Escuela Superior de Tecnología y Ciencias Experimentales
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Tesis Doctoral

Estudio integral de los mecanismos de resistencia inducida. Inductores frente a estrés biótico y abiótico

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CONTENTS

Introducción, objetivos, metodología y resumen ..... ….5

CHAPTER 1 .....……19

General Introduction

CHAPTER 2 .....….55

Ammonium enhances resistance to salinity stress in citrus plants

CHAPTER 3 .....……93

NH_4^+ induces antioxidant cellular machinery and provides resistance to salt stress in citrus plants

CHAPTER 4 125

NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation

CHAPTER 5 169

Hexanoic acid treatment prevents the systemic movement of MNSV in *Cucumis melo* plants by priming callose deposition and hormonal signaling

CONCLUSIONES 218

Introducción, objetivos, metodología y resumen

INTRODUCCIÓN

Las plantas a lo largo de su vida tienen que enfrentarse a complejas situaciones en las que interviene diversos factores ambientales. La exposición a estreses bióticos y abióticos tiene un gran impacto en su metabolismo y para minimizar los daños biológicos causados por los mismos, las plantas han desarrollado sofisticados mecanismos de resistencia que les permiten adaptarse y sobrevivir a dichas condiciones adversas. Se conocen algunas respuestas genéricas y comunes a ambos tipos de estrés como son: cambios en el trasporte de iones, acumulación de especies reactivas de oxígeno (ROS), activación de cascadas MAMPKK, así como la activación de la síntesis de hormonas tales como ácido abscísico (ABA), ácido salicílico (SA), ácido jasmónico (JA), y etileno (ET) (Rejeb et al., 2014). Además, estas estrategias defensivas desarrolladas por las plantas, pueden ser activadas mediante diferentes mecanismos con el fin de preparar a las mismas ante la llegada del estrés, mecanismo que se conoce como resistencia inducida (IR). Estos sistemas defensivos pueden ser activados por el ataque de un patógeno, produciéndose resistencia sistémica adquirida o SAR; por la colonización de la raíces por microorganismos radiculares no patógenos, activándose respuesta sistémica inducida o ISR; por la pre-exposición a un estrés abiótico, activándose la aclimatación sistémica adquirida o SAA; mediante el tratamiento con productos químicos naturales o sintéticos; por la alteración del metabolismo primario o por la percepción de ciertos compuestos orgánicos volátiles (Pieterse et al 2000; Oostendorp et al., 2001; Durrant et al., 2007; Pozo et al., 2008). Entre los compuestos inductores de resistencia, hay algunos que basan su modo de acción en la activación de las respuestas mediante un mecanismo conocido como “priming”. El “priming” es un estado fisiológico que permite a las plantas responder de forma más rápida y robusta a un estrés biótico o abiótico, sin coste alguno sobre su

desarrollo (Prime-A-Plant Group et al., 2006; Aranega-Bou et al., 2014). La activación de SAR suele estar relacionada con la prevención de infecciones y los mecanismos mediados por SAA suelen conferir resistencia frente a estreses abióticos (Baxter et al., 2014). Sin embargo, estudios recientes apuntan a una interacción entre estos mecanismos, por lo que una aclimatación a un estrés abiótico puede inducir resistencia frente al ataque de un patógeno (Karpinski et al., 2013). En todos estos procesos de señalización sistémica en situaciones de estrés, juegan un papel muy importante las especies reactivas de oxígeno (ROS). Recientemente, se ha descubierto que existe una señalización dependiente de H_2O_2 a larga distancia que se induce frente a diversos estímulos de origen abiótico (Mitller et al., 2009). Las iniciadoras de la propagación de esta señal sistémica célula a célula son las NADPH oxidasas RBOH, la activación de las cuales es condición indispensable para que se produzca la acumulación de H_2O_2 en el espacio extracelular iniciándose entonces lo que se conoce como "ola ROS" (Mitller et al., 2011). Por tanto se considera que es esta "ola ROS" la que se propaga célula a célula actuando como señal de priming y la que alerta a los tejidos sistémicos de la presencia de una situación de estrés. Además se ha demostrado que la SAA en situaciones de estrés abiótico está mediada por la interacción espacio-temporal de la "ola ROS" con hormonas y aminoácidos relacionados con respuesta a estrés en tejidos sistémicos (Baxter et al., 2014).

Uno de los compuestos naturales capaces de inducir resistencia mediante la activación del mecanismo de priming es el ácido hexanoico (Hx). Estudios previos realizados en nuestro grupo de investigación han demostrado su efectividad en plantas de tomate frente a *Botrytis cinerea* y *Peudomonas syringae* pv tomato DC3000 (*Pst*), en *Arabidopsis thaliana* frente a *B. cinerea* y en mandarino Fortune frente a *Alternaria alternata*. En la actualidad, se considera un tema de vital importancia el desarrollo de

sistemas alternativos al uso de fitosanitarios en la lucha frente a enfermedades y otras condiciones adversas. Por ello, además de la investigación en el modo de acción de Hx y de otros compuestos naturales como inductores de resistencia frente a estrés, el grupo de investigación en el que se desarrolla la presente tesis doctoral se centra en la búsqueda de nuevos inductores de resistencia que sean compuestos naturales. Dada la experiencia en fertilización nitrogenada, se plantea como uno de los objetivos de la tesis, testar el amonio (NH_4^+) como inductor de resistencia y comprobar su efectividad frente a un estrés biótico y abiótico. Es conocido que el NH_4^+ es un nutriente esencial para las plantas, aunque muchas de ellas son sensibles al mismo cuando se aplica como única forma de nitrógeno, desarrollando lo que comúnmente se conoce como "síndrome de NH_4^+" (Britto et al., 2001, Britto y Kronzucker, 2002 y Horchani et al., 2011). Sin embargo, estudios recientes apuntan a que el NH_4^+ podría reducir el impacto de algunos estreses (Hessini et al., 2013), aunque poco se conoce acerca de este proceso. En el presente trabajo, se pretende estudiar el mecanismo de acción de la resistencia inducida por NH_4^+ (NH_4^+-IR) en plantas de cítricos *citrange Carrizo* (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L.) sometidas a estrés salino, así como el impacto del mismo sobre la maquinaria antioxidante activada frente a dicho estrés. Una vez demostrada la eficacia de la NH_4^+-IR en cítricos frente a estrés salino, se plantea comprobar la efectividad del NH_4^+ como inductor de resistencia frente a estrés biótico. Para ello se utilizarán plantas de tomate (*Solanum lycopersicum* Mill. cv. Ailsa Craig) inoculadas *Pst* y se estudiará su modo de acción. Por último, en este trabajo se propuso estudiar la resistencia inducida por el ácido hexanoico (Hx-IR) en otro patosistema, concretamente en plantas de melón (*Cucumis melo* L. "galia") inoculadas con el *virus de las manchas necróticas del melón* (MNSV) y además se analizarán los mecanismos de defensa basales en el mismo.

OBJETIVOS

Teniendo en cuenta los antecedentes anteriormente descritos, en este trabajo se pretende estudiar:

1) El mecanismo de acción de la resistencia inducida por NH_4^+ (NH_4^+-IR) en plantas de cítricos sometidas a estrés salino.
2) El impacto del NH_4^+ sobre la maquinaria antioxidante activada frente a estrés salino en plantas de cítricos.
3) El mecanismo de acción de la NH_4^+-IR en plantas de tomate inoculadas con el patógeno hemibiótrofo *Pst*.
4) Los mecanismos de defensa basales en plantas de melón inoculadas con el virus de las manchas necróticas del melón MNSV.
5) La resistencia inducida del ácido hexanoico (Hx-IR) en plantas de melón inoculadas con MNSV.

METODOLOGÍA Y PLAN DE TRABAJO

Material vegetal y tratamientos

Para llevar a cabo este trabajo, se han utilizado tres especies vegetales diferentes: plantas de *citrange Carrizo* (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L.), suministradas por Beniplant (Valencia, Spain) germinadas y crecidas en vermiculita; plantas de tomate, *Solanum lycopersicum* Mill. cv. Ailsa Craig germinadas y crecidas en vermiculita y plantas de melón *Cucumis melo* L. "galia" germinadas y crecidas en turba. Los tres cultivos se crecieron en condiciones controladas de cámara de cultivo.

Estreses aplicados

a) Salinidad: para aplicar el estrés salino, se añadió 90 mM de NaCl a la solución hidropónica y ésta fue renovada dos veces por semana.

b) Infección por *Pst*: la bacteria se creció en medio King B a 28ºC. Para la inoculación, se transfirió la bacteria a media King B líquido y se preparó el inóculo en $MgSO_4$ (10 mM) más Silwet-L77 a 0.01%, ajustando la concentración a $5*10^5$ unidades formadoras de colonias por mililitro. La inoculación se realizó por inmersión de las plantas en el inóculo.

c) Infección por *B. cinerea*: este hongo necrotrófo se creció en placas de PDA (potato dextrose agar). Las esporas se obtuvieron de una placa de 15 días de edad y se ajustó la concentración a $1x10^6$ esporas/ml para la preparación del inóculo.

d) Infección con MNSV: El aislado de MNSV utilizado en este estudio se obtuvo de plantas de campo de melón infectadas con el virus. El tejido de la hoja infectada por MNSV se homogeneizó con 30 mM de tampón de fosfato (pH 7,0) que contiene mercaptoetanol 20 mM, y el extracto bruto se usó como inóculo.

Tratamientos con NH_4^+ y Hx

Para la aplicación de los tratamientos de NH_4^+ y Hx se procedió de la siguiente manera:

a) Cítricos: después de la germinación y durante 6 semanas, las plantas se regaron con la solución nutritiva de Hoagland carente de N, complementada 1 mM NH_4NO_3 (plantas control) y 5mM de NH_4^+ (tratamiento de NH_4^+).

b) Tomate: se crecieron durante tres semanas con solución Hoagland (plantas control), o con solución Hoagland carente de nitrógeno y suplementada con 20 mM de KNO_3^- (para el tratamiento de NO_3^-); 2, 5 o 8 mM de NH_4^+ [$(NH_4)_2SO_4$)] (para el tratamiento de NH_4^+).

c) Melón: el Hx se aplicó mediante tratamientos radiculares por saturación de la maceta con 1L de solución de Hx 25mM 3 y 1 día antes de la inoculación.

Procedimiento experimental

a) Microscopía

El H_2O_2 y la calosa se detectaron mediante tinción con 3,3'-diaminobenzidina y azul de anilina respectivamente, y se visualizaron en microscopio de epifluorescecia en campo claro para el H_2O_2 o bajo luz UV para la calosa. La cuantificación se realizó mediante recuento de píxeles marrones o amarillos (H_2O_2 o calosa) sobre fotografías tomadas de las visualización al microscopio utilizando para ello el programa de manipulación de imágenes GIMP.

b) RT-qPCR

Mediante esta técnica de PCR a tiempo real, se analizó la expresión de los transcritos de los genes seleccionados tras la extracción de RNA mensajero total, su conversión a cDNA y posterior amplificación con cebadores específicos. Para cada cultivo se seleccionó un control interno y unos genes marcadores específicos de las rutas de señalización implicadas en cada tipo de estrés. Mediante el análisis de la curva de melting se comprobó la pureza del producto amplificado.

c) Análisis de los niveles de hormonas y poliaminas

La cuantificación de hormonas y poliaminas se llevo a cabo mediante cromatografía de HPLC-MS, según lo descrito en Flors et al. (2008) y Sánchez-López et al. (2009), respectivamente.

RESUMEN

En este trabajo se ha demostrado que el NH_4^+ actúa como inductor de resistencia frente a estrés salino en cítricos, observándose acumulación de ABA, poliaminas (PAs), H_2O_2 y prolina, mecanismos directamente relacionados con resistencia frente a salinidad. Asimismo, se ha observado que las plantas crecidas con NH_4^+ presentan una disminución de Cl^-, tanto en hoja como en raíz, reduciéndose así el efecto tóxico de este ion, así como una mayor expresión del gen hidroperóxido glutation peroxidasa (*PHGPx)*, demostrándose la activación de la maquinaria antioxidante que podría aliviar el estrés oxidativo inducido por la sal. El crecimiento de los cítricos en estas condiciones resultó ser óptimo, mostrando las plantas un mayor crecimiento de raíces secundarias sin verse afectada la longitud de la raíz principal. Este fenotipo es característico de la respuesta morfogenética específica inducida por estrés conocida como SIMRs y que está relacionada con procesos de aclimatación. Basándonos en estos resultados se propuso la siguiente hipótesis: la nutrición basada en NH_4^+ produciría un estrés leve crónico subletal que activaría los mecanismos de defensa, induciendo así resistencia al estrés salino aplicado posteriormente. Con objeto de profundizar en el modo de acción del NH_4^+ frente a estrés salino se planteó estudiar el impacto del mismo sobre la maquinaria antioxidante activada frente a dicho estrés. En este trabajo se ha demostrado que, en ausencia de sal, el NH_4^+ induce la acumulación de altos niveles de H_2O_2, la inhibición de las enzimas superóxido dismutasa (SOD) y glutatión reductasa (GR), así como una mayor acumulación de glutatión oxidado (GSSG), confirmando el estrés leve que se ha producido en los cítricos. Sin embargo, una vez aplicado el estrés salino, las plantas crecidas con NH_4^+ mostraron una reducción de los niveles de H_2O_2, junto con un incremento de las actividades enzimáticas de catalasa (CAT), SOD y GR, así como altos niveles de glutatión reducido (GSH). Los

análisis de expresión génica mostraron una inducción de los genes glutatión-S-transferasa (*GST)* y *PHGPx* en estas condiciones. Estos resultados indican que la activación de la maquinaria antioxidante inducida por el estrés leve crónico producido por NH_4^+ podría ser un mecanismo clave en la resistencia inducida por este ion, en condiciones de estrés salino.

También se ha demostrado que plantas de tomate crecidas con NH_4^+ muestran una reducción de los síntomas de la enfermedad producida por *Pst*. El estudio del modo de acción de la NH_4^+-IR reveló que los mecanismos inducidos en las plantas en respuesta a NH_4^+, tales como la acumulación de ABA, PAs y H_2O_2 (siendo este último probablemente el mensajero del estrés) son los mecanismos clave para la inducción de aclimatación sistémica adquirida (SAA) que confiere a las plantas de tomate resistencia frente a *Pst.* Además de la activación de SAA, se observó que el NH_4^+ induce el cierre estomático que podría reducir la entrada de la bacteria al mesófilo, así como acumulación de ácido ferúlico, un compuesto fenólico asociado con defensa frente a patógenos. Aunque no se encontraron evidencias claras de la activación de la ruta del SA, comúnmente relacionada con resistencia a organismos biótrofos, pudimos demostrar el papel clave de las PAs, concretamente putrescina (Put) en la resistencia basal frente a *Pst*.

Por último, se ha demostrado que el virus MNSV induce en plantas de melón una compleja red hormonal de respuesta, así como la acumulación de calosa y ROS alrededor del sitio de infección, tanto a nivel local como sistémico. El Hx ha resultado ser efectivo como inductor de resistencia frente a virus en plantas de melón, ya que los tratamientos radiculares han impedido en el 100% de las plantas testadas el paso del virus al floema y por tanto su dispersión a otros órganos de la planta. Los análisis del modo de acción de este inductor natural demostraron la importancia de la acumulación de SA en la Hx-IR, así como del OPDA y JA-Ile en la primera fase de la infección.

También se ha demostrado que la rápida acumulación de calosa y la reducción de ROS a nivel de cotiledón son procesos clave de la Hx-IR frente a MNSV.

APORTACIONES ORIGINALES

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CHAPTER 1
General Introduction

PLANT RESPONSES TO ABIOTIC AND BIOTIC STRESS

Plants are subjected to various and complex types of interactions that involve numerous environmental factors. As sessile organisms, they have evolved specific mechanisms that allow them to adapt and survive stressful events. Exposure of plants to adverse environment situations has an impact on plant metabolism, which involves physiological costs, and thus leads to reduced health, and ultimately to lower productivity (Shao et al., 2008; Massad et al., 2012). Stress situations, produced by either abiotic stress (e.g. strong light, UV, high and low temperatures, freezing, drought, salinity, heavy metals and hypoxia) or biotic pressures (insect attack or infection by a fungus, bacterium or virus), are responsible for severe losses in the field which, in some cases, can cause losses of over 50% (Wang et al., 2003). The correct activation of complex signaling cascades of defense in a rapid efficient manner depends on the early perception of stress to a great extent (Abou et al., 2009; Andreasson et al., 2010).

General responses to salinity

Salinity is a major stress that limits increased demand for food crops. More than 20% of cultivated land worldwide is affected by salt stress, and this amount is increasing day by day. This complex environmental stress has three different components: an ionic component linked to the accumulation of ions, which become toxic at high salt concentrations (mainly Na^+ and Cl^-) in the cytoplasm, and lead to ionic imbalance; an osmotic component due to the compartmentalization of this toxic ion in the vacuole, this being a oxidative stress that is common to other abiotic stress situations. Exposure to desiccation, salt stress and low temperature is generally accompanied by an increase in endogenous abscisic acid (ABA). ABA accumulation can alleviate the inhibitory effect of salinity on photosynthesis, growth and

translocation of assimilates (Popova et al., 1995; Jeschke, 1997). The effect of ABA accumulation and salinity tolerance can be attributed to the accumulation of K^+, Ca^{2+} and compatible solutes, such as proline and sugars, in vacuoles of roots, which counteract with Na^+ and Cl^- uptake (Chen et al., 2001; Gurmani et al., 2011). The activation of ABA-mediated signaling pathways is also required to activate the expression of a number of salt and water deficit-responsive genes (Gupta et al., 2014). High polyamine (PAs) levels imply another event that occurs in plants exposed to abiotic stress (Gill and Tuteja 2010; Alcázar et al., 2010). Benefits of PA have been associated with membrane integrity maintenance, regulation of gene expression for the synthesis of osmotically active solutes, reduced reactive oxygen species (ROS) production, and the control of the accumulation of Na^+ and Cl^- ions in different organs (Takahashi et al., 2010). The third piece of the puzzle is oxidative stress induced by salinity. Salinity and other abiotic stresses induce the disruption of electron transport chains (ETC) in chloroplasts and mitochondria (Gupta et al., 2014). Under these conditions, molecular oxygen (O_2) acts as an electron acceptor, which gives rise to ROS accumulation. Singlet oxygen ($^1O_2$), hydroxyl radical (OH^-), the superoxide radical (O_2^-), and hydrogen peroxide (H_2O_2), are all strongly oxidizing compounds and are, therefore, potentially harmful for cell integrity (Groß et al., 2013). To alleviate the toxic effect of these molecules, antioxidant enzymes and non-enzymatic compounds are activated at cells to detoxify ROS. Salinity tolerance has been linked directly to the activation of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR), and also to the accumulation of non-enzymatic antioxidant compounds (Asada et al., 1999; Gupta et al., 2005).

General responses to pathogens: immunity system

To defend themselves from all these different types of pathogens, plants have evolved sophisticated strategies to perceive their attacker and to translate this perception into an effective immune response. The primary objectives of basal defenses have been developed to face biotic and abiotic stress, and are found in the plant before pressure arrives. The first of these barriers is the passive protection provided by the waxy cuticle and the accumulation of antimicrobial compounds like saponins, piretrines (Osbourn, 1996) and other secondary metabolites. However, plants have also evolved sophisticated defense mechanisms to perceive pathogen attacks and to translate this perception into an adaptive response. Two tiers of recognition by the innate immune system have been defined. The first branch is triggered by the recognition of highly conserved microbe-associated molecular patterns (MAMPs) by host cell transmembrane proteins that function as pattern recognition receptors (PRRs) which, in turn, activate MAMP-triggered immunity (MTI) (Jones and Dangl, 2006). To resist non pathogenic microbes, and probably some pathogens, the activation of MTI is normally sufficient. However, adapted pathogens are able to introduce virulence effector proteins into cells to promote plant susceptibility, which leads to MTI responses. For this reason, plants have evolved a second branch to recognize microbial effectors inside plant cells via nucleotide-binding site-leucine-rich repeat (NB-LRR) resistance (R) proteins This produces the activation of effector-triggered immunity (ETI), and is associated with programmed cell death, known as the hypersensitive response (HR) (Jones and Dangl, 2006). The HR lesion is a stronger form of defense and limits microbial spread by killing infected plant cells. In this struggle, the end ultimately depends on plants' ability to recognize and activate defensive

mechanisms efficiently, and on the pathogen to attempt to suppress the plant immune system.

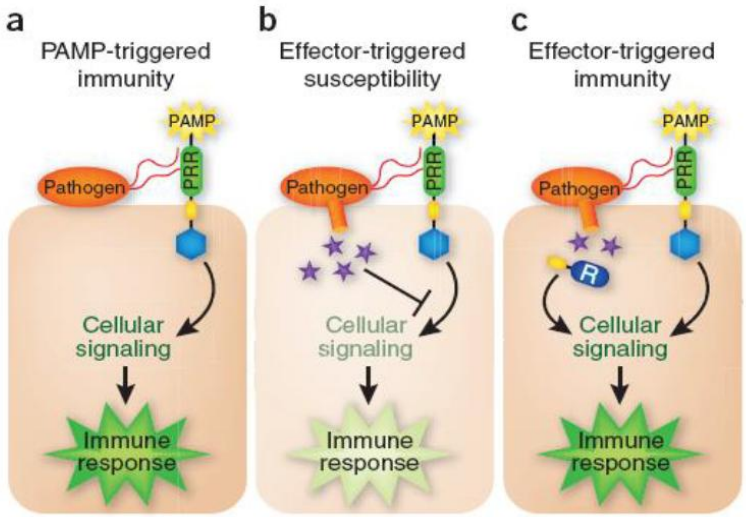

Fig.1. Representation of plant immune system. (a) Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signaling cascade that leads to PAMP-triggered immunity (PTI). (b) Virulent pathogens have acquired effectors (purple stars) that suppress PTI, resulting in effector-triggered susceptibility (ETS). (c) In turn, plants have acquired resistance (R) proteins that recognize these attackerspecific effectors, resulting in a secondary immune response called effectortriggered immunity (ETI) (Pieterse et al., 2009).

The initial defense response after pathogen recognition is the alkalinization of the growth medium through drastic changes in the fluxes of H^+, K^+, Cl_2 and Ca^{2+} ions across the plasma membrane (Garcia-Brugger et al., 2006). Ca^{2+} accumulation at the cytosol is a critical step in the MTI response and is mediated by Ca^{2+} accumulation in the thylakoid lumen of chloroplasts, followed by an increased Ca^{2+} influx in the cytoplasm (Nomura et al., 2012). Ca^{2+} accumulation performs different functions, like controlling ROS, the activated salicylic acid (SA) pathway or inducing stomatal closure

(Muthamilarasan et al., 2013). Another of the earliest events after pathogen detection is the production of reactive nitrogen intermediates (RNIs) and ROS in the cells that participate in plant defense activation (Muthamilarasan et al., 2013). In higher plants, callose, a b-1,3-glucan polymer with b-1,6-branches, is present ubiquitously in different tissues and appears to play diverse roles in plant growth, development and biotic stress responses as a common indicator of MTI (Oide et al., 2013). Callose deposition is an important factor for plants' penetration resistance against invading pathogens. Besides being a very important component of papillae, it rapidly synthesizes and deposits immediately around the sites of attempted pathogen penetration, and has long since been considered a key event for plant resistance (Huckelhoven, 2007). Different studies based on the *Arabidopsis* powdery mildew resistant 4 (*pmr4*) mutant, whose wound- and pathogen-induced callose depositions are abolished, have demonstrated that this mutant shows increased susceptibility against *Blumeria graminis* (Jacobs et al., 2003), and to different fungal and oomycete pathogens (Ton and Mauch-Mani, 2004; Adie et al., 2007), which indicates the major role of callose in disease resistance.

MTI also involves the biosynthesis of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Mishina and Zeier 2007; Tsuda et al., 2008), which are indispensable for both local and systemic acquired resistances (Durrant and Dong 2004). However, the activated hormonal network depends on the lifestyle and infection strategy of the plant pathogen, which varies among cultivars (De Vos et al., 2005). Generally if plants respond to a biotrophic pathogen, the SA response pathway is typically activated (Glazebrook 2005). SA is a phenolic compound that can be synthesized from two distinct enzymatic pathways, one that involves phenylalanine ammonia lyase (PAL) and the other implicates isochorismate synthase (ICS/SID2) (Garcion and

M´etraux 2006). The signalling downstream of SA is controlled largely by regulatory protein NONEXPRESSOR OF *PR* GENES1 (NPR1), which upon activation by SA, acts as a transcriptional coactivator of a large set of defense-related genes (Moore et al., 2011), for example, in *PR* genes like *PR-1*, which is often used as a robust marker for SA-responsive gene expression. Many WRKY transcription factor genes are also SA-inducible. WRKY transcription factors activate or repress SA responses, which highlights their role in both SA-mediated resistance and the feedback control of the SA signaling pathway (Rushton et al., 2010). However, if plants are attacked by necrothrophic pathogens or insects, JA-dependent signaling pathways are normally activated (Lorenzo et al., 2003). JA, and its structurally related metabolites, is lipid-derived compounds that are rapidly synthesized via the oxylipin biosynthesis pathway (Gfeller et al., 2010). After synthesis, JA can be either metabolized to methyl jasmonate (MeJA) or conjugated to amino acids such as isoleucine via JAR1 to obtain jasmonoyl-isoleucine (JA-Ile) (Seo et al., 2001; Fonseca et al., 2009). The F-box protein CORONATINE INSENSITIVE1 (COI1) is a key regulator of the JA signaling pathway. Together with the JASMONATE ZIM (JAZ) domain transcriptional repressor proteins, COI1 functions as a JA-Ile receptor (Sheard et al., 2010). Binding of JA-Ile to COI1 leads to the ubiquitinylation and subsequent degradation of JAZ repressor proteins via the proteasome (Pauwels and Goossens 2011).

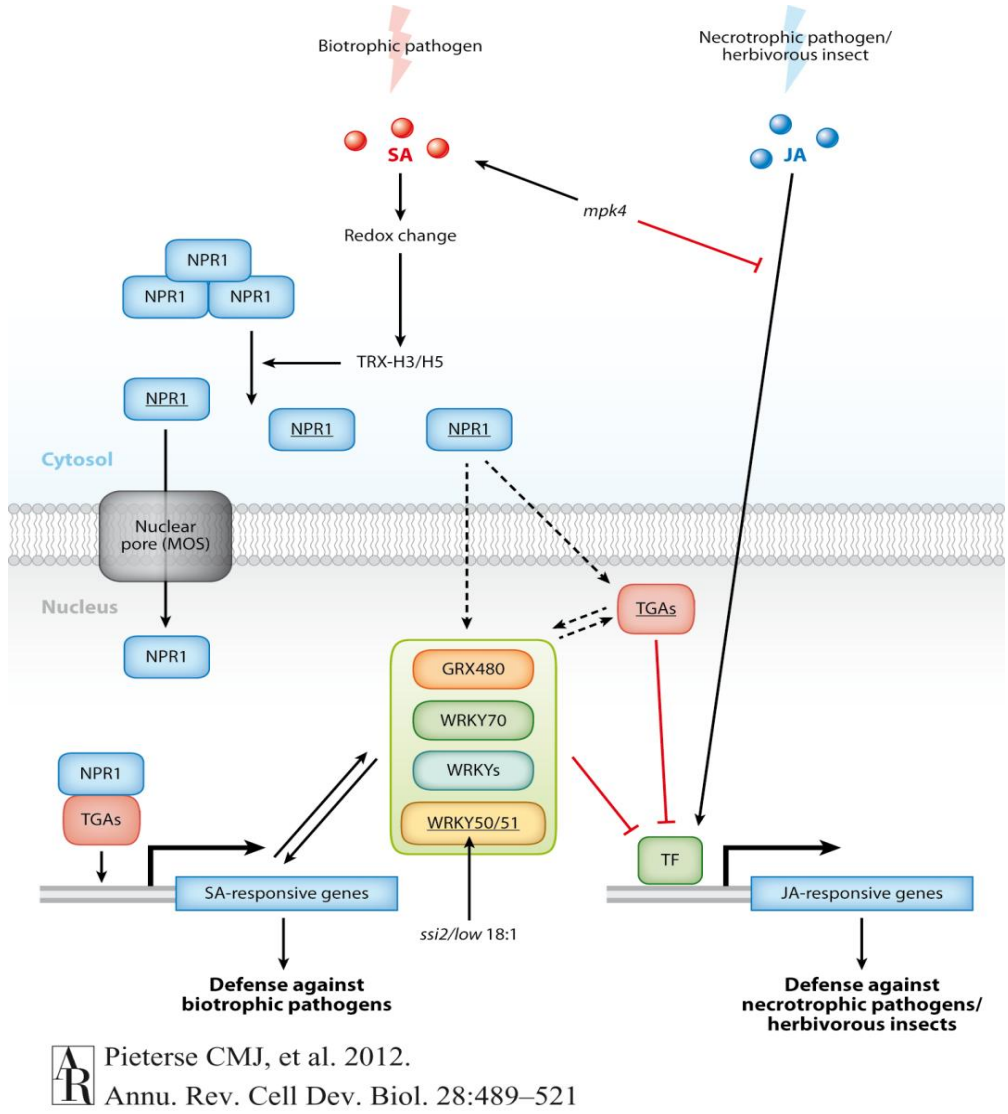

Fig.2. Schematic representation of the molecular components involved in the SA and JA interation pathways (Pietersen et al., 2012).

INDUCED RESISTANCE MECHANISMS

Plants are subjected to a variety of external factors that adversely affect their growth and development, and adaptation to these environmental stresses is essential for their survival and propagation (Rasmann et al., 2012). In the last few years, research has been directed to understand the response of plants to individual stresses. However, it is important to elucidate the mechanisms of response to combined stresses (Fujita et al., 2006). Plants have evolved sophisticated acclimation and defense mechanisms that have improved their resistance to subsequent stress. Higher plants are capable of demonstrating some stress 'memory', or stress imprinting, after stress exposure (Bruce et al., 2007). Stress imprinting is usually defined as genetic or biochemical modifications, induced by a first stress exposure, that lead to enhanced resistance to subsequent stress. Preliminary stress exposure is indeed known to boost the stress tolerance of plants through the induction of acclimation responses (Bruce et al., 2007). Exposing plants to mild chronic stress can cause the induction of a specific stress-induced morphogenic response (SIMR) (Potters et al., 2007). The SIMR forms part of a general acclimation strategy characterized by a blockage of cell division in the main meristematic tissues, inhibition of elongation, redirected outgrowth of lateral organs (Potters et al., 2009), and also by the activation of antioxidants that prevent the damage caused by ROS and the accumulation of foliar anthocyanin, which acts as a modulator of stress signals (Steyn et al., 2002; Gould and Lister, 2006).

Induced resistance (IR)-related mechanisms lead to various types of systemic resistance throughout plants. IR is based on the activation of defense responses in systemic tissue after local stimuli, which implies the activation of systemic responses, but only when pathogens or abiotic stimuli reach these sites. Systemic responses are initiated in response to many different signals,

including pathogens, wounding and abiotic stresses (Mitller et al., 2015). The IR triggered by pathogens can be divided depending on pathogen type: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Piettersen et al., 2014). The term SAR is often associated with acquired resistance in healthy systemic tissues in response to a localized infection in another place of the plant (Ryals et al., 1996) through the activation induced by SA-dependent signaling pathways, and is effective against biotrophic pathogens. Another defensive response triggered by the action of nonpathogenic microbes (Alstrom, 1991; Vanpeer et al., 1991) was discovered in the 90s and was named induced systemic resistance (ISR). Subsequent studies have shown that bacteria and fungi like *Pseudomonas, Serratia, Bacillus* strains and non pathogenic *Fusarium oxysporum*, *Trichoderma,* and symbiotic arbuscular mycorrhizal fungi are able to trigger ISR (Piettersen et al., 2014). In recent years, particular attention has been paid to the IR mediated by mycorrhizae since its effectiveness to abiotic stresses, such as drought, salinity, or presence of heavy metals, against soil-borne fungal and bacterial pathogens, nematodes, root-chewing insects, and also against shoot and aerial pathogens, has been demonstrated (Jung et al., 2012). While SAR depends on plant hormone salicylic acid (SA), along with the expression of pathogenesis-related (PR) proteins, and has been recently related to the involvement of others small metabolites, including methyl salicylate, the abietane diterpenoid dehydroabietinal, the lysine catabolite pipecolic acid, a glycerol-3-phosphate-dependent factor, and dicarboxylic and azelaic acids (Shah et al., 2013), rhizobacteria-mediated ISR in *Arabidopsis* neither needs SA signaling nor is associated with the expression of known defense-related genes (Pieterse et al., 1996, 1998; Van der Ent et al., 2008). Instead ISR requires responsiveness to JA and ET, but is not

associated with endogenous increases in these hormones (Pieterse et al., 2000).

The activation of defense or acclimation mechanisms in systemic or non-challenged tissues in response to abiotic stimuli, such as high light, heat, cold, UV, osmotic stress or salinity, is termed systemic acquired acclimation (SAA) (Karpiński et al., 2013). A systemic response to mechanical stress, which can be caused by biotic (e.g., insect feeding) or abiotic stress (e.g., wind or heavy downpour), is termed systemic wound response. These responses are typically measured by the enhanced accumulation of defense or acclimation transcripts and proteins, and/or by demonstrating an enhanced level of tolerance or resistance to subsequent abiotic or biotic stress applied to systemic tissues (Karpiński et al., 2013; Suzuki et al., 2013; Shah et al., 2013). It is commonly accepted that achieving an acclimation stage induced by prior abiotic stimuli induces resistance against subsequent abiotic stress. For example, the prior exposure of plants to cold and drought stress has resulted in a stronger successful response to subsequent cold or drought stress (Lang et al., 1992; Knight et al., 1998). Prior exposure of *Arabidopsis* plants to drought stress or ABA has resulted in freezing tolerance in these plants (Mantyla et al., 1995).

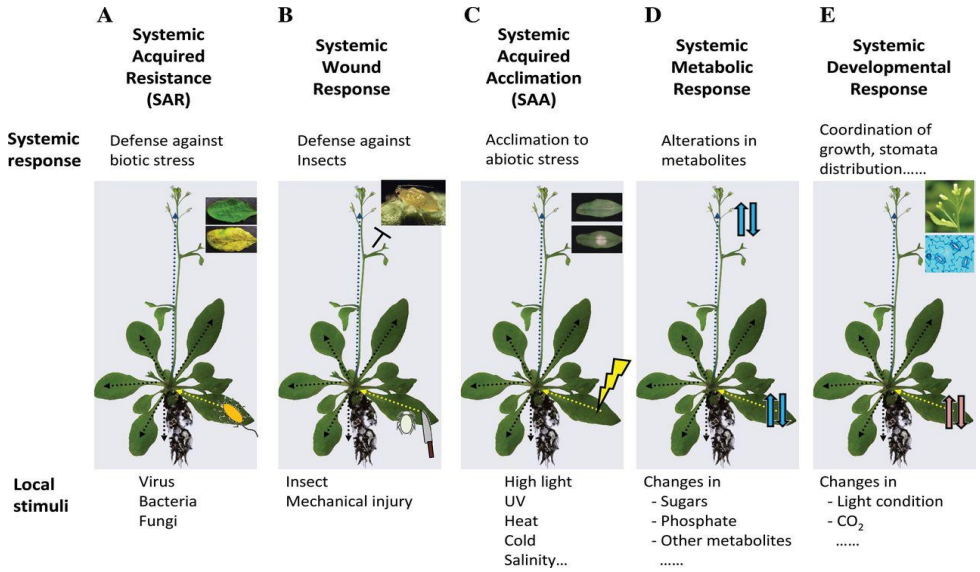

Fig.3. Systemic signaling in plants. (A) Systemic acquired resistance (SAR) triggered by pathogens (viruses, bacteria, and fungi). The signal enhances resistance of systemic tissues to pathogens. (B) Systemic wound response triggered by insects and mechanical injury. The signal activates defence mechanisms in systemic tissues against insect attack. (C) Systemic acquired acclimation (SAA) triggered by abiotic stimuli such as high light, UV light, heat, cold, or salinity. The signal enhances tolerance of systemic tissues to abiotic stress. (D) Systemic metabolic responses triggered by changes in the level of sugars, phosphate, and other metabolites. The signal alters the metabolic state in systemic tissues. (E) Systemic developmental responses activated by changes in light conditions and atmospheric CO_2. Growth and stomatal distribution are coordinated in new developing leaves (Baxter et al., 2014).

Beside all this, recent studies have shown that SAA might induce resistance against biotic pressure. For example, this related process has been demonstrated in osmotic and proton stress by inducing callose-containing papillae formation to block fungal growth, and by acting as an inducer of resistance in barley against powdery mildew (Weise et al., 2004). Achuo et al. (2006) demonstrated that ABA accumulation induced by drought stress on tomato leaves increased resistance against *B. cinerea*, and salt stress reduced susceptibility against *Odium neolycopersici.* Recent studies have revealed

that previous drought acclimation in *Nicotiana benthamiana* plants imparts tolerance to necrotrophic fungus *Sclerotinia sclerotiorum*, and also to hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv. tabaci probably mediated by ROS generated during drought acclimation (Ramegowda et al., 2013). Moreover, changes in sugars or phosphates as primary metabolites, or in external conditions that have an impact on photosynthetic processes, could induce several changes which, in turn, could induce systemic signalling that might modifies the immunity system (Baxter et al., 2014).

All these acclamatory mechanisms share a common event: ROS as a messenger after stimuli detection. ROS not only have the potential to cause oxidative damage by reacting with biomolecules, as described above, but it is widely accepted that ROS also play key roles in systemic signaling during plant immunity, wound response or SAA acclimation (Alvarez et al.,1998, Orozco-Cardenas and Ryan 1999; Karpinski et al., 1999). Mitller et al. (2011) have demonstrated that RBOHD is required for the initiation and propagation of a rapid cell-to-cell systemic signal, which is dependent on H_2O_2 accumulation in extracellular spaces to generate a 'ROS wave'. Specifically in abiotic stress, Baxter et al. (2014) have suggested that the ROS wave functions as a general priming signal to alert systemic tissues about the occurrence of localized abiotic stress. ROS signals or ROS waves, along with other signals such as hormones and small peptides, can prime neighboring cells to defense (Mittler et al., 2011). The afore-mentioned ROS signal waves are sensed by specific receptors, which can transfer the message to activate other networks through phosphorylation cascades using mitogen-activated protein kinases (MPKs) (Colcombet and Hirt, 2008).

Priming stage induced by natural or quemical compounds

Priming is a mechanism which leads to a physiological state that enables plants to respond more rapidly and/or more robustly after exposure to biotic or abiotic stress. The "primed" state has been related to the increased, more efficient activation of the defense response, and also to enhanced resistance to challenging stress, and it correlates with no or minimal gene induction (Prime A Group et al., 2006; Aranega-Bou et al., 2014). It is commonly accepted that treatment with natural or synthetic chemicals can induce the primed state. Chemical priming inducers can be divided into two groups: xenobiotic and endogenous compounds. Xenobiotic compounds include β-amino butyric acid (BABA), Benzothiadiazole (BTH) and 2,6-Dichloroisonicotinic acid (INA), which are the most widely studied. The chemical priming of *Arabidopsis* plants with (BABA) increases drought and salt stress tolerance through accelerated stress gene expression, and stomatal closure is mediated by ABA. These findings suggest the possibility of increased plant tolerance to abiotic stresses through the effective priming of pre-existing defense pathways (Jakab et al., 2005). BABA has been reported to prime defenses against *Hyaloperonospora arabidopsidis* through an SA- and an NPR1-independent signaling pathway (Zimmerli et al., 2000; Ton et al., 2005). BABA-IR involves increased callose deposition at the site of attempted infection (Zimmerli et al., 2001; Ton and Mauch-Mani, 2004). The other group, endogenous plant compounds, or functional analogs, are synthesized by plants in response to biotic stress signals, such as SA (Kauss and Jeblick, 1995), JA (Frost et al., 2008) and azelaic acid (Jung et al., 2009). Other natural compounds include oligosaccharides, glycosides, amides, vitamins, carboxylic acids and aromatic compounds (Aranega-Bou et al., 2014). In general, natural compounds tend to be better tolerated by plants than most tested synthetic compounds, but there is still concern about toxicity

(Iriti et al., 2010; Noutoshi et al., 2012). The molecular basis of priming has recently started to be unraveled, but is still poorly understood.

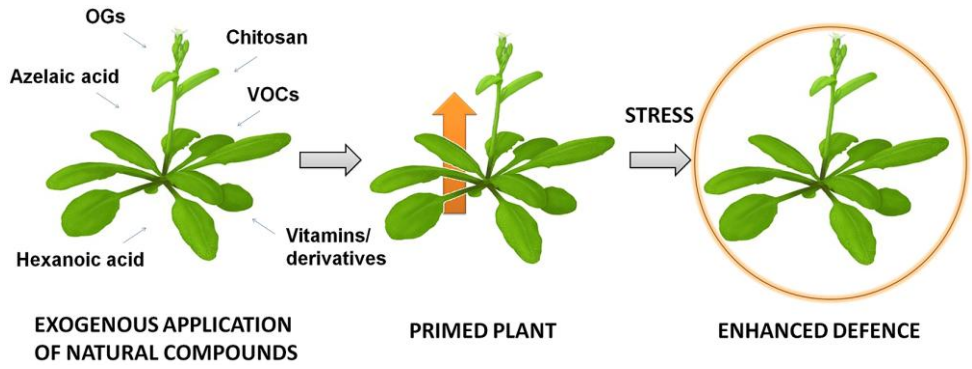

Fig.4. Effect of treatment with natural compounds on increased plant protection to subsequent stress (Aranega-Bou et al., 2014).

In recent years, our research group has focused on studying inducers of resistance, such as natural 6C monocarboxylic hexanoic acid (Hx), which displays effectiveness in tomato plants against necrotrophic fungi *B. cinerea* (Leyva et al., 2008; Vicedo et al., 2009). Treating the roots of 4-week-old plants with Hx at concentrations below 1 mM for 48 h prior to infection has been reported to significantly reduce disease incidence (Vicedo et al., 2009). Having tested the direct effect of this concentration on fungal development, these authors found no effect, which strongly supports the inducer effect of this priming agent. Studying the mode of action has demonstrated that Hx treatment induces callose accumulation upon *B. cinerea* infection, probably by inducing cell wall fortification since this process is a key resistance event in the IR of other chemical inducers like BABA or BTH (Kohler et al., 2002). Another work has demonstrated that Hx-treated plants also display caffeic acid accumulation, which supports the hypothesis that Hx-IR is based on cell

wall fortification. Hx treatment has also been shown to impact oxylipin pathways (Aranega-Bou et al., 2014) since bioactive signal JA-Ile (Chico et al., 2008) and Oxylipin12-oxo-phytodienoic acid (OPDA) sharply increase in Hx-primed tomato plants after *Botrytis* inoculation. An analysis of marker genes, induced in response to *B. cinerea* in tomato plants, has shown that Hx treatment significantly primes *LoxD*, a LOX involved in the oxylipins pathway, and leads to OPDA and JA accumulation (Cohn and Martin, 2005; Flors et al., 2007). Vicedo et al., 2009 have revealed that ABA-deficient mutant *flacca* is impaired in Hx-IR and this failed Hx protection correlates with the absence of callose deposition after *B. cinerea* infection. This fact implies a positive role of ABA in Hx-IR by enhancing callose deposition, as previously described for BABA-IR in *Arabidopsis* (Ton and Mauch-Mani, 2004). In tomato *B. cinerea*, the effectiveness of Hx treatment as an inducer of resistance has been tested in other pathosystems to reveal enhancement against necrotrophs *Alternaria brassicicola* in *Arabidopsis* (Kravchuk et al., 2011) and *Alternaria alternata* in Fortune mandarin (Llorens et al., 2013). In Fortune Mandarin, Llorens et al. (2013) have demonstrated that JA-signaling and callose priming are required for Hx-IR. Moreover, Hx treatment has been reported to protect tomato plants against hemi-biotrophic bacterium *P.syringae* pv tomato DC3000 (Vicedo et al., 2009). Recently, some studies have revealed that in this pathosystem, Hx treatment seems to counteract the negative effect of pathogen coronatine (COR), which requires JA-Ile on the SA pathway (Scalschi et al., 2013). Interestingly, Hx treatment has enhanced high OPDA accumulation, as seen in both tomato and Arabidopsis against *B.cinerea*, which suggests that this molecule might play a role *per se* in Hx-IR (Aranega-Bou et al., 2014). Therefore, Hx displays plasticity in its responses since it induces different plant responses depending on the host plants and against the lifestyles of the pathogen.

NH_4^+: nutrient as a novel inducer of resistance.

As previously mentioned, nutrient level changes or primary metabolites can induce systemic signs, which are likely to impact defensive machinery against subsequent stress. Recent studies into the possible effect of ammonium (NH_4^+) nutrition as an inducer of resistance have been published demonstration that NH_4^+ nutrition is able to improve the depressive effects of high salinity in barley (Kant et al., 2007) and halophytes (Kudo and Fujiyama 2010; Hessini et al., 2011). Hessini et al. (2013) observed the benefits of NH_4^+ nutrition on halophyte *Spartina alterniflora* under saline conditions, which seems to be associated with high antioxidant enzyme activities, together with low MDA content, electrolyte leakage and H_2O_2 concentrations. Further studies need to be conducted to elucidate how NH_4^+ nutrition enhances plants' ability to improve a subsequent salt stress situation, and the relationship of this induces resistance with ROS metabolism alteration (Patterson et al., 2010) and/or with its assimilation products (e.g. glutamine or glutamate) as a stress signal (Misra and Gupta, 2006). The inducer effect of this nutrient is probably based on the fact that many plants display toxicity symptoms when grown with NH_4^+ as a sole N source since the so-called 'ammonium syndrome' is produced (Britto and Kronzucker, 2002). This may include leaf chlorosis, lower plant yield production and root/shoot ratio, lower cation content, acidification of the rhizosphere, and changes in several metabolite levels, such as amino acids or organic acids (Britto and Kronzucker, 2002; Bittsánszky et al., 2015). The NH_4^+ nutrition affects on various cellular mechanisms, like cell wall stability and synthesis, C and N primary metabolism, phytohormone and signaling molecules, have displayed major changes in their levels of N-containing compounds, such as proteins, amino acids or polyamines (Barth et al., 2010; Houdusse et al,. 2008, Ueda et al., 2008, Ariz et al., 2011; Ariz et al., 2013).

One of the physiological changes induced by NH_4^+ nutrition is shortened roots (Li et al., 2014). Li et al. (2013) observed that contact between the root tip and NH_4^+ is both necessary and sufficient to inhibit primary root elongation. NH_4^+ supply increased lateral root initiation and branching (Lima et al., 2010). Studies on the quadruple NH_4^+-transporter insertion line (qko, the *amt1;1 amt1;2 amt1;3 amt2;1* mutant) have revealed that this mutant fails in the lateral root formation induced by NH_4^+ supplies, and that this phenotype is independent of cumulative NH_4^+ uptake. These results suggest that NH_4^+ acts as a signal to activate lateral root formation (Lima et al., 2010). In addition to root elongation and branching, root gravitropism is also affected by NH_4^+. Although moderate NH_4^+ can enhance root gravitropism in *Arabidopsis*, high NH_4^+ levels can diminish root gravitropism (Zou et al., 2012). Reduced shoot biomass and chlorosis of leaves are other major symptoms that plants display upon ammonium toxicity. Leaves are sensitive to NH_4^+ when they come into direct contact with NH_4^+ in agar-plate growth systems (Li et al., 2011). Experimental procedures based on chlorotic phenotypes that displayed *Arabidopsis* thaliana leaves upon NH_4^+stress, have identified the NH_4^+ overly sensitive 1 (*amos1*) and *amos2* mutants (Li et al., 2012; 2014). Genetically approaches have revealed that amos1 is an allelic mutation of EGY1, which encodes a plastid metalloprotease, and is required for normal chloroplast development and ethylene-dependent gravitropism (Li et al., 2012; 2013). An analysis of the amos1 mutant has shown how the AMOS1/EGY1-dependent plastid retrograde signaling pathway is required for chloroplast functionality maintenance upon NH_4^+ toxicity (Loque et al., 2009). It is commonly accepted that ABA and ROS accumulate after NH_4^+ exposure as a sole nitrogen form (Patterson *et al*., 2010; Fernández-Crespo et al., 2012).

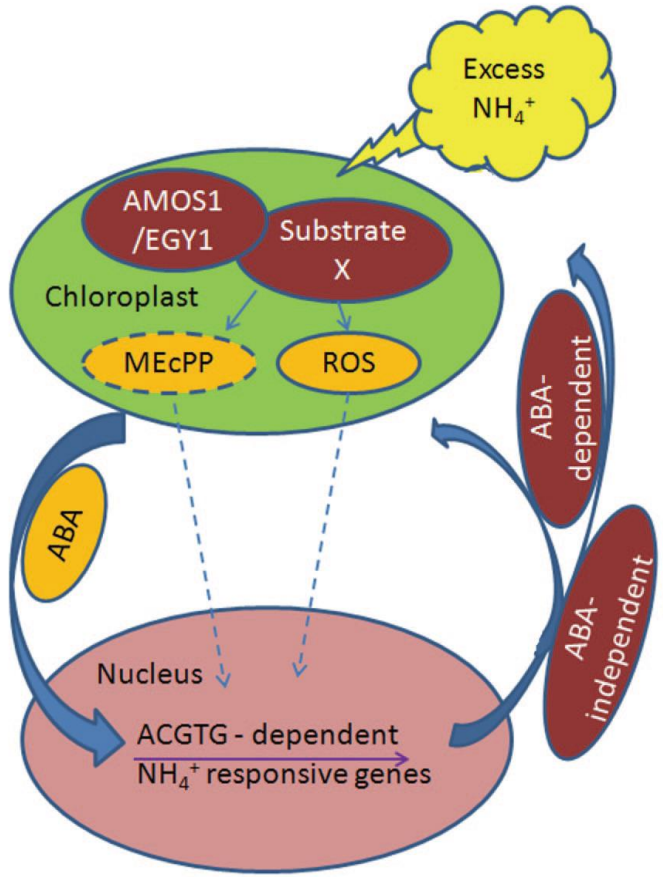

Fig.5. Model for AMOS1/EGY1-dependent plastid retrograde signaling during ammonium stress. X, the unknown substrate of AMOS1/ EGY1. All dashed lines indicate areas that require further research. During ammonium stress, AMOS1/EGY1 activates X to regulate the level of ROS or MEcPP, triggering plastid retrograde signaling, which is required for the expression of ammonium-stress-responsive nuclear genes including those encoding ABA-dependent proteins and others, such as small heat shock proteins. Most of these genes contain the 'ACGTG' regulatory element in their promoter region. Further, AMOS1/EGY1-dependent plastid retrograde signaling can recruit ABA signaling to enhance the expression of ammonium-stress-responsive ABA-dependent nuclear genes. These proteins cooperate to enhance the resistance of leaves to high ammonium and help maintain chloroplast functionality (Li et al., 2013).

Based on these results, Li et al. (2012) proposed a model to clarify responses to ammonium toxicity: under NH_4^+ stress, the chloroplast receives the stress signal that triggers ROS-mediated AMOS1/EGY1-dependent retrograde signaling as a plausible messenger that recruits downstream of ABA signaling to regulate the expression of NH_4^+ -responsive genes in the nucleus and to prevent NH_4^+ toxicity.

Although NH_4^+assimilation requires less energy than that of NO_3^-, in most species the combination of NH_4^+ and NO_3^- increases plant growth and yield, and the optimum NH_4^+/NO_3^- ratio varies according to species and environmental conditions (Britto et al., 2002). In recent years, the detrimental effects of NO_3^- fertilization have been demonstrated since NO_3^- loss in the field produces soil and water pollution, and releases emissions of nitrous oxide due to incomplete capture and poor nitrogen fertilizer conversion (Sarasketa el at., 2014). Given these negative NO_3^- nutrition effects on ecosystems (Gruber and Galloway, 2008), the potential of NH_4^+ as a nitrogen source has been reconsidered and advances in plant nutrition research goes in this direction as well as in the to improvement of N use efficiency (NUE) of crops (IPCC, 2007). To this ecological need for using NH_4^+ as an N source to act as an NO_3^- alternative, we add its potential effect as an inducer of resistance by either ROS-mediated retrograde signaling or by the accumulation of signal molecules. More studies are needed to elucidate the molecular effects that underlie NH_4^+ nutrition to better understand these processes in order to make the most of this phenomenon in the sustainable agriculture context.

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CHAPTER 2
Ammonium enhances resistance to salinity stress in citrus plants

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ABSTRACT

In this work, we demonstrate that NH_4^+ nutrition in *citrange Carrizo* plants acts as an inducer of resistance against salinity conditions. We investigated its mode of action and provide evidence that NH_4^+ confers resistance by priming abscisic acid and polyamines, just as enhancing H_2O_2 and proline basal content. Moreover it observed a diminished Cl^- uptake as well as an enhanced *PHGPx* expression after salt stress. Control and N-NH_4^+ plants have shown optimal growth, however it was observed that N-NH_4^+ plants have displayed greater dry weight and total lateral roots than control plants, but that differences are not seen for primary roots length. Our results reveal that N-NH_4^+ treatment induces a similar phenotypical response to the recent stress-induced morphogenetic response (SIMRs). The hypothesis is that N-NH_4^+ treatment triggers mild chronic stress in *citrange Carrizo* plants, which might explain the SIMR observed. Moreover, we observed modulators of stress signaling, such as H_2O_2 in N-NH_4^+ plants, which could acts as an intermediary between stress and the development of the SIMR phenotype. This observation suggests that NH_4^+ treatments induce a mild stress condition that primes the *citrange Carrizo* defense response by stress imprinting and confers protection against a subsequent salt stress.

INTRODUCTION

Nitrate (NO_3^-) and ammonium (NH_4^+) are the main inorganic nitrogen (N) resources absorbed by the roots of higher plants. Nitrogen is used to form glutamine, a precursor of many amino acids, like nucleic acids, alkaloids and polysaccharides, as well as secondary metabolites like PAs (Bagh et al., 2004). It is generally accepted that many plants display optimal growth and development if nitrogen is available in the form of NO_3^- (Coruzzi and Bush, 2001). Although NO_3^- uptake consumes more energy than NH_4^+, only a few plant species display optimal growth when N is available in only the NH_4^+ form (Marschner, 1995). Camañes et al., (2009) demonstrated that citrus plants prefer to absorb NH_4^+ more than NO_3^- when both N forms are present in the nutrient solution, which is probably due to less energy in the assimilation ion process. NH_4^+ is a paradoxical nutrient ion because, despite being a major N source and an important intermediate in many metabolic reactions, there are reports that high concentrations of this ion in either soil or the nutrient solution may lead to an "ammonium syndrome". This may include leaf chlorosis, lower plant yield production and root/shoot ratio, lower cation content, acidification of the rizosphere and changes in several metabolites levels, such as amino acids or organic acids (Britto and Kronzucker, 2002). In spite of the information available about the appearance of toxic symptoms due to NH_4^+ nutrition, different studies have produced contradictory results. This could be explained by each plant's specific and varietal characteristics, and by experimental conditions. Thus, there is a wide range of plant responses to NH_4^+ nutrition; there are some species that are tolerant to high NH_4^+ doses, such as rice (Wang et al., 1993), and some very sensitive species which practically cannot survive under NH_4^+ nutrition, such as tomato or barley (Britto et al., 2001).

Salinity is amongst the most significant environmental factors responsible for substantial losses in agricultural production worldwide and it is one of the serious problems confronting sustainable agriculture in irrigated production systems in arid and semiarid regions (Marschner 1995; Ravindran et al., 2007). Nearly 20% of the world's cultivated area and about half of the world's irrigated lands are affected by this stress (Munns and Tester, 2008). This is a critical problem especially in citrus since they are one of the most globally important horticultural crops considered salt sensitive (Al-Yassin 2005). Salinity causes several injuries in citrus such as tissue burning, loss of yield, leaf abscission and finally plant death (Romero-Aranda et al., 1998). Identifying successful strategies that enhance salinity resistance to this plant species is of both agronomic and economic interest. This complex environmental stress presents three different components: an ionic component linked to the accumulation of ions, which become toxic at high salt concentrations (mainly Na^+ and Cl^-) in the cytoplasm, leading to ionic imbalance; an osmotic component due to the compartmentalization of this toxic ion in the vacuole. When this compartmentalization occurs in cells, the cytosol water potential must be lowered to balance the low-external water potential, thus ensuring water intake in the plant cell and avoiding macromolecule damage (Ellouzi et al., 2011). Apart from the toxic and osmotic effects of salinity, a high cellular NaCl concentration causes enhanced formation of reactive oxygen species ROS (Hernandez and Almansa, 2002). ROS are highly reactive and, in the absence of any scavenging mechanism, they can provoke major alterations in normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Foyer and Noctor, 2005). However, transient ROS formation apart from causing oxidative damage when present at high concentrations can play a signaling protective role in the short term (Dat et al., 2000). The role of some

ROS, such as signal molecules in biotic or abiotic stress, is of biological significance because the production of these molecules could benefit the plants brought into a state of acclimation (Foyer et al., 1997, Jubany-Marí et al., 2009). Similarly, plant hormones play an important role in response to unfavorable environmental conditions. They are involved in signaling response to drought and salinity by the activation of acclimation processes such as stomatal closure, regulation of hydraulic conductivity and regulation of developmental processes that affect stress tolerance, such as senescence abscission (Sakamoto et al., 2008). Plants are sessile organisms that have developed an extensive array of defensive responses. An important aspect related to response to a range of biotic and abiotic stress is the phenomenon of priming (Van der Ent et al., 2009). Preliminary stress exposure or stress imprinting is indeed necessary to induce priming, which makes the plants more resistant to future biotic or abiotic stress (Conrath et al., 2006; Bruce et al., 2007; Galis et al., 2009). Priming state can be induced by different biological or chemical stimuli. Some quemical inducers are 2,6-dichloro isonicotinic acid (INA), benzo-(1,2,3)-thiadiazole-7-carbotionic acid S-methyl ester (BTH), β-aminobutyric acid (BABA) (Oostendorp et al., 2001; Conrath et al., 2002) and hexanoic acid, which by root treatment protects tomato plants and Arabidopsis against *Botrutis cinerea* (Vicedo et al., 2009; Kravchuk et al., 2011). Regarding abitotic stress, BABA has been shown to confer plant protection against salinity and drought (Jakab et al., 2005; Macarisin et al., 2009). Moreover, stress can also boost plant stress tolerance through induction of acclimation responses. Tolerance can be linked to an array of morphological, physiological and biochemical responses which lower the stress exposure limit and damage, or facilitate the repair of damaged systems (Mittler 2002). After exposure to stress, various changes take place, leading to different phenotypes depending on the type of stress, its

duration or experimental conditions. However, a common response in all these responses occurs, which is known as "stress-induced morphogenic responses" (SIMRs). Exposure of plants to mild chronic stress could cause induction of these specific SIMRs. These responses are characterized by blockage of cell division in the main meristematic tissues, inhibition of elongation and redirected outgrowth of lateral organs. Furthermore, it is believed that this process brings about a rise in ROS species and alters different hormones (Potters et al., 2007). The induced resistance against abiotic stress in citrus and other woody species has not been explorer, and understanding the molecular mechanisms beneath this process will provide the necessary insights to exploit this phenomenon in sustainable agriculture.

In this work, we demonstrate that NH_4^+ nutrition in *citrange Carrizo* plants enhanced resistance to salinity conditions. We investigated its mode of action, and provide evidence that NH_4^+ primes *citrange Carrizo*'s defenses by enhancing abscisic acid (ABA), polyamines (PAs) and potentiating H_2O_2 and proline basal content as well as diminished Cl^- uptake.

MATERIAL AND METHODS

Plant material, growth conditions and nutrition treatments

citrange Carrizo seeds (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L.) (Beniplant, Valencia, Spain) were allowed to germinate in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 20/24°C, light intensity of 200 μmol m^{-2} s^{-1} and relative humidity of 70%. Seeds were irrigated twice a week with distilled water. After 6 weeks, seedlings were irrigated for two months with Hoagland solution lacking nitrogen (Hoagland and Arnon, 1950) complemented with 1 mM NH_4NO_3 (control treatment) and 5 mM of N-NH_4^+ [$(NH_4)_2SO_4$)] (NH_4^+ treatment). Then 1.5 mM K_2SO_4 and 3 mM $CaSO_4$

were added to compensate for the absence of K^+ and Ca^{2+}. The pH of the nutrient solution was adjusted to 6.0 with 1 mM KOH.

Prior to the experiments, 3-month-old plants with a single shoot were selected for uniformity of size, and transferred to an aerated complemented Hoagland solution for 7 days in hydroponic culture devices.

To salt stress, 90 mM NaCl were added to the hydroponic solution and renewed twice weekly. Samples were taken for individual analysis at 2 h and 14 d after addition of salt to the hydroponic solution. At the end of the experiment, the phenotype was determined by the percentage of leaves with symptoms of necrosis or burns.

Growth and damage of salt values

The DW and length primary roots were measured at each sampling. The lengths of the individual primary roots of individual seedling were measured directly. Dry weight refers to the total roots (primary, secondary and tertiary) of the individual seedlings.

To measure the damage provoked by high salinity in the medium soil, we also established three damage levels: healthy leaves, chlorotic leaves (level 1), leaves with necrosis (level 2) and burnt leaves (level 3).

Quantitative RT-qPCR analysis

Gene expression by quantitative real-time PCR (RT-qPCR) was performed using RNA samples extracted from leaf tissue using the Total Quick RNA cells and tissues kit (Talent; http://www.spin.it/talent). Citrus leaves tissue samples for RNA isolation were collected at 2 h and 14 d after NaCl treatment. Leaf tissue was collected from treated and untreated plants. The RT-qPCR conditions were those described by Flors et al. (2007). A list

of the primers used in the RT-qPCR is shown in Table 1, using *GAPDH* gene expression of citrus how an internal standard.

Primer name	Forward primer	Reverse primer
GAPDH	5′- ggaaggtcaagatcggaatcaa - 3′	5′ cgtccctctgcaagatgactct -3′
AOS	5′- cgaatttcaatccccaagaa-3′	5′- ttggtgggttgttcatcaga-3′
PHGPx	5′- catcacagtgtggcttgacc -3′	5′- tgctggatttcagatgcttg -3′
PR5	5′- tgggggactactccaatgtc -3′	5′- atcctcctggaaccctcaat-3′
RD22	5′- ttggaaaaggacttgcatcc-3′	5′- atgccagcgtcttcacactc-3′

Table 1 Primers sequences

Chromatographic analysis

Hormone extraction and quantification were performed as described in Flors et al. (2008). Briefly, fresh material was frozen in liquid nitrogen. Before extraction, a mixture of internal standards containing 100 ng [^{2}H6]-ABA and 100 ng [^{2}H4]-SA was added. Dry tissue (0.05 g) was immediately homogenized in 2.5 mL of ultrapure water. After centrifugation (5000 g, 40 min), the supernatant was recovered and adjusted to pH 2.8 with 6% acetic acid, and subsequently partitioned twice against an equal volume of diethyl ether. The aqueous phase was discarded, the organic fraction evaporated in a Speed Vaccuum Concentrator (Eppendorf; www.eppendorf.com) at room temperature, and the solid residue re-suspended in 1 mL of a water/methanol (90:10) solution and filtered through a 0.22 μm cellulose acetate filter. A 20 μl aliquot of this solution was then directly injected into the HPLC system.

For PAs analysis, fresh material was frozen in liquid nitrogen. Before extraction, according to the method of (Sánchez-López et al., 2009) a mixture of internal standards containing $^{13}C_4$-putrescina and 1,7-diamineheptane, was added. Dry tissue (0.02 g) was homogenized in 2 mL of 2% perchloric acid. After centrifugation (5000 g, 40 min), the supernatant was separated 2 mL of

2% perchloric acid were add to the pellet and centrifugation was repeated. Then both supernatants were collected and a mixture of 10% MeOH and HFBA 25 mM was added until 6 mL. Next 1 mL of the mixture was taken and filtered through a 0.45 μm cellulose acetate filter. A 20 μl aliquot of this solution was directly injected into the HPLC system. Analyses of hormone and PAs sample were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100 x 2 mm i.d.; 5 mL; Scharlab, Barcelona, Spain; http:// www.scharlab.es). The chromatographic system was interfaced to a Quatro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and plant samples.

Chloride analyses

Chloride measurements were taken by automatic titration using a chloridemeter (Model 926, Sherwood Scientific Ltd., Cambridge, UK), as described in López-Climent et al. (2008). Briefly, ground plant material was incubated overnight in a mixture of 0.1 N HNO_3 (Panreac, Barcelona, Spain) and 10% glacial acetic acid (Panreac) under continuous shaking. The supernatant was filtered through Whatmann #1 filter paper and 0.5 mL used for determinations.

Proline analyses

Leaf proline content was determined by a spectrophotometric assay, as described in Bates et al. (1973). Briefly, 50 mg of frozen plant material was extracted in 5mL of 3% sulphosalicylic acid (Panreac). After centrifugation at 4000×g and 4 ºC, 1 mL of supernatant was combined with 1 mL of glacial

acetic acid (Panreac) and 1 mL of ninhydrin (Panreac) solution. The combined solution was incubated at 80 ºC in a water bath for 1 h and the resulting mixture was partitioned against 2 mL of toluene after a cooling period. Absorbance at 520 nm was read in the organic layer against a blank. Determinations were performed using commercial proline as a standard (Sigma-Aldrich, Madrid, Spain).

DAB staining and H_2O_2 quantification

N-NH_4^+ and control plants were exposed to 90 mM NaCl for 2 h, and the salt-stressed leaves were stained in 1 mg of DAB per milliliter at pH < 3 for 24 h in the dark and were subsequently destained in 95% ethanol. Later, samples were rehydrated with distilled water. DAB staining intensities were quantified from digital photographs by the number of dark-brown DAB pixels in relation to the total pixels corresponding to plant material, using GIMP2 program.

Statistical analysis

Statistical analysis was carried out using the Statgraphics software support. Data are expressed as means and standard error. Mean values were compared by an LSD (least significant difference) test. All experiments were repeated at least three times.

RESULTS

Development of the *citrange Carrizo* plants grown under the NH_4^+ condition

Three-month-old *citrange Carrizo* plants were grown for 2 months with Hoagland solution lacking nitrogen, but complemented with 1 mM NH_4NO_3 (control plants) and 5 mM $(NH_4)_2SO_4$ (N-NH_4^+plants). Although other studies have shown that some species develop toxic symptoms when only NH_4^+ nutrition is applied (Gerendás et al., 1997; Lasa et al., 2001), the *citrange Carrizo* N-NH_4^+ plants displayed optimal growth, estimated on the basis of biomass production (Fig. 1A). Moreover, we observed that N-NH_4^+ plants developed a darker green color, and their chlorophyll content was 13.12% higher than in the control plants (data not shown). Likewise, the N-NH_4^+ plants showed vigorous root growth, estimated on the basis of the DW of total roots, which was higher in the N-NH_4^+ plants if compared with the control plants (Fig. 1C). However, primary root length did not differ between the control and the NH_4^+ treated plants (Fig. 1B). The secondary and tertiary lateral roots of the N-NH_4^+ plants developed more than control plants. NH_4^+ treatment increased the number of lateral roots per DW of the total roots of the individual seedlings, as well as the number of total roots per primary root length (Figs. 1D and E).

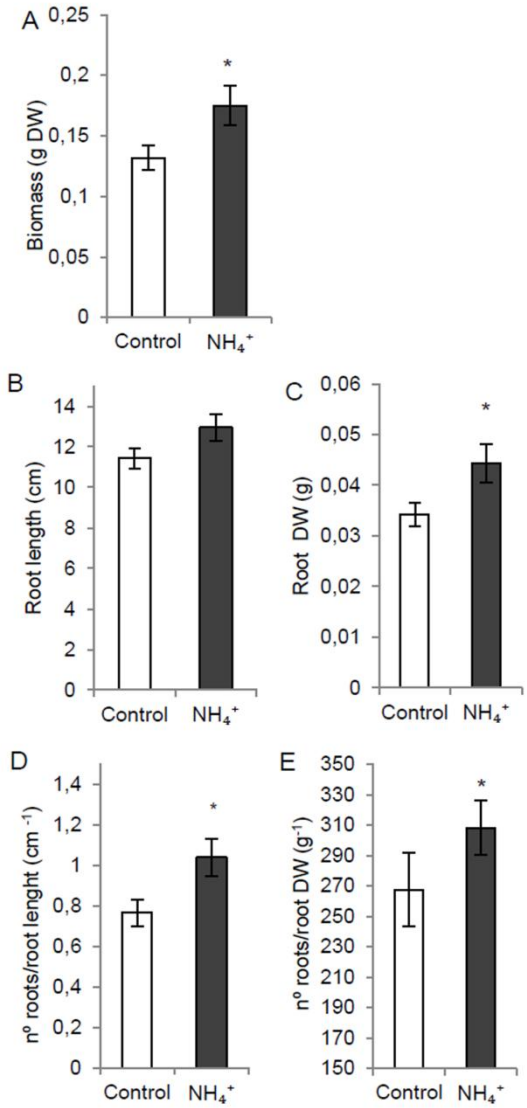

Fig.1. Effect of NH_4^+ nutrition on the growth of *citrange Carrizo* plants. (A) Biomass production, (B) root length, (C) root DW, lateral roots development expressed as: (D) number of lateral roots/root DW and (E) number of lateral roots/root length. Data are from a representative experiment that was repeated three times with similar results. Values are the mean of 50 seedlings. Asterisk indicates statistically significant differences ($p< 0.05$).

NH_4^+ treatment enhances *citrange Carrizo* resistance to salt stress

The increased levels of NaCl in the watering solution led to different levels of damage in plants. To achieve salt stress, 90 mM NaCl were added to the hydroponic solution to the control and the N-NH_4^+ plants over a 14-day period. In order to check how NH_4^+ nutrition could affect the response of *citrange Carrizo* plants to salt stress, necrosis and burns on leaves were estimated. The result was expressed as percentage of damaged leaf in relation to the total leaves per plant by establishing the following damage levels: healthy leaves, chlorotic leaves (level 1), leaves with necrosis (level 2) and burnt leaves (level 3) (Fig. 2). In this case, significant differences between the control and the N-NH_4^+ salt-treated plants were noted as the N-NH_4^+ plants displayed 30% more healthy leaves than the control plants. Both the control and the N-NH_4^+ plants had a similar percentage of chlorotic and necrotic leaves but, interestingly, the level of burnt leaves was higher in the control plants.

NH_4^+ treatment reduced *citrange Carrizo* toxic and osmotic stress

Salinity tolerance in *Citrus* is strongly related to leaf chloride accumulation. It is well-known that Cl^- toxicity, rather than Na^+ toxicity, is the primary factor involved in the molecular responses of citrus plant leaves to salinity (Brumós et al., 2009). We observed that the Cl^- concentration in leaves increased in both treatments, in the control and the N-NH_4^+ plants upon salt stress (Fig. 3A). The highest leaf Cl^- concentration occurred in the control plants at 14d, at which time the Cl^- concentration noted for the leaves of the N-NH_4^+ plants had reduced by 24% when compared with the control plants leaves.

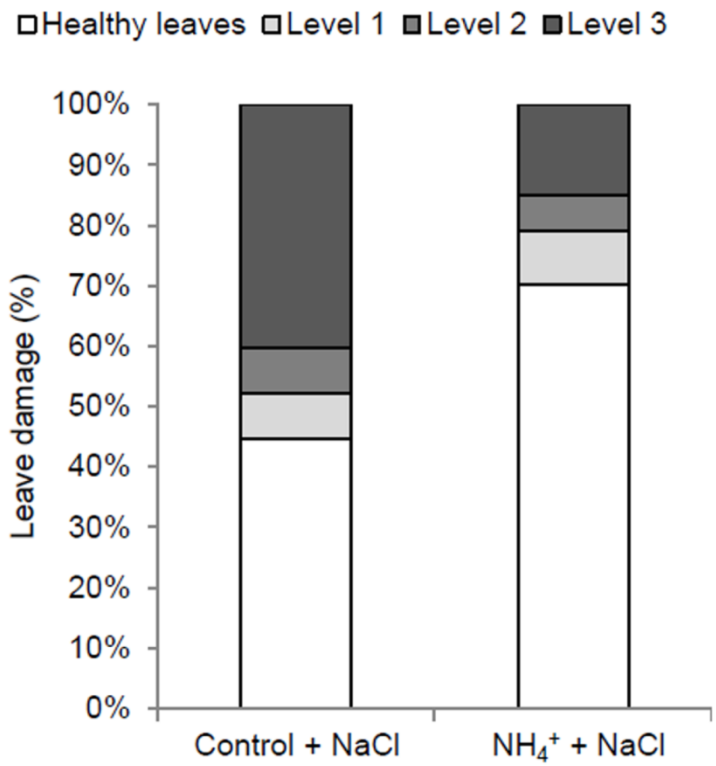

Fig.2. Effect of NH_4^+ treatment on the *citrange Carrizo* plants treated with NaCl (90 mM) for 14 d. The result is expressed as % of damage at different levels: healthy leaves, chlorotic leaves (level 1), intermediate leaf necrosis (level 2) and burnt leaves (level 3). Data are from a representative experiment that was repeated three times with similar results. Values are the mean of 50 seedlings.

It is well-known that soil with high salt concentrations is virtually dry because the available water is trapped by ions. Proline has been considered to play an important role in plant response to salinity (Gaspar et al., 2002) since it acts as a compatible solute that adjusts the osmotic potential in the cytoplasm (Bartels and Sunkar, 2005). In order to assess the effectiveness of $N\text{-}NH_4^+$ treatment against osmotic stress induced by NaCl, we tested proline content in leaves. The basal proline content differed between the control and the $N\text{-}NH_4^+$ plants (Fig. 3B).

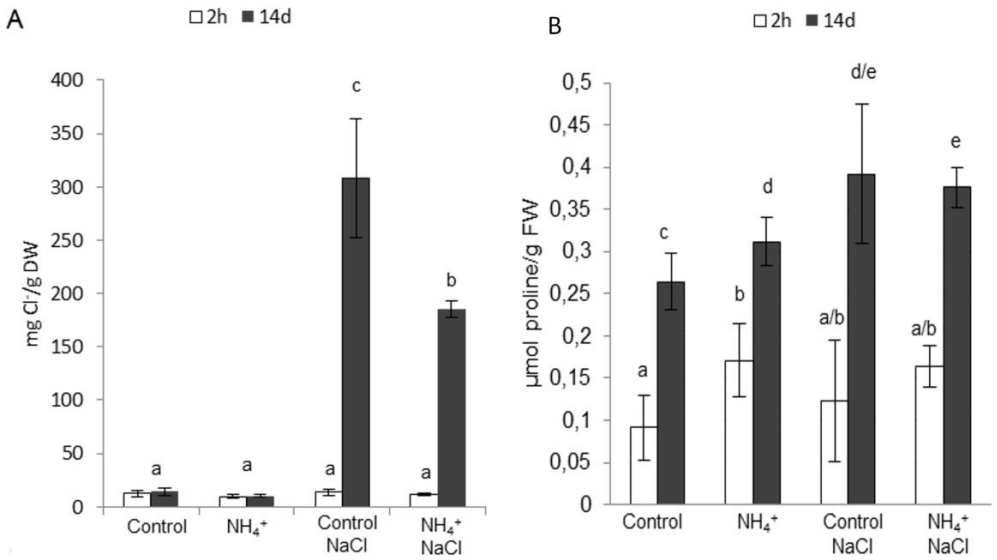

Fig.3. Effect of NH_4^+ treatment on the Cl^- and proline content in the *citrange Carrizo* plants treated with NaCl (90 mM) for 2 h and 14 d. (A) Cl^- content expressed in mg Cl^- g^{-1} DW and (B) Proline accumulation expressed in µmol proline g^{-1} FW. Data show the average of three independent experiments of a pool of 30 plants ± SE. Letters indicate statistically significant differences ($p < 0.05$).

The N-NH_4^+ plants had higher proline content at 2 h and 14 d in the absence of salt if compared with the control plants. Interestingly the proline content of both treatments significantly increased after salinity, with no statistically significant differences between the control and the N-NH_4^+-treated plants. Proline content increased by 19.53% in the control plants upon salinity treatment, while it increased by only 9.30% in the N-NH_4^+ plants.

NH_4^+ treatments enhance H_2O_2 accumulation

In this work, we used DAB staining to establish how NH_4^+ treatment affects cellular oxidative stress. The H_2O_2 staining based on the *in vivo* reaction of H_2O_2 with DAB allows a rapid estimation of H_2O_2 accumulation in leaves (Thordal-Christensen et al., 1997). Our results indicate that the N-

NH_4^+ plants show higher initial levels of H_2O_2 accumulation than the control plants in the absence of salt stress. Increased H_2O_2 accumulation was noted 2 h after treatment with 90 mM NaCl. Although higher H_2O_2 accumulation was seen in the control plants in response to salt stress, the highest levels of H_2O_2 accumulation were observed for the N-NH_4^+ plants treated with NaCl (Fig. 4)

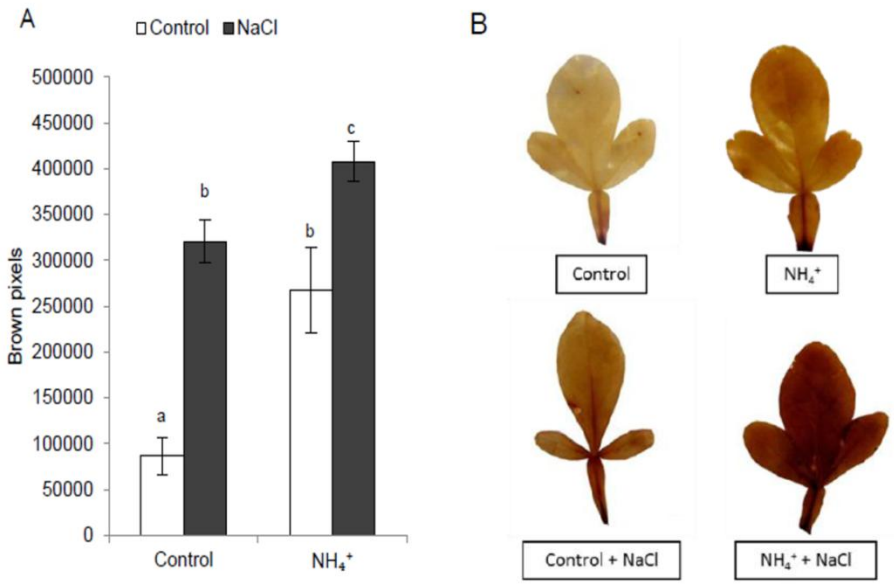

Fig.4. H_2O_2 staining, estimated by using DAB staining in the leaves of the control and the N-NH_4^+ *citrange Carrizo* treated with NaCl (90 mM) for 2 h. (A) Quantitative H_2O_2 measurement on the basis of brown pixels from digital photographs and (B) Brownish areas are indicative of H_2O_2 accumulation. Data are from a representative experiment that was repeated three times with similar results. Values are the mean of 10 seedlings. Letters indicates statistically significant differences ($p < 0.05$).

NH_4^+ treatment induces the main hormone signaling pathways

In order to establish whether enhanced resistance of NH_4^+ is mediated by the induction of the ABA-, salicylic acid- (SA) and jasmonic acid - (JA) signaling pathways, the *RD22*, *PR5* and *AOS* marker genes expressions were analyzed by RT-qPCR (Fig. 5). These genes have been previously reported to

be salt stress inducible in different species (Zhu et al., 1995; Nylander et al., 2001; Pendranzani et al., 2003). We observed that NH_4^+ treatments induced *RD22* mRNA accumulation in the absence of salt stress and that *RD22* mRNA accumulation in response to salt stress lightly increased after salinity in both treatments (Fig. 5A). NH_4^+ treatment induced *PR5* mRNA accumulation in the absence of salt stress, however, upon salinity stress both treatments induced the *PR5* expression at 14 d, but greater inductions were observed in the control plants than in the N-NH_4^+ plants (Fig. 5B). We also checked JA-dependent signaling pathway transduction after NH_4^+ treatment in *citrange Carrizo* plants. NaCl treatment induced *AOS* mRNA accumulation in both treatments, but the N-NH_4^+ plants exhibited greater accumulation upon salinity when compared with the control plants (Fig. 5C). The expression patterns of the markers genes for the ABA, SA and JA pathways indicate that all the pathways were more induced in the NH_4^+ treated plants than in the control ones. In order to further confirm the possible role of the different signaling pathways in NH_4^+ resistance, we analyzed the hormonal levels in both the control and the N-NH_4^+ treated plants at 14 d after salinity stress. The basal ABA levels differed between control and the N-NH_4^+ plants in the absence of salt, but were higher in the N-NH_4^+ plants (Fig. 6A). The control plants revealed increased ABA accumulation 14 d after salt stress, as expected, but no such increase was observed in the N-NH_4^+ plants upon salt stress. These initial ABA accumulations suggest that the resistance induced by NH_4^+ treatment could be mediated by this hormone, which plays a role in defense signaling in osmotic and salt stresses (Jakab et al., 2005). The basal SA levels did not differ between the control and the NH_4^+-treated plants before salinity, but ranged between 25 ng g^{-1} and 38 ng g^{-1} FW (Fig. 6B). Interestingly, the control plants displayed a significant increase in SA accumulation at 14 h after salinity which was not observed in

the N-NH_4^+-treated plants upon salinity. No differences in JA levels were observed in either the control or the NH_4^+-treated plants in the absence or the presence of salt stress (data not shown).

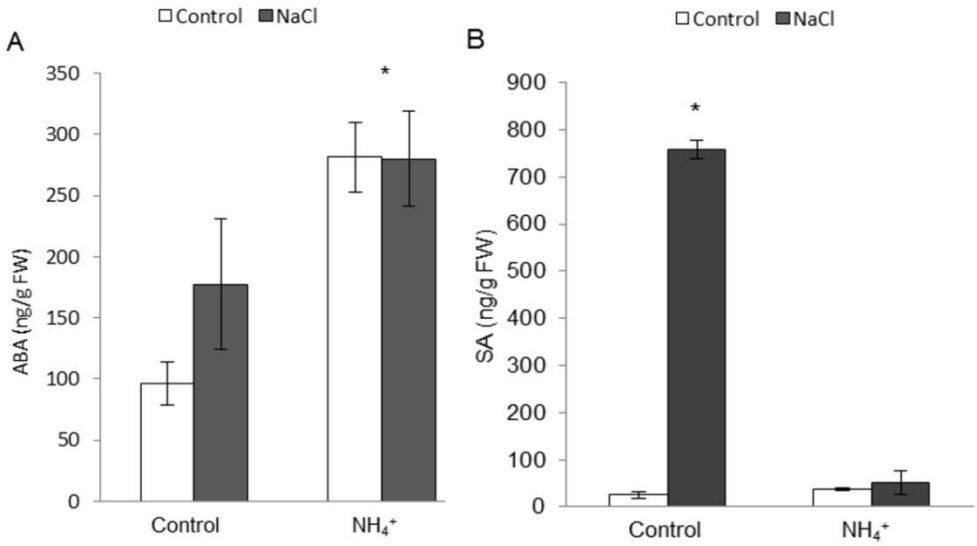

Fig.5. Hormone levels in the control and N-NH_4^+ *citrange Carrizo* plants upon salinity. Leaves were collected at 14 d after addition of NaCl (90 mM). (**A)** ABA and (B) SA levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 30 plants per experiment ± SE. Asterisk indicates statistically significant differences ($p < 0.05$).

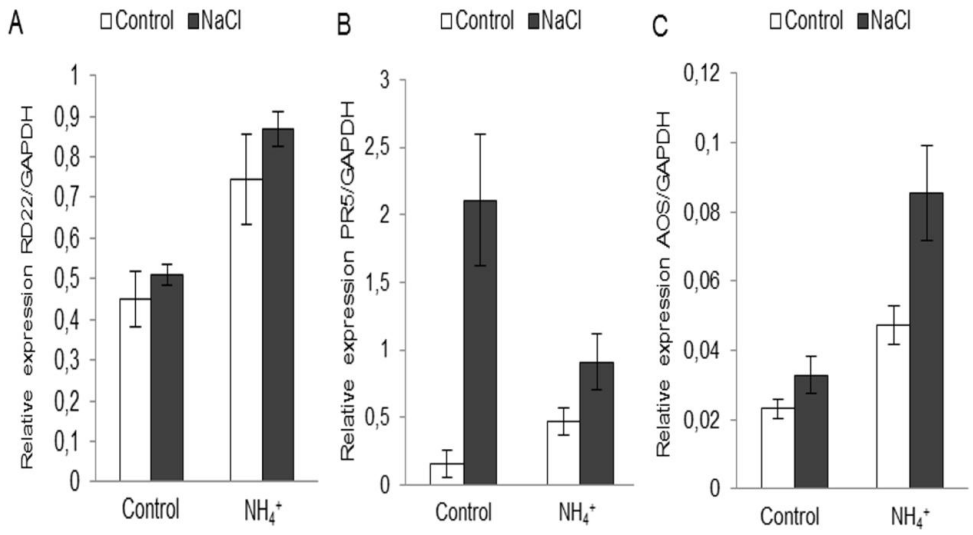

Fig.6. Effect of NH_4^+ treatment on the gene expression in *citrange Carrizo* plants upon salt stress. Total RNA was isolated from leaves at 14 d after addition of NaCl (90 mM) converted into cDNA, and was subjected to a RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The relative level of (A) *RD22*, (B) *PR5* and (C) *AOS* were analyzed in the control and the N-NH_4^+ citrus plants. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. The experiment was repeated three times with similar results.

NH_4^+ treatments reduced the oxidative damage caused by salt stress. Polyamines content and *PHGPx* expression

PAs play a key role in plant responses to salinity. These compounds have been tested not only as antioxidants, but also as osmoprotectors under salinity conditions (Groppa et al., 2001; Chattopadhayay et al., 2002; Kakkar and Sawhney, 2003). In order to determine whether NH_4^+ treatment affects PAs content, leaf samples were analyzed at 14 d in the control and the N-NH_4^+ plants, and also in these plants after salt stress. In the N-NH_4^+ plants, the concentrations of putrescine (Put), spermidine (Spd), and spermine (Spm) were higher than in the control plants in the absence of salt. It is interesting to note that the Put titer increase was especially important. The Put

concentration in the control plants was 6.16 ng mg $^{-1}$ FW, while it was 146.98 ng mg $^{-1}$ FW in the N-NH_4^+ plants. However, the Put, Spd and Spm contents remained unaffected in the control and the N-NH_4^+ plants after salt stress (Table 2). Put accumulations in N-NH_4^+ plants suggest that the resistance induced by NH_4^+ treatment could be mediated by this polyamine, which reduces salt-inducible oxidative damage (Groppa and Benavides, 2008).

	Put		**Spd**		**Spm**	
Control	6.16 ± 1.9	a	29.51 ± 1.41	b/c	11.76 ± 2.57	a
NH_4^+	146.98 ± 27.87	b	36.06 ± 1.93	c	19.83 ± 2.36	b
Control + NaCl	3.04 ± 1.12	a	24.35 ± 2.99	a/b	12.89 ± 2.61	a
NH_4^+ + NaCl	160 ± 14.68	b	30.65 ± 3.43	b/c	18.77 ± 4.02	a

Table 2. Polyamine levels expressed in µg g^{-1} FW in control and N-NH_4^+ plants upon salinity. Leaves were collected at 14 days after addition of NaCl (90 mM). Levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 30 plants per experiment ± SE. Letters indicates statistically significant differences ($p < 0.05$).

In order to establish whether the enhanced resistance of N-NH_4^+ treatment to salt stress is mediated by the induction of antioxidant activity pathways, the *PHGPx* gene expression was analyzed by RT-qPCR (Fig. 7). It has been previously reported that *PHGPx* is a unique intracellular antioxidant enzyme that directly reduces phospholipid hydroperoxides produced in cell membranes under salt conditions, and has been considered the main line of enzymatic defense against oxidative biomembrane damage in mammalian cells (Chun-Juan et al., 2009). Although the *PHGPx* expression was unaffected in the absence of salt, N-NH_4^+ treatment enhanced this expression at 14 d after salt stress.

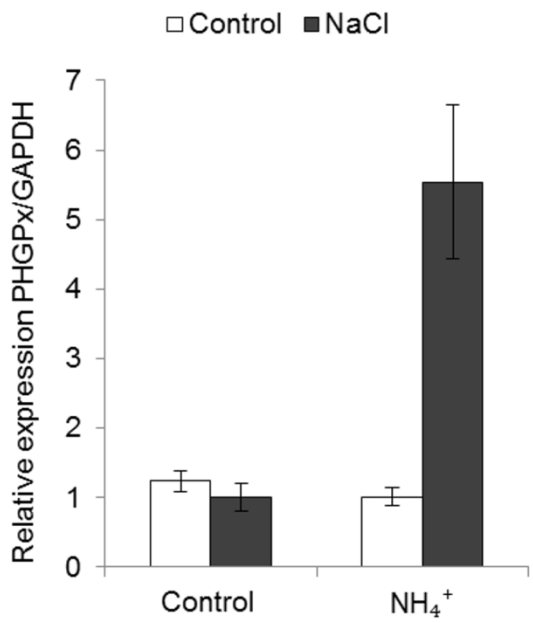

Fig. 7. Effect of NH_4^+ treatment on the *PHGPx* expression in the *citrange Carrizo* plants upon salt stress. Total RNA was isolated from leaves at 14 d after addition of NaCl (90 mM) converted into cDNA, and was subjected to a RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. The experiment was repeated three times with similar results.

DISCUSSION

In this study, we have analyzed influences of NH_4^+ nutrition on *citrange Carrizo* plants undergoing 90 mM NaCl. *citrange Carrizo* plants were grown with 1 mM NH_4NO_3 (control plants) and 5 mM de N-NH_4^+ (N-NH_4^+ plants), they showed optimal growth in both treatments. However, we observed that the N-NH_4^+ plants had greater DW and total lateral roots than the control plants; yet these differences were not noted for primary roots length. It is commonly accepted that the root system is critical for nutrient and water uptake from soil, and that it displays considerable plasticity in response to development and environment signals (Li et al., 2010). Primary root growth

is often diminished in stressful soil environments, such as those deficient in phosphate (Svistoonoff et al., 2007) or with excess aluminum (Jones and Kochian, 1995). Previous results have shown that stunted root systems are a significant symptom of NH_4^+ toxicity and confirmed that NH_4^+ in soil inhibits primary root growth, being cell elongation but not cell division, the principal target in NH_4^+ inhibition (Li et al., 2010). We have demonstrated that NH_4^+ treatment induces lateral root development in *citrange Carrizo* plants. This is supported by the findings of Yang et al. (2011), who observed that NH_4^+ stimulated root hair branches in Arabidospsis which may be directly due to a response to NH_4^+ toxicity or NH_4^+-induced stress signals. Moreover, it has been suggested that ROS may be an NH_4^+-induced stress signal leading to the formation of hair branching. Moreover, it demonstrates that *citrange Carrizo* plants grown under N-NH_4^+ conditions are more resistant to salinity stress. Salinity stress was induced by the addition of 90 mM NaCl for 14 d to the control and the N-NH_4^+ plants. After checking for any damage induced by salinity, we found a differential response to salt stress, and the N-NH_4^+ plants presented less damage than the control plants. These data suggest that N-NH_4^+ treatment produces some response mechanism which benefits *citrange Carrizo* plants to better tolerate exposure to 90 mM NaCl. Salinity is a complex environmental stress that presets three different components: an ionic component linked to the accumulation of ions, mainly Cl^- in citrus plants (Brumós et al., 2009); an osmotic component due to compartmentalization of this toxic ion in the vacuole, which triggers accumulation of low molecular-weight osmolytes (Zhu et al., 1998); and increased ROS formation, which is considered the primary event under a variety of stress conditions (Hernandez and Almansa, 2002). N-NH_4^+-treated plants are capable of reduced Cl^- leaves accumulation after 14 d of salt exposure. The high shoot Cl^- level in the salt-treated control plants indicates

this ion's poor capacity to prevent translocation to shoots. However, N-NH_4^+ treatment helps avoid leaves from accumulating Cl^-, probably by the inhibition of chlorid channel-like (CLC) proteins, as observed in barley (Lopes and Araus, 2008). Moreover, soil with high salt concentrations is practically dry because ions trap any available water. To overcome this problem, citrus responds by overproducing compatible osmolites such as proline (Bañuls and Primo-Millo, 1992). For a long time, proline has been considered an inert compatible osmolyte which protects subcellular structures and macromolecules upon osmotic stress (Kishor et al., 2005). Several studies have shown that proline is a potent ROS scavenger associated with the prevention of apoptotic-like PCD (Chen and Dickman, 2005). Proline content in the N-NH_4^+ plants at 2 h and 14 d in the absence of salt is higher than in the control plants. Yet under salt conditions, both treatments showed an increased proline accumulation. The highest basal proline content in the N-NH_4^+ plants could confer initial protection to salt stress since proline accumulation in stressed plants has been associated with enhanced tolerance to abiotic stress conditions (Szabados and Savouré, 2009). Furthermore, it is well-known that salinity increases cellular ROS accumulation (Hernández and Almansa, 2002). Although ROS can induce severe cellular damage, these molecules are important in signaling, since control, among others, the expression of stress tolerance (Foyer and Noctor, 2005). Control and N-NH_4^+ *citrange Carrizo* plants display considerably increased H_2O_2 accumulation 2 h after salinity stress. It is noteworthy that the initial H_2O_2 levels were higher in the N-NH_4^+ plants than in the control ones. This result supports the idea that H_2O_2 could act as a stress signal in the N-NH_4^+-treated plants.

Several studies have suggested that NH_4^+ nutrition induces a stress response in several species (Lasa et al., 2001). Here, we confirmed that N-NH_4^+ treatment enhance resistance to salt stress. Moreover, it also found that

$N\text{-}NH_4^+$ treatment induced a similar phenotypical response to the recently stress-induced morphogenetic response (SIMRs) (Potters et al., 2007, 2009). We hypothesize that $N\text{-}NH_4^+$ treatment triggers mild chronic stress in *citrange Carrizo* plants which may account for the SIMRs noted. SIMRs is part of a general acclimation strategy characterized by blockage of cell division in main meristematic tissues, inhibition of elongation, redirected outgrowth of lateral organs (Potters et al., 2009), increase in antioxidants that prevent damage caused by ROS, and accumulation of foliar molecules which act as modulators of stress signals (Gould and Lister, 2006). This work demonstrates that the $N\text{-}NH_4^+$-treated plants clearly provoke an increase in lateral organs by augmenting the weight and number of lateral roots. $N\text{-}NH_4^+$ plants do not increase primary root length, however these plants showed an increase of modulators of stress signaling, such as H_2O_2, which could be intermediated between stress and the development of the SIMRs phenotype (Potters et al., 2007). This observation suggests that NH_4^+ treatment results in an enhanced resistance to salinity, possibly due to plants being previously exposed to mild stress which could be the prime *citrange Carrizo* defenses by stress imprinting, thus conferring plants resistance (Bruce et al., 2007).

On the one hand, we also investigated the effect of NH_4^+ nutrition on the expression of the *RD22, PR5*, and *AOS* marker genes involved in the stress response. We noted that $N\text{-}NH_4^+$ treatment increase the accumulation of the three marker genes. These results may indicate that the $N\text{-}NH_4^+$ plants have a more active defense pathway than the control plants. Moreover, salt treatment mainly increased *PR5* accumulation in the control plants, but the expression of the other marker genes in the control and the $N\text{-}NH_4^+$ plants was practically unaffected, which may be directly due to acclimated stage that NH_4^+ nutrition confers to citrus plants.

The analysis of hormones and metabolites in relation to plant responses to salinity reveal that ABA plays a role in the response against salt stress in the N-NH_4^+ plants. In this work, we found that the N-NH_4^+ plants have higher ABA levels than the control plants in the absence of salt. This fact is supported by the findings of Lopes and Araus (2008), who studied the gene expression profiles of barley seedlings fertilized with NH_4^+, NH_4^+ and NO_3^-, or with NO_3^- they observed that an epoxycarotenoid dioxygenase gene (involved to ABA synthesis) was upregulated in NH_4^+, probably due to NH_4^+ treatments which may invoke stress responses. Previous results have shown that stomatal closure occurs when barley plants are exposed to NH_4^+ for long periods. The fact that NH_4^+ nutrition increases ABA accumulation in leaves may induce ABA-signaling. ABA signaling plays an important role in adaptation to abiotic stress and in the regulation of several genes, thought to be involved in dehydratation or salt tolerance as well as in stomatal closure (Zhu 2002). However, signaling in response to salinity seems to not depend solely on ABA. SA has long since been known to be a signal molecule in inducing defense mechanisms in plants (Shah 2003, Halim et al., 2007). Under our experimental conditions, the control plants exhibit SA accumulation at 14 d after salt stress; this accumulation correlates well with the SA-marker gene, *PR5*, since it was overexpressed in the control plants at the same time. On the other hand, several studies support that SA binds directly to the catalase enzymes inhibiting its activity in several plants species (Sanchez-Casas and Klessig, 1994; Horvath et al., 2002). This inhibition of catalase activity has been proposed to explain an increased H_2O_2 level upon SA accumulation (Chen et al., 1993), and H_2O_2 is responsible for ROS accumulation and induction of cell death (Overmyer et al., 2003). This fact can explain our results since the control plants, with greater SA accumulation, were more affected by salinity. Jackab et al. (2005) observed

that BABA-induced salt stress tolerance mediated by ABA-dependent signaling in Arabidopsis and this response is independent of functional SA signaling. In this work, we also show antagonism between ABA and SA since the N-NH_4^+ plants treated with NaCl showed a faster, stronger ABA accumulation which could inhibit SA accumulation. Hence, salinity resistance in the N-NH_4^+ plants might be mediated by ABA accumulation, which is a regulator of salt tolerance. N-NH_4^+ treatment could increase ABA accumulation in leaves, thus conferring *citrange Carrizo* plants resistance to later salinity conditions.

The analysis of PAs in our study has determined that N-NH_4^+ treatment induces a faster, stronger Put accumulation at 2 h (data not shown) and at 14 d in the absence of salt. It is commonly accepted that some species develop toxic symptoms when NH_4^+ nutrition is applied (Gerendás et al., 1997; Lasa et al., 2001), while a negative effect on plant growth has been observed with this kind of nutrition (Claussen and Lenz, 1999; Walch-Liu et al., 2000). Ammonium nutrition decreases essential cations content (Britto and Kronzucker, 2002), probably due to competition with NH_4^+ in the uptake process. Although the uptake of cations other than NH_4^+ is sometimes reduced, NH_4^+ uptake usually increases under NH_4^+ nutrition. Finally, plants may have excessive total cation content in comparison with anion content (Clark 1982). Gerendás et al. (1997) suggest that plants could accumulate PAs to compensate for the lack of some cations other than NH_4^+, hence they could contribute to cellular ionic balance maintenance. Furthermore PAs mainly Put, has an important role in abiotic stress since it reduces salt-induced oxidative damage by increasing the activities of antioxidant enzymes and by lowering lipid peroxidation (Tang and Newton, 2005). In this work, we also reveal that NH_4^+ treatment leads to a greater induction of gene *PHGPx*. *PHGPx* is a unique intracellular antioxidant enzyme that directly

reduces the phospholipid hydroperoxides produced in cell membranes, and has been considered the main line of enzymatic defense against oxidative biomembrane damage in mammalian cells (Chun-Juan et al., 2009). Furthermore, *PHGPx* gene expression levels have been recorded to increase in plant tissues in response to pathogen infections (Criqui et al., 1992), high salinity (Li et al., 2001), heavy metals (Li et al., 2001), and extreme temperatures (Chen et al., 2004), suggesting the important roles that play in the defense responses of plants to biotic and abiotic stresses. Transient expression of *LePHGPx* protects tobacco leaves from salt and heat stress, and suppresses the apoptotic pathway induced by Bax (Chen et al., 2004). Our results suggest that the resistance to salinity that we found in the N-NH_4^+-treated plants could be mediated by a stronger accumulation of Put and the *PHGPx* transcript, which might induce resistance to the oxidative damage induced by salinity.

In conclusion, collectively these results indicate that NH_4^+ treatment enhances *citrange Carrizo* defense against salinity stress. This suggests that NH_4^+ treatment produces mild chronic stress and therefore induces the SIMRs in *citrange Carrizo*. Activation of the response related to SIMRs due to NH_4^+ toxicity led to the "acclimation stage", which leads to better adaptation to subsequent salt stress. This response initially brings about increased H_2O_2 accumulation which could act as a modulator of stress signal. Besides this, NH_4^+ treatment lowers Cl^- accumulation in leaves reducing its toxic effect and produces a higher basal proline content which might confer initial protection against salt stress. Moreover, the N-NH_4^+ treated plants have more active defense pathways than the control plants, and have activated ABA accumulation, which could prime ABA-signaling and PAs, mainly Put, which, in turn, could contribute to cellular ionic balance maintenance and reduce salt-induced oxidative damage. Furthermore, the N-NH_4^+ citrus

seedlings display enhanced antioxidant machine activity, thus increasing *PHGPx* transcription. Together, this observation suggests that NH_4^+ treatments induce a mild stress condition that primes the *citrange Carrizo* defense response by stress imprinting and confers protection against a subsequent salt stress. The use of nutritional compounds like NH_4^+ could be an interesting alternative to the use of chemical compounds to induce plant resistance. In addition, this fact may help to alleviate the toxicity caused by salinity, one of the major problems currently on citrus crop.

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CHAPTER 3
NH_4^+ induces antioxidant cellular machinery and provides resistance to salt stress in citrus plants

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ABSTRACT

NH_4^+ nutrition in *Carrizo citrange* (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L) plants acts as an inducer of resistance against salinity conditions. NH_4^+ treatment triggers mild chronic stress that primes plant defence responses by stress imprinting and confers protection against subsequent salt stress. In this work, we studied the influence of NH_4^+ nutrition on antioxidant enzymatic activities and metabolites involved in detoxification of reactive oxygen species (ROS) to clarify their involvement in NH_4^+-mediated salt resistance. Our results showed that NH_4^+ nutrition induces in citrus plants high levels of H_2O_2, strongly inhibits superoxide dismutase (SOD) and glutathione reductase (GR) activities, and leads to higher content of oxidized glutathione (GSSG) than in control plants in the absence of salt, thus providing evidence to confirm mild stress induced by NH_4^+ nutrition. However, upon salinity, plants grown with NH_4^+ (N-NH_4^+ plants) showed a reduction of H_2O_2 levels in parallel to an increase of catalase (CAT), SOD, and GR activities compared with the control plants. Moreover, N-NH_4^+ plants were able to keep high levels of reduced glutathione (GSH) upon salinity and were able to induce glutathione-S-transferase (*GST*) and phospholipid hydroperoxide glutathione peroxidise (*PHGPx*) mRNA accumulation. Based on this evidence, we confirm that sublethal concentrations of NH_4^+ might act as a mild oxidative stressor, which triggers antioxidant cellular machinery that can provide resistance to subsequent salt stress.

INTRODUCTION

Salinity is amongst the most significant environmental factors responsible for substantial losses in agricultural production worldwide, and it is one of the serious problems confronting sustainable agriculture in irrigated production systems in arid and semiarid regions (Ravindran et al. 2007; Marschner et al. 2012). Nearly 20% of the world's cultivated area and approximately half of the world's irrigated lands are affected by salinity stress (Munns and Tester, 2008). This is a critical problem especially for citrus plants, as they are one of the most globally important horticultural crops that are considered to be salt-sensitive (Al-Yassin, 2005). Damages caused by high salinity are often associated with three different mechanisms (Ievinsh, 2006): ion toxicity caused by the excessive accumulation of Na^+ and Cl^- in the cytoplasm, leading to an ionic imbalance. Second, even if massive ion compartmentalisation occurred in the vacuole, the cytosol water potential must also be lowered to balance the low external water potential, thus allowing water intake in the plant cell and preventing macromolecular damage. Moreover, high cellular NaCl concentration induces oxidative stress in plants through enhanced generation of ROS (Khanna-Chopra and Selote, 2007), which is considered the primary event for a variety of stress conditions (Foyer and Noctor, 2005). It is known that oxidative stress results from the disruption of the cellular homeostasis of ROS production. ROS accumulation induces oxidative damage in membrane lipids, nucleic acids, and proteins (Mittler, 2002). A causal relationship has been established between high or increased activities of antioxidant enzymes and the degree of protection from salt-associated oxidative damage (Mittova et al. 2004; Stepien and Klobus, 2005). Moreover, ROS have been implicated in a plethora of physiological processes in plants, from seed germination to cell death (Arc et al. 2011; Filippou et al. 2011). In addition, the imposition of

abiotic and biotic stresses causes the overproduction of ROS, which ultimately inflicts a secondary oxidative and nitrosative stress, leading to various signalling responses (Mittler et al. 2011). ROS steady-state levels are often altered in plants under various stress conditions and play a dual role: at mild concentrations, ROS act as signal molecules involved in acclimatory signalling and trigger tolerance against various stress conditions, and at high concentrations, ROS orchestrate programmed cell death (Asada, 2006). H_2O_2 has been suggested to be a signalling molecule in defence and adaptive responses such as increase tolerance to chilling in maize (Prasad et al. 1994), tolerance to paraquat in cucumber seedlings (Xia et al. 2009), and tolerance to chilling in tomato plants (Zhou et al. 2012).The damaging effects of ROS have caused plant cells to cope with oxidative stress by triggering complex redox homeostatic antioxidative mechanisms. These ROS-scavenging antioxidative mechanisms include specific antioxidant enzymes such as CAT, SOD, ascorbate peroxidase (APX), peroxidase, and some other low-molecular-weight antioxidants such as ascorbate and reduced glutathione (Iannone et al. 2012).

NH_4^+ nutrition is of interest as an alternative to that of nitrate. However, NH_4^+ nutrition turns out to be stressful to many plants, including some important crops, leading to a reduced growth (Britto and Kronzucker, 2002). In addition to growth reduction, several characteristics of plant metabolism – lower content of mineral cations and organic anions and higher levels of amino acids – are altered, resulting in the so-called 'ammonium syndrome". However, we demonstrated that *Carrizo citrange* plants prefer to absorb NH_4^+ more than NO_3^- if both N forms are present in the nutrient solution (Camañes et al. 2009). Although other studies have shown that some species develop toxic symptoms when only NH_4^+ nutrition is applied (Gerendás et al. 1997; Lasa et al. 2001), the *Carrizo citrange* plants displayed optimal growth

when they were grown with NH_4^+ as the sole nitrogen source (Fernandez-Crespo et al. 2012). Besides, in Fernandez-Crespo et al. (2012) it was also demonstrated that NH_4^+ treatment confers protection against subsequent salt stress by reducing Cl^- uptake and decreasing its toxicity, by priming abscisic acid and polyamines, and enhances H_2O_2 and proline basal content and they concluded that N-NH_4^+ treatment triggers mild chronic stress, which may account for the noted stress-induced morphogenetic responses (SIMRs). SIMR is a part of a general acclimation strategy that is characterised by the blockage of cell division in the main meristematic tissues, the inhibition of elongation, redirected outgrowth of lateral organs (Potters et al. , 2009), an increase in the antioxidants that prevent damage caused by ROS, and the accumulation of foliar molecules that act as modulators of stress signals (Gould and Lister, 2006). Activation of the response related to SIMRs due to NH_4^+ toxicity led to the "acclimation stage", which leads to better adaptation to subsequent salt stress. Preliminary stress exposure or stress imprinting is indeed necessary to induce priming, which makes the plants more resistant to future biotic or abiotic stress (Conrath et al. 2006, Bruce et al. 2007, Galis et al. 2009). Recent studies regarding NH_4^+ inducer effect indicate that NH_4^+ or one of its assimilation products (e.g. glutamine or glutamate) may serve as a stress signal and that NH_4^+ -grown plants operate metabolic pathways which induce the accumulation of ROS (Misra and Gupta, 2006). Several studies demonstrated that NH_4^+ nutrition is able to improve the depressive effects of high salinity in barley (Kant et al., 2007) and in halophytes (Kudo and Fujiyama 2010; Hessini et al. 2011). Hessini et al. (2013) observed the benefits of NH_4^+ nutrition on the halophyte *Spartina alterniflora* under saline conditions which seems to be associated with high antioxidant enzyme activities, together with low MDA content, electrolyte leakage, and H_2O_2 concentration. Further studies need to be done to elucidate how NH_4^+

nutrition enhances plants ability to improve a subsequent salt stress situation and the relationship of this induced resistance with ROS metabolism. For this reason, the main aim of this work has been investigated the biochemical mechanisms underlying NH_4^+-mediated resistance to high salt concentrations in *Carrizo citrange* plants and its relationship with the antioxidant cellular machinery.

MATERIAL AND METHODS

Plant material, growth conditions and nutrition treatments

Carrizo citrange seeds (Beniplant, Valencia, Spain) were allowed to germinate in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 20/24 ºC and RH of 70%. Seeds were irrigated twice a week with distilled water, and after 6 weeks, seedlings were irrigated for two months with Hoagland solution lacking nitrogen (Hoagland and Arnon, 1950) complemented with either 1 mM NH_4NO_3 (control treatment) or 5 mM of N-NH_4^+ [$(NH_4)_2SO_4$)] (NH_4^+ treatment). Prior to the experiments, 3-month-old plants with a single shoot were selected for uniformity of size and transferred to an aerated, complemented Hoagland solution for 7 days in hydroponic culture devices. Salt stress was induced by adding 90 mM NaCl to the hydroponic solution and was renewed twice weekly. Samples were taken for individual analysis at 14 d after addition of salt to the hydroponic solution.

DAB staining and H_2O_2 quantification.

N-NH_4^+ and control plants were exposed to 90 mM NaCl for 2 h, and the salt-stressed leaves were stained in 1 mg of DAB per millilitre at pH < 3 for 24 h in the dark and subsequently destained in 95% ethanol. Later, samples were rehydrated with distilled water. DAB staining intensities were

quantified from digital photographs (Nikon Eclipse 11000, Tokyo) by the number of dark-brown DAB pixels relative to total pixels corresponding to plant material, using the GIMP2 program.

Antioxidant enzymatic activity determination

Superoxide dismutase assay

Samples (0.05 g of tissue) were resuspended in 200 µL extraction buffer (Tris-HCl, pH 8.5) and a cocktail of protease inhibitors 1X (Roche) and vortexed for 30 s on three occasions. Extracts were centrifuged at 955 x g for 3 min at 4 ºC and the supernatant was used for protein quantification and gel electrophoresis analysis. The protein concentration was quantified for each extract using the Bradford protein assay from Bio-Rad. Protein samples were separated on 10% native acrylamide gel electrophoresis as described in Gamero-Sandemetrio et al. (2013).

Catalase assay

Samples (0.05 g of tissue) were homogenised in 0.2 mL of 50 mM phosphate buffer at pH 7.0 and a mix of protease inhibitors (200 µM phenylmethylsulfonyl fluoride (PMSF), 20 M µTPcK, 200 µM pepstatin A) and centrifuged at 15300 x g for 10 min at 4 ºC. Catalase activity was assayed in microplates by adding 1-10 µL of supernatant to 0.2 mL of 50 mM phosphate buffer pH 7.0 and 80 mM H_2O_2 (Jakubowski et al. 2000). The absorbance decrease of H_2O_2 was measured at 240 nm, and enzyme activity was calculated using an extinction coefficient of 43.66 $M^{-1}cm^{-1}$. The protein concentration was quantified for each extract using the Bradford protein assay from Bio-Rad. Catalase activity was expressed as µmol of H_2O_2 min^{-1} mg of $protein^{-1}$ (U/mg prot).

Glutathione reductase assay

Samples (0.1 g of tissue) were homogenised in 0.5 mL of 50 mM MES/KOH buffer at pH 6.0 and centrifuged at 20800 x g for 10 min at 4 ºC. The supernatant was diluted (10-40 µL) in a final volume of 0.2 mL of 50 mM Hepes buffer at pH 8.0 with 0.5 mM EDTA. GR activity was assayed in microplates and was measured spectrophotometrically as NADPH oxidation at 340 nm in the presence of 0.25 mM NADPH (Murshed et al. 2008). GR reaction was started by the addition of 5 µL of 20 mM GSSG to each well. The specific activity was calculated from the 6.22 $mM^{-1}cm^{-1}$ extinction coefficient. GR activity was defined as micromoles of substrate consumed per minute per milligram of protein. The protein concentration was quantified for each extract using the Bradford protein assay from Bio-Rad.

Glutathione determination

Measurements of total glutathione (GSH, reduced form; GSSG, oxidised form) were carried out using 5,5´-dithiobis-(2-nitrobenzoic) acid (DTNB), according to the method of Griffith (1980). 0.1 g of tissue was homogenised in 1 mL ice-cold 8 mM HCl with 1.3% (w/v) 5-sulphosalicylic acid. Samples were centrifuged at 18000 x g for 15 min at 4°C, and supernatants were used for glutathione determination. For total glutathione determination, the supernatant was directly diluted in 0.2 mL of 0.4 M Mes, 0.1 M sodium phosphate buffer pH 7.4, 2 mM EDTA, pH 6.0. GSSG content was measured after the removal of GSH by 2-vinyl pyridine derivatisation for 1 h at room temperature. 0.2 mL of sample was mixed with 0.12 mL of NADP (0.4 mg mL^{-1}), glucose-6-phosphate (0.16 mg mL^{-1}), glucose-6-phosphate dehydrogenase (3 µg mL^{-1}), glutathione reductase (1 mU) and 0.48 mL of 0.2 mM DTNB. The mixture was incubated at room temperature for 20 min under agitation and dark conditions, and absorbance was measured at 412

nm. For calculation of glutathione contents a standard curve prepared with GSSG was used. Glutathione levels are expressed as µmol per mg FW.

Quantitative RT-qPCR analysis

Gene expression by quantitative real-time PCR (RT-qPCR) was performed using RNA samples extracted from leaf tissue using the Total Quick RNA cells and tissues kit (Talent; http://www.spin.it/talent). Citrus leaf tissue samples for RNA isolation were collected at 2 h and 14 d after NaCl treatment. Leaf tissue from five plants each of the NH_4^+ treated and untreated plants was collected. For quantitative Real time PCR experiments, 1.5 µg of total RNA was digested using 1 unit of RNase-Free DNase (Promega; http://.www.promega.com) in 1 µl of DNase buffer and up to 10 µl of Milli-Q water and was incubated for 30 min at 37 ºC. After the incubation, 1 µl of RQ1 DNase stop buffer was added, and the solution was incubated again at 65 ºC for 10 min to inactivate the DNase. Highly pure RNA was used for the RT reaction. The RT reaction was performed by adding 2 µl of RT buffer, 2 µl of 5 mM dNTP, 2 µl of 10 µM oligo$(dT)_{15}$ primer (Promega), 1 µl of 10 U μl^{-1} RNasin RNase inhibitor (Promega) and 1 µl of Omniscript reverse transcriptase (QIAGEN; http://www.qiagen.com/). The reaction mixture was incubated at 37 ºC for 60 min. Forward and reverse primers (0.3 µM) were added to 12.5 µl of QuantiTect^{TM} SYBR Green PCR reaction buffer (QIAGEN), as were 2 µl of cDNA and Milli-Q sterile water up to 25 µl total reaction volume. Quantitative PCR was carried out using the Smart Cycler II instrument (Cepheid; http://www.cepheid.com). A list of the primers used in the RT-qPCR is shown in Table 1, the *GAPDH* gene expression was used an internal standard. Melting-curve analysis was performed at the end of the PCR reaction to confirm the products' purity. Differences in cycle numbers during the linear amplification phase between samples containing cDNA

from treated and untreated plants were used to determine differential gene expression. The corresponding RT-qPCR efficiencies were calculated according to the equation $E=10^{[-1/slope]}$ and E was in all case in the range of 1.8–2.2. Triplicate analyses were performed on all occasions using the cDNA samples derived from three independent experiments.

Statistical analysis

Statistical analysis was carried out using the StatGraphics software support. The data are expressed as the means and standard error. Mean values were compared by an LSD (least significant difference) test. All experiments were repeated at least three times.

RESULTS

NH_4^+ nutrition induces H_2O_2 accumulation in *Carrizo citrange* plants

In this work, we used DAB staining to establish how NH_4^+ treatment affects cellular oxidative stress. H_2O_2 staining based on the *in vivo* reaction of H_2O_2 with DAB allows a rapid estimation of the H_2O_2 steady-state levels in leaves (Thordal-Christensen et al. 1997). Our results indicate that the N-NH_4^+-treated plants show higher basal levels of H_2O_2 than the control plants in the absence of salt stress (Fig. 1). Increased H_2O_2 levels were noted at 2 h after treatment with 90 mM NaCl for both the control and N- NH_4^+ conditions. However, the highest levels of H_2O_2 were observed in the N-NH_4^+ plants treated with NaCl. Interestingly, after 14 d of salt addition, the control plants showed higher H_2O_2 steady-state levels than the N-NH_4^+-treated plants, and these plants did not show significant changes between the salt-treated and untreated plants after 14 d.

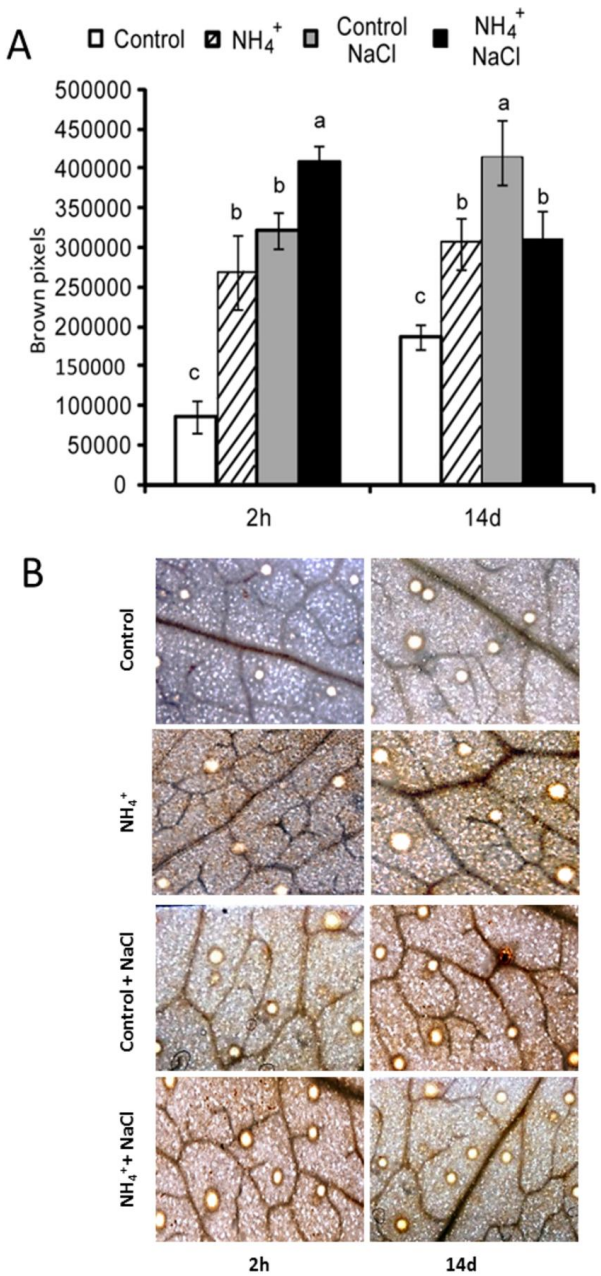

Fig.1. H_2O_2 staining, estimated by using DAB staining in the leaves of the control plants and the N-NH_4^+ *Carrizo citrange* plants treated with NaCl (90 mM) for 2 h and 14 d. (A) Quantitative H_2O_2 measurement on the basis of brown pixels from digital photographs. (B) Representative pictures of H_2O_2 production. Data show a representative experiment that was repeated three times; each point is the average of

a pool of 10 plants ± standard error (SE) (n=10). Letters indicates statistically significant differences between treatments within each time points ($p < 0.05$).

NH_4^+ nutrition modify antioxidants enzymatic activity in salt stress conditions

To establish how NH_4^+ nutrition affects the response of citrus plants to salt-induced oxidative stress, several antioxidant enzyme activities were determined after 2 h and 14 d and after salt stress for both the control and the N-NH_4^+-treated plants. Among different cellular free radical scavengers, superoxide dismutase (SOD) plays an important role as an antioxidant defence and protects cellular components from being oxidised by ROS. When acclimating to increased levels of oxidative stress, SOD concentrations typically increase with the degree of the stress condition. Therefore, we analysed SOD activity as a marker of oxidative stress under our experimental conditions (Fig. 2). The basal SOD activities differed between the control and the N-NH_4^+-treated plants. Both of the SOD (Cu/Zn-SOD and Mn-SOD) isoforms displayed less activity in the N-NH_4^+ plants in the absence of salt stress when compared to the control plants, suggesting a possible antioxidant function for NH_4^+. In the control plants, Mn-SOD and Cu/Zn-SOD activity remained unaffected after both 2 h and 14d of salt stress. Similar results were observed for the N-NH_4^+ plants after 2 h salt treatment for both of the SOD isoforms (Fig. 2A and 2B).

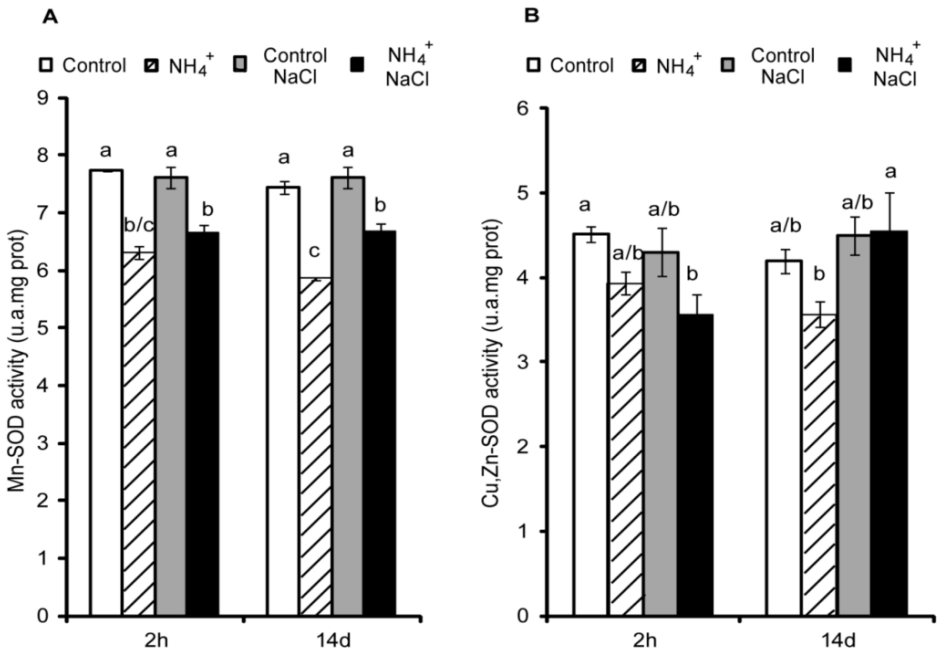

Fig.2. Effect of NH_4^+ treatment on Mn-SOD (A) and Cu, Zn-SOD (B) in the *Carrizo citrange* plants treated with NaCl (90 mM) for 2 h and 14 d. Data show the average values of three independent experiments from a pool of 30 plants per experiment ± SE (n=30).. Letters indicates statistically significant differences between treatments within each time points ($p < 0.05$).

However, SOD activity levels in the N-NH4$^+$ salt-treated plants were significantly lower than in salt-treated control plants at 2 h. Interestingly, after 14 days of salt stress, the N-NH_4^+ plants displayed increased Mn-SOD and Cu/Zn-SOD activities, but these increases were not observed in the salt-treated control plants.

Another antioxidant enzyme is catalase (CAT), which plays an important role as an H_2O_2 scavenger. To determine how NH_4^+ nutrition affects CAT activity, leaf samples were analysed for both the control and N-NH_4^+ plants (Fig. 3A). In the absence of salt, significant differences between treatments were noted: the N-NH_4^+ plants showed higher CAT activity than the control plants. After 14 days, N-NH_4^+ plants exhibited a 23% increase in CAT activity in response

to salt stress; however, no such increase was observed in the control plants upon salt stress.

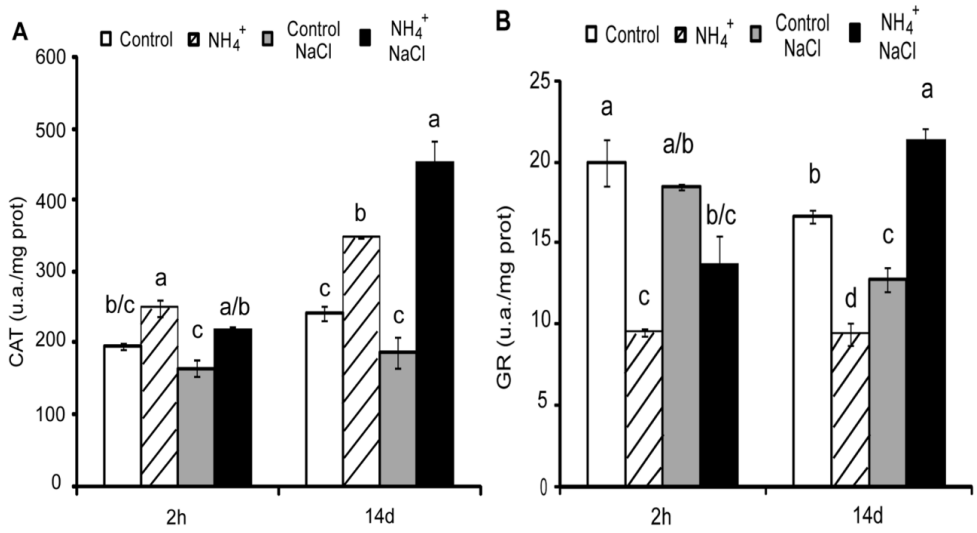

Fig.3. Effect of NH_4^+ treatment on CAT (A) and GR (B) activities in the *Carrizo citrange* plants treated with NaCl (90 mM) for 2 h and 14 d. Data show the average of three independent experiments from a pool of 30 plants per experiment ± SE (n=30). Letters indicates statistically significant differences between treatments within each time points ($p < 0.05$).

We also observed that NH_4^+ treatment induced a strong inhibition of glutathione reductase (GR) activity compared with the control plants (Fig. 3B). Under control conditions, plants did not show significant variation in the GR activity after 2 h of NaCl exposure. However, NH_4^+ treatment significantly increased the GR activity after 2 h of salt addition. Interestingly, after 14d of salt treatment, control plants showed a significant decrease in the GR activity, while the N-NH_4^+salt-treated plants were able to increase GR activity 56% when compared to the salt-treated control plants. N-NH_4^+ plants are capable of increasing GR activity in presence of salt stress, although the initial activity was much lower than the control plants.

NH_4^+ nutrition induces changes in glutathione metabolism

To evaluate the contributions of antioxidant metabolites on NH_4^+ salt mediated-resistance, glutathione content was evaluated (Fig. 4). In the absence of salt, N-NH_4^+ plants displayed higher GSH and GSSG basal content without significant changes in the GSSG oxidation state. At 14 days after salt stress, we observed that GSH and GSSG content significantly decreased in both control and t N-NH_4^+ plants. However, N-NH_4^+ plants displayed higher GSH content if compared with control salt treated plants (Fig 4A). Moreover GSSG oxidation state was higher in control plants after 14 d of salinity stress (Fig 4B).

These results point to the fact that the oxidised cellular environment displayed by control plants upon salinity stress may be due to their inability to synthesize GSH upon stress situation. Nevertheless, the GSSG oxidation state remained unaffected in the presence of salt stress in N-NH_4^+ plants (Fig 4B), probably due to the significant increase in GSH content (Fig 4A) that may attenuate NaCl cell disturbances.

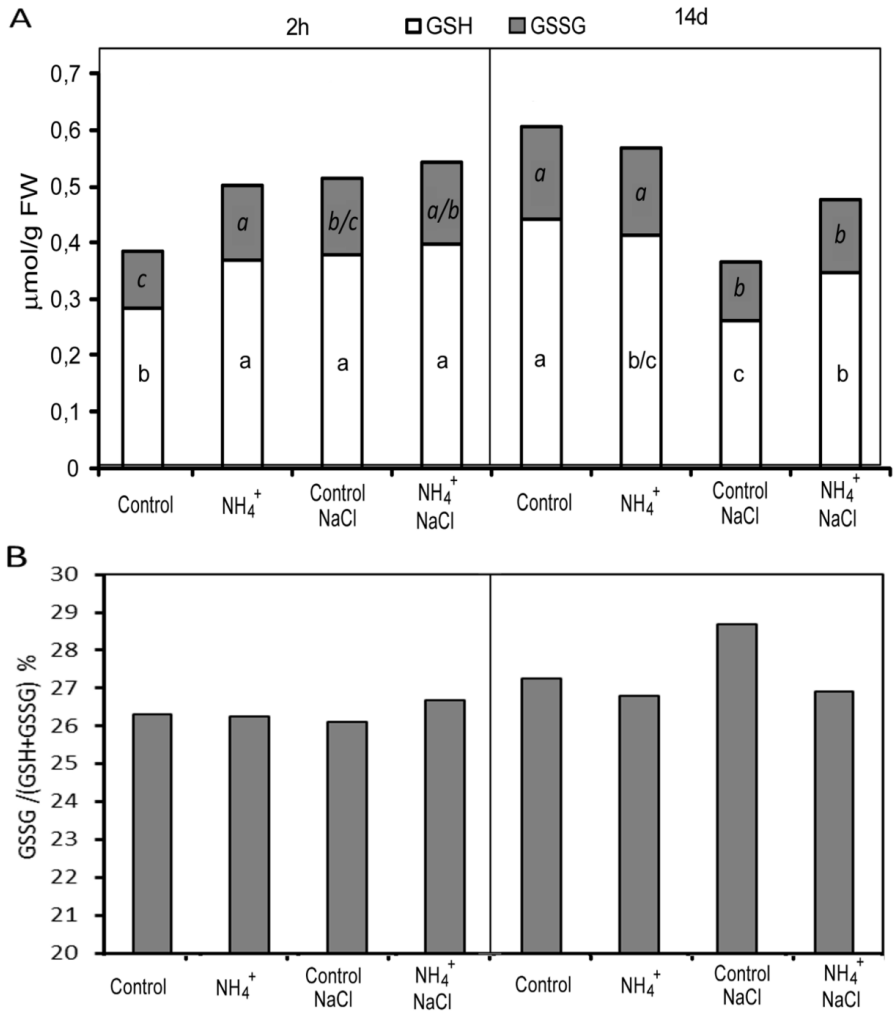

Fig. 4. Effect of NH_4^+ treatment on GSH content in the *Carrizo citrange* plants treated with NaCl (90 mM) for 2 h and 14 d. (A) GSH (white bars) and GSSG content (black bars). (B) GSSG oxidation state [100 GSSG/(GSH+GSSG)]. Data show the average of three independent experiments from a pool of 30 plants per experiment ± SE (n=30). Different letters indicates statistically significant differences in GSH content between treatments within each time points ($p < 0.05$). Different italic letters indicates statistically significant differences in GSSG content between treatments within each time points ($p < 0.05$).

NH_4^+ nutrition induces oxidative stress-related gene expression in salt response

To establish whether NH_4^+ nutrition affects the oxidative stress-related gene expression, the gene expression of glutathione-S-transferase (*GST)*, the phospholipid hydroperoxide glutathione peroxidase (*PHGPx)* and ascorbate peroxidase (*APX2)* were analysed by RTq-PCR as markers of oxidative stress (Fig. 5). *GST* mRNA accumulation did not differ between the control and the N-NH_4^+-treated plants either before or after salt treatment (Fig. 5A). However, *GST* mRNA accumulation was induced by salinity after 14 d in both treatments, and the highest *GST* mRNA accumulation was observed in the N-NH_4^+-treated plants. We also checked *PHGPx* expression and observed that NaCl induced *PHGPx* accumulation after 2 h in the control plants. Although the *PHGPx* expression was unaffected in the absence of salt, NH_4^+ treatment significantly enhanced this expression after 14 d of salt stress (Fig. 5B). We also analysed *APX2* expression (Fig. 5C). NH_4^+ treatment induced *APX2* mRNA accumulation in the absence of salt stress, especially transcript accumulation after 14d. NaCl treatment also induced *APX2* mRNA accumulation in the control plants. However, the combination of both treatments, N-NH_4^+-NaCl plants, exhibited a strong reduction in mRNA accumulation upon salinity. In summary, the largest differences in oxidative stress gene markers were observed after long-term salinity treatment, in which NH_4^+ nutrition significantly increased both *GST* and *PHGPx* gene expression and reduced *APX* gene expression. The obtained results for glutathione metabolism gene expression also correlated with higher GR activity and the maintenance of the GSSG oxidised state observed in the N-NH_4^+ salt-treated plants compared with the salt-treated control plants.

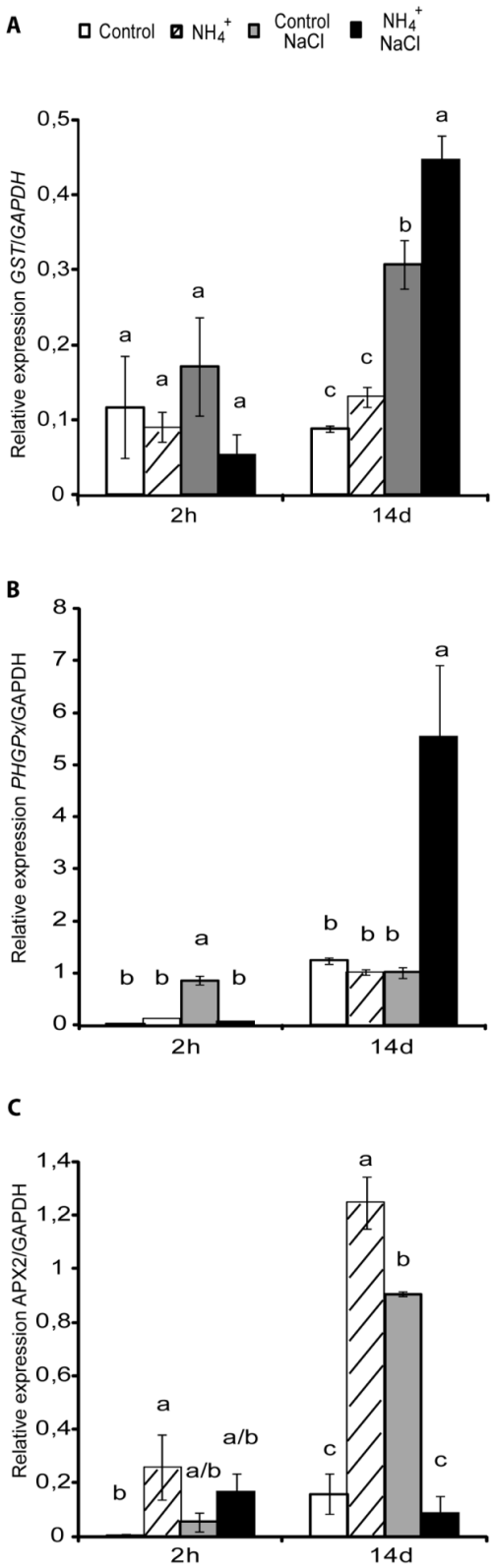

Fig.5. Effect of NH_4^+ treatment on the gene expression in *Carrizo citrange* plants upon salt stress. Total RNA was isolated from leaves at 2 h and 14 d after the addition of NaCl (90 mM) and was converted into cDNA and subjected to a RT-qPCR analysis. The results were normalised to the *GAPDH* gene expression measured in the same samples. The relative level of (A) *GST*, (B) *PHGPx* and (C) *APX2* were analysed in the control and the N-NH_4^+ plants. The data show the average of three independent experiments obtained with a pool of 30 plants per point ±SE (n=30). Letters indicates statistically significant differences between treatments within each time points ($p < 0.05$).

DISCUSSION

In this study, the role of NH_4^+ nutrition on *Carrizo citrange* responses to NaCl-induced oxidative stress has been analysed. In a previous work, we found a differential response to salt stress between control and N-NH_4^+-treated plants, which displayed strong salt resistance probably mediated by high levels of H_2O_2, reduced Cl^- uptake, putrescine and proline content observed in N-NH_4^+ salt-treated plants. NH_4^+ treatment triggers mild chronic stress in *Carrizo citrange* plants which leads to the acclimation stage and to a better adaptation to subsequent salt stress (Fernandez-Crespo et al. 2012). To gain further insight into other biochemical mechanisms related to NH_4^+-mediated salt resistance in *Carrizo citrange* plants and its relationship with acclimation stage mediated by H_2O_2 accumulation, we studied the influence of this treatment on antioxidant enzymatic activities and metabolites involved in control of cellular redox stage. ROS have been proposed to be a central component of plant adaptation to both biotic and abiotic stresses. We show that both the control and N-NH_4^+-treated *Carrizo citrange* plants display considerably increased H_2O_2 accumulation 2 h after salinity stress. It is noteworthy that the initial H_2O_2 levels were higher in the N-NH_4^+-treated plants than in the control ones, supporting the idea that H_2O_2 acts as a stress signal in the N-NH_4^+-treated plants. An increase in H_2O_2 content has been well studied in acclimated plants, and exogenous H_2O_2 significantly increases the tolerance against salt stress mediated by induction of antioxidant defences (Gondim et al. 2012). Tanou et al. (2009) demonstrated that pre-exposure to H_2O_2 induces acclimation to subsequent treatment with NaCl in citrus plants. In our experimental procedure system, NH_4^+ treatment may acts as a mild stressor that induces H_2O_2 accumulation and leads the plants to the acclimation stage. However, at 14 d after salt stress NH_4^+ plants displayed lower H_2O_2 steady-state levels than control plants, probably due to a proper

activation of the antioxidant machinery. These results are in concordance with the obtained by Hessini et al. (2013), who demonstrated that NH_4^+ nutrition benefits halophyte plants subjected to salt stress due to the efficiently activation of different antioxidants enzymes, resulting in physiologically acceptable levels of H_2O_2 in the leaves. To counteract the negative effect of H_2O_2 steady-state levels accumulated in stress situation the plants activate antioxidant cellular machinery. In this work, we observed that NH_4^+ nutrition provokes an inhibition of the two isoforms activity especially of Mn-SOD activity. This inhibitory effect was probably due to the mild stress induced by NH_4^+ treatment. These data are supported by the findings of Rodríguez-Serrano et al. (2009), who observed that the downregulation of Mn-SOD and Cu/Zn-SOD in Cd stress conditions resulted in the overproduction of the H_2O_2 and O_2^- in *Pisum sativum*. However, N-NH_4^+-treated plants were able to increase the SOD activity upon salt stress. The up regulation of SODs is implicated in combating oxidative stress caused by biotic and abiotic stress and plays a critical role in the survival of plants in stressful environments. Several studies have suggested that both the overexpression of Mn-SOD in transgenic Arabidopsis plants and Cu/Zn-SOD overexpression in transformed tomato plants produced increased salt tolerance (Wang et al. 2004; 2007). Catalases are the principal scavenging enzymes which can directly decompose H_2O_2 and are indispensable for ROS detoxification during stress (van Breusegem et al. 2001). In the present study, we show that salt-treated N-NH_4^+ plants exhibited an increase in CAT activity compared to the control plants. In previous work, we demonstrated that salicylic acid (SA) accumulation occurred after 14 d of salt stress in the control plants, but not in the N-NH_4^+ plants (Fernández-Crespo et al. 2012). Several studies have suggested that SA binds directly to the catalase enzymes inhibiting its activity in several plants species (Sánchez-Casas and Klessig,

1994; Horvath et al. 2002). These results support the ones obtained for CAT activity in our experimental system since control plants were unable to increase CAT activity after 14 d of salt stress; while NH_4^+ treatment leads to the correct function of the CAT antioxidant enzyme, which could avoid the injuries of ROS accumulation. For this reason, we conclude that NH_4^+ nutrition induced resistance against salinity may be partially due to the increase in SOD and CAT activities, allowing plants to resist potential oxidative salt induced damage. GR catalyses the reduction of GSH, molecule involved in many metabolic regulatory and antioxidant processes in plants. GR and GSH together play a crucial role in determining the tolerance of a plant for various stress situations (Chalapathi et al. 2008). In the present work we observed that NH_4^+ treatment inhibited GR activity. The strong inhibition of GR activity might be caused by the toxic effect of NH_4^+ treatment on *Carrizo citrange* plants. Another study showed that GR activity was inhibited in *Phaseolus vulgaris* primary leaves after cooper accumulation, resulting in a shift from GSH towards its oxidised form GSSG (Cuypers et al. 2000). In despite of GR activity reduction by NH_4^+ treatment, we show that GR activity is enhanced in these plants after salt stress when compared with control plants after 2 h and 14 d. This correlation between GR activity and higher tolerance to salt stress under N-NH_4^+ nutrition is consistent with data from several crops where increases in GR activity during salt stress have been reported (Gueta-Dahan et al. 1997; Sumithra et al. 2006) and also over-expression of GR in plants which leads to increased resistance to oxidative stress (Kocsy et al. 2001).

Collectively, NH_4^+ nutrition induces a strong inhibition of the SOD and GR activities likely due the NH_4^+ toxic effect which also induces H_2O_2 accumulation. This increase in circulating peroxide could act as a trigger to signalling defence pathways and leads to *Carrizo citrange* plants to a better

adapt to subsequent salt stress. Indeed, after 14 d of salt stress, N-NH_4^+ salt-treated plants that more efficiently activated the antioxidant machinery showed a higher capacity for ROS-scavenging compared with the control plants, demonstrating NH_4^+ nutrition beneficial effect in citrus plants upon salinity conditions.

We also investigated the effect of NH_4^+ nutrition on the other components of glutathione pathway. In the control plants, we observed an increase of the GSSG oxidation state as consequence of salinity. However, in N-NH_4^+-treated plants, the GSSG oxidation state remained unaffected by salt stress due to a significant increase in the total glutathione and GSH contents that allows for minimised NaCl-induced cell disturbances. Mittova et al. (2003) demonstrated that salt tolerance is linked to the ability to upregulate enzymes for glutathione synthesis and this activity is absent from the salt-sensitive species. Higher concentrations of glutathione would confer better antioxidative protection and would be considered to indicate acclimation. We further investigated the effect of NH_4^+ on *GST* gene expression, which have an important role in different detoxification process (Dixon et al. 2010). We noted that N-NH_4^+ nutrition in *Carrizo citrange* plants induced higher levels of *GST* mRNA accumulation after 14 d of salinity than in the control plants. It has also been found that *GST* overexpression enhances plant tolerance to various abiotic stresses (Gill and Tuteja, 2010). Transgenic rice seedlings that co-express both *GST* and *CAT* displayed a greater increase in CAT and SOD activity and also showed markedly enhanced tolerance to salt stress compared with non-transgenic controls upon 200 mM NaCl treatment in a greenhouse (Zhao et al. 2006). Our results suggest that the higher GR activity, *GST* expression and GSH accumulation in the salt-treated N-NH_4^+ plants leads to an increase in the antioxidant capacity through the induction of a more

reduced glutathione environment that minimises disturbances to the glutathione redox-based system.

In addition, we also investigated the effect of NH_4^+ nutrition on the expression of others oxidative marker genes, as *PHGPx* and *APX2* involved in the oxidative stress response. Although APX is an important enzyme for removing intracellular H_2O_2, little is known about the role of *APX2* gene expression in abiotic stress tolerance. In our experimental conditions, we found that NH_4^+ nutrition induces strong *APX2* mRNA accumulation in the absence of salt. However, *APX2* was strongly induced in control, but not in N-NH_4^+ plants 14 d after salt stress. Previous results in tobacco plants suggest that under stress conditions, APX was completely inactivated and that high CAT activity reduced H_2O_2 instead of APX in the chloroplasts (Shikanai et al. 1998). Thus, we can conclude that the higher CAT and SOD activities in N-NH_4^+ salt treated plants seem to be enough to reduce the H_2O_2 steady levels without a concurrent induction of *APX2*. This fact would reveal a secondary role of APX2 activation in the NH_4^+ induced resistance against salinity. In this work, we also discovered that NH_4^+ treatment led to a greater induction of the *PHGPx* gene after 14d of salt treatment. Chen et al. (2004) demonstrated that plant *PHGPx* genes that are expressed under various stresses may function as cytoprotectors against oxidative damage and play an important role in scavenging ROS such as lipid hydroperoxides (Chun-Juan et al. 2009).

In summary, we have provided evidence for several mechanisms underlying the resistance to salinity mediated by NH_4^+ treatment in *Carrizo citrange* plants, and also biochemical support for our previous hypothesis that NH_4^+ treatments would induce a mild stress condition that primes defence response system by stress imprinting, then conferring protection against subsequent salt stress. Moreover, we showed that NH_4^+-induced acclimation stage

significantly improves salinity tolerance and this enhancement of resistance was associated with a higher H_2O_2 basal content. Such evidences come from the analysis of the enzymatic and the non-enzymatic ROS scavenging machinery in both control and N-NH_4^+-treated plants upon exposure to salinity. In the absence of salt, we observed higher levels of H_2O_2, stronger inhibition of the SOD and GR activities and higher GSSG content in the N-NH_4^+ plants than in the control plants At early time points, 2 h after salt treatment, both the control and N-NH_4^+ treated plants displayed few changes on activation of antioxidant machinery against salinity stress. However, upon exposure for 14 d to salinity, N-NH_4^+-treated plants showed a reduction in the H_2O_2 levels, increased enzymatic antioxidant activity and the induction of the *GST* and *PHGPX* genes. Moreover, N-NH_4^+ plants were able to maintain high levels of the GSH form upon exposure to salinity, which provided the cells with the ability to counteract the negative effects of oxidative stress syndrome. Together, these results reveals that sublethal concentrations of NH_4^+ can act as mild oxidative stressors that trigger the antioxidant cellular machinery thus providing resistance to subsequent salt stress.

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CHAPTER 4
NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation

Adapted From:

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ABSTRACT

NH_4^+ nutrition provokes mild toxicity by enhancing H_2O_2 accumulation, which acts as a signal activating systemic acquired acclimation (SAA). Until now, induced resistance mechanisms in response to an abiotic stimulus and related to SAA, were only reported for exposure to a subsequent abiotic stress. Herein we provide the first evidence that this acclimation of abiotic stimulus induces resistance to later pathogen infection since, we demonstrated the NH_4^+ nutrition induces-resistance (NH_4^+-IR) against *Pseudomonas syringae* pv tomato DC3000 (*Pst*) in tomato plants. N-NH_4^+ plants displayed basal H_2O_2, abscisic acid (ABA) and putrescine (Put) accumulation. H_2O_2 accumulation acted as a signal to induce ABA-dependent signalling pathways required to prevent NH_4^+ toxicity. This acclimatory event provoked an increase of resistance against later pathogen infection. N-NH_4^+ plants displayed basal stomatal closure produced by H_2O_2 derived of enhanced *CuAO* and *rboh1* activity that may reduce the entry of bacteria into the mesophyll, diminishing the disease symptoms as well as inducing strongly oxidative burst upon *Pst* infection, favouring the NH_4^+-IR. Experiments with inhibitors of Put accumulation and ABA-deficient mutant *flacca* demonstrated that Put and ABA downstream signalling pathways are required to complete NH_4^+-IR. Metabolic profile revealed that N-NH_4^+ infected plants showed greater ferulic acid accumulation compared with control plants. Although classical salicylic acid (SA) dependent responses against biotrophic pathogens were not found, the important role of Put in the resistance of tomato against *Pst* was demonstrated. Moreover, this work revealed the cross-talk between abiotic stress acclimation (NH_4^+ nutrition) and resistance to subsequent *Pst* infection.

INTRODUCTION

Plants employ diverse constitutive and inducible defence strategies to counteract colonization by microbial pathogens (Spoel and Dong, 2012). One of the earliest cellular responses following pathogen-associated molecular patterns (PAMP) elicitation is the oxidative burst produced by NADPH oxidases, cell wall peroxidases or polyamine oxidases (Yoda *et al*., 2006). In addition to the classical salicylic acid (SA) and jasmonic acid (JA)/ethylene-defence pathways, plant immunity to microbial pathogens is regulated by distinct pathways related by nitrogen (N) compounds such as amino acids and polyamines (PAs) (Takahashi and Kakehi, 2010; Zeier, 2013). PAs, including putrescine (Put), spermidine (Spd), and spermine (Spm) are positively charged small metabolites implicated in physiological processes, including organogenesis, embryogenesis, floral initiation and development, leaf senescence, pollen tube growth, fruit development and ripening (Tiburcio *et al*., 2014). PAs are synthesized from amino acids by decarboxylation of ornithine, or arginine by ornithine decarboxylase (ODC), or arginine decarboxylase (ADC) respectively (Walters, 2003). In addition to free PAs, some PAs conjugates to hydroxycinnamic acids, and the products of PAs oxidation participate in the response to abiotic and biotic stresses (Tiburcio *et al*., 2014). Oxidation of PAs by Copper Amine Oxidases (CuAOs) contributes to the regulation of PAs homeostasis and generates catabolic products with biological functions. CuAOs are homodimeric enzymes with high affinity for oxidizing the primary amino groups of Put and cadaverine and lower affinity for Spd and Spm (Moschou *et al*., 2012). It is commonly accepted that H_2O_2 produced by CuAO in Put oxidation has an important role in stress-induced cell wall stiffening, in stomata movement, and in programed cell death (Angelini *et al*., 2008). Although several studies have demonstrated a role of PAs in protection against abiotic stresses

(Bouchereau *et al*., 1999; Kasinathan and Wingler, 2004) little is known about how they act under conditions of biotic stress.

Plants are frequently exposed to a myriad of biotic and abiotic stress that can act in succession or simultaneously. Being sessile organisms, plants have developed sophisticated acclimation and defence mechanisms to cope with different stress situations (Boyko and Kovalchuk, 2011; Bray *et al*., 2000; Reddy *et al*., 2011). These can be activated in the initial tissue exposed to stress as well as in systemic tissues that have not yet been exposed to the stress. Looking at the source of the stimulus, several kinds of induced resistance can be distinguished. If the induced resistance is achieved by treatment with a chemical or natural compound, this phenomenon is known as priming. Priming enables cells to respond to low levels of a stimulus in a more rapid and robust manner than is found in nonprimed cells (Conrath, 2011). Thus, when plants are primed and subsequently challenged by pathogens or abiotic stresses, they show a faster and/or stronger activation of defence responses (Prime-A-Plant Group *et al*., 2006). Recently, it has been demonstrated that hexanoic acid (Hx) induces resistance in *Solanum lycopersicum* and *Arabidopsis thaliana* plants against *Botrytis cinerea* (Kravchuk *et al*., 2011; Vicedo *et al*., 2009) and against *Pst* (Schalschi *et al*., 2013) as well as in citrus plants against the fungus *Alternaria alternata* (Llorens *et al*., 2013). The activation of defence or acclimation mechanisms in systemic nonchallenged tissues is normally termed systemic acquired resistance (SAR) – if the local stimuli is induced by virus, bacteria or fungi – or systemic acquired acclimation (SAA) – if the initial stimuli is an abiotic stress situation – and serves an important role in preventing further infection or damage to the entire plant (Baxter *et al*., 2014). Recent studies have demonstrated that the reactive oxygen species (ROS) wave functions as a general priming signal in plants, warning systemic tissues what is happening

is a localized abiotic stress stimulus (Mittler *et al*., 2011). Upon abiotic stress, SAA is mediated by temporal-spatial interactions of the ROS wave with hormone or amino acid signals activated in systemic tissues (Suzuki *et al*., 2013). Specifically, recent reports demonstrated that acclimation-induced cross-tolerance in tomato plants is largely attributed to Respiratory Burst Oxidase Homologue (RBOH1)-dependent H_2O_2 production at the apoplast, which may subsequently activate MPK1/2 to induce stress responses (Zhou *et al*., 2014). In recent years there has been evidence to suggest that plants with increased activation of response mechanisms by acclimation to abiotic stimulus can respond better to biotic stress, although little is known about the mechanisms underlying this type of induced resistance. Mild chronic stress situations can also boost plant stress resistance through induction of acclimation responses. Factors such as light, temperature, drought, mineral concentrations, and biotic infection are all capable of causing extensive damage to plants as well as inducing short- and long-term acclimation responses (Gordon *et al*., 2013).

NH_4^+ is a fundamental substrate for amino acids, nucleic acids, alkaloids, and polysaccharides, as well as for secondary metabolites such as PAs (Bagh *et al*., 2004) in all living organisms (von Wirén and Merrick, 2004). The downstream molecular events produced by NH_4^+ nutrition have been extensively studied and are related mainly to cell-wall stability and biosynthesis, carbon metabolism and energy, primary N metabolism, phytohormone, and signalling molecules (Ariza *et al*., 2013). However, NH_4^+ is toxic to cells when it is present at high concentrations in the soil or the nutrient solution because it causes the so-called 'ammonium syndrome'. This may include leaf chlorosis, lower plant yield production and root/shoot ratio, lower cation content, acidification of the rhizosphere, and changes in several metabolite levels, such as amino acids or organic acids (Britto and

Kronzucker, 2002; Bittsánszky *et al*., 2015). Despite this, recent studies regarding the possible effect of NH_4^+ nutrition as an inducer indicate that NH_4^+ or one of its assimilation products (e.g. glutamine or glutamate) may serve as a stress signal and, in NH_4^+-grown plants, operate metabolic pathways that induce the accumulation of ROS (Misra and Gupta, 2006). Specifically, Fernández-Crespo et al. (2012) demonstrated that NH_4^+ nutrition confers protection against subsequent salt stress by reducing Cl^- uptake and decreasing its toxicity by priming ABA and PAs accumulation, and by enhancing the basal content of H_2O_2 and proline in citrus plants. The authors concluded that NH_4^+ nutrition triggers mild chronic stress, which may account for the noted stress-induced morphogenetic responses (SIMRs) as part of a general acclimation strategy. The induction of the 'acclimation stage' leads to better adaptation to subsequent salt stress. Moreover, Fernández-Crespo et al. (2014) demonstrated that H_2O_2 and the manipulation of antioxidant machinery act as intermediaries between mild stress induced by NH_4^+ nutrition and the development of the acclimation stage.

In this work, we test the effectivity of NH_4^+ nutrition as inducer of resistance against a biotic stress, selecting for this purpose the pathogen *Pst*. We provide evidences that NH_4^+ nutrition induce resistance against *Pst* in tomato plants and performed assays to determine his mode of action. We concluded that NH_4^+ nutrition provokes mild toxicity in tomato plants, inducing H_2O_2 accumulation, which acts as a signal that can activate SAA and thus impart resistance to subsequent biotic stress. Moreover, we demonstrated the importance of Put and ABA downstream signalling pathways in NH_4^+-IR against *Pst* infection.

MATERIAL AND METHODS

Plant material, growth conditions and nutrition treatments

Four-week-old tomato plants of *Solanum lycopersicum* Mill. cv. Ailsa Craig were germinated in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 24/18°C, light intensity of 200 μmol m^{-2} s^{-1} and relative humidity of 60%. Seeds were irrigated twice a week with distilled water. Seedlings were irrigated for three weeks with Hoagland solution (Hoagland and Arnon, 1950) (control plants) or with Hoagland solution lacking N complemented with 20 mM of KNO_3^- (NO_3^-plants); 2, 5 or 8 mM of NH_4^+ [$(NH_4)_2SO_4$)] ($N\text{-}NH_4^+$ plants). Then, K_2SO_4 and $CaSO_4$ were added to compensate for the absence of K^+ and Ca^{2+}. The pH of the nutrient solution was adjusted to 6.0 with 1 mM KOH.

Tomato genotypes used in our study were: wild-type Ailsa Craig, Moneymaker and Castlemart. The authors are grateful to Jonathan Jones (John Innes Centre, Norwich, Norfolk, UK) for seeds of the SA-deficient *NahG* tomato plant in the background Moneymaker, and to G. Howe (Michigan State University, East Lansing, MI, USA) for the JA pathway mutant *def1* in the background Castlemart. The ABA pathway mutant used was the ABA-deficient mutant *flacca* in the background Ailsa (LA3613), which was provided by the Tomato Genetics Resource Center (TGRC), University of California, Davis, CA, USA.

Treatments with Put and PAs biosynthesis inhibitors (DFMA and DFMO)

The chemical PAs biosynthesis inhibitors difluoromethylarginine (DFMA) and difluoromethylornithine (DFMO), obtained from Dr Altabella, Centre for Research in Agricultural Genomics (CRAG), were dissolved in water and an inhibitor solution containing 2 mM of DFMA and 5 mM of DFMO. Four

treatments were performed, with the inhibitor solution applied directly to each pot during the week prior to *Pst* inoculation. For Put treatment, four-week-old plants were treated with 0.5 mM Put or mock solution (water) using foliar sprays applied 48 h before *Pst* infection. Tomato plants were maintained in the same culture conditions and inoculated as described above.

Pseudomonas syringae and *Botrytis cinerea* bioassays

Pst was grown in King's B medium (KB) (King *et al*., 1954) at 28°C. Rifampicin was added to KB at a concentration of 50 mg mL^{-1}. The coronatineless strain of *Pst* (*CmaA*) (COR-) (Brooks *et al*., 2005) was grown in KB with rifampicin (50 mg mL) and kanamycin (25 mg mL^{-1}). For inoculation, *Pst* was grown in KB at 28°C for 24 h. Bacterial suspensions were adjusted to 5×10^5 colony-forming units (c.f.u.) mL^{-1} in sterile $MgSO_4$ (10 mM) containing 0.01% of the surfactant Silwet L-77 (Osi Specialties, Danbury, CT, USA), as described previously (Katagiri *et al*., 2002). Pathogen inoculation was performed by dipping the third and fourth leaves into the bacterial suspension. The disease rate was scored at 72 hours post inoculation (hpi) by determining the percentage of dark-brown spots on the leaf surface. For molecular and hormonal analyses, the samples were taken at 3, 24 and 48 hpi. At least three samples for colony counting and 20 samples for disease rate scoring were taken for each treatment over a three-day period. Each experiment was independently conducted at least three times.

B. cinerea CECT2100 was routinely cultured on potato dextrose agar at 24°C. *B. cinerea* spores were collected from 10- to 15-day-old cultures with sterile water containing 0.01% (vol/vol) Tween- 20, which was then filtered, quantified with a hemacytometer, and adjusted at 1×10^6 ml^{-1}.

Biomass, chlorophyll content and photosynthetic rate (A_N)

Four-week-old tomato plants (control and N-NH_4^+ plants) were collected and dried in an oven during two days at 65 ºC. Plant tissues dried were weighted and the dry weight (DW) of 10 plants was obtained and expressed as biomass.

The chlorophyll level of the leaves of four-week-old tomato plants was measured using a chlorophyll meter (SPDA; Minolta, Tokyo, 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 of the 10 plants per treatment were averaged to represent one observation. The results were obtained as SPAD values (arbitrary units). Although the equations used to convert the SPAD value into the chlorophyll concentration have been obtained for other species like *Quercus serrata* (Hoshino, 1996), there is no such equation known for tomato plants to date.

For the net photosynthetic rate (A_N) LCpro+ portable infrared gas analyser (ADC BioScientific Ltd., Hoddesdon, UK) was utilized under ambient CO_2 and humidity. Supplemental light was provided by a PAR lamp at 1000 μmol m^{-2} s^{-1} photon flux density and air flow was set at 150 μmol mol^{-1}. After instrument stabilization, measurements were taken on three mature leaves (from an intermediate position on the stem) in each of the 10 replicate plants.

H_2O_2 determination, microscopy analysis and quantification

Samples of 10 leaves were collected for 3'3-diaminobenzidine (DAB) staining at 3, 24 and 48 hpi. Leaves were cut and put immediately in DAB 1 mg mL^{-1} at pH<3 for 24 h in the dark and were subsequently destained in 96% ethanol and rehydrated in distilled water. DAB staining intensities were quantified in micrographies by the number of dark-brown DAB pixels in

relation to the total pixels corresponding to plant material using the GIMP program (version 2.6.12).

H_2O_2 accumulation in leaves was quantified using the xylenol orange method (Kim et al., 2013a). Ten leaf discs (0.5 cm^2) were floated on 1 mL of distilled water for 1 h, centrifuged for 1 min at 12,000g, and 100 µL of supernatant was immediately added to 1 mL of xylenol orange assay reagent. The mixture was incubated for 30 min at room temperature. A standard curve for H_2O_2 was generated from measurements obtained from a serial dilution of 100 nM to 100 mM of H_2O_2. H_2O_2 was quantified by measuring by a spectrophotometer the A_{560}.

Analysis of gene expression by quantitative real time-polymerase chain reaction (qRT-PCR)

Gene expression by qRT-PCR was performed using RNA samples extracted from leaf tissue using the Total Quick RNA cells and tissues kit (Talent; http://www.spin.it/talent), according to the manufacturer's instructions. Tomato leaf tissue samples for RNA isolation were collected at 3 and 48 hpi. Leaf tissue from 10 plants each of the NH_4^+ treated and untreated plants was collected. A total of 1.5 µg of total RNA was digested using 1 unit of RNase-Free DNase (Promega; http://.www.promega.com) in 1 µL of DNase buffer and up to 10 µL of Milli-Q water and was incubated for 30 min at 37ºC. After the incubation, 1 µL of RQ1 DNase stop buffer was added, and the solution was incubated again at 65ºC for 10 min to inactivate the DNase. Highly pure RNA was used for the RT reaction. The RT reaction was performed according to the manufacturer's instructions Omniscript reverse transcriptase kit (QIAGEN; http://www.qiagen.com/). The reaction mixture was incubated at 37ºC for 60 min. Forward and reverse primers (0.3 µM) were added to 12.5 µL of QuantiTect™ SYBR Green PCR reaction buffer (QIAGEN), as were 2

μL of cDNA and Milli-Q sterile water up to a total reaction volume of 25 μL. Quantitative PCR was carried out using the Smart Cycler II instrument (Cepheid; http://www.cepheid.com). A list of the primers used in the qRT-PCR is shown in Table S1, levels of *EF1α* gene expression were used an internal house-keeping control. Melting-curve analysis was performed at the end of the PCR reaction to confirm the purity of the amplified products.

The amplification efficiency for each primer pair was calculated using serial cDNA dilutions. Differences in cycle numbers during the linear amplification phase between samples from treated and untreated plants were used to determine differential gene expression. At least three independent experiments were performed to confirm the results. In each experiment, three biological replicates were used to generate means and determine the statistical significance.

Stomatal aperture analysis

Tomato plants were maintained in the same culture conditions and treated as described above. The third and fourth leaves were inoculated with *Pst* collected from the plants at 1 and 3 hpi and placed on glass slides with the adaxial epidermis in contact with dental resin. The dental resin mold was filled with nail polish to create a cast that was examined by light microscopy (Scalschi *et al.*, 2013). For stomatal aperture analysis, images of random regions were taken using a Leica IRB microscope equipped with a LeicaDC300F camera (Leica Microsystems CMS GmbH, Wetzlar, Germany). The stomatal aperture was analysed using the Eclipse-Net software of the Laboratory imaging program (http://www.laboratory-imaging.com). Approximately 50 stomata from each leaflet were measured.

Chromatographic analysis

For hormonal analysis, fresh material was frozen in liquid N, ground and freeze dried. Fresh tissue (0.5 g) was immediately homogenized in 2.5 mL of ultrapure water, and a mixture of internal standards ($^{2}H_6$-ABA, $^{2}H_4$-SA, dihydrojasmonic acid and propylparaben) was added at 100 ng mL^{-1} prior to extraction. After extraction, a 20-□L aliquot was injected directly into the high-performance liquid chromatography (HPLC) system. For PAs analysis, fresh material was frozen in liquid N. Before extraction, according to the method described by Sánchez-López et al. (2009) a mixture of internal standards containing $^{13}C_4$- putrescine and 1,7-diaminoheptane was added. A 20-□L aliquot of this solution was directly injected into the HPLC system.

Analyses of hormone and PAs samples were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100mm×2mm i.d.; 5 µm; Scharlab, Barcelona, Spain; http://www.scharlab.es). The chromatographic system was interfaced to a Quatro LC (quadrupole–hexapole–quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and plant samples.

Statistical analysis

Statistical analysis was carried out using a one-way analysis of variance in the Statgraphics-plus software of Windows V.5 (Statistical Graphics Corp., Rockville, MD, USA). The means are expressed with standard errors and compared using a Fisher's least-significant difference test at the 95% confidence interval. All of the experiments were repeated at least three times.

RESULTS

NH_4^+ nutrition enhances tomato resistance against *Pst* infection

To investigate the role of NH_4^+ nutrition in induced resistance against *Pst*, four-week-old tomato plants of *S. lycopersicum* were germinated in vermiculite under growth chamber-controlled conditions. Seeds were irrigated twice weekly with Hoagland complete solution (control plants) or with Hoagland solution lacking N completed with different NO_3^- and NH_4^+ concentrations [20 mM of KNO_3^-, N-NO_3^- plants and 2, 5 and 8 mM of $(NH_4)_2SO_4$; N-NH_4^+plants]. All plant groups were inoculated with *Pst* by dipping leaves into the bacterial suspension. Statistically significant reductions in disease symptoms (Fig 1A) and in the size of the bacterial population (Fig 1B) were found at 72 hpi under all N nutrition conditions. This reduction was more pronounced in plants grown with 5 mM of NH_4^+, demonstrating that the inducer effect of NH_4^+ nutrition is concentration dependent. This direct correlation between NH_4^+ concentration and *Pst* resistance is lost at high concentrations of this ion since clear toxicity symptoms were observed in tomato plants grown with 8 mM of NH_4^+ (data not shown). To gain further insight into biochemical and molecular mechanisms related to NH_4^+-mediated resistance to *Pst*, the 5 mM nutrition condition was selected for use in this study. This concentration was also tested against a necrotrophic pathogen, *B. cinerea*, and a similar effect was observed (Fig S2).

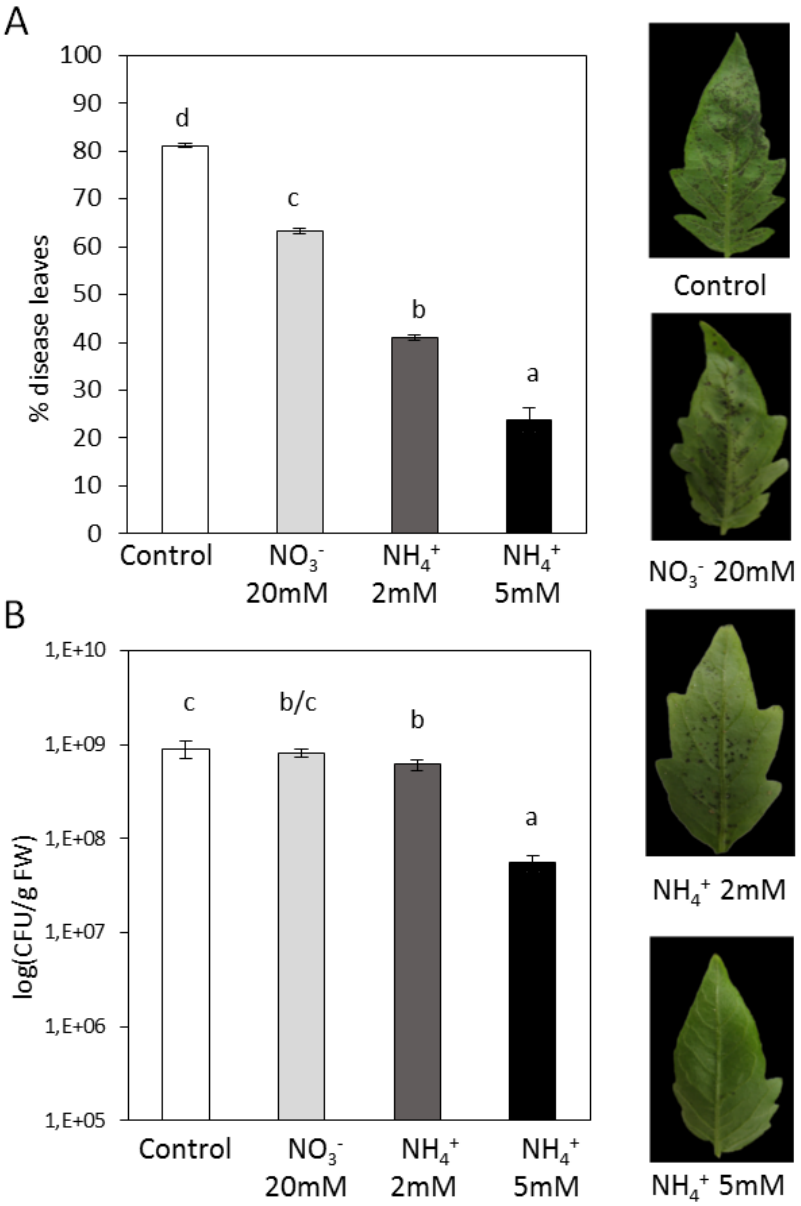

Fig.1. Effect of different N treatments on the resistance of tomato plants to *Pst*. Four-week-old tomato plants growth with different NO_3^- and NH_4^+ concentrations were inoculated by dipping them in a bacterial suspension of *Pst* at 5×10^5 c.f.u. mL^{-1}. After 72 hpi, the disease rating was scored by measuring the percentage of infected leaves in relation to the total number of analysed leaves (A) and by recounting of bacterial populations by plating in agar–KB medium (B). The photograph shows a representative picture of disease symptoms in the different N

treatments. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences ($p < 0.05$; least-significant difference test).

NH_4^+ nutrition induces mild toxicity, the first step of acclimation stage

NH_4^+ is a paradoxical nutrient ion because, despite being a major N source for many metabolic reactions, it can provoke toxic symptoms in sensitive species. In this study, the influence of NH_4^+ nutrition on plant growth, chlorophyll content and photosynthetic rate (A_N) (Table 1) was analysed. As expected, plants grown with NH_4^+ as the sole source of N showed a 27% reduction in their growth, when compared with control plants. Moreover, it was observed that NH_4^+ nutrition provoked a significant increase in chlorophyll content and induced A_N inhibition in tomato plants.

Table 1. Effect of NH_4^+ nutrition on growth, chlorophyll content and photosynthetic rate (A_N)

Treatments	Biomass (g DW)	Cholorophyll content (SPAD Units)	A_N (µmol m^{-2} s^{-1})
Control	0,480 ± 0,117 [b]	37,573 ± 0,578 [b]	21,565 ± 0,841 [b]
NH_4^+	0,345 ± 0,031 [a]	43,300 ± 0,716 [a]	13,543 ± 0,663 [a]

Put accumulation is required to NH_4^+-IR against *Pst*

To determine whether NH_4^+ nutrition affects PAs content, ornithine, Put, Spm and Spd levels were determined at 3, 24, 48 hpi in control and N-NH_4^+ plants (Fig 2 and Fig S1). N-NH_4^+ plants displayed higher basal levels of ornithine and Put content when compared with control plants. However, upon infection, no changes in ornithine content were observed in control plants; while, N-NH_4^+ infected plants showed a marked decrease in ornithine content, which was apparently transformed into Put. Regarding Put accumulation, although both control and N-NH_4^+ plants displayed an increase

in Put content at 24 and 48 hpi, the increase was higher in N-NH_4^+ infected plants (Fig 2). No changes were observed in the Spm and Spd content under any experimental condition (Fig S1). Collectively, these results suggest the involvement of PAs metabolism, specifically Put, in NH_4^+-IR.

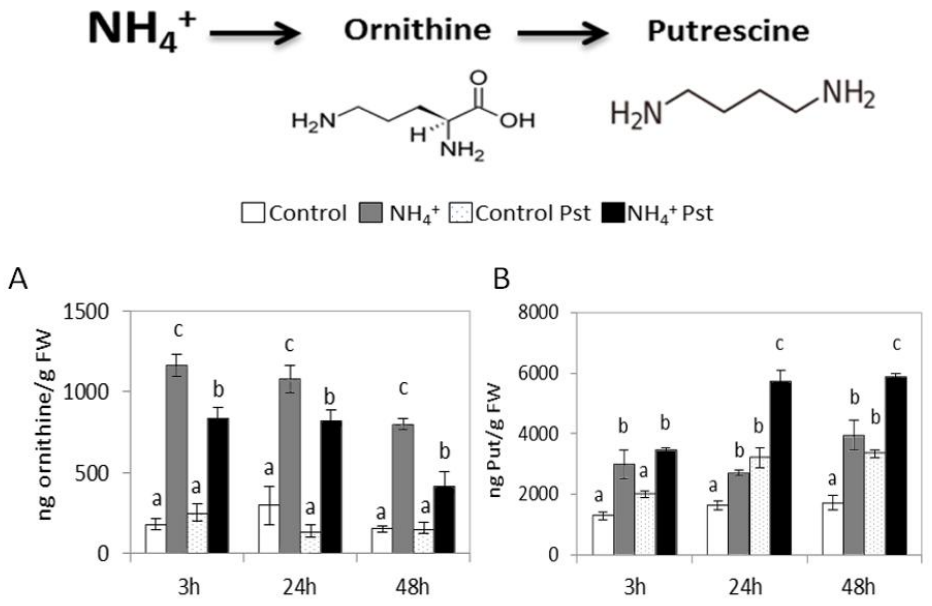

Fig.2. PAs content in control and N-NH_4^+ tomato plants in *Pst* infection. Four-week-old tomato plants were grown under control conditions or with 5 mM of NH_4^+, and inoculated by dipping them in a bacterial suspension of *Pst* at 5× 10^5 c.f.u. mL^{-1}. Leaves were collected at various time points and ornithine (A) and Put (B) 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. Letters indicate statistically significant differences ($p < 0.05$; least-significant difference test).

To assess the importance of Put accumulation, DFMA and DFMO were used as irreversible inhibitors of ADC and ODC enzymes, respectively (Fallon and Phillips, 1988), with the purpose of reducing cellular Put accumulation induced by NH_4^+ nutrition (Fig 3). As expected, N-NH_4^+ plants treated with inhibitor solution displayed a reduction of 35.3% in Put content in the absence of *Pst* (Fig. 3C).

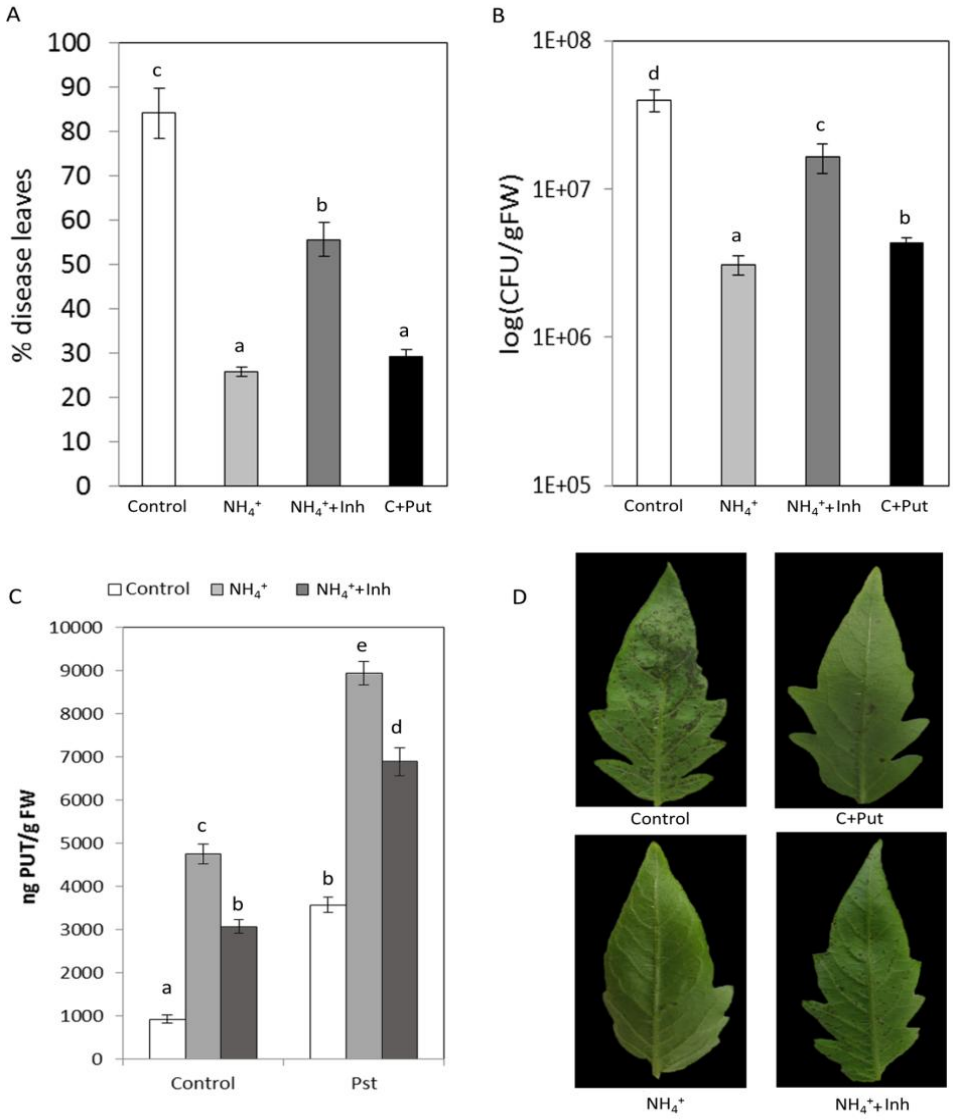

Fig.3. Influence of Put accumulation on NH_4^+-IR against *Pst*. Plants were grown as described in Figure 2, and one week prior to inoculation, N-NH_4^+ plants were treated with inhibitor solution (containing DFMO and DFMA; NH_4^++Inh) and control plants were treated with 0.5 mM of Put 48h before infection (C + Put), and inoculated by dipping them in a bacterial suspension of *Pst* at 5×10^5 c.f.u. mL^{-1}. The disease rating was scored by measuring the percentage of infected leaves in relation to the total number of analysed leaves (A) and by recounting of bacterial populations by plating in agar–KB medium (B) at 72 hpi. Put level in control, NH_4^+ and NH_4^++Inh plants was analysed at 48 hpi (C). The photograph shows a representative picture of disease symptoms in control, NH_4^+, NH_4^++Inh and C+Put)

tomato leaves at 72 hpi (D). Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicates statistically significant differences ($p < 0.05$; least-significant difference test).

Surprisingly, N-NH_4^+ treated with the inhibitor solution were more susceptible to *Pst* when compared to N-NH_4^+ plants untreated plants (Fig 3A, 3B and 3D). This shows that reduced Put accumulation reverts the resistance phenotype induced by NH_4^+ nutrition, revealing an important role of Put accumulation. To confirm the effect of Put on *Pst* infection, control plants were treated with 0.5 mM of Put by foliar spray 48 h before infection. It was observed that, in control plants, Put induced statistically significant reductions in disease symptoms (Fig 3A) and bacterial population size (Fig. 3B). These experiments demonstrate that Put accumulation is a crucial event in the resistance of tomato plants to *Pst*.

Oxidative burst induced by NH_4^+ nutrition enhances the resistance against *Pst*

Early accumulation of ROS is one of the first biochemical responses of the plant to pathogen attack. To clarify how NH_4^+ nutrition affects cellular oxidative burst, and to determine its relationship with NH_4^+-IR against *Pst*, H_2O_2 accumulation was evaluated by means of DAB staining (Fig 4). H_2O_2 accumulation was examined at 3 and 48 hpi in control and N-NH_4^+ plants by digital quantification of DAB intensity (Fig 4A, 4B and 4C). This analysis revealed that N-NH_4^+ plants showed higher basal levels of H_2O_2 accumulation than control plants. Upon infection with *Pst*, no changes in H_2O_2 accumulation were observed at 3 hpi. However, at 48 hpi *Pst* induced strong oxidative bursts in control and N-NH_4^+ plants, although the highest levels of H_2O_2 accumulation occurred in the later ones.

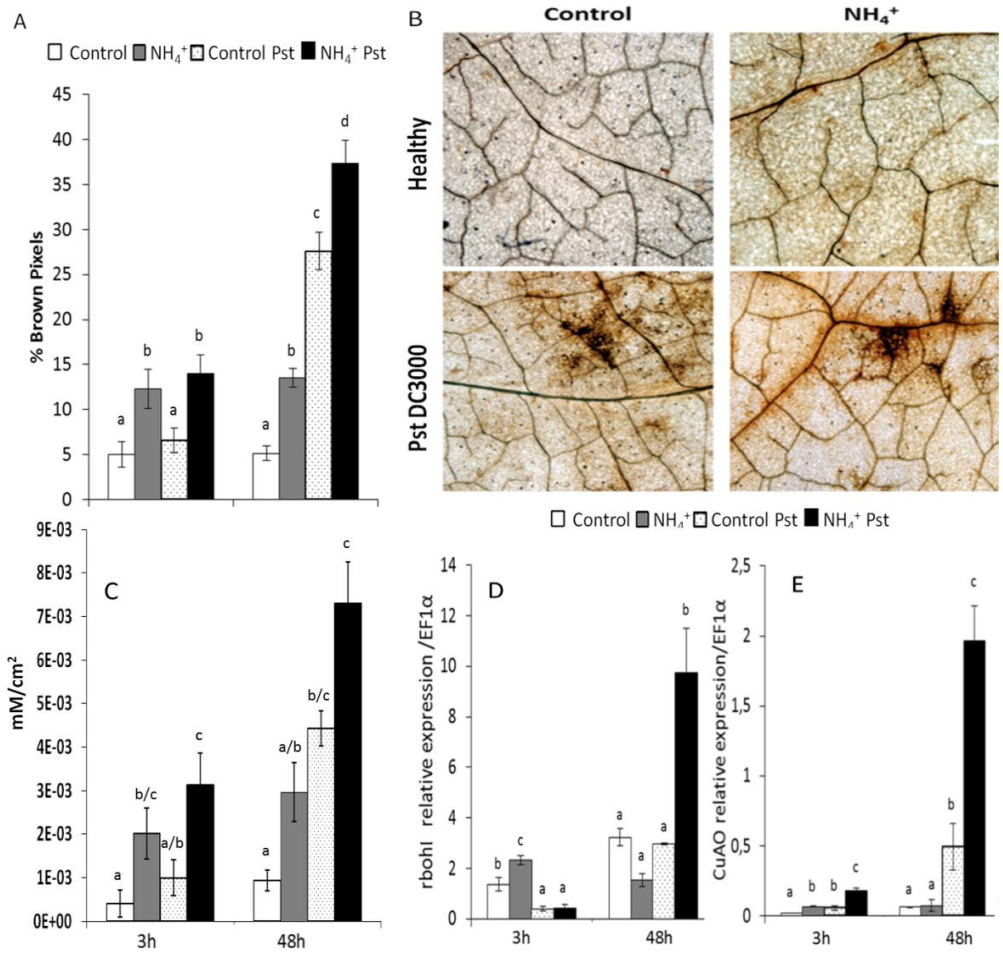

Fig.4. Effect of NH_4^+ nutrition on the oxidative burst upon *Pst* infection. Plants were grown and inoculated as described in Figure 2. H_2O_2 accumulation was visualized by DAB staining. Quantification was performed by determining the number of brown pixels on digital photographs of leaves at 3 and 48 hpi (A). Representative photographs were taken of H_2O_2 accumulation in control and N-NH_4^+ plants in the absence of pathogens, as well as 48 hpi (B). Data show average values standard error (n = 20) of the relative number of brown or yellow pixels per photograph. H_2O_2 concentrations were quantified by xylenol orange analysis (C). Total RNA was isolated from leaves at 3 and 48 hpi and was converted into cDNA and subjected to a RT-qPCR analysis. The relative level of (D) *rboh1* and (E) *CuAO* was analysed in the control and N-NH_4^+ plants. The results were normalised to the *EF1α* gene expression measured in the same samples. Letters indicate significant differences between treatments in each time point ($p < 0.05$; least-significant difference test).

These results indicate that cellular oxidative stress could have an important role in NH_4^+-IR against *Pst*. Thus, respiratory burst oxidase *rboh1* and *CuAO* gene expression was analysed at 3 and 48 hpi by qRT-PCR (Fig 4D and 4E). In N-NH_4^+ plants, higher *rboh1* mRNA accumulation was observed at 3 h in the absence of *Pst*. At 48 hpi, N-NH_4^+ plants displayed strong *rboh1* induction when compared with control plants. Similar results to those obtained for *rboh1* were observed in the analysis of *CuAO*.

NH_4^+ nutrition induces changes in the hormonal profile of tomato plants

To determine whether the main signalling pathways are involved in NH_4^+-IR, the levels of hormones and ferulic acid were analysed simultaneously in control and N-NH_4^+ plants at 3, 24 and 48 hpi (Fig 5). N-NH_4^+ plants displayed higher basal ABA and SA levels compared to control plants. On infection with *Pst*, no changes in ABA and SA content were observed in treated plants during the experiment. In the oxylipin pathway, a significant increase was observed in the OPDA content of the N-NH_4^+ infected plants at 24 and 48 hpi compared to the control plants. A fast increase of JA level in response to *Pst* at 3 hpi was found in the N-NH_4^+ condition, whereas no changes in control and infected plants were found. These results showed that NH_4^+ nutrition induces fast activation of oxylipin pathways in tomato plants, which leads to significant OPDA accumulation. Despite that, an abolished production of JA-Ile levels was observed in these plants (data not shown). Moreover, an increase in the ferulic acid content was observed at 48 hpi, which was higher in the N-NH_4^+ infected plants.

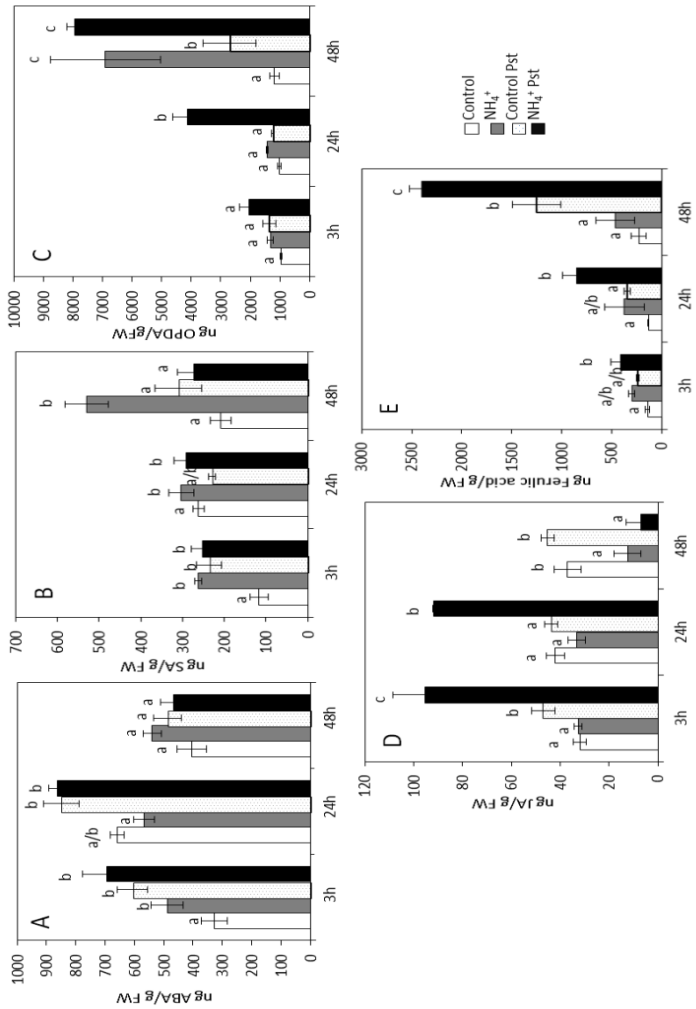

Fig.5. Hormonal profile in control and the N-NH_4^+ tomato plants upon *Pst* infection. Plants were grown and inoculated as described in Figure 2. Leaves were collected at different time-points and ABA (A), SA (B), OPDA (C), JA (D) and ferulic acid (E) levels were determined by HPLC–MS. The concentration of the hormones was determined in all samples by normalizing the chromatographic area for each compound with the fresh weight of the corresponding sample. Data show the average of three independent experiments of a pool of 20 plants per experiment ± SE. Letters indicate statistically significant differences in each time point ($p < 0.05$; least-significant difference test).

NH_4^+-IR against *Pst* involves ABA signalling pathways

To confirm the changes produced by NH_4^+ nutrition in the hormonal balance, the expression patterns of marker genes for ABA (*Asr1*), SA (*PR1* and *PR5*), JA (*LoxD, JMT*) and ethylene (*ACCOx*) signalling pathways were analysed in the control and N-NH_4^+ plants at 48 hpi (Fig 6). NH_4^+ nutrition induces *Asr1* mRNA accumulation at 48 hpi although an increase in ABA levels was not observed at this time point. However, in absence of infection N-NH_4^+ plants displayed higher basal levels of ABA at early time points. This initial ABA accumulations and the strong induction of *Asr1* suggest that the ABA pathway has an important role in NH_4^+-IR. Regarding SA pathways, although N-NH_4^+ plants showed higher basal SA levels compared to control plants, upon infection, no significant increase in SA content was observed. However, control and N-NH_4^+ plants displayed larger increases in *PR1* and *PR5* mRNA accumulation in response to *Pst* infection, being more pronounced in control infected plants. The expression patterns of marker genes related to oxylipin pathways revealed that *Pst* infection induces *LoxD* expression at 48 hpi, but its expression was higher in N-NH_4^+ plants. Regarding *JMT* expression, both control and N-NH_4^+ plants displayed an increase in mRNA accumulation at 48 hpi without significant changes between treatments. Moreover, we extended our analyses to *ACCox* marker gene related to ethylene pathway and observed that, in control and N-NH_4^+ plants, *Pst* infection enhances *ACCox* mRNA accumulation, although this was higher in control plants. Based on these results, we can conclude that the higher induction of *Asr1* and *LoxD* genes points to the fact that NH_4^+-IR might be due to the activation of ABA- and JA-defence signalling pathways.

To gain further insight into the mechanisms behind NH_4^+-IR against *Pst*, tomato mutants impaired in the SA, ABA or JA pathways were analysed (Fig 7). NH_4^+ nutrition did not protect ABA-deficient mutant *flacca* plants against

Pst (Fig 7A and 7B). N-NH_4^+ *flacca* plants displayed significant increases in disease symptoms, as well as in the size of bacterial population, compared with control *flacca* plants, indicating a requirement for this hormonal pathway in NH_4^+-IR. Transgenic *NahG* plants displayed a basal susceptibility to *Pst* due to their reduced SA levels. *NahG* plants displayed intact NH_4^+-IR, which supports the theory that SA does not play an important role in NH_4^+-IR (Fig 7C and 7D). The mutant *def1,* which is deficient in JA biosynthesis and acts downstream of OPDA formation, showed intact NH_4^+-IR (Fig 7E and 7F). This result supports the possible implication of oxylipin molecules upstream OPDA in NH_4^+-IR.

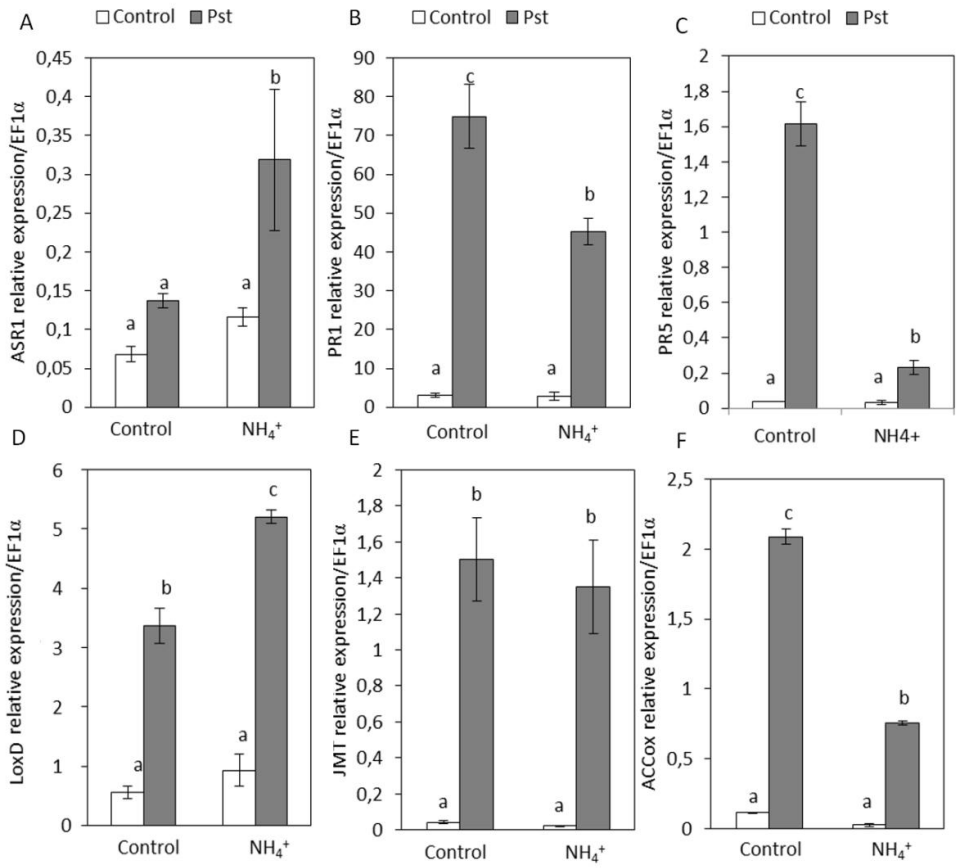

Fig.6. Gene expression profile of plant defence pathways in control and the N-NH_4^+ tomato plants upon *Pst* infection. Plants were grown and inoculated as described in Figure 2. The expression of genes representing key components of ABA (*Asr1*) (A), SA (*PR1* and *PR5*) (B and C), JA (*LoxD, JMT*) (D and E) and ethylene (*ACCOx*) (F) signalling pathways were analysed in cDNA from leaves of control and N-NH_4^+ plants at 48 hpi. The results were normalized to the *EF1α* gene expression measured in the same samples. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences ($p < 0.05$; least-significant difference test).

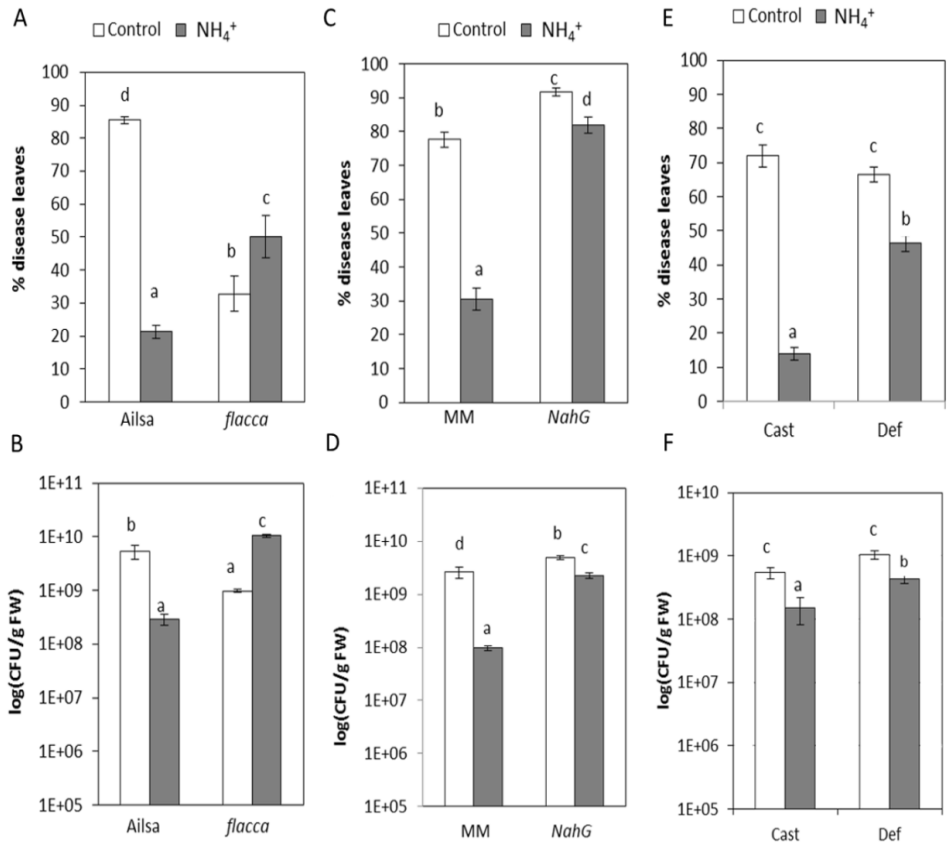

Fig.7. Influence of ABA, SA and JA signalling pathways on NH_4^+-IR against *Pst*. Four-week-old tomato mutants impaired in these signalling pathways were grown under control and NH_4^+ treatments and inoculated by dipping in a bacterial suspension of *Pst*. The disease rating was scored for wild-type tomato plants of Ailsa Craig (Ailsa), Moneymaker (MM) and Castlemart (Cast) and their respective ABA-impaired mutant *flacca* (A, B), SA-impaired mutant *NahG* (C, D) and JA-

impaired mutants *def1* (E, F) at 72 hpi. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences ($p < 0.05$; least-significant difference test).

NH_4^+ nutrition induces basal stomatal closure in tomato plants

A cellular response induced upon PAMP recognition is the closure of stomata within a few hours to restrict pathogen spread to other plant tissues. Therefore, in order to invade the apoplast and cause disease, *Pst* produces the bacterial phytotoxin, COR to impede stomatal closure and/or trigger stomatal reopening. To study the possible effect of NH_4^+ on this process, we measured stomatal aperture at 0, 1 and 3 hpi in control and N-NH_4^+ plants (Fig 8). In both treatments it was observed that plants closed the stomata to restrict pathogen entry to the apoplast at 1 hpi. Moreover, at 3 hpi, both control and N-NH_4^+ plants showed stomatal re-opening, probably induced by COR without significant changes between treatments (Fig 8A). Curiously, N-NH_4^+ plants displayed basal stomatal closure when compared with control plants (Fig 8B) which might contribute to the NH_4^+-IR against *Pst*. Thus, the relevance of the virulence factor COR, a JA-Ile mimic on NH_4^+-IR was further tested. Therefore control and N-NH_4^+ plants were infected with the *Pst* strain *cmaA*, which lacks COR (COR-). A reduction in bacterial growth was observed in plants infected with the mutant strain. Under NH_4^+ nutrition a statistically significant reduction in disease symptoms was observed when compared to the control plants (Fig 8C). Although no significant changes were found in the size of the bacterial population (Fig 8D), the reduction in disease symptoms and the normal re-opening at 3 hpi in N-NH_4^+ plants, revealed that this nutrition does not directly interfere with COR action.

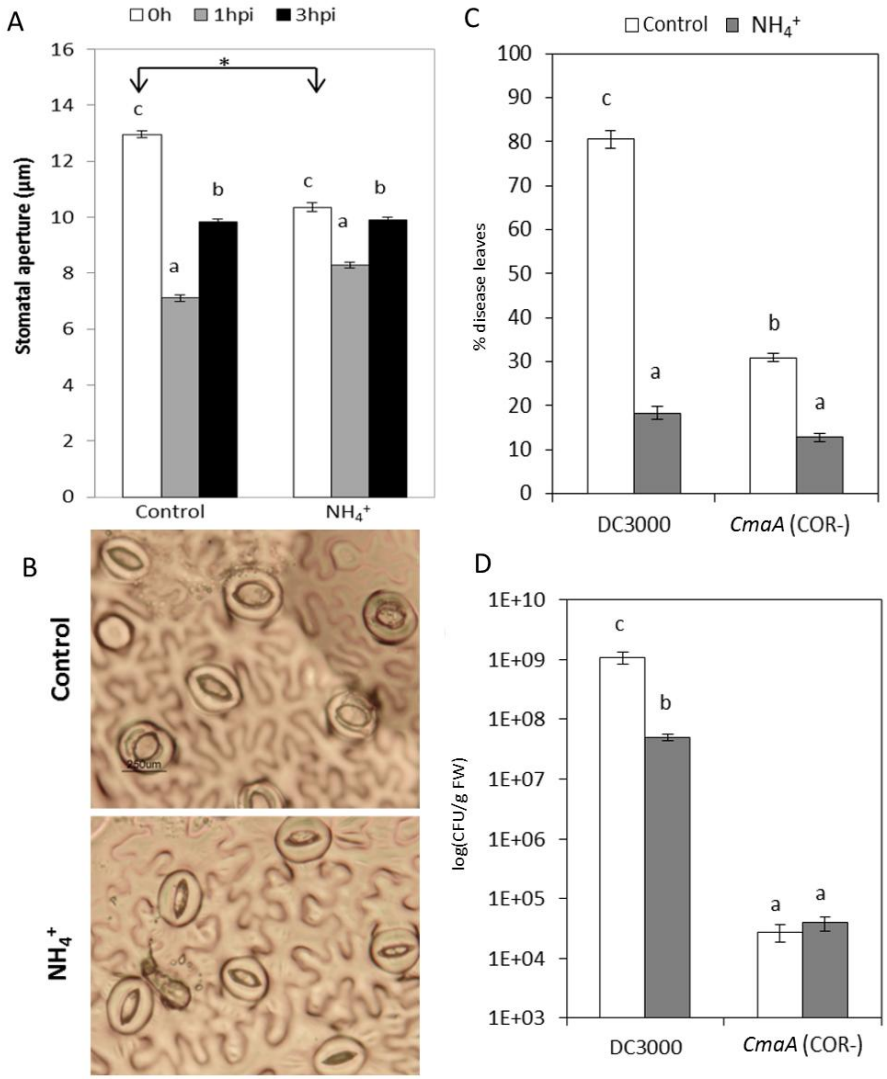

Fig.8. NH_4^+ treatment induces basal stomatal closure and NH_4^+-IR against *Pst* is independent of COR toxin effect. Tomato plants were grown, treated and inoculated as described in Figure 2. Stomatal apertures were analysed 'in situ' in leaflets of control and N-NH_4^+ plants at 0, 1 and 3 hpi (A). Results are means ± standard error (SE) (n > 50 stomata). Representative photographs of basal stomatal closure induced by NH_4^+ treatment (B) were taken. Tomato plants were infected by dipping them in a bacterial suspension of *Pst* and the coronatineless strain of *Pst* (*CmaA* COR^-). The disease rating was scored by measuring the percentage of infected leaves (C) and by recounting of bacterial populations (D) at 72 hpi. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences ($p < 0.05$; least-significant difference test).

DISCUSSION

The role of NH_4^+ as an essential macronutrient and signalling molecule has been extensively studied (Castaings *et al*., 2011), but its impact on plant defence responses is still obscure. In this study, we clarified the complex relationship between SAA mechanisms and the NH_4^+-IR against *Pst* by observing that changes in the N nutrition status, specifically NH_4^+ applied as the sole N source, resulted in an increased resistance against *Pst* in tomato plants. NH_4^+ nutrition protects tomato plants in a concentration-dependent manner, and this resistance is especially important for plant growth under conditions of 5 mM of NH_4^+. Extensive studies investigating the underlying mechanisms of NH_4^+ toxicity have been reported in plants, but how plants acclimated to high levels of NH_4^+ are able to more efficiently induce the mechanisms of resistance against subsequent stress situation is poorly understood. Plants accumulate PAs to compensate for the lack of some cations other than NH_4^+, besides serving as a sink for excess NH_4^+ to reduce toxicity (Gerendás *et al*., 1997). Although long known for their implication in abiotic stress responses, there has been little investigation of defence responses of PAs during pathogen infection. In this work, we observed that N-NH_4^+ plants displayed a higher basal content of ornithine and Put. Upon infection N-NH_4^+ plants showed a marked decrease in ornithine content, which was apparently transformed into Put. Interestingly, N-NH_4^+ tomato plants displayed more susceptibility to *Pst* when plants were treated with DFMA and DFMO inhibitors, and Put content was reduced. Moreover, Put treatment induces resistance against this pathogen in control tomato plants, revealing the importance of PAs, specifically Put, in the resistance of tomato plants to a biotic stress. According to these findings, it can be confirmed that signalling derived of changes to the ornithine pool and its conversion in Put, leading to high Put accumulation, are directly linked with NH_4^+ nutrition and

are key events in NH_4^+-IR against *Pst*. Kim et al. (2013b) observed that an *adc2* knock-out mutant displayed reduced Put content, reduced expression of *PR1* and enhanced susceptibility against *Pst*. Disease susceptibility of the *adc2* mutant was recovered by the addition of exogenous Put, revealing its direct impact on resistance against this pathogen. Another common response to the toxic effect of NH_4^+ nutrition is ROS accumulation and the modification of redox cell state (Patterson *et al*., 2010). Recent studies identified plastid retrograde signalling derived responses as key in plants against NH_4^+ stress (Li *et al*., 2012; 2013). These authors propose that, under NH_4^+ stress, the chloroplast receives the stress signal (mediated by ROS) and activates retrograde signalling pathways, recruiting downstream ABA signalling to regulate the expression of NH_4^+-responsive genes in the nucleus and prevent NH_4^+ toxicity. In this study, we confirmed the mild toxic effect of NH_4^+ nutrition on tomato plants since growth retardation, increased chlorophyll content, reduced photosynthetic rate and basal H_2O_2 accumulation were observed in N-NH_4^+ plants. The plastid retrograde signalling induced by H_2O_2 accumulation is probably related to the expression of nuclear genes to prevent NH_4^+ toxicity. The activation of this defensive pathway might induce the establishment of SAA in leaves of tomato plants, allowing them to better withstand a subsequent *Pst* infection. This hypothesis is in accordance with our previous works, in which we demonstrated that NH_4^+ nutrition in citrus plants triggers mild chronic stress, induces H_2O_2 accumulation and acts as a signal, which primes plant defence responses by stress imprinting and confers protection against subsequent salt stress (Fernández-Crespo *et al*., 2012; 2014). Although little is known about how prior exposure of plants to abiotic stress improves their capacity to effectively respond to biotic stress, recent studies suggest that ROS is closely associated with this adaptive process. For example, high light exposure-

induced ROS accumulation, and this signal is required for the SAA of plants, enhancing tolerance of plants to *Pst*, indicating that there is cross-talk between abiotic stress acclimation and pathogen responses (Karpinski *et al.*, 2013). For this reason, it was concluded that H_2O_2 accumulation derived of NH_4^+ mild chronic stress acts as a signal and primes plant defence responses, probably mediated by the activation of downstream plastid retrograde signalling. The signalling derived from the chloroplast could be related to the establishment of SAA in tomato plants, enhancing the resistance against subsequent *Pst* infection.

To further investigate the role of ROS in the SAA induced by NH_4^+ nutrition, *rboh1* and *CuAO* gene expression as H_2O_2 producers was analysed. As expected, NH_4^+ nutrition induced in tomato plants mRNA accumulation of both genes. RBOHs are required for the initiation and self-propagation of systemic signals by H_2O_2 accumulation to generate a 'ROS wave' (Mittler *et al*., 2011). Suzuki *et al*. (2013) demonstrated that SAA of plants to heat stress was correlated with activation of the ROS wave and the transient accumulation of ABA in systemic tissues, and these responses were suppressed in a mutant lacking RBOHD. According to these findings, the higher basal induction of *rboh1* accompanied with the higher basal H_2O_2 accumulation induced by NH_4^+ nutrition, might be the key event in the acclimation stage induced in tomato plants. As for CuAO, it is commonly accepted that H_2O_2 produced as a catabolite by CuAO action is involved in ABA-induced stomatal closure in *Vicia faba* (An *et al*., 2008). Therefore, H_2O_2 produced by CuAO and RBOH1 could have a dual role, first may play an important role in ABA-mediated basal stomatal closure, which was found in N-NH_4^+ tomato plants, and secondly the H_2O_2 wave could be used as an amplifier for signalling related to SAA mechanisms. N-NH_4^+ plants displayed a strong oxidative burst, probably mediated by the highest induction of *rboh1*

and *CuAO* gene. This response which leads to limited pathogen spread might play a key role in NH_4^+-IR against *Pst*.

To understand the effect of NH_4^+ nutrition on defence, signalling pathways were analysed. The hormonal profile revealed that NH_4^+ nutrition induces higher basal levels of SA in tomato plants, but upon *Pst* infection no significant changes in SA content between treatments were found. *PR1* and *PR5* gene expression are less induced in the NH_4^+ infected plants and *NahG* plants displayed intact NH_4^+-IR. This evidence supports the hypothesis that SA-signalling pathways are not the main pathways required for NH_4^+-IR against *Pst*. Regarding oxylipin pathways, a faster and stronger accumulation of JA in N-NH_4^+ plants at 3 hpi was observed. However, throughout the infection, decreased JA levels occurred accompanied with a higher increase in OPDA content and a strong induction of *LoxD* in N-NH_4^+ plants. The role of JA and OPDA in the NH_4^+-IR were tested with JA-deficient *def1* plants impaired in the JA as well as OPDA accumulation, and showed an intact resistance against *Pst*, revealing the possible implication of oxylipins upstream OPDA in the NH_4^+-IR against *Pst*. Regarding the phenylpropanoid pathway, NH_4^+ infected plants displayed higher accumulation of ferulic acid, which points to the implication of this pathway in NH_4^+-IR. It is probable that shikimate derived from tyrosine compete with SA accumulation synthesized via phenylalanine ammonia lyase, and thus, significant changes in free SA as well as reduced induction of *PR1* and *PR5* in N-NH_4^+ plants were not observed.

As expected, N-NH_4^+ plants displayed higher basal ABA accumulation and, on infection, NH_4^+ nutrition primed the induction of *Asr1*. To clarify the role of ABA-dependent signalling pathways, the effect of NH_4^+ nutrition on the responses of ABA-deficient *flacca* mutants was analysed. It was demonstrated that *flacca*- NH_4^+ treated plants were more susceptible to *Pst*,

and displayed impaired NH_4^+-IR expression. The fact that NH_4^+-IR was absent in *flacca* supports the idea that an intact ABA signalling pathway is required, at least in part in NH_4^+-IR against *Pst.* ABA has also emerged as a complex modulator of plant defence responses, since it can function as a positive or negative regulator, depending on the plant-pathogen interaction analysed (Ton *et al*., 2009). Specifically, ABA was found to be a key regulator of the pathogen-mediated stomatal closure (Melotto *et al*., 2006). One mechanism of *Pst* pathogenesis is to produce the effector COR necessary for stomatal re-opening in the process of bacterial infection (Melotto *et al*., 2006). To confirm the role of NH_4^+ nutrition on the stomatal movement on *Pst* infection, the stomatal aperture was analysed in treated and untreated plants at 0, 1 and 3 hpi. As expected, it was observed, that bacteria induced closure of stomata at 1 hpi, and stomata re-opened in a COR-dependent manner at 3 hpi in control and N-NH_4^+ plants (Tsai *et al*., 2011). However, N-NH_4^+ plants displayed more closed stomata than control plants, probably due to the higher basal ABA accumulation. These findings reveal that NH_4^+ does not have a direct effect on stomatal movement induced by COR, but the basal stomatal closure observed in NH_4^+ plants was sufficient to reduce the entry of bacterial into the mesophyll and reduce the disease symptoms. In addition to its role in stomatal movement, ABA and its role in PAs homeostasis has been largely studied. Toumi et al. (2010) showed that ABA induced PAs accumulation and secretion into the apoplast, where they were oxidized by CuAOs producing H_2O_2. Based on these findings, it seems clear that activation of ABA-dependent signalling pathways mediated by NH_4^+ nutrition may activate PAs biosynthesis and catabolism. For this reason, we conclude that ABA could act as a positive regulator of NH_4^+-IR since it was able to strengthen Put biosynthesis, which has a direct effect on the resistance to *Pst*, and induced PAs catabolism by enhancing CuAO

activity, carrying H_2O_2 accumulation and therefore provoking basal stomatal closure.

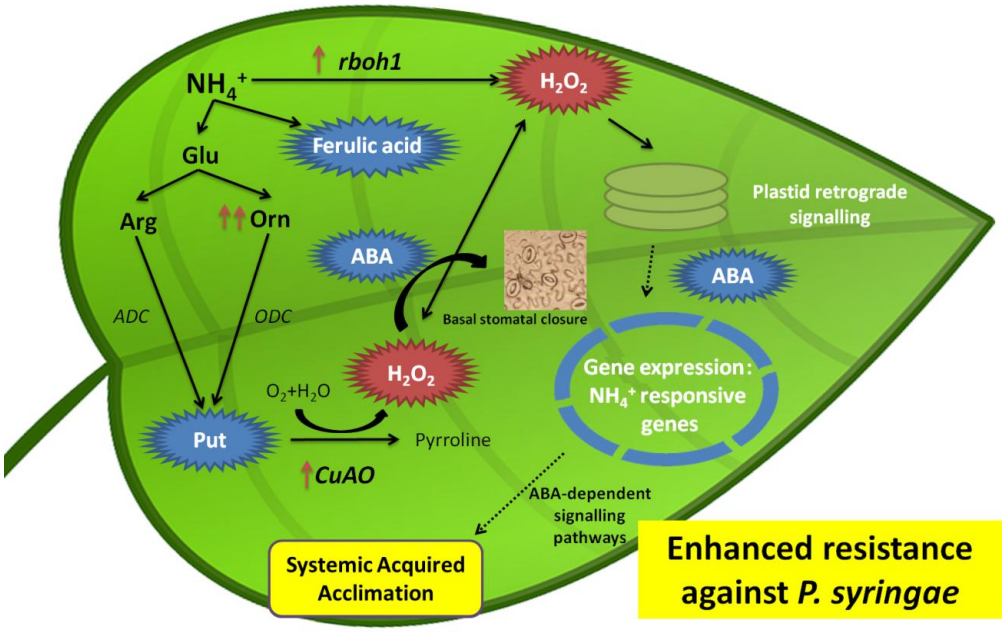

Fig.9. Model of SAA achievement induced by NH_4^+ nutrition. Tomato plants grown in NH_4^+ as a sole N source develop different responses to relieve the mild toxicity effect. These responses play an important role in achieving SAA and in the resistance against *Pst*. A common response against NH_4^+ nutrition is the accumulation of PAs to compensate for the lack of some cations other than NH_4^+ besides serving as a sink for excess NH_4^+ to reduce the toxicity. Here, basal metabolic changes induced by NH_4^+ nutrition were observed, specifically Put, ABA and ferulic acid accumulation. Another response against the mild toxic effect of NH_4^+ nutrition is ROS accumulation and consequently, the modification of redox cell state. This modification occurs by the basal induction of *CuAO* and *rboh1* gene observed in N- NH_4^+ plants. Moreover, it was found that basal stomatal closures are probably produced by H_2O_2 derived of enhanced *CuAO* activity, induced by ABA-mediated NH_4^+ responses. Chloroplast receives the stress signal (mediated by ROS) and activates retrograde signalling pathways, recruiting downstream ABA signalling to regulate the expression of NH_4^+-responsive genes in the nucleus and prevent NH_4^+ toxicity. The activation of this defensive pathway might induce the establishment of SAA in tomato plants leaves, allowing them to better withstand a subsequent *Pst*.

Collectively, these results indicate that NH_4^+ nutrition provokes mild chronic stress that leads to the activation of SAA responses that prime tomato defence pathways and induce resistance against subsequent biotic stress. NH_4^+ nutrition enhanced H_2O_2 accumulation, which acts as a signal to induce ABA-dependent responses, reducing the NH_4^+ toxicity. These ABA-dependent signalling pathways induce PAs biosynthesis and catabolism, which in turn enhance H_2O_2 accumulation, favouring stress signal amplification. Moreover, NH_4^+ nutrition induces Put accumulation and it was demonstrated that compromising Put accumulation provokes increased susceptibility to *Pst*. Conversely, the basal stomatal closure observed in N-NH_4^+ plants was probably produced by H_2O_2 derived of enhanced CuAO activity induced by ABA-mediated NH_4^+ responses. The basal stomatal closure observed in NH_4^+ plants may reduce the entry of bacteria into the mesophyll, reducing the diseased symptoms favouring the NH_4^+-IR. Besides these functions, we speculate that H_2O_2 acts as a signal of NH_4^+ stress and primes plant defence responses against subsequent pathogen infection. Although classical SA dependent responses against biotrophic pathogens were not found, we observed other defence mechanisms related to the resistance of these lifestyle pathogens as strong and fast oxidative bursts, probably mediated by *rboh1* and *CuAO* genes induction, which directly reduce the spread of pathogens in the plant (Fig. 9). The study of mechanism of action of NH_4^+ as an inducer of resistance, showed that N-NH_4^+ plants displayed basal responses related to the establishment of acclimation state in systemic leaves through SAA-related mechanisms. This activation allows the plant to trigger more efficient and effective specific responses to prevent pathogen spread, and therefore induce resistance to disease. The NH_4^+-IR mediated by SAA mechanisms is especially important because it has been shown to be effective in other species against abiotic stress (Fernandez-

Crespo *et al*., 2012) and against two different life style pathogens as *Pst* and *B. cinerea*. The study of events underlying this kind of induced resistance, which is effective against biotic and abiotic stress, provides knowledge necessary to exploit this phenomenon in the context of sustainable agriculture.

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SUPLEMENTARY DATA

Table S1. Primers sequences

Gene	GenBank accession number	Tm	Primers
EF1α	NM_001247106.1	60	Fwd: 5′-GACAGGCGTTCAGGTAAGGA-3′Rev: 5′ -GGGTATTCAGCAAAGGTCTC-3′
PR1	EU589238.1	54	Fwd: 5′-CCGTGCAATTGTGGGTGTC-3′ Rev: 5′-GAGTTGCGCCAGACTACTTGAGT-3′
PR5	AY257487.1	58	Fwd: 5′-GAGGTTCATGCCAAACTGGTC-3′ Rev: 5′-TCAACCAAAGAAATGTCC-3′
LoxD	U37840.1	58	Fwd: 5′-GGCTTGCTTTACTCCTGGTC-3′ Rev: 5′-AAATCAAAGCGCCAGTTCTT-3′
ACCox	NP_001233928.1	54	Fwd: 5′-CCATGTCCTAAGCCCGATTTGAT-3′ Rev: 5′-TCACTTT GTCATCTTGGAACAGA-3′
JMT	Scalschi et al., 2013	55	Fwd: 5′-GGTTCAAAGTGCATGAGAGCT-3′ Rev: 5′-TACACCACACACTGAAGGAAA-3′
Asr1	NM_001247703.1	54	Fwd: 5′-ACACCACCACCACCTGT-3′ Rev: 5′-GTGTTTGTGTGCATGTTGTGGA-3′
rboh1	AF148534.1	60	Fwd: 5′-AAGCCGGAACTTGAGTCAGA-3′ Rev: 5′-GTCTCAGCAGCACCCTTAGC-3′
CuAO	AJ871578.1	52	Fwd: 5′-ATATCCCGGGTCATGTTTGA-3′ Rev: 5′-TCCATCAGGTGCCACAAATA-3′

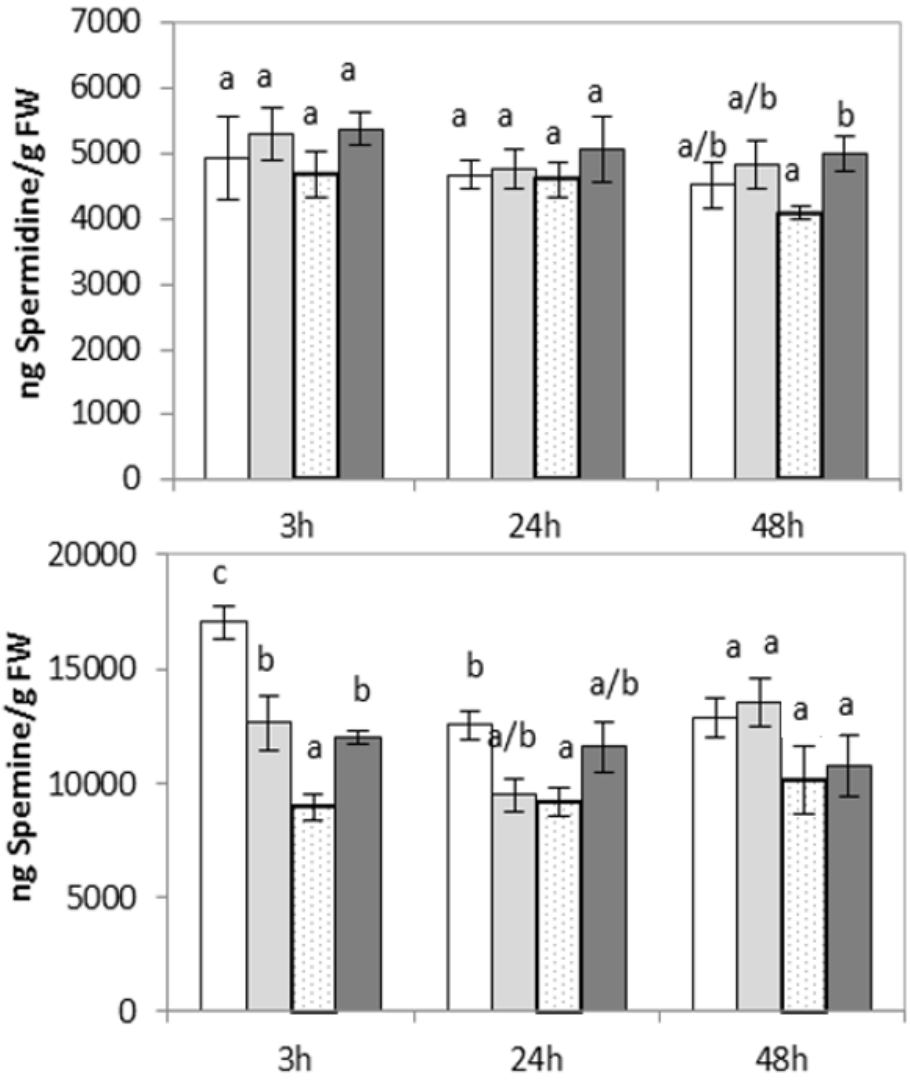


Fig.S1. PAs content in control and N-NH_4^+ tomato plants in *Pst* infection. Four-week-old tomato plants were grown in control conditions or in 5 mM of NH_4^+ and were inoculated by dipping them in a bacterial suspension of *Pst* at 5×10^5 c.f.u. mL^{-1}. Leaves were collected at various time points and spermidine (A) and spermine (B) 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. Letters indicate statistically significant differences ($p < 0.05$; least-significant difference test).

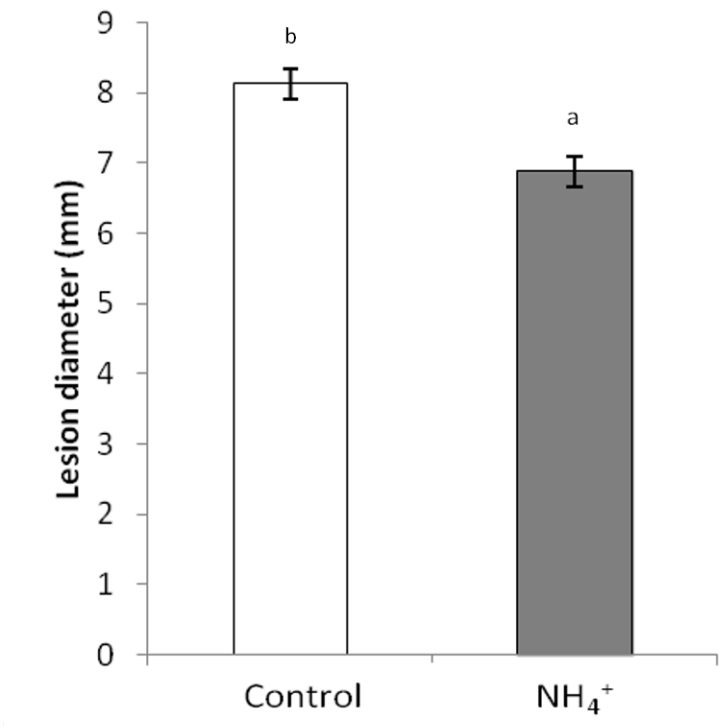


Figure S2. Effect of NH_4^+ treatment on tomato plants infected with *B. cinerea*. Fourweek-old tomato plants were grown in control conditions or in 5 mM of NH_4^+ and lesion diameter was measured after 72 h of inoculation. The data show the lesion diameter (mm) ± standard error (SE) (n = 20).

CHAPTER 5

Hexanoic acid treatment prevents the systemic movement of MNSV in *Cucumis melo* plants by priming callose deposition and hormonal signaling

ABSTRACT

We studied the basal response of melon (*Cucumis melo*) to *Melon necrotic spot virus* (MNSV) and demonstrated the efficacy of the hexanoic acid priming that blocks the virus systemic spread. We analyzed callose deposition and ROS production, as well as the hormonal profile and gene expression at the whole-plant level. This provided the hormonal homeostasis in melon roots, cotyledons, hypocotyls, stems and leaves involved in the basal and Hx-induced resistance (Hx-IR) to MNSV. We also demonstrated the relevant role of SA in response to MNSV but also of the oxylipins 12-oxo-phytodienoic acid (OPDA) and Jasmonic-Isoleucine (JA-Ile) and of the phenolic compound ferulic acid. The complexity of the hormonal balance and metabolites depending on timing and location associated to basal and Hx-induced defenses evidences the reprogramming of plant metabolism in MNSV inoculated plants at local and systemic tissues. Our results demonstrate an interesting correlation between callose deposition, oxidative environment and hormone homeostasis in this pathosystem and contribute to understand the host defenses in response to viruses beyond model species. In addition, they provide valuable evidences to unravel the complex mechanisms of the priming phenomenon by natural compounds.

INTRODUCTION

When a plant recognizes an invading pathogen, a wide range of responses is induced to avoid infection. The understanding of the mechanisms by which plants activate host defenses in response to viruses is still incomplete. Plant viruses can interfere and/or compete for a substantial amount of host resources, which can disrupt host physiology to cause disease (Culver and Padmanabhan, 2007; Pallás and García, 2011; Mandadi and Scholthof, 2013). A large body of evidence has recently shown that virus infection disturbs the delicate hormonal balance that governs plant life by mainly taking into account the highly relevant involvement of cross-talk among different hormone pathways in antiviral defenses (Alazem et al., 2015; García and Pallas, 2015). Thus the *Tobacco mosaic virus* replicase interacts with and alters the subcellular localization of several members of the auxin/indole acetic acid (Aux/IAA) protein family (Padmanabhan et al., 2006). Viral CPs interfere with modulate hormone-signaling pathways. Recently, Rodriguez et al. (2014) showed that the expression of the CP of TMV strain Cg that infects Arabidopsis (Cg-CP) stunts plant growth and delays floral transition timing. Remarkably, Cg-CP expression negatively regulates the SA-mediated defense pathway by stabilizing DELLA proteins during Arabidopsis thaliana viral infection. This suggests that Cg-CP alters the stability of DELLAs as a negative modulation mechanism of antiviral defense responses. This effect is similar to that previously reported for the P2 outer capsid protein of the phytoreovirus *Rice dwarf virus*, which interacts with the ent-kaurene oxidase, a key factor in the biosynthesis of gibberellins, to cause dwarf symptoms (Zhu et al., 2005).

The three phytohormones that influence the regulation of plant-virus interactions the most are salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA). SA is an essential phytohormone for the establishment of local and systemic resistance since both SA biosynthesis and signaling are activated upon the recognition of viral effectors by *R* gene products, the hallmarks of incompatible interactions (Vlot et al., 2009; Carr et al., 2010; Alazem et al., 2014). In compatible interactions, SA has been seen to improve plant basal immunity by delaying the onset of viral infection and disease establishment (Mayers et al., 2005). JA is also involved in compatible interactions, but seems to act in a phase-specific mode, induced in early infection stages (Pacheco et al., 2012; Garcia-Marcos et al., 2013). In general, virus infections inhibit JA-induced gene expression. This is particularly relevant in geminivirus infection since both pathogen localization and jasmonate synthesis occur preferentially in phloem cells, which makes the suppression of the jasmonate response a feasible target during infection (Hanley-Bowdoin et al., 2013). ABA has been observed to play a defensive role in plant-virus interactions through the inhibition of basic β-1,3-glucanase, which is responsible for the degradation of β-1,3-glucan (callose), which, in turn, is deposited on plasmodesmata and strengthens them against virus movement (Mauch-Mani and Mauch, 2005). Remarkably, JA precursor cis-(+)-12-oxo-phytodienoic (OPDA) has been seen to be involved in callose deposition during the activation of defense responses against *B. cinerea* (Scalschi et al., 2015). There is evidence to indicate that jasmonates OPDA, JA, and methyl jasmonic acid (MeJA) act as active systemic signaling molecules (Tamogami et al., 2012; Zhu et al., 2014; Wasternack et al., 2010). The ABA pathway has been recently shown to have multifaceted effects on *Bamboo mosaic virus* accumulation, and can disrupt the antagonistic effect of ABA and SA cascades (Alazem et al., 2014).

On a genome-wide scale, the gene expression analysis in *Arabidopsis thaliana* has provided relevant information on compatible plant-virus interactions, which indicates that the transcriptome of host cells undergoes significant reprogramming during infection (Rodrigo et al., 2012). Studies that go beyond model species are needed to provide valuable information on crop protection and disease management (Carr et al., 2010).

Many natural compounds prime plant defenses, including oligosaccharides, glycosides, amides, vitamins, carboxylic acids and aromatic compounds (Aranega et al., 2014). Among them, hexanoic acid (Hx) is a potent priming agent within a wide range of host plants and pathogens (Aranega et al., 2014). Treating roots of tomato plants with Hx protects them against necrotrophic fungi *B. cinerea* (Vicedo et al., 2009) and the hemibiotrophic bacterium *Pseudomonas syringae* pv tomato (Scalschi et al., 2013). Hx can early activate broad-spectrum defenses by inducing callose deposition, in addition to the SA and oxylipin pathways. It can also prime pathogen-specific responses in each particular case according to the pathogen and its lifestyle, and has an anti-oxidant protective effect (Finiti et al., 2014; Camañes et al., 2015).

Research into priming resistance against viruses by natural compounds is scarce. Some non natural resistance inducers are effective against viruses, such as (2,1,3)-benzothiadiazole (BTH), a functional analog of SA that protects tobacco against tobacco mosaic virus (Friedrich et al. 1996) and tomato plants against cucumber mosaic virus (CMV)(Anfoka, 2000).

In the present work, we studied the basal response and Hx-IR of melon plants to *Melon necrotic ringspot virus* (MNSV). This plant virus belongs to the genus *Carmovirus* in the family *Tombusviridae,* and causes systemic necrotic spots on leaves and streaks on stems of melon, cucumber and watermelon, and occasionally total plant collapse (“sudden death”) (Hibi and Furuki,

1985). The MNSV genome is a single-stranded RNA molecule of 4.3 kb with positive polarity that encodes at least five different proteins (Genoves et al., 2006). The open reading frame (ORF) at the 5′ end terminates in an amber codon to yield two proteins involved in replication: p29 and p89. Cell-to-cell viral movement is supported by two proteins, p7A and p7B, encoded by two small centrally-located ORFs (Genoves et al., 2009, 2010; Serra-Soriano et al., 2014). The ORF at the 3´end encodes coat protein (CP) p42, which is also involved in the systemic transport of the virus and is a symptom determinant. At present, the only resistance found to MNSV in melon is that controlled by single recessive gene *nsv* (Nieto et al., 2006). As with most plant viruses, MNSV systemic spread in melon plants occurs through phloem tissue (Gosalvez-Bernal et al., 2008; Pallas et al., 2011). MNSV is transmitted naturally in soil by the zoospores of the chytrid fungus *Olpidium bornovanus* (Lange and Insunza, 1977) when it attaches to the spore outer covering (Ohki et al., 2010). Studies previously performed in our laboratory have shown that melon roots act as a reservoir for MNSV (Gosálvez et al., 2003).

The present study aimed to characterize the molecular mechanisms that underlie the basal and Hx-IR in melon plants inoculated with MNSV. Although hormonal homeostasis has been studied in different plant-virus interactions, most of these studies have been carried out at the tissue-specific level, with very few done at the whole-plant level. We herein investigated the hormonal response in MNSV-infected melon plants in cotyledons, roots, hypocotyls, stems and leaves. We also demonstrated the efficacy of priming agent Hx for preventing virus systemic movement. Our results revealed an interesting correlation among callose deposition, oxygen reactive species (ROS) environment and hormone homeostasis in this pathosystem, and contribute to understand host defenses in response to viruses beyond model species. They also provide valuable evidence to unravel the complex

mechanisms of the priming phenomenon by natural compounds and reveal the role of OPDA, in addition to SA, pathogen-induced callose deposition and H_2O_2 accumulation in the local response to prevent the virus from spreading.

MATERIALS & METHODS

Virus source

The MNSV-Al isolate, used herein, was originally obtained from field melon plants (*Cucumis melo* L.) collected in Murcia (Spain), and was kindly provided by Dr. F. Botella (Miguel Hernandez University, Alicante, Spain) (Gosálvez *et al.*, 2003). MNSV-infected leaf tissue was homogenized with 30 mM phosphate buffer (pH 7.0) that contained 20 mM mercaptoethanol, and the crude extract was used as an inoculum. Subsequently the virus was purified, essentially as described by Díez *et al.* (1998) for carmoviruses, and was used to propagate infection by serial mechanical transmissions to melon plants (*Cucumis melo* L. "galia").

Plant material and bioassays

Thirteen inoculation plus sampling experiments were performed. Each experiment consisted in growing 36 melon plants in 12 different pots (3 plants per pot), of which 10 pots (30 plants) were mechanically inoculated in the stage when there was no true leaf present. The other 2 pots (6 plants) were either mock-inoculated or kept healthy as controls.

Bioassays were performed in a climatic chamber with a day/night period of 16/8 h. Temperatures were 25 ºC and 18 ºC, respectively, with irradiance of 210 µmol photons m-2 s-1. The mock-inoculated and healthy control plants were similarly treated.

Hx treatments

To test the effectiveness of Hx as an inducer of resistance in melon plants against MNSV, three experiments were performed. In the first one, Hx acid was applied by foliar sprays at 16 mM to saturation, by soil drench with 1 L of Hx at 4 mM at 3 and 1 days before infection, and 24 h after infection as a curative treatment. For the second experiment, we selected two sequential Hx treatments (25 mM) applied by soil drench at 72 h and 24 h before MNSV inoculation, and another application at 24 h post-inoculation. In the third experiment, we selected the same experiment template, but removed the post-inoculation treatment.

H_2O_2 determination, microscopy analysis and quantification

Samples of 10 cotyledons were collected for 3'3-diaminobenzidine (DAB) staining at 3, 24 and 48 hpi. Cotyledon and leaves were cut and placed immediately in DAB 1 mg mL^{-1} at pH<3 for 24 h in the dark. They were subsequently destained in 96% ethanol and rehydrated in distilled water. DAB staining intensities were quantified in micrographies by the number of dark brown DAB pixels compared to the total pixels that corresponded to the plant material using the GIMP program (version 2.6.12).

Callose deposition and Co-localization

Callose deposition in response to MNSV infection was quantified from the digital photographs of the aniline blue-stained cotyledons, as described by Flors et al. (2007). Observations were made with a Leica IRB epifluorescence microscope equipped with a Leica DC300F camera (Leica Microsystems, http://www.leica-microsystems.com). The yellow spots that

corresponded to the stained callose were analyzed for number of pixels with the GNU Image Manipulation program (GIMP, http://www.gimp.org/). Callose intensity was expressed as the % of yellow pixels per total pixels on the digital photographs of the infected areas (n=10). For the double staining of H_2O_2 and callose, plant material was stained with DAB, and was subsequently stained with aniline-blue, as described above.

Gene expression analysis by quantitative real time-polymerase chain reaction (qRT-PCR)

Gene expression by qRT-PCR was performed with the RNA samples extracted from leaf tissue using the Total Quick RNA cells and tissues kit (E.Z.N.A. mini kit; http://omegabiotek.com), according to the manufacturer's instructions. The melon tissues samples for RNA isolation were collected at 24 hpiand 5dpi. The leaf tissue from 10 plants each of the NH_4^+-treated and untreated plants was collected. Then 1.5 µg of total RNA was digested using 1 unit of RNase-Free DNase (Promega; http://.www.promega.com) in 1 µL of DNase buffer, and up to 10 µL of Milli-Q water, and was incubated for 30 min at 37ºC. After incubation, 1 µL of RQ1 DNase stop buffer was added, and the solution was incubated again for 10 min at 65ºC to inactivate DNase. Highly pure RNA was used for the RT reaction. The RT reaction was performed according to the manufacturer's instructions included in the Omniscript reverse transcriptase kit (QIAGEN; http://www.qiagen.com/). The reaction mixture was incubated at 37ºC for 60 min. Forward and reverse primers (0.3 µM) were added to 12.5 µL of QuantiTect™ SYBR Green PCR reaction buffer (QIAGEN), as were 2 µL of cDNA and Milli-Q sterile water up to a total reaction volume of 25 µL. Quantitative PCR was carried out in a Smart Cycler II (Cepheid; http://www.cepheid.com). A list of the primers used in the qRT-PCR is found in Table S1. The *Actine* gene expression levels

were used as an internal house-keeping control. A melting-curve analysis was performed at the end of the PCR reaction to confirm the purity of the amplified products.

The amplification efficiency for each primer pair was calculated using serial cDNA dilutions. Differences in cycle numbers in the linear amplification phase between the samples from the treated and untreated plants were used to determine differential gene expression. At least three independent experiments were performed to confirm the results. In each one, three biological replicates were used to generate means and to determine statistical significance.

Chromatographic analysis

For the hormonal analysis, fresh material was frozen in liquid N, ground and freeze-dried. Fresh tissue (0.5 g) was immediately homogenized in 2.5 mL of ultrapure water, and a mixture of internal standards ($^{2}H_{6}$-ABA, $^{2}H_{4}$-SA, dihydrojasmonic acid and propylparaben) was added at 100 ng mL^{-1} prior to extraction. After extraction, a 20-µL aliquot was injected directly into the ultra-high-performance liquid chromatography (UPLC) system. Hormone analyses were carried out using aliquid Waters Acquity system (Milford, MA, USA) in a nucleosil ODS reversed-phase column (100mm×2mm i.d.; 5 µm; Scharlab, Barcelona, Spain; http://www.scharlab.es). The chromatographic system was interfaced to Waters TQD triple quadrupole mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software, version 4.1 (Micromass), was used to process the quantitative data from the calibration standards and plant samples.

Statistical analysis

A statistical analysis was carried out using a one-way analysis of variance in the Statgraphics-plus software of Windows V.5 (Statistical Graphics Corp., Rockville, MD, USA). Means were expressed with standard errors and compared using a Fisher's least-significant difference test at the 95% confidence interval. All the experiments were repeated at least 3 times.

RESULTS

Effect of MNSV infection on pathogen-induced callose and the redox environment in melon plants

To understand the plant response in melon plants inoculated with MNSV, cytological observations were made at infection sites. Lesions started to appear on cotyledons at 2 or 3 days post-infection, and had developed at 5 dpi (Fig.1a). Virus systemic movement started at 5 dpi and necrotic symptoms were clearly observed on primary leaves at 12 dpi (Fig. 1b). The effect of MNSV infection on ROS production was also determined by analyzing hydrogen peroxide (H_2O_2) accumulation by the 3,3'-diaminobenzidine (DAB) staining of inoculated cotyledons at 5dpi (Fig. 1c). Dark spots were present mainly around the local infection site, which reflected the oxidative stress associated with this viral infection. A histological analysis, performed by aniline blue staining, demonstrated that cotyledons accumulated pathogen-induced callose at the infection site (Fig. 1e), while no callose was present in the non inoculated plants (data not shown). We also checked the tissue location of H_2O_2 accumulation in relation to callose deposition by DAB and aniline blue double staining. As shown in Fig. 1g, both H_2O_2 and callose accumulated on the outer edge of lesions as part of the plant response to viral infection. The analysis of ROS and callose in leaves at 18 dpi demonstrated that both accumulated in leaves on the edges of infected sites, which is indicative of the activation of these defenses in the

systemic response of melon plants to MNSV (Fig. 1d,f). In this case, H_2O_2 accumulation was clearly less intense than at the local infection sites. Co-location of callose and H_2O_2 was also observed in systemic tissues at 18dpi (Fig. 1h).

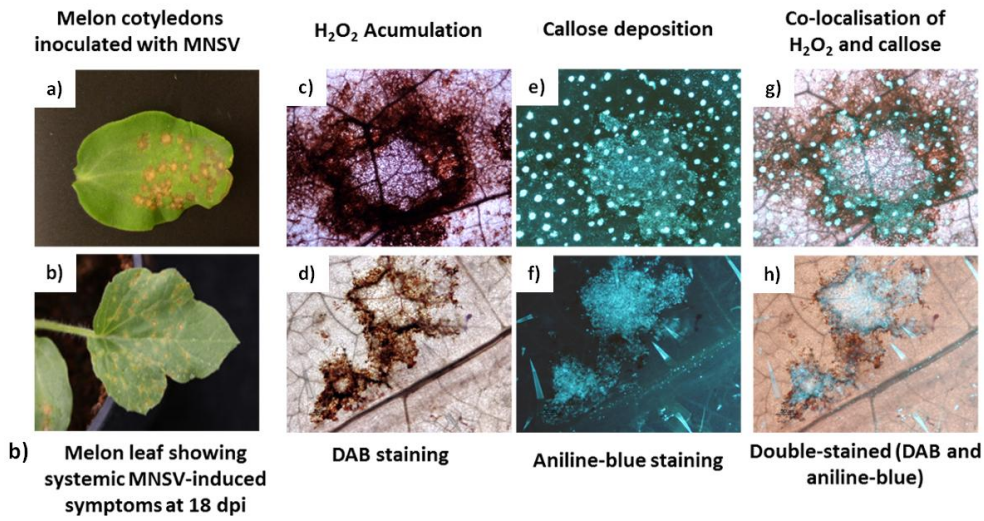


Fig.1. MNSV symptoms in inoculated cotyledons and systemically infected leaves. Localization of H_2O_2 and callose deposition. *C. melo* plants were infected by mechanical inoculation of fully expanded cotyledons with purified virions of MNSV-Al isolate. **(a)** Typical MNSV necrotic lesions in the inoculated cotyledons at 5 dpi. **(b)** Necrotic lesions in the systemic leaves at 18 dpi. **(c)** and **(e)** localization of H_2O_2 accumulation and callose deposition in inoculated cotyledons at 5dpi, respectively. **(g)** Co-localization of H_2O_2 and callose deposition at the sites of infection. **(d)** Localization of H_2O_2 accumulation and **(f)** callose deposition on MNSV infected leaves at 18 dpi. **(h)** Co-localization of H_2O_2 and callose deposition in systemically infected leaves at 18 dpi.

Changes in the accumulation of defense-related metabolites of MNSV-inoculated melon plants

The metabolic analysis during the interaction of melon plants with MNSV showed a different hormonal and metabolite balance according to timing and location. At 5 dpi, when systemic symptoms were still not observed, the metabolic profile in the necrotized inoculated cotyledons reflected a significant increase in SA and ferulic acid compared to the mock-inoculated

tissue. Interestingly, the accumulation of the oxylipin OPDA was associated with reduced JA-Ile content (Fig. 2a).

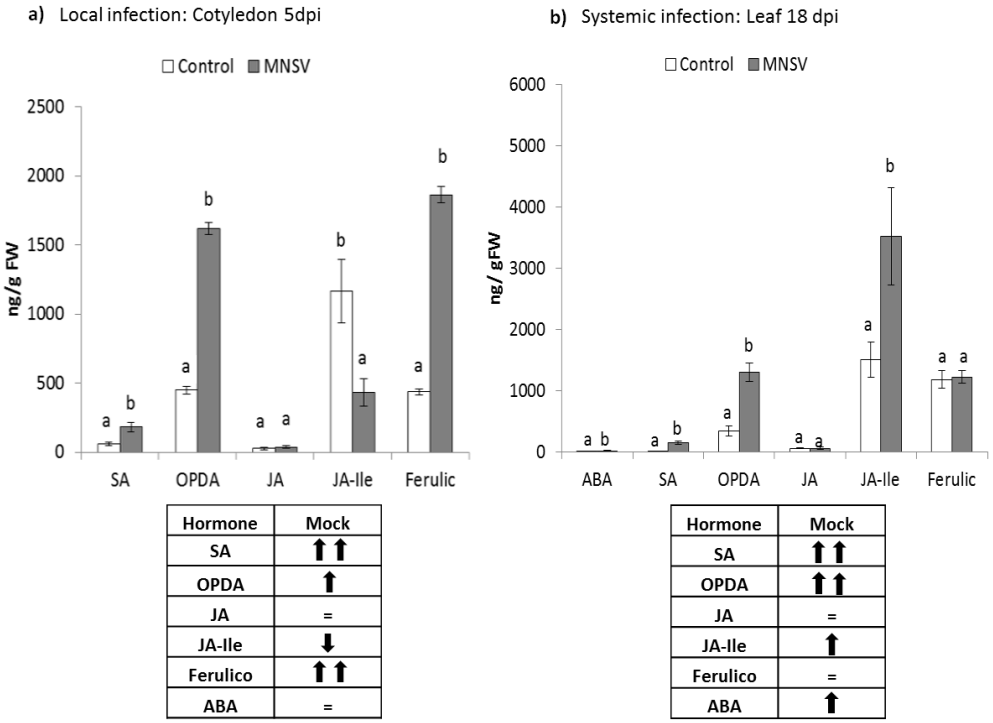


Fig.2. Metabolic profile in melon plants upon MNSV infection. Plants were grown and inoculated as described in Material and Methods section. **(a)** Cotyledons were collected at 5dpi (local infection) and SA, OPDA, JA, JA-Ile and ferulic acid levels were determined by UPLC–MS. **(b)** Leaves were collected at 18dpi (systemic infection) and ABA, SA, OPDA, JA, JA-Ile and ferulic acid levels were determined by UPLC–MS. The concentration of the hormones and metabolites was determined in each sample by normalizing the chromatographic area for each compound with the fresh weight of the corresponding sample. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences between mock and inoculated plants ($p < 0.05$; least-significant difference test).

At 18 dpi, when systemic infection was established and primary leaves showed evident symptoms of necrosis, the metabolic profile in this tissue displayed significant SA and OPDA accumulation, plus a major increase in JA-Ile and slight ABA accumulation (Fig. 2b). These results evidenced the reprogramming of plant metabolism in the MNSV-inoculated plants in local

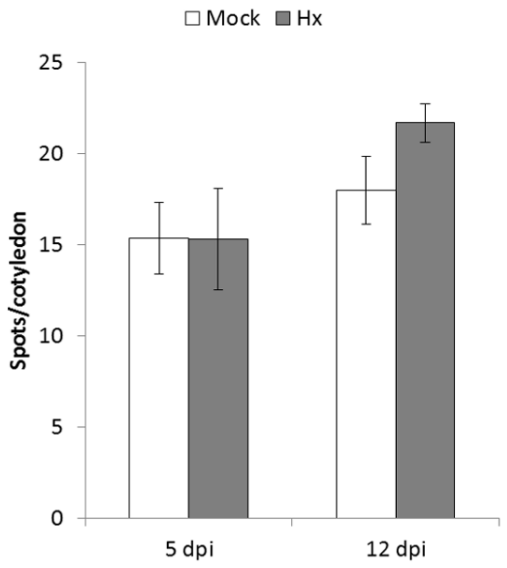
and systemic tissues. These data demonstrated the relevant role of SA in the plant response to this virus, but also the complexity of the hormonal balance and metabolites associated with defense in this pathosystem.

Treating melon plants with hexanoic acid suppresses MNSV systemic movement

a)

Treatment	Spots/cotyledon	% Plants with systemic infection
Mock	17.31	50
Spray	8.25	25
Soil treatment	19.22	0

b)



c)

Treatment	Mock	Hx
% Plants with systemic infection	100	0

Fig.3. Effect of hexanoic acid on melon plants infected with MNSV. **(a)** One week-old melon plants were treated with 25 mM Hx by spray or by soil drench and subsequently mechanically infected with MNSV-Al isolate. Treatments were applied 72 h and 24 h before MNSV inoculation and another additional application 24 hours after inoculation. Severity of infection was expressed as spots/cotyledon (5dpi), and by counting the number of plants with systemic symptoms (18 dpi). **(b)** Quantification of MNSV local infection of melon plants treated with 25 mM Hx applied by soil drench at 72 h and 24 h before MNSV inoculation by counting spots/cotyledon at 5 and 12 dpi, and **(c)** systemically infection by counting plants with symptoms at 18 dpi.

We tested the efficacy of Hx treatment as an inducer of resistance in melon plants against MNSV. Based on previous data of Hx-IR in tomato plants against *B. cinerea* and *P. syringae* (Leyva et al., 2008; Vicedo et al., 2009; Scalschi et al., 2013), we set up Hx treatments in melon plants, which were applied by either foliar spray or soil drench. We checked different concentrations and patterns of treatment applications (Fig. S1). This allowed the selection of two sequential Hx treatments (25 mM) at 72 h and 24 h before MNSV inoculation, and an additional application a 24 hpi. Under these conditions, Hx application by foliar spray led to statistically significant reductions in MNSV local and systemic infection symptoms (Fig. 3a). Interestingly, the Hx treatments by soil drench prevented MNSV systemic infection, with no significant changes noted in local symptoms (spots/cotyledons) (Fig. 3a). Given the importance of this finding, and in order to gain further insight into the biochemical and molecular mechanisms related to Hx-IR against MNSV, we selected soil drench with 25 mM Hx to do further studies. A slight delay in plant growth and chlorotic symptoms observed in the treated plants versus the mock plants were due to the post-inoculation treatment. Once eliminated, treated plants quickly recovered from the mild stress associated with Hx treatment (data not shown). No changes in local infection (spots/cotyledons) were found in the plants treated at 72 h and 24 h before inoculation at 5 and 12 dpi compared to the mock plants (Fig. 3b), but no systemic infection occurred. Thus virus entry into the phloem sap was prevented (Fig. 3c). This was determined by a dot blot analysis at 24 hpi and 5 dpi, when disease symptoms were still not observed in leaves. For further studies, we selected the mock plants in which systemic movement was confirmed at these time points.

Suppression of virus-systemic spread in the Hx-treated plants coupled to enhanced callose deposition and reduced ROS accumulation at the inoculation site

To gain further insight into the nature of Hx-IR against the MNSV of melon plants that suppressed virus systemic movement, cytological observations were made at the local infection sites. Pathogen-inducible callose deposition significantly increased at 5 dpi in the cotyledons of the Hx-treated plants compared to the infected untreated plants (Fig. 4 a, b). This supports a relevant role of callose priming for Hx-IR in melon plants against MNSV, as it has been previously reported in other pathosystems (Aranega et al., 2014).

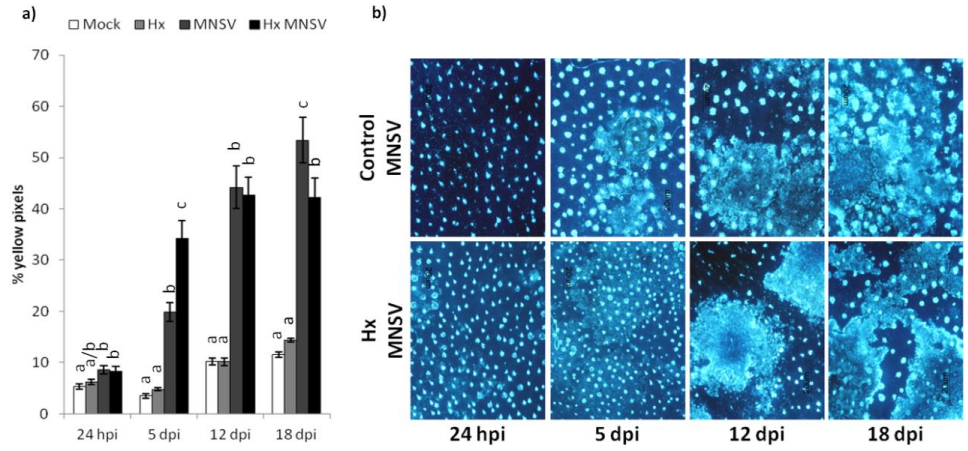


Fig.4. Contribution of callose deposition to Hx-IR against MNSV. Plants were grown and inoculated as described in Fig. 3b. To determine callose deposition in mock and Hx treated plants after MNSV infection melon cotyledons were sampled at 24 hpi and at 5, 12 and 18 dpi and then stained with aniline blue and analyzed by epifluorescent microscopy. **(a)** Quantification performed by determining the % of yellow pixels related to total pixels of photographs. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate significant differences between treatments in each time point ($p < 0.05$; least-significant difference test). **(b)** Representative pictures of callose deposition in mock (control MNSV) and Hx-treated plants upon MNSV infection (Hx MNSV).

Hx treatment did not boost callose deposition when infection was absent (Fig. 4a), which demonstrates the priming effect of the inducer on this defense mechanism.

The effect of Hx treatment on pathogen-induced ROS production was also determined. It showed that hydrogen peroxide (H_2O_2) accumulation reduced in the inoculated cotyledons at 5 dpi compared to the infected untreated plants (Fig. 5a).

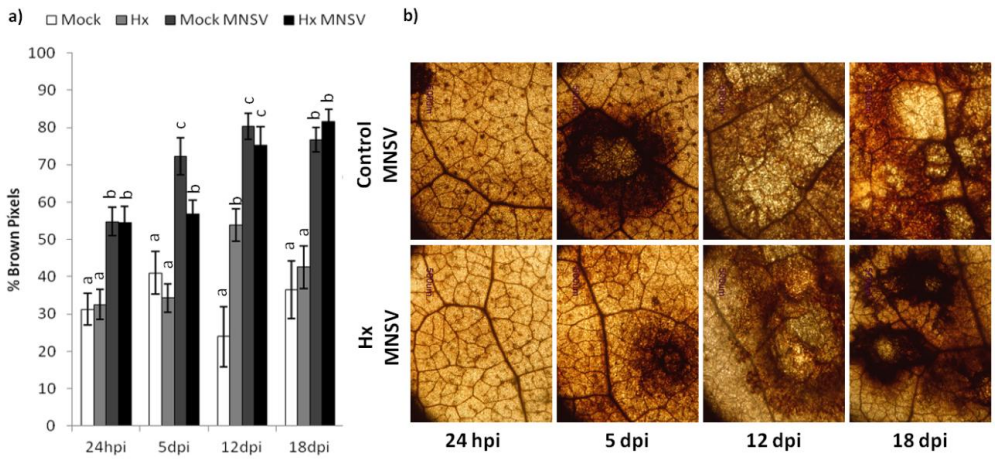


Fig.5. Effect of Hx treatment on the ROS production after MNSV infection. Plants were grown and inoculated as described in Fig. 3b. Melon cotyledons were sampled at 24 hpi and at 5, 12 and 18 dpi and then H_2O_2 accumulation was visualized by DAB staining. **(a)** Quantification was performed by determining the number of brown pixels on digital photographs. Data show average values ± SE (n = 10) of the relative number of brown pixels per photograph. Letters indicate significant differences between treatments in each time point ($p < 0.05$; least-significant difference test). **(b)** Representative photographs of H_2O_2 accumulation taken in mock and Hx-treated plants upon MNSV infection.

This alteration in ROS production was also associated with Hx-IR in the tomato plants infected with *B. cinerea* (Finiti et al., 2014). Hx treatment did not alter the co-location of both defensive responses (Fig. S2). Therefore, it seemed that the inducer prevented virus systemic movement by priming

pathogen-induced callose and reducing H_2O_2 accumulation at the infection site.

Changes in the main signaling pathways at the priming onset and the response of the Hx-treated melon plants against MNSV

The analysis of the Hx-treated melon plants in the absence of infection revealed slight metabolic changes, which were associated with priming effect establishment (Table 1). A significant reduction in JA-Ile and SA was observed at 24 hpi in hypocotyls in this priming phase. At this time point, Hx treatment induced a JA accumulation in the cotyledon as well as SA, JA and ferulic acid increase in roots. At 5 dpi, OPDA and SA increased in cotyledons, while ferulic acid slightly increased in hypocotyls and roots, and SA slightly increased in roots at this point. Hence the metabolic reprogramming at priming onset in melon plants was associated mainly with changes in SA and oxylipin-signaling. Ferulic accumulation also suggested a cell wall reinforcement target. Upon infection, the metabolic profile changed as part of the basal resistance and Hx-IR. Hx-MNSV increased OPDA, JA-Ile and ferulic acid in cotyledons at 24 hpi (Fig. 6a). One of the most significant biochemical changes noted in the early response (24 hpi) of MNSV plants was the reduction in the free SA levels in roots and hypocotyls (Fig. 6 b,c). SA, JA and ferulic acid increased in hypocotyls, but JA-Ile reduced in the roots of Hx-MNSV plants at 24 hpi (Fig. 6 b,c).

Table 1. Hormonal changes induced by Hx treatment at 24 hpi and 5 dpi.

	24hpi			5 dpi		
Cotyledon	**Mock**	**Hx**		**Mock**	**Hx**	
SA	22,5 ± 4,3	43,5 ± 19,9		91,27 ± 23,31	21,15 ± 14,08	↑
OPDA	92 ± 15,5	158,9 ± 60		nd	102,49 ±16,88	↑
JA	27,7 ± 1,3	50,5 ± 1,1	↑	9,74 ± 0,83	9,73 ± 1,08	
JA-Ile	622,9 ± 52,2	672,7 ± 215,7		1013,47 ± 94,61	712,66 ± 211,87	
Ferulic acid	117,4 ± 19,7	241,9 ± 75		461,91 ± 50,86	515,27 ± 50,56	
ABA	1,3 ± 0,3	nd		nd	nd	
Hypocotyl	**Mock**	**Hx**		**Mock**	**Hx**	
SA	132,92 ± 24,05	11,15 ± 6,46	↓	82,42 ± 17,25	21,15 ± 14,08	
OPDA	11,87 ± 1,87	nd		41,18 ± 6,39	102,49 ±16,88	↓
JA	39,01 ± 11,67	54,20 ± 1,97		13,78 ± 3,32	9,73 ± 1,08	
JA-Ile	1794,66 ± 423,09	705,33 ± 166,29	↓	16,26 ± 5,17	6,80 ± 2,08	
Ferulic acid	27,25 ± 7,37	93,45 ± 39,48		23,35 ± 11,37	135,15 ± 39,12	↑
ABA	nd	nd		nd	nd	
Root	**Mock**	**Hx**		**Mock**	**Hx**	
SA	64,28 ± 3	106,43 ± 4,43	↑	17,92 ± 7,92	63,85 ± 13,85	↑
OPDA	10,58 ± 2,3	nd		nd	nd	
JA	nd	21,21 ± 1,60		8,69 ± 1,18	2,62 ± 1,36	
JA-Ile	367,26 ± 27,86	278,24 ± 75,82		190,35 ± 102,57	136,074 ± 53,67	
Ferulic acid	55,88 ± 0,88	107,05 ± 33,33		46,37 ± 0,82	97,55 ± 28,29	↑
ABA	nd	nd		nd	nd	

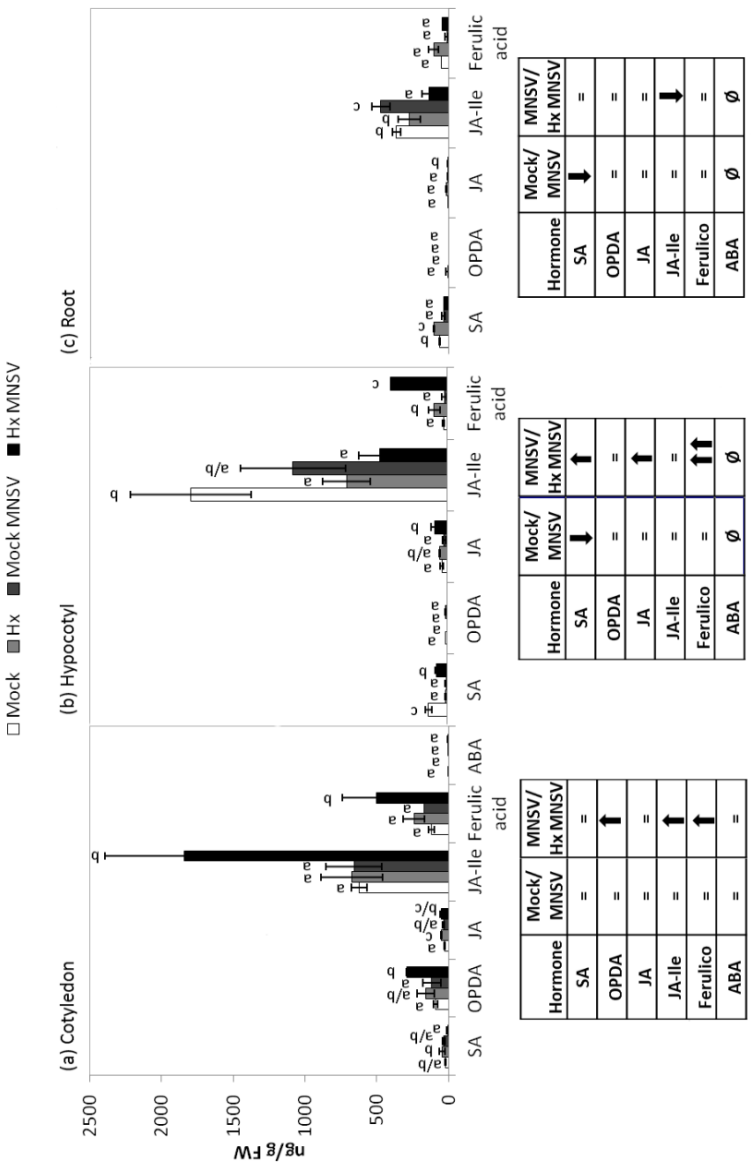


Fig.6. Metabolic profile in mock and Hx treated plants upon MNSV infection at early stages of infection. Plants were grown and inoculated as described in Fig. 3b. Different tissues were collected at 24 hpi and SA, OPDA, JA, JA-Ile, ferulic acid and ABA levels were determined by UPLC–MS. **(a)** Cotyledon, **(b)** hypocotil and **(c)** root metabolic profile**.** The concentration of the hormones and metabolites was determined in each sample by normalizing the chromatographic area for each compound with the fresh weight of the corresponding sample. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences for each hormone ($p < 0.05$; least-significant difference test).

At 5 dpi, MNSV plants displayed accumulated SA in cotyledons and roots (Fig. 7 a,c). The accumulation of OPDA in MNSV cotyledons, roots and hypocotyls at this time point was noteworthy. Ferulic acid accumulated in both cotyledons and roots, while JA-Ile reduced in cotyledons and increased in hypocotyls, and JA increased in roots. Hx-MNSV plants enhanced OPDA accumulation in cotyledons and roots, but it lowered in hypocotyls *vs.* MNSV plants (Fig. 7 a,b,c). SA reduced in the roots and hypocotyls of the Hx-MNSV plants, while JA increased in hypocotyls (Fig. 7 a,b,c). At 12 dpi (Fig. 8), when systemic infection was established in MNSV plants, leaves and stems showed increased SA. Leaves also had increased ferulic acid and ABA content, while stems had increased JA-Ile (Fig 8c). At this time, cotyledons showed highly increased SA, JA and ferulic acid, along with OPDA and ABA, but JA-Ile decreased (Fig 8a). Roots had increased JA and JA-Ile (Fig. 8d). Interestingly in this infection step, cotyledons of the Hx-MNSV plants, in which no systemic movement took place, completely necrotized and considerably accumulated OPDA, but displayed reduced SA compared to MNSV (Fig, 8a). Leaves and roots of Hx-MNSV plants, which exhibited no virus movement, reflected reduced JA and JA-Ile compared to the MNSV plants. The Hx-MNSV leaves also contained low SA, ferulic acid and ABA (Fig. 8c).

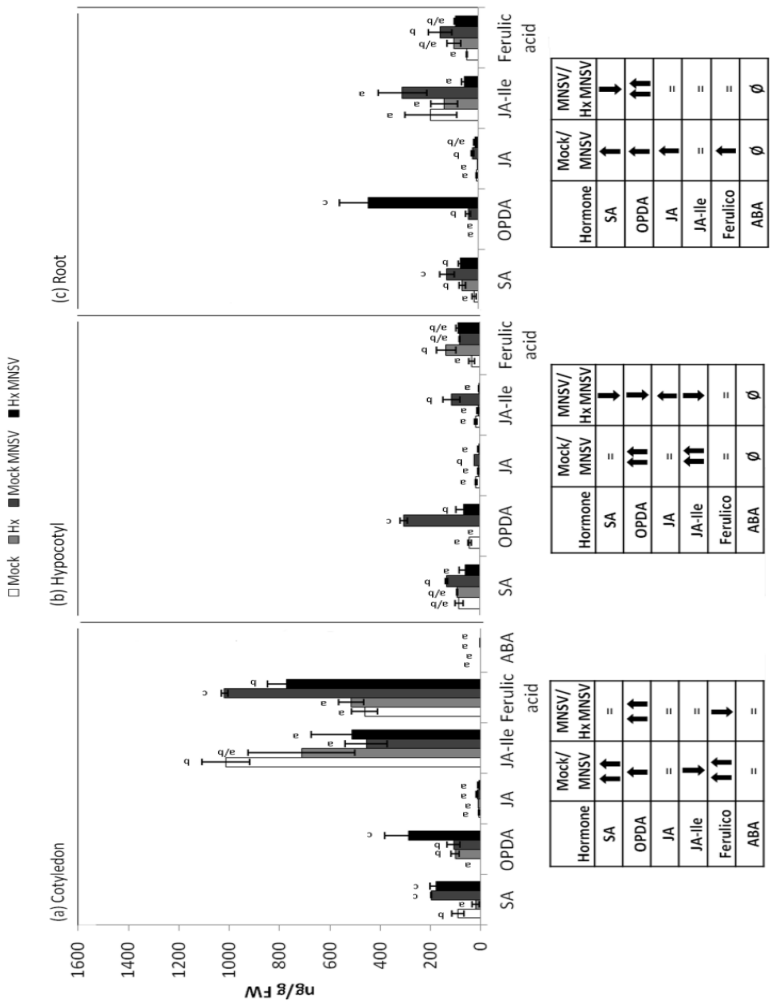
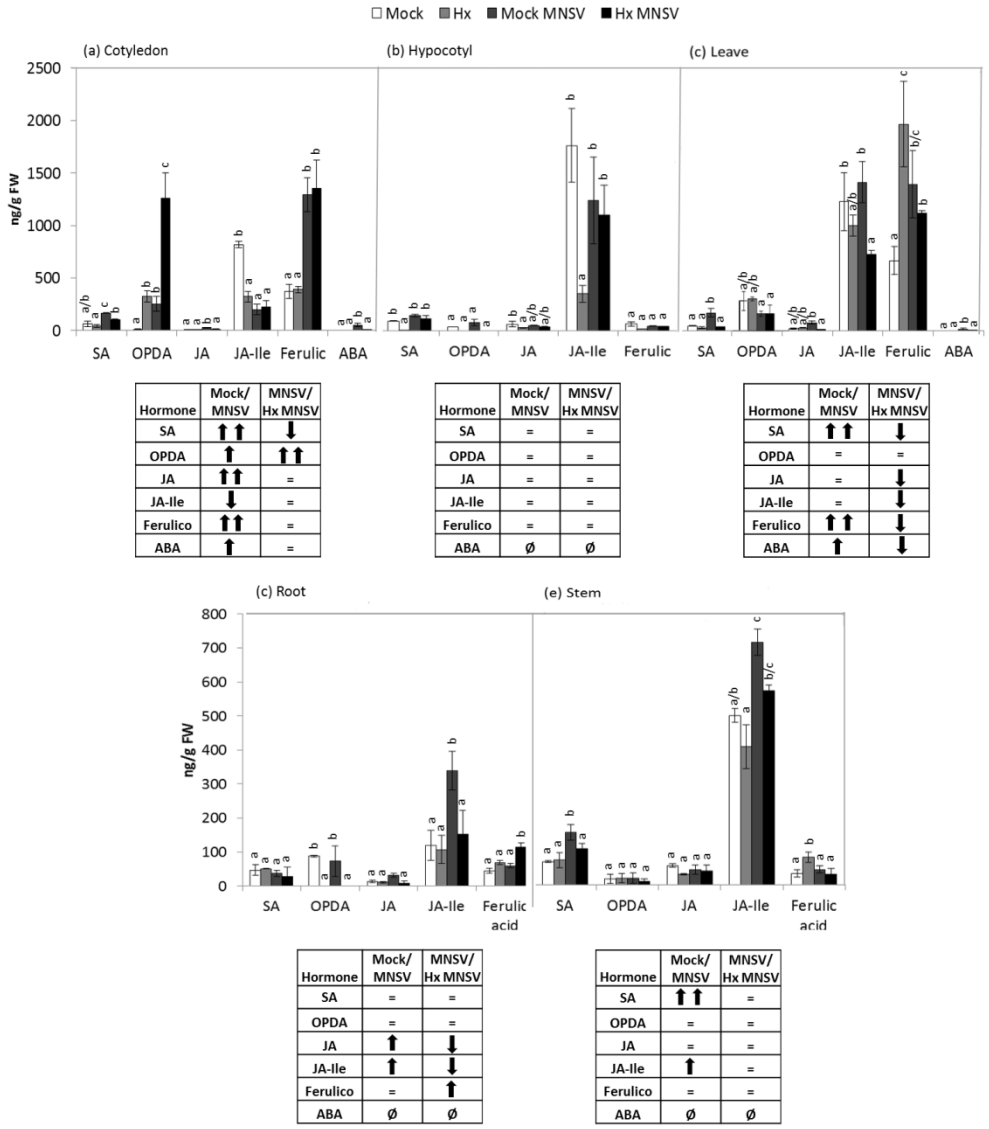


Fig.7. Metabolic profile in mock and Hx treated plants at 5 days after MNSV infection. Plants were grown and inoculated as described in Fig. 3b. Different tissues were collected at 5 dpi and SA, OPDA, JA, JA-Ile, ferulic acid and ABA levels were determined by UPLC–MS. **(a)** Cotyledon, **(b)** hypocotil and **(c)** root metabolic contents**.** The concentration of the hormones and metabolites was determined in each sample by normalizing the chromatographic area for each compound with the fresh weight of the corresponding sample. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences for each hormone ($p < 0.05$; least-significant difference test).



Hormone	Mock/ MNSV	MNSV/ Hx MNSV
SA	↑↑	↓
OPDA	↑	↑↑
JA	↑↑	=
JA-Ile	↓	=
Ferulico	↑↑	=
ABA	↑	=

Hormone	Mock/ MNSV	MNSV/ Hx MNSV
SA	=	=
OPDA	=	=
JA	=	=
JA-Ile	=	=
Ferulico	=	=
ABA	Ø	Ø

Hormone	Mock/ MNSV	MNSV/ Hx MNSV
SA	↑↑	↓
OPDA	=	=
JA	=	↓
JA-Ile	=	↓
Ferulico	↑↑	↓
ABA	↑	↓

Hormone	Mock/ MNSV	MNSV/ Hx MNSV
SA	=	=
OPDA	=	=
JA	↑	↓
JA-Ile	↑	↓
Ferulico	=	↑
ABA	Ø	Ø

Hormone	Mock/ MNSV	MNSV/ Hx MNSV
SA	↑↑	=
OPDA	=	=
JA	=	=
JA-Ile	↑	=
Ferulico	=	=
ABA	Ø	Ø

Fig.8. Metabolic profile in mock and Hx treated plants upon MNSV infection at late stages of infection. Plants were grown and inoculated as described in Fig. 3b. Different tissues were collected at 12 dpi and SA, OPDA, JA, JA-Ile, ferulic acid and ABA levels were determined by UPLC–MS. **(a)** Cotyledon, **(b)** hypocotil and **(c)** leaf**, (d)** root and **(e)** stem metabolic contents**.** The concentration of the hormones and metabolites was determined in each sample by normalizing the chromatographic area for each compound with the fresh weight of the corresponding sample. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences for each hormone ($p < 0.05$; least-significant difference test).

Changes in gene expression during the basal and Hx-primed response of melon plants to MNSV

In order to verify the connection between the basal response and Hx priming with callose metabolism, we analyzed the expression of the *CalSS* gene by a real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). In cotyledons, the transcript levels for the callose synthase gene were induced following MNSV inoculation, which significantly increased at 5 dpi (Fig. 9a). No changes were observed in roots upon infection. However, the infected Hx-treated plants contained increased *CalSS* in roots at 24 hpi and 5 dpi, while the expression of this gene reduced in cotyledons. The transcript that coded for β-1,3-Glucanase was induced in cotyledons at 24 hpi and at 5 dpi. Hx-treatment increased this transcript at 24 hpi in roots, but reduced it in cotyledons (Fig. 9b). *PDLP6*, which coded for a protein involved in callose deposition at plasmodesmata, was induced in cotyledons at 5 dpi after MNSV infection (Fig. 9c). Hx-priming increased this transcript at 5 dpi, but reduced it in cotyledons. These findings support the notion that Hx-priming alters the transcriptional regulation of callose metabolism.

In order to gain insight into the complexity of the hormonal network found herein, we analyzed the expression pattern of the marker genes for SA (*ICS2*) and oxylipins (*AOS, HSP17.4, JAZ10* and *COI1*) by RT-qPCR in both roots and cotyledons at 24 hpi and 5 dpi (Fig. 9 and 10). We also determined the expression of *WRKY70* in relation to SA-JA cross-talk: pathogen-responsive gene *PAL* involved in phenylpropanoids metabolism; *rbohC* involved in oxidative burst and *GST* for ROS detoxification.

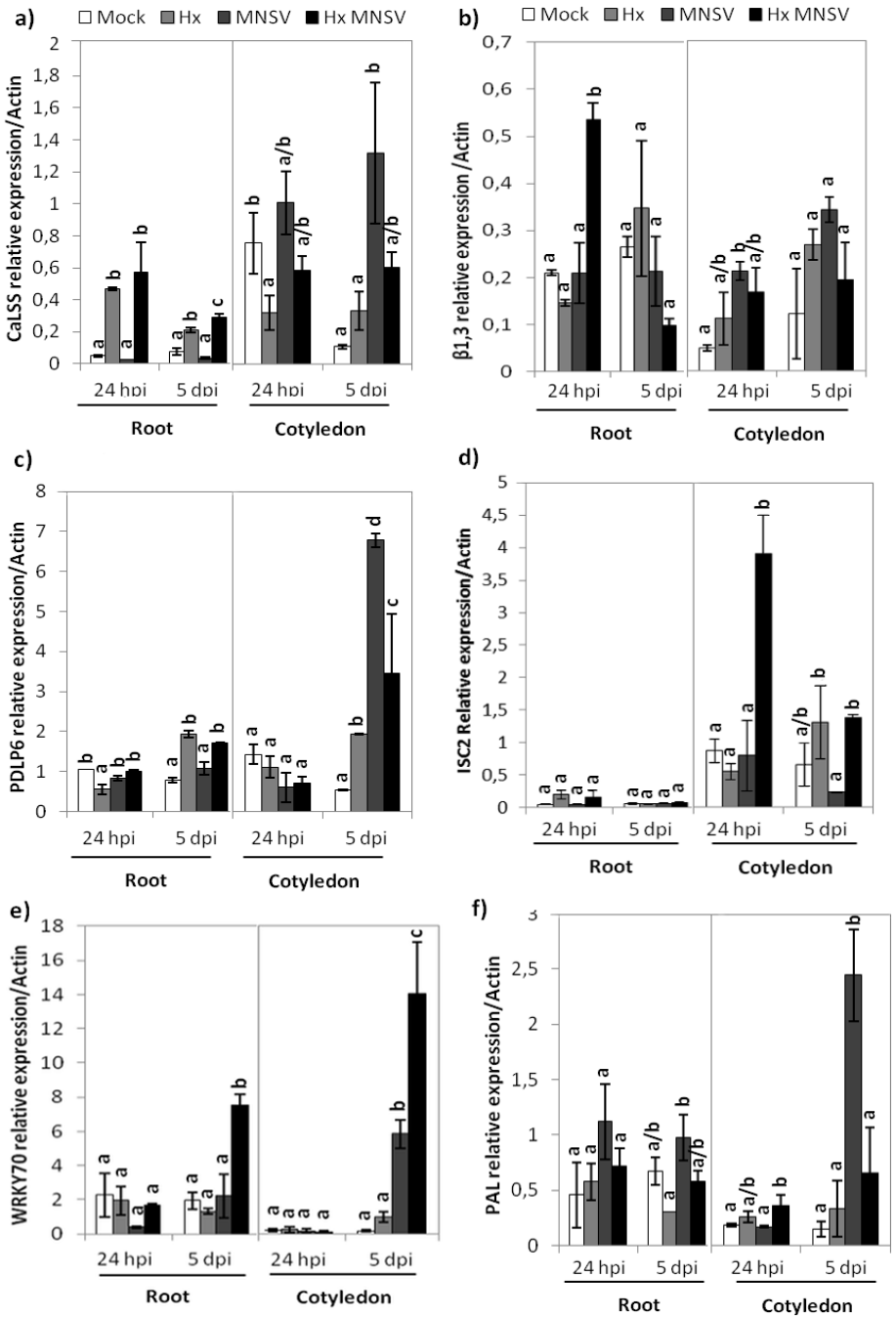


Fig.9. Expression profile of genes involved in plant defence pathways in mock and Hx-treated plants upon MNSV infection. Plants were grown and inoculated as described in Fig. 3c. Total RNA was isolated from roots and leaves at 24 hpi and 5 dpi, converted to cDNA and subjected to qRT-PCR analysis. Results were normalized to the Actin gene expression measured in the same sample. The expression of genes representing key

components of callose metabolism: **(a)** *CaLSS*, **(b)** *β-1-3 endoglucanase* and **(c)** *PDLP6*; SA signaling pathways: **(d)** *ICS2*; phenilpropanoid metabolism: **(e)** PAL and **(f)** WRKY70 trascriptor factor were analysed. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences between treatments in each time point ($p < 0.05$; least-significant difference test).

ICS2, involved in SA synthesis, was not induced by MNSV in cotyledons or roots, despite this hormone accumulating at 5 dpi in both tissues. The Hx-MNSV cotyledons considerably increased this transcript at 24 hpi (Fig 9d). *WRKY70* was also strongly induced in the roots and cotyledons of the Hx-MNSV plants at 5dpi (Fig. 9e). Collectively, these results suggest the involvement of the SA-pathway in Hx-IR against MNSV. The *PAL* transcript increased at 5 dpi in the MNSV cotyledons, but reduced in Hx-MNSV (Fig 9f).

Regarding oxylipins, we observed that MNSV activated this pathway in cotyledons by inducing *COI1* at 24 hpi and 5dpi in cotyledons, *JAZ10* at 24 hpi in roots and at 5 dpi in cotyledons. The Hx-MNSV plants reduced *COI1* and *JAZ10* at 5 dpi in cotyledons and at 24 hpi in roots (Fig. 10 b,c). These results reflect the attenuation of JA-signaling by Hx. *HSP17.4,* a marker of OPDA, was induced in the Hx-MNSV roots only at 24 hpi (Fig. 10d). This supports the relevance of OPDA in Hx-IR, and reflects the complexity of melon basal and induced resistance against MNSV. The analysis of the genes related to oxidative environment showed the induction of *GST* at 5dpi in the cotyledons of the MNSV-infected plants, which Hx primed at 24 hpi and boosted at 5 dpi in the Hx-MNSV cotyledons (Fig 10e). The *rbohC* transcript also increased in the Hx-MNSV cotyledons at 5 dpi (Fig. 10f).

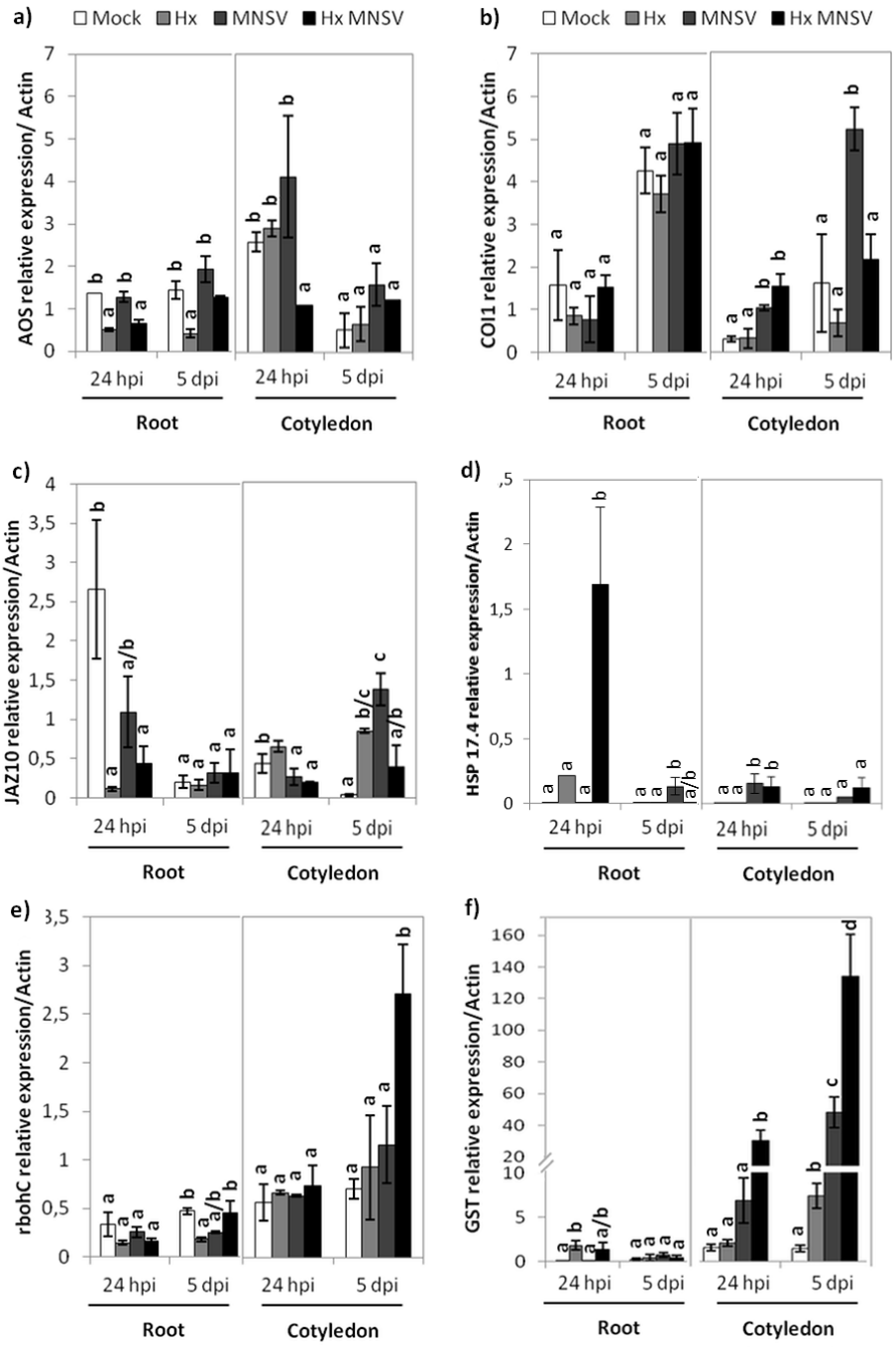


Fig.10. Expression profile of genes involved in plant defence pathways in mock and Hx-treated plants upon MNSV infection. Plants were grown and inoculated as described in Fig. 3c. Total RNA was isolated from roots and leaves at 24 hpi and 5 dpi, converted to cDNA and subjected to qRT-PCR analysis. Results were normalized to the Actin gene

expression measured in the same sample. The expression of relevant genes of the oxylipins metabolism: **(a)** *AOS*, **(b)** *COI1*, **(c)** *JAZ10* and **(d)** *HSP17.4*; and markers of oxidative metabolism: **(e)** *rbohC* and **(f)** *GST* were analysed. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Letters indicate statistically significant differences between treatments in each time point ($p < 0.05$; least-significant difference test).

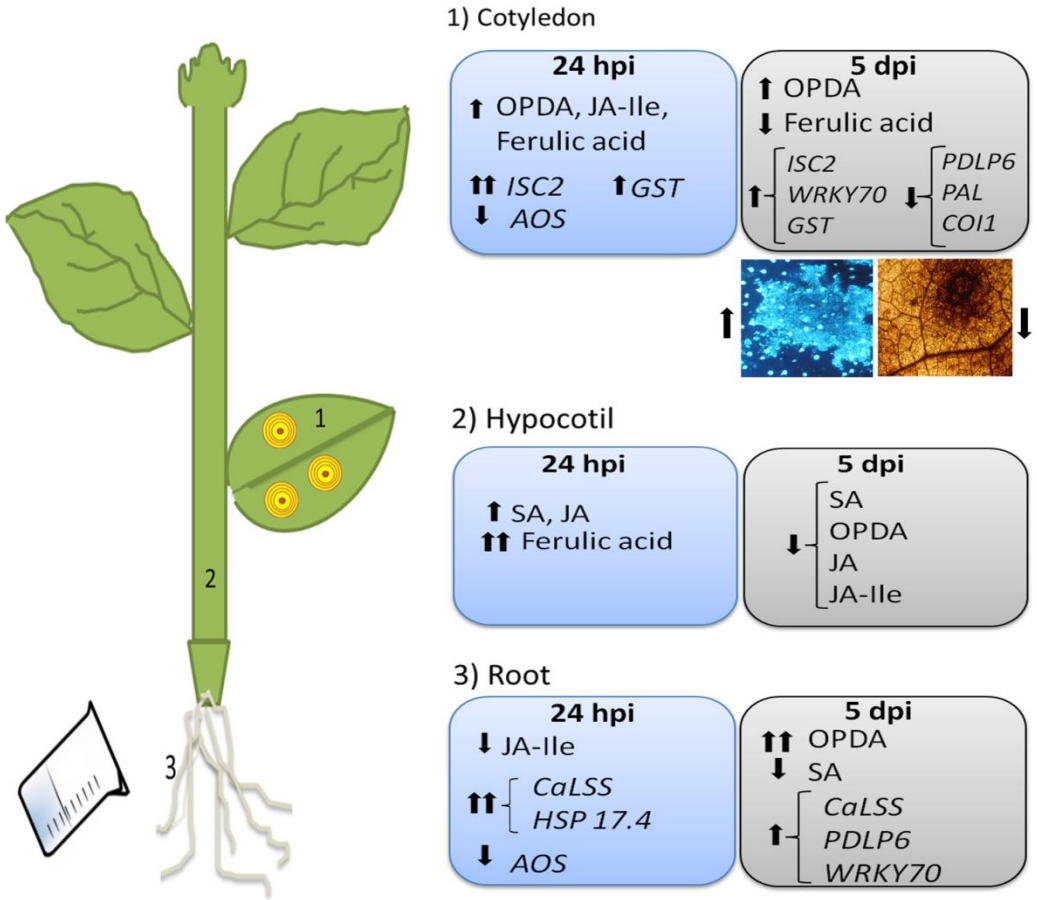


Fig.11. Model for Hx-IR in melon plants against MNSV. Hx treatment induced several changes that impede the systemic spread of MNSV. At early stages of infection (24 hpi), a significant OPDA, JA and ferulic acid accumulation occurred in cotyledons in addition to a high induction of *ICS2*, *AOS* and *GST* genes. In hypocotil, Hx induced SA, JA, and ferulic acid accumulation. Upon infection, Hx-treated roots displayed a reduction of JA-Ile and the *AOS* inhibition along with a strong induction of *CalSS* and *HSP17.4* genes, involved in the callose metabolism and OPDA-signaling respectively. Moreover, at a second phase of infection (5 dpi), when MNSV moves to the phloem sap, Hx primed callose accumulation

and the reduction of H_2O_2 accumulation at the infection site, that contributed to block the systemic movement of the virus. Hx also primed OPDA in the infected local tissue and a reduction of ferulic acid. Regarding the marker genes, treated and infected cotyledons displayed a high induction of *ICS2, WRKY70* and *GST*, but a reduction of the expression of *PAL, PDLP6* and *COI1*.

DISCUSSION

In this study we have analyzed the mechanisms of resistance of melon plants against MNSV infection and the effectiveness and mode of action of Hx-IR in melon-MNSV interaction. MNSV infects a narrow range of host plants, being largely restricted to species belonging to the Cucurbitaceae family. In melon, the virus causes necrotic brown lesions in the inoculated cotyledons and, as the infection progresses, the virus systemically infects the first leaf, also producing necrotic spots and necrosis in the stem (Bellés et al., 2008). First, we analyzed the basal response of melon plants to MNSV inoculation in cotyledons at 5 dpi and in leaves at 18 dpi. Melon plants responded to MNSV infection increasing callose deposition and H_2O_2 accumulation and changing the hormone homeostasis.

Callose accumulation is important in the plant response against virus infection (Li et al., 2012). Cell-to-cell movement of a plant virus through plasmodesmata (PD) is a prerequisite for systemic infection (Luca et al., 2004). Callose deposition decreases the permeability of PD and therefore virus movement through PD (Luna et al., 2011; Zavaliev et al., 2011). Our results confirm the relevance of this defense polysaccharide in the local and systemic response of melon plants to MNSV. Another common response after virus perception is the H_2O_2 accumulation. In plant-virus incompatible interactions, the generation of H_2O_2 is associated with resistance to virus (Hafez et al., 2012). However, in compatible interactions the increase of H_2O_2 is more noteworthy in the virus susceptible plant than in the resistant plant (Díaz-Vivanco et al., 2006). In our experimental conditions, we

observed a significant H_2O_2 accumulation both in local and in systemic tissues. Moreover, we demonstrated the co-location of callose and H_2O_2 in melon-MSNV interaction as previously described in other pathosystems such as Tomato-*B. cinerea* (Angulo et al., 2015). The analysis of the hormonal responses to MNSV revealed that MNSV-infected cotyledons (local response) displayed higher SA, OPDA and ferulic acid accumulation than healthy plants. Melon leaves with systemically disease symptoms, displayed a different hormonal balance showing an increase of SA, OPDA and JA-Ile accumulation. It´s commonly accepted that in compatible interaction the over-expression of SA biosynthesis genes or application of SA (or its analogues) often improves plant basal immunity by delaying the onset of viral infection and disease establishment (Ishihara et al., 2008; Mayers et al., 2005; Peng et al., 2013). It highlights the accumulation of the oxylipin OPDA in local and systemic tissue of MNSV-infected melon plants. OPDA, a precursor of JA can act in a JA-independent manner (Park et al., 2013). Little is know about JA-Ile and OPDA functions in plant-virus interaction, but recent reports demonstrated the important role of OPDA in the plant response to other pathogens with different infection strategies like *B. cinerea* and *P. syringae* (Vicedo et al., 2009; Scalschi et al., 2013). In addition, recent reports showed the role of OPDA in the pathogen-induced callose accumulation (Scalschi et al., 2015). Based on these evidences, we speculate that OPDA *per se* migh have an important role in the basal response of melon plants against MNSV.

In this work we also studied the induced resistance against MNSV in melon plants. We analyzed the efficacy of Hx, a potent natural priming agent in a wide range of host plants and pathogens (Aranega et al., 2014), in this pathosystem. Treatment of melon plants by soil drench with 25 mM Hx at 72 and 24 hour before inoculation prevented the systemic movement of MNSV,

confining the virus at the inoculation site. Based on the efficacy of the Hx-IR in these experimental conditions, we investigated the mechanisms underlying the priming effect against MNSV.

Hx-treated melon plants displayed an increase of callose accumulation in cotyledons at 5 dpi, if compared with mock plants after MNSV infection. This supports the role of callose in the local plant response to viruses and confirms the relevant role of this defensive polymer in the Hx-IR (Aranega et al., 2014). Seo et al. (2014) demonstrated that soybean plants treated with 2-deoxy-D-Glc, a callose synthesis inhibitor, resulted in the failure to arrest viruses at initial inoculated sites. To go in depth on the relationship between callose deposition and melon basal and Hx-induced resistance, we analyzed the expression pattern of genes related to callose metabolism as *CalSS* (Vaten et al., 2011) and *PDLP6* (Lee et al., 2011) in roots and cotyledons at 24 hpi and 5 dpi. We found that *CaLSS* and *PDLP6* induced in cotyledons of MNSV-infected plants at 5 dpi when pathogen-induced callose is accumulated. However, the increase in callose accumulation associated to the Hx-IR was not associated with the local induction of both genes upon infection. MNSV did not induce this gene in roots but Hx treatment primed this gene at 24 hpi and 5 dpi. The fast activation of *CaLSS* in roots and the high callose accumulation in cotyledon suggest the complexity of the regulation of this structural defenses and reveals that primed callose deposition induced by Hx could contribute to the Hx-IR against MSNV infection.

In previous studies it was shown that Hx priming influences the plant oxidative environment (Finiti et al., 2014; Camañes et al., 2015).We analyzed H_2O_2 production in local and systemic tissue which is usually produced during plant-virus compatible interactions (Riedle-Bauer et al., 2000) altough the cell responses regulated by H_2O_2 are largely unknown (Xu et al., 2011).

We observed that MNSV infection was associated with a significant H_2O_2 accumulation in cotyledons. At 5 dpi, Hx-treated and infected plants accumulated less H_2O_2 than untreated plants. These results suggested that Hx priming might reduce the oxidative damaged associated to viral infection in melon plants as previously demonstrated in tomato plants challenged with *B. cinerea* (Finiti et al., 2014). Interestingly, the timing of the reduction in ROS accumulation in Hx-primed plants correlates with that of the increase in callose accumulation in the local response, what could contribute to prevent the virus systemic movement. In support of this hypothesis Hx treatment induced *GST* transcript accumulation in cotyledons at 24 hpi and 5dpi relative to untreated plants. This demonstrates that Hx-IR alleviates the oxidative stress induced by MNSV by activating the antioxidant machinery. This finding suggests that Hx priming could act by damping the fluctuations of the redox equilibrium to provide a less stressed environment in melon plants infected by MNSV.

For better understanding of melon defensive mechanisms against MNSV, we analyzed the hormonal profile and gene expression at the whole-plant level. This provided the hormonal homeostasis in melon roots, cotyledons, hypocotyls, stems and leaves involved in the basal and Hx-induced resistance (Hx-IR) to MNSV. These analyses confirmed that the SA/JA-signaling pathways play a role in the basal and inducible defense against MNSV in melon plants. Moreover, in the priming phase, we observed this joint accumulation of SA and JA induced only by Hx treatment in MNSV absence. Althought slight metabolic changes induced by Hx treatment were observed, the metabolic change were particularly important in the roots at 24 hpi since displayed SA, JA and ferulic acid in the absence of infection. These results demonstrate the importance of the root as marker tissue and the priming effect of this natural inducer of resistance, since Hx primed defenses for

faster and stronger activation after the pathogen perception. In addition, we demonstrated the induction of the SA pathway along with the induction of components of the oxylipin pathway in Hx treated and inoculated plants. At 24 hpi, in mock-MNSV infection did not observe metabolic changes. However, faster and stronger accumulation of OPDA, JA-Ile and ferulic acid in cotyledons of Hx-MNSV plants was observed. Moreover, hypocotils of these plants displayed an increase of ferulic acid accumulation. Phenolic compounds play a role in cell wall fortification, and also show antimicrobial and antioxidant activity (Taheri and Tarighi, 2010, 2011). At the first step of melon-MNSV interaction, gene expression analysis revealed that at 24 hpi Hx-MNSV cotyledons accumulated higher levels of *ICS2* and *PAL* transcripts than mock-MNSV plants, revealing the importance of SA and phenilpropanoid pathways in Hx-IR against MNSV. Moreover, we also observed a strongly induction of *HSP17.4*, a marker of OPDA, in roots at the first step of infection in Hx-MNSV plants demonstrating the key role of this oxilipin in Hx-IR against MNSV. These findings reveal that Hx-IR might protect melon plants by the combined effect of SA/JA pathways and by the OPDA accumulation.

At 5 dpi, when the systemically movement of virus started, SA and ferulic acid were accumulated in cotyledons in mock and Hx- treated plants. Besides this, Hx-MNSV plants displayed a significant accumulation of OPDA at cotyledons and roots. Recent studies demonstrated that OPDA might play an intrinsic role in plant defenses and in induced resistance processes in tomato and Arabidopsis against necrotrophic and hemibiorophic pathogens (Vicedo et al., 2009; Kravchuk et al., 2011; Scalschi et al., 2013). Scalchi et al. (2015) observed that the transgenic tomato plants SiOPR3, silenced in the 12-oxophytodienoato reductase 3, displayed reduced levels of OPDA accumulation and increased susceptibility to *B. cinerea.* These authors

demonstrated that this transgenic line was defective in pathogen-callose accumulation and this phenotype was recovered by the addition of exogenous OPDA, revealing its direct impact on resistance against this pathogen. It is now well established that viruses exploit plasmodesmata as cell-to-cell symplastic connections to achieve spreading local and systemic infection in host plants (Benitez-Alfonso et al., 2010). Moreover, SA was recently related with the induction of callose deposition in plasmodesmata to impede the pathogen spread (Wang et al., 2013). Based on these evidences, we are tempting to speculate that a combined effect of SA and OPDA in melon plants might have an essential role in the early callose deposition in cotyledons that impede the MNSV movement to phloem sap.

To gain in depth on the relationship between SA and JA pathway in melon-MNSV interaction, genes markers of these pathways were analyzed. We selected *COI1* as a gene marker of the JA pathway and *WRKY70* as a positive regulator of SA signaling (Li et al., 2004). At 24 hpi, we observed a significant induction of *COI1* upon MNSV infection in mock and in Hx-treated plants. However, at 5dpi Hx-treated and inoculated plants displayed a *COI1* inhibition compared with mock infected plants supporting a repression of the JA-signaling. Moreover, at 5 dpi, Hx-MNSV plants displayed a strong induction of *WRKY70*. In *A. thaliana, WRKY70* transcription factor plays a key role in determining the balance between SA- and JA-dependent defense signaling pathways (Li et al., 2006; Eulgem et al., 2007). Over-expression of *WRKY70* results in constitutive expression of SA-induced PR genes and suppression of JA-induced defense genes, such as *PDF1.2* (Li et al., 2004). Moreover, Ando et al., 2014 demostrated a crucial role of *WRKY70* on the suppression of *Cucumber mosaic virus* in Arabidopsis. In our experimental conditions, the *WRKY70* transcript accumulation in root and cotyledon of Hx-treated at 5dpi and *COI1* inhibition at this time point revealed the activation

of the SA-dependent and repression of the JA-dependent signaling pathways. This fact revealed that Hx primes at the first stage of infection JA-dependent signaling pathways being inhibited at 5dpi. This inhibition correlated with a strongly activation of SA-dependent responses. The importance of this sequential activation of JA and SA-dependent pathways to complete resistance against viral agents was demonstrated by Garcia-Marcos et al.(2013), who observed that JA treatment at early stages of PVY–PVX double infection enhanced resistance, but later application increased susceptibility. Moreover, other work supported this hypothesis since treatments with JA or SA on *N. benthamiana* enhanced the systemic resistance to TMV, and that resistance was further enhanced by pretreatment with JA followed by SA (Zhu et al., 2014).

To complete the global view of the underlying metabolite and genetic changes induced by Hx treatment that impede de MNSV spread, a late stage of infection was analyzed in this study. At 12 dpi we observed that MNSV induced several hormonal changes at cotyledons, as accumulation of SA, OPDA, JA, ferulic acid and ABA. This response is common in susceptible plants, since viral infection results in hormonal disruption, which manifests as the simultaneous induction of several antagonistic hormones (Alazem et al., 2014b). On the other hand, at this time point, hexanoic treated plants did not display major changes in hormonal levels. However, it is important to note that Hx-MNSV plants in which no systemic movement took place displayed high accumulation of OPDA but reduction of SA levels at cotyledons.

In summary, we demonstrated that at early stages of infection Hx primed a significant accumulation of OPDA, JA and ferulic acid and a high induction of *ICS2*, *AOS* and *GST* genes in cotyledons. In hypocotils, Hx induced SA, JA, and ferulic acid accumulation. In the roots, Hx-treated plants after

infection displayed a reduction of JA-Ile levels in addition to the *AOS* inhibition and the strong induction of *CalSS* and *HSP17.4* genes related with callose synthesis and OPDA signaling. It seems clear that the activation of the SA/JA dependent signaling pathways and the OPDA accumulation, jointly with the activation of *CalSS* that might induce the subsequent cotyledon callose accumulation, and the activation of *GST* were key events to reduce the MNSV severity. Moreover, at the second phase of infection, the key moment for MNSV movement to the phloem sap, Hx induced several changes that prevented the spread of MNSV at other melon tissues. For example, at cotyledons Hx induced OPDA accumulation and ferulic acid reduction compared with control plants. Regarding the marker genes, primed cotyledons displayed high induction of *ICS2, WRKY70* and *GST* but reduction of the expression of *PAL*, *PDLP6* and *COI1*. Moreover, the high callose accumulation around the site of infection and the reduction of the oxidative environment mediated in the blockade of virus. These facts reveal that plant protection required an active SA-dependent signaling pathway while JA signaling was repressed. At hypocotils, in which the virus moves to roots, a reduction of SA OPDA, JA and JA-Ile was observed. At roots, we observe a high increase of OPDA but a reduction of SA. Moreover, this tissue seems to have an important role in the activation of the defense pathways since displayed a strong induction of *CaLSS, PDLP6* and *WRKY70*. Collectively, this results demonstrated that the Hx-IR in melon plants against MNSV depends on the sequential SA/JA-signaling pathways and the induction of OPDA accumulation, that probably triggered the high callose accumulation at the site of infection in addition to alleviate the the oxidative damage induced by MNSV infection. In this work, it has been provided valuable evidences to unravel the complex mechanisms of the priming

phenomenon by natural compounds and the importance of Hx as a broad-spectrum inducer of resistance.

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SUPPLEMENTARY DATA

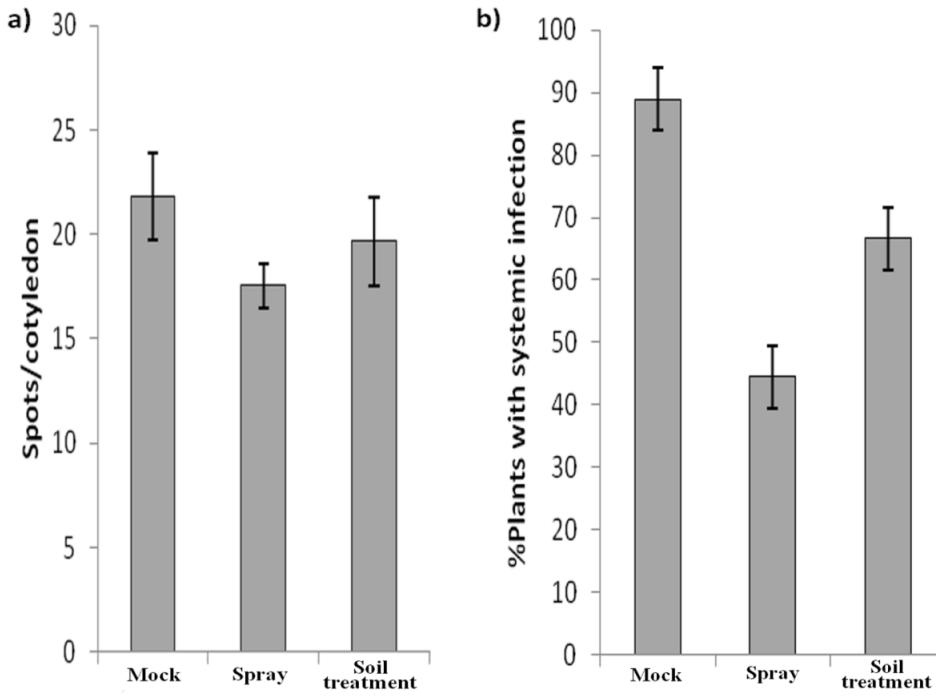

Fig.S1. Effect of Hx treatments on MNSV disease severity. One week-old melon plants were treated with 16 mM Hx by foliar spray and with 4 mM Hx by soil drench at 3 and 1 day before, and 1d after virus inoculation. **(a)** Spots/cotyledon at 5dpi, **(b)** Counting of the number of plants with systemic symptoms at18 dpi.

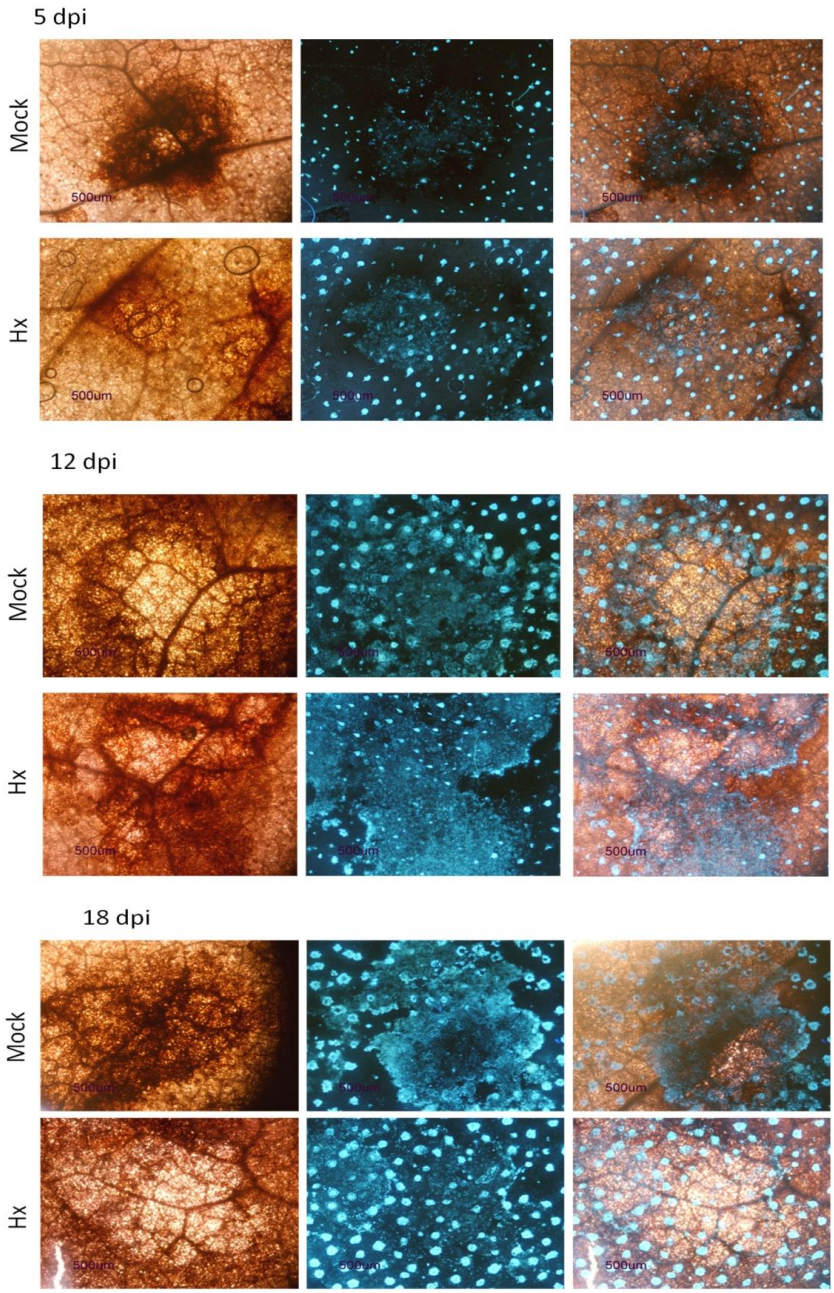

Fig. S2. Co-localization of H_2O_2 and callose deposition in cotyledons at 5, 12 and 18 dpi. Representative photographs of H_2O_2 and callose accumulation in mock and Hx-treated plants upon MNSV infection, obtained as described in Material and Methods section.

Table S1. Primers sequences.

Gene	GenBank accession number	Primers
Actin	XM_008462689.1	Fwd: 5′- GGAGCTGAGAGATTCCGTTG - 3′ Rev: 5′ - GGTGCAACGACCTTGATTTT -3′
CalSS	XM_008462676.1	Fwd: 5′- TCTTTGCTGGCTTCAACTCA - 3′ Rev: 5′- ATGCGGAAAAAGTCAAAACG-3′
β-1,3;1,4-Glucanase	XM_008440507.1	Fwd: 5′- CAAAATCGCGGTCCTTTTTA - 3′ Rev: 5′ - GAGCTTCAACCACAGGCTTC -3′
PAL	XM_008451017.1	Fwd: 5′- CAACTTCCAAGGGACTCCAA - 3′ Rev: 5′ - ATTGCGATCTCAGCACCTTT -3′
ISC2	XM_008454959.1	Fwd: 5′- TGCTTGCTGCTACTGTTGCT - 3′ Rev: 5′-GCACGTTCCCAGAATGTTTT -3′
AOS	AF081954.1	Fwd: 5′- ACCGTCGGTTGTACGACTTC - 3′ Rev: 5′ - GTCGGGTGTGGAGATTCACT -3′
COI1		Fwd: 5′- AGCTTGATGCGCTTACTCGT - 3′ Rev: 5′- GACAATCATACGCCGGAAGT -3′
JAZ10	XM_008461681.1	Fwd: 5′- GCTTGATTTCTTCGGTCTCG - 3′ Rev: 5′ - TTGGGAGTAGGAGGCTGAGA -3′
WRKY70	XM_008458746.1	Fwd: 5′- CAAGCCACGAAGCAAGTACA - 3′ Rev: 5′ - CTCTCCCACCTTGATTGCAT -3′
rbohC	XM_008466944.1	Fwd: 5′- CCGCCTTAATCATGGAAGAA - 3′ Rev: 5′ - TATGATGGGGTTGGTTTCGT -3′
GST	XM_008450384.1	Fwd: 5′- TTTTGGCCAAGTCCGTTTAG - 3′ Rev: 5′ - GAAATGGGTTTTCCATGGTG-3′
PDLP6	XM_008468883.1	Fwd: 5′- CGCTTCCTTAGACACCTTCG - 3′ Rev: 5′- AGGCCGTAGATGGTGTTTTG -3′

CONCLUSIONES

En esta tesis doctoral se ha llevado a cabo el estudio integral de los mecanismos de resistencia inducida. Inductores frente a estrés biótico y abiótico.

Las conclusiones que se han obtenido han sido las siguientes:

1. El NH_4^+ produce un estrés leve crónico subletal que activa los mecanismos de defensa en plantas de cítricos, induciendo así resistencia al estrés salino aplicado posteriormente. Los cítricos crecidos en estas condiciones, presentan un fenotipo característico de la respuesta morfogenética específica inducida por estrés conocida como SIMRs, relacionada con procesos de aclimatación.
2. El mecanismo de NH_4^+-IR frente a salinidad se basa en la acumulación de ABA, PAs, H_2O_2 y prolina, eventos directamente relacionados con resistencia frente a dicho estrés. Además se ha demostrado, que los cítricos crecidos con NH_4^+ presentan una disminución de Cl^-, tanto en hoja como en raíz, reduciéndose así el daño por la toxicidad del mismo y una mayor expresión de *PHGPx,* gen relacionado con la disminución del daño oxidativo producido por la sal.
3. El estudio del efecto del NH_4^+ sobre la maquinaria antioxidante mostró que el este ion *per se* induce acumulación de altos niveles de H_2O_2, inhibición de SOD y GR así como una mayor acumulación de glutatión oxidado (GSSG), datos que confirman el estrés leve producido por el mismo.
4. Los cítricos crecidos con NH_4^+ y sometidos a salinidad muestran una reducción de los niveles de H_2O_2 circulante junto con un incremento

de las actividades enzimáticas de CAT, SOD, GR, altos niveles de GSH así como una inducción de los genes *GST* y *PHGPx*. La activación de la maquinaria antioxidante inducida por el estrés leve crónico producido por NH_4^+ podría ser un mecanismo clave en la NH_4^+-IR frente a salinidad.

5. Se ha demostrado que el NH_4^+ actúa como inductor de resistencia frente a *Pst* mediante la acumulación de ABA, PAs y H_2O_2, metabolitos clave para la activación de la aclimatación sistémica adquirida (SAA).
6. El NH_4^+ induce en plantas de tomate cierre estomático basal, hecho que podría reducir la entrada de la bacteria en el mesófilo. Además se observó una mayor acumulación de ácido ferúlico en hoja, un compuesto fenólico directamente relacionado con la defensa de las plantas frente al ataque de patógenos.
7. Se ha demostrado la importancia del papel de las poliaminas, concretamente de putrescina, en la NH_4^+-IR frente a *Pst*, así como en la respuesta basal de plantas de tomate frente a este hemibiótrofo.
8. El estudio de la caracterización de los mecanismos de resistencia de plantas de melón frente al virus MNSV ha revelado la compleja red hormonal inducida tras la inoculación con el virus, así como la acumulación de callosa y ROS alrededor del sitio de infección.
9. El Hx, un agente natural de priming, demostró tener una gran efectividad como inductor de resistencia frente a virus en plantas de melón ya que el tratamiento radicular impide en el 100% de las plantas testadas el paso del virus al floema y por tanto, su dispersión a otros órganos de la planta.

10. El análisis del balance hormonal demostró la importancia de la acumulación de SA en Hx-IR, así como la de OPDA y JA-Ile en la primera fase de la infección en el patosistema melón-MNSV.
11. Los estudios relacionados con la deposición de callosa y ROS alrededor del sitio de infección revelaron la importancia de la rápida acumulación de callosa y la reducción de ROS a nivel de cotiledón, siendo procesos clave en la Hx-IR frente a un biótrofo obligado como es MNSV.