Tesis Doctoral

New approaches to control *Pseudomonas syringae* disease in tomato plants

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PD. El orden de los factores no altera el producto, cada pieza es importante y sin todas ellas, esta experiencia no hubiese sido como ha sido.

A mi familia y a
la memoria de mis abuelos

Contents

Abbreviations

1-MT: 1-methyltrypthophan

2-OG: 2-oxoglutarate

3-PGA: 3-phosphoglyceric acid

ABA: abscisic acid.

AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

ADC: arginine decarboxylase

AHL: acylated homoserine lactone

Al: aluminium

Ala: alanine

AMT: ammonium transporters

ANOVA: analysis of variance

AOC: allene oxide cyclase

APX: ascorbate peroxidase

Arg: arginine

ASN: asparagine synthase

Asn: asparagine

Asp: aspartate

ASR1: ABA stress ripening

AUX: auxin influx transporters

***AvrPtoB*:** gene responsible for effector synthesis

B: boron

C4: Aspartate + Asparragine

C5: Glutamate + Glutamine

CAT: catalase

CFA: coronafacic acid

Cfl: coronafacate ligase

Cfu: colony forming units

CMA: coronamic acid

COI1: coronatine Insensitive1

COR: coronatine

Cu: copper

Cys: cysteine

DAB: 3',3-diaminobenzidine

DAMP: damage associated molecular patterns

DAO: diamine oxidase

DI: disease incidence

DS: disease severity

DW: dry weight

EBDC: mancozeb

EF: elongation factor

ELS: external local supply

ERF1: ethylene response factor 1

ET: ethylene

ETI: effector triggered immunity

ETS: effector triggered susceptibility

Fe: iron

fliC: flagellin

FW: fresh weight

G6PDH: glucose-6-phosphate dehydrogenase

GABA: γ-aminobutyric acid

GDH: glutamate dehydrogenase

GFP: green fluorescent protein

Gln: glutamine

Glu: glutamate

Gluc: glucose

Gly: glycine

GOGAT: glutamine-oxoglutarate aminotransferase

GPX: glutathione peroxidase

GR: glutathione reductase

GS: glutamine synthase

GSH: gluthatione synthetase

H_2O_2: hydrogen peroxide

HATS: high affinity transporters

His: histidine

HPLC-MS: Liquid chromatography–mass spectrometry

HR: hypersensitive response

HrpA: marker gene of the type III secretion system-associated pilus

***HrpL*:** marker genes of the type III secretion system

Hx: hexanoic acid

IAA: indole-3-acetic acid

ICP-OES: inductively coupled plasma optical emission spectrometry

IS: internal status

ISC/SID2: isochorismate synthase

ISR: induced systemic resistance

JA: jasmonic acid

Ja-Ile: jasmonoyl-isoleucine

JAZ: jasmonate Zim

Kan: kanamycin

KB: King B medium

LATS: low affinity transporters

LAX: auxin influx transporters

LB: Luria-Bertani medium

Leu: leucine

LR: lateral root

LSD: least significant difference

Lys: lysine

MAMP: microbe associated molecular patterns

MAPK: mitogen-activated protein kinases

MeJA: methyl jasmonate

Met: methionine

Mg: magnesium

Mo: molybdenum

Mn: manganese

MS: Murashige and Skoog

NADPH: nicotinamide adenine dinucleotide phosphate

NB-LRR: nucleotide-binding site-leucine-rich repeat

NH_4^+: ammonium

Ni: nickel

NO: nitric oxide

NO_3^-: nitrate

NPR: non-expressor of the pathogenesis related genes

NiR: nitrite reductase

NPTII: kanamycin encoding gene

NR: nitrate reductase

NRT: nitrate transporter

NUE: nutrient use efficiency

ODC: ornithine decarboxylase

OPDA: 12-oxo-phytodienoic acid

P: phosphorus

P19: RNA silencing suppresor

PA: polyamine

PAL: phenylalanine ammonia lyase

PAMP: pathogen associated molecular patterns

PAO: polyamine oxidase

PCA: principal component analysis

PCD: programmed cell death

PEPC: phosphoenolpyruvate carboxylase

Phe: phenylalanine

Pi: post-inoculation

PIN: PIN-FORMED auxin efflux carriers

PK: pyruvate kinase

PR: pathogenesis related or primary root

Pro: proline

PRR: pattern recognition receptor

***PsyI*:** Quorum sensing establishment-related genes

PTI: PAMP-triggered immunity

Put: putrescine

pv.: pathovar

qPCR: quantitative polymerase chain reaction

QS: quorum sensing

***RecA*:** bacterial interal reference gene

RH: relative humidity

RNS: reactive nitrogen species

ROS: reactive oxygen species

RSA: root system architecture

S: sulfur

SA: salicylic acid

SAR: systemic acquired resistance

SAA: systemic acquired acclimation

SAM: S-adesylmethionine

SAMDC: S-adesylmethionine decarboxylase

SDS: sodium dodecyl sulfate

SE: standard error

Ser: serine

SIMR: stress-induced morphogenic response

SIR: sulfur induced resistance

SOD: superoxide dismutase

TCA: tricarboxylic acid

SIMR: stress-induced morphogenetic response

Thr: threonine

Trp: tryptophan

TTSS: type III protein secretion system

Tyr: tyrosine

UDP: uridine diphosphate

UPLC-MS: ultra performance liquid chromatography - mass spectrometry

YFP: yellow fluorescent protein

Zn: zinc

Summary

Plants are sessile organisms, so they have to cope with environmental situations that could be biotic and abiotic stresses. These stresses strongly impact the plant metabolism, but plants have developed sophisticated resistance mechanisms to avoid them, and to survive unfavourable conditions. These defence mechanisms can be activated by several ways, such as treatment with chemical or natural compounds that could act as defence inducers against biotic and abiotic stresses or by systemic acquired acclimation (SAA) produced by a previous exposure to abiotic stress, among others.

Presently, looking for new alternatives to using pesticides to fight against plant diseases is considered a priority issue. One of today's approaches is to use natural compounds that do not incur any risk to the environment and can induce defence mechanisms in plants.

Based on the research group's background about induced resistance, two experimental approaches were proposed. The first was to propose a new formulation to induce natural plant defences based on a complex between copper (Cu) and heptagluconic acid as an alternative use to pesticides in the tomato-*Pseudomonas syringae* pathosystem. This complex has been proposed to reduce free-Cu application against pathogens avoiding this way its toxicity for organisms and the environment. The second approach was to go in-depth into the action mechanisms of two compounds, 1-methyltrypthophan (1-MT) and ammonium (NH_4^+), in the tomato-*P. syringae* pathosystem, because their effectiveness as resistance inducers against *P. syringae* in tomato plants has been recently found.

Within the first approach, the application of Cu heptagluconate was proposed to treat the tomato plants subsequently infected with the bacterium *P. syringae* due to its easy absorption and diffusion in plants. It was observed that this complex was most effective in controlling and preventing bacteria attack, and symptoms reduced by more than 50%. The application of this new formulation also induced the accumulation of phenolic compounds such as caffeic acid and chlorogenic acid related to antioxidant activity. These compounds were related to cell wall reinforcement that could inactivate the enzymes produced by the pathogen or inhibit the synthesis of specific toxins. Infected

plants showed lower reactive oxygen species (ROS) levels, which can delay or deteriorate the pathogenesis machinery or induce defensive plant responses.

As discussed above, the second approach was to study the 1-MT effect against *P. syringae* because a previous study of the research group showed a 33-fold enhancement of this compound in tomato plants infected with this pathogen. Moreover, 1-MT treatment was effective against this pathogen when it was applied to the roots. Therefore, one aim of this work was to examine in-depth the mode of action of 1-MT in tomato plants against the pathogen *P. syringae* by analysing the changes produced in both the plant and bacteria. This work confirms that 1-MT acts as a resistance inducer against stress in tomato plants by modifying apoplast content. It also demonstrates that abscisic acid (ABA) can be implicated in this resistance mechanism. ABA can inhibit the stomatal aperture caused by bacterial coronatine (COR), which can prevent bacteria from entering the mesophyll. Moreover, 1-MT treatment might block the oxylipine pathway in plants due to the reduction observed in plant jasmonic acid levels (JA), which may pose a difficulty for salicylic acid (SA) pathway manipulation for bacteria with shown enhancement in the *PR5* gene expression that can help plants to defend themselves against pathogen infection. At last, 1-MT treatment is demonstrated to affect bacteria by reducing its motility and also the expression of the gene responsible for flagellum synthesis (*fliC*).

On the other hand, the action mechanism of NH_4^+ in the tomato-*P. syringae* pathosystem was studied. Previous results showed that treatment with NH_4^+ in tomato plants reduces the symptoms caused by the bacteria *P. syringae*, where ABA plays a fundamental role, as do polyamines (PAs) and H_2O_2 in the systemic acquired aclimation (SAA) process that acclimate plants to a subsequent stress. Therefore, an in-depth study into the mechanism of action of induced NH_4^+-mediated resistance against *P. syringae* was proposed, and whether nutritional balance and the primary metabolism of C and N are involved in this mechanism. Regarding nutritional balance, an accumulation of S, P, Zn and Mn was observed in the leaves of the plants infected and treated with NH_4^+, these being nutrients required for the proper functioning of ROS detoxification enzymes. In addition, primary metabolism reprogramming was observed in both leaves and roots, along with a marked increase in amino acid Arginine, one of the precursors of the biosynthesis of putrescine (Put). These findings suggest the importance of the glutamate pathway as a metabolic key point in the plants treated with NH_4^+ and

infected. As mentioned above, the important role of Put in resistance induced against *P. syringae* was pointed out. This polyamine is synthesised through two different enzymatic pathways: one catalysed by ornithine decarboxylase (ODC) and the other by arginine decarboxylase (ADC). Generating tomato plants silenced in both pathways (ADC and ODC) in an independent manner was proposed to elucidate the role of both pathways in induced resistance in the tomato-*P. syringae* pathosysthem. The *ADC*- and *ODC*-silenced plants showed a marked reduction in gene expression and increased susceptibility to *P. syringae* in the heterozygous plants. It suggests that Put synthesis is required for a proper plant defence response. These transgenic plants will be a very useful tool for unveiling the role that Put can play in the induced resistance mechanism against various stresses. Another partial objective was to study the cellular localisation of both proteins (ADC and ODC) through transient transformation in *Nicotiana benthamiana* plants. The results revealed the presence of ADC in the cytoplasm, and that of ODC in both the cytoplasm and nucleus.

Finally, as mentioned above, several changes in nutritional balance and in C and N metabolisms, were observed in the roots of the tomato plants treated with NH_4^+. Therefore, the final goal of this work was to elucidate how the N source can affect both root development and N assimilation at the transcriptional level in tomato plants. Moreover, whether ABA plays a role in root development under different N sources was also investigated. The results showed that the tomato roots grown with NH_4^+ as the only N source displayed less root development than when NO_3^- was present in the medium. It was proven that addition of C compounds (glucose or 2-oxoglutarate) to NH_4^+ treatment alleviated the negative effect of this ion on root development. Lastly, the effect of the N source on auxin transport, N uptake and assimilation and ROS detoxification processes was also analysed at the transcriptomic level. The results showed that tomato root reprogrammes gene transcription according to the N source, and the assimilation process was affected by the N source and induced by lack of ABA. Moreover, NH_4^+ nutrition induced several of the enzymes involved in ROS scavenging processes.

In summary, we conclude that Cu heptagluconate, 1-MT and NH_4^+ are good alternatives to induce resistance in tomato plants against the pathogen *P. syringae*, avoiding the excessive use of pesticides.

Resumen

Las plantas son organismos sésiles, por lo que a lo largo de su vida tienen que enfrentarse a diferentes estreses ambientales, tanto de carácter biótico como abiótico, lo que supone un gran impacto sobre su metabolismo. Para minimizar este impacto, las plantas han desarrollado una serie de mecanismos de defensa que les permite hacer frente a estas situaciones desfavorables. Estos mecanismos pueden ser activados de distintas formas, ejemplos de ellos son, el tratamiento con productos químicos o naturales que pueden actuar como inductores de defensa frente a estreses tanto abióticos como bióticos o la aclimatación sistémica adquirida (SAA) ante la preexposición a un estrés abiótico, entre otros.

En la actualidad, se considera un tema prioritario buscar alternativas al uso de pesticidas en la lucha frente a las enfermedades de las plantas. Una de las aproximaciones actuales es el uso de compuestos naturales que no producen ningún riesgo para el medioambiente y pueden inducir los mecanismos de defensas en la planta.

Basándonos en los trabajos previos del grupo de investigación sobre resistencia inducida, se propusieron dos aproximaciones experimentales. La primera fue proponer una nueva formulación para inducir las defensas naturales de las plantas, basada en el complejo de cobre con el ácido heptaglucónico en el patosistema tomate-*Pseudomonas syringae*. Esta combinación se propuso con la finalidad de restringir el uso del Cu en forma libre frente a patógenos, evitando así la contaminación tanto de los organismos vivos como del medio ambiente. La segunda aproximación fue profundizar en el mecanismo de acción de dos compuestos, el 1-metiltriptófano (1-MT) y el amonio (NH_4^+), en el patosistema tomate-*P. syringae*, habiéndose demostrado recientemente su efectividad como inductores de resistencia en este patosistema.

Dentro de la primera aproximación, debido a su fácil absorción y difusión en la planta, se planteó la aplicación del heptagluconato de cobre a plantas de tomate que posteriormente se infectaron con la bacteria *Pseudomonas syringae* pv. tomato DC3000. Se observó que este complejo es altamente efectivo para el control y prevención del ataque de la bacteria, mostrándose una reducción de los síntomas de la infección en más de un 50%. Además, la aplicación de esta nueva formulación induce la acumulación de los compuestos fenólicos de plantas como son el ácido cafeico y el ácido clorogénico,

compuestos con actividad antioxidante relacionados con el refuerzo de la pared celular que podrían inactivar los enzimas producidos por el patógeno o inhibir la síntesis de toxinas específicas. Además, se reduce la acumulación de las especies reactivas de oxígeno (ROS) en plantas infectadas, lo que puede causar un retraso o deterioro en la maquinaria de patogénesis o una inducción de respuestas defensivas de la planta.

Dentro de la segunda aproximación, se perseguía profundizar en el mecanismo de acción del 1-MT porque en estudios previos del grupo, se encontró un aumento de más de 33 veces de este metabolito en plantas de tomate infectadas con *P. syringae* y era efectivo como tratamiento vía radicular frente a este patógeno. Por ello, otro de los objetivos de esta tesis, ha sido profundizar en su modo de acción y analizar los cambios producidos tanto a nivel de planta como de bacteria. En este trabajo, se ha confirmado que el 1-MT actúa como inductor de resistencia frente a dicho estrés modificando el contenido del apoplasto, y se ha demostrado que el ácido abscísico (ABA) podría estar implicado en el mecanismo de resistencia. Éste podría causar la inhibición de la apertura estomática producida por la coronatina (COR) de la bacteria, pudiendo así prevenir la entrada de la bacteria en el mesófilo. Además, el tratamiento con 1-MT podría estar bloqueando la ruta de las oxilipinas, puesto que se observan niveles reducidos de ácido jasmónico (JA) en la planta, lo que dificultaría la manipulación por parte de la bacteria de la ruta del ácido salicílico (SA), mostrándose elevados niveles de expresión del gen *PR5* que ayudarían a la planta a defenderse del patógeno. Finalmente, el tratamiento con 1-MT parece también afectar a la bacteria porque se reduce su motilidad, observándose además, una reducción en la expresión del gen encargado de la correcta síntesis del flagelo (*fliC).*

Por otra parte, se profundizó en el mecanismo de acción del NH_4^+ en el patosistema tomate-*P. syringae*. Los resultados previos mostraron que el tratamiento con NH_4^+ en plantas de tomate, reducen los síntomas causados por la bacteria *P. syringae*, jugando el ABA un papel fundamental, así como las poliaminas y el H_2O_2 en el proceso de SAA (aclimatación sistémica adquirida) que aclimata a la planta frente a un estrés posterior. Por ello, se propuso profundizar en el mecanismo de acción de la resistencia inducida mediada por NH_4^+ frente a *P. syringae* y estudiar si el balance nutricional y el metabolismo primario del C y N están implicados en dicho mecanismo. Respecto al balance nutricional, se ha observado una acumulación de S, P, Zn y Mn en hojas de

plantas infectadas y tratadas con NH_4^+, nutrientes necesarios para el correcto funcionamiento de los enzimas implicados en la detoxificación de ROS. Además, se observa una reprogramación del metabolismo primario tanto a nivel de hoja como de raíz. Concretamente, se observa un marcado incremento del aminoácido Arginina, uno de los precursores de la biosíntesis de la putrescina (Put). Esto sugiere la importancia de la ruta del glutamato como punto clave del control metabólico en las plantas tratadas con NH_4^+ e infectadas. Además, en trabajos previos del grupo ya se había destacado el papel clave de la Put en la resistencia inducida frente a *P. syringae*. Esta poliamina se sintetiza a través de dos vías enzimáticas, una catalizada por la ornitina descarboxilasa (ODC) y la otra por la arginina descarboxilasa (ADC), por lo que se propuso dilucidar el papel de ambas rutas en la resistencia inducida en el patosistema tomate-*P. syringae*. Para ello, se han generado plantas silenciadas en ambas vías de forma independiente. Las plantas silenciadas en cada una de las rutas de los genes *ADC* y *ODC*, han mostrado una reducción acusada en la expresión de los mismos y una mayor susceptibilidad frente a *P. syringae* en plantas heterocigotas, lo que indica que la síntesis de Put es necesaria para que la planta se defienda correctamente del patógeno. Las plantas silenciadas en estas rutas serán una herramienta muy útil para poder desvelar el papel que puede desempeñar la Put en el mecanismo de resistencia inducida frente a diversos estreses. Por otra parte, otro objetivo parcial planteado ha sido estudiar la localización celular de ambas proteínas (ADC y ODC) mediante una transformación transitoria en plantas de *Nicotiana benthamiana*. Los resultados han mostrado la presencia de ADC en el citoplasma y de ODC en citoplasma pero también en el núcleo celular.

Finalmente, al observarse cambios en el balance nutricional y en el metabolismo primario del C y N en la raíz de las plantas de tomate tratadas con NH_4^+, se planteó dilucidar cómo la fuente de N puede afectar al desarrollo de la raíz y a la asimilación del N a nivel transcripcional en plantas de tomate. Además, se ha investigado si el ABA juega un papel en el desarrollo de la raíz crecidas con diferentes fuentes de N. Los resultados han mostrado que las raíces de las plantas de tomate crecidas con NH_4^+ como única fuente de N presentan un desarrollo radicular menor que cuando se tratan con NO_3^-. Además, se ha comprobado que la adición de compuestos carbonados (glucosa o 2-oxoglutarato) al tratamiento de NH_4^+ alivia el efecto negativo que tiene este ión en el desarrollo radicular.

Por último, también se analizó a nivel transcriptómico qué efecto tiene la fuente de N en los procesos de transporte de auxinas, absorción y asimilación de N y detoxificación de ROS. Los resultados han mostrado que la raíz reprograma la transcripción de los genes en función de la fuente de N y que el proceso de asimilación está afectado por la fuente e inducido por la ausencia del ABA. Además, la nutrición por NH_4^+ induce varios enzimas implicados en el proceso de detoxificación de ROS.

En resumen, teniendo en cuenta todos los resultados descritos, se propone el heptagluconato de cobre, el 1-MT y el NH_4^+ como buenas alternativas para inducir resistencia en plantas de tomate contra el patógeno *P. syringae*, evitándose el uso abusivo de pesticidas.

GENERAL INTRODUCTION

Introduction

The world population will reach around 9 billion in the next thirty years (United Nations, 2015). Thus food production will necessarily increase to cover the food demand of the world's population (Godfray *et al*., 2010). To fulfil this objective, a large amount of agrochemicals will be required, with consequent effects on both the environment and our health without taking into account the recommendation proposed by Food and Agriculture Organization about food security (www.fao.org). Thus, research to find new environmentally friendly compounds is paramount to prevent these negative effects.

Pesticides are agrochemical compounds used in agriculture to control plant diseases. The widespread use of some of them poses a health and environment risk (Bassil *et al*., 2007; Aktar *et al*., 2009). For years, farmers have applied large amounts of these products to ensure production, but the pesticides used in excess have produced environmental pollution (Wilson and Tisdell, 2001). Moreover, several studies show increased resistance against the use of pesticides (Maino *et al*., 2018; Dermauw *et al*., 2018; Hawkins *et al*., 2018). For this reason, a set of strategies is required to minimize the environmental effects of these compounds, such as adjusting the amount of them supplied to crops or using enviro-friendly compounds (Damalas and Eleftherohorinos, 2011, Nicolopoulou-Stamati *et al*., 2016).

The vast amounts of N agrochemical fertilisers, mainly in the NO_3^- form, is another environmental problem, as only 50% of the N supplied to soil is used by plants (Hodge *et al*., 2000; Robertson and Vitousek, 2009). NO_3^- is highly soluble and it can be leached into groundwater to produce water eutrophication and is also released to the atmosphere as nitrous oxide (Sánchez-Pérez *et al*., 2003). Hence, NH_4^+-based fertilisation is a more enviro-friendly alternative to NO_3^-, as it can be adsorbed into the soil particles, which would reduce the possibility of leaching to groundwater.

Based on this scenario, further studies on the search for green alternatives are necessary to increase crop productivity and decrease applications of pesticides and fertilisers in order to achieve sustainable agriculture.

The tomato-*Pseudomonas syringae* pv tomato DC3000 system

Tomato (*Solanum lycopersicum* L.) is a dicotyledonous and perennial herbaceous plant that belongs to the *Solanaceae* family. It requires a warm climate of around 26 ºC during the day and 15 ºC at night with a relative humidity of 60% (OEDC, 2017). Nowadays, tomato is one of the most extensively produced and consumed vegetable crops (Zorzoli *et al*., 2007) and offers many beneficial metabolites that can contribute to healthy diet (Goff and Klee, 2006; Tohge *et al*., 2014). Tomato production has increased in recent years, its world production is about 182 million tons from 4,848 million ha, and Spain is the eighth largest tomato-producing country in the world (www.fao.org/faostat/en). However, tomato plant production is reduced by the attack of a variety of pests and diseases (Table 1).

Table 1. Examples of pests and diseases of tomato plants (Adapted from: OEDC, 2017).

Insects	Bacteria	Oomycetes/Fungi
● *Agrotis ipsilon* Hufnagel ● *Bemisia argentifolii* Bellows and Perring ● *Keiferia lycopersicella* Wallshingham ● *Leptinotarse decemlineata* Say ● *Manduca sexta* L. ● *Spodoptera exigua* Hübner ● *Tuta absoluta* Meyrick ● *Thrips tabaci* Linderman ● *Trioza* spp. ● Aphids	● *Clavibacter michiganense* Smith ● *Ralstonia solanacearum* Smith ● *Pseudomonas syringae* van Hall pv. tomato ● *Xanthomonas campestris* Pammel	● *Alternaria alternate* Keissler ● *Alternaria alternata* f. sp.lycopersici Grogan *et al*. ● *Alternaria solani* Jones and Grout ● *Botrytis cinerea* *Cladosporium fulvum* ● *Fusarium oxysporum* f. sp. *lycopersici* Vawdrey and Peterson ● *Fusarium solani* (Mart.) Sacc. ● *Phytophtora infestans* (Mont.) de Bary ● *Sclerotinia sclerotiorum* (Lib.) de Bary

The tomato genome was sequenced seven years ago (The Tomato Genome Consortium, 2012). It is diploid specie and it has a genome size of around 950 Mb (Michaelson *et al*., 1991), which permitted several improvements in biotechnology or genetic engineering experiments (Aflitos *et al*., 2014; Tranchida-Lombardo *et al*., 2018).

P. syringae is a Gram-negative bacterium with flagellum that causes major diseases and economical loss in a wide range of species such as tomato producing the bacterial speck disease in leaves, stems or fruits, mainly caused by the pathovar tomato DC3000 (Zhao *et al*., 2000; Mansfield *et al*., 2012). *P. syringae* is a hemibiotrophic pathogen that requires a living host in the first stage to take nutrients (biotroph) and then causes cell death (necrotroph). *P. syringae* pathogenesis comprises epiphytic colonisation, enters through stomata or wounds, grows in the apoplast using the nutrients present in the plant cell and ends in chlorosis and black speck disease symptoms (Fig. 1). The change from the epiphytic to the endophytic phase seems cell density-dependent through the detection of their own molecular signals in coordinating gene expression by what is known as quorum-sensing (QS) (Venturi, 2006). In Gram-negative bacteria such as *P. syringae*, the most common signal molecule is the acylated homoserine lactone (AHL) molecule (Li and Nair, 2012). When these compounds are found at an adequate concentration in the apoplast, the virulence mechanisms of bacteria are activated. Moreover, the pathogenesis process will be successful or not depending on the secretion of effectors and other virulence factors, host defence suppression and the alteration of the nutritional and physiological status (Melotto and Kunkel, 2013; Xin and He, 2013) (Fig. 2). The required conditions for disease development are high leaf humidity and temperatures around 15-25ºC (Silva and Lopes, 1995). Pathogens have developed several mechanisms to interfere with plant immunity, such as the effector delivery from pathogen to plant through the requirement of a type III protein secretion system (TTSS) (Alfano and Collmer, 1997; Galán and Collmer, 1999; Jones and Dangl, 2006; Büttner and He, 2009). This secretion system constitutes a multiprotein complex with a needle-like structure that injects different types of proteins inside plant cells (McCann and Guttman, 2008; Block *et al*., 2008; Büttner and He, 2009; Xin and He, 2013). These proteins are called Hop proteins (from the Hrp outer proteins) and are divided into helper and effector proteins. The first type of protein (helper) helps the injection of the second type of proteins (effectors) by degrading the plant cell wall to facilitate contact with the plasma membrane (Hauser, 2009). Effector proteins target different plant cell machinery components by producing host metabolism adjustments to acquire nutrients (Cunnac *et al*., 2009). One common example of the virulence effectors produced by *P. syringae* strains to manipulate plant hormonal balance is the phytotoxin coronatine (COR) (Zheng *et al.*, 2012), which also suppresses PTI and induces stomata opening (Li *et al.*, 2005; Melotto *et al.*, 2006).

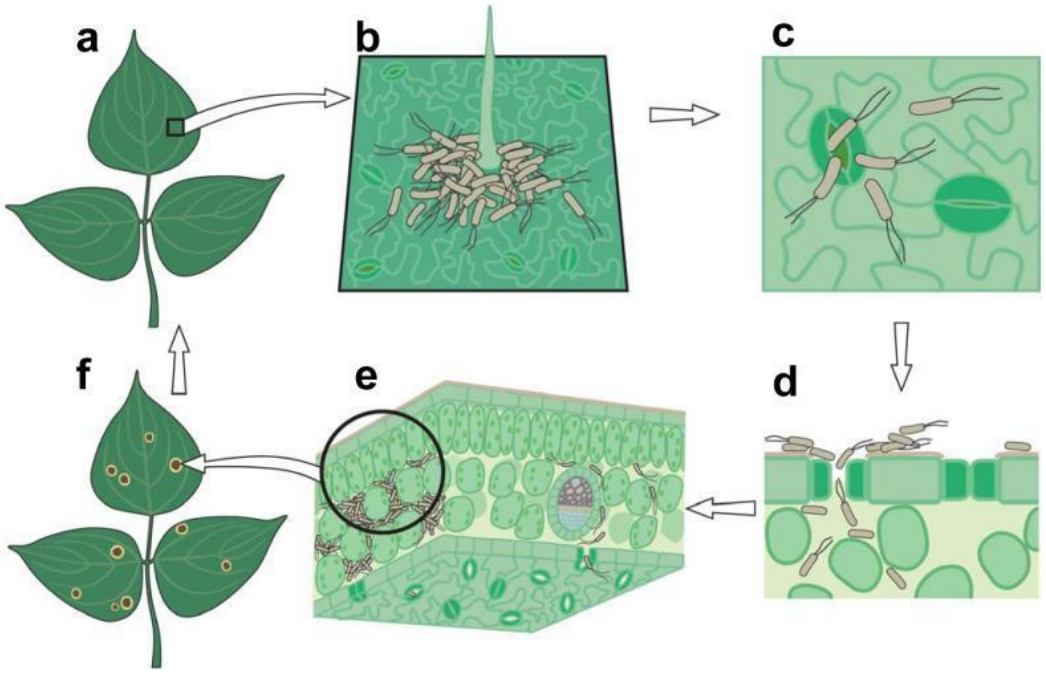

Figure 1. The development of *P. syringae* life cycle follows the next steps: epiphytic colonisation (a, b); entry into the plant mesophyll thought stomata or wounds (c, d); establishment and multiplication in the plant apoplast and the secretion and delivery of effectors through TTSS (e) and production of chlorosis and black speck disease symptoms (f). Source: Melotto *et al*. (2008).

Plant defence mechanisms

Plants are sessile organisms and they have developed a complex coordinated system to fight against different biotic and abiotic stresses. Plants have their own constitutive barriers which they use to cope external conditions, and have developed a sophisticated defence system. Constitutive or preformed physical or chemical barriers are located on the leaf surface (wax layer, cutin, cell wall, trichomes, bark, secondary metabolites, etc.) and constitute a previous step to prevent pathogen penetration in plants (McDowell and Dang, 2000; Thordal-Christensen, 2003; Walters, 2011; Uma and Podile, 2015). However, when a pathogen is able to overcome these barriers, a set of signalling cascades are activated leading to the induced defence (Walters, 2011) that depends on the pathogen nutrition and lifestyle (Walters *et al.*, 2013).

- **Plant immune system**

When the pathogen has overcome the constitutive barriers, the next step for plants is to identify the pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs), such as lipopolysaccharides, peptidoglycans, elongation factor Tu (EF-Tu) and flagellin in

bacteria (Zipfel *et al*., 2006; Gust *et al*., 2007; Pieterse *et al.*, 2009), or chitin and xylanase in fungus (Ron and Avni, 2004; Miya *et al.*, 2007; Wan *et al.*, 2008). These molecules are recognised by plasma membrane receptors called Pattern Recognition Receptors (PRRs), while PAMP-triggered immunity (PTI) is activated to avoid pathogen activity by the alteration of the ionic flux through the plasmatic membrane, reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) production, MAPKs activation, callose accumulation or pathogenesis-related proteins (PRs) synthesis (Dangl and Jones, 2001; Jones and Dangl, 2006; García-Brugger *et al.*, 2006; Nguyen *et al*., 2010; Monaghan and Zipfel, 2012; Muthamilarasan and Prasad, 2013) (Fig. 2a). Plants also recognise and respond when faced with their own molecules, or with cell wall or cuticle fragments released by the action of pathogen enzymes. These signal molecules are called Damage-Associated Molecular Patterns (DAMPs) (Ferrari *et al*., 2007; Boller and Felix, 2009). However, pathogens can interfere with the recognition of PAMPs by reducing PTI efficacy, and they produce and translocate effectors into the plant cell to hamper plant immunity, a phenomenon known as effector-triggered susceptibility (ETS) (Cui *et al.*, 2015; Andersen *et al*., 2018) (Fig. 2b). Thus plants recognise pathogen effectors through resistance proteins (PR) or the nucleotide-binding site-leucine-rich repeat proteins (NB-LRR) synthesised in the endoplasmic reticulum and transported to the plasma membrane (Frescatada-Rosa *et al.*, 2015). These resistance proteins are specific of each plant-pathogen interaction and activate effector triggered immunity (ETI) (Pieterse *et al*., 2009; Dodds and Rathjen, 2010; Andolfo and Ercolano, 2015) (Fig. 2c). ETI is a stronger enduring response accompanied by ROS or NO production as a signalling molecule to activate other defence mechanisms, such as the hypersensitive response (HR), which produces programmed cell death (PCD) around the infection site by limiting pathogen spread and protecting other tissues from further damage (Hammond-Kosack and Jones, 1996; Jones and Dangl, 2006; Rosebrock *et al.*, 2007). Plant response to pathogen infection includes changes in the photosynthesis process, PR proteins synthesis, cytoskeleton organisation, ROS production and cell apoptosis (Kumudini *et al*., 2018).

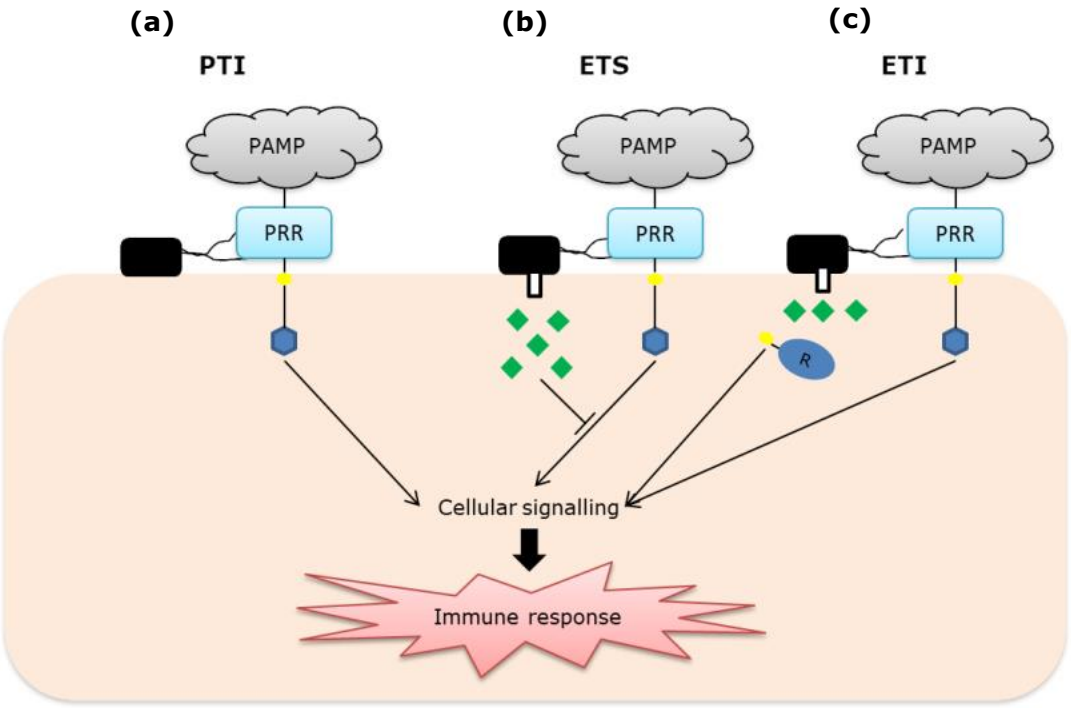

Figure 2. A schematic overview of plant immunity responses. (a) Upon pathogen attack, PAMPs activate PRRs in the plant by producing a downstream cascade that leads to PTI. (b) Virulent pathogens have acquired effectors (purple stars) to suppress PTI that result in ETS. (c) So plants have acquired resistance proteins that recognise these pathogen-specific effectors, which results in a secondary immune response called ETI. (Adapted from Pieterse *et al*., 2009).

On the other hand, several authors have revealed the role of primary metabolism in plant-pathogen interactions, which changes depending on the type of pathogen (Scharte *et al*., 2005; Rolland *et al*., 2006; López-Gresa *et al*., 2010; Duan *et al*., 2013), but further studies are required. Primary metabolism supports the cellular energy requirements for the plant defence response by inducing respiratory activity, among others (Bolton, 2009; Kangasjarvi *et al*., 2012; Rojas *et al*., 2014). Sugar signalling is produced by photosynthesis and C metabolism, and causes the feedback regulation of primary metabolism against pathogen attack (Scharte *et al*., 2005; Rolland *et al*., 2006). It is known that glucose, fructose and sucrose are the main C sources in leaves, followed by organic acids and amino acids (Lindow and Brandl, 2003; Vorholt, 2012). Plant could reprogrammed primary metabolism depending on the environmental stresses, since amino acid biosynthesis requires C skeletons from TCA cycle (Temple *et al*., 1998; Corzzi and Last *et al*., 2000) and also several amino acids could be metabolized into TCA intermediates (Miflin and Habash, 2002). Moreover, it has been described that amino acid metabolism is the key point for the biosynthesis of protective plant natural

compounds, having a role in plant immune mechanisms (Návarová *et al*., 2012; Kim *et al*., 2013).

- **Induced resistance**

Induced resistance is described as "a physiological state of enhanced defensive capacity elicited by specific environmental stimuli, whereby the plant's innate defences are potentiated against subsequent biotic challenges" (Vallad and Goodman, 2004). In this way, after stress has been recognised, besides the local response the plant is able to induce defensive responses in systemic tissues by protecting the parts that are still not exposed to damage. In the last years, it has been demonstrated that this enhanced physiological status can also be induced by mild stress, the application of natural and chemical compounds or beneficial microbes, mycorrhiza and endophytes (Pieterse *et al*., 2000; van Wees *et al*., 2008; Jung *et al*., 2012; Bastias *et al*., 2017; Llorens *et al*., 2017).

Depending on the stimuli that induce the systemic response in plants, induced resistance has been described as systemic acquired resistance (SAR), induced systemic resistance (ISR) and systemic acquired acclimation (SAA) (Glazebrook, 2005; Mittler and Blumwald, 2015). SAR and ISR are induced against biotic stresses, and SAA is activated in response to abiotic stimuli.

- Systemic Acquired Resistance, Induced Systemic Resistance, and Systemic Acquired Acclimation

SAR is characterised by its broad spectrum against pathogens and durability over both time and generations. This systemic resistance is associated with an increase in salicylic acid (SA) (Vallad and Goodman, 2004). The SAR response is initiated in infected tissues as a mobile signal is produced and then transferred to uninfected areas. The signalling transport from infected tissues to the distal and unchalleged ones is produced through the vascular system, especially by the phloem (Shah *et al*., 2014). Long-distance signalling is carried out by mobile compounds like methyl salicylate (MeSA), dehydroabietinal, azelaic acid, pipecolic acid or a glycerol-3-phosphate-derived molecule (Park *et al*., 2007; Chanda *et al*., 2011; Chatuverdi *et al*., 2012; Wang *et al*., 2014; Shan and He, 2018). This systemic resistance can be activated in the plant by the attack of biotrophic pathogens or by the treatment with chemical or natural compounds

such as benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl-ester [benzothiadiazole (BTH); acibenzolar-S-methyl (ASM)] or methyl salicylate, respectively (Conrath, 2009) (Table 2).

SA is a small phenolic molecule synthesised from either phenylalanine ammonia lyase (PAL) or isochorismate synthase (ICS/SID2) (Garcion and Métraux, 2006). It plays an important role in plant resistance against biotic stress due to its role in induced resistance activation, HR or SAR induction (Loake and Grant, 2007; Vlot *et al*., 2009), and SA exogenous application induces resistance in several crops (Malamy and Kleesig, 1992). An increase in an endogenous SA concentration induced by pathogen presence produces several changes in the plant redox status that lead to important signalling processes, such as the activation of PR genes like *PR1*, and a decrease in the non-expressor of the PR Genes1 (*NPR1*) protein (Durrant and Dong, 2004; Spoel *et al*., 2009). Then *NPR1* controls SA signalling downstream and acts as a transcriptional coactivator of several defence-related genes (Moore *et al*., 2011), such as PR genes, which are considered SA-inducible gene expression markers.

The induction of ISR constitutes a physiological state of the enhanced defensive capacity produced by environmental stimuli through which innate defences are enhanced against subsequent biotic stresses by relying on jasmonic acid (JA) and ethylene (ET) pathways (Choudhary *et al*., 2007). It produces protection against different non-pathogenic organisms, pathogen attack or treatments with chemical and natural compounds (Zeller, 2006; van der Ent *et al*., 2009; Pieterse *et al*., 2014).

JA is a lipid-derived compound that is synthesised through the oxylipins pathway (Gfeller *et al*., 2010). It could be metabolised to methyl jasmonate (MeJA) or conjugated to amino acids, such as isoleucine, to obtain jasmonoyl-isoleucine (JA-Ile) (Seo *et al*., 2001; Fonseca *et al*., 2009). JA regulates the induction of several defence genes through the interaction with F-box protein Coronatine Insensitive1 (COI1), which leads to the degradation of Jasmonate Zim (JAZ) repressor proteins via the proteasome, and also to the release of transcription factors like MYC2 or ERF1, which modify defence gene expression (Staswick, 2008; Fonseca *et al*., 2009; Koo and Howe, 2009; Pauwels and Goossens, 2011). However, there is complex crosstalk between SA and JA signalling pathways (Gimenez-Ibanez and Solano, 2013), which is especially consistent in mutual synergistic effects (Mur *et al*., 2006; Koorneef *et al*., 2008), and ET can crosstalk with both pathways (Verma *et al*., 2016). Other hormones, such as cytokinins,

auxins, ABA, gibberellins or brassinoesteroids, have been described for their function in the response to plant stresses (Bari and Jones, 2009).

SAA is described as systemic acquired acclimation induced by abiotic stress in non-challenged tissues (Mittler and Blumwald, 2015). Despite the mechanism of SAA not being fully described, several studies have suggested that SAA against heat, cold, UV, osmotic stress, salinity and high light stress includes the activation of a ROS wave (Mittler *et al*., 2011; Karpinski *et al*., 2013; Suzuki *et al*., 2013) (Table 2). For instance, it has been reported that a ROS wave is required for SAA activation in response to local stimuli, including heat or excess light (Suzuki *et al.*, 2013; Zandalinas *et al*., 2018). ROS play an important role in plant defence against different pathogens and the excess of ROS production (superoxide, hydrogen peroxide and hydroxyl radicals) produces the so-called oxidative burst, one of the primary stress responses. On the other hand, ROS species are toxic compounds, so they have to be detoxified through the plant antioxidant machinery (Bhardwaj *et al*., 2013). Apart from the ROS wave, other factors have been described as mediators of the SAA response, such as calcium waves, electric signals and hormones (Mittler and Blumwald, 2015; Choi *et al*., 2017), and these results have been confirmed through the accumulation of transcripts, proteins or enhanced levels of tolerance or resistance to later stress (Karpinski *et al*., 2013; Shah and Zeier, 2013). It has been demonstrated that wounding increases salt stress tolerance in tomato mediated by systemin, JA synthesis and cadmodulin-like activity (Capiati *et al*., 2006). Nevertheless, studies have suggested that plants with induced SAA pathways to abiotic stimuli can respond better against later biotic stress. For example, Achuo *et al.* (2006) have shown that drought stress results in enhanced ABA and reduced *Botrytis cinerea* infection, and also in the suppression of *Oidium neolycopersici* in tomato; Arasimowicsz-Jelonek *et al*. (2014) have revealed that the enhanced defence responses observed in susceptible potato cultivars under aluminium (Al) stress in roots correlate with a reduction in disease symptoms after leaf inoculation with *Phytophthora infestans*; and Fernández-Crespo *et al.* (2015) have reported that NH_4^+ nutrition leads to subsequent resistance to *Pseudomonas syringae* through the accumulation of H_2O_2 and Put.

Table 2. Systemic signalling processes in plants (Adapted from: Baxter *et al*., 2014)

Systemic signalling	**Systemic Acquired Resistance (SAR)**	**Systemic Wound Response**	**Systemic Acquired Acclimation (SAA)**	**Systemic Metabolic Response**	**Systemic Developmental Response**
Primary stimuli	Microorganism (bacteria, virus, fungi)	Insect bite, wounds	High light, heat, cold, etc.	Modification in sugars and other metabolites	Changes in light, CO_2, *etc*.
Systemic response	Biotic stress defence	Insect defence	Abiotic stress acclimation	Metabolites modification	Growth coordination, *etc*.

- Induction of resistance

To enhance plant immunity inducers of resistance are also used, these compounds confer plant protection against pathogen attack by inducing plant defence mechanisms. Table 3 shows several examples of resistance inducers used in *Solanaceae* plants against different pathogens with different lifestyles.

Table 3. Examples of inducers of resistance in *Solanaceae* plants (Adapted from Alexandersson *et al*., 2016).

Compound	**Plant**	**Pathogen**	**Reference**
Aluminium	Potato	*Phytophthora infestans*	Arasimowicz-Jelonek *et al*. (2014)
Copper	Tobacco	*virus Y-vein necrosis strain (PVY(N))*	Li *et al*. (2009)
β-Aminobutyric acid (BABA)	Pepper	*Phytophthora capsici*	Lee *et al*. (2000)
	Tomato	*Clavibacter michiganensis*	Hassan and Buchenauer (2008)
	Tomato	*Ralstonia solanacearum*	Hassan and Abo-Elyousr (2013)
Benzo-thiadiazole-7-carbothioic acid S-methyl ester (BTH)	Tomato	*Botrytis cinerea*	Mehari *et al*. (2015)
Hexanoic acid	Tomato	*Pseudomonas syringae*	Scalschi *et al*. (2013)
	Tomato	*Botrytis cinerea*	Vicedo *et al*. (2009)

Table 3 (cont). Examples of inducers of resistance in *Solanaceae* plants (Adapted from Alexandersson *et al*., 2016).

Compound	Plant	Pathogen	Reference
Indole-3-acetic acid (IAA)	Tomato	*Fusarium oxysporum*	Sharaf and Farrag (2004)
Sulphur	Tobacco	*Tobacco mosaic virus*	Király *et al*. (2012)
	Tomato	*Oidium neolycopersici*	Llorens *et al*. (2016)

Traditionally, inorganic formulations of copper such as Cu hydroxide or Cu sulphate have been used in agriculture in large amounts as a pesticide (www.mapa.gob.es) or as inducer of resistance (Table 3). This compound is a heavy metal and it could be bioaccumulated in plants and leached into the groundwater producing environmental and health problems. Nowadays, its use is limited in agriculture and other alternative formulations could be the application of Cu in complexing compounds, which are characterized by its easy absorption and diffusion in plants. In this way, the applied Cu concentration to plants could be reduced, together with the reduction of the pollution.

In the search of new natural compounds as inducer of resistance, hexanoic acid has been one of the proposed in the last decade (Table 3). This compound acts as an inducer of plant defenses by means of a priming mechanism against pathogens with different lifestyles (Vicedo *et al*., 2009; Llorens *et al*., 2013; Scalschi *et al*., 2013). Priming is described as the exposure to certain stimuli that can induce a stress memory which is persistent and prepare the plant for better and faster response in later events. It is characterized by a lag phase that separates the priming activation from the second later stress, without physiological costs (Conrath *et al*., 2006; Jung *et al*., 2009; Martínez-Medina *et al*., 2016) and it could be maintained to the next generations through epigenetic changes (Luna et al., 2012; Luna, 2016).

Camañes *et al.* (2015) studied the metabolome of tomato plants treated with the hexanoic acid and infected with the pathogens *P. syringae* or *B. cinerea*. They showed an increase of 33-fold of 1-MT metabolite in *P. syringae* infected compared to control tomato plants. Moreover, these authors have also demonstrated that the root application of 1-MT significantly reduces the infection of both pathogens but the resistance mechanisms in this interaction were still lacking. In addition, in recent years, other tryptophan derivates have emerged as relevant defence mechanisms, which have

conferred resistance to the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis thaliana* (Sánchez-Vallet *et al.* 2010, Gamir *et al.* 2012).

NH_4^+ nutrition as a mild abiotic stress to acclimate plants

Apart from the environmental pollution due to pesticides, the use of excesive fertilizer constitutes another pollution cause. NH_4^+-based fertilizers are a less environmentally harmful compared to NO_3^- fertilization due to the decrease of NO_3^- leaching and nitrous oxide emissions. NH_4^+ nutrition produces a mild toxicity known as *ammonium syndrome* that depends on NH_4^+ concentration and crop species (Domínguez-Valdivia *et al*., 2008; Cruz *et al*., 2011). Thus, this mild toxicity induces different symptoms such as leaf chlorosis, biomass reduction, ionic imbalances, acidification of the rhizosphere and changes in root architecture, among others (Britto and Kronzucker, 2002; Esteban *et al*., 2016; da Silva *et al*., 2016; Liu and von Wirén, 2017; Xuan *et al*., 2017). Moreover, NH_4^+ is considered a primary stimulus that increases stress resistance or tolerance to later stress through the induction of acclimation responses (Fernández-Crespo *et al*., 2012; Fernández-Crespo *et al*., 2015). Firstly, as aforementioned, NH_4^+ nutrition confers resistance against *Pseudomonas syringae* by enhancing H_2O_2 accumulation, acting as a signal and activating SAA mediated by ABA and Put. Put is a polyamine that could be synthetized by two different paths, from Arg via Arginine Decarboxylase (ADC) or from Orn via Ornithine Decarboxylase (ODC). Polyamines play a role in several physiological processes (Couée *et al.*, 2004; Liu *et al*., 2015) and in plant defence against abiotic or biotic stresses (Walters, 2003; Pang *et al.*, 2007; Hussain *et al.*, 2011; Fernández-Crespo *et al*., 2015). Secondly, it has been already described that NH_4^+ induces stress-induced morphogenic response (SIMR) in Carrizo plants (Fernández-Crespo *et al*., 2014). SIMR was decribed as a general acclimation strategy characterised by cell division blocking, reduced elongation and the redirected outgrowth of lateral roots induced by a plant exposure to mild chronic stress (Potters *et al*., 2007; 2009). Moreover, the abundance of N compounds in soil fluctuates and can affect different plant processes, such as root architecture, leaf development or flowering (Walch-Liu *et al*., 2000; Nacry *et al*., 2013; Guilbaud *et al*., 2014). Thus plants have to organise their development and growth rate in response to environmental cues, since roots are responsible for the uptake of water and nutrients, apart from plant anchorage and biomolecule storage (Marschner, 2012). It has already been shown that root

development is strongly affected by nutrient and water availability, soil density, salinity, temperature and drought (Mansoorkhani *et al.*, 2014; Julkowska *et al.*, 2014; Senthilkumar *et al.*, 2017), this root adaptation is called root plasticity. External and internal nutrient status plays a role in several root processes, such as primary root (PR) growth or lateral root (LR) formation (Mansoorkhani *et al.*, 2014). Thus the root architecture is modulated by not only local nutrient availability, but also by the internal nutrient status, which respectively gives way to local or systemic signalling (Fig. 3) (Giehl *et al.*, 2014). Moreover, it is known that root growth processes are orchestrated by the regulation of several plant hormones that are transported along the plant, such as auxin, ABA, cytokinin, gibberellins or ethylene, and other hormones such as brassinosteroids, JA, SA, NO or strigolactones (Santner *et al.*, 2009; Santner and Estelle, 2009).

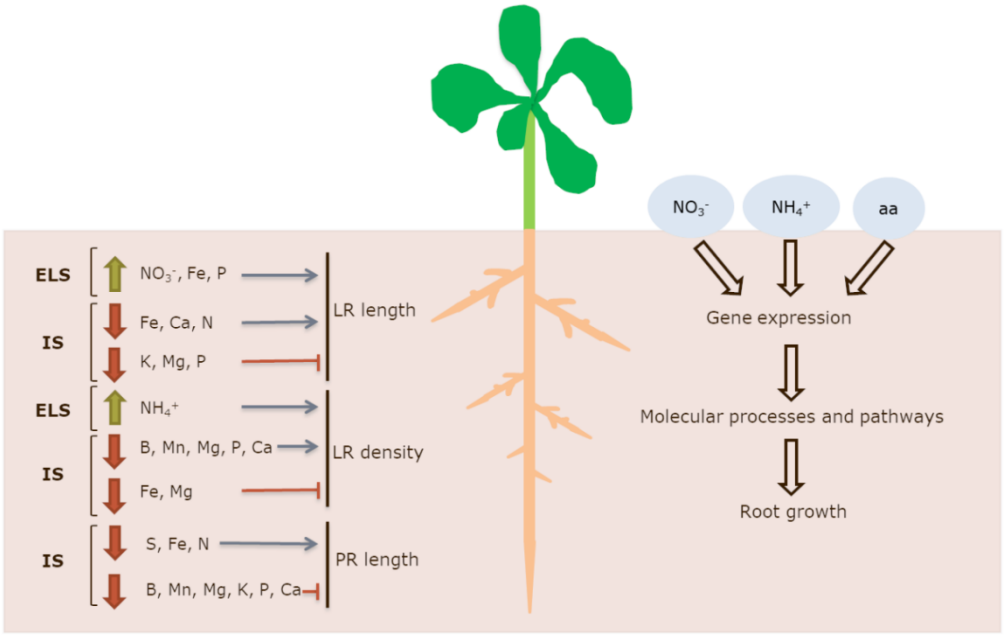

Figure 3. Overview of the main effects of nutrients on root system development in relation to their availability for plants (ELS: external local supply; IS: internal status). Moreover, the sensing and signalling of N compounds give rise to changes in gene expression and root plasticity to N supply. NO_3^-: nitrate; NH_4^+: ammonium; B: boron; Fe: iron; Mg: magnesium; Mn: manganese; P: phosphorus. Adapted from: Giehl *et al.* (2014) and Vidal and Gutiérrez (2008).

Based on the background and interest of the Biochemistry and Biotechnology research group (Universitat Jaume I) in the search for resistance inducers against biotic and abiotic stresses that not suppose any risk for the environment, we proposed the next

approaches. One of them was the use of complex compounds with copper, since Cu was traditionally used in agriculture and it is very effectively against pathogens. However, this compound causes several environmental risks when it is supplied in large amounts. Thus, we proposed to study the action mechanisms of Cu heptagluconate against *P. syringae* in tomato plants due to its efficacy as pesticide together with the interest of Idai Nature S.L. Company to test green compounds with Cu formulations.

To go deeper into the search of natural compounds against pathogens and based on previous results in the study of hexanoic acid treated plants metabolome that showed an accumulation of 1-MT metabolite in tomato plants infected with *P. syringae*, led us to study the effect of this compound in induced resistance. Moreover, a reduction of disease severity in tomato plants treated with this compound against *P. syringae* and *B. cinerea* was also observed by Camañes *et al*. (2015). Thus, we proposed to elucidate how 1-MT action mechanisms in treated and infected plants against *P. syringae* and study if it has a direct antimicrobial effect on the pathogen.

At last, previous works of the research group have shown that NH_4^+ nutrition causes "*ammonium syndrome*" inducing different metabolic changes and thereby provide resistance against *Pseudomonas syringae* infection through the activation of SAA mediated by ABA and Put (Fernández-Crespo *et al.*, 2015), we focused our attention in this compound. Hence, we proposed to study the mechanisms underlying the SAA mediated by NH_4^+, the changes in nutrient balance, C and N skeletons and their implication in the resistance against *P. syringae*. Moreover, the previous knowledge point to indicate that Put could be involved in induced resistance, thus we proposed to silence the Put synthesis pathways (ADC and ODC) in tomato plants in order to confirm the Put role against *P. syringae*.

At the same time, it was proposed to characterize the NH_4^+ nutrition effect in root development to elucidate how the N source affects to root development and N assimilation at transcriptional levels in tomato seedlings. Moreover, we investigated whether ABA plays any role in N-dependent root development. On the other hand, we have hypothesized that exogenous C compounds addition (Gluc or 2-OG) to NH_4^+ treatment might alleviate NH_4^+ toxicity.

REFERENCES

Achuo EA, Prinsen E, Höfte M. 2006. Influence of drought, salt stress and abscisic acid on the resistance of tomato to *Botrytis cinerea* and *Oidium neolycopersici*. Plant Pathology 55, 178-186.

Aflitos S, Schijlen E, Jong H, Ridder D, *et al*. 2014. Exploring genetic variation in the tomato (*Solanum* section *Lycopersicon*) clade by whole-genome sequencing. Plant J 80, 136-148.

Alexandersson E, Mulugeta T, Lankinen Å, Liljeroth E, Andreasson E. 2016. Plant Resistance Inducers against Pathogens in Solanaceae Species-From Molecular Mechanisms to Field Application. Int J Mol Sci. 17(10), 1673.

Alfano JR, Collmer A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. Journal of Bacteriology 179(18): 5655–5662.

Aktar MW, Sengupta D, Chowdhury A. 2009. Impact of pesticide use in agriculture: their benefits and hazards. Interdisc. Toxicol. 2(1), 1-12.

Andersen EJ, Ali S, Byamukama E, Yen Y, Nepal MP. 2018. Disease Resistance Mechanisms in Plants. Genes 9(7): 339.

Andolfo G, Ercolano MR. 2015. Plant innate immunity multicomponent model. Frontiers in Plant Science 6: 987.

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Drzewiecka K, Chmielowska-Bąk J. 2014. Aluminum induces cross-resistance of potato to *Phytophthora infestans*. Planta 239, 679.

Bari R, Jones JDG. 2009. Role of plant hormones in plant defence responses. Plant Mol Biol 69 (4), 473-488.

Bassil KL, Vakil C, Sanborn M, Cole DC, Kaur JS, Kerr KJ. 2007. Cancer health effects of pesticides. Systematic review. *Can. Fam. Physician* 53, 1704–1711.

Bastias DA, Martínez-Ghersa MA, Ballaré CL, Gundel PE. 2017. *Epichloë* fungal endophytes and plant defenses: not just alkaloids. Trends in Plant Sci. 22(11), 939-948.

Baxter A, Mittler R, Suzuki N. 2014. ROS as key players in plant stress signalling. J Exp Bot 65(5), 1229–1240.

Bhardwaj G, Pareek SS, Gupta P, Ravi I, Sharma V. 2013. Reactive Oxigen Species (ROS): role in plant defense. Biotech Today 3(2), 39-44.

Block A, Li G, Fu ZQ, Alfano JR. 2008. Phytopathogen type III effector weaponry and their plant targets. Current Opinion in Plant Biology 11, 396–403.

Boller T, Felix G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology 60, 379–406

Bolton MD. 2009. Primary metabolism and plant defense – fuel for the fire. *Molecular Plant Microbe Interaction* 22, 487–497.

Britto DT, Kronzucker HJ. 2002. NH_4^+ toxicity in higher plants: a critical review. Journal of Plant Physiology 159, 567–584.

Büttner D, He SY. 2009. Type III Protein Secretion in Plant Pathogenic Bacteria. Plant Physiology 150(4), 1656-1664.

Camañes G, Scalschi L, Vicedo B, González-Bosch C, García-Agustín P. 2015. An untargeted global metabolomic analysis reveals the biochemical changes underlying basal resistance and priming in *Solanum lycopersicum*, and identifies 1-methyltryptophan as a metabolite involved in plant responses to *Botrytis cinerea* and *Pseudomonas syringae*. The Plant Journal 84(1), 125–139.

Capiati DA, País SM, Téllez-Iñón MT. 2006. Wounding increases salt tolerance in tomato plants: evidence on the participation of calmodulin-like activities in cross-tolerance signalling. *J. Exp. Bot.* 57, 2391–2400.

Chanda B, Xia Y, Mandal MK, Yu K, Sekine KT, Gao QM, Selote D, Hu Y, Stromberg A, Navarre D, *et al.* 2011. Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. Nat Genet 43(5), 421–427.

Chaturvedi R, Venables B, Petros RA, Nalam V, Li M, Wang X, Takemoto LJ, Shah J. 2012. An abietane diterpenoid is a potent activator of systemic acquired resistance. Plant J. 71, 161–172.

Choi WG, Miller G, Wallace I, Harper J, Mittler R, Gilroy S. 2017. Orchestrating rapid long-distance signaling in plants with Ca^{2+}, ROS and electrical signals. Plant J. 90(4): 698–707.

Choudhary DK, Prakash A, Johri BN. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian J Microbiol 47, 289-297.

Conrath U. 2009. Priming of Induced Plant Defense Responses. In: VanLoon LC, ed. Plant Innate Immunity. London: Academic Press Ltd-Elsevier Science Ltd, 361–95.

Conrath U, Beckers GJM, Flors V, *et al.* 2006. Priming: Getting ready for battle. *Mol Plant-Microbe Interact* 19, 1062–1071.

Corzzi G, Last R. 2000: Assimilation of inorganic nitrogen into N-transport amino acids. In: Buchanan BB, Cruissem W, Jones RL, eds. Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, 362–379.

Couée I, Hummel I, Sulmon C, Gouesbet G, El Amrani A. 2004. Involvement of polyamines in root development. Plant Cell, Tissue and Organ Culture 76(1), 1–10.

Cruz C, Domínguez-Valdivia MD, Aparicio-Tejo PM, Lamsfus C, Bio A, Martins-Louçao MA, Moran JF. 2011. Intra-specific variation in pea responses to ammonium nutrition leads to different degrees of tolerance. Environmental and Experimental Botany 70, 233-243

Cui H, Tsuda K, Parker JE. 2015. Effector-triggered immunity: from pathogen perception to robust defense. Annual Review of Plant Biology 66, 487–511.

Cunnac S, Lindeberg M, Collmer A. 2009. *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. Current Opinion in Microbiology 12, 53–60.

Da Silva GP, Prado R de M, Ferreira RPS. 2016. Absorption of nutrients, growth and nutritional disorders resulting from ammonium toxicity in rice and spinach plants. Emirates Journal of Food and Agriculture 28, 882–889.

Damalas CA, Eleftherohorinos IG. 2011. Pesticide exposure, safety issues and risk assessment indicators. Int J Environ Res Public Health 8(5), 1402-1419.

Dangl JL, Jones JD. 2001. Plant pathogens and integrated defence responses to infection. Nature 411, 826–833.

Dermauw W, Pym A, Bass C, Van Leeuwen T, Feyereisen R. 2018. Does host plant adaptation lead to pesticide resistance in generalist herbivores? Current Opinion in Insect Science 26, 25-33.

Dodds PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. Nature Reviews Genetics 11, 539–548.

Domínguez-Valdivia MD, Aparicio-Tejo PM, Lamsfus C, Cruz C, Marins-Louçao MA, Moran JF. 2008. Nitrogen nutrition and antioxidant metabolism in ammonium-tolerant and -sensitive plants. Physiology Plantarum 132, 359-369.

Duan G, Christian N, Schwachtje J, Walther D, Ebenhöh O. 2013. The metabolic interplay between plants and phytopathogens. Metabolites 3, 1–23.

Durrant WE, Dong X. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol 42, 185-209.

Esteban R, Ariz I, Cruz C, Moran JF. 2016. Mechanisms of ammonium toxicity and the quest for tolerance. Plant Science 248, 92–101.

Food and Agriculture Organization of the United Nations (FAO). http://www.fao.org [accesed November 2018]

FAOSTAT. http://www.fao.org/faostat/en/#data/QC [accesed January 2019]

Fernández-Crespo E, Camañes G, Garcia-Agustin P. 2012. Ammonium enhances resistance to salinity stress in citrus plants. Journal of Plant Physiology 169, 1183–1191.

Fernández-Crespo E, Gómez-Pastor R, Scalschi L, Llorens E, Camañes G, García-Agustín P. 2014. NH_4^+ induces antioxidant cellular machinery and provides resistance to salt stress in citrus plants. Trees 28, 1693.

Fernández-Crespo E, Scalschi L, Llorens E, García-Agustín P, Camañes G. 2015. NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation. Journal of Experimental Botany 66(21), 6777-6790.

Ferrari S, Galletti R, Denoux C, De Lorenzo G, Ausubel FM, Dewdney J. 2007. Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. Plant Physiology 144, 367–379.

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R. 2009. (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol. 5(5), 344-350.

Frescatada-Rosa M, Robatzek S, Kuhn H. 2015. Should I stay or should I go? Traffic control for plant pattern recognition receptors. Current Opinion in Plant Biology 28, 23–29.

Galán JE, Collmer A. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284, 1322-1328.

Gamir J, Pastor V, Cerezo M, Flors V. 2012. Identification of indole-3-carboxylic acid as mediator of priming against Plectosphaerella cucumerina. Plant Physiology and Biochemistry 61, 169–179.

García-Brugger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B, Wendehenne D, Pugin A. 2006. Early signalling events induced by elicitors of plant defences. Molecular Plant Microbe Interaction 19(7), 711-724.

Garcion C, Métraux JP. 2006. Salicylic acid. In: Hedden P, Thomas SG, eds. Plant hormone signaling. Annual Plant Reviews 24, 229–257.

Gfeller A, Dubugnon L, Liechti R, Farmer EE. 2010. Jasmonate biochemical pathway. Sci Signal 3, 109.

Giehl RFH, Gruber BD, von Wirén N. 2014. It's time to make changes: modulation of root system architecture by nutrient signals. Journal of Experimental Botany 65, 769–778.

Gimenez-Ibanez S, Solano R. 2013. Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens. Front Plant Sci. 4, 72.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43, 205-227

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C. 2010. Food security: the challenge of feeding 9 billion people. Science 327(5967), 812-818.

Goff SA, Klee HJ. 2006. Plant volatile compounds: sensory cues for health and nutritional value? Science 311, 815-819.

Guilbaud CSE, Dalchau N, Purves DW, Turnbull LA. 2014. Is "peak N" key to understanding the timing of flowerin in annual plants? New Phytol. 205, 918-927.

Gust A, Biswas R, Lenz HD, Rauhut T, Ranf S, Kemmerling B, *et al*. 2007. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. Journal of Biological Chemistry 282, 32338–32348.

Hammond-Kosack KE, Jones JD. 1996. Resistance gene-dependent plant defense responses. Plant Cell 8(10), 1773–1791.

Hassan MAE and Buchenauer HJ. 2008. Enhanced control of bacterial wilt of tomato by DL-3-aminobutyric acid and the fluorescent *Pseudomonas* isolate CW2. Plant Dis Prot 115, 199.

Hassan MAE, Abo-Elyousr KAM. 2013. Activation of tomato plant defence responses against bacterial wilt Caused by *Ralstonia Solanacearum* using Dl-3-aminobutyric Acid (BABA). European journal of plant pathology 136(1), 145-157.

Hauser AR. 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nature Reviews Microbiology 7, 654–665.

Hodge A, Robinson D, Fitter A. 2000. Are microorganisms more effective than plants at competing for nitrogen? Trends Plant Sci 5, 304-308.

Hussain SS, Ali M, Ahmad M, Siddique KHM. 2011. Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. Biotechnol. Adv. 29, 300–311.

Jones JDG, Dangl JL. 2006. The plant immune system. Nature 444, 323–329.

Julkowska MM, Hoefsloot HCJ, Mol S, Feron R, de Boer GJ, Haring MA, Testerink C. 2014. Capturing Arabidopsis root architecture dynamics with ROOT-FIT reveals diversity in responses to salinity. Plant Physiology 166, 1387–1402.

Jung SC, Martínez-Medina A, Lopez-Raez JA, Pozo MJ. 2012. Mycorrhiza-induced resistance and priming of plant defences. J Chem Ecol. 38, 651-664.

Jung HW, Tschaplinski TJ, Wang L, Glazebrook J, Greenberg JT. 2009. Priming in systemic plant immunity. Science 324(80), 89–91.

Kangasjarvi S, Neukermans J, Li S, Aro EM, Noctor G. 2012. Photosynthesis, photorespiration, and light signalling in defence responses. Journal of Experimental Botany 63, 1619–1636.

Karpinski S, Szechynska-Hebda M, Wituszynska W, Burdiak P. 2013. Light acclimation, retrograde signalling, cell death and immune defences in plants. Plant, Cell and Environment 36, 736–744.

Kim SH, Kim SH, Yoo SJ, Min KH, Nam SH, Cho BH, Yang KY. 2013. Putrescine regulating by stress-responsive MAPK cascade contributes to bacterial pathogen defense in *Arabidopsis*. Biochemical and Biophysical Research Communications 437, 502–508.

Király L, Künstler A, Höller K, Fattinger M, Juhász C, Müller M, Gullner G, Zechmann B. 2012. Sulfate supply influences compartment specific glutathione metabolism and confers enhanced resistance to tobacco mosaic virus during a hypersensitive response. Plant Physiol. Biochem. 59, 44–54.

Koo AJK, Howe GA. 2009. The wound hormone jasmonate. Phytochemistry 70(13-14), 1571–1580.

Koornneef A, Leon-Reyes A, Ritsema T, Verhage A, Den Otter FC, Van Loon LC, Pieterse CMJ. 2008. Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant Physiol. 147(3), 1358-1368.

Kumudini BS, Jayamohan NS, Patil SV, Govardhana M. 2018. Primary Plant Metabolism During Plant–Pathogen Interactions and Its Role in Defense. In: Ahmad P, Ahanger MA, Singh VP, Tripathi DK, Alam P, Alyemeni MN. Plant Metabolites and Regulation Under Environmental Stress. Academic Press: 215-229.

Lee Y, Hong H, Hippe-Sanwald S, Hwang B. 2000. Histological and ultrastructural comparisons of compatible, incompatible and DL-β-amino-n-butyric acid-induced resistance responses of pepper stems to *Phytophthora capsici*. Physiol. Mol. Plant Pathol. 57, 269-280.

Li X, Gu JJ, Zhao XX, Li LM, Wu YH. 2009. A study on the mechanism of copper-induced resistance to potato virus Y-vein necrosis strain (PVY(N)) in tobacco. Bing Du Xue Bao 25(3), 226-230.

Li X, Lin H, Zhang W, Zou Y, Zhang J, Tang X, Zhou JM. 2005. Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. Proceedings of the National Academy of Sciences of the United States of America 102, 12990-12995.

Li Z, Nair SK. 2012. Quorum sensing: how bacteria can coordinate activity and synchronize their response to external signals? Protein Science 21, 1403-1417.

Lindow SE, Brandl MT. 2003. Microbiology of the phyllosphere. Applied and Environmental Microbiology 69, 1875–1883.

Liu JH, Wang W, Wu H, Gong X, Moriguchi T. 2015. Polyamines function in stress tolerance: from synthesis to regulation. Front Plant Sci. 6, 827.

Liu Y, von Wirén N. 2017. Ammonium as a signal for physiological and morphological responses in plants. Journal of Experimental Botany 68, 2581–2592.

Llorens E, Fernandez-Crespo E, Vicedo B, Lapeña L, García-Agustín P. 2013. Enhancement of the citrus immune system provides effective resistance against *Alternaria* brown spot disease. Journal of Plant Physiology 170, 146–154.

Llorens E, Agustí-Brisach C, González-Hernández AI, Troncho P, Vicedo B, Yuste T, Orero M, Ledó C, García-Agustín P, Lapeña L. 2016. Bioassimilable sulphur provides effective control of *Oidium neolycopersici* in tomato, enhancing the plant immune system. Pest Management Science 73(5), 1017-1023.

Llorens E, García-Agustín P, Lapeña L. 2017. Advances in induced resistance by natural compounds: towards new options for woody crop protection. Sci. Agric. 74(1), 90-100.

Loake G, Grant M. 2007. Salicylic acid in plant defence-the players and protagonists. Curr Opin Plant Biol 10(5), 466-472.

López-Gresa MP, Maltese F, Bellés JM, Conejero V, Kim HK, Choi YH, Verpoorte R. 2010. Metabolic response of tomato leaves upon different plant– pathogen interactions. Phytochemical Analysis 21, 89–94.

Luna E, Bruce TJA, Roberts MR, Flors V, Ton J. 2012. Next-Generation Systemic Acquired Resistance. Plant Physiol 158, 844–53.

Luna E. 2016. Using green vaccination to brighten the agronomic future. Outlooks on Pest Management 27(3), 136-140.

Maino JL, Umina PA, Hoffmann AA. 2018. Climate contributes to the evolution of pesticide resistance. *Global Ecol Biogeogr*. 27, 223–232.

Malamy J, Klessig DF. 1992. Salicylic acid and plant disease resistance. The Plant Journal 2(5), 643-654.

Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA, Toth I, Salmond G, Foster GD. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular Plant Pathology 13, 614–629.

Mansoorkhani FM, Seymour GB, Swarup R, Bagheri HM, Ramsey RJL, Thompson AJ. 2014. Environmental, developmental, and genetic factors controlling root system architecture. Biotechnology and Genetic Engineering Reviews 30(2), 95-112.

Marschner H. 2012. Marschner's Mineral Nutrition of Higher Plants, 3rd Edn London: Academic Press.

Martinez-Medina A, Flors V, Heil M, *et al*. 2016. Recognizing Plant Defense Priming. Trends Plant Sci 21, 818–822.

McDowell JM, Dangl JL. 2000. Signal transduction in the plant immune response. Trends in Biochemical Sciences 25, 79–82.

Mehari ZH, Elad Y, Rav-David D, Graber ER, Harel YM. 2015. Induced systemic resistance in tomato (*Solanum lycopersicum*) against *Botrytis cinerea* by biochar amendment involves jasmonic acid signaling. Plant Soil. 395(1–2), 31-44.

Melotto M, Underwood W, He SY. 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. Annual review of phytopathology 46, 101-122.

Melotto M, Underwood W, Koczan J, Nomura K, He SY. 2006. Plant stomata function in innate immunity against bacterial invasion. Cell 126, 969-980.

Melotto M, Kunkel BN. 2013. Virulence strategies of plant pathogenic bacteria. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F. (eds) The Prokaryotes. Springer, Berlin, Heidelberg.

Michaelson MJ, Price HJ, Ellison JR, Johnston JS. 1991. Comparison of plant DNA contents determined by Feulgen microspectrophotometry and laser flow cytometry. Am J Bot 78, 183–188.

Miflin BJ, Habash DZ. 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. Journal of Experimental Botany. 53, 979-987.

Ministerio de Agricultura Alimentación y Medio Ambiente, Registro de Productos Fitosanitarios - Agricultura https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/productos/forexi.asp?e=0andplagEfecto=28andculUso=010401040101000andambUti=01andsolEsp=

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F. 2011. ROS signaling: the new wave? Trends in Plant Science 16, 300–309.

Mittler R, Blumwald E. 2015. The Roles of ROS and ABA in Systemic Acquired Acclimation. The Plant Cell 27(1), 64-70.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 104, 19613–19618

Monaghan J, Zipfel C. 2012. Plant pattern recognition receptor complexes at the plasma membrane. Current Opinion in Plant Biology 15(4), 349–357.

Moore JW, Loake GJ, Spoel SH. 2011. Transcription dynamics in plant immunity. The Plant Cell 23(8), 2809-2820.

Mur LAJ, Kenton P, Atzorn R, Miersch O, Wasternack C. 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. Plant Physiol 140, 249–262.

Muthamilarasan M, Prasad M. 2013. Plant innate immunity: An updated insight into defense mechanism. J. Biosci. 38(2), 433-449.

Nacry P, Bouguyon E, Gojon A. 2013. Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil 370(1-2), 1-29.

Nguyen HP, Chakravarthy S, Velásquez AC, McLane HL, Zeng L, Nakayashiki H, Park DH, Collmer A, Martin GB. 2010. Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. Molecular Plant-Microbe Interactions 23(8), 991–999.

Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L. 2016. Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. Front Public Health 4, 148.

OECD. 2017. Safety Assessment of Transgenic Organisms in the Environment. OECD Consensus Documents, OECD Publishing, Paris 7, 69-104.

Pang XM, Zhang ZY, Wen XP, Ban Y, Moriguchi T. 2007. Polyamine, all-purpose players in response to environment stresses in plants. Plant Stress 1, 173–188.

Park SW., Kaimoyo E, Kumar D, Mosher S, Klessig DF. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science 318, 113–116.

Pauwels L, Goossens A. 2011. The JAZ Proteins: A Crucial Interface in the Jasmonate Signaling Cascade. The Plant Cell 23 (9), 3089-3100.

Pieterse CMJ, Van Pelt JA, Ton J, Parchmann S, Mueller MJ, Buchala AJ, Métraux JP, Van Loon LC. 2000. Rhizobacteria-mediated induced resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. 57, 123-134.

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM. 2009. Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5(5), 308–316.

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker PAHM. 2014. Induced Systemic Resistance by Beneficial Microbes. Annual Review of Phytopathology 52(1), 347-375.

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK. 2007. Stress-induced morphogenic responses: growing out of trouble? Trends in Plant Science 12, 98–105.

Potters G, Pasternak TP, Guisez Y, Jansen MAK. 2009. Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant, Cell and Environment 32, 158–169.

Robertson GP, Vitousek PM. 2009. Nitrogen in agriculture: balancing the cost of an essential resource. Annu. Rev. Environ. Resour. 34, 97-125.

Rojas CM, Senthil-Kumar M, Tzin V, Mysore KS. 2014. Regulation of primary plant metabolism during plant–pathogen interactions and its contribution to plant defense. Frontiers in Plant Science 5, 17.

Rolland F, Baena-González E, Sheen J. 2006. Sugar sensing and signaling in plants: conserved and novel mechanisms. Annual Review of Plant Biology 57, 675–709.

Ron M, Avni A. 2004. The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. Plant Cell 16, 1604–1615.

Rosebrock TR, Zeng L, Brady JJ, Abramovitch RB, Xiao F, Martin G.B. 2007. A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448, 370–374.

Sánchez-Pérez JM, Antigüedad I, Arrate I, García-Linares C, Morell I. 2003. The influence of nitrate leaching through unsaturated soil on groundwater pollution in an agricultural area of the basque country. Sci. Total Environ. 317, 173–187.

Sánchez-Vallet A, Ramos B, Bednarek P, López G, Piślewska-Bednarek M, Schulze-Lefert P, Molina A. 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. The Plant Journal 63(1), 115-127.

Santner A, Calderón-Villalobos LI, Estelle M. 2009. Plant hormones are versatile chemical regulators of plant growth. Nature Chemical Biology 5(5), 301-307.

Santner A, Estelle M. 2009. Recent advances and emerging trends in plant hormone signalling. Nature 459, 1071–1078.

Scalschi L, Vicedo B, Camanes G, Fernandez-Crespo E, Lapena L, Gonzalez-Bosch C, García-Agustín P. 2013. Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways. Molecular Plant Pathology 14(4): 342-355.

Scharte J, Schon H, Weis E. 2005. Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with *Phytophthora nicotianae*. Plant, Cell and Environment 28, 1421–1435.

Senthilkumar M, Sadashiva AT, Laxmanan V. 2017. Impact of water stress on root architecture in tomato (*Solanum lycopersicum* Mill). International Journal of Current Microbiology and Applied Sciences 6(7), 2095-2103.

Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD. 2001. Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proceedings of the National Academy of Sciences, USA 98, 4788–4793.

Shah J, Chatuverdi R, Chowdhury Z, Venables B, Petros RA. 2014. Signaling by small metabolites in systemic acquired resistance. Plant J. 79, 645-658.

Shah J, Zeier J. 2013. Long-distance communication and signal amplification in systemic acquired resistance. Frontiers in Plant Science 4, 30.

Shan L, He P. 2018. Pipped at the post: pipecolic acid derivative identified as SAR regulator. Cell 173(2): 286-287.

Sharaf EF, Farrag AA. 2004. Induced resistance in tomato plants by IAA against Fusarium oxysporum lycopersici. Pol J Microbiol 53(2), 111–116.

Silva VL, Lopes CA. 1995. Populações epifíticas de *Pseudomonas syringae* pv. tomato em cultivo comercial de tomateiro industrial. Fitopatologia Brasileira 20, 179-183.

Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X. 2009. Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell 137(5), 860-72.

Staswick PE. 2008. JAZing up jasmonate signaling. Trends Plant Sci. 13, 66–71.

Suzuki N, Miller G, Salazar C, Mondal HA, Shulaev E, Cortes DF, Shuman JL, Luo X, Shah J, Schlauch K, *et al*. 2013. Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants. Plant Cell 25, 3553–3569.

The Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485, 635–641.

Thordal-Christensen H. 2003. Fresh insights into processes of non host resistance. Current Opinion in Plant Biology 6(4), 351–357.

Tohge T, de Souza LP, Fernie AR. 2014. Genome-enabled plant metabolomics. Journal of Chromatography B 966, 7–20.

Tranchida-Lombardo V, Aiese Cigliano R, Anzar I, *et al*. 2018. Whole-genome re-sequencing of two Italian tomato landraces reveals sequence variations in genes associated with stress tolerance, fruit quality and long shelf-life traits. DNA Res. 25(2), 149-160.

Uma B, Podile AR. 2014. Overlapping sets of transcripts from host and non-host interactions of tomato are expressed early during non-host resistance. Plant Omics Journal 7(1), 19-27.

United Nations Sustainable Development Summit 2015 https://sustainabledevelopment.un.org/post2015/summit

Vallad GE, Goodman RM. 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci. 44, 1920–1934.

Van der Ent S, Van Wees SCM, Pieterse CMJ. 2009. Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. Phytochemistry 70(13-14), 1581–1588.

Van Wees SCM, Van der Ent S, Pieterse CMJ. 2008. Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol. 11(4), 443–448.

Venturi V. 2006. Regulation of quorum sensing in *Pseudomonas*. FEMS Microbiology 30(2), 274–291.

Verma V, Ravindran P, Kumar PP. 2016. Plant hormone-mediated regulation of stress responses. BMC Plant Biol 16, 86.

Vicedo B, Flors V, Leyva MD, Finiti I, Kravchuk Z, Real MD, Garcia-Agustin P, Gonzalez-Bosch C. 2009. Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. Molecular Plant-Microbe Interactions 22(11), 1455–1465.

Vidal EA, Gutiérrez RA. 2008. A systems view of nitrogen nutrient and metabolite responses in *Arabidopsis*. Current Opinion in Plant Biology 11, 521–529.

Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol 47, 177-206.

Vorholt JA. 2012. Microbial life in the phyllosphere. Nature Reviews Microbiology 10, 828–840.

Walch-Liu P, Neumann G, Bangerth F, Engels C. 2000. Rapid effects of nitrogen form on leaf morphogenesis in tobacco. Journal of Experimental Botany 51, 227–237.

Walters DR, Ratsep J, Havis ND. 2013. Controlling crop diseases using induced resistance: challenges for the future. Journal of Experimental Botany 64(5), 1263–1280.

Walters DR. 2011. Plant defense: warding off attack by pathogens, herbivores, and parasitic plants. Blackwell Publishing Ltd, 236.

Walters DR. 2003. Polyamines and plant disease. Phytochemistry 64(1), 97–107.

Wan J, Zhang XC, Neece D, Ramonell KM, Clough S, Kim SY, Stacey MG, Stacey G. 2008. A LysM Receptor-Like Kinase Plays a Critical Role in Chitin Signaling and Fungal Resistance in *Arabidopsis*. Plant Cell 20(2), 471–481.

Wang C, El-Shetehy M, Shine MB, Yu K, Navarre D, Wendehenne D, Kachroo A, Kachroo P. 2014. Free radicals mediate systemic acquired resistance. Cell Rep. 7(2), 348–355.

Wilson C, Tisdell C. 2001. Why farmers continue to use pesticides despite environmental, health and sustainability costs. Ecological Economics 30, 449-462.

Xin XF, He SY. 2013. *Pseudomonas syringae* pv. tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. Annual Review of Phytopathology 51, 473-498.

Xuan W, Beeckman T, Xu G. 2017. Plant nitrogen nutrition: sensing and signaling. Current Opinion in Plant Biology 39, 57–65.

Zandalinas SI, Mittler R, Balfagón D, Arbona V, Gómez-Cadenas A. 2018. Plant adaptations to the combination of drought and high temperatures. Physiol. Plant 162, 2–12.

Zeller W. 2006. Status on induced resistance against plant bacterial diseases. Fitosanidad 10(2): 99-103.

Zhao Y, Damicone JP, Demezas DH, Rangaswamy V, Bender CL. 2000. Bacterial leaf spot of leafy crucifers in Oklahoma caused by *Pseudomonas syringae* pv. *maculicola*. Plant Disease 84, 1015–1020.

Zheng XY, Spivey NW, Zeng W, Liu PP, Fu ZQ, Klessig DF, He SY, Dong X. 2012. Coronatine promotes Pseudomonas syringae virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. Cell Host Microbe 11(6), 587–596.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125, 749–760.

Zorzoli R, Pratta GR, Rodríguez GR, Picardi LA. 2007. Advances in biotechnology: tomato as a plant model system. Functional Plant Science and Biotechnology 1(1), 146-159.

OBJECTIVES

Objectives

In order to propose new alternatives to reduce the use of pesticides, and to study in depth the mechanisms of induced resistance based on treatments with Cu heptagluconate, 1-methyltryptophan (1-MT) and the NH_4^+ in the tomato-*P. syringae* pathosystem model, the following objectives were set out:

1. Proposal of a new formulation based on the combination of heptagluconic acid with copper as an inducer of resistance against *P. syringae*.

2. The study of the mode of action and the modifications in the apoplastic content produced by 1-MT to go in-depth into the resistance mechanisms against *P. syringae* and to check their possible direct effect on the bacteria.

3. The analysis of the NH_4^+ effect on the nutritional balance and the primary metabolism in the pathosystem model.

4. The generation of transgenic silenced plants in the two putrescine biosynthesis pathways (ADC and ODC) in an independent manner to study their response in the induced resistance against *P. syringae*. At the same time, the cellular localisation of proteins SlADC and SlODC will be studied.

5. The study of how N sources can affect the root growth parameters and the expression of the genes involved in auxin transport and N assimilation. Moreover, we propose to study whether the addition of carbon sources can mitigate mild NH_4^+ toxicity, and if ABA could play a role in root development in response to different N sources.

OBJETIVOS

Objetivos

Con el fin de proponer nuevas formulaciones alternativas para reducir el uso de pesticidas y, profundizar en el estudio de los mecanismos de resistencia inducida basados en los tratamientos con heptagluconato de Cu, 1-metiltriptófano (1-MT) y el NH_4^+ en el patosistema modelo tomate-*P. syringae*, se plantearon los siguientes objetivos:

1. Propuesta de una nueva formulación basada en la combinación del ácido heptaglucónico con cobre, como inductor de resistencia frente a *P. syringae*.

2. Estudio del modo de acción del 1-MT y de los cambios producidos por éste en el contenido del apoplasto para profundizar en el conocimiento del mecanismo de resistencia frente a *P. syringae* y, testar su posible efecto directo sobre la bacteria.

3. Análisis del efecto del NH_4^+ sobre el balance nutricional y el metabolismo primario en el patosistema modelo.

4. Generación de plantas silenciadas en las dos rutas de biosíntesis de la putrescina (ADC y ODC) de forma independiente para ver su respuesta en la resistencia inducida frente a *P. syringae*. Al mismo tiempo, se estudiará la localización celular de las proteínas SlADC y SlODC.

5. Estudio de cómo la fuente de N puede afectar a los parámetros de crecimiento de la raíz y sobre la expresión de genes implicados en el transporte de auxinas y asimilación de N. Asimismo, se estudiará si la adición de fuentes de carbono puede mitigar la toxicidad del NH_4^+ y se determinará si el ABA puede jugar un papel en el desarrollo de la raíz en respuesta a distintas fuentes de N.

PLAN DE TRABAJO Y METODOLOGÍA

Plan de trabajo y metodología

PLAN DE TRABAJO

Material vegetal

Para llevar a cabo este trabajo, se han utilizado plantas de tomate (*Solanum lycopersicum* Milll.) cv. Ailsa Craig en todos los experimentos, excepto para el silenciamiento de los genes *ADC* y *ODC* que se han utilizado plantas de tomate cv. Moneymaker debido a que la transformación en este cultivar es más eficiente. Para la sobreexpresión transitoria de los genes *ADC* y *ODC* donde se han utilizado plantas de *Nicotiana benthamiana*. Además, para los experimentos de desarrollo de raíz, se ha utilizado el mutante *flacca* (background Ailsa Craig). Todas las plantas fueron crecidas en cámara de cultivo en condiciones controladas de temperatura (26/18 ºC día/noche), foperiodo 16/8 h (día/noche), humedad (60 %) y luz (200 μmol m^{-2} s^{-1}).

Microorganismos utilizados

El principal patógeno utilizado en este trabajo ha sido *Pseudomonas syringae* pv. tomato DC3000. Además, para los experimentos de localización de la bacteria en hoja de tomate de plantas tratadas con 1-MT se utilizó *P. syringae* 775EGFP (DC3000 marcada con GFP). Las bacterias se crecieron en medio King B líquido a 28ºC en oscuridad. Para la inoculación, la tercera y cuarta hoja fueron sumergidas en la solución bacteriana de $5 \cdot 10^5$ unidades formadoras de colonias (ufc) por mL en $MgSO_4$ 10 mM y Silwet-L77 0,01%. El daño causado por la bacteria se cuantificó a 72 horas después de la infección mediante el conteo de ufc y por síntomas visuales determinando el porcentaje de hoja infectada.

Tratamientos aplicados

Los tratamientos se han aplicado siguiendo el siguiente plan de trabajo:

1. Aplicación del compuesto heptagluconato de cobre: plantas de tomate cv. Ailsa Craig de cuatro semanas de edad fueron tratadas con heptagluconato de cobre (IDAI Cobre) proporcionada por la empresa Idai Nature S.L. (La Pobla de Vallbona, Valencia, España). La concentración que se aplicó fue la recomendada por la empresa para su uso en campo (6 ml/l). El tratamiento preventivo fue aplicado vía

radicular por riego con 20 ml de la solución por planta 72 horas antes de la inoculación y el tratamiento curativo fue aplicado por riego con 20 ml de la solución o por spray con 5 ml de la solución por planta 24 horas después de la infección. (Método descrito en el Capítulo 1)

2. Aplicación del compuesto 1-MT: plantas de tomate cv. Ailsa Craig de cuatro semanas de edad fueron tratadas vía radicular 72 horas antes de la infección con 20 ml de 1-MT (5 mM) a pH=6 disuelto en solución Hoagland. (Método descrito en el Capítulo 2)

3. Tratamiento con NH_4^+: una vez germinadas las semillas, las plantas de tomate cv. Ailsa Craig fueron regadas durante tres semanas con solución Hoagland carente de N y suplementada con distintas fuentes de N. Las plantas control se regaron con la solución 10 mM de N en forma de KNO_3 o de $(NH_4)_2SO_4$ como única fuente de N. En la solución de NH_4^+, K_2SO_4 y $CaSO_4$ fueron añadidos para evitar la carencia de K^+ y Ca^{2+} y la sal sódica MES como solución tampón. (Métodos descritos en los Capítulos 3 y 4)

4. Tratamiento con NO_3^-, NH_4^+ y distintas proporciones 2:2 y 1:3 (NO_3^-:NH_4^+). Las semillas de tomate cv. Ailsa y el mutante *flacca* fueron germinadas en placas con agar en oscuridad durante 72 h. A continuación, las semillas germinadas se traspasaron a placas con medio nutritivo con las diferentes fuentes de nitrógeno y carbono. El nitrógeno fue aplicado como NO_3^- en forma de KNO_3 y $Ca(NO_3)_2$ para las plantas tratadas con NO_3^- o como NH_4^+ en forma de $(NH_4)_2SO_4$. En el tratamiento con NH_4^+ y en las proporciones, el K y el Ca fueron suplementados en forma de K_2SO_4 y $CaSO_4$. En los tratamientos con las proporciones 2:2 y 1:3, el N fue suministrado en forma de KNO_3 y $(NH_4)_2SO_4$ y suplementado con $CaSO_4$ y K_2SO_4. Además, la sal sódica MES fue añadida en todos los tratamientos con el fin de mantener el pH, el cual fue ajustado a 5.8-6.0. Por otra parte, diferentes fuentes de C fueron adicionadas en concentraciones fisiológicas al tratamiento NH_4^+ 10 mM en forma de glucosa al 1% (NH_4^++ Gluc) o 2-oxoglutarato al 0,004 % (NH_4^++ 2-OG). (Método descrito en el Capítulo 5)

METODOLOGÍA

1. Determinación y cuantificación de H_2O_2

Se tomaron muestras de hojas para la tinción con 3′,3-diaminobenzidina (DAB) a las 48 horas después de la infección siguiendo el procedimiento descrito por Fernández-Crespo *et al*. (2015). (Método descrito en el Capítulo 1)

2. Análisis de hormonas, aminoácidos y azúcares

El análisis de hormonas se llevó a cabo mediante HPLC-MS y la cuantificación con la ayuda del programa MASSLYNX, según lo descrito por Llorens *et al*. (2016). (Método descrito en los Capítulos 1 y 2)

El análisis de aminoácidos se llevó a cabo por dos métodos, UPLC-MS sin derivatizar (método descrito en el Capítulo 2) y, otro por derivatización siguiendo el procedimiento indicado por Hilo *et al*. (2017). (Método descrito en el Capítulo 3)

El análisis de azúcares fue llevado a cabo siguiendo el procedimiento de Cebolla-Cornejo *et al.* (2012) y de Hilo *et al*. (2017). (Métodos descritos en los Capítulos 2 y 3, respectivamente)

3. Análisis de ácidos orgánicos

La cuantificación de estos metabolitos se llevó a cabo mediante cromatografía iónica-MS según lo descrito por Ghaffari *et al*. (2016). (Método descrito en el Capítulo 3)

4. Análisis de nutrientes

Para determinar la concentración de nutrientes, las muestras fueron digeridas con ácido nítrico en un ultraclave. Posteriormente, fueron diluidas en H_2O mQ y se realizó un análisis con la técnica de espectroscopía de emisión óptica de plasma acoplado inductivamente (ICP-OES). (Método descrito en el Capítulo 3)

5. Análisis de expresión génica

Esta técnica se utilizó para analizar la expresión de los trascritos de los genes seleccionados. Para ello, se extrae el RNA mensajero total de las muestras, se retrotranscribe a cDNA (retrotranscripción) y posteriormente se amplifica con la ayuda

de los primers específicos por qPCR. El protocolo seguido ha sido el descrito por Scalschi *et al*. (2014). (Métodos descritos en los Capítulos 2, 4 y 5)

6. Extracción de apoplasto

La extracción de los compuestos presentes en el apoplasto, se llevó a cabo 48 horas después de la inoculación siguiendo el método descrito por O'Leary *et al.* (2014). (Método descrito en el Capítulo 2)

7. Cuantificación de la apertura estomática

La epidermis adaxial de las hojas muestreadas se puso en contacto con una resina dental según lo descrito por Delgado *et al.* (2011). El análisis de la apertura estomática fue llevado a cabo según lo descrito por Scalschi *et al*. (2013). (Método descrito en el Capítulo 2)

8. Transformación estable en plantas de tomate

Para iniciar el procedimiento, se diseñaron los primers adecuados para silenciar las rutas de la síntesis de putrescina (ADC y ODC). Se amplificaron las secuencias de interés *ADC* u *ODC* y, posteriormente, el fragmento amplificado fue introducido en el vector intermedio pDNOR207 y finalmente en el pBIN19, vector final transferido a *Agrobacterium tumefaciens* LBA4404. El Agrobacterium fue precultivado para infectar los cotiledones. Una vez inoculados, los cotiledones fueron transferidos a un medio de cocultivo durante dos días con el objetivo de facilitar la transformación. Transcurrido ese periodo de tiempo, los cotiledones fueron lavados y transferidos a un medio no selectivo sin kanamicina (- kan) y, posteriormente, a un medio selectivo con kanamicina (+ kan). Una vez que las plántulas seleccionadas y originadas a partir de un callo, alcanzaron un tamaño de aproximadamente dos centímetros, fueron transferidas a medio de enraizamiento. Una vez crecida la raíz, se dispusieron en maceta en cámara de cultivo y, transcurridos quince días aproximadamente, se llevaron al invernadero para la obtención de semillas de la primera generación (T1) (Scalschi *et al*., 2014). (Método descrito en el Capítulo 4)

9. Transformación transitoria en plantas de *Nicotiana benthamiana*

Para iniciar el procedimiento, se diseñaron los primers adecuados para amplificar las secuencias completas de los genes *ADC* u *ODC*. Posteriormente, el fragmento

amplificado fue introducido en el vector intermedio pDNOR207 y finalmente en el pEarleyGate 101, vector final transferido a *Agrobacterium tumefaciens* LBA4404. Una vez insertada la secuencia de interés (*ADC* u *ODC*) en el plásmido pEarleyGate 101 en *Agrobacterium tumefaciens* LBA4404, se estableció un precultivo de dichas bacterias que contienen las construcciones de interés de forma independiente y otro con el p19, un supresor de silenciamiento del gen. Una vez alcanzada una densidad óptica (λ=600 nm) de 0.3-0.6, la solución que contiene las células bacterianas se centrifugó a 5000 rpm y el precipitado fue resuspendido en una solución de $MgCl_2$ y MES hasta ajustarlo una DO de 0.3. Se indujeron los genes *vir* con la adición de acetosiringona y se procedió a infiltrar las hojas con ayuda de una jeringa. Tres días después, las plantas agroinfiltradas fueron observadas por microscopia confocal con el fin de observar la localización de las proteínas ADC y ODC. (Método descrito en el Capítulo 4)

10. Análisis estadístico

Los experimentos realizados en todos los capítulos, se repitieron al menos tres veces y cada réplica constaba de, al menos, 8 plantas. Los resultados de las distintas réplicas fueron analizados de forma conjunta a través de un análisis de varianza ANOVA ($P>0,05$) o por el test LSD de Fisher ($P\geq0,05$). El procesado de los datos se realizó con el programa estadístico Statgraphics Centurion XVI.

Los heatmaps fueron generados por transformación logarítmica usando el paquete de gplots del software estadístico R (versión 3.4.3). (Método descrito en el Capítulo 2)

El análisis de componentes principales (PCA) se realizó con el software MetaboAnalyst 3.0 software. Los resultados fueron normalizados por mediana, transformados por raíz cúbica, distribuidas por el método de Pareto y procesados con un análisis multivariante PCA. (Método descrito en el Capítulo 3)

REFERENCIAS

Cebolla-Cornejo J, Valcárcel M, Herrero-Martínez JM, Roselló S, Nuez F. 2012. High efficiency joint CZE determination of sugars and acids in vegetables and fruits. Electrophoresis 33(15), 2416–2423.

Delgado D, Alonso-Blanco C, Fenoll C, Mena M. 2011. Natural variation in stomatal abundance of *Arabidopsis thaliana* includes cryptic diversity for different developmental processes. Annals of botany 107(8), 1247–58.

Fernández-Crespo E, Scalschi L, Llorens E, García-Agustín P, Camañes G. 2015. NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation. Journal of Experimental Botany 66(21), 6777-6790.

Geisler M, Nadeau J, Sack FD. 2000. Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the too many mouths mutation. The Plant Cell 12(11), 2075–86.

Ghaffari MR, Shahinnia F, Usadel B, Junker B, Schreiber F, Sreenivasulu N, Hajirezaei MR. 2016. The metabolic signature of biomass formation in barley. Plant Cell and Physiology 57(9), 1943-1960.

Hilo A, Shahinnia F, Druege U, Franken P, Melzer M, Rutten T, von Wirén N, and Hajirezaei MR. 2017. A specific role of iron in promoting meristematic cell division during adventitious root formation. Journal of Experimental Botany 68(15), 4233-4247.

Llorens E, Agustí-Brisach C, González-Hernández AI, Troncho P, Vicedo B, Yuste T, *et al.* 2016. Bioassimilable sulphur provides effective control of *Oidium neolycopersici* in tomato, enhancing the plant immune system, Pest Management Science 73(5), 1017-1023.

O'Leary BM, Rico A, McCraw S, Fones HN, Preston, GM. 2014. The infiltration-centrifugation technique for extraction of apoplastic fluid from plant leaves using *Phaseolus vulgaris* as an example. Journal of Visualized Experiments 94, 1-8.

Scalschi L, Vicedo B, Camanes G, Fernandez-Crespo E, Lapena L, Gonzalez-Bosch C, Garcia-Agustin P. 2013. Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways. Molecular Plant Pathology, 14 (4), 342–355.

Scalschi L, Camañes G, Llorens E, Fernández-Crespo E, López MM, García-Agustín P, Vicedo B. 2014. Resistance inducers modulate *Pseudomonas syringae* pv. tomato strain DC3000 response in tomato plants. PloS one 9, e106429.

Scalschi L, Sanmartín M, Camañes C, Troncho P, Sánchez-Serrano JJ, García-Agustín P, Vicedo B. 2014. Silencing of *OPR3* in tomato reveals the role of OPDA in callose deposition during the activation of defense responses against *Botrytis cinerea*. Plant Journal, 81: 304-315

APORTACIONES ORIGINALES

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PUBLICACIONES

1. **Ana I. González-Hernández**, Eugenio Llorens, Carlos Agustí-Brisach, Begonya Vicedo, Teresa Yuste, Antonio Cerveró, Carlos Ledó, Pilar García-Agustín and Leonor Lapeña. 2018. Elucidating the mechanism of action of copper heptagluconate on the plant immune system against *Pseudomonas syringae* in tomato (*Solanum lycopersicum* L). **Pest Management Science**, 74(11): 2601-2607. doi: 10.1002/ps.5050.
2. Loredana Scalschi, Eugenio Llorens, **Ana I. González-Hernández**, Mercedes Valcarcel, Jordi Gamir, Pilar García-Agustín, Begonya Vicedo and Gemma Camañes. 2018. 1-Methyltryptophan modulates tomato resistance against *Pseudomonas syringae*. **Frontiers in Microbiology**, 9: 2056. doi: 10.3389/fmicb.2018.02056.
3. **Ana I. González-Hernández**, Emma Fernández-Crespo, Loredana Scalschi, Mohammad-Reza Hajirezaei, Nicolaus von Wirén, Pilar García-Agustín and Gemma Camañes. 2019. Ammonium mediated changes in carbon and nitrogen metabolisms induce resistance against *Pseudomonas syringae* in tomato plants. (Manuscrito enviado a **Journal of Plant Physiology**)
4. **Ana I. González-Hernández**, Loredana Scalschi, Pilar García-Agustín and Gemma Camañes. 2019. Changes in root development and N assimilatory pathways are ABA-dependent in tomato plants grown under different N sources. (Manuscrito enviado a **Plant Physiology and Biotechnology**)

COMUNICACIONES A CONGRESOS:

Pósters:

1. **Ammonium nutrition modifies root system architecture in tomato plants**

   Autores: **Ana I. González-Hernández**, Loredana Scalschi, Emma Fernández-Crespo, Eugenio Llorens, Begonya Vicedo, Pilar García-Agustín and Gemma Camañes

   Nombre del congreso: XIV RBMP

   Ciudad de celebración: Salamanca, España

   Fecha de celebración: 04/07/2018 Fecha de finalización: 06/07/2018

Entidad organizadora: SEFV

2. **Copper heptagluconate as eco-friendly compound enhancing the plant immune system of *Solanum lycopersicum* against *Pseudomonas syringae*, causal agent of bacterial speck**

   Autores: **Ana I. González-Hernández**, Eugenio Llorens, Carlos Agustí Brisach, Begoña Vicedo, Teresa Yuste, Antonio Cerveró, Carlos Ledó, Pilar García-Agustín and Leonor Lapeña.

   Nombre del congreso: 18th International Conference on Organic Fruit Growing

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3. **Ammonium nutrition changes C/N metabolism and induces resistance against *Pseudomonas syringae***

   Autores: **Ana I. González-Hernández**, Emma Fernández-Crespo, Loredana Scalschi, Eugenio Llorens, Leonor Lapeña, Begonya Vicedo, Mohammad Reza Hajirezaei, Nicolaus von Wirén, Pilar García-Agustín and Gemma Camañes Querol

   Nombre del congreso: XIV Solanaceae and 3rd Cucurbitaceae Joint Conference

   Ciudad de celebración: Valencia, Comunidad Valenciana, España

   Fecha de celebración: 03/09/2017 Fecha de finalización: 06/09/2017

4. **NH_4^+ nutrition downstream metabolites are involved in the induced resistance in tomato plants against *Pseudomonas syringae***

   Autores: Emma Fernández Crespo, **Ana I. González-Hernández**, Loredana Scalschi, Eugenio Llorens, Leonor Lapeña, Begonya Vicedo, Pilar García-Agustín and Gemma Camañes

   Nombre del congreso: Nitrogen2016

   Ciudad de celebración: Montpellier, Francia

   Fecha de celebración: 22/08/2016 Fecha de finalización: 26/08/2016

   Entidad organizadora: EMBO

5. **Tryptophan derivatives involved in *Solanum lycopersicum* responses to *Pseudomonas syringae***

   Autores: **Ana I. González-Hernández**, Loredana Scalschi, Emma Fernández-Crespo, Eugenio Llorens, Leonor Lapeña, Begonya Vicedo, Pilar García-Agustín and Gemma Camañes

Nombre del congreso: Workshop New Frontiers in Plant Biology

Ciudad de celebración: Madrid, Comunidad de Madrid, España

Fecha de celebración: 15/06/2016 Fecha de finalización: 17/06/2016

Entidad organizadora: CBGP (Madrid, España)

Comunicaciones orales:

1. **NH_4^+ nutrition produces several changes in primary metabolism which protect tomato plants against *Pseudomonas syringae***

   Autores: **Ana I. González-Hernández**, Emma Fernández-Crespo, Loredana Scalschi, Eugenio Llorens, Begonya Vicedo, Mohammad-Reza Hajirezaei, Nicolaus von Wiren, Pilar García-Agustín and Gemma Camañes

   Nombre del congreso: XIV Reunión Nacional Metabolismo del Nitrógeno

   Autor de correspondencia: Sí

   Ciudad de celebración: Segovia; España

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   Entidad organizadora: Centro de Biología Molecular Severo Ochoa

   Ciudad entidad organizadora: Madrid

CHAPTER 1

Elucidating the mechanism of action of Copper heptagluconate on the plant immune system against *Pseudomonas syringae* in tomato (*Solanum lycopersicum* L)

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ABSTRACT

Phytopathogenic problems caused by the bacterial pathogen *Pseudomonas syringae* in tomato have been becoming more serious by the emergence of resistant strains to classical pesticides. This situation has propelled the research for new formulations with lower environmental problems. One of the most promising alternatives to the use of classical pesticides is the induction of natural plant defences. New formulations based on Cu complexed with heptagluconic acid are able to induce plant innate defences which could be an alternative to the classical treatments based on inorganic Cu against bacterial speck. In order to study the efficacy of this compound in tomato against *P. syringae*, we tested its systemic effect applied the treatments via radicular and infected tomato leaves. Treated plants showed lower infection development and lower number of viable bacteria in leaves. We also observed better performance in parameters involved in plant resistance such as antioxidant response and accumulation of phenolic compounds. Thus, results showed that soil drench applications can be highly effective for the prevention and control of bacterial speck in tomato plants, showing a reduction of symptoms around 50%. Moreover, the application of Cu heptagluconate induced accumulation of plant polyphenols caffeic and chlorogenic acids and reduced the amount of ROS in infected plants.

KEYWORDS: Copper heptagluconate, induced resistance, *Pseudomonas syringae*, *Solanum lycopersicum*

INTRODUCTION

Pseudomonas syringae is a hemibiotrophic pathogen with a high degree of specialization and host specificity. According to that specificity, the different *P. syringae* strains have been classified in pathovars (pv.) which only can infect a certain number of plant species or a certain plant species (Xin and He, 2013). *P. syringae* pv. tomato (Okabe, 1933) Young, Dye and Wilkie, 1978 is considered as one of the most devastating pathogens of tomato plants (*Solanum lycopersicum* L.) over the world. This pathogen causes lesions on the stems, as well as on the petioles and fruits, which could reduce the production of the plant by 60%. The typical symptoms are black necrotic specks surrounded by a yellow halo. The bacteria are able to survive on the plant leaf in an epiphytic lifestyle and enter into the apoplastic space through natural openings or wounds. If the bacterial population is high enough, the presence of elevated levels of

homoserine lactone (AHL) will induce the expression of virulence factors and secondary metabolites that mediate colonization of the host. This coordinated expression of virulence factors depending on the population density is known as quorum-sensing (QS) (Scalschi *et al.*, 2014). As a hemibiotrophic pathogen, *P. syringae* can multiply very fast in a susceptible host under favorable conditions. However, this initial multiplication occurs in the apoplast, with the absence of apparent plant cell death. At the later stage of pathogenesis, plant cells die and infected tissues show necrotic spots (Xin and He, 2013). Despite the large number of investigations about the pathogenicity of *P. syringae* (Xin and He, 2013; Bashan and De-Bashan, 2002), disease control is still based on preventive chemical spray applications. Nowadays, around 100 compounds including strains of non-pathogenic bacteria, plant extracts, essential oils, antibiotics, etc. have been tested to control bacterial diseases. However, only 20 of them have been tested against *P. syringae* (McLeod *et al*., 2017). But, these alternative compounds are not generalized or allowed in crop protection reducing the number of available compounds mainly to inorganic formulations of copper (Cu), such as Cu-hydroxide or Cu-sulphate, or a mixture of Cu-based and ethylene bis-dithiocarbamates (EBDC; commonly called mancozeb or maneb) (magrama.gob.es). In addition, resistant commercial tomato cultivars to *P. syringae* have not been obtained yet (McLeod *et al*., 2017).

On the other hand, the generalized use of Cu-based compounds has lead the appearance of Cu resistant strains that reduced dramatically the effectiveness of classical treatments (Griffin *et al*., 2017). Moreover, the use of combinations of Cu-based compounds with EBDC are restricted to the pre-harvesting period since it can last for up to eight weeks in tomato crops. Additionally, there are many environmental concerns about the accumulation of both Cu and EBDC in the soil. The extensive use of Cu-based fungicides is widely spread in agriculture reporting contamination not only in tomato fields but also in hop fields, apple and especially in vineyards (Schramel *et al*., 2000; Komárek *et al*., 2010). In order to avoid these environmental problems, there is an increasing interest in developing compounds able to induce the plant natural defences as a potential alternative to chemicals. Likewise, it has been demonstrated that the application of some natural and chemical compounds induces a plant innate resistance that, usually, is enough to overcome the stress and survive (Scalschi *et al*., 2013; Llorens *et al*., 2015). Moreover, some resistant inducers showed a persistent effect up to

several months, which can contribute to reduce the amount of residues in the vegetables and fruits (Llorens *et al*., 2017; Llorens *et al*., 2015).

The study of new compounds based on organic formulations of Cu and its double effect as an inductor of the plant innate defences would provide a valuable alternative to farmers to control pathogens such as *P. syringae*. Cu heptagluconate is a compound characterized by the great Cu absorption and diffusion over the plant, and it is safer for the environment compared with others traditional inorganic Cu-based fungicides (Zhang *et al*., 2013). Nevertheless, biochemical studies elucidating the mechanism of action of Cu-heptagluconate against *P. syringae*, and its compatibility and lack of phytotoxicity in tomato have not been performed yet. Therefore, the main goal of this study was to elucidate the efficacy of Cu enhancing the plant immune system in tomato against *P. syringae*. For this purpose, the pathosystem *P. syringae* vs. tomato was set up as a model to study the biochemistry and physiology of plant-microbe-Cu heptagluconate interaction. In order to know the mechanism of action of the treatment, infection development, physiological parameters, and several parameters enrolled in plant immune system were monitored.

MATERIAL AND METHODS

Plant material and growth chamber conditions

Tomato seeds of *Solanum lycopersicum* cv. Ailsa Craig were sowed in individual plastic pots (100 ml) filled with Sphagnum peat as substrate (Jiffy products, Kristiansand, Norway) and maintained in distilled water until the firsts true leaves were developed. Subsequently, seedlings were irrigated with Hoagland`s solution (Hoagland and Arnold, 1950). They were grown and pre-conditioned under growth chamber conditions at temperatures ranging from 18 to 26°C with a 16/8 h day/night photoperiod and 60% relative humidity (RH). Four-week old tomato plants were used for the experiments performed in this study. When the experiments were conducted, plants were placed in humid chambers (plastic containers, 59 × 40 × 35 cm, 80% RH), maintained in the growth chamber, and irrigated with Hoagland`s solution until the end of the experiment. The experiments were conducted in the growth chamber of the Department of Agricultural and Environmental Sciences of Universitat Jaume I (UJI) of Castellon (Spain).

Bacterial strain and inoculum preparation

The *P. syringae* strain DC3000, rifampicin resistant (Cuppels, 1986) was used in this study. The bacterial strain was grown in King B medium (KB) (King *et al*., 1954) with rifampicin added (50 mg ml^{-1}; Sigma Aldrich, San Luis, MO, USA) at 28ºC in darkness for 24 h. The bacterial suspension used for leaves inoculation was obtained from this culture, by resuspending the bacteria in sterile $MgSO_4$ (10 mM) solution containing 0.01% of the surfactant Silwet L-77 (Osi Specialties, Danbury, CT, USA) and adjusted to 5×10^5 colony-forming units (cfu) ml^{-1} using spectrophotometer (Fernández-Crespo *et al*., 2015).

Effect of Cu heptagluconate on plant growth and plant immune system

Copper heptagluconate, treatments, and bacterial inoculation

Liquid Cu heptagluconate (Cu 6.0% p/p = 8.22% p/v, IDAI Cobre) was provided by the company Idai Nature S.L. (La Pobla de Vallbona, Valencia, Spain).

Applications of Cu heptagluconate were performed when seedlings were at three-four true leaf stage (four-week old tomato plants) with a solution at 6 ml l^{-1} as recommended by the manufacturer for field applications. Preventive treatments were performed by irrigating 20 ml of the solution per plant 72 h before inoculation, and curative treatments were performed by irrigating 20 ml of the solution per plant, 24 h after inoculation or by spraying 5 ml of the solution per plant, 24 h after inoculation. Pathogen inoculation was performed by dipping the third and fourth leaves into the bacterial suspension described above for 3 sec. Additionally, plants treated with Cu heptagluconate and non-inoculated, non-treated and inoculated (positive control), and non-treated and non-inoculated (negative control) were included in the experiment for comparative purposes. During the experiment, all plants were maintained in humid chambers and irrigated as described above. There were 10 replicated plants per treatment (4 treatments), 40 plants in total. The experiment was conducted three times.

Disease severity, disease incidence and plant growth

To determine the disease severity (DS), third and fourth leaves of each plant and treatment were selected at 72 h post-inoculation (pi) and evaluated by visual observations determining the percentage of dark-brown spots developed on the leaf

surface. Disease incidence (DI) was determined by counting the cfu of *P. syringae* strain DC3000 per gr of leaves developed in KB medium. To this end, three leaves samples of 50 mg of each infected treatment were washed with sterile water, ground and resuspended in 50 ml of $MgSO_4$ 10 mM. Serial dilutions from these suspensions were plated in KB medium with rifampicin (50 mg ml^{-1}). Cfu were counted after 24 h at 28º C in darkness.

To evaluate the effect of the product on plant growth, total growth was evaluated on non-inoculated plants at 30 days after Cu heptagluconate treatment measuring the plant length from soil surface to the apical shoot. The plant length of non-treated plants (control) was also measured. Moreover, the roots and shoots of the 10 plants of each treatment (Cu heptagluconate and control) were separated and dried in an oven at 65 ºC for 2 days. Subsequently, dried plant tissues were weighed, and the dry weight (DW) was expressed as biomass.

Determination and quantification of H_2O_2

Samples of 10 leaves per treatment were collected for 3′,3-diaminobenzidine (DAB) staining at 48 h pi. Leaves were cut and put immediately in 1 mg ml^{-1} DAB at pH <3 for 24 h in darkness and were subsequently discolored 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) (Llorens *et al*., 2016).

Evaluation of hormones and phenolic compounds related to plant defense by chromatographic analysis

Fresh material (10 leaves per treatment and experiment; 1 leaf per plant in each treatment and experiment) 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 standard deuterated abscisic acid ([$^2H_6$] ABA), deuterated salicylic acid ([$^2H_4$] SA), dihydrojasmonic acid (dhJA) and propylparaben was added at 100 ng ml^{-1} prior to extraction in order to quantify the level of hormones 12-oxo-phyto dienoic acid [OPDA], SA and ABA and phenolic compounds (Caffeic and chlorogenic acids), respectively. After extraction, a 20 µl aliquot was injected directly into the high-performance liquid chromatography (HPLC) system.

For both hormones and phenolic compounds measurements, analyses were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100 mm × 2 mm, i.d. 5 μm; Scharlab, Barcelona, Spain; http://www.scharlab.com). 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 (Llorens *et al*., 2016).

Statistical analysis

All experiments were conducted at least two times. Data from different repetitions was analysed together due to the fact that analysis of variance (ANOVA) did not show significant differences ($P > 0.05$) between repetitions in each experiment. Subsequently, for each assessment, ANOVA was performed with DI, DS, plant growth, H_2O_2, hormones or phenolic compound levels as dependent variables and treatment (plants treated or non-treated with Cu heptagluconate and/or inoculated or non-inoculated with *P. syringae* DC3000 as independent variable). All data of this study were tested for normality, homogeneity of variances, and residual patterns. Mean values were compared using the Fisher's protected least significant difference (LSD) test at $P = 0.05$ (Steel and Torrie, 1986). Statistical analyses were performed by using the software Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA, USA).

RESULTS

Effect of Cu heptagluconate on disease severity, plant growth and bacterial populations

The preventive treatment with Cu heptagluconate by soil drench reduced markedly the development of black necrotic specks triggered by *P. syringae* in tomato leaves (Fig 1). Plants treated by Cu heptagluconate develop only a few symptoms of the disease on leaves 72 h pi with *P. syringae* strain tomato DC3000 (Fig. 1B) in comparison with the visible damage observed in leaves from non-treated and inoculated plants (positive control) (Fig. 1A). Significant differences in *P. syringae* infection (%) and viable *P. syringae* populations (cfu mg^{-1} of leaf) were observed between the positive control, and treated and inoculated plants ($P = 0.0003$ and $P=0.0022$, respectively). At 72 h pi,

leaves from plants treated with Cu heptagluconate showed 31.7% of leaf surface covered by black necrotic specks, whereas the positive control had affected leaf surface > 70.51%, which represents a reduction in the percentage of lesions by 55.1% by the use of Cu heptagluconate (Fig. 2A). The control obtained with the preventive treatment is higher than the observed with curative applications which showed a reduction of symptoms around 25% (Fig. S1). In the same way, the preventive treatment also significantly reduced the viable *P. syringae* populations in tomato leaves compared with those from the positive control (Fig. 2B). The analysis showed a bacterial population around 1.14×10^8 cfu and 8.80×10^8 g^{-1} of leaf for inoculated treated and non-treated plants (control), respectively. This result indicates that preventive soil drench applications with Cu heptagluconate are reduces the presence of viable bacteria populations *in planta* by 85.9%, which supports the reduction observed in the percentage of lesions on leaf surface. Due to the fact that the preventive treatment showed better results reducing the bacterial symptoms, we focused the analysis of the mechanism of action in this treatment.

Figure 1. Effect of Cu heptagluconate on tomato against *P. syringae*. (A) Leaf of tomato from non-treated plants 72 hpi with *P. syringae* strain DC3000 at 5×10^5 cfu ml^{-1} showing bacterial specks; (B) Leaf of tomato from treated plants with Cu heptagluconate by irrigation, 72 hpi with *P. syringae* strain tomato DC3000 as described before.

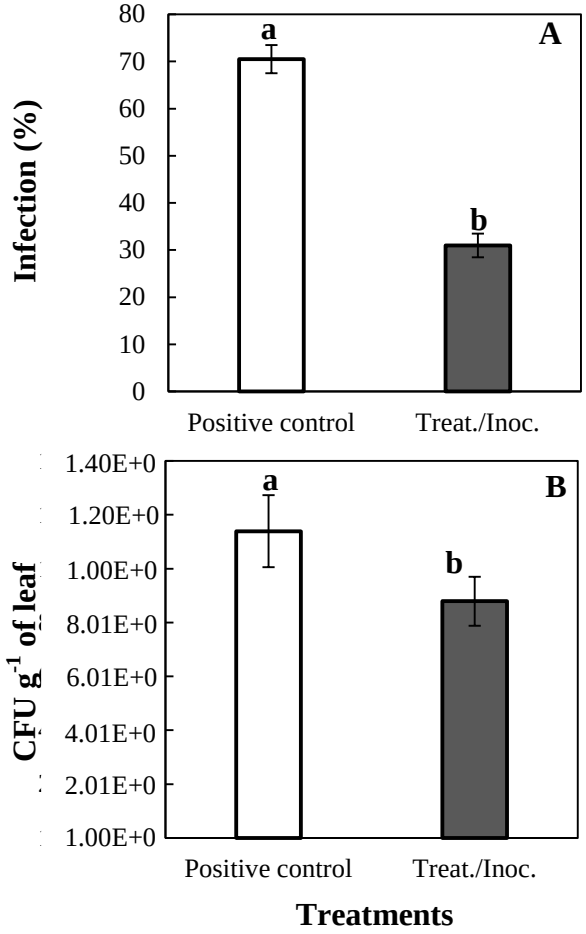

Figure 2. Effect of Cu heptagluconate on the resistance of tomato plants 72 hpi with *P. syringae* strain DC3000 at 5 $\times 10^5$ cfu ml^{-1}. (A) Infection rate scored measuring the percentage leaf covered by bacterial specks, and (B) bacterial population assessed using KB medium, for the treatments Positive control: Non-treated and inoculated plants; and Treat. Inoc.: Treated and inoculated plants. Columns represent the means of three independent experiments of 10 replicated plants per treatment. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

Physiological parameters

Even though plants treated with Cu heptagluconate showed higher values of plant height, and shoot and root dry weight than control plants, ANOVA did not show significant differences (Plant height: $P = 0.5239$; Shoot dry weight: $P = 0.3804$; Root dry weight: $P = 0.4112$) between both lots of plants. This indicates that the uptake of Cu by the plant is not causing phytotoxicity so it does not compromise the development of the plant.

Effect of Cu heptagluconate on the levels of H_2O_2

In general, the formation of a dark brown insoluble precipitate was observed after leaf staining in 3,3'-DAB for 24 h in all leaf samples tested, indicating H_2O_2 accumulation. Nevertheless, ANOVA showed significant differences in the levels of H_2O_2 between the four treatments tested (P = 0.0002). Samples collected from plants treated with Cu heptagluconate and inoculated with *P. syringae* showed fewer dark brown precipitate than that observed in samples from positive control plants. This result indicates that treatments with Cu heptaglunate decrease markedly the levels of H_2O_2 in treated plants. In fact, the level of H_2O_2 in positive control plants was 30% higher than that observed in plants treated with Cu heptagluconate and inoculated with *P. syringae*. Interestingly, the effect of Cu heptagluconate was also visible in the batch of plants which were treated but non-inoculated, also showing a reduction of ROS levels ≈30 times compared with positive and negative (non-treated and non-inoculated plants) controls (Fig. 3).

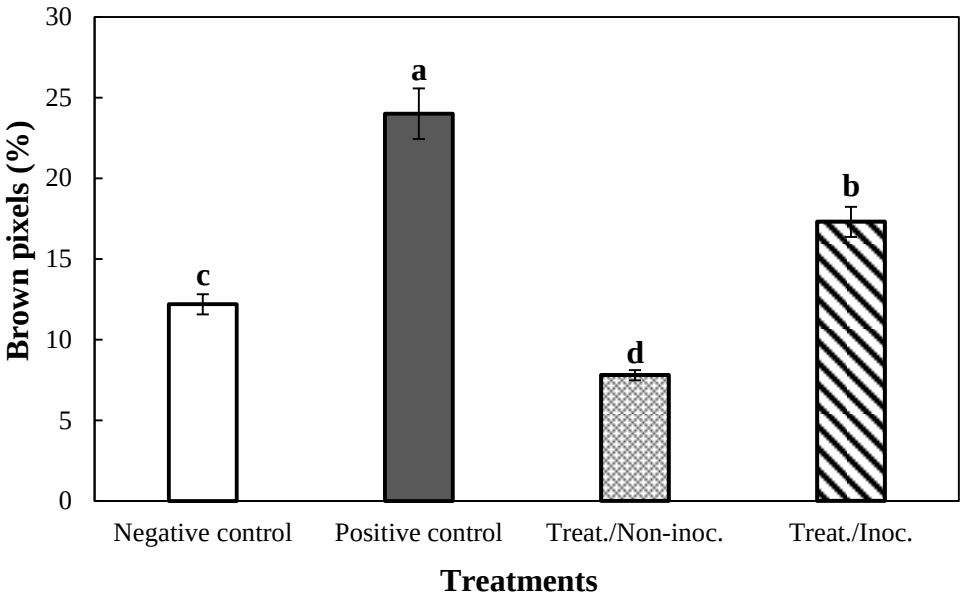

Figure 3. H_2O_2 staining represented as % of leaf area, estimated by using DAB staining in the leaves of infected tomato plants. The following treatments were evaluated: Negative control (Non-treated and non-inoculated plants); Positive control (Non-treated and inoculated plants); Treat. Non-inoc. (Treated and non-inoculated plants); and Treat. Inoc. (Treated and inoculated plants). Columns represent the means of three independent experiments of 10 replicated plants per treatment. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

Effect of Cu heptagluconate on the enhancement of hormones related to plant defence

For all hormones tested, their levels were higher in inoculated plants in both treated and non-treated lots of plants with Cu heptagluconate, with the exception of SA. Levels of OPDA were higher in inoculated plants without significant differences (P = 0.0858) between positive control (437.6 ± 138.1 ng g^{-1} of leaf) and treated and inoculated plants (336.7 ± 12.1 ng g^{-1} of leaf) (Fig. 4A). On the other hand, the results obtained for SA showed a different pattern to that observed for OPDA. The highest level of SA was observed in treated and inoculated plants (1206.3 ± 417.0 ng g^{-1} of leaf) showing no significant differences between the rest of the treatments tested (P = 0.5290). Moreover, the SA level of the positive control was lower (820.9 ± 281.2 ng g^{-1} of leaf) than that observed for treated and non-inoculated plants (998.7 ± 388.5 ng g^{-1} of leaf), but ANOVA did not show significant differences in SA level between these two treatments (P = 0.5290) (Fig. 4B). Finally, the levels of ABA were similar in plants from the four treatments tested without significant differences in ABA level between treatments (P = 0.8705). Despite the lack of significant differences between treatments in this last case, ABA levels were higher in both lots of inoculated plants (Positive control: 3479.6 ± 531.5 ng g^{-1} of leaf; Treated and inoculated plants: 3786.6 ± 349.7 ng g^{-1} of leaf) (Fig. 4C).

Effect of Cu heptagluconate on the levels of phenolic compounds

The measurement of the accumulation of phenolic compounds in leaves showed that soil drench treatments with Cu heptagluconate induce the accumulation of caffeic and chlorogenic acids. Accumulation of caffeic acid was induced in response to the inoculation with *P. syringae* in both plants, treated with Cu heptagluconate (18161.0 ± 2184.9 ng g^{-1} of leaf) and nontreated plants (17011.2 ± 2277.1 ng g^{-1} of leaf), without significant differences between treatments (P = 0.7341). Interestingly, treated and non-inoculated plants also showed a significantly high level of caffeic acid (14798.5 ± 3042.6 ng g^{-1} of leaf) compared to non-treated plants, but without significant differences between the two lots of inoculated plants (P = 0.6541) (Fig. 5A). On the other hand, accumulation of Chlorogenic acid was not observed in both lots of non-treated plants (negative and positive control), whereas the treatment with Cu heptagluconate induced the accumulation of this phenolic compound in both inoculated (5802.4 ± 3515.5 ng g^{-1}

of leaf) and non-inoculated (5720.9 ± 5431.9 ng g^{-1} of leaf) plants, without significant differences between them (P = 0.4625) (Fig. 5B).

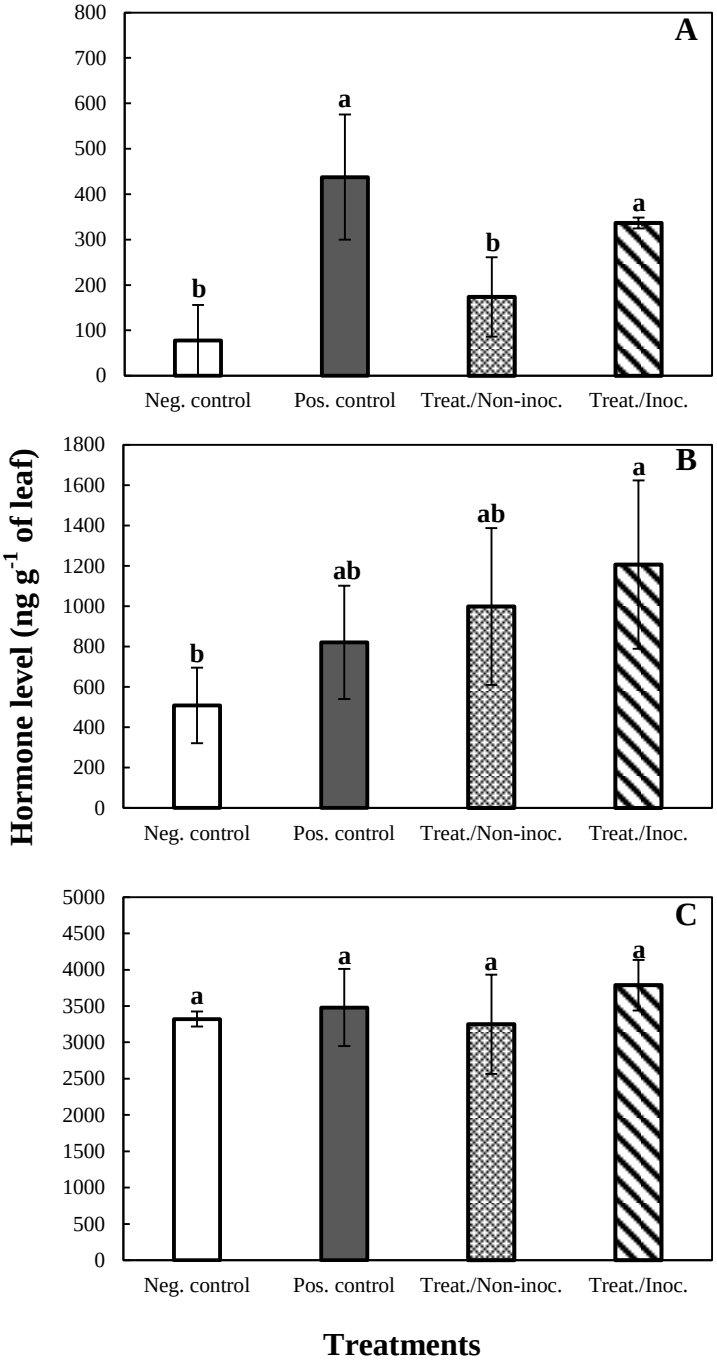

Figure 4. Hormone levels in tomato plants subjected to the following treatments: Negative control (Non-treated and non-inoculated plants); Positive control (Non-treated and inoculated plants); Treat. Non-inoc. (Treated and non-inoculated plants); and Treat. Inoc. (Treated and inoculated plants). Levels of (A) OPDA, (B) SA and (C) ABA were determined in freeze-dried material by HPLC. Columns represent the means of three experiments with 10 replicated plants per treatment. Vertical bars are the standard error of

the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

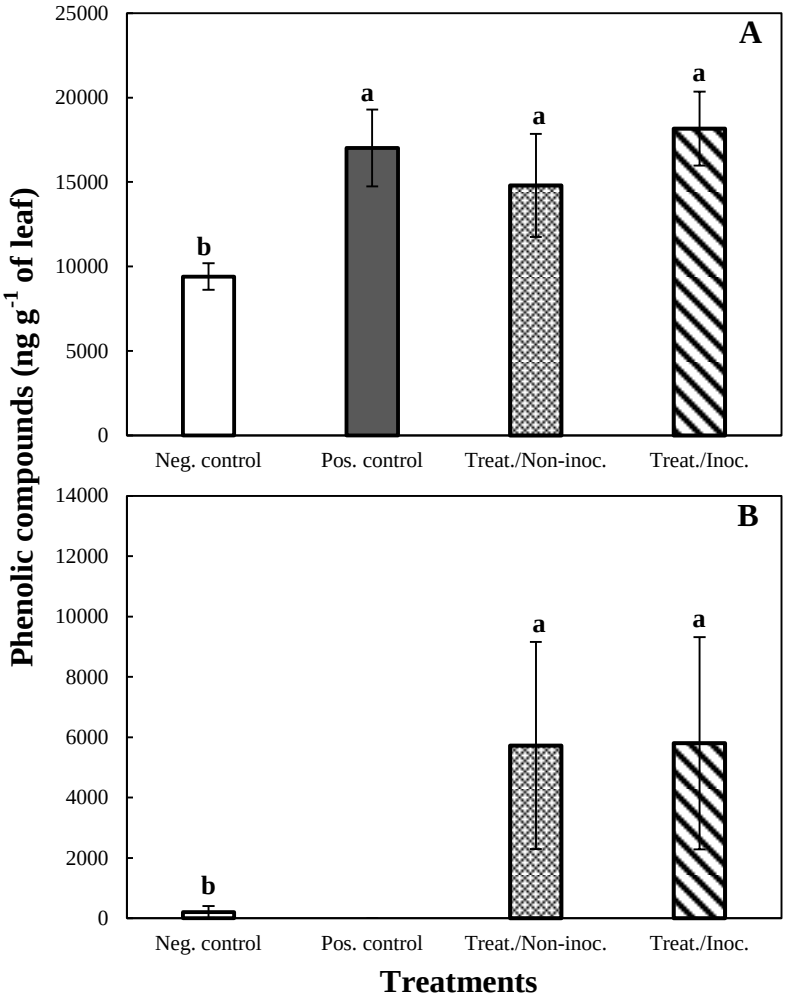

Figure 5. Levels of phenolic compounds tomato plants subjected to the following treatments: Negative control (Non-treated and non-inoculated plants); Positive control (Non-treated and inoculated plants); Treat. Non-inoc. (Treated and non-inoculated plants); and Treat. Inoc. (Treated and inoculated plants). Levels of (A) caffeic acid and (B) chlorogenic acid were determined in freeze-dried material by HPLC. Columns represent the means of three experiments with 10 replicated plants per treatment. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

DISCUSSION AND CONCLUSIONS

Copper-based fungicides are among the oldest pesticides used in agriculture. Since the discovery of the "Bordeaux mixture" in the nineteen century, the use of copper against plant diseases have been maintained until nowadays. However, the excessive use of inorganic formulations for the lasts 140 years has brought with it a number of problems.

The first of these problems is the toxic levels of Cu due to anthropogenic activities (Yruela, 2009; Micó *et al*., 2006). The excessive use of Cu based pesticides to control plant diseases has resulted in Cu accumulation in the surface layer of agricultural soils (Mackie *et al*., 2012; Michaud *et al*., 2007). In Europe, continuous spray of Cu based pesticides, such as Bordeaux mixture has drastically increased the Cu pollution of soils. In agricultural soils, normal Cu concentration varies from 5 to 30 mg kg^{-1} depending on soil type, but the soils of vineyards, which are commonly sprayed with this kind of pesticides, contain Cu that ranges from 200 to 500 mg kg^{-1} (Brun *et al*., 1998). The excess of Cu may cause toxicity to the environment and for the crops. Is well known that photosynthetic reactions, both photochemical and biochemical ones, are usually inhibited many heavy metals and in particular Cu (Küpper *et al*., 2009). This excess of metal in the soil can result in decreasing final fruit number, dry root weight, and plant height (Sonmez *et al*., 2006).

On the other hand, the excessive use of copper in agriculture leads to the appearance of resistant pests. Since 1986, an increasing number of studies have reported Cu resistance in *P. syringae* (Bender and Cooksey, 1986) and other related pathogens such as *Xanthomonas* spp. (Griffin *et al*., 2017; Behlau *et al*., 2013) and the subsequently reduced efficacy of Cu-based products for controlling these pathogens. In this way, it has been demonstrated that the application of Cu is ineffective in managing tomato bacterial speck in South Africa, probably due to the presence of Cu resistant *P. syringae* populations in the region (McLeod *et al*., 2017). Therefore, alternative management strategies must be developed in order to combat those diseases and minimize the damage caused by the excessive use of Cu.

In this study, we evaluated the effectiveness of a formulation of Cu complexed with heptagluconic acid, which is characterized by high assimilability by the plant. The results obtained showed that, although the formulation of this compound is absorbed highly by the plant, there were no symptoms of phytotoxicity. A single treatment prior to bacterial inoculation was able to reduce disease symptoms and the number of viable bacteria in leaves by more than 50%. This control is higher than observed in treatments with Cu hydroxide or other inorganic formulations in tomato, which can range from 40% (Iacobellis *et al*., 2003) to no control in others (Ji *et al*., 2006). The lack of control of regular inorganic formulations has forced to the use of alternatives or combinations of compounds such as Cu with mancozeb or Cu with Acibenzolar-s-methyl (ASM) (Ji *et*

al., 2006; Huang *et al*., 2012). Moreover, the content of copper in the classical formulations can range from 20% of Cu in the Bordeaux mixture to 50% of Cu in copper oxychloride formulations (Ferreira *et al*., 2007), whereas the content of copper in the Cu heptagluconate formulation is only 6%. According to the recommended doses of application, this reduction of Cu in the formulation would represent a reduction between 40 and 65% compared with classical formulations. In this way, under field conditions, Dagostin *et al*. (2011) demonstrated that heptagluconic acid controlled *P. viticola* at the same levels than copper hydroxide reducing the amount of metallic copper per ha per year by 40% in comparison to the control treatments.

On the other hand, our results also showed a reduction of ROS in treated and infected plants. Generation of ROS during pathogen attack is part of the defensive response and is involved in the stimulation of hypersensitive cell death (Apel and Hirt, 2004). However, the accumulation of ROS produced by the disruption of the cellular homeostasis under pathogen attacks also produces oxidative damage in membrane lipids, proteins, and nucleic acids (Mittler, 2002). The production of ROS in plants after *P. syringae* infection is induced by the non-host-specific phytotoxin coronatine (COR) (Ishiga *et al*., 2009). COR is a toxin formed by the coronafacic acid (CFA) and coronamic acid (CMA) which acts as a structural and functional analog of plant signal molecules, such as jasmonic acid (JA) (Ishiga *et al*., 2009; Uppalapati *et al*., 2005). Presence of COR promotes stomatal aperture, which facilitates the entry of the bacteria into the mesophilic, and induce the JA pathway and antagonize SA-mediated defence responses during *P. syringae* infection, preparing the plant for a successful pathogen colonization. Our results showed that levels of OPDA are lower in plants treated with Cu than those observed in untreated controls, and therefore, the SA is not repressed. This lack of hormonal alteration expected by the presence of COR after *Pseudomonas* inoculation and the reduced levels of ROS may indicate that the application of Cu via soil drench could cause a delay or impairment in the pathogenesis machinery or an induction of plant defensive responses. In order to ascertain if the application of the treatment is inducing the plant natural defences we measured the phenolic compounds. These metabolites have been found throughout the plant kingdom and possess antioxidant activity and its induction has been described as part of the active defence of the plant (Hammerschmidt, 2014; Baker *et al*., 2015). Besides its direct antimicrobial activity, phenolic compounds are also able to inactivate microbe produced enzymes that

are involved in pathogenesis or inhibit the synthesis of specific toxins (Bostock *et al*., 1999; Wojciechowska *et al*., 2014). Our results show the application of Cu in soil drench induces accumulation of chlorogenic and caffeic acids in absence of inoculation. In this way, previous studies showed that the application of resistance inducers or priming agents such as acibenzolar-S-methyl, isonitroacetophenone, lipopolysaccharide, flagellin or chitosan resulted in the accumulation of phenolic compounds (Hammerschmidt, 2014) which could indicate that the application of Cu is protecting the plant not only by a direct antimicrobial activity but also by the induction of plant defences. Previous studies about the effect of resistance inducers demonstrated that the application of priming compounds is able to affect the behavior of the bacteria in the plant. Scalschi *et al*. (2014) observed that the reduction of bacterial population provoked by the resistance inducer application prevent bacteria from reaching the quorum sensing, delaying in this way the production of COR and the subsequent colonization of the plant.

As conclusion of this work, the application of Cu heptagluconate by irrigation manages to activate the defence mechanisms of the plant reducing the incidence of *P. syringae* by more than 50%, by the direct antimicrobial activity of Cu, together with the increase of the phenolic compounds an activation of this mechanism against the pathogen. It should be noted that the activation of plant innate immunity combined with the direct effect of Cu against the pathogen, could prevent the bacteria from reaching the quorum sensing and, thus, reduce the amount of COR produced by the bacteria with the subsequent reduction of oxidative damage and avoiding the hormonal balance disruption.

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REFERENCES

Adriano DC, Copper, Trace Elements in Terrestrial Environments, Springer New York, New York, NY, pp. 499–546 (2001).

Apel K and Hirt H, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, Annu Rev Plant Biol 55: 373–399 (2004).

Baker CJ, Mock NM, Smith JM, and Aver'yanov AA, The dynamics of apoplast phenolics in tobacco leaves following inoculation with bacteria, Front Plant Sci 6: 649, Frontiers Media SA (2015).

Bashan Y and De-Bashan LE, Protection of tomato seedlings against infection by *Pseudomonas syringae* pv. tomato by using the plant growth-promoting bacterium *Azospirillum brasilense*. Appl Environ Microbiol 68: 2637–2643, American Society for Microbiology (ASM) (2002).

Behlau F, Hong JC, Jones JB, and Graham JH, Evidence for Acquisition of Copper Resistance Genes from Different Sources in Citrus-Associated *Xanthomonas*, Phytopathology 103: 409–418, Scientific Societies (2013).

Bender CL and Cooksey DA, Indigenous plasmids in *Pseudomonas syringae* pv. tomato: conjugative transfer and role in copper resistance., J Bacteriol 165: 534–541, American Society for Microbiology (ASM) (1986).

Bostock RM, Wilcox SM, Wang G, and Adaskaveg JE, Suppression of *Monilinia fructicolacutinase* production by peach fruit surface phenolic acids, Physiol Mol Plant Pathol 54: 37–50 (1999).

Brun L, Maillet J, Richarte J, and Herrmann P, Relationships between extractable copper, soil properties and copper uptake by wild plants in vineyard soils, Environmental 102(2-3): 151-161 (1998).

Cuppels DA, Generation and Characterization of Tn5 Insertion Mutations in *Pseudomonas syringae* pv. tomato, Appl Environ Microbiol 51: 323–327, American Society for Microbiology (ASM) (1986).

Dagostin S, Schärer H-J, Pertot I, and Tamm L, Are there alternatives to copper for controlling grapevine downy mildew in organic viticulture?, Crop Prot 30: 776–788 (2011).

Ishiga Y, Uppalapati SR, Ishiga T, Elavarthi S, Martin B, and Bender CL, Involvement of coronatine-inducible reactive oxygen species in bacterial speck disease of tomato, Plant Signal Behav 4: 237–239, Taylor and Francis (2009).

Fernández-Crespo E, Scalschi L, Llorens E, García-Agustín P, and Camañes G, NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation, J Exp Bot 66(21): 6777-6790 (2015).

Ferreira ICFR, Barros L, Soares ME, Bastos ML, and Pereira JA, Antioxidant activity and phenolic contents of *Olea europaea* L. leaves sprayed with different copper formulations, Food Chem 103: 188–195, Elsevier (2007).

Griffin K, Gambley C, Brown P, and Li Y, Copper-tolerance in *Pseudomonas syringae* pv. tomato and *Xanthomonas* spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review, Crop Prot 96: 144–150 (2017).

Hammerschmidt R, Chlorogenic acid: A versatile defense compound, Physiol Mol Plant Pathol 88:iii–iv (2014).

Hoagland and Arnon D, The water-culture method for growing plants without soil, Circ Calif Agric Exp Stn 347 (1950).

Huang C-H, Vallad GE, Zhang S, Wen A, Balogh B, Figueiredo JFL, *et al.*, Effect of application frequency and reduced rates of acibenzolar-*S*-methyl on the field efficacy of induced resistance against bacterial spot on tomato, Plant Dis 96: 221–227, The American Phytopathological Society (2012).

Iacobellis NS, Collmer A, Hutcheson SW, Mansfield JW, Morris CE, Murillo J, *et al.*, *Pseudomonas syringae* and related pathogens : biology and genetic, Springer Science+Business Media (2003).

Ji P, Campbell HL, Kloepper JW, Jones JB, Suslow TV, and Wilson M, Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria, Biol Control 36: 358–367 (2006).

King EO, Ward MK, and Raney DE, Two simple media for the demonstration of pyocyanin and fluorescin, J Lab Clin Med 44: 301–307 (1954).

Komárek M, Čadková E, Chrastný V, Bordas F, and Bollinger J-C, Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects, Environ Int 36: 138–151 (2010).

Küpper H, Götz B, Mijovilovich A, Küpper FC, and Meyer-Klaucke W, Complexation and toxicity of copper in higher plants. I. Characterization of copper accumulation, speciation, and toxicity in *Crassula helmsii* as a new copper accumulator., Plant Physiol 151: 702–714, American Society of Plant Biologists (2009).

Llorens E, Agustí-Brisach C, González-Hernández AI, Troncho P, Vicedo B, Yuste T, *et al.*, Bioassimilable sulphur provides effective control of *Oidium neolycopersici* in tomato, enhancing the plant immune system, Pest Manag Sci 73(5): 1017-1023 (2016).

Llorens E, García-Agustín P, and Lapeña L, Advances in induced resistance by natural compounds: Towards new options for woody crop protection, Sci Agric 74(1): 90-100 (2017).

Llorens E, Scalschi L, Fernández-Crespo E, Lapeña L, and García-Agustín P, Hexanoic acid provides long-lasting protection in 'Fortune' mandarin against *Alternaria alternata*, Physiol Mol Plant Pathol 91: 38–45 (2015).

Llorens E, Vicedo B, López MM, Lapeña L, Graham JH, and García-Agustín P, Induced resistance in sweet orange against *Xanthomonas citri* subsp. citri by hexanoic acid, Crop Prot 74: 77-84 (2015).

Li W, Zhang M, and Shu H, Distribution and fractionation of copper in soils of apple orchards, Environ Sci Pollut Res - Int 12: 168–172, Ecomed (2005).

Mackie K, Müller T, and Kandeler E, Remediation of copper in vineyards–a mini review, Environ Pollut 167: 16-26 (2012).

McLeod A, Masimba T, Jensen T, Serfontein K, and Coertze S, Evaluating spray programs for managing copper resistant *Pseudomonas syringae* pv. tomato populations on tomato in the Limpopo region of South Africa, Crop Prot 102: 32–42 (2017).

Michaud A, Bravin M, Galleguillos M, and Hinsinger P, Copper uptake and phytotoxicity as assessed in situ for durum wheat (*Triticum turgidum durum* L.) cultivated in Cu-contaminated, former vineyard soils, Plant and Soil 298(1-2): 99-111 (2007).

Micó C, Recatalá L, Peris M, and Sánchez J, Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis, Chemosphere 65(5): 863-872 (2006).

Ministerio de Agricultura Alimentación y Medio Ambiente, Registro de Productos Fitosanitarios - Agricultura - magrama.gob.es. http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/productos/forexi.asp?e=0andplagEfecto=28andculUso=0104010401010000andambUti=01andsolEsp= [accessed 12 September 2017].

Mittler R, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci 7: 405–410 (2002).

Scalschi L, Camañes G, Llorens E, Fernández-Crespo E, López MM, García-Agustín P, *et al.*, Resistance inducers modulate *Pseudomonas syringae* pv. tomato strain DC3000 response in tomato plants, PLoS One 9(9): e106429, Public Library of Science (2014).

Scalschi L, Vicedo B, Camanes G, Fernández-Crespo E, Lapena L, González-Bosch C, *et al.*, Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways, Mol Plant Pathol 14: 342–355 (2013).

Schramel O, Michalke B, and Kettrup A, Study of the copper distribution in contaminated soils of hop fields by single and sequential extraction procedures, Sci Total Environ 263: 11–22 (2000).

Sonmez S, Kaplan M, Sonmez NK, Kaya H, and Uz I, High level of copper application to soil and leaves reduce the growth and yield of tomato plants, Sci Agric 63: 213–218, Scientia Agricola (2006).

Steel RGD and Torrie JH, Bioestadistica: principios y procedimientos, 2nd ed., McGraw-Hill, Mexico (1986).

Xin X-F and He SY, *Pseudomonas syringae* pv. tomato DC3000: A model pathogen for probing disease susceptibility and hormone signaling in plants, Annu Rev Phytopathol 51: 473–498, Annual Reviews (2013).

Uppalapati SR, Ayoubi P, Weng H, Palmer DA, Mitchell RE, Jones W, *et al.*, The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato, Plant J 42: 201–217 (2005).

Wojciechowska E, Weinert CH, Egert B, Trierweiler B, Schmidt-Heydt M, Horneburg B, *et al.*, Chlorogenic acid, a metabolite identified by untargeted metabolome analysis in resistant tomatoes, inhibits the colonization by *Alternaria alternata* by inhibiting alternariol biosynthesis, Eur J Plant Pathol 139: 735–747, Springer Netherlands (2014).

Yruela I, Copper in plants: acquisition, transport and interactions, Funct Plant Biol 36: 409, CSIRO PUBLISHING (2009).

Zhang Y, Mao R, Zheng J, Xiao Y, Tang M, and Quan J, Laboratory Bioassay and Field Evaluation of copper gluconate as a new potential bactericide against *Xanthomonas campestris* pv. citri (Hasse) Dye, J Agric Sci 5: 23 (2013).

SUPPLEMENTARY DATA

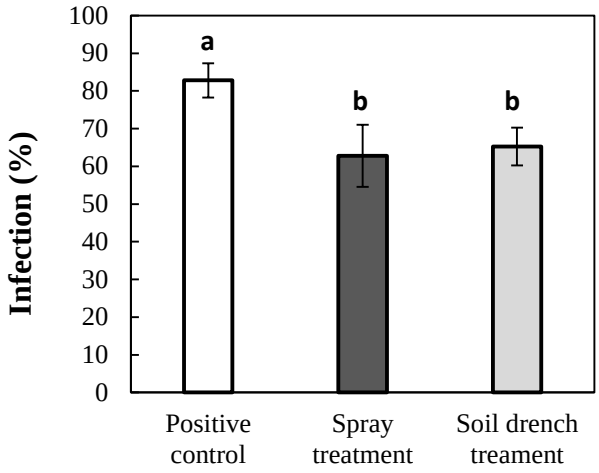

Figure S1. Curative effect of Cu heptagluconate on tomato plants 72 h pi with *P. syringae* strain tomato DC3000 at 5 $\times 10^5$ cfu ml^{-1}. (A) Infection rate scored measuring the percentage leaf covered by bacterial specks, for the treatments Positive control: Non-treated and inoculated plants; Spray: Cu heptagluconate applied in spray 24 h pi and Soil drench: Cu heptagluconate applied in soil drench 24 h pi. Columns represent the means of three independent experiments of 10 replicated plants per treatment. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

CHAPTER 2

1-Methyltryptophan modifies apoplast content in tomato plants improving resistance against *Pseudomonas syringae*

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ABSTRACT

Plants are able to produce numerous natural products, many of which have been shown to confer protection against microbial attack. In this way, we identified 1-methyltryptophan (1-MT), a natural compound produced by tomato plants in response to *Pseudomonas syringae* attack, whose application by soil drench provided protection against this pathogen. In the present work, we have studied the mechanisms underlying this protection. The results demonstrated that 1-MT can be considered a new activator of plant defense responses that acts by inhibiting the stomatal opening produced by coronatine (COR), which could prevent bacteria entering the mesophyll. Besides, 1-MT acts by blocking the jasmonic acid (JA) pathway, which could avoid manipulation of the salicylic acid (SA) pathway by a bacterium, and thus hinder its growth. Although the concentration of 1-MT reached in the plant did not show antimicrobial effect, we cannot rule out a role of 1-MT by itself because it affects the expression of the *fliC* gene, involved in the synthesis of the flagellum, and produces reduced bacterium motility and, therefore, its infective capacity. The results highlight the effect of a tryptophan derivative on induced resistance in plants.

KEYWORDS: Induced resistance, 1-methyltryptophan, *Pseudomonas syringae*, *Solanum lycopersicum*, apoplast

INTRODUCTION

Plants have developed a variety of chemical and physical basal defense mechanisms to cope with environmental changes and pathogenic invasions. When pathogens penetrate the superficial layer of plant leaves, they encounter early-acting post-invasive defense systems. The ability to detect and activate a defense response against potentially pathogenic microorganisms is important for stopping disease progression. The activation of these defense systems is linked to the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides and flagellin in gram-negative bacteria, or chitin and ergosterol in higher fungi (Nürnberger *et al*. 2004). Besides, in response to the delivery of pathogen effector proteins, plants produce resistance proteins (R proteins) to either directly or indirectly monitor the presence of the pathogen effector proteins (Chisholm *et al*. 2006).

The plant pathogen interaction is a system that depends on the lifestyle of the microorganism and plant species. The pathosystem *Pseudomonas syringae* vs. tomato is a widely used model to study the plant-pathogen interaction: firstly given its genetic tractability and pathogenicity, *Pseudomonas syringae* pv. tomato DC3000 is an appropriate strain used to investigate plant-microbe interactions (Cuppels 1986, Elizabeth and Bender 2007, Uppalapati *et al*. 2008); secondly, tomato is an economically important plant and its study could be extended to other species closely related to it that are important for agriculture.

Plant immunity against *Pseudomonas* involves multiple responses (Xin and He 2013). Among them, plant hormones play an important role in modulating plant resistance to *P. syringae*. Salicylic acid (SA) acts at different levels of plant defense against biotrophic and hemibiotrophic pathogens and is associated with the induction of PR (pathogenesis-related) genes expression (Vlot *et al*. 2009). Jasmonic acid (JA) is known to protect plants against necrotrophic pathogens and herbivores (Thomma *et al*. 2001). Although previous works have shown the antagonistic interactions between the SA- and JA-mediated signaling pathways (Niki *et al*. 1998, Koornneef *et al*. 2008, Pieterse *et al*. 2012), synergistic interactions have also been described (Mur *et al*. 2006, Halim *et al*. 2009, Scalschi *et al*. 2013).

Abscisic acid (ABA) is also involved in biotic stress responses in a complex manner. Several authors have shown that ABA promotes *Pseudomonas* infection (Thaler and Bostock, 2004; Cao *et al*., 2011; Robert-Seilaniantz *et al*., 2011). Nevertheless, ABA is the hormone responsible for guard cell closure control, which is a very important aspect to prevent the entrance and reproduction of pathogens in the mesophyll (Melotto *et al*., 2006; Gudesblat *et al*., 2009a). In relation to this, Fernández-Crespo *et al*. (2015) have shown that tomato plant grown with NH_4^+ as the only source of N displays higher basal ABA accumulation and more closed stomata than control plants, which reduce the entry of Pseudomonas in the mesophyll apoplast.

P. syringae pv. tomato DC3000 is a bacterial pathogen with two lifestyles: an initial epiphytic phase on the leaf surface and an endophytic phase in the apoplastic space, which it can access via wounds or natural plant openings, such as stomata (Beattie and Lindow 1995, Melotto *et al*. 2008). *P. syringae* survival within the plant depends on several factors, such as its motility and ability to enter the mesophyll, the availability of

nutrients in the apoplast, and the ability to cope with host defense systems. *P. syringae* uses flagellar motility to locate at optimal sites for nutrient acquisition or to avoid toxic substances. Therefore, flagella play a role by either stimulating host defense or in disease causation (Xin and He 2013). This bacterium also possesses many virulence factors, like proteinaceous effectors, which are secreted through the type III secretion system directly into host cells, and a polyketide phytotoxin called coronatine (COR), which structurally mimics plant hormone jasmonate isoleucine and triggers the activation of JA-dependent defence responses to lead to the suppression of SA-dependent defence responses (Laurie-Berry *et al*. 2006).

P. syringae develops its pathogenic phase in the apoplast. The apoplastic space is the plant cell compartment outside the cell membrane, where the first interaction between plants and pathogens takes place. It is an environment that is acidic, low in nitrogen, and rich in plant-derived sugars, such as fructose and glucose (Rico and Preston 2008). It is known that both abiotic and biotic stresses are able to change its content. Therefore the apoplast can be considered one of the primary lines of defense against pathogen invasion because it may contain antimicrobial molecules (Bednarek *et al*. 2010) and reactive oxygen species (Torres *et al*. 2006, Torres 2010), which might directly affect the pathogen or serve as plant response inducers. For this reason, an analysis of the changes that occur in the apoplast during pathogen infection and in plant-induced defense is important to gain a better understanding of the plant-pathogen interaction and disease control in early development stages.

An alternative system to use pesticides in agriculture is to induce plant defense against pathogens by treatments with natural compounds. The search for such compounds that are effective in protecting crops against pathogens allowed us to determine the effectiveness of hexanoic acid (Hx). Hx acts as an inducer of plant defenses by means of a priming mechanism against pathogens with different lifestyles (Vicedo *et al*., 2009; Llorens *et al*., 2013; Scalschi *et al*., 2013). In order to characterize the priming mechanism conferred by Hx, a metabolic profile of tomato plants infected by *Botrytis cinerea* or *P. syringae*, and treated with Hx, has been performed (Camañes *et al*. 2015). In that study, compound 1-methyltryptophan (1-MT) was detected and its presence was associated with tomato-*P. syringae* and tomato-*B. cinerea* interactions, and with Hx-induced resistance. Moreover, the same work demonstrated that the root application of 1-MT significantly reduces the infection of both pathogens. In recent years, other

tryptophan-derivates have emerged as relevant defene mechanisms, which have conferred resistance to the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis thaliana* (Sanchez-Vallet *et al*. 2010, Gamir *et al*. 2012).

The aim of this work was to study the modifications produced by 1-MT in the plant that improve its resistance against the pathogen and its possible direct effect on bacteria. For this purpose, we treated tomato plants with 1-MT by soil drenching. Then the metabolic and transcriptomic profiles were analyzed in the plant and apoplast extracts. We also analyzed the transcriptomic changes in the genes related to the pathogenicity and virulence of bacteria.

MATERIAL AND METHODS

Microbial strains, growth conditions and plant material

P. syringae pv. tomato strains used in the present study were DC3000 and 775EGFP (DC3000 labeled with GFP, Río-Álvarez *et al.*, 2014). Rifampicin and kanamycin respectively were added to King B medium (KB) at 50 µg mL^{-1}. Tomato seeds (*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 60 % relative humidity. Seeds were irrigated with distilled water for a week and the next 3 weeks with Hoagland solution (Hoagland and Arnon 1950). The pH of the nutrient solution was adjusted to 5.8 - 6.0 with 1 mM KOH.

P. syringae bioassays

Four-week-old tomato plants were treated with nutrient solution or 20 mL of 1-MT (5 mM) at pH=6 dissolved in the nutrient solution 72 hours before inoculation. *P. syringae* pv tomato DC3000 was grown in KingB (KB) medium at 28 ºC for 24 h. Bacterial suspensions were adjusted to 5 x 10^5 cfu mL^{-1} in sterile $MgSO_4$ (10 mM) with 0,01 % of Silwet L-77 surfactant (Osi Specialties, Danbury, CT, USA). Tomato plants were challenged by dipping with *P. syringae* and the disease rate was scored as described by Camañes *et al*., (2015). The third and fourth leaves of 10 plants for every treatment were sampled at different time points (6, 24 and 48 hpi) and frozen at -80 ºC.

Chromatographic analysis

Leaves were frozen in liquid N_2, ground, and lyophilized. For hormonal analysis, dry tissue (0.05 g) was homogenized in 2.5 mL of ultrapure water and 100 ng mL^{-1} of internal standards (d^6-ABA, d^4-SA and dihydrojasmonic acid) was added. The samples were centrifuged at 5.000 rpm for 45 min at 4 ºC. The supernatant was partitioned against diethylether, dried in a speed vacuum and resuspended in 90:10 H_2O:MeOH. For amino acids and 1-MT analysis, dry tissue (0.1 g) was homogenized with 750 µl of extraction solution composed by: 80 µl of distilled water, 200 µl of chloroform and 470 µl of methanol per sample. Moreover, a mixture of internal standards was added prior to extraction (100 ng of Phe $^{13}C_9{}^{15}N$ and 100 ng of Thr $^{13}C_4{}^{15}N$). In both cases, a 20 µl aliquot was injected into an Acquity ultra-performance liquid chromatography system (UPLC) with an ACQUITY UPLC BEH C18 column (1.7 µm 2.1x50 mm) (Waters, Mildford, MA, USA), which was interfaced with a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The solvent gradient used was 95% H_2O: 5% MeOH: 0.1% CHOOH to 5% H_2O: 95% MeOH: 0.1% CHOOH over 8 min.The MASSLYNX NT software version 4.1 (Micromass; www.micromass.co.uk) was used to process the quantitative data from calibration standards and plant samples.

Hormonal, amino acids and 1-MT analyses were also performed for the apoplast extracted at 48 hpi from control and treated plants, infected and non-infected. 100 ng mL^{-1} of internal standards were directly added to the apoplast after extraction and further processed with the UPLC as described above.

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

RNA was extracted from frozen tomato leaves using the E.Z.N.A® Plant RNA Kit (www.omegabiotek.com) according to the manufacturer's instructions. Leaf tissue from 10 treated and untreated plants were collected at the specified time points post-inoculation. A total of 1 µg of total RNA was digested using 1 U of RNase-free DNase (Thermofisher, www.thermofisher.com) and incubated for 30 min at 37 ºC. For each sample, cDNA was synthesized using 1 µg of total RNA in 10 µL total reaction volume with oligo dT primer and primescript RT enzyme mix 1 (Primescript RT reagent kit, TaKaRa). Forward and reverse primers (10 µM) were added to 5 µl of Maxima SYBR Green/ROX qPCR Master Mix (Thermofisher), as well as 1 µl of diluted cDNA and

Milli-Q sterile water up to a total reaction volume of 10 µl. Quantitative PCR was carried out using the StepOne™ Real-Time PCR System (www.thermofisher.com). A list of the primers used for the quatitative Real Time-PCR is shown in Table S2. Levels of *EF1α* gene expression were used as an internal housekeeping control. 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.

To extract bacterial RNA from infected plants we used a protocol for extracting RNA from *P. syringae* recovered from infected leaves as described by Yu *et al.* (2013). Briefly, for isolation of RNA, the Qiagen RNeasy Bacteria Mini Kit (www.qiagen.com) was used according to manufacturer's instructions. Reverse transcription and Quantitative PCR were carried out as described above. Primers used for the assay are described in Table S3. Relative levels of the monitored genes were normalized with *recA* that was used as an internal reference.

Apoplast extraction

Apoplast extraction was carried out 48h after *Pseudomonas syringae* inoculation by using the infiltration-centrifugation method as described by (O'Leary *et al.* 2014). Leaflets of the third and fourth true leaves of 10 one-month old plants were used for each treatment. Briefly, this technique is a two-step method that essentially involves replacement of the apoplastic air space with sterile distilled water, which mixes with the native apoplastic fluid, followed by recovery of the infiltration/apoplastic mixture by gentle centrifugation of the leaves. The cytoplasmic contamination of apoplast was estimated by comparison of G6PDH activity in apoplast extracts and leaf homogenates as is described in Rico and Preston (2008). G6PDH was not detected in apoplast extract. Prior to subsequent analyzes, the apoplast extract was diluted twice in distilled water and filtered on a cellulose filter in syringe filter pore size 0.2 µm, in order to avoid bacterial contamination. Four biological replicates of apoplast extracted from control and treated plants, infected and non-infected were performed.

Stomatal aperture analysis

Tomato plants were maintained in the same culture conditions and treated as described for the *P. syringae* bioassays. The third and fourth leaves were collected and placed on

glass slides with the adaxial epidermis in contact with dental resin (Geisler *et al.* 2000, Delgado *et al.* 2011). Stomatal aperture analysis was performed as described by Scalschi *et al*. (2013).

Analysis of bacteria presence in the leaves with confocal microscopy

For confocal microscopy analysis, *P. syringae* pv tomato 775EGFP strain, transformed with pUFZ15 containing the GFP fluorescent protein (Río-Álvarez *et al.* 2014) was used. In order to check 775EGFP strain response to 1-MT treatment, bacteria were inoculated as described above. Results showed a similar effect as for DC3000 strain (data not shown). For the confocal analysis, inoculated tomato leaves from treated and non-treated plants were examined at different time periods in order to check for the presence of the bacteria on the surface and in the mesophyll. The GFP signal and chlorophyll autofluorescence were collected on an Inverted Confocal Microscope Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) using 488 nm ray line of the argon laser for their excitation. GFP fluorescence was collected between 500 and 540 nm by a HyD detector while the fluorescence emitted by the chlorophyll was collected between 650 and 700 nm by a PMT detector. The same gain and offset settings were used for the different treatments. The images were processed using the LAS X (Leica Microsystems). Five leaves were observed for each treatment and for each time point.

Determination of sugar concentration

Fructose, glucose, and sucrose concentration was determined following the method described by Cebolla-Cornejo *et al.* (2012) with some modifications using an Agilent 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany). Prior to use, uncoated fused silica capillaries (67 cm total length, 60 cm effective length, 375 µm outside diameter, 50 µm internal diameter) from Polymicro Technologies (Phoenix, AZ, USA) were conditioned at 50 ºC with NaOH 1 N (5 min), NaOH 0.1 N and MilliQ water (10 min). Before each working session, the capillary was rinsed for 30 min with the running buffer (20 mM PDC and 0.1% w/v HDM at pH 12.1). Between runs, the capillary was flushed with 60 mM SDS (3 min), MilliQ water (1 min) and the running buffer (2 min). Samples were diluted ½ with MilliQ water, filtered (0.2 µm) and then injected hydrodynamically at 6900 Pa during 30 s. Separations were performed at -25 kV and 20 ºC, with indirect detection at 214 nm.

In vitro bacterial growth assay

We tested 1-MT effect against *P. syringae pv tomato* DC3000 in LB medium to which, the compound was added at a final concentration of 0.5, 1, 2.5 and 5 mM. 1-MT was prepared in distilled water adjusted to pH=6, and sterilized by filtration. 2xLB was prepared and diluted with 1-MT and water until reaching the concentration of use. The inoculum was obtained as described above. The growth assay was carried in a microtiter plate, in a total volume of 250 µl LB with or without 1-MT, using an initial bacterial concentration of about 5×10^5 cfu mL^{-1}. Bacterial growth was monitored by measuring optical density in a microplate reader (MB-580, Heales) at 20 hpi in the medium. Eight independent replicates were performed for each condition.

Bacterial growth assays were also performed in extracted apoplast from treated or untreated plants, infected or non-infected using an initial bacterial concentration of about 1×10^6 cfu mL^{-1}.

Swimming assays

To analyze the effect of the treatment on the mobility of the bacteria in vitro, *P. syringae* inoculum was obtained as described above. Five microliters of the bacterial suspension were inoculated onto KB agar plates (containing 50% KB and 0.25% agar) with or without 1-MT at 1.5 or 5 mM, with a sterile pipette tip. The plates were incubated at 28 ºC. Five plates by treatments were used. The diameter of the culture was measured at 72 hpi.

Statistical analysis

All experiments were conducted at least three times. Data from different repetitions was analyzed together due to the fact that analysis of variance (ANOVA) did not show significant differences ($p<0.05$) between repetitions in each experiment. All data of this study were tested for normality, homogeneity of variances, and residual patterns. When ANOVA showed significant differences between variables, mean values were compared using the Fisher's least significant difference (LSD) test at the 95% confidence. Statistical analyses were performed using the software Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA, USA). Heatmaps were generated with the levels of aminoacids, hormones and sugars following log transformation using the gplots package of R statistical program (version 3.4.3).

RESULTS

We have previously reported that 1-MT protects tomato plants against *P. syringae* and *B. cinerea* (Camañes *et al.* 2015). The present paper checked the protection of tomato plants by 1-MT against *P. syringae.* The results obtained were similar to those described previously by Camañes *et al*. (2015), we observed an average reduction closed to 40 % in disease symptoms and an average drop of 80 % in the number of colony-forming units (cfu) after. Therefore, we extended our analyses to study the mechanisms underlying the resistance against *P. syringae* conferred by 1-MT. For this purpose, metabolic and transcriptomic analyses of leaf samples were performed in tomato plants.

Changes in the hormonal pattern of the 1-MT-treated tomato plant upon *P. syringae* infection

In order to further confirm the possible role of the different signaling pathways in 1-MT-induced resistance, we analyzed the hormonal levels in the control and the 1-MT-treated tomato plants at three time points after *P. syringae* infection.

ABA levels significantly increased at 48 hours post-inoculation (hpi) in both the treated and infected plants compared to the untreated infected ones (Fig. 1A). Besides ABA, SA levels also increased in the treated and infected plants at this time point (Fig. 1B), but no significant differences were observed compared to the untreated infected plants. Interesting results about the oxylipin pathway were obtained. Even though oxylipin 12-oxo-phytodienoic acid (OPDA) and JA increased in the infected plants, at 24 hpi this remarkable increase in both the treated and untreated plants was observed only in the untreated infected plants at 48 hpi (Fig. 1C and D). These results indicate that 1-MT could block the oxylipin pathway.

1-MT induced the expression of the genes involved in defense pathways

As 1-MT treatment changed the hormonal profile, the next step was to study the expression pattern of the marker genes for the ABA (*ASR1*), SA (*PR1* and *PR5*) and JA (*AOC*) signaling pathways in the leaf samples taken from the treated and untreated plants at 6, 24 and 48 hpi.

The results show that 1-MT increased *ASR1* gene expression at 48 hpi (Fig. 2A). This finding correlated with the accumulation of ABA observed at this time point, which suggests that the ABA pathway might play a role in 1-MT-induced resistance.

Regarding the SA pathway, no differences in the expression levels of *PR1* were observed between the treated and untreated plants upon infection, consistently with the hormonal analysis (Fig. 2B). However, *PR5* gene expression was induced by treatment at 48 hpi (Fig. 2C). This result indicates a possible role of this protein in the protection mediated by 1-MT. Despite the fact that the OPDA and JA levels were lower in the treated and infected plants, no differences in the expression levels of the marker genes of the oxylipin pathway were observed in the treated *versus* the untreated plants upon infection (Fig. 2D).

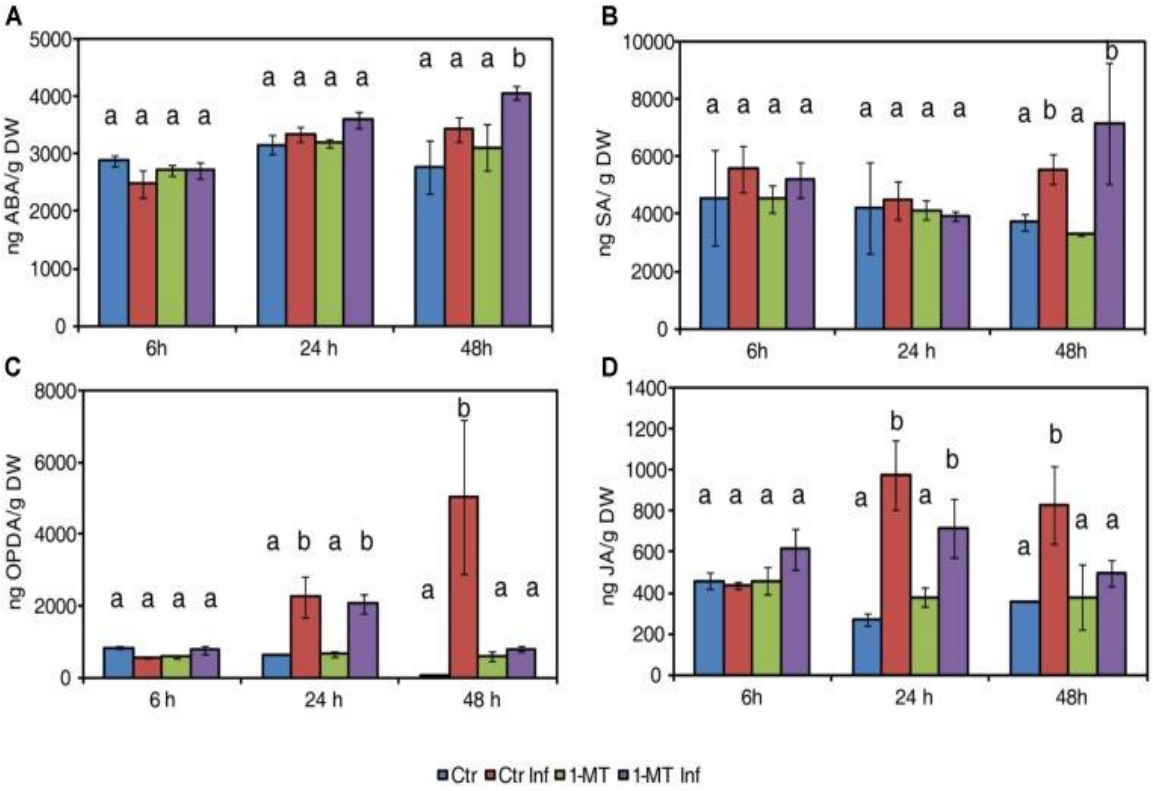

Figure 1. Hormone levels in water- and 1-MT-treated tomato plants on *P. syringae* infection. Leaves were collected at 6, 24 and 48 hpi and ABA (A), SA (B), OPDA (C), and JA (D) levels were determined by ultra-performance liquid chromatography (UPLC)-mass spectrometry. Data show the average of three independent experiments of a pool of 10 plants per experiment ± SE. Different letters indicate statistically significant differences between treatments at the same time point ($p<0.05$; least-significant difference test). Ctr, untreated and uninfected plants; Ctr Inf, untreated and inoculated plants; 1-MT, treated plants; 1-MT Inf, treated and inoculated plants.

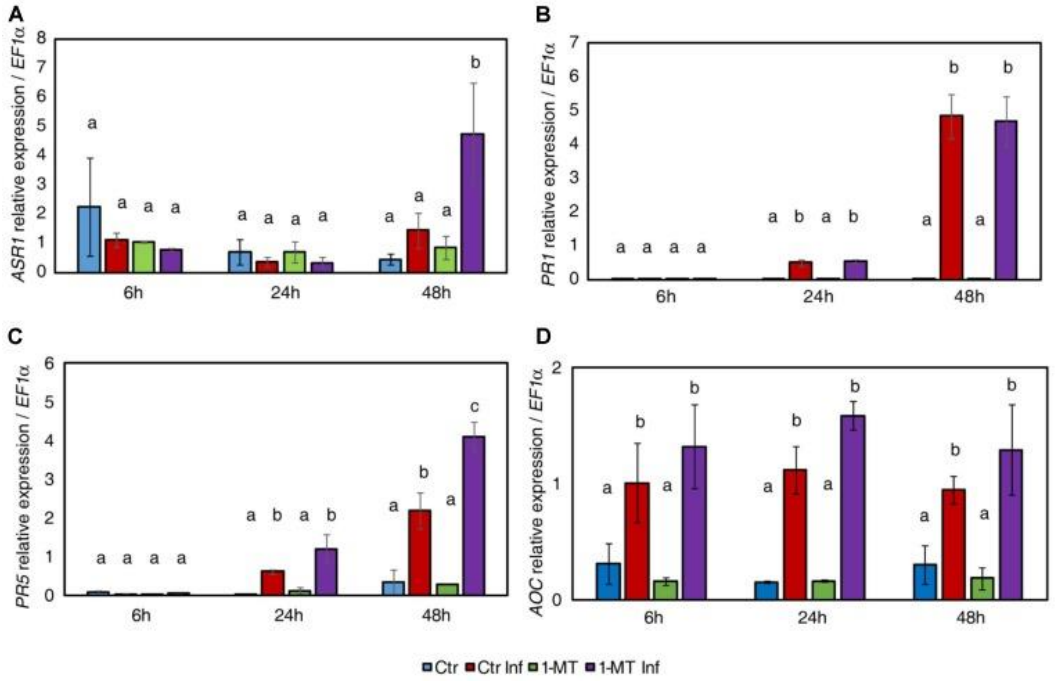

Figure 2. Gene expression profile of plant defense pathways in water- and 1-MT-treated tomato plants on *P. syringae* infection. Leaves were collected at 6, 24 and 48 hpi and expression levels of marker genes of ABA (*ASR1*) (A), SA (*PR1* and *PR5*) (B and C) and JA (*AOC*) (D) signaling pathways were analyzed. 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. Statistical analysis was carried out between samples collected at the same time point. Different letters indicate statistically significant differences between treatments ($p<0.05$; least-significant difference test). Ctr, untreated and uninfected plants; Ctr Inf, untreated and inoculated plants; 1-MT, treated plants; 1-MT Inf, treated and inoculated plants.

1-MT inhibits stomatal opening and reduces bacterial mesophilic colonization

It is well-known that ABA plays key roles in regulating stomatal closure. In this context, the increase of ABA levels in plants treated and inoculated, indicated the possibility of 1-MT treatment acting by blocking the re-opening of stomata promoted by COR. Therefore, the treatment effect on stomatal opening was studied. The results showed that both the treated and untreated infected plants had more closed stomata than the uninfected ones in the early hours of infection (data not shown). Nevertheless, the stomata of the treated and infected plants remained more closed at 24 h, which could affect the entry of the bacterium and its establishment in the mesophyll. These results, together with those observed for the induction of ABA and the ABA marker gene,

support the idea that 1-MT treatment might regulate stomatal closure and make plant defense more effective (Figs. 3A and 3B).

In order to study how the bacterium colonized the mesophyll, confocal microscopy studies were carried out using a *P. syringae* pv tomato DC3000 strain labeled with GFP (Río-Álvarez *et al.* 2014). Pictures were taken at 3, 24 and 72 hpi. They showed that although bacteria were observed on the leaf surface at 3 hpi, at 24 h their presence on the leaf surface decreased in both the treated and untreated plants. Moreover, at 24 hpi, small groups of bacteria began to appear in the intercellular space in the control plants, whereas only isolated bacterial cells were seen in the treated plants (Fig. 3C). At longer time points, the number of bacteria considerably increased in the mesophyll of the control plants, but it was difficult to see clusters in the treated plants.

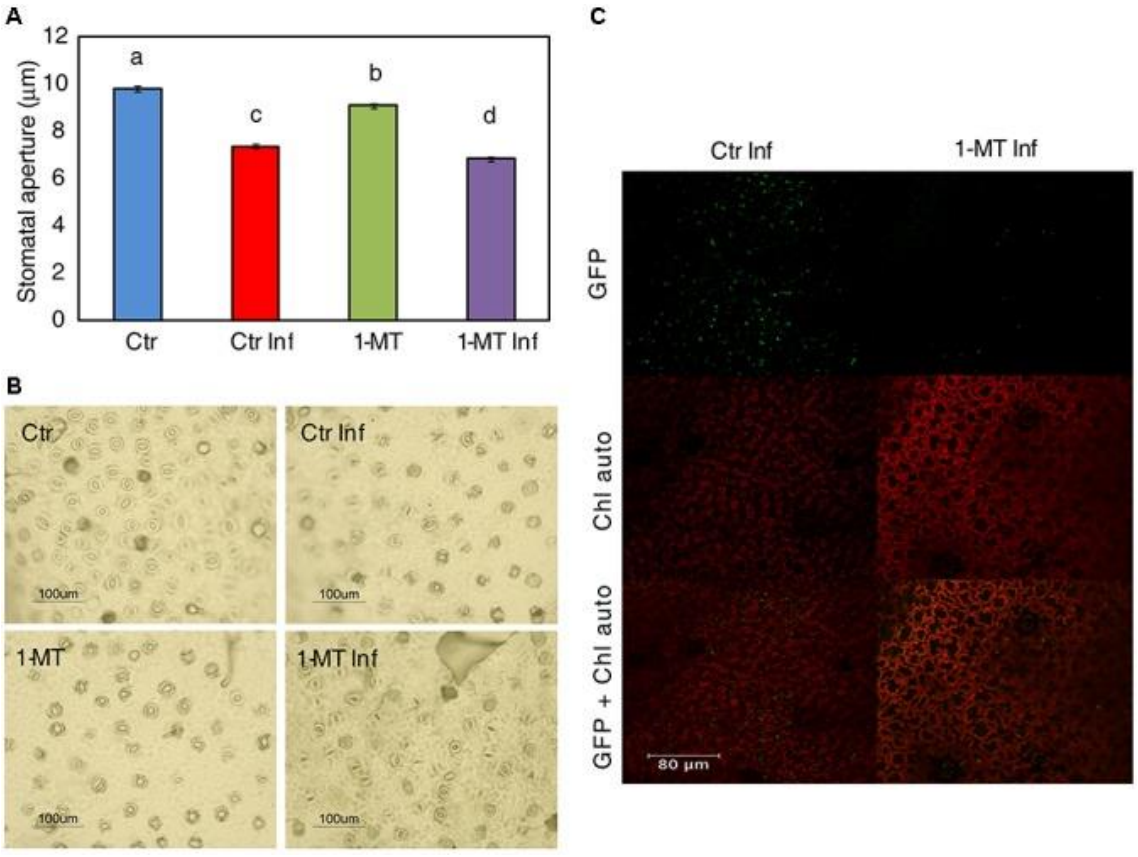

Figure 3. 1-MT-treatment affects stomatal opening. (A) Stomatal apertures were analyzed '*in situ*' in leaflets of water (Ctr) and 1-MT treated tomato plants at 24 hpi. Results are average ± standard error (SE) (n<50 stomata). Different letters represent statistically significant differences (p<0.05; least-significant difference test) (B) Representative photographs of stomatal aperture taken after 1-MT treatment. C) Confocal images showing a low number of bacteria in 1-MT treated plants and small colonies formed by a greater number of cells in nontreated plants 24 hpi.

Apoplastic changes induced by 1-MT affect *P. syringae* survival *in vitro*

Since the endophytic phase of bacteria takes place in the apoplast, its composition might be another factor that affects the bacterial colonization of the mesophyll. To study this hypothesis, apoplast extraction was performed 48 hpi from both the treated and untreated plants, as previously described by Rico and Preston (2008). In order to assess the apoplast's capacity of inhibiting bacterial growth, a bacterial suspension of 10^6 cfu mL^{-1} was let grow on the extracted apoplast and its development was monitored during 24 hpi. The results showed significantly less growth of the bacterial populations inoculated in the apoplast extracted from the treated plants than in the apoplast extracted from the untreated plants (Fig. 4). However, no differences in bacterial growth were found when using apoplast extracted from the treated plants with or without infection. Therefore, we conclude that the changes in leaf apoplast caused by 1-MT treatment affected the survival of bacteria independently of infection.

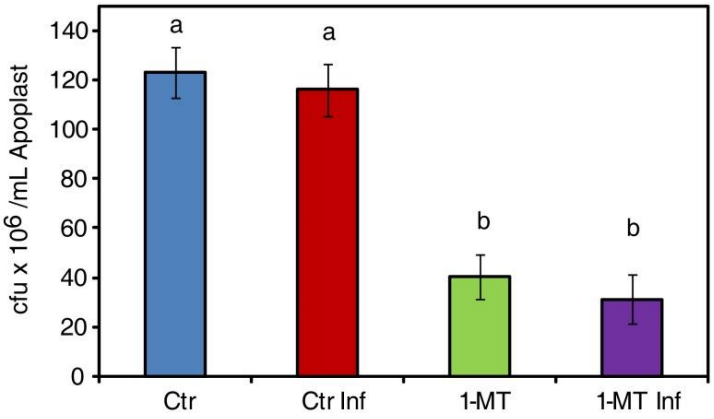

Figure 4. Survival of *P. syringae* in apoplast extract. 10^6 cfu mL^{-1} of bacteria initial concentration was maintained in apoplast extract from uninfected (Ctr) infected (Ctr Inf) treated (1-MT) and treated and infected (1-MT Inf) plants. Bacterial growth was measured at 24h. Data show the average of 8 samples per condition of an experiment ± SE. The experiment was repeated three times with similar results. Letters indicate statistically significant differences ($p<0.05$, least-significant difference test).

Changes in hormonal pattern, sugars and amino acid content in the apoplast upon *P. syringae* infection

Having observed the ability of the apoplast to inhibit bacterial growth, we compared the metabolic responses between the apoplast of the treated and untreated plants, with or without infection (Fig. 5 and Table S1). Firstly, we performed the analysis of the levels

of the plant hormones involved in plant defense processes. The results showed that the hormone levels in the apoplast were much lower than those detected inside cells. Moreover, the levels of most of the analyzed hormones were significantly higher in the infected plants than in the uninfected ones, regardless of treatment. This finding suggests that elevated levels are associated with the presence of the pathogen. Therefore, it appears that no studied hormone itself was able to exert an effect on the control of bacterial growth in the apoplast. The amino acid content was also analyzed, and the results showed that threonine (Thr) levels were higher in the infected and uninfected treated plants. After infection, its levels lowered in the untreated plants, and tryptophan (Trp) levels were higher only in the treated and infected plants. No significant differences were found between the treated and untreated plants for the other analyzed amino acids.

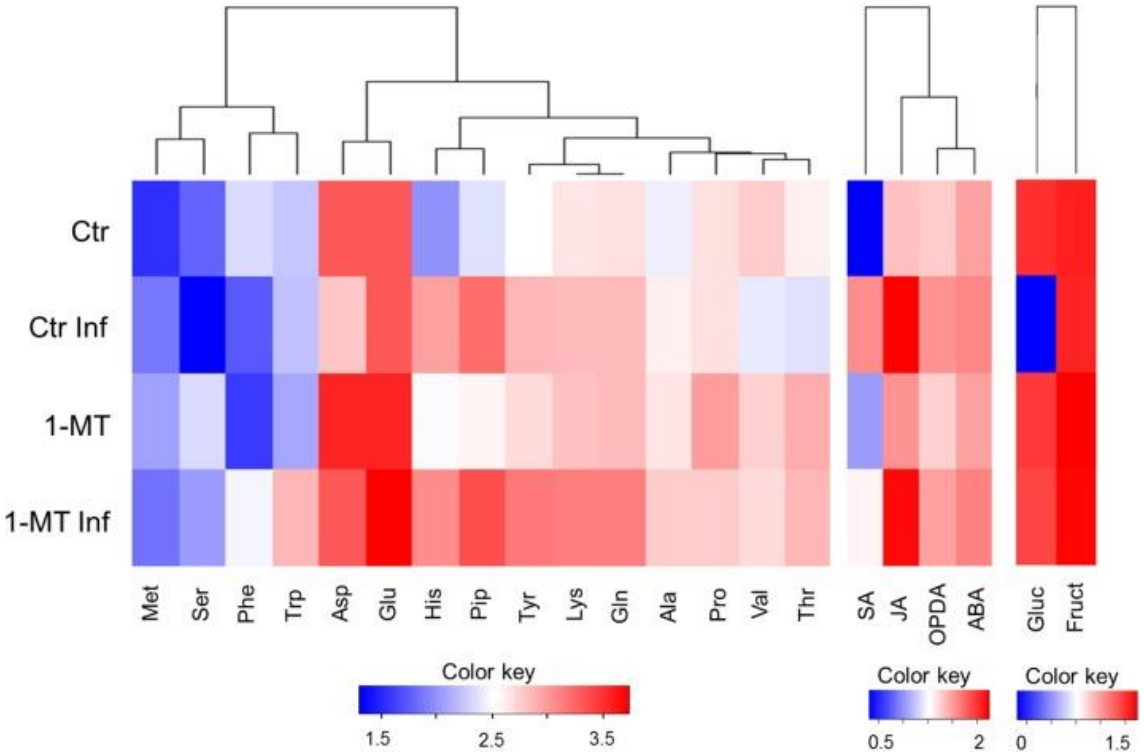

Figure 5. Heatmap visualization of the content of metabolites in the apoplast in water- and 1-MT-treated tomato plants on *P. syringae* infection. Apoplast extract was collected at 48 hpi and hormone, amino acid and sugar content were analyzed. Ctr, untreated and uninfected plants; Ctr Inf, untreated and inoculated plants; 1-MT, treated plants; 1-MT Inf, treated and inoculated plants. Columns present the different metabolites analyzed; Color key indicates log transformation of metabolite concentration, blue: Lowest, red: highest.

Finally, sucrose, fructose, and glucose levels were determined. While glucose and fructose were detected in all the experiments, be it at very low levels, no sucrose was detected in any of them. Glucose was observed in the apoplast extracted from the infected control plants, in which higher bacterial populations were found. This sugar was probably not detected because it was metabolized by bacteria. This is consistent with the amounts of glucose detected in the apoplast of the treated and infected plants, which appeared to inversely correlate with the amounts of bacteria. Interestingly, the absence of glucose did not negatively affect the growth of the bacteria inoculated in the apoplast extracted from the untreated and infected plants (Fig. 4). Besides, their growth was similar to that of the bacteria inoculated in the apoplast extracted from the control plants, and was higher than the growth of the bacteria inoculated in the apoplast of the treated and treated and infected plants. Fructose levels were also higher in the apoplast of the infected and uninfected treated plants, but no significant differences were observed compared with the untreated plants.

P. syringae growth is dependent on 1-MT concentration

In order to test the direct 1-MT effect against *P. syringae*, growth of the bacterium was checked in both the absence and presence of different 1-MT concentrations of (0.5, 1, 2.5 and 5 mM) in LB medium. The results showed that of all the tested concentrations, only the 5 mM 1-MT one produced a 15% inhibition of bacterial growth. The addition of 1-MT at the 0.5, 1 and 2.5 mM concentrations did not reduce the growth of the bacteria compared with the control (Fig. 6).

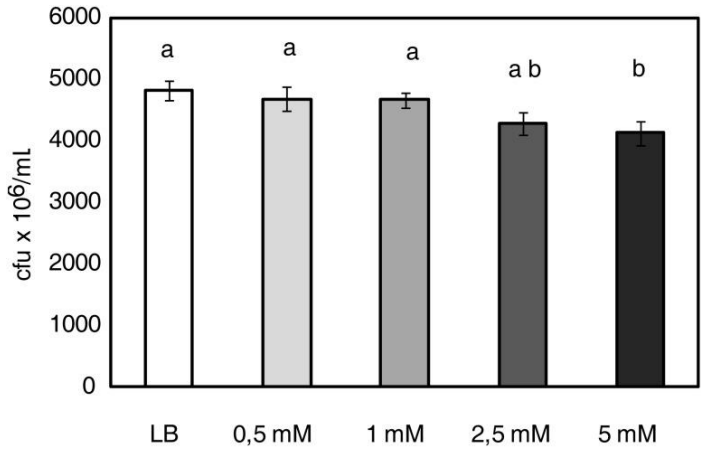

Figure 6. *P. syringae* growth is dependent on 1-MT concentration. $5x10^5$ cfu mL-1 of bacterial initial concentration was grown in LB or LB supplemented with 0.5, 1, 2.5 and 5 mM 1-MT. Bacterial growth was monitored by measuring optical density in a microplate

reader (at 20 h after inoculation in the medium). Data show the average of 8 samples per condition of an experiment ± SE. The experiment was repeated three times with similar results. Letters indicate statistically significant differences ($p<0.05$, least-significant difference test).

As the root treatment concentration (5 mM) had an effect on bacterial growth, the content of this compound in leaves and the apoplast was analyzed. The results (Table 1) showed that the concentration in both the apoplast and plant did not exceed 2 mM and was, therefore, below the concentration that could affect bacterial growth.

Table 1. 1-MT concentration determined in leaves or in apoplast.1-MT concentration was determined by UPLC-MS^2 in leaves of tomato plants or in apoplast extract in Ctr, untreated and uninfected plants; Ctr Inf, untreated and inoculated plants; 1-MT, treated plants; 1-MT Inf, treated and inoculated plants. Data show the average of three independent experiments ± SE. Letters indicate statistically significant differences ($p<0.05$, least-significant difference test).

In planta	Control	n.d.
	Infected plant	n.d.
	1-MT	$1,8 \pm 0,5^{a}$ mM
	1-MT infected plants	$1,2 \pm 0,2^{a}$ mM

Apoplast	Control	n.d.
	Infected plant	n.d.
	1-MT	$0,007 \pm 0,03^{b}$ mM
	1-MT infected plants	$0,008 \pm 0,03^{b}$ mM

nd: no detected

1-MT affects *P. syringae* motility

To test whether bacterial virulence was altered by treatment, we analyzed the expression of the genes related to the pathogenesis and survival of *P. syringae* in the bacteria extracted from both the treated and untreated plants at 72 hpi. The selected genes and their functions are shown in Table S2. The results indicated that only *fliC* expression, which encodes flagellin, was significantly lower in the bacteria extracted from the 1-MT-treated plants than in the bacteria extracted from the untreated ones (Fig. 7A). No differences were observed in the other analyzed genes (Fig. S1). These results suggest

that 1-MT might affect the motility of bacteria by disrupting flagellum synthesis or function.

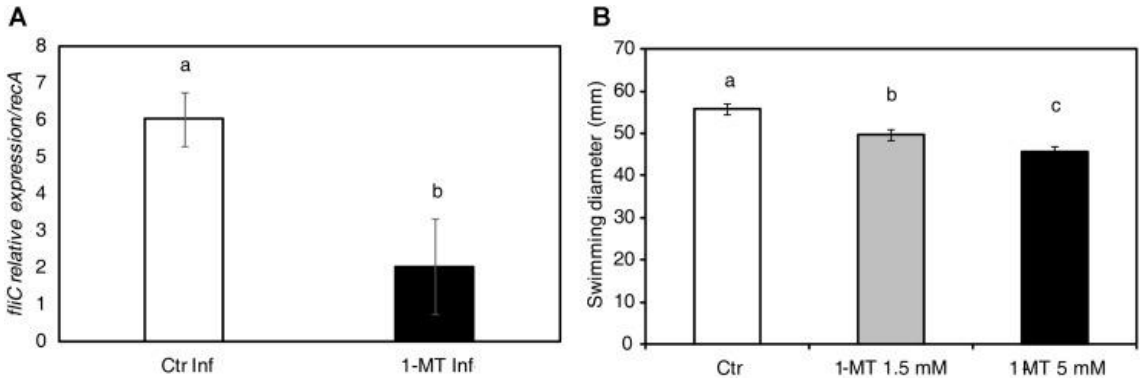

Figure 7. 1-MT affects the motility of *P. syringae*. (A) RNA extraction was performed from bacteria extracted from treated and untreated plants 72 hpi. Relative expression of *fliC* during bacterial growth was analyzed. The *recA* gene was used as an endogenous reference gene. (B) Swimming diameter was determined 72 hpi in KB agar plates (containing 50% KB and 0.25% agar) with or without 1-MT at 1.5 or 5mM where 5 µl of $5x10^5$ cfu mL^{-1} bacterial suspension was inoculated. The plates were incubated at 28 ºC and 5 plates by treatment were measured. The results represent the average of three independent experiments ± SE. Letters indicate statistically significant differences ($p<0.05$, least-significant difference test).

Given that *fliC* gene expression seemed to be affected by the 1-MT treatment, we assayed *P. syringae* swimming motility at 72 hpi (Fig. 7B). The swimming motility of the bacterium reduced in the cells incubated on the plates that contained 1.5 and 5 mM of 1-MT compared to the control cells. These results suggest that *P. syringae* swimming motility in the *in vitro* assays was affected in a concentration-dependent manner by 1-MT because a more marked reduction in motility was seen at higher concentrations of this compound. These results confirm our hypothesis that 1-MT affects the synthesis of flagellar components.

DISCUSSION AND CONCLUSIONS

In a previous work, the characterization of tomato plant response to Hx treatment by a metabolomic approach allowed us to detect 1-MT to be one of the molecules that might be involved in defense against *P. syringae*. Its protective effect has also been demonstrated (Camañes *et al.* 2015). In the present study, we investigated the mode of action of 1-MT in tomato plants against *P. syringae* by analyzing both the changes produced in the plant and the bacterial response after treatment.

The *importance* of *SA* in defense against *P. syringae* in tomato has long since been established. This pathway plays a key role in the systemic acquired resistance (SAR) signaling and synthesis of resistance proteins (PR) (Van Loon 1997) and for the regulation of defense responses, such as stomatal closure (Alvarez 2000, Melotto *et al.* 2008). It is already known that antagonistic cross-talk exists between SA and JA, which consists in inhibiting the SA-dependent responses by the presence of JA (Pieterse *et al.* 2009). Bacterial virulence factor COR stimulates the JA pathway by suppressing the SA pathway in Arabidopsis and tomato (Brooks *et al.*, 2005; Uppalapati *et al.*, 2005). In this work, we observed that treatment with 1-MT reduced the oxylipin pathway after infection. This reduction could avoid the manipulation of the SA pathway by bacteria by allowing the plant to induce the SA response through *PR5*. Although the SA and *PR1* gene expression levels did not significantly differ between the treated and untreated infected plants, the observed levels sufficed to enhance the pathogen recognition mechanism by the plant cell (Katagiri *et al.* 2002). Previous studies have shown that besides *PR5* responding to SA, it can also respond to either ethylene or MeJA (Reymond and Farmer 1998). It should be noted that SA is not only a major signal transducer after recognizing pathogen attack, but can also enhance pathogen recognition sensitivity at low levels (Shirasu *et al.* 1997). One role played by SA in gene-for-gene resistance may be to enhance the recognition mechanism. In this way, only those gene-for-gene interactions with relatively low sensitivities might be strongly affected by a lower SA level (Katagiri *et al.* 2002). It is also known that besides SA, other hormones may be involved in defense against *P. syringae*, although their role is not entirely clear (Gimenez-Ibanez and Rathjen 2010). In fact, ABA signaling could play a role in the protection against *P. syringae* mediated by 1-MT because the 1-MT-treated plants displayed both higher ABA accumulation and the induction of the *ASR1* marker gene at 48 hpi. Previous studies have uncovered ABA as an important *regulator* of plant *defense* responses, which can function positively or negatively depending on the analyzed plant-pathogen interaction (Ton *et al.* 2009). Specifically, ABA has been found to be a key regulator of pathogen-mediated stomatal closure (Melotto *et al.* 2008). One *P. syringae* pathogenesis mechanism is stomatal reopening activation mediated by effector COR, which allows bacteria to enter the mesophyll (Melotto *et al.* 2006). The ability of bacteria to successfully colonize the apoplastic space is crucial for successful infection. This colonization depends on the ability of bacteria to firstly access the apoplast and to secondly survive and reproduce once inside. As for the first requirement

to successfully carry out colonization, it is known that stomata effectively function as part of the plant innate immunity (Melotto *et al.* 2008). In the present work, we observed that the 1-MT treated and infected plants displayed more closed stomata than the control plants, probably due to greater ABA accumulation. This effect of 1-MT treatment on stomatal opening could hinder bacteria entering the mesophyll by reducing disease symptoms in this way. Several elicitors and resistance inducers, such as chitosan and Hx, have been respectively reported to trigger stomatal closure and to, thus, contribute to control disease (Klusener *et al.*, 2002; Gudesblat *et al.*, 2009b; Scalschi *et al.*, 2013).

The second requirement for infection to occur is for bacteria to establish in the apoplastic space. This depends on several factors, like the ability of bacteria to tolerate preformed defense molecules, to import and metabolize available nutrients, and, ultimately, to express pathogenicity and virulence factors that modulate host defenses and host metabolism by inducing the release of nutrients and water from inside plant cells. Once inside the apoplast, bacteria consume the nutrients present in it and, through virulence factors, they suppress or evade plant defense molecules (Katagiri *et al.* 2002, Rico and Preston 2008). When monitoring the distribution of bacteria over time in the mesophyll of the treated and untreated plants, by confocal microscopy we observed how a very small number of bacteria invaded the intercellular space of the treated plants. This could indicate not only more closed stomata, but also greater difficulties for bacteria to reproduce themselves.

Moreover, the inability of bacteria to colonize the leaf apoplast of the 1-MT-treated plants could also be due to the presence of antimicrobial factors in the apoplast (Katagiri *et al.*, 2002), a hypothesis that is supported by bacterial growth *in vitro* being lower in the apoplast extracted from the treated plants. This evidence led us to run a metabolomic analysis of its content. This analysis allowed us to identify differences between glucose concentrations and of a few amino acids in the treated and untreated plants. Nevertheless, the lower glucose levels found in the infected plants did not affect the growth of the bacterium *in vitro*, which might indicate that the higher glucose concentrations detected in the treated plants could be involved in increasing plant defense, which agrees with other authors (Rojas *et al.* 2014). Regarding amino acid content, we highlight an increase in the treated and infected plants of Thr, which could positively affect plant protection, as mentioned before with other biotrophs (Stuttmann

et al. 2011). Moreover, the levels of Trp, which is considered a modulator of defense (Ward *et al.* 2010), were clearly higher in the treated plants. As previously described by other authors (Dominguez and Carrari 2015), higher Thr and Trp levels could be a consequence of the increase observed in *ASR1* gene expression in the treated plants. As expected, 1-MT levels were also higher in the treated plants. In view of this result, we tested the direct effect of 1-MT on the bacterium. For this purpose, an *in vitro* study was carried out and demonstrated that at the 5 mM concentration applied to roots, cell division was inhibited, but no effect was observed at similar lower concentrations to those detected in leaves and the apoplast. These findings demonstrate that the present concentrations were much lower than those that affected bacterial growth.

Therefore, having ruled out the bactericidal effect of 1-MT at the concentration present in the plant, the possible effect of treatment on the genes involved in the pathogenicity of the bacterium was analyzed because another important factor for the successful colonization of bacteria is their ability to synthesize virulence factors (Rico and Preston 2008). The analysis of the expression of these genes indicated that treatment did not seem to affect the virulence of bacteria, but affected their motility because the *fliC* gene, which is involved in the synthesis of flagellin, one of the main components of the bacterial flagellum, showed a low expression in the treated plants. These results indicate a malfunction of the flagellum, which was confirmed by the swimming assays in a semisolid medium in the absence or presence of 1-MT. The motility of bacteria is essential for both their entry and establishment in the mesophyll apoplast and also for their capacity to move inside it. Previous works have shown that the flagella of bacteria are involved in other functions, like forming a secretory system (Young *et al.* 1999, Haiko and Westerlund-Wikström 2013). Therefore, their absence could hinder the colonization of the apoplastic space by bacteria.

A model of the 1-MT mode of action based on the results obtained herein is provided in Figure 8. We conclude that the effectiveness of 1-MT treatment can be due, on the one hand, to the inhibition of stomatal opening caused by COR since the treated plants showed higher ABA levels and more closed stomata, which could prevent, or at least hinder, the entry of bacteria to the mesophyll, a crucial step for successful colonization to take place. Besides, 1-MT appeared to act by blocking the JA pathway, which could avoid the manipulation of the SA pathway by the bacterium. On the other hand, the entry of bacteria into the mesophyll could also be hindered by the presence of 1–MT

itself in the plant. Although the 1-MT levels detected in the plant did not seem to have any antimicrobial effect, the possibility of them being high enough to avoid, or at least affect flagellum formation, cannot be ruled out. By affecting flagellum formation, it directly affects the mobility of bacteria in the mesophyll and, together with it, their capacity to reach the nutrients present in the apoplast. However, further metabolic and proteomic studies are needed to identify other compounds with antimicrobial activity which could help us to explain the reduced bacterial growth observed in the apoplast extracted from the treated plants.

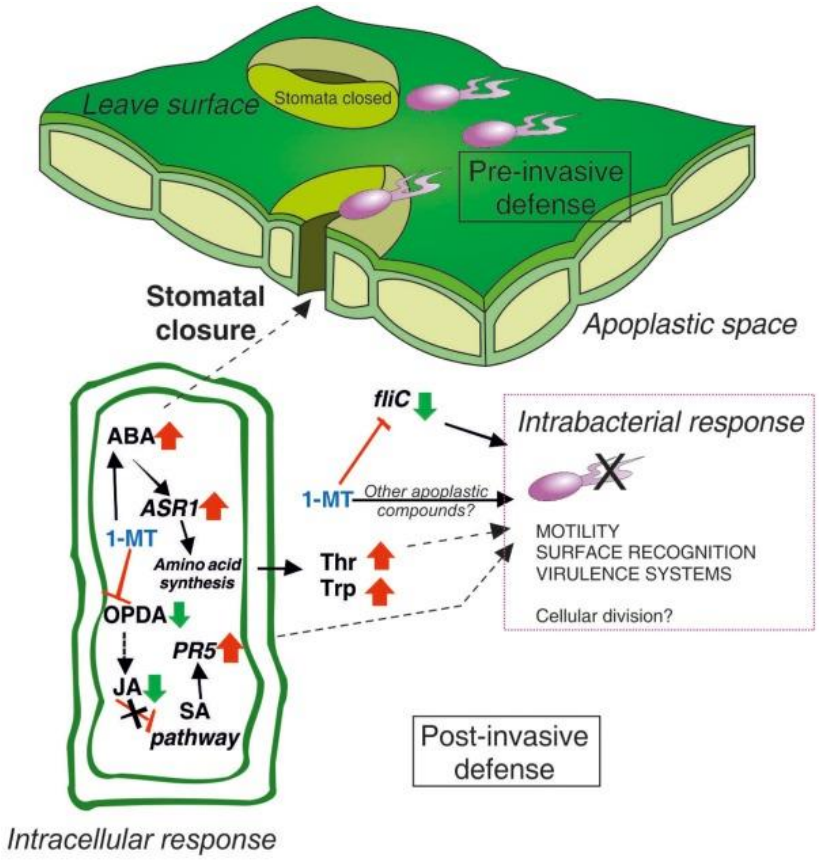

Figure 8. The involvement of 1-MT in tomato–*Pseudomonas syringae* pathosystem. ABA plays a positive role in pre-invasive stomatal immunity by induction of stomatal closure to prevent pathogen entry. 1-MT blocks JA pathway activating this way SA pathway through the expression of *PR5*. The increase in the concentration of Thr and Trp together with 1-MT could be affecting the cellular division of the bacteria. In addition, 1-MT reduces the expression of *flic* gene involved in the synthesis of the flagellum, causing a reduction in the motility of the bacteria and therefore a reduction in its capacity of infection.

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REFERENCES

Alvarez, M.E., 2000. Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant molecular biology*, 44 (3), 429–42.

Beattie, G.A. and Lindow, S.E., 1995. The secret life of foliar bacterial pathogens on leaves. *Annual Review of Phytopathology*, 33 (1), 145–172.

Bednarek, P., Kwon, C., and Schulze-Lefert, P., 2010. Not a peripheral issue: secretion in plant–microbe interactions. *Current Opinion in Plant Biology*, 13 (4), 378–387.

Brooks, D.M., Bender, C.L., and Kunkel, B.N., 2005. The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Molecular Plant Pathology*, 6 (6), 629–639.

Camañes, G., Scalschi, L., Vicedo, B., González-Bosch, C., and García-Agustín, P., 2015. An untargeted global metabolomic analysis reveals the biochemical changes underlying basal resistance and priming in *Solanum lycopersicum*, and identifies 1-methyltryptophan as a metabolite involved in plant responses to *Botrytis cinerea* and *Pseudomonas syringae*. *The Plant Journal*, 84 (1), 125–139.

Cao, F.Y., Yoshioka, K., and Desveaux, D., 2011. The roles of ABA in plant–pathogen interactions. *Journal of Plant Research*, 124 (4), 489–499.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J., 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, 124 (4), 803–814.

Cuppels, D.A., 1986. Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. tomato. *Applied and environmental microbiology*, 51 (2), 323–7.

Delgado, D., Alonso-Blanco, C., Fenoll, C., and Mena, M., 2011. Natural variation in stomatal abundance of *Arabidopsis thaliana* includes cryptic diversity for different developmental processes. *Annals of botany*, 107 (8), 1247–58.

Dominguez, P.G. and Carrari, F., 2015. ASR1 transcription factor and its role in metabolism. *Plant Signaling and Behavior*, 10 (4), e992751.

Elizabeth, S. V. and Bender, C.L., 2007. The phytotoxin coronatine from *Pseudomonas syringae* pv. tomato DC3000 functions as a virulence factor and influences defence pathways in edible brassicas. *Molecular Plant Pathology*, 8 (1), 83–92.

Fernández-Crespo, E., Scalschi, L., Llorens, E., García-Agustín, P., and Camañes, G., 2015. NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation. *J Exp Bot*, 66 (21), 6777-6790.

Gamir, J., Pastor, V., Cerezo, M., and Flors, V., 2012. Identification of indole-3-carboxylic acid as mediator of priming against *Plectosphaerella cucumerina*. *Plant Physiology and Biochemistry*, 61 (0), 169–179.

Geisler, M., Nadeau, J., and Sack, F.D., 2000. Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the too many mouths mutation. *The Plant cell*, 12 (11), 2075–86.

Gimenez-Ibanez, S. and Rathjen, J.P., 2010. The case for the defense: plants versus *Pseudomonas syringae*. *Microbes and Infection*, 12 (6), 428–437.

Gudesblat, G.E., Torres, P.S., and Vojnov, A.A., 2009a. *Xanthomonas campestris* overcomes *Arabidopsis* stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. *Plant physiology*, 149 (2), 1017–27.

Gudesblat, G.E., Torres, P.S., and Vojnov, A.A., 2009b. Stomata and pathogens: Warfare at the gates. *Plant signaling and behavior*, 4 (12), 1114–6.

Haiko, J. and Westerlund-Wikström, B., 2013. The role of the bacterial flagellum in adhesion and virulence. *Biology*, 2 (4), 1242–67.

Halim, V.A., Altmann, S., Ellinger, D., Eschen-Lippold, L., Miersch, O., Scheel, D., and Rosahl, S., 2009. PAMP-induced defense responses in potato require both salicylic acid and jasmonic acid. *The Plant Journal*, 57 (2), 230–242.

Hoagland and Arnon, D., 1950. The water-culture method for growing plants without soil. *Circular. California Agricultural Experiment Station*, 347.

Katagiri, F., Thilmony, R., and He, S.Y., 2002. The *Arabidopsis Thaliana-Pseudomonas Syringae* Interaction. *The Arabidopsis Book*, 1, e0039.

Klusener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V., and Schroeder, J.I., 2002. Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in *Arabidopsis* guard cells. *Plant physiology*, 130 (4), 2152–2163.

Koornneef, A., Pieterse, C.M.J., Conconi, A., and Ryan, C.A., 2008. Cross talk in defense signaling. *Plant physiology*, 146 (3), 839–44.

Laurie-Berry, N., Joardar, V., Street, I.H., and Kunkel, B.N., 2006. The *Arabidopsis thaliana JASMONATE INSENSITIVE 1* gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions*, 19 (7), 789-800.

Llorens, E., Fernández-Crespo, E., Vicedo, B., Lapeña, L., and García-Agustín, P., 2013. Enhancement of the citrus immune system provides effective resistance against *Alternaria* brown spot disease. *Journal of Plant Physiology*, 170 (2), 146-154.

Van Loon, L.C., 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology*, 103 (9), 753–765.

Melotto, M., Underwood, W., and He, S.Y., 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. *Annual review of phytopathology*, 46, 101–122.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S.Y., 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell*, 126 (5), 969–980.

Mur, L.A.J., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C., 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant physiology*, 140 (1), 249–62.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., and Ohashi, Y., 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of Pathogenesis-Related (*PR*) protein genes in wounded mature tobacco leaves. *Plant and Cell Physiology*, 39 (5), 500–507.

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L., 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological reviews*, 198, 249–66.

O'Leary, B.M., Rico, A., McCraw, S., Fones, H.N., and Preston, G.M., 2014. The infiltration-centrifugation technique for extraction of apoplastic fluid from plant leaves using *Phaseolus vulgaris* as an example. *Journal of Visualized Experiments*, 94.

Pieterse, C.M.J., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S.C.M., 2012. Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, 28, 489-521.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C.M., 2009. Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology*, 5 (5), 308–316.

Reymond, P. and Farmer, E.E., 1998. Jasmonate and salicylate as global signals for defense gene expression. *Current opinion in plant biology*, 1 (5), 404–411.

Rico, A. and Preston, G.M., 2008. *Pseudomonas syringae* pv. tomato DC3000 uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. *Molecular Plant-Microbe Interactions*, 21 (2), 269–282.

Río-Álvarez, I., Rodríguez-Herva, J.J., Martínez, P.M., González-Melendi, P., García-Casado, G., Rodríguez-Palenzuela, P., and López-Solanilla, E., 2014. Light regulates motility, attachment and virulence in the plant pathogen *Pseudomonas syringae* pv tomato DC3000. *Environmental Microbiology*, 16 (7), 2072–2085.

Robert-Seilaniantz, A., Grant, M., and Jones, J.D.G., 2011. Hormone crosstalk in plant disease and defense: more than just JASMONATE-SALICYLATE antagonism. *Annual Review of Phytopathology*, 49 (1), 317–343.

Rojas, C.M., Senthil-Kumar, M., Tzin, V., and Mysore, K.S., 2014. Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. *Frontiers in plant science*, 5, 17.

Sánchez-Vallet, A., Ramos, B., Bednarek, P., López, G., Piślewska-Bednarek, M., Schulze-Lefert, P., and Molina, A., 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. *The Plant Journal*, 63 (1), 115-127.

Scalschi, L., Vicedo, B., Camañes, G., Fernández-Crespo, E., Lapena, L., González-Bosch, C., and García-Agustín, P., 2013. Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways. *Mol Plant Pathol*, 14 (4), 342–355.

Shirasu, K., Nakajima, H., Rajasekhar, V.K., Dixon, R.A., and Lamb, C., 1997. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *The Plant Cell online*, 9 (2), 261–270.

Stuttmann, J., Hubberten, H.-M., Rietz, S., Kaur, J., Muskett, P., Guerois, R., Bednarek, P., Hoefgen, R., and Parker, J.E., 2011. Perturbation of *Arabidopsis* amino acid metabolism causes incompatibility with the adapted biotrophic pathogen *Hyaloperonospora arabidopsidis*. *The Plant Cell*, 23 (7), 2788–2803.

Thaler, J.S. and Bostock, R.M., 2004. Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects. *Ecology*, 85 (1), 48–58.

Thomma, B.P., Penninckx, I.A., Broekaert, W.F., and Cammue, B.P., 2001. The complexity of disease signaling in *Arabidopsis*. *Current opinion in immunology*, 13 (1), 63-68.

Ton, J., Flors, V., and Mauch-Mani, B., 2009. The multifaceted role of ABA in disease resistance. *Trends in Plant Science*, 14 (6), 310–317.

Torres, M.A., 2010. ROS in biotic interactions. *Physiologia Plantarum*, 138 (4), 414–429.

Torres, M.A., Jones, J.D.G., and Dangl, J.L., 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiology*, 141 (2), 373–378.

Uppalapati, S.R., Ayoubi, P., Weng, H., Palmer, D.A., Mitchell, R.E., Jones, W., and Bender, C.L., 2005. The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *The Plant Journal*, 42 (2), 201–217.

Uppalapati, S.R., Ishiga, Y., Wangdi, T., Urbanczyk-Wochniak, E., Ishiga, T., Mysore, K.S., and Bender, C.L., 2008. Pathogenicity of *Pseudomonas syringae* pv. *tomato* on tomato seedlings: phenotypic and gene expression analyses of the virulence function of coronatine. *Molecular Plant-Microbe Interactions*, 21 (4), 383–395.

Vicedo, B., Flors, V., Leyva, M.D., Finiti, I., Kravchuk, Z., Real, M.D., García-Agustín, P., and González-Bosch, C., 2009. Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. *Molecular Plant-Microbe Interactions*, 22 (11), 1455–1465.

Vlot, A.C., Dempsey, D.A., and Klessig, D.F., 2009. Salicylic Acid, a multifaceted hormone to combat disease. *In*: *Annual Review of Phytopathology*. 177–206.

Ward, J.L., Forcat, S., Beckmann, M., Bennett, M., Miller, S.J., Baker, J.M., Hawkins, N.D., Vermeer, C.P., Lu, C., Lin, W., Truman, W.M., Beale, M.H., Draper, J., Mansfield, J.W., and Grant, M., 2010. The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. *The Plant Journal*, 63 (3), 443–457.

Xin, X.-F. and He, S.Y., 2013. *Pseudomonas syringae* pv. *tomato* DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. *Annual Review of Phytopathology*, 51 (1), 473–498.

Young, G.M., Schmiel, D.H., and Miller, V.L., 1999. A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, 96 (11), 6456–6461.

Yu, X., Lund, S.P., Scott, R.A., Greenwald, J.W., Records, A.H., Nettleton, D., Lindow, S.E., Gross, D.C., and Beattie, G.A., 2013. Transcriptional responses of *Pseudomonas syringae* to growth in epiphytic versus apoplastic leaf sites. *Proceedings of the National Academy of*

Sciences of the United States of America, 110 (5), E425-34.

SUPPLEMENTARY DATA

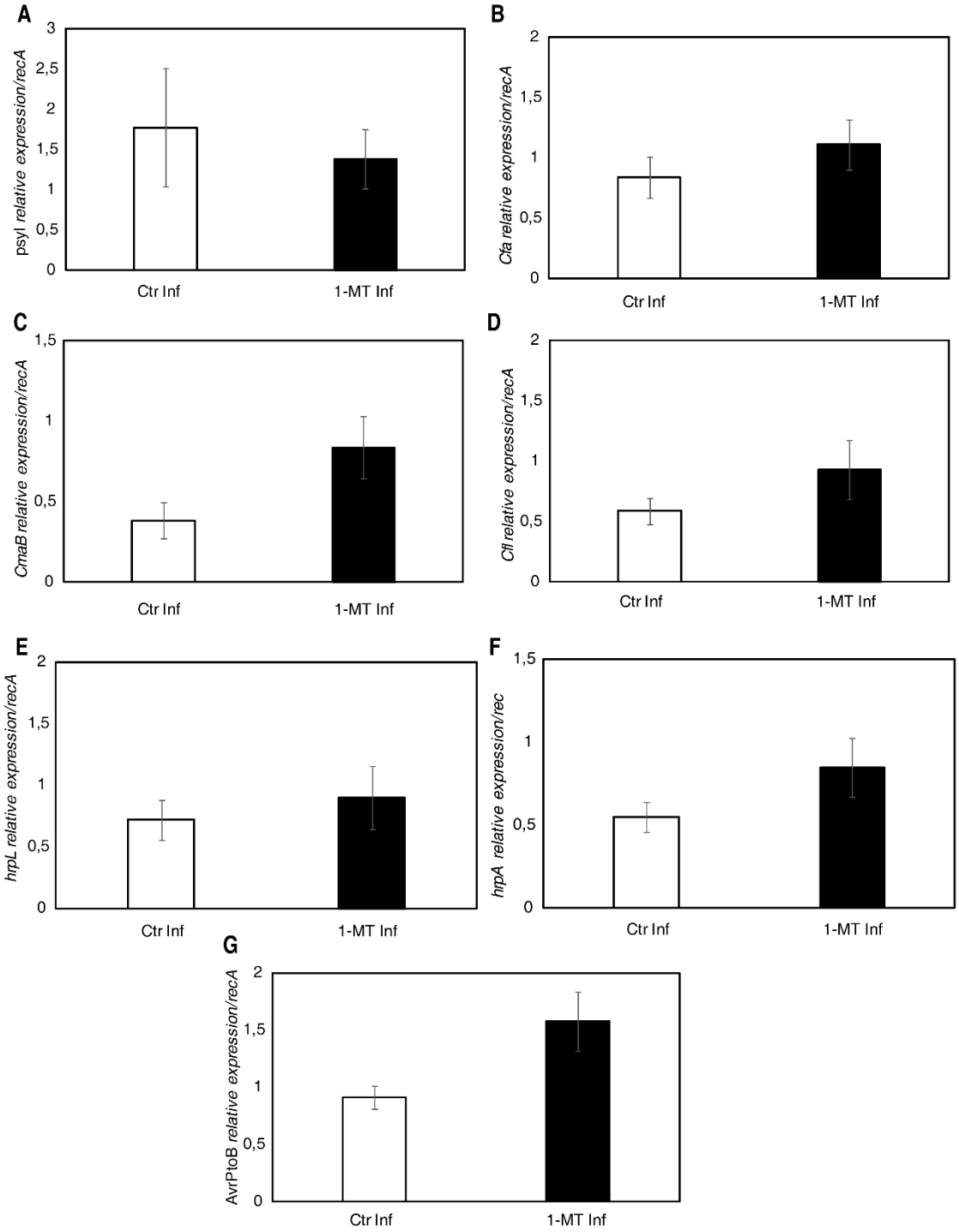

Figure S1. Bacterial RNA extraction was performed from *P. syringae* extracted from infected leaves. Relative expression of following genes were analyzed during bacterial growth (A) Quorum sensing establishment-related genes (*psyI*), COR synthesis-related genes [(B) *cfa1*, (C) *cmaB* and (D) *cfl*], marker genes of the type III secretion system and the type III secretion system-associated pilus, respectively [(E) *hrpL* and (F) *hrpA*], (G) gene responsible for effector synthesis (*avrPtoB*). The recA gene was used as an endogenous reference gene.

Table S1. Amino acids, hormone and sugar content in apoplast extract. The values are the average of three independent experiments ± SE. Apoplast amino acid and hormone concentration are expressed in ng mL^{-1} and sugar concentration is expressed in µg mL^{-1}.

	Ctr	1-MT	Ctr Inf	1-MT Inf
Thr	406 ± 156	845 ± 244	235 ± 65	737 ± 247
Val	592 ± 156	559 ± 258	255 ± 96	496 ± 118
Pro	489 ± 47	1014 ± 39	495 ± 109	601 ± 92
Ser	59 ± 28	222 ± 81	20 ± 10	111 ± 26
Ala	271 ± 75	456 ± 142	417 ± 100	616 ± 156
Trp	180 ± 72	124 ± 59	164 ± 125	750 ± 335
Phe	225 ± 182	53 ± 15	38 ± 15	298 ± 259
Glu	2031 ± 736	3639 ± 887	2045 ± 327	5503 ± 1910
Asp	2054 ± 408	3590 ± 252	655 ± 185	2056 ± 667
Tyr	341 ± 112	511 ± 195	753 ± 102	1486 ± 291
His	103 ± 39	307 ± 22	964 ± 361	1154 ± 345
Met	35 ± 29	121 ± 29	76 ± 24	71 ± 12
Gln	478 ± 231	715 ± 137	722 ± 253	1385 ± 543
Lys	463 ± 254	687 ± 185	721 ± 273	1384 ± 632
Pip	230 ± 40	396 ± 42	1606 ± 223	2275 ± 970
ABA	38 ± 8	38 ± 3	48 ± 10	49 ± 4
SA	2 ± 2	8 ± 7	45 ± 27	19 ± 10
OPDA	27 ± 1	26,1 ± 0,1	42 ± 12	38 ± 7
JA	29 ± 5	42 ± 11	143 ± 42	127 ± 43
Fructose	50 ± 1	64,5	48	59 ± 11
Glucose	44 ± 7	40 ± 13	n.d.	37

Table S2. Primers used for plant gene expression analyses.

Function	Gene	Primer
Abscisic stress-ripening protein 1	*ASR1*	F 5'- ACACCACCACCACCTGT -3' R 5'- GTGTTTGTGTGCATGTTGTGGA -3'
Pathogenesis-related protein 1	*PR1*	F 5'- CCGTGCAATTGTGGGTGTC -3' R 5'- GAGTTGCGCCAGACTACTTGAGT -3'
pathogenesis-related protein 5	*PR5*	F 5'- GAGGTTCATGCCAAACTGGTC -3' R 5'- TCAACCAAAGAAATGTCC -3'
Allene oxide cyclase	*AOC*	F 5'- GCACGAAGAAGAGAAGAAAGGAGA -3' R 5'- CGGTGACGGCTAGGTAAGTTT -3'
Elongation factor 1-alpha	*EF1α*	F 5'- GACAGGCGTTCAGGTAAGGA-3' F 5'- GGGTATTCAGCAAAGGTCTC-3

Table S3. Primers used for bacterial gene expression analyses.

Function	Gene	Primer
Coronatine synthesis	*cfa1*	F 5'-AAAACCATCGTCGACATTCTG-3' R 5'-GTTGGCGTTGAGGTCGATA-3'
	cmaB	F 5'-AATTCGACACCCGACAAGAC-3' R 5'-ACTAGGGGCTTCAGGTCCAT-3'
	cfl	F 5'-ACAGCTGAAGCAGCACTTGA-3' R 5'-CGAGGATCTCTCGGTAGTCG-3'
Type III secretion system, type III secretion system-associated pilus and effector synthesis	*hrpL*	F 5'-TCTCCAGTGCGTGTTTCTTG-3' R 5'-AGCTTTCCTGATACGGCTGA-3'
	hrpA	F 5'-CCTCCAAACTCACCAACCTT-3' R 5'-CGGACTCTTTACTGGCCTTG-3'
	avrPtoB	F 5'-ACCCTATCGCGTCACAATTC-3' R 5'-CATGAACGCCAGGTCCTTAT-3'
Quorum sensing establishment	*psyI*	F 5'-GGCTTGAATGGAATGTTCGT-3' R 5'-CAGGTGTTGATCAGCCGTAA-3'
Flagellin synthesis	*fliC*	F 5'-ATCTGAACGGCAAGAACCTG-3' R 5'-TGCGCTCAAAGTCAGAGAGA-3'
Internal reference	*recA*	F 5'-CGGCAAGGGTATCTACCTCA-3' R 5'-CTTTGCAGATTTCCGGGTTA-3'

CHAPTER 3

Ammonium mediated changes in carbon and nitrogen metabolisms induce resistance against *Pseudomonas syringae* in tomato plants

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CHAPTER 4

Putrescine biosynthesis pathways are involved in tomato plant defence against *Pseudomonas syringae*

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IN PREPARATION

CHAPTER 5

Changes in root development and N assimilatory pathways are ABA-dependent in tomato plants grown under different N sources

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GENERAL DISCUSSION

General discussion

Tomato is one of the most important vegetable crops in the world. Today's tomato production is about 182 million tons, obtained from 4,848 million ha, and Spain is the world's eighth producing country (www.fao.org/faostat/en). Among the factors leading to tomato cultivation loss is attack of pathogens like *Pseudomonas syringae* pv. tomato DC3000. To control pathogen attack, pesticides need to be employed. The use of these agrochemical compounds may pose environmental pollution and health problems, which is why researchers are searching for enviro-friendly strategies to mitigate pathogen attacks. In this work, new strategies such as using Cu heptagluconate, 1-MT and NH_4^+ are proposed for controlling pathogen spread in tomato crops and reducing pesticide use.

Traditionally, large amounts of Cu have been applied as a pesticide, which has caused environmental pollution and led to search for alternatives that reduce its application. One such strategy is using complexing organic compounds, characterised by greater absorption, like Cu heptagluconate are allowed in sustainable agriculture and play a double role as a pesticide and fertiliser, but are applied in smaller amounts. In **Chapter 1**, the efficacy of the complexing organic compound between heptagluconic acid and Cu was tested in tomato plants against *P. syringae*. The analyses showed a reduction of more than 50% in bacterial populations in Cu-heptagluconate-treated and inoculated plants, because plant defence mechanisms were activated via enhancement in phenolic compounds. Several authors have previously described the relation of Cu treatment with increased lignin biosynthesis or antimicrobial substances such as flavonoids, which are compounds that play a role in plant disease resistance (Harker *et al*., 1990; Aziz *et al*., 2006; Chmielowska *et al*., 2010). Our results also showed lower ROS production after infection. This event has also observed by Chmielowska *et al*., 2010 in *Capsicum anuum* against *Verticillium dahliae* through the up-regulation of peroxidase after supplying Cu inorganic forms. Besides, *in vitro* assays have indicated the antimicrobial effect of Cu on pathogen development. It should be noted that the activation of plant innate immunity, in combination with the direct effect of Cu against the pathogen, could be an effective treatment for preventing disease in the field as it would not only avoid using inorganic Cu forms, but would diminish pollution.

Use of resistance inducers is another strategy to fight against pathogen attack. In the last decade, Hx has been one of the resistance inducers used in tomato plants against *P.* syringae. This compound acts as an inducer of plant defences by a priming mechanism against pathogens with different lifestyles (Vicedo *et al*., 2009; Llorens *et al*., 2013; Scalschi *et al*., 2013). This priming mechanism is characterised by a metabolic profile of *Botrytis cinerea*- or *P. syringae*-infected tomato plants, and is treated with Hx. In this study, the authors showed an increase in 1-MT, a natural compound produced by tomato plants infected with *Pseudomonas syringae* attack. Application of 1-MT to tomato roots lowers disease symptoms close to 40 % (Camañes *et al*. 2015). In **Chapter 2**, this compound's mode of action was studied by metabolic and transcriptomic profiles in tomato plant tissue against *P. syringae* and in the apoplast extract as this pathogen develops its pathogenic phase in this space. The transcriptomic changes in genes related to the pathogenicity and virulence of bacteria were studied. After 1-MT treatment and inoculation, ABA and the *ASR1* marker gene and stomatal closure increased and bacterial entry in the mesophyll diminished. Several authors have previously described the role of ABA in stomata closure control and its relation with *Pseudomonas* infection (Melotto *et al*., 2006; Melotto *et al*., 2008; Cao *et al*., 2011). Our results also showed increased SA content in the 1-MT-treated and infected plants, while the oxylipin pathway reduced, which means that manipulation of the SA pathway by bacteria could be avoided, which would allow better induction of the SA response through *PR5* by plants. It has been previously shown that *PR5* can respond to SA, or to either ethylene or MeJA (Reymond and Farmer, 1998), but SA is the main signal that appears after recognising pathogen attack (Shirasu *et al*., 1997). As the endophytic phase of bacteria takes place in the apoplast, its composition could be another factor that affects the bacterial colonisation of the mesophyll. This led us to study apoplast content and to hypothesise that reduction in bacteria could be due to the presence of antimicrobial factors in the apoplast as previously described by Katagiri *et al*. (2002). We found that several metabolites in the apoplast increased, 1-MT and amino acids such as Thr or Trp, which have been previously described as defence modulators (Stuttmann *et al*., 2011; Ward *et al*., 2010). Moreover, we found that bacterial growth was dependent on the 1-MT concentration and that this compound affected *P. syringae* motility, with a decrease in the *fliC* expression that encodes flagellin. Hence, this treatment had an effect on plant defence, but also repressed bacterial motility by hindering bacteria from entering and establishing in the apoplast, as well as their capacity to move inside it. In short, 1-MT is

a natural compound produced by tomato plants that might constitute an alternative to pesticide use given it's observed capacity to induce resistance against *Pseudomonas syringae* attack. However, further studies are required to determine its application in the field.

In an attempt to search for other approaches to control bacterial speck damage, we proposed elucidating the effect of NH_4^+ nutrition against this disease. The large amounts of N fertilisers, mainly in the NO_3^- form constitute another agriculture problem. This compound is highly soluble, can be released to the atmosphere as nitrous oxide and can be leached into groundwater to produce water eutrophication (Sánchez-Pérez *et al.*, 2003). Therefore, NH_4^+-based fertilisation might be a better enviro-friendly alternative to NO_3^-. NH_4^+ nutrition triggers mild chronic stress, which induces different plant changes and therefore confer resistance to later stress through the activation of systemic acquired acclimation (SAA) (Fernández-Crespo *et al.*, 2013; Fernández-Crespo *et al.*, 2015). Hence, in order to investigate the mechanisms underlying SAA mediated by NH_4^+, the changes in nutrient balance, C and N compounds and their implication in resistance against *P. syringae* were studied (**Chapter 3**). When tomato plants were growing under NH_4^+ nutrition as the sole N source, the concentration of the positively charged macronutrients general lowered and anion uptake increased as described previously by Da Silva *et al*. (2006) and Roosta and Schjoerring (2007) in other crops. An increase in Mn, Zn, S and P was observed, which acted mainly as cofactors of antioxidant enzymes to reduce the oxidative stress produced by NH_4^+ nutrition as Carlisle *et al*. (2012) observed in wheat plants. Regarding primary metabolism, our results showed a general reprogramming of the primary metabolism in leaves and roots. Thus the concentration of N-containing compounds and the levels of Glu metabolism-derived amino acids rise. These results agree with Frechilla *et al*. (2002) and Ueda *et al*. (2008), who observed an increase in Glu and Asn in soybean and pea plants under NH_4^+ nutrition. Our results suggest the strong biosynthesis of Gln together with Asn, which are amino acids involved in alleviating the stress produced by this cation. In addition, an accumulation in Arg was observed, which accumulated 16-fold in the NH_4^+-fed plants. Arg is a Put precursor and the importance of this polyamine in tomato plant defence has been previously reported by Fernández-Crespo *et al*. (2015) and in *Arabidopsis* by Kim *et al*. (2013). In view of these results, we proposed generating transgenic silenced tomato plants on the Put biosynthesis pathways to test their role against the pathogen in

order to demonstrate the role of polyamines in plant defence against *P. syringae* (**Chapter 4**). The T1 knock-down lines were generated and the expressions of genes *ADC* and *ODC* notably reduced, while plants became more susceptible to *P. syringae* infection. This indicates that Put could be involved in defence mechanisms against *P. syringae* in tomato plants. In this way, Kim *et al.* (2013) observed how the Put concentration and PR expression lowered, which led to *Arabidopsis ADC* knockout susceptibility to *P. syringae*. Altogether, this suggests that NH_4^+ constitutes a fertiliser alternative to substitute NO_3^--based fertilisers by playing a double role as both a fertiliser and pesticide. So its use could be included in the fertilisation programmes and could become a very effective easy-to-use fertiliser.

It is already known that abiotic stress could trigger mild chronic stress inducing stress-induced morphogenetic responses (SIMRs) as part of a general acclimation strategy, which affects the root architecture (Potters *et al*., 2009). As NH_4^+ produces mild stress and from the results obtained in Chapter 3, where several changes in the primary metabolism in the NH_4^+-fed tomato plants were observed, we were motivated to investigate if it had any effect on tomato root development and its role in the assimilation process (**Chapter 5**). Firstly in this chapter, we proposed adding different NO_3^- proportions and C to alleviate the mild toxic effect of NH_4^+ (Britto and Krozucker, 2002; Garnica *et al.*, 2009). In this work, no differences were observed in PR length and LR number under NH_4^+ nutrition. However in other crops such as *Arabidopsis* or *Lotus japonicus*, NH_4^+ brings about an increase in LR development and inhibits PR and LR length, respectively (Lima *et al*., 2010; Rogato *et al*., 2010; Giehl *et al*., 2014). These results indicate the different responses of crops to NH_4^+ nutrition during root development. Secondly, it is known that crosstalk takes place between the C and N pathways because of the use of C compounds by assimilatory processes (Nunes-Nesi *et al*., 2010). C sources, such as glucose or 2-oxoglutarate, were supplied to NH_4^+ treatment, and alleviation in mild NH_4^+ toxicity was observed during root development. Besides, N uptake and assimilation was done by specialised transporters located in plant roots (Miller and Crammer, 2004). For this purpose, the genes encoding for the NO_3^- (*NRT*) and NH_4^+ (*AMT*) low- and high- affinity transport systems (LATS and HATS) were studied as markers of the uptake process. The study of N transporters resulted in a general decrease in *NRT* and an increase in *AMT* gene expressions, while only *ASN1* and the anaplerotic gene *PEPC* were up-regulated in roots of plants fed with NH_4^+

nutrition during the N assimilation process. Moreover, auxins are essential phytohormones involved in the regulation of PR development and LR emergence (Overvoorde *et al.*, 2010), which are facilitated by auxin influx transporters (LAX) and auxin efflux transporters (PIN) (Giehl *et al.*, 2014). We proposed studying the expression of genes *LAX* and *PIN* genes in the root development of the plants treated with NH_4^+, and a decrease in *LAX4* and *PIN4* was observed. Accordingly, previous studies conducted with *Arabidopsis* and wheat have shown lower IAA content linked to NH_4^+ nutrition by inducing root development inhibition (Kudoyarova *et al.*, 1997; Li *et al.*, 2014). Moreover, ABA also plays a role in root growth (Peuke *et al.*, 1998). In tomato plants, NO_3^--fed *flacca* seedlings showed a significant decrease in the root parameters compared to the NO_3^--wild type, while the NH_4^+-fed *flacca* seedlings showed a reduction only in PR length. These changes seemed to be related with auxin transport, and also with N uptake and assimilation.

In summary, when we compared the defence mechanisms of the three studied compounds, we found that the treatment of Cu heptagluconate and NH_4^+ induced the metabolism of phenolic compounds, but it did not seem to be involved in this pathway in 1-MT. Besides, 1-MT and NH_4^+ induce ABA accumulation and stomatal closure by avoiding bacterial entry in mesophyll cells, whereas this increase in ABA was not observed in the plants undergoing Cu heptagluconate treatment. In addition, 1-MT and Cu have a direct effect on the bacterium, while this phenomenon has not yet been studied in NH_4^+. As shown in this work, the different treatments also induced other specific defence mechanisms in tomato plants against *P. syringae*.

To conclude, the use of Cu heptagluconate, 1-MT and NH_4^+ are proposed as an alternative to reduce pesticide use and they could be considered in phytosanitary programmes currently used in agriculture, as they can control attack of pathogens, specifically *P. syringae* in tomato plants avoiding todays' loss production. In the future, the use of these compounds can be extended to other pathosystems of interest to finally transfer its application to the field.

REFERENCES:

Aziz A, Trotel-Aziz P, Dhuicq L, Jeandet P, Couderchet M, Vernet G. 2006. Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. Phytopathology 96(11), 1188–1194

Britto DT, Kronzucker HJ. 2002. NH_4^+ toxicity in higher plants: a critical review. Journal of Plant Physiology 159, 567–584.

Camañes G, Scalschi L, Vicedo B, González-Bosch C, García-Agustín P. 2015. An untargeted global metabolomic analysis reveals the biochemical changes underlying basal resistance and priming in *Solanum lycopersicum*, and identifies 1-methyltryptophan as a metabolite involved in plant responses to *Botrytis cinerea* and *Pseudomonas syringae*. The Plant Journal 84(1), 125–139.

Cao FY, Yoshioka K, Desveaux D. 2011. The roles of ABA in plant–pathogen interactions. Journal of Plant Research 124(4), 489–499.

Carlisle E, Myers S, Raboy V, Bloom A. 2012. The effects of inorganic nitrogen form and CO_2 concentration on wheat yield and nutrient accumulation and distribution. Frontiers in Plant Science 3, 1–13.

Chmielowska J, Veloso J, Gutiérrez J, Silvar C, Díaz J. 2010. Cross-protection of pepper plants stressed by copper against a vascular pathogen is accompanied by the induction of a defense response. Plant Sci. 178, 176-182.

Da Silva GP, Prado R de M, Ferreira RPS. 2016. Absorption of nutrients, growth and nutritional disorders resulting from ammonium toxicity in rice and spinach plants. Emirates Journal of Food and Agriculture 28, 882–889.

FAOSTAT. http://www.fao.org/faostat/en/#data/QC [accesed January 2019]

Fernández-Crespo E, Gómez-Pastor R, Scalschi L, Llorens E, Camañes G, García-Agustín P. 2013. NH_4^+ induces antioxidant cellular machinery and provides resistance to salt stress in citrus plants. Trees 28, 1693.

Fernández-Crespo E, Scalschi L, Llorens E, García-Agustín P, Camañes G. 2015. NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation. Journal of Experimental Botany 66(21), 6777-6790.

Frechilla S, Lasa B, Aleu M, Juanarena N, Lamsfus C, Aparicio-Tejo PM. 2002. Short-term ammonium supply stimulates glutamate dehydrogenase activity and alternative pathway respiration in roots of pea plants. Journal of Plant Physiology 159, 811–818.

Garnica M, Houdusse F, Zamarreño AM, Garcia-Mina JM. 2010. The signal effect of nitrate supply enhances active forms of cytokinins and indole acetic content and reduces abscisic acid in wheat plants grown with ammonium. Journal of Plant Physiology 167, 1264–1272.

Giehl RFH, Gruber BD, von Wirén N. 2014. It's time to make changes: modulation of root system architecture by nutrient signals. Journal of Experimental Botany 65, 769–778.

Harker CL, Ellis TH, Coen ES. 1990. Identification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2(3), 185–194.

Katagiri F, Thilmony R, He SY. 2002. The *Arabidopsis Thaliana-Pseudomonas Syringae* Interaction. The Arabidopsis Book 1, e0039.

Kim SH, Kim SH, Yoo SJ, Min KH, Nam SH, Cho BH, Yang KY. 2013. Putrescine regulating by stress-responsive MAPK cascade contributes to bacterial pathogen defense in Arabidopsis. Biochemical and Biophysical Research Communications 437, 502–508.

Kudoyarova G, Farkhutdinov R, Veselov S. 1997. Comparison of the effects of nitrate and ammonium forms of nitrogen on auxin content in roots and the growth of plants under different temperature conditions. Plant Growth Regulation 23, 207-208.

Li B, Li G, Kronzucker HJ, Baluška F, Shi W. 2014. Ammonium stress in *Arabidopsis*: signaling, genetic loci, and physiological targets. Trends in Plant Science 19, 107–114.

Lima JE, Kojima S, Takahashi H, von Wirén N. 2010. Ammonium triggers lateral root branching in *Arabidopsis* in an AMMONIUM TRANSPORTER 1;3-dependent manner. Plant Cell 22, 3621–3633.

Llorens E, Fernandez-Crespo E, Vicedo B, Lapeña L, García-Agustín P. 2013. Enhancement of the citrus immune system provides effective resistance against *Alternaria* brown spot disease. Journal of Plant Physiology 170, 146–154.

Melotto M, Underwood W, He SY. 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. Annual review of phytopathology 46, 101-122.

Melotto M, Underwood W, Koczan J, Nomura K, He SY. 2006. Plant stomata function in innate immunity against bacterial invasion. Cell 126, 969-980.

Miller AJ, Cramer MD. 2005. Root nitrogen acquisition and assimilation. Plant Soil 274, 1–36.

Nunes-Nesi A, Fernie AR, Stitt M. 2010. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions Mol. Plant, 3, 973-996.

Overvoorde P, Fukaki H, Beeckman T. 2010. Auxin control of root development. Cold Spring Harb. Perspect. Biol. 2, a001537.

Peuke AD, Jeschke WD, Dietz KJ, Schreiber L, Hartung W. 1998. Foliar application of nitrate and ammonium as sole nitrogen supply in *Ricinus communis*. I. Carbon and nitrogen uptake and inflows. New Phytologist 138, 675–687.

Potters G, Pasternak TP, Guisez Y, Jansen MAK. 2009. Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant Cell Environ. 32, 158–169.

Reymond P, Farmer EE. 1998. Jasmonate and salicylate as global signals for defense gene expression. Current opinion in plant biology 1(5), 404–411.

Rogato A, D'Apuzzo E, Barbulova A, Omrane S, Parlati A, Carfagna S, Costa A, Lo Schiavo F, Esposito S, Chiurazzi M. 2010. Characterization of a developmental root response caused by external ammonium supply in *Lotus japonicus*. Plant Physiology 154(2), 784-795.

Roosta HR, Schjoerring JK. 2007. Effects of Ammonium Toxicity on Nitrogen Metabolism and Elemental Profile of Cucumber Plants. Journal of Plant Nutrition 30, 1933–1951.

Scalschi L, Vicedo B, Camanes G, Fernandez-Crespo E, Lapena L, Gonzalez-Bosch C, García-Agustín P. 2013. Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways. Molecular Plant Pathology 14(4): 342-355.

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C. 1997. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. The plant cell online 9(2), 261–270.

Sánchez-Pérez JM, Antigüedad I, Arrate I, García-Linares C, Morell I. 2003. The influence of nitrate leaching through unsaturated soil on groundwater pollution in an agricultural area of the basque country. Sci. Total Environ. 317, 173–187.

Stuttmann J, Hubberten HM, Rietz S, Kaur J, Muskett P, Guerois R, Bednarek P, Hoefgen R, Parker JE. 2011. Perturbation of *Arabidopsis* amino acid metabolism causes incompatibility with the adapted biotrophic pathogen *Hyaloperonospora arabidopsidis*. The Plant Cell 23(7), 2788–2803.

Ueda S, Ikeda M, Yamakawa T. 2008. Provision of carbon skeletons for amide synthesis in non-nodulated soybean and pea roots in response to the source of nitrogen supply. Soil Science and Plant Nutrition 54, 732–737.

Vicedo B, Flors V, Leyva MD, Finiti I, Kravchuk Z, Real MD, Garcia-Agustin P, Gonzalez-Bosch C. 2009. Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. Molecular Plant-Microbe Interactions 22(11), 1455–1465.

Ward JL, Forcat S, Beckmann M, Bennett M, Miller SJ, Baker JM, Hawkins ND, Vermeer CP, Lu C, Lin W, Truman WM, Beale MH, Draper J, Mansfield JW, Grant M. 2010. The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. The Plant Journal 63(3), 443–457.

CONCLUSIONS

CONCLUSIONS

Based on the proposed objectives described herein, the following conclusions can be drawn:

Objective 1. Proposal of a new formulation based on the combination of heptagluconic acid with copper as an inducer of resistance against *P. syringae*.

Conclusion:

1.a. The new Cu heptagluconate formulation, applied via roots in tomato plants, is effective in preventing and controlling the bacterial speck (*Pseudomonas syringae* pv. tomato DC3000) by inducing caffeic and chlorogenic acid accumulation, and by reducing ROS accumulation in infected plants.

Objective 2. The study of the mode of action and the modifications in the apoplastic content produced by 1-MT to go in-depth into the resistance mechanisms against *P. syringae* and to check their possible direct effect on the bacteria.

Conclusion:

2.a. 1-MT treatment acts as a resistance inducer against *P. syringae*, since an increase in abscisic acid (ABA) levels was observed, inhibiting stomatal opening and avoiding bacterium entry. Moreover, 1-MT might block the oxylipin pathway by avoiding the manipulation of the SA pathway by bacteria, which could help plants to defend themselves from the pathogen.

2.b. 1-MT directly impacts the bacterium because its motility diminishes and the expression of *fliC* gene, responsible for the correct flagellum synthesis, lowers.

Objective 3. The analysis of the NH_4^+ effect on the nutritional balance and the primary metabolism in the pathosystem model.

Conclusion:

3.a. Under infection, NH_4^+ increases macronutrient (S, P) and micronutrient (Zn, Mn) concentrations in infected leaves, which can act as cofactors of antioxidant enzymes to help reduce oxidative stress.

3.b. Absorption of NH_4^+, as the sole N source, reduces the TCA cycle intermediates and increases the level of amino acids to avoid the toxicity caused by NH_4^+. Moreover, the increase of Arg, a precursor of putrescine biosynthesis that forms part of the systemic acquired acclimation process seems to be required for a better plant resistance to a subsequent infection.

Objective 4. The generation of transgenic silenced plants in the two putrescine biosynthesis pathways (ADC and ODC) in an independent manner to study their response in the induced resistance against *P. syringae*. At the same time, the cellular localisation of proteins SlADC and SlODC will be studied.

Conclusion:

4.a. *In vitro* tests with putrescine show that it has no direct antibacterial effect.

4.b. The transgenic lines *SiADC-1*, *SiADC-2*, *SiODC-1* and *SiODC-2*, show a lower expression of genes *ADC* and *ODC*, respectively, in relation to greater susceptibility to infection.

4.c. For the first time, the fluorescence analysis reveals that the SlADC protein is located in the cytosol, while the SlODC is found in both the cytosol and nucleus.

Objective 5. The study of how N sources can affect the root growth parameters and the expression of the genes involved in auxin transport and N assimilation. Moreover, we propose to study whether the addition of carbon sources can mitigate mild NH_4^+ toxicity, and if ABA could play a role in root development in response to different N sources.

Conclusion:

5.a. NH_4^+ treatment represses almost all the root development parameters (lateral root number, root density and fresh weight), except for principal root length.

5.b The presence of NO_3^- or addition of C compounds to NH_4^+ treatment alleviates the mild toxicity that this ion causes.

5.c Auxin transporters genes *LAX4* and *PIN4* might be involved in root development under NH_4^+ treatment and, specifically, *LAX4* is ABA-dependent.

5.d. The N assimilation process is affected by the N source and induced by the absence of ABA.

CONCLUSIONES

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Las conclusiones obtenidas de los objetivos propuestos en el presente trabajo han sido las siguientes:

Objetivo 1. Propuesta de una nueva formulación basada en la combinación del ácido heptaglucónico con cobre, como inductor de resistencia frente a *P. syringae*.

Conclusión:

1.a. La nueva formulación, heptagluconato de cobre, aplicada vía radicular en plantas de tomate, ha resultado ser efectiva para la prevención y control de la peca bacteriana (*Pseudomonas syringae pv. tomato* DC3000), induciendo la acumulación de ácido cafeico y clorogénico y reduciendo la acumulación de ROS en plantas infectadas.

Objetivo 2. Estudio del modo de acción del 1-MT y de los cambios producidos por éste en el contenido del apoplasto para profundizar en el conocimiento del mecanismo de resistencia frente a *P. syringae* y, testar su posible efecto directo sobre la bacteria.

Conclusión:

2.a. El tratamiento con 1-MT actúa como inductor de resistencia frente a *P. syringae*, observándose una acumulación de ácido abscísico (ABA) que inhibe la apertura estomática y evita de este modo la entrada de la bacteria. Además, el 1-MT podría estar bloqueando la ruta de las oxilipinas, dificultando la manipulación por parte de la bacteria de la ruta del SA lo que podría ayudar a la planta a defenderse del patógeno.

2.b. El tratamiento con 1-MT tiene un efecto directo sobre la bacteria reduciendo su motilidad. Además, se observa una reducción de la expresión del gen encargado de la correcta síntesis del flagelo (*fliC*).

Objetivo 3. Análisis del efecto del NH_4^+ sobre el balance nutricional y el metabolismo primario en el patosistema modelo.

Conclusión:

3.a. El NH_4^+ incrementa los niveles de macronutrientes (S, P) y micronutrientes (Zn, Mn) en hoja infectada, que pueden actuar como cofactores de enzimas antioxidantes implicadas en la reducción del estrés oxidativo.

3.b. La absorción de NH_4^+ como única fuente de N provoca una reducción en los intermediarios del ciclo TCA y un aumento del nivel de aminoácidos de Gln y Asn para evitar la toxicidad causada por el NH_4^+. Además, también se observa una acumulación de Arg, precursor de la ruta de síntesis de putrescina, como parte del proceso de aclimatación sistémica adquirida que prepara a la planta para resistir mejor a una infección posterior.

Objetivo 4. Generación de plantas silenciadas en las dos rutas de biosíntesis de la putrescina (ADC y ODC) de forma independiente para ver su respuesta en la resistencia inducida frente a *P. syringae*. Al mismo tiempo, se estudiará la localización celular de las proteínas SlADC y SlODC.

Conclusión:

4.a. Los ensayos *in vitro* con putrescina han demostrado que ésta no tiene efecto antibacteriano directo.

4.b. Las líneas silenciadas *SiADC1, SiADC2, SiODC1 y SiODC2*, mostraron una reducción en la expresión de los genes de ADC y ODC, respectivamente, relacionándose con una mayor susceptibilidad a la infección.

4.c. El análisis por fluorescencia revela por primera vez que la proteína SlADC está localizada en la célula en el citosol, mientras que la SlODC está en el citosol y en el núcleo.

Objetivo 5. Estudio de cómo la fuente de N puede afectar a los parámetros de crecimiento de la raíz y sobre la expresión de genes implicados en el transporte de auxinas y asimilación de N. Asimismo, se estudiará si la adición de fuentes de carbono puede mitigar la toxicidad del NH_4^+ y se determinará si el ABA puede jugar un papel en el desarrollo de la raíz en respuesta a distintas fuentes de N.

Conclusión:

5.a. El NH_4^+ reprime gran parte de los parámetros de desarrollo de la raíz (el número de raíces laterales, la densidad radicular y el peso fresco de la raíz), a excepción de la longitud de la raíz principal.

5.b. La presencia de NO_3^- o la adición de compuestos carbonados al tratamiento de NH_4^+ alivia la leve toxicidad causada por este ión.

5.c. Los genes de transporte de auxinas *LAX4* y *PIN4* estarían implicados en el desarrollo de la raíz bajo el tratamiento de NH_4^+, y concretamente *LAX4* es dependiente de ABA

5.d. El proceso de asimilación de N está afectado por la fuente de N e inducido por la ausencia de ABA.