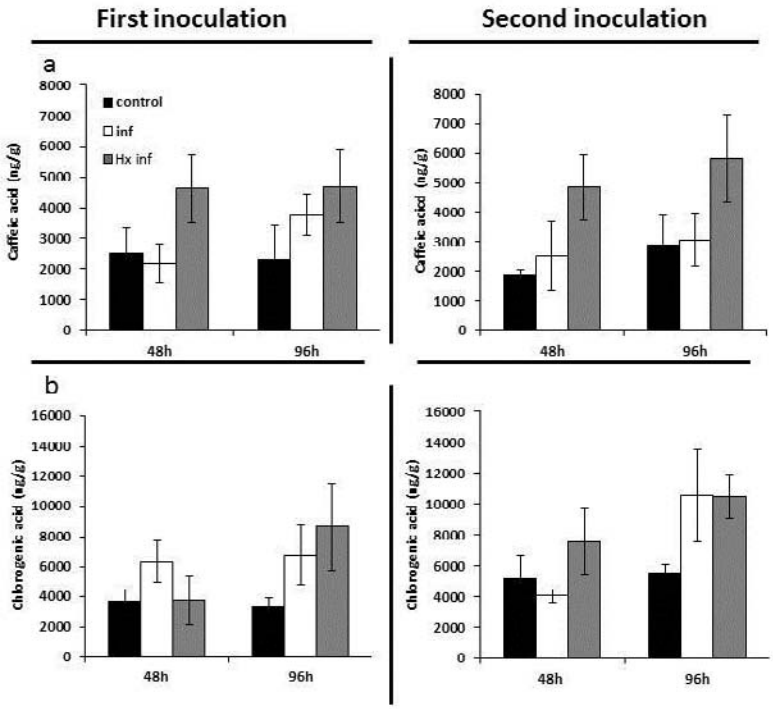

Figure 3. Phenolic compound levels in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated after infection. Leaves were collected at 48 and 96 h after each inoculation. The a) caffeic and b) chlorogenic levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE.

Hormone response-induced Hx differs after the second inoculation

Resistance to necrotrophic pathogens is dependent on complex signaling pathways, which involve the major plant phytohormones JA, ethylene, and ABA. However, SA does not have a major role in resistance against these pathogens (Flors, et al., 2008).

The analyzed hormones relating to plant-pathogen interactions confirmed that the levels of JA and OPDA increase after *A. alternata* inoculation in treated 'Fortune' mandarin trees. As previously described in our laboratory (Vicedo, et al., 2009; Llorens, et al., 2013; Scalschi, et al., 2013), the accumulation of OPDA was higher in Hx-treated plants compared with in non-treated plants, suggesting that in the first step of infection the JA pathway plays an important role in the protection of plants (Fig 4). At 48 h after the first inoculation, the levels of JA and OPDA

were higher compared with in untreated plants, indicating an enhancement in disease defenses, which prepare the plants to fight infection. However, after the second inoculation, no significant differences were observed between treated and untreated plants. The results obtained in the hormone analysis were corroborated by a study of the AOS gene. After the first inoculation (Fig. 5), an enhancement of AOS expression at 48 h only in treated plants was observed. However, after the second inoculation, the expression level of this gene is higher in both treated and untreated plants than in control plants, showing a constant up-regulation of the JA pathway.

Our results after the first inoculation showed a high ABA response at 48 hpi in treated and untreated plants. In previous work (Llorens, et al., 2013), a detailed study of hormone responses showed an enhancement of ABA mediated by Hx at 12 hpi. However, at 48 and 96 h, no difference between treatments was observed. Nevertheless, after the second inoculation, levels of ABA increased more slowly than after the first inoculation and were higher at 96 h, but without significant differences between treated and untreated plants.

The role of the JA pathway in resistance against necrotrophs has been widely studied (Kazan and Manners, 2008), and its implication in Hx-IR was previously reported by Vicedo (2009). Our results showed a known role of the JA/ABA pathway in response to necrotrophic pathogens, which was enhanced after the first inoculation by the application of Hx. The increased levels of JA and OPDA observed after the second inoculation promote a high level of protection in plants compared with after the first inoculation. However, the lower incidence of infection in Hx-treated plants after the second inoculation could be due to a synergistic combination of phenolic compound accumulation, expression of PGIP, and the up-regulation of the JA pathway.

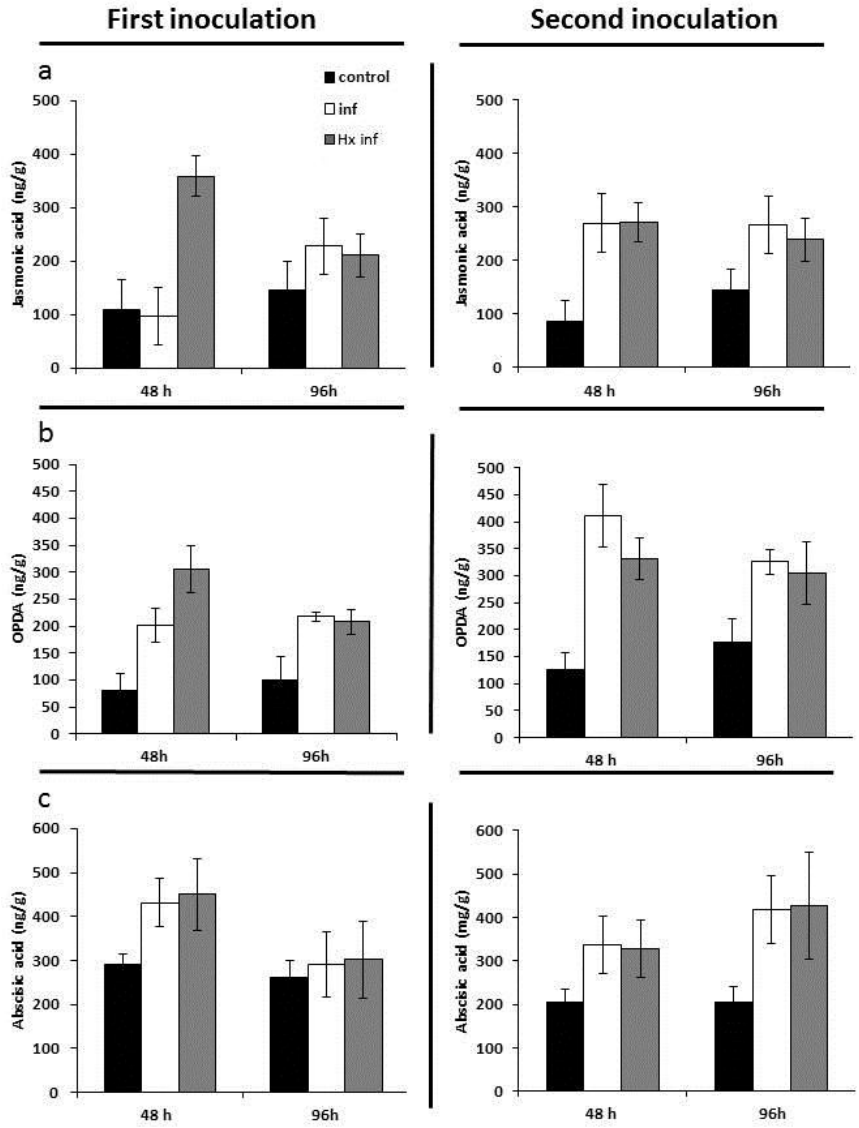

Figure 4. Hormone levels in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated after infection. Leaves were collected at 48 and 96 h after each inoculation. The a) JA, b) OPDA, and c) ABA levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE.

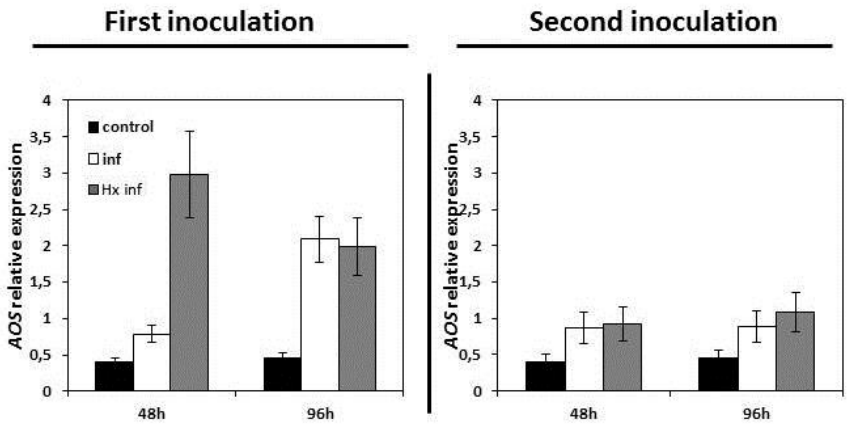

Figure 5. Relative levels of allene oxide synthase (*AOS*) analyzed in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated. Total RNA was isolated from leaves at 48 and 96 h after each inoculation, converted into cDNA, and subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The data show the average of three independent experiments obtained with a pool of 10 plants per point ± SE.

CONCLUSIONS

Results obtained corroborate that the application of Hx is effective to reduce the incidence of *A. alternata* in 'Fortune' mandarin trees, and its effect is long lasting enough to protect the plants for 2 months with only one application. The observed effect of Hx after the first inoculation indicates the early enhancement of the JA pathway, leading to callose deposition. After the second inoculation, defensive pathways are up-regulated in both treated and untreated plants, suggesting a remaining effect of the first inoculation, which is observed in the reduction of lesions in all treatments. However, Hx remains active in the plant, leading to an enhancement of *PGIP* expression levels and defensive barriers, including phenolic compounds. This enhancement of defensive barriers provides an effective reduction in lesions, achieving a lower susceptibility against *A. alternata* than observed in untreated plants. The reduction observed in the rate of infection could be enough to protect citrus plants against low to mid inoculum pressure, providing a long-lasting alternative to classical control measures. The use of this natural compound in an integrated pest management system could reduce the applications of Cu, making them necessary only against the threat of high inoculum pressure and optimal conditions for infection.

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Chapter 3

Induced resistance in sweet orange against *Xanthomonas citri* subsp. *citri* by hexanoic acid

ABSTRACT

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is a serious and widespread disease of citrus, causing loses in fruit yield and quality. There are no highly effective citrus canker disease control measures. Repeated spray applications of copper are often employed to protect fruit from bacterial infection with consequences for copper phytotoxicity and accumulation in the soil. Alternatively, innate plant defense mechanisms can be enhanced by plant treatments with specific natural and synthetic inducers for control of bacterial diseases. In this study, hexanoic acid applied as a soil drench or foliar spray on 9-month-old potted citrus trees reduced lesions in leaves by 50% compared with control plants. Disease reducing activity lasted up to 50 days after application. Induction of resistance mediated by hexanoic acid was demonstrated by enhanced expression of PR genes and callose deposition in treated and infected plants. These findings indicated that hexanoic acid applications trigger a defensive response in the plants. The application of this natural compound may have potential for management of citrus canker in conjunction with other disease control measures and may reduce the frequency or rate of copper bactericides.

INTRODUCTION

Citrus canker, caused by the bacterial pathogen, *Xanthomonas citri* subsp. *citri* (Xcc; syn. *X. axonopodis* pv. *citri*), is a serious disease of commercial citrus cultivars including grapefruit (*Citrus paradisi* Macf.) and early mid-season oranges (*C. sinensis* (L.) Osb) for juice processing. The pathogen causes necrotic, erumpent lesions on leaves, stems, and fruits that create a range of symptoms including defoliation, blemished fruit, premature fruit drop, and twig dieback to general tree decline (Graham and Myers 2011).

Currently, citrus canker is present in wet subtropical citrus-producing regions of the world, but it has not been reported from areas dominated by Mediterranean climates such as southern Europe, where it is considered a quarantine pathogen (EPPO 2014). Rapidly expanding leaves and continuously growing fruit tissues are most vulnerable to infection, and field resistance of trees to Xcc is directly related to tissue juvenility (Graham et al., 1992). Citrus cultivars and species with greater frequency, size, and duration of leaf flushes and duration of fruit growth are more field-susceptible to Xcc than less vigorous cultivars or those whose foliage matures more rapidly (Gottwald et al., 1993).

There are no highly effective canker disease treatments when susceptible cultivars are growing in areas with favorable conditions for bacterial multiplication. Copper reduces bacterial populations on leaf surface, but multiple applications are needed to protect fruit of susceptible citrus varieties. Disadvantages of long term use of copper bactericides include induced copper resistance in xanthomonad populations (Behlau et al., 2012) and accumulation in soils with potential phytotoxic and adverse environmental effects. The protective activity of copper is diminished by wind-blown rain that introduces bacteria directly into stomata (Behlau et al., 2008). However, other bactericides such as antibiotic based products are not as effective as copper because they lack sufficient residual activity to protect leaf and fruit surfaces for extended periods (Graham et al., 2006; Behlau et al., 2008). On the other hand, the lack of effective means of control of this bacterium promoted new lines of investigation focused on the development of transgenic plants with genes that confers resistance to Xcc (Mendes et al., 2010; Cardoso et al., 2010).

Plant-pathogen interaction during infection induces signal cascades which activate a cellular response to minimize lesions. Once the pathogen attack is recognized by the plant, it activates cell wall-associated defense such as the callose deposition, the rapid accumulation of reactive oxygen species (ROS) which is the principal initial barrier against *Xanthomonas* infection in citrus plants (Enrique et al., 2011). In addition to these physical barriers, innate plant defenses trigger a

broad spectrum of metabolic and hormonal responses (Lorenzo et al., 2004; Durrant and Dong 2004).

In last years, various synthetic compounds including β-aminobutyric acid (BABA) and acibenzolar-S-methyl (ASM) have been investigated for systemic disease control without expression of a direct toxic effect on the pathogen (Jakab et al., 2001). In addition, plant extracts of neem (*Azadirachta indica*), ginger (*Zingiber officinale* Roscoe) and curcuma rhizomes (*Curcuma longa* L.) (Vechet et al., 2009) have been reported to be capable of controlling plant disease without directly inhibiting the pathogen.

Acibenzolar- *S*-methyl (ASM; Actigard or Bion; Syngenta Crop Protection), a functional homolog of salicylic acid (SA), is the most widely known commercial inducer of induced resistance (Tally et al., 1999). Although ASM has been extensively evaluated as a component for plant disease control in the field, it's effectiveness in disease management has been questioned due to variability of control (Walters and Fountaine 2009). Field studies showing promise for control of bacterial diseases have been conducted with foliar sprays of ASM either alone or in combination with copper on tomato and pepper (Romero et al., 2001; Louws et al., 2001; Ortuno et al., 2008). Recently, reductions in foliar infection and canker-induced defoliation on young non-bearing grapefruit trees were measured after soil applications with the neonicotinoids (imidacloprid and thiamethoxam) and ASM (Graham and Myers 2011). Expression of the PR protein (β-1,3 glucanase) gene, *PR2*, in citrus increased in response to soil drenches of ASM and neonicotinoids (Francis et al., 2009). Moreover, reduction of lesions was sustained for weeks with soil drenches, whereas after foliar spray of ASM, *PR2* activity and disease control lasted only weeks.

Similarly our research group has demonstrated the efficacy of soil applications of carboxylic acids for protecting tomato plants against *Alternaria solani* and *Phytophthora citrophthora* (Flors et al., 2003). More recently, we found that hexanoic acid (Hx) can protect Arabidopsis and tomato plants against *Botrytis cinerea* (Vicedo et al., 2009; Kravchuk et al., 2011) and citrus plants against *Alternaria alternata* (Llorens et al., 2013). This natural short-chain monocarboxylic acid displays antimicrobial activity and can also induce plant defense responses when used as a priming agent. Post-infection, oxylipin (1,2-oxo-phytodienoic acid; OPDA) and the bioactive molecule jasmonate-isoleucine (JA-Ile) were significantly induced in treated plants. Additionally, abscisic acid (ABA) acted as a positive regulator of Hx-induced resistance (Hx-IR) by enhancing callose accumulation (Vicedo et al., 2009).

Plant disease control based on systemic resistance induced by a compound like Hx with low toxicity could potentially be integrated with copper for citrus canker control as recently proposed for other inducers of systemic acquired resistance (Graham and Myers 2013). Hence, the aim of this work was to evaluate the efficacy of Hx as an inducer of resistance in citrus against Xcc and to compare the disease control and resistance responses with those obtained after treatment of ASM. In addition, method of application, and longevity of the systemic activity was assessed to determine whether Hx as an inducer of resistance has potential for sustained of control of Xcc.

MATERIAL AND METHODS

Bacterial strains, culture media and growth conditions

The Xcc strain X2002-0014 used in this study was isolated in 2002 from sweet orange in Dade County, FL and was routinely grown on Luria Bertani broth (LB) (10 g tryptone, 5 g yeast extract and 5 g sodium chloride per liter) or on LB plates (1.5% bacteriological agar) at 27ºC for 48 h.

Bacterial growth assay

Growth of Xcc was measured in LB broth adjusted to pH 7 with addition of MES buffer and amended with Hx (Sigma-Aldrich, St. Louis, MO ref.153745) at 0, 0.06, 0.6, 1.5, 3.0, 6.0, 10, and 20 mM. Xcc was grown in LB broth overnight and the bacterial suspension was centrifuged, washed and resuspended in 10mM $MgSO_4$. The growth assay was carried out in a total volume of 300µL in microtiter wells using an initial bacterial density of 1.5×10^3 colony-forming units (cfu) mL^{-1} adjusted with a spectrophotometer set at A_{600nm}. Bacteria were incubated on a rotary shaker for 96 h at 26℃ and optical density of the suspension was measured every 10 min using a Bioscreen C Reader (Labsystems Oy, Helsinki, Finland) set at A_{600nm}. After the growth assay, a LIVE/DEAD® (Life Technologies Corp, Carlsbad, CA, USA) test was performed to asses cell mortality caused by Hx acid.

Inoculum preparation, plant treatment and inoculation procedures

Xcc inoculum was prepared in nutrient broth and grown at 28°C for 24 h to log phase. Bacterial suspension was centrifuged at 10,000g for 20 min, re-suspended in saline phosphate buffer (PBS; 40 mM Na_2HPO_4 + 25 mM KH_2PO_4), and

adjusted to 10^4 cfu mL^{-1} for attached leaf inoculations and 10^5 cfu mL^{-1} for detached leaf inoculations as previously described (Francis et al., 2010) .

Nine-month old 'Pineapple' sweet orange plants growing in 2.5 L containers of a general purpose peat based soil (Pro-Mix BX; Premier Horticulture, Red Hill, PA) were maintained in a greenhouse located at the Citrus Research and Education Center in Lake Alfred, FL. Four weeks prior to the treatments, seedlings were cut back to approximately 40 cm, and only one shoot per plant was allowed to grow to approximately 20–30 cm in order to obtain 4–5 immature leaves (75% expanded) suitable for treatment and/or inoculation.

Test compounds were applied in a single application as a soil drench (500ml of solution per pot) or as a foliar spray (100 mL of solution per plant) using an airbrush (Crown Spra-Tool, Aervoe Industries, Inc.). The timing of treatments and their rates were chosen based in previous reports (Francis et al., 2009; Llorens et al., 2013) of effective dosages and timing of applications. In brief: acibenzolar-S-methyl (ASM; Actigard® 50WG, Syngenta Crop Protection) as a positive control was applied as a foliar spray at 1mM 4 days before inoculation or as a soil drench applied 7 days before inoculation; hexanoic acid was applied as a foliar spray at 1mM or 3 mM one day before inoculation or as a soil drench at 1mM 4 days before inoculation. In all the experiments, untreated and inoculated plants were included as untreated checks (UTC). Inoculation of all plants was made on the same day.

For Xcc inoculation, immature leaves (75% expanded) were injection-infiltrated in the abaxial side with 10^4 or 10^5 colony forming units (cfu) mL^{-1} using a tuberculin syringe (1.0 cm^3) with no needle as previously described (Francis et al., 2009). A 6 mm diameter area of the leaf was infiltrated with approximately 2 μL of bacterial suspension. Three injections were performed on each side of the leaf mid-vein.

Detached leaf assay

After spray applications, five leaves per treatment were collected from greenhouse plants in the morning, rinsed and disinfested as previously described (Francis et al., 2010). Leaves were rinsed three times with sterile distilled water in the same plastic bags to remove any debris or spray residues, dipped in 70% ethanol for 30 s, immersed in 0.5% sodium hypochlorite for 30 s, and then were immediately rinsed three times with sterile distilled water. Leaves handled by the petiole end were placed on a sterile paper towel and inoculated with 10^5 cfu ml^{-1} as described above. Excess inoculum was wiped from the leaf surface with a sterile

paper towel. Inoculated leaves were placed on the surface of soft water agar (0.5%) with the abaxial side up. The petiole was removed and the leaf pressed onto the agar surface with a plastic spreader to obtain as much contact as possible. Petri dishes were sealed with Parafilm and incubated in an environmentally controlled growth chamber under fluorescent light at 60 μmol m^{-2} s^{-1} for 12 h photoperiod at 28°C. Symptoms on the inoculated detached leaves were assessed 7, 10 and 14 days post inoculation (dpi). The experiment was conducted three times with six replications per treatment.

Attached leaf assay

After spray or soil drench treatments, 4 leaves per plant were injection-infiltrated with inoculum on the abaxial surface of the leaf. Four plants per treatment were inoculated with Xcc and a buffer control was mock inoculated with sterile PBS.

Greenhouse inoculations were performed from 9:00 to 12:00 h when stomata were fully open with 10^4 cfu ml^{-1} as described above. Inoculated shoots were immediately enclosed in plastic bags for 48 h. After removal of the bags, plants were rotated on the greenhouse bench twice weekly. Samples of leaves were taken at 10 and 20 dpi. Lesions on the inoculated leaves were counted under a hand lens (×10) at 20 dpi.

To determine the persistence of the treatment effect over time, after the first set of inoculations, all the leaves were removed to force a new flush. Shoots were allowed to grow another 2 weeks until 4–6 new leaves developed. When the leaves achieved the requisite size, a second set of inoculations was performed (5 weeks after initial treatment) without an additional chemical application. The experiment was conducted three times with six replications per treatment.

Quantification of Xcc

Viable bacteria in the inoculated areas were estimated at 14 dpi. Leaf disks (6 mm diameter), from three infiltrated sites were excised and ground with 1.0 mL of PBS buffer using a glass homogenizer. Serial dilutions of suspension were plated on KCC medium (nutrient agar plus kasugamycin 16.0 mg L^{-1}, cephalexin 16.0 mg L^{-1}, and chlorothalonil 12.0 mg L^{-1}) as previously decribed (Francis et al., 2010). Total bacterial colonies were expressed as log cfu per inoculation site. Total Xcc populations per inoculation site were quantified by quantitative real-time PCR (Q-PCR) as previously described (Francis et al., 2009). In brief, leaf

disks (6 mm diameter), from three infiltrated sites were excised processed for DNA extraction with the mini DNA kit for plant tissue (QIAGEN Sciences Inc., Germantown, MD). Q-PCR assays were carried out using primers and probe for the *pth* gene that occurs universally in Xcc (Francis et al., 2009).

Reverse transcription (RT-) PCR analysis of plant gene expression

Three discs of 6mm diameter were collected 10 and 20 days per plant and treatment after each set of inoculations and frozen in liquid nitrogen and stored at −80°C until processed. Each plant was processed as a different biological sample. RNA was extracted using an RNeasy® Plant Mini Kit (QIAGEN) following the manufacturer's instructions. Reverse transcription (RT) and real-time quantitative PCR (Q-PCR) were performed as previously described (Francis et al., 2009). The primers used in the RT-qPCR were *pth* and *PR2* previously described by Francis et al. (2009), CALs1 described by Enrique et al (2011) and *AOS* and *PR5* described by Fernández-Crespo et al. (2012). Actin and 18S gene expression were used as an internal standard (Yan et al., 2012) (supplementary table 1: primer sequences).

Callose deposition

Callose deposition was determined in control and infected leaves at different time-points after inoculation as described by Flors et al. (2007). Leaves were collected at the timepoints indicated and incubated in 95% ethanol at room temperature. De-stained leaves were washed in 0.07 M phosphate buffer (pH7), incubated for 15min in 0.07 mM phosphate buffer containing 0.01% aniline blue at room temperature, and then incubated in 0.1% aniline blue one week at room temperature. Observations were performed with an epifluorescense microscope. Callose deposition was quantified from digital photographs of aniline blue-stained leaves. Blue spots corresponding to stained callose were analysed for number of pixels using ADOBE PHOTOSHOP CS4 software. Callose intensity was expressed as the average of yellow pixels/million pixels on digital photography.

Statistical analysis

Treatments were analysed by one-way ANOVA using Statgraphics centurion XVI.I software (Statistical Graphycs Corp.), and means were separated using Fisher's least significant difference (LSD) at 95%. Treatments were 1) non-inoculated untreated plants, 2) inoculated untreated plants, 3) inoculated Hx treated plants and 4) inoculated ASM treated plants. All experiments were

repeated three times with six plants per treatment. Figures show the average of three independent experiments.

RESULTS

Characterisation of antibacterial activity of hexanoic acid

To characterise the effect of hexanoic acid on Xcc *in vitro*, bacterial growth was measured for 96 h in LB medium amended with increasing concentrations of Hx (0.06, 0.6, 1.5, 3.0, 6.0, 10 and 20 mM). Hx at 0.06 mM did not affect the growth of Xcc compared with the non-amended control while 0.6, 1.5 and 3.0 mM Hx inhibited bacterial growth by 8%, 11% and 16%, respectively (Fig. 1). At these concentrations, Hx reduced rate of bacterial growth because entry into the lag phase was significantly ($P<0.05$) delayed compared with the control and 0.06 mM treatments. Concentrations greater than 3.0 mM completely inhibited Xcc growth. Hx at 0.6, 1.5 and 3mM mM apparently had a temporary bacteriostatic effect which may explain the reduction in rate of bacterial growth. Assay with the Live/Dead Cell Viability kit indicated that concentrations greater than 3.0 mM Hx did not kill Xcc cells.

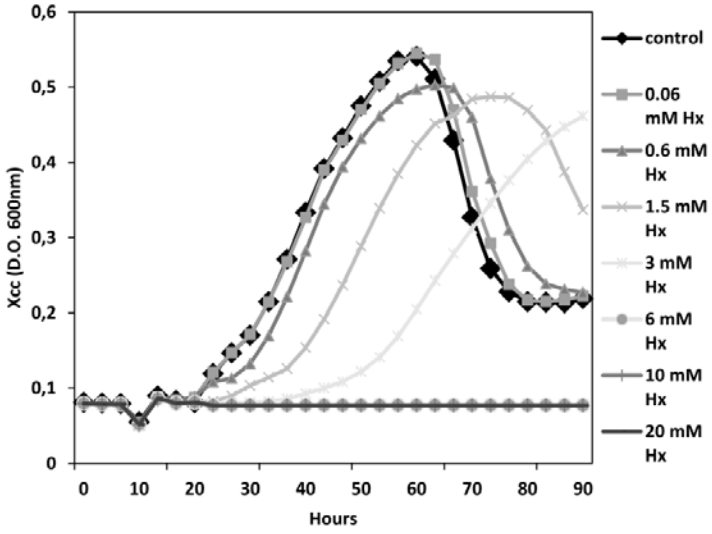

Figure 1. Growth of *Xanthomonas citri* subsp. *citri* (Xcc) in Luria Bertani broth amended with different concentrations of hexanoic acid (Hx) along 96 hours.

Lesion development and bacterial populations in a detached leaf assay

Treatments with Hx and ASM of detached leaves inoculated with Xcc almost completely prevented development of lesions compared with untreated checks (UTC) at 15 days post-inoculation (dpi) (Fig. 2). Based quantification of total Xcc populations at 15 dpi by Q-PCR Hx at 1.0 mM and 3.0 mM reduced Xcc by 1.41 log units (6.58 log) and 0.91 log units (7.08 log), respectively, compared to the UTC (7.99 log), whereas ASM reduced the populations 1.57 log units (6.42 log; Table 1).

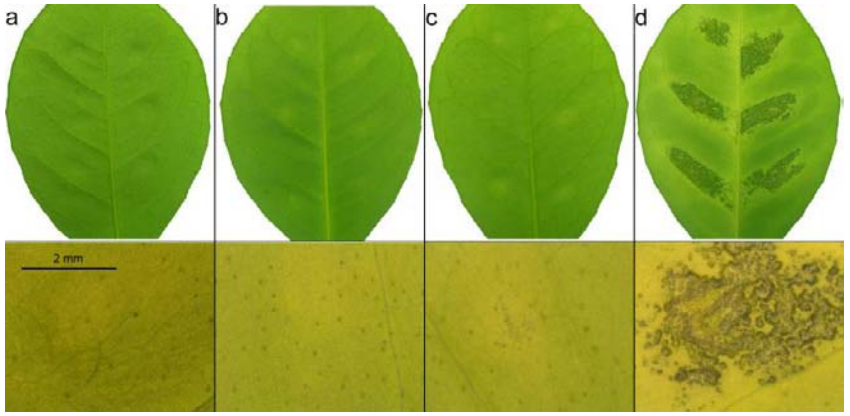

Figure 2. Effect of a single spray application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) on development of citrus canker lesions in detached sweet orange leaves at 15 days after inoculation with *Xanthomonas citri* subsp. *citri* (Xcc) at 10^5 cfu mL^{-1} in the detached leaf assay. A) Hx at1.0 mM, B) Hx at 3.0 mM, C) ASM,1mM D) untreated control.

Recovery of viable bacteria was 2.32 log units lower in the 1.0 mM Hx-treated leaves (3.97 log), 1.81 log units lower in the 3.0 mM Hx-treated leaves (4.48 log) and 2.51 log units lower in the ASM-treated leaves (3.78 log) compared with the UTC leaves (6.29 log) (Table 1).

For subsequent experiments, 1.0 mM Hx was chosen because, *in vitro*, this concentration significantly reduced infection and bacterial population development without causing direct toxicity to Xcc.

Table 1. Effect of a single spray application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) on bacterial population at 15 days after inoculation with *Xanthomonas citri* subsp. *citri* (Xcc) at 10^5 cfu mL−1 in the in vitro assay. Means followed by the same letter are not significantly different at P≤0.05 according to LSD test. P value lower than 0.05 indicates differences between groups

	Log viable Xcc*	**Log total Xcc****
UTC	6.29a	7.99a
Hx 1 mM	3.97b	6.58b
Hx 3 mM	4.48b	7.08ab
ASM	3.78b	6.42b
P-value	0.0001	0.047

*Xcc recovered per inoculation site on KCC semi-selective medium

**Xcc detected per inoculation site by QPCR

Lesion development and bacterial populations in an attached leaf assay

At 20 days after the initial inoculation of attached leaves with Xcc, soil drench and foliar spray treatments with Hx reduced lesions by 50.7% and 47.4%, respectively, compared to the UTC (Table 2). Hx treatments also significantly reduced the viable Xcc populations in leaves when compared with the UTC plants (Table 2). Hx treatments reduced the number of lesions to similar levels to those obtained in plants treated with the ASM in spray. However, this reduction was lower than produced by the ASM soil drench. After the second inoculation of the treated plants, Hx soil and foliar treatments reduced lesions by 68.5% and 65.5%, respectively, compared to UTC. Soil and foliar Hx reduced Xcc populations by more than 50%. The magnitude of bacterial population control was similar to that obtained after treatment with ASM. After the second inoculation, the reductions produced by Hx treatments were greater than that for ASM spray, but less than that for ASM soil drench. The Xcc populations estimated by Q-PCR in the treated leaves after the first and second inoculations did not differ from the UTC (Table 2).

Table 2. Effect of spray or soil drench application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) at 20 days after first (A) and second (B) inoculation on development of citrus canker lesions or bacterial populations on sweet orange leaves inoculated with *Xanthomonas citri* subsp. *citri* (Xcc) at 104 cfu mL−1 in the greenhouse assay. Values represent the average of three experiments. Numbers followed by the same letter are not significantly different at P≤0.05 according to the LSD test. P value lower than 0.05 indicates differences between groups

A	**Day 20 after first inoculation**		
	Lesion number	**Log viable Xcc***	**Log Total Xcc****
UTC	116a	6.96a	8.42a
Hx	57b	6.27a	8.85a
Hx spray	61b	6.67a	8.75a
ASM	36c	5.55a	8.85a
ASM spray	40bc	6.71a	8.43a
P value	0.0158	0.4465	0.7360

B	**Day 20 after second inoculation**		
	Lesion number	**Log viable Xcc***	**Log Total Xcc****
UTC	138a	4.276a	8.33a
Hx	67b	3.67b	8.26a
Hx spray	71b	3.60b	8.39a
ASM	38c	3.48b	8.30a
ASM spray	60b	3.66b	8.35a
P value	0.0248	0.046	0.7091

*Xcc recovered per inoculation site on KCB semi-selective medium

**Xcc detected per inoculation site by QPCR

Expression analysis of defense-related genes after Xcc inoculation

Transcription of the *PR2, PR5* and *AOS* genes was monitored in leaves below the inoculation point. After the first inoculation of plants at 10 dpi, the expression of *PR5* was unaffected, and *AOS* and *PR2* slightly increased. At 20 dpi, expression of *AOS* and both of the *PR* genes was significantly increased by Hx and ASM applied as spray or in soil compared to the untreated and infected control (Fig. 3a and c).

After the second inoculation at 10 dpi, *AOS* expression was promoted by all the treatments (Fig. 3b), but a lower effect was observed for the Hx soil treatment. Expression of *PR2* (Fig. 3d) was induced by Hx and ASM, and this enhancement was much greater than observed after the initial inoculation. Unlike the *AOS* and *PR2* gene responses, *PR5* only responded at 20 days after the first inoculation but showed no significant differences from UTC after the second inoculation (Fig. 3e and f). The treated non-inoculated control did not show changes in the gene expression compared with the untreated non-inoculated plants (Fig. 3).

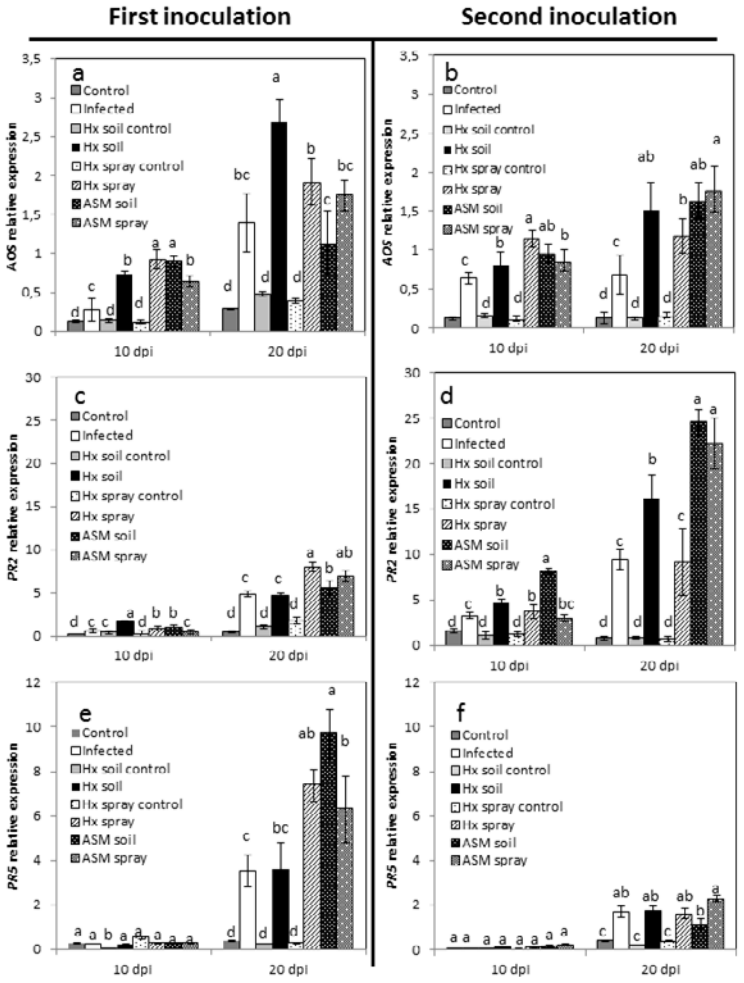

Figure 3. Effect of spray or soil drench application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) at 10 days and 20 days after first and second inoculations on *AOS*, *PR2* and *PR5* gene expression in sweet orange leaves inoculated with *Xanthomonas citri* subsp. *citri* at 104 cfu mL−1 in the greenhouse assay. Bars represent the ■ Control, □ UTC, ▒ Hx soil control, ■ Hx soil infected, ▨ Hx spray control, ▨ Hx spray infected, ▪ ASM soil, ▩ ASM spray infected. Values represent the average of three experiments, bars represent standard error of the mean values, and different letters represent significant differences for each time point at P≤0.05 according to LSD test

Callose deposition

In order to assess the mechanism of resistance induced by Hx, callose formation was observed at the Xcc infection site. Callose accumulation significantly increased upon infection in the plants treated with soil-applied Hx after the first inoculation (Fig. 4a), whereas all the treatments produced significant differences after the second inoculation. Callose accumulation was 4 times higher than the other treatments. A higher *CALS1* gene expression in the Hx-soil treated plants confirmed the in situ callose response (Fig. 4b). In contrast, Hx applied with spray and the ASM treatments did not enhance callose.

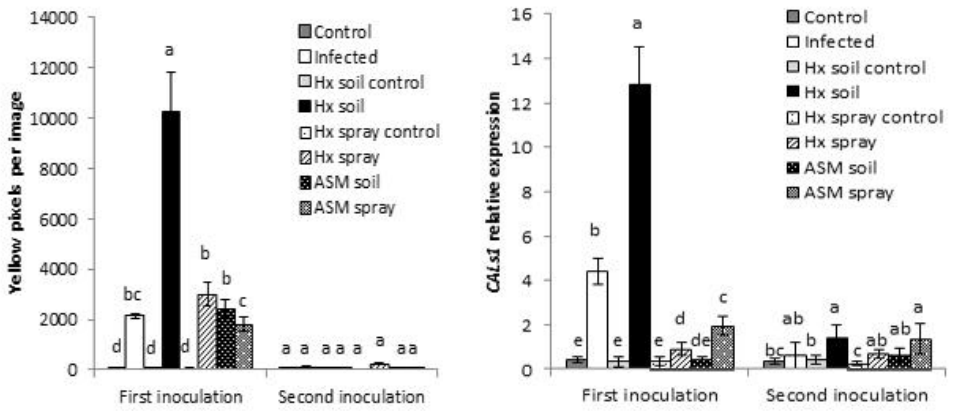

Figure 4. Effect of spray or soil drench application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) at 20 days after first and second inoculation on (a) callose accumulation (Readings were calculated from the average number of blue pixels in the images), (b) CALS1 gene expression in sweet orange leaves inoculated with *Xanthomonas citri* subsp. *citri* at 10^4 cfu mL−1 in the greenhouse assay. Bars represent the ■ Control, □ UTC, ▤ Hx soil control, ■ Hx soil infected, □ Hx spray control, ▨ Hx spray infected, ▪ ASM soil, ▩ ASM spray infected. Values represent the average of three experiments, bars represent standard error of the mean values, and different letters represent significant differences in each time point at $P \leq 0.05$ according to LSD test

DISCUSSION

Hx is a natural monocarboxylic acid produced by several plants including strawberry (Zabetakis et al., 2000) and *Arbutus unedo* (Soufleros et al., 2005). Hx is also detected in butter and butter oil (Peterson and Reineccius 2003) and in cheeses (Morales et al., 2006), and contributes to their aromatic character. This acid has been tested as a resistance inducer in crop plants such as tomato against *B. cinerea* (Vicedo et al., 2009), *P. syringae* (Scalschi et al., 2013), and in citrus against the fungus *Alternaria alternata* (Llorens et al., 2013). In the present work we evaluated the effectiveness of Hx for host-mediated resistance against Xcc, the cause of citrus canker, the direct activity against the bacterium, and the longevity of the protective effect.

In vitro, Hx concentrations over 0.6 mM in LB medium inhibited Xcc growth. Results indicated that Hx delayed entry of the bacterium into the stationary phase, but did not reduce the size of bacterial population. The LIVE/DEAD® assay demonstrated that the growth inhibition was a bacteriostatic effect. In detached citrus leaf inoculations, Hx at1.0 and 3.0 mM reduced lesion number and viable and total Xcc populations. In attached leaf inoculation of plants, both soil and foliar Hx spray applications reduced the number of lesions produced by Xcc. Hx produced a similar reduction in lesions and the bacterial population as ASM. These disease control effects are consistent with those reported by Francis et al. (2009), who demonstrated that soil drenches with ASM reduced lesions and Xcc populations in leaves and differences in response between the spray and soil applications. Hx and ASM significantly reduced canker symptoms for up to 45 days after treatment, but disease control activity was longer-lasting after soil drenching.

Activation of defense pathways was confirmed by elevated expression of PR genes. Wang et al. (2012) demonstrated that the exogenous application of SA promoted the expression of *PR* genes and reduced the occurrence of canker disease. Moreover, Francis *et al* (2009) observed a correlation between the expression of *PR2* and lesion reduction. Gene expression responses suggest that Hx activates both the *AOS* and PR responses. Similar results were obtained by Fu et al. (2011) using transgenic sweet orange plants overexpressing spermidine synthase, which induced high constitutive levels of *PR* gene expression and resistance to Xcc. These authors (Fu et al., 2011) observed an induction of *AOS* after Xcc infection, which implies that JA synthesis is involved in resistance. A relationship between the induction of JA/SA pathways is supported by previous results for Hx activity in tomato against *P. syringae* (Scalschi et al., 2013).

The activation of defense pathways also shows differences in accordance with the mode of application and the time that elapsed after treatment. Hx and ASM produced a quicker *PR2* response in the spray-treated plants, but gene expression was higher in the soil-treated plants after the second inoculation. This elevated *PR2* expression after the ASM application was previously described by Francis et al. (2009).

Enhanced callose deposition in Hx soil-treated plants after the first inoculation was correlated with the *CALS1* gene expression. This accumulation was not observed in the Hx spray- and ASM-treated plants. Callose acts as a physical barrier that concentrates antimicrobial compounds at fungal penetration sites (Huang et al., 2006). Lee et al. (2009) proposed that callose also contributes to resistance against invading bacterial pathogens by providing a physical barrier. Recently, Enrique et al. (2011) demonstrated in callose-silenced citrus plants, that lowering callose levels weakened this barrier and led to enhanced Xcc susceptibility compared to wild-type plants. Moreover, Yun et al. (2006) found that callose is required for resistance to Xcc and that suppressing callose deposition induces susceptibility to Xcc in *N. benthamiana* and *Arabidopsis*. In contrast, no accumulation of callose in the Hx spray-treated plants was detected after the first inoculation or in any of the treatments after the second inoculation. This lack of callose accumulation correlated with a higher *PR2* expression, as previously found by Kaliff (2007) for the *Arabidopsis abi 1-1* mutant.

In conclusion, Hx application by spray or soil drench systemically reduced citrus canker lesions and viable Xcc populations with similar effectiveness to the commercial inducer ASM. Hx is a natural plant product with low phytotoxicity that has been demonstrated to be effective under greenhouse conditions, and now should be tested in field trials as previously reported for ASM (Graham and Myers 2011,13). At this time, hexanoic acid has been patented and commercialized in Spain (Induct, Salquisa; Patent nº 200501535/0) for systemic resistance in tomato against Botrytis. Based on our results, the application of hexanoic acid could be used to augment current chemical inducers for citrus canker control to possibly reduce the number frequency of applications of copper bactericides.

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Supplementary Table 1. Primers and probe used in real time-PCR reactions

Primers Sequence	**(5′→3)'**	**Target**
J-RTpth3 forward	ACCGTCCCCTACTTCAACTCAA	pth gene from *Xanthomonas citri* sbsp. *citri*
J-RTpth4 reverse	CGCACCTCGAACGATTGC	
J-Taqpth2a	FAM-ATGCGCCCAGCCCAACGCMGB	
***Actin* forward**	CCAATTCTCTCTTGAACCTGTCCTT	Beta actin
***Actin* reverse**	GAAGACCGTCAAGAGTAGTCAGT	
***18S* forward**	TCGGGTGTTTTCACGTCTCA	18SrRNA
***18S* reverse**	TGGATGCCGCTGGGAAGC	
***PR2* forward**	TTCCACTGCCATCGAAACTG	PR-2 (β-1,3-glucanase) *Citrus sinensis*
***PR2* reverse**	TGTAATCTTGTTTAAATGAGCCTCTTG	
***PR5* forward**	TGGGGGACTACTCCAATGTC	PR5, osmotin-like protein *Citrus sinensis*
***PR5* reverse**	ATCCTCCTGGAACCCTCAAT	
***AOS* forward**	CGAATTTCAATCCCCAAGAA	Allene oxide synthase *Citrus sinensis*
***AOS* reverse**	TTGGTGGGTTGTTCATCAGA	
***CALs1* forward**	ACGGCTTAATGTTTGCTCGC	Callose synthase
***CALs1* reverse**	TACGTGAAGGCAACGTCACT	

Chapter 4

Metabolomics approach on the effect of hexanoic acid in citrus

Hexanoic acid is a short natural effective monocarboxylic acid as a resistance inducer in tomato plants against *Botrytis cinerea* and *Pseudomonas syringae*, and in citrus against *Alternaria alternata* and *Xanthomonas citri* subsp. *citri*. Previous studies have suggested that induced resistance by applying this acid can be mediated by the jasmonic acid pathway, callose accumulation and the implication of phenolic compounds. In this work, we studied the metabolic response produced by Hx acid application in response to the challenge pathogen *Alternaria alternata*. An analysis of volatile compounds showed that an emission of more than 15 compounds was related with the mevalonic and linoleic pathways. Moreover, the results obtained with the metabolomics assay indicate the enhancement of more than 200 metabolites that were induced differentially by applying the compound. Both pathways start in the Acetyl-CoA, which suggests that the effect of Hx acid as a resistance inducer may be noted at this level. Studying the application of hexanoic acid marked with ^{13}C has demonstrated that this molecule remains in roots and does not mobilise to leaves.

INTRODUCTION

Plants are static organisms that have been forced to develop mechanisms to adapt and survive different stresses and unfavourable environments. To face the enormous number of microorganisms that can hurt them, they arm themselves with molecular shields against their attackers. The innate resistance of plants is based on constitutive physical and chemical barriers that are able to avoid infection from many challenge pathogens. When plants are attacked, batteries of inducible defenses, as well as constitutive barriers, are activated to interfere in colonization. These are the results of a complex signalling network in which hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are involved. This innate immunity can be stimulated to achieve an enhanced level of resistance that leads to a phenomenon known as induced resistance (IR).

The term induced resistance describes the state of resistance in plants triggered by a biological or chemical agent which protects non-exposed plant parts against future attacks by pathogenic microbes or herbivorous insects (Kuc, 1982). Hence resistance is expressed not only locally at the induction site, but also systemically in other parts separated from the inducer. Depending on the pathogen kind and the subsequent plant response, this induced resistance can be divided into systemic acquired resistance (SAR), herbivore-induced resistance (HIR), and induced systemic resistance (ISR) (Pieterse et al., 2014).

SAR has been extensively studied in the last few years (Vlot et al., 2009; Spoel and Dong, 2012). This process is characterised by increased levels of SA, the activation of pathogenesis-related (*PR*) genes, many of which encode PR proteins with antimicrobial activity, hypersensitive response (HR), trailing necrosis, cell wall strengthening by callose and lignin rich in papillae deposition (Van Loon, 1997; Van Loon and Van Strien, 1999). ISR is the name given to the resistance induced by non-pathogenic microbes in the rhizosphere (Alstrom, 1991). These two mechanisms of resistance are mediated by SA-independent signalling pathway, which leads to JA pathway activation (De Vleesschauwer and Hofte, 2009; Pieterse, et al., 2014). The response of JA-dependent plants is also related to the pathogen type that activates the different branches of this pathway. Infection by necrotrophic pathogens entails ethylene accumulation (ET) that acts synergistically on the expression of the ethylene response factor branch (ERF) of the JA pathway associated with resistance to necrotrophs (Lorenzo et al., 2003; Anderson et al., 2004; Lorenzo et al., 2004; Pré et al., 2008). Upon wounding or herbivory attack, abscisic acid acts synergistically on the expression of the MYC branch of the JA response pathway, while it antagonises the ERF branch (Anderson, et al., 2004). This action results in the prioritisation of the immune

signalling network towards the MYC branch of the JA pathway, which is associated with resistance to herbivory (Anderson, et al., 2004; Dombrecht et al., 2007; Fernández-Calvo et al., 2011). Besides the direct accumulation of defense proteins, indirect defense involves the production of a blend of volatiles that attracts the predatory or parasitic enemies of herbivores. Plant volatiles can also play a role in the control of defense gene expression, probably as a signal mediator within and between plants (Farmer, 2001). We can find examples of this in *Arabidopsis thaliana* (Seo et al., 2001), lima beans (Ozawa et al., 2000; Arimura et al., 2002), tomatoes (Farmer and Ryan, 1990) and citrus (Gomi et al., 2003).

Plants also have the ability to prepare themselves to face pathogenic attacks. This phenomenon is called priming and it is a physiological condition that leads to a faster and stronger response of the basal plant defenses to biotic or abiotic stress (Achuo et al., 2004; Conrath, 2009; Jung et al., 2009). The primed state of plants can be stimulated by exogenously applied inorganic or organic compounds. The most widely studied priming inducers are acibenzolar-s-methyl, β-aminobutiric acid, azelaic acid, chitosan or volatile organic compounds.

In recent years, carboxylic acids have been extensively studied as a priming inducer (Flors et al., 2001; Flors et al., 2003; Vicedo et al., 2006). Hexanoic is a monocarboxylic acid that can induce plant defense responses when used as a priming agent. In the last few years, several studies in our lab have demonstrated that hexanoic acid (Hx) can protect tomato and Arabidopsis plants against *Botrytis cinerea* (Vicedo et al., 2009; Kravchuk et al., 2011) and citrus plants against *Alternaria alternata* (Llorens et al., 2013). These studies suggest the enhancement of oxylipin (1,2-oxo-phytodienoic acid; OPDA) and bioactive molecule jasmonate-isoleucine (JA-Ile) in treated plants with the challenge pathogen after inoculation. Abscisic acid (ABA) also acts as a positive regulator of Hx-induced resistance (Hx-IR) by enhancing callose accumulation (Vicedo, et al., 2009).

High performance chromatographic techniques enable the identification of the metabolites induced in the priming phase and involved in defense. In this way, several secondary metabolites have been recently identified as possible players in plant priming, such as azelaic acid (Jung, et al., 2009), indol-3-carboxilic acid (Gamir et al., 2012) or pipecolic acid (Návarová et al., 2012). The combination of non-targeted and targeted quantitative analyses provide us with the possibility to conduct an in-depth study of the alterations produced by both the priming agent and pathogen response. The characterisation of the elements participating in plants defense and the targets and changes produced by the priming agent will allow us to develop more accurate systems to protect plants against different stresses. For this reason, the present work aims to determine the metabolic changes produced by

hexanoic acid in citrus plants, and to study the different responses of 'Fortune' trees mandarin inoculated with *Alternaria alternata*.

MATERIALS AND METHODS

Plant material and pathogen inoculation

For all the experiments, we used 2-year-old 'Fortune' mandarin plants grafted onto citrange Carrizo plants (*Citrus sinensis* L. Osbeck x *Poncirus trifoliata* Blanco) (Beniplant, Valencia, Spain), grown in a greenhouse in 10-L pots using peat as the substrate (Vivercitrus, Alcanar, Spain). One month before each experiment commenced, leaves were removed to encourage uniform sprouting.

For the ^{13}C assay, 4-month-old citrange Carrizo seedlings with a single shoot were used.

Spores of *Alternaria alternata* were collected from 10- to 15-day-old cultures with sterile water containing 0.02% (v/v) Tween-20. Solutions were filtered, quantified with a haemocytometer and adjusted to 10^5 spores/mL. Infection was carried out by dispensing 5 µL of the spore solution onto each leaf. Leaf and root samples were collected at 96 h post-infection for the LC-ESI full scan mass spectrometry measurements and fresh material was collected in an Erlenmeyer flask for the volatile compound analysis.

Chlorophyll content

The chlorophyll level in leaves was measured by a chlorophyll meter (SPDA; Minolta, Tokio, Japan). Three measurements were taken per leaf on each side of the central vein with 10 plants per treatment. The three SPAD readings taken on one leaf for each treatment plant were averaged to represent one observation. The results were obtained as SPAD values (S, dimensionless).

Photosynthetic parameters

Determinations were made *in situ* on the apical part of the leaves of the untreated and treated plants that were not infected. Leaves of the same age were measured. The gas exchange analysis was carried out using a portable open system with an LCpro+ infrared gas analyser (ADC Bioscientific Ltd., Hoddesdon, UK) in ambient CO_2 and humidity. Light was provided by a photosynthetically active radiation lamp at 1,000 mol m^{-2} s^{-1} photon flux density. The photosynthetic rate

(μmol CO_2 $m^{-2}s^{-1}$), transpiration rate (mol H_2O $m^{-2}s^{-1}$) and stomatal conductance (mol H_2O $m^{-2}s^{-1}$) were recorded for one leaf per plant.

Volatile compound analysis

Extraction was performed as described by Beltran et al. (2006), but was modified for the citrus assays. The system consisted in a 500-mL Erlenmeyer flask attached to a glass cap with two connexion tubes; the inlet was connected to a dry N_2 gas supply and the outlet to a Tenax trap. Dry nitrogen (99.7%) was used for the purge process and flowed into the flask at 1 L min^{-1}. A citrus sample with 5% (w/w) $CaCl_2$ and 50 μL 15 mg mL^{-1} methyl salicylate-D_4 (surrogate/internal standard) was magnetically stirred (350 rpm) and heated at 35°C for 120 min to allow volatile analytes to be retained in the Tenax trap (ambient temperature). $CaCl_2$ was added to deactivate the enzyme systems. The trap was removed and eluted with 3.5 mL hexane–ether (1:1). The final extract volume was adjusted to 1 mL by a gentle nitrogen stream.

GC-MS analysis

A Varian CP-3800 gas chromatograph coupled with a mass spectrometric detector (Saturn 4000, Varian) was used to identify and quantify the volatile compounds in the citrus extract. Analytes were separated in a 30 m×0.25 mm DB-5MS (0.25 μm film thickness) Varian capillary column. Helium at 1 mL min used as the carrier gas. The temperature programme was: 45°C for 5 min, increased to 96°C at 3° min−1, then to 150°C at 6° min−1, and finally to 240°C at 30° min−1, with a final 1.5-minute isothermal stage (the total chromatographic analysis time was 36 min). The gas chromatograph was directly interfaced with the Varian 4000 mass-spectrometer (ion trap) in the external ionization mode. Quantitation of the analytes in the sample extracts was performed using a calibration graph to obtain a plotting peak area in relation to that of internal standard methyl salicylate-D4 against concentration (ng mL^{-1}). The m/z ratios used for quantification and confirmation were those described by Beltran et al (2006). The quantification ion used for the internal standard methyl salicylate-D4 was 155.

LC-ESI full scan mass spectrometry (Q-TOF instrument)

Extraction was performed as described by Llorens et al. (in press). Aliquots of 20 μL were injected into a UPLC (Waters, Mildford, MA, USA) coupled to a quadrupole-time of flight mass spectrometer (QTOF Premier) through an

electrospray ionization source. The LC was developed for 25 min in a standard C18 column using a standard variable H_2O:MeOH gradient. Mass detection was performed with 25 V of cone energy. Nitrogen was the drying gas and the nebulizing gas. The desolvation gas flow and the cone gas flow were set at 600 L/h and 60 L/h, respectively. A 20 V cone voltage and a 3.3 kV capillary voltage were used in the negative ionisation mode. The nitrogen desolvation temperature was set at 350°C and the source temperature was set at 120°C. The instrument was calibrated within the *m*/*z* 50–1000 range with a 1/1 mixture of 0.01 M NaOH/1% HCOOH, which was diluted 10-fold with acetonitrile/water (80/20, v/v).

^{13}C assay

Four-month-old citrange 'Carrizo' seedlings with a single shoot were selected for their uniformity of size and were transferred to aerated complemented Hoagland solution for 7 days on hydroponic culture devices. After adaptation to the hydroponic system, Hoagland solution was amended with 1mM of Hx acid labelled with ^{13}C on the carboxylic end to simulate treatment. After 48 h and 96 h of labelling, roots, stems and leaves were separated and dried for 48 h at 65°C, crushed in a hammer, milled and weighed. The ^{13}C analysis was performed using an integrated system for continuous flow isotope ratio mass spectrometry (Euro-EA elemental analyser; EuroVector S.P.A., Milan, Italy) and an Isoprime mass spectrometer (GV Instruments, Manchester, UK). The mass of ^{13}C was calculated from the fractional abundance (F) and total C content using a value of 0.0112372 for the absolute isotope ratio of the Pee Dee Belemite (international ^{13}C standard), the measured values of ^{13}C (‰PDB) and the relationship (Stewart and Metherell, 1999).

Bioinformatics and statistical analysis

The raw data obtained with the MASSLYNX software were transformed into the .CDF format using the DataBridge programme provided with the MASSLYNX software. The .CDF data were processed with R for statistical computing using the XCMS package for relative quantification (Smith et al., 2006). A partial least squares analysis was performed as described at http://www.numericaldynamics.com/ to define the major changes occurring in the metabolome of the plant under the priming conditions during fungal infections. For the heat map construction and the clustering of the metabolite, the MarVis Filter and MarVis cluster (http://marvis.gobics.de/; (Kaever et al., 2012) softwares

were used. In order to identify metabolites. The results obtained with QTOF were matched by comparing the exact mass with the Metlin (http://metlin.scripps.edu/index.php), KEGG (www.genome.jp/kegg/) and MetaCyc databases (MetaCyc.org).

Statistical analyses were performed with the Statgraphics software. Mean values were compared by the LSD (least significant difference) test. All the experiments were repeated at least 3 times.

RESULTS

Physiological parameters are enhanced by hexanoic acid application

The results of the physiological parameters indicate that chlorophyll content increased in the Hx-treated plants throughout the experiment to achieve 10% higher values than the untreated plants at the end of experiment (Table 1). The gas exchange parameters also showed differences for the treated plants, with increases of almost 30% in the photosynthetic rate and of 8% in the transpiration rate. A higher photosynthesis rate, as compared to transpiration, indicated better water use efficiency (W.U.E.). This rate was at least 25% higher in the treated plants at the end of experiment. W.U.E. is a parameter that relates CO_2 absorption and water loss in leaves.

Table 1. Effect of hexanoic acid treatment on the physiological parameters. Asterisks indicate a statistically significant difference (P<0.05)

	Control	**Hexanoic**	***Sig.***
Transpiration (H_2O/m^2s^{-1})	2.71 ± 0.14	2.95 ± 0.10	*
Photosynthesis rate (μ mol CO_2 m^{-2} s^{-1})	2.16 ±0.28	3.17 ± 0.33	*
Stomatal conductance (mol/m^2 s^{-1})	0.25 ± 0.06	0.36 ± 0.03	*
WUE mmol (CO_2)·mol^{-1}(H_2O)	0.80± 0.07	1.08± 0.14	*
Chlorophyll (SPAD units)	30.12 ± 0.16	33.47 ± 0.36	*

Our results indicate a better W.U.E. in the Hx-treated plants, which suggest better CO_2 assimilation per mol of water. Stomatal conductance is the speed at which water evaporates from plant pores, and it relates directly to relative stomatal aperture size. In the stomatal conductance study, the maximum values were given in the treated plants. The conductance values obtained under these conditions were 0.25 and 0.36 $mol/m^2 \cdot s^{-1}$ (Table 1).

Production of volatile compounds is enhanced by treatment with Hx acid.

The GC-MS analysis allowed us to determine and characterise the most important volatile compounds released by leaves. In our experiment, we detected 27 of the 47 possible compounds (Table 1, Supplementary Material). The emission of 16 of these detected compounds was strongly affected during priming phase by applying Hx acid to both the infected and non-infected plants (Fig. 1). Among the compounds, only Z-3-hexenol and E-2-heptenal were induced in absence of challenge pathogen. Figure 1 shows that linalool and 2-carene were induced the most with a response above 10^5. R-limonene, 1-octanol and α-Pinene also presented a response over 10^4, while the rest obtained a response of 10^3 or lower. Only beta ionone was induced by infection in the treated and untreated plants. No significant differences between treatments were found for the other detected compounds.

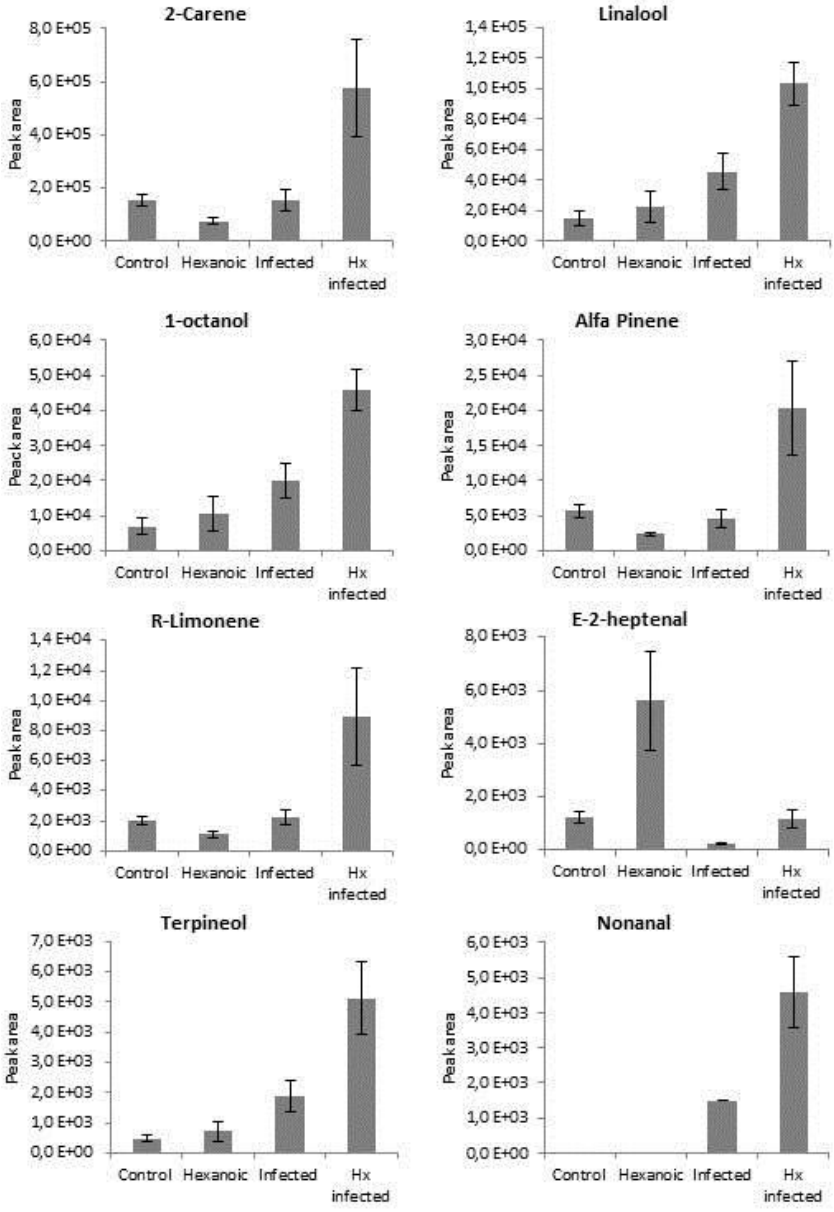

Figure 1. Relative intensity of the volatile compounds detected by GC-MS

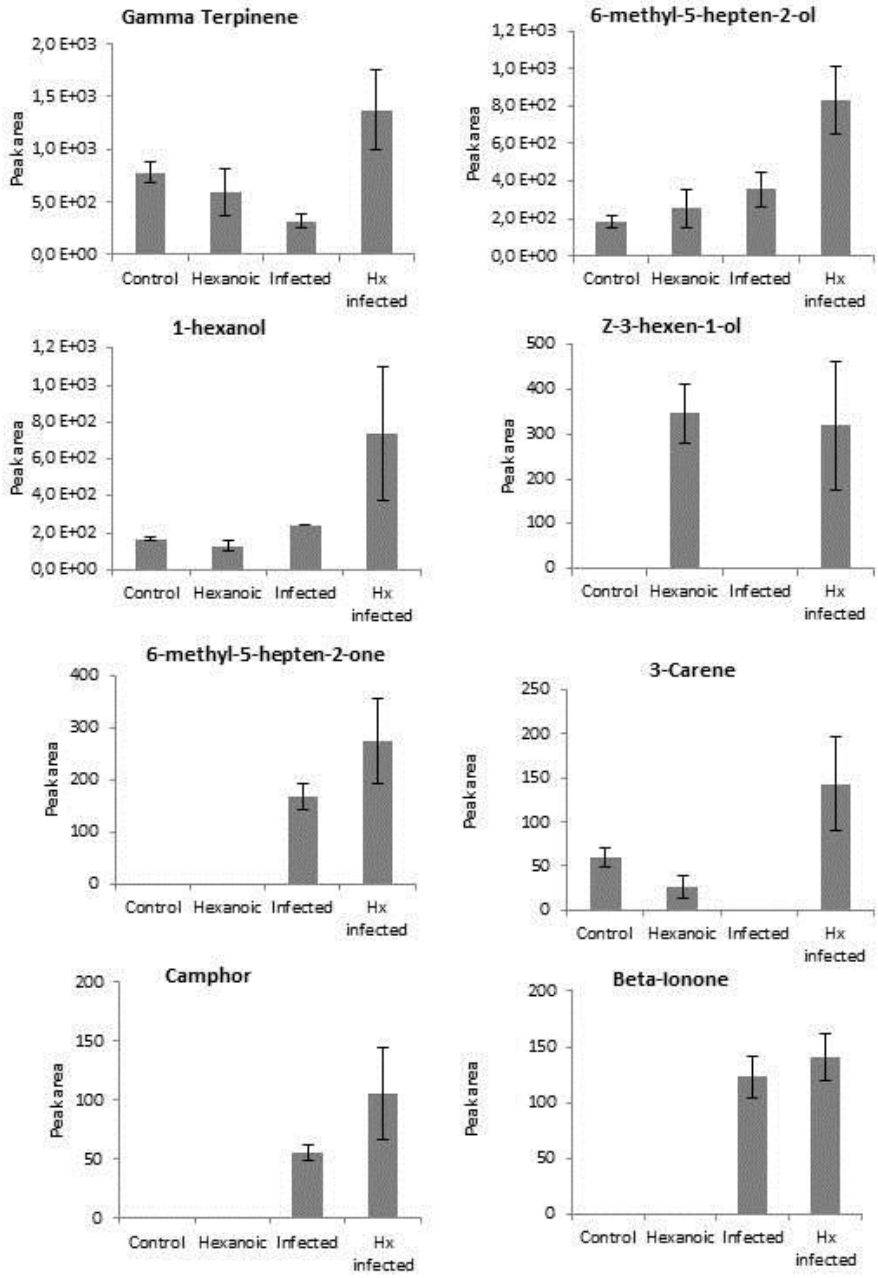

Figure 1. (continuation). Relative intensity of the volatile compounds detected by GC–MS

Metabolic profile changes after infection and Hx application.

As a first approach to account for the overall variability between Hx treatment and time, the normalised area values were subjected to the partial least squares (PLS) method. This method instead of PCA was chosen to avoid multicollinearity problems (Vega-Vilca and Guzmán, 2011). The PLS analysis of the principal components indicated high variability at earlier times in both the infected and uninfected plants. Figure 2 reveals that, at 96 h, the global behaviour of the compounds in control, infected, treated and treated and infected, induce qualitative different metabolites, easily visualized in the cluster. Signals found in treatment do not overlap with control, but are in the same axe, wich confirm that application of Hx provoke changes but does not lead to major changes compared with control.

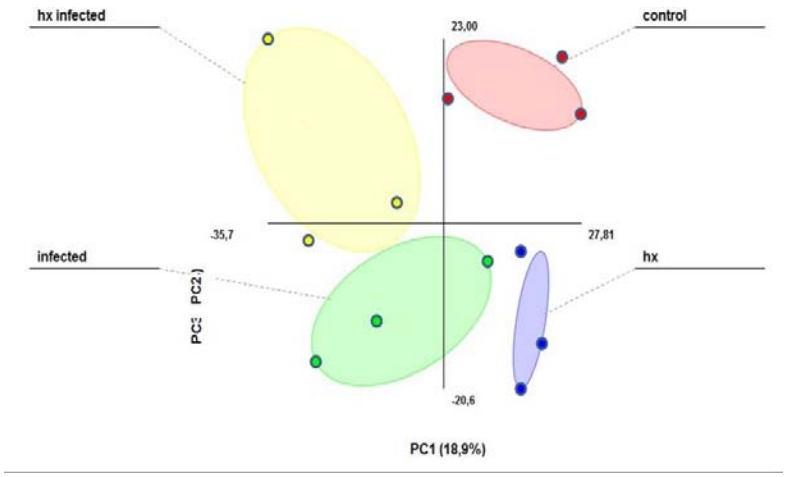

Figure 2. The LS plot showing two major sources of variability among the control, infected, hexanoic acid-treated, and treated and infected plants after LC-MS in the positive mode 96 h after infection.

Treatment with Hx acid and inoculation with Alternaria alternata induced changes in the metabolism of roots and leaves.

The metabolomic data obtained by LC-ESI full scan mass spectrometry were processed and submitted to the bioinformatics and statistical analyses of the signals. The results obtained after the Kruskal-Wallys test ($p<0.05$) indicate that at least 224 compounds were differentially altered in leaves in the positive electrospray ionisation mode, whereas 131 compounds were differentially altered in leaves in the negative electrospray ionisation mode. In roots, 137 compounds differentially altered in leaves in the positive electrospray ionisation mode,

whereas only 72 were found in compounds in the negative electrospray ionisation mode.

These compounds were grouped depending on the treatment that produced the alteration. The numbers of compounds enhanced in each treatment are listed in Figure 3. We observe that in leaves, many compounds were altered by Hx application in both the positive (51 compounds) and negative (26 compounds) ionisation modes, but only 14 and 4 compounds, respectively, were induced by this treatment application in roots.

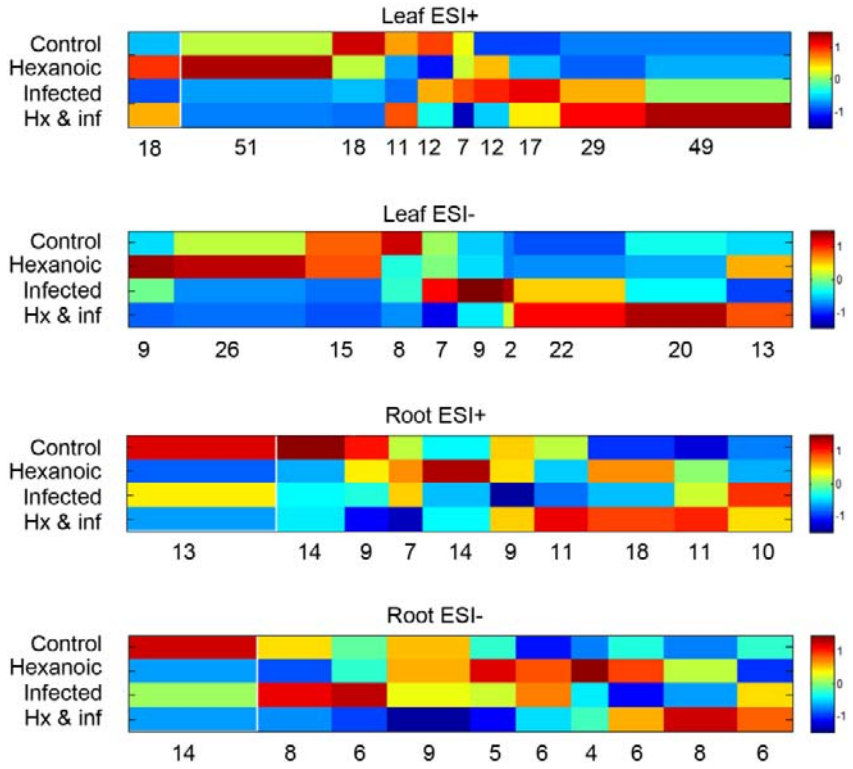

Figure 3. Cluster plots when comparing the four groups generated from the major sources of the variable signals obtained in ESI+ and ESI− by the non-targeted HPLC-QTOF MS analysis of the four groups: Control, Hexanoic, Infected, and Hexanoic and infected. The numbers under each group indicate the number of compounds included. Cluster analyses were performed using the Marvis Filter and Cluster packages following a Kruskal-Wallys test ($p < 0.05$).

When we observed the compounds altered with infection by *Alternaria alternata* in leaves 17 and 16 compounds were altered in ESI+ and ESI-, respectively, and only 10 and 6 compounds were observed in ESI+ and ESI-, ionization in roots.

The combined effect of treatment and infection on the metabolomics profile was strong. In leaves 49 and 20, compounds were enhanced only in the Hx and infected plants in the positive and the negative ionisation, respectively, and 29 and 22 were enhanced in both the infected and Hx-infected plants.

Putative identification of compounds

With the GC-MS analysis results, we did a database search to study the pathways implied in the synthesis of the detected volatile compounds. The obtained results showed that most of the detected volatiles derived from gernanyl diphosphate and linoleic acid (Figs. 4 and 5). The in-deep study that compared the synthesis pathway of both compounds with the metabolomic assay results allowed us to tentatively identify some metabolites (Fig. 6). Beginning with the detected volatile compounds, the intermediates of both synthesis pathways can be found to be between the exact mass of the metabolites altered by the application of Hx and/or inoculation with *Alternaria alternata.*

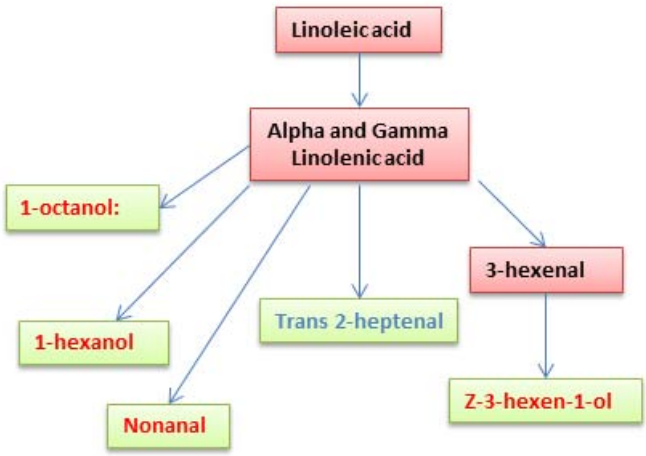

Figure 4. Details of the detected volatiles deriving from linoleic acid. Green boxes indicate that the compound was detected by GC-MS. The compounds printed in red were induced by Hx and inoculation. The compounds printed in blue were induced by Hx in the absence of inoculation.

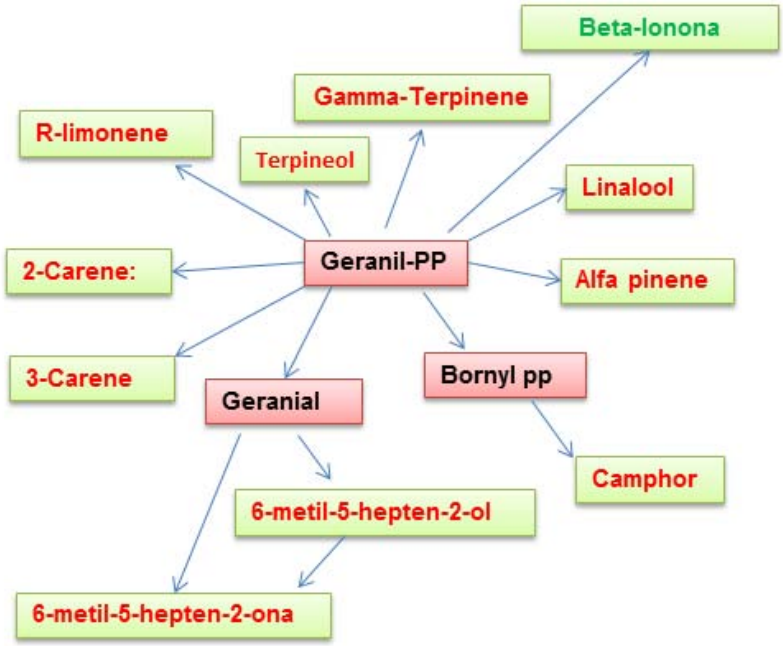

Figure 5. Detail of the detected volatiles deriving from Geranyl diphosphate. Green boxes indicate that the compound was detected by GC-MS. The compounds printed in red were induced by Hx and inoculation. The compounds printed in green were induced by inoculation.

The comparison of our results with the mass databases allowed us to identify some compounds from the linolenic acid pathway, such as malonyl-CoA, linoleic acid, as well as three compounds with the same m/z, but with a different retention time that could match the three isomers of HpOTrE (Fig. 6). The bloxplots of these compounds indicated that this pathway was strongly induced in both the treated and inoculated plants. In the mevalonic acid pathway, the application of Hx in the absence of inoculation enhanced the compounds that matched the m/z of mevalonic acid and mevalonate-5P, while the compounds located downstream were enhanced in the Hx-treated and inoculated plants (Fig. 6).

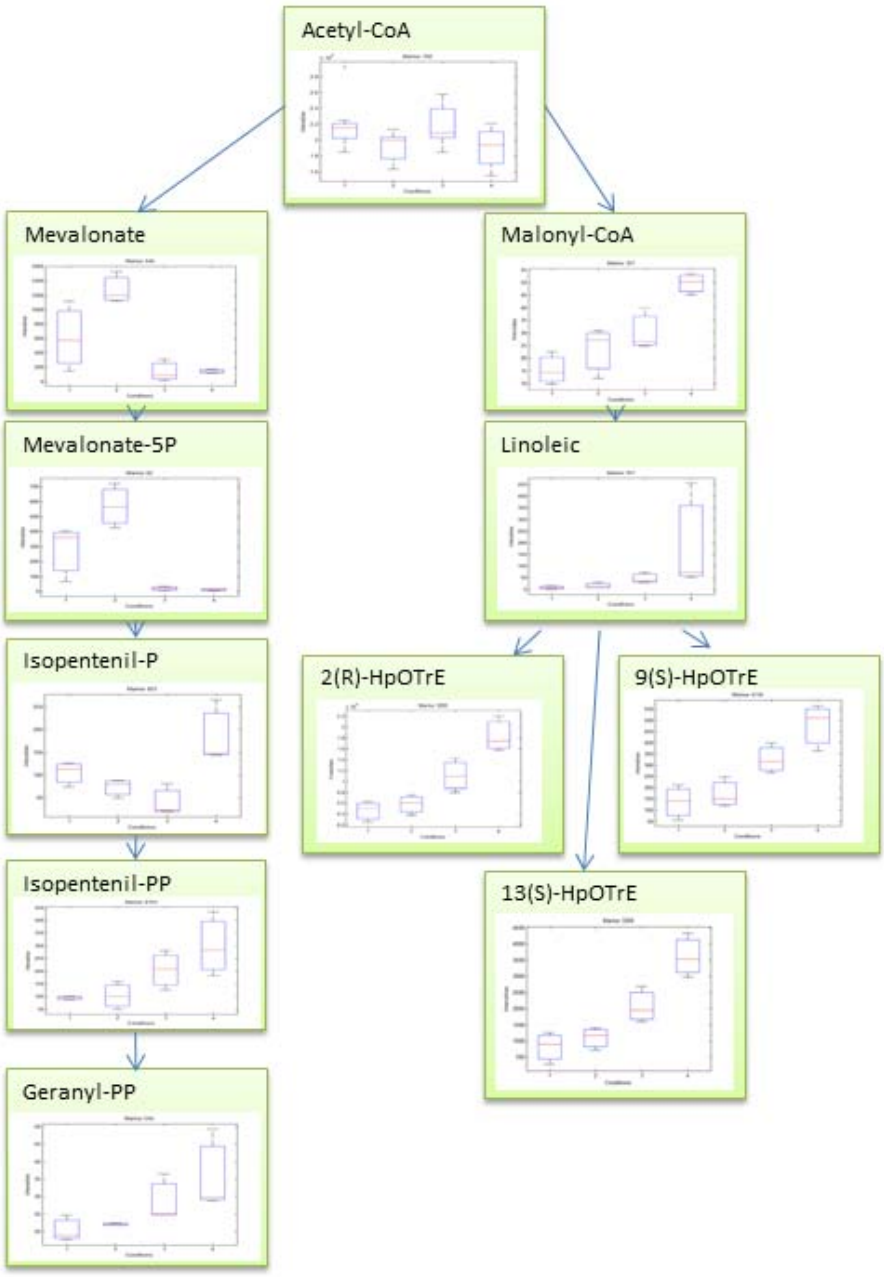

Figure 6. Detail of the compounds detected by exact mass. Boxplots represent the intensity of the control, Hx-treated, infected and Hx-treated and infected plants.

Detection of hexanoic acid in plants after treatments.

In order to determine the location of hexanoic acid in the plant after treatment, we treated plants with Hx labelled with ^{13}C on the carboxylic end. The accuracy of the isotope analyzer allowed us to detect the plant part where a higher concentration of ^{13}C from Hx accumulated. The obtained results showed that after 48 h, considerable ^{13}C accumulation was observed in citrus roots, but the level of labelled carbon was slightly lower at 96 h. Surprisingly, no ^{13}C accumulation was observable in either stems or roots (Fig. 7).

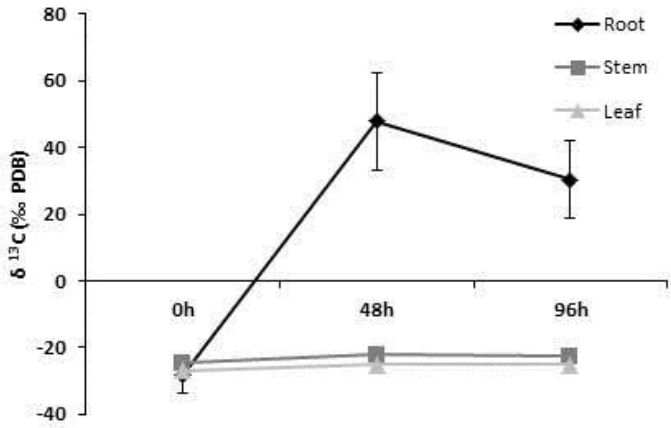

Figure 7. Recovery of ^{13}C in roots, stems and leaves at 0 h, 48 h and 96 h after ^{13}C pulse labelling. The data show the average of 10 plants per experiment±SE.

DISCUSION

Very little knowledge of how hexanoic acid operates in plants is available. To date, we only know that this compound can induce resistance against necrotrophic pathogens by enhancing the jasmonic acid pathway, which leads to enhanced callose deposition to surround inoculation (Vicedo, et al., 2009; Llorens, et al., 2013). However, against biotropohic pathogens application of this acid is also capable of enhancing the salicylic acid pathway (Scalschi et al., 2013). Most studies conducted into defense inducers in plants have focused on the protective effects and defensive pathways activated, and very few have studied the benefits

and costs of aspects like the plant physiological state (van Hulten et al., 2006). To assess this aspect in our study, we analysed the changes that take place at the physiological parameters level to determine the effect of hexanoic acid treatment on citrus plants. Chlorophyll detection is considered a rapid indicator of photosynthetic efficiency and a good tool for the early detection of stress effects in plants. Nevertheless, we must bear in mind that chlorophyll measurements alone cannot be used to establish the processes affected by stress (Lorenzini et al., 1997), so they were combined with gas exchange parameters. In our experiment, an increase in the photosynthetic rate, transpiration rate, stomatal conductance and water use efficiency in the treated plants was observed if compared to the untreated plants. This result indicates that Hx enhances these physiological parameters.

A link between volatile compounds and defensive responses, such as enhanced phytoalexins secretion, incorporation of hydroxycinnamic acid esters into the cell wall, enhanced oxidative burst or augmented induction of defense genes, is well-known (Conrath, 2009). Our experiment detected two big families of volatile compounds. The first is produced from 13-hydroperoxides of linoleic or linolenic acid as one of the branches of the phytooxylipin pathway (Hatanaka, 1993). These results agree with a previous work on citrus (Llorens, et al., 2013) and indicate that the linolenic pathway might play a major role in Hx-induced resistance. Interestingly, only the volatiles Z-3-hexenol and E-2-hepetnal deriving from this pathway were enhanced with Hx application in the absence of inoculation. Suggesting that this pathway is strongly primed by the treatment.

The volatiles deriving from fatty acids also form during the hypersensitive response against pathogens (Croft et al., 1993) or by exogenous JA application (Avdiushko et al., 1995). It is known that the volatiles deriving from linoleic can prime plants for a more robust defense response by increasing total volatiles emission and endogenous JA content after detecting an elicitor (Engelberth et al., 2004). They also seem to increase sensitivity to methyl jasmonate (MeJA), the methyl ester of JA (Hirao et al., 2012), and (Z)-3-hexen-1-ol apparently plays a twofold role by priming and modulating the behaviour of herbivorous insects. It has also been reported that treatment with (E)-2-hexenal, (Z)-3-hexenol or (Z)-3-hexenyl acetate induces several defense-related genes, such as chalcone synthase (*CHS*), *AOS* and *LOX2* in A. thaliana (Bate and Rothstein, 1998), *PR*s, *LOX* or *PAL* in lima beans (Arimura et al., 2001), and *AOS*, *LOX* and *HPL* in citrus (Gomi, et al., 2003) (Supplementary material, Fig. 1).

The second largest group of plant volatiles comprises the isoprenoids deriving from geranyl diphosphate. These compounds belong to the mevalonate pathway and they are produced mainly from damaged plant leaves (Loughrin et al., 1994; Rose et al., 1996). They also serve to attract pollinators, fruit-dispersing animals and enemies of herbivorous arthropods (Pichersky and Gershenzon, 2002). Some of these volatile compounds are able to activate the expression of a number of defense-related genes, such as *PR2* and *PAL*. All these findings suggest that volatile compounds may act as a signal to facilitate defense responses. Engelberth et al. (2004) also demonstrated that the volatile compounds emitted from herbivore-infested corn plants can prime intact plants against insect herbivore attacks. Despite the implication of mevalonate derivatives being widely known as players in plant resistance, herein we show for the first time that it might be involved in hexanoic acid induced resistance

Enhancement in volatile compounds emission suggests that both pathways can be altered by hexanoic acid application. For this reason, we attempted to detect the precursors of these two routes enhanced by hexanoic acid treatment. In this way we compared the data obtained in the metabolomic profile and those in several metabolite databases. A tentative metabolites identification was performed by using the exact mass between the compounds altered with significance at $p<0.05$. The obtained result shows the metabolites enhanced by hexanoic acid application, inoculation with *Alternaria alternata*, or both, with an exact mass that matches the compounds present in both pathways. With the enhanced metabolites we can follow both paths up to common metabolites, in our case, Acetyl-CoA. However, none of the masses detected in leaves in the metabolomic assay corresponded to Hx. As this acid is not found in leaves, it suggests that the effect of Hx is due to a derivative or a systemic effect triggered by this molecule.

The accuracy of newly developed analysis techniques allowed us to conduct an in-depth study of the changes caused by this compound in plants during infection. These recent studies demonstrate the potential for using either MS or NMR to obtain detailed information on the relative isotopomer abundance of a wide range of metabolites following labelling with ^{13}C -substrates. The ability to take relevant measurements to date, and the application of steady-state labelling strategies to determine metabolic flux, has been largely exploited in plants. Several studies have applied the labelling method to study the transformation undergone by a compound in a pathway (Rowan et al., 1999; Kruger and von Schaewen, 2003; Opitz et al., 2014). The results obtained after treatment with hexanoic labeled with ^{13}C on the carboxylic extreme provided us the surprising result that the label of this acid accumulated and remained in roots, and was not detected in either stems or leaves.

In order to clarify this point, we performed a metabolomic analysis of citrus roots. Once again, the exact mass of hexanoic acid was not detectable 96 h after treatment. However, major changes in the metabolic profile were observed. The fact that a group of metabolites in roots was altered by the inoculation of *Alternaria alternata* in both the treated and untreated plants is a remarkable finding, and suggests a strong coordination between distal and proximal plants from the challenge infection. Several studies have indicated that some compounds travel systemically in the plant to activate defenses (Shah and Zeier, 2013). As we demonstrate, the hexanoic acid label remained in the root, and at this time point, we were unable to detect the exact compound mass. This finding suggests that hexanoic application to the soil drench brings about changes in roots that can travel systemically to leaves to induce resistance.

As stated above, both pathways implied a start in Acetyl-CoA. However, this metabolite did not seem to be vastly altered by hexanoic application, which suggests that hexanoic acid action can take place near this compound. It is known in animal cells that the application of hexanoic acid has an effect on Acetyl-CoA carboxylase (Hillgartner and Charron, 1997). It has also been suggested that hexanoic acid and the rest of the medium chain fatty acid enter the cell rapidly by simple diffusion without implicating specific transporters. Then hexanoic acid is rapidly transformed into an acyl-CoA derivative through the action of a specific acyl-CoA synthetase (Akpa et al., 2010). As a substrate, the presence of hexanoic acid in plants has been extensively studied in *Cannabis sativa*, where it is transformed into hexanoyl-CoA (Stout et al., 2012). Taken together, these results indicate that once inside the plant, hexanoic acid can be transformed into a compound that triggers the priming state. Nevertheless, more studies are required to determine the target of hexanoic acid in plants.

In conclusion, the results obtained in this study reveal that hexanoic acid is absorbed by citrus roots, but remains there and does not translocate to other plant parts. Application of this acid to induce resistance against *Alternaria alternata* provokes the alteration of more than 100 compounds and the emission of 16 volatile compounds, which suggests that the effect of hexanoic acid may act upstream, near the primary metabolism. With these data, we highlight the alteration of the linolenic and mevalonic pathways, which produce most of the compounds induced by Hx described in this work and in previous ones. Taken together, the results obtained herein suggest that the effect of hexanoic acid can take place near Acetyl-CoA, probably as a transformation into another compound that triggers the priming state. However, more experimentation is necessary to clarify this issue

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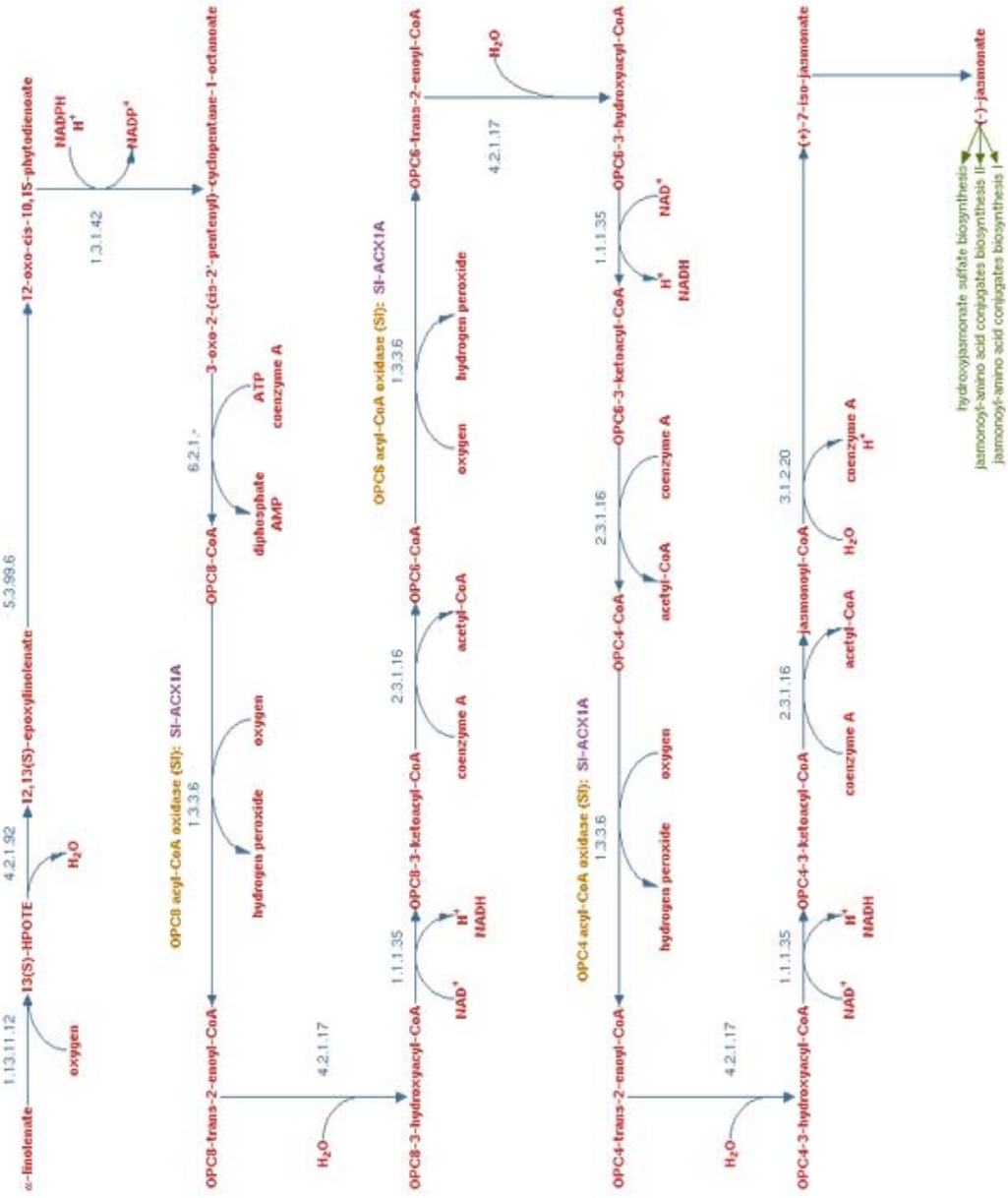
Supplementary Table 1. List of detected compounds in the GS-MS analysis.

Compound	Detected	Compound	Detected
Camphor	+	**6-Methyl-5-hepten-2-one**	+
E-2-HexenylAcetate	+	**Trans-2-hexen-1-al**	+
3-Carene	+	**Methyl salicilate**	+
Alfa Pinene	+	**Hexanal**	-
Terpineol	+	**Guaiacol**	+
3-Methyltiopropionaldehyde	-	**Ethyl salicilate**	+
E-2-Hexen-1-ol	-	**2-Mehtyl-1-butanol**	-
1-hexanol	+	**Trans,trans-2,4-hexadienal**	-
Isoamyl Acetate	-	**Salicilaldehyde**	-
Gamma Terpinene	+	**1-Octanol**	+
E-2-Octenal	-	**Trans,trans-2,4-heptadienal**	-
Z-3-hexen-1-ol	+	**Trans,trans-2,4-decadienal**	+
R-Limonene	+	**Beta-cyclocitral**	-
Citral	+	**2-Octanone**	-
Geranyl Acetone	+	**2-Heptanone**	-
Nonanal	-	**Benzaldehyde**	+
2-Isobutylthiazole	-	**Butyl acetate**	+
2-Carene	+	**2,6-Dimethyl-6-hepten-2-ol**	-
E-2-heptenal	+	**4-Methoxyphenol**	-
6-methyl-5-hepten-2-ol	+	**Diphenyl-ether**	+
Beta-Ionone	+	**Methyl-jasmonate**	-
Eugenol	-	**Methyl-dihydrojasmonate**	-
Linalool	+	**Damascenone**	-
Phenethyl Alcohol	+		



Supplementary Figure 1. Jasmonic acid biosynthesis (Source: Metacyc.org)

Conclusions

CONCLUSIONS

En aquesta tesi doctoral s'ha dut a terme l'estudi de la resistència induïda en cítrics mediada per àcid hexanoic i l'aproximació al seu mecanisme d'acció.

Les conclusions que s'han obtingut son les següents:

1. L'àcid hexanoic indueix resistència en plantes de mandarina 'Fortune' davant del fong necròtrof *Alternaria alternata* reduint la grandària i el nombre de lesions en les fulles, a través del mecanisme conegut com "priming" que indueix la defensa en presència del patogen.

2. Els principals mecanismes involucrats en la defensa de 'Fortune' davant d'*A. alternata* són la ruta de l'àcid jasmónic, l'acumulació de proteïnes inhibidores de poligalacturonases i la deposició de callosa a la zona d'infecció.

3. L'àcid hexanoic indueix resistència en cítrics enfront d' *A. alternata* proporcionant protecció a llarg termini, mostrant elevats nivells de compostos fenòlics i de proteïnes inhibidores de poligalacturonases (increment de l'expressió del gen PGIP)

4. L'Hx protegeix a les plantes de cítrics davant del bacteri biotròfic *Xanthomonas citri* reduint el nombre de lesions per fulla a través d'un increment de l'expressió del gen *AOS*, el *PR2* i el *PR5*, la qual cosa indica una implicació de les rutes dels àcids jasmònic i salicílic. A més l'aplicació del inductor proporciona una protecció a llarg termini enfront de *Xanthomonas citri* intervingut per l'augment de l'expressió dels gens *AOS* i *PR2*.

5. L'anàlisi de paràmetres fisiològics va mostrar que l'àcid hexanoic no solament no modifica, sinó que incrementa la taxa fotosintètica, la transpiració i l'ús eficient de l'aigua. Això indica que el procés de resistència induïda per Hx no comporta un elevat cost energètic.

6. L’Hx indueix resistència en cítrics davant d’*Alternaria alternata* promovent canvis importants en el perfil metabòlic, la qual cosa provoca l'increment de més de 200 metabòlits. A més, indueix l'emissió de 15 compostos volàtils fonamentalment de les rutes dels àcids linoleic i mevalònic. Això suggereix que l’inductor pot actuar aigües a dalt d'aquestes rutes relacionat amb el metabolisme primari.

7. L’inductor (Hx) és absorbit per les arrels però que no és mobilitzat a altres parts de la planta. Aquesta observació suggereix que l'aplicació de l’Hx en el sòl pot produir canvis en compostos que es desplaçarien de forma sistèmica cap a les fulles.

8. Aquest estudi revela, per primera vegada, una relació entre la resistència induïda per l'àcid hexanoic i l'emissió de compostos volàtils.

CONCLUDING REMARKS

In this thesis has been carried out the study of induced resistance in citrus mediated hexanoic acid and the approach on its mechanism of action.

The conclusions that have been obtained are as follows:

1. Hexanoic acid induces resistance in ‘Fortune’ mandarin plants against the necrotrophic fungus *Alternaria alternata* reducing the size and the number of lesions per leaf, based on a mechanism known as "priming" through induction of defense mechanism in presence of the pathogen.

2. The principal mechanisms involved in the defense of 'Fortune' against *A. alternata* are the jasmonic acid pathway, the accumulation of polygalacturonase-inhibiting proteins and callose deposition at the site of infection.

3. Hexanoic acid induces resistance in citrus against *A. alternata* providing a long lasting protection, showing high levels of phenolic compounds and polygalacturonase inhibiting proteins (increase PGIP gene expression).

4. Hx protects citrus plants against the biotrophic bacteria *Xanthomonas citri* reducing the number of lesions per leaf by an enhanced expression of the *AOS* gene, the *PR2* and *PR5* gene and callose deposition, which indicate an implication of jasmonic and salicylic pathways. Moreover, the application of the inductor provides a long lasting protection against *Xantomonas citri* mediated by the enhancement of *PR2* and *AOS* gene expression.

5. Physiological analysis show that hexanoic acid not only does not modify, even increase, photosynthetic rate, transpiration and the water use efficiency. This indicates that the plants are not making a higher energetic cost in the Hx-IR.

6. Hx induced resistance in citrus against *Alternaria alternata*, promoting major changes in the metabolic profile, causing the enhancement of more than 200 metabolites. Moreover, induces the emission of 15 volatile compounds mainly

from the linoleic acid and mevalonic acid pathways. These findings suggest that the inductor could act upstream these pathways related with primary metabolism.

7. This study reveals that the inductor is absorbed by roots, but is not translocated to other plant parts. This finding suggests that Hx application to soil could produce changes in compounds that could travel systemically to the leaves.

8. This study reveals, for first time, a relation between the resistance induced by hexanoic acid and the emission of volatile compounds.

CONCLUSIONES

En esta tesis doctoral se ha llevado a cabo el estudio de la resistencia inducida en cítricos media por el ácido Hexanoico y la aproximación a su mecanismo de acción.

Las conclusiones que se han obtenido han sido las siguientes:

1. El ácido hexanoico induce resistencia en plantas de mandarino "Fortune" frente al hongo necrótrofo *Alternaria alternata* reduciendo el tamaño y el número de lesiones en las hojas, a través del mecanismo conocido como "priming" que induce la defensa en presencia del patógeno.

2. Los principales mecanismos involucrados en la defensa de "Fortune" frente a *A. alternata* son la ruta del ácido jasmónico, la acumulación de proteínas inhibidoras de la poligalacturonasa y el depósito de callosa en la zona de infección.

3. El ácido hexanoico induce resistencia en cítricos frente a *A. alternata* proporcionando protección a largo plazo, mostrando elevados niveles de compuestos fenólicos y de proteínas inhibidoras de la poligalacturonasa (incremento de la expresión del gen PGIP).

4. El Hx protege a las plantas de cítricos frente a la bacteria biótrofa *Xanthomonas citri* reduciendo el número de lesiones por hoja a través de un incremento de la expresión del gen *AOS*, el *PR2* y el *PR5*, lo que indica una implicación de las rutas de los ácidos jasmónico y salicílico. Además la aplicación del inductor proporciona una protección a largo plazo frente a *Xanthomonas citri* mediado por el aumento de la expresión de los genes *AOS y PR2.*

5. El análisis de parámetros fisiológicos mostró que el ácido hexanoico no solo modifica, sino que incrementa la tasa fotosintética, la transpiración y el uso eficiente del agua. Esto indica que el proceso de resistencia inducida por Hx no conlleva un elevado coste energético.

6. El Hx induce resistencia en cítricos frente a *Alternaria alternata* promoviendo cambios importantes en el perfil metabólico, lo que provoca el incremento de más de 200 metabolitos. Además, induce la emisión de 15 compuestos volátiles fundamentalmente de las rutas de los ácidos linoleico y mevalónico. Esto sugiere que el inductor puede actuar aguas arriba de estas rutas relacionadas con el metabolismo primario.

7. El inductor (Hx) es absorbido por las raíces pero que no es movilizado a otras partes de la planta. Esta observación sugiere que la aplicación del Hx en el suelo puede producir cambios en compuestos que se desplazarían de forma sistémica hacia las hojas.

8. Este estudio revela, por primera vez, una relación entre la resistencia inducida por el ácido hexanoico y la emisión de compuestos vol