University of Granada

Doctoral Programme in Fundamental and Systems Biology

Signaling in mycorrhizal symbiosis: regulation of colonization and mycorrhiza-induced resistance against pests in tomato

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Se volvió a oruga, la mariposa,
cansada de volar y no poder
arrastrarse al fondo de las cosas
a ver si dentro puede comprender

Tercer movimiento: Lo de dentro
Extremoduro

The butterfly became again a larvae,

tired of flying and not being able

to crawl to the depths of things

maybe inside it can understand

Tercer movimiento: Lo de dentro
Extremoduro

Esta Tesis Doctoral ha sido realizada en el Departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín (EEZ) del Consejo Superior de Investigaciones Científicas (CSIC) de Granada, dentro del grupo de investigación de Micorrizas.

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Ethylene signaling is essential for Mycorrhiza Induced Resistance against chewing insects in tomato

J. Lidoy, J. Rivero, Ž. Ramšak, M. Petek, V. Flors, K. Gruden, A. Martínez-Medina, M. J. Pozo

2021 Group of Mycorrhizas. Estación Experimental del Zaidín. Granada. **Spain**

La señalización de etileno es esencial para la MIR frente a insectos masticadores en tomate

J. Lidoy, J. Rivero, Ž. Ramšak, M. Petek, V. Flors, K. Gruden, A. Martínez-Medina, M. J. Pozo

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Priming of ethylene signaling is essential for mycorrhiza induced resistance against generalist and specialist chewing insects in tomato

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Unraveling the role of strigolactones and flavonoids in the AM symbiosis

J. Lidoy, C. Montalbán, C. Rial, M. J. Pozo, F. A. Macías, J. A. López-Ráez

2019 II National Meeting about Carotenoids in Microorganisms, Plants, Nutrition and Health. National Network of Carotenoids (RNC). Granada. **Spain**.

Relevance of apocarotenoids in plant-microorganism communication: an opportunity for sustainable agriculture

J. A. López-Ráez, J. Lidoy, J. M. García, M. J. Pozo

2019 COST Action FA1405 Final Meeting. Thessaloniki, **Greece.**

Towards understanding of the molecular signaling mechanisms in multiway interactions of crops with arthropods and microorganisms

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2018 CSIC-UJI-UV Coordination meeting. Granada, **Spain**.

Phytaspases: the prosystemin breakers

J. Lidoy, J.A. López-Ráez, M.J. Pozo

2018 COST Action FA1405 3rd Annual Meeting. University of Malta, Valletta Campus. **Malta.**

Defense signaling regulates mycorrhizal root colonization and its impact on Spodoptera exigua

J. Lidoy, C. M. Amate, J. Prieto, J. M. García, J. A. López Ráez, C. Azcón-Aguilar, M. J. Pozo

2017 National Meeting about Carotenoids in Microorganisms, Plants, Nutrition and Health. **Spain**.

Importancia de las estrigolactonas y otros apocarotenoides en la comunicación planta-microorganismo: una nueva estrategia para agricultura sostenible

J.A. López-Ráez, J. Lidoy, E. Berrio, M.J. Pozo

2017 National congress Microbiología de Plantas. Salamanca, **Spain.**

Abiotic stress or aboveground activation of plant defenses differentially impacts root colonization by different arbuscular mycorrhizal fungi

J. Lidoy, C. M. Amate, J. M. García, C. Azcón-Aguilar, M. J. Pozo

2016 International Congress on Invertebrate Pathology and Microbial Control Society for Invertebrate Pathology. Tours, **France.**

Priming of plant defenses against herbivores by arbuscular mycorrhizal fungi

M. J. Pozo, J. Rivero, J. Lidoy, V. Flors

2016 Induced Resistance Meeting. Workshop. University of Pécs, **Hungary.**

Hormonal regulation of AM fungal colonization and plant response

J. Lidoy, C. M. Amate, J. M. García, C. Azcón-Aguilar, M. J. Pozo

2016 FA1405 "Plant-mediated communication between above and belowground foodwebs". Workshop COST Action. Leipzig, **Germany.**

Priming of plant defenses against chewing insects by arbuscular mycorrhizal fungi

M. J. Pozo, J. Rivero, J. Lidoy, J. Pozo, A. Martínez-Medina, S. Herrero, V. Pastor, V. Flors

Poster presentations (*presenter underlined)*

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Unraveling the role of flavonoids and strigolactones in the arbuscular mycorrhizal symbiosis

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2017 National congress SEFV Spanish Society of Plant Physiology. Barcelona, Spain.

Regulation of root colonization by different arbuscular mycorrhizal fungi under different stress conditions

J. Lidoy, C. M. Amate, J. Prieto, J. M. García, C. Azcón-Aguilar, M. J. Pozo

Working groups meetings

2018 COST Action FA1405 Working Group 2 Meeting. Granada, **Spain.**

Analysis database "Mechanisms in Crop-Arthropod-Microbe (CAMo) Interactions"

2018 COST Action FA1405 Working Group 2 Meeting. University of Thessaly, Larissa, **Greece.**

Updating and analyzing a database on signaling pathways involved in CAMo

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Resumen

Summary

Resumen

Existe una creciente conciencia social sobre el impacto de los agroquímicos en el medio ambiente y en la salud, lo que ha incrementado la demanda de alternativas agronómicas más sostenibles y respetuosas con el medio ambiente. De hecho, en los últimos años ha aumentado considerablemente el uso de microorganismos beneficiosos como inoculantes que actúan como biofertilizantes y agentes de bioprotección. Entre estos microorganismos beneficiosos destacan los hongos formadores de micorrizas arbusculares (MA). Estos forman asociaciones mutualistas, llamadas micorrizas, con más del 70% de las plantas vasculares, incluyendo la mayoría de las especies de interés agronómico (Barea et al., 2005; Brundrett & Tedersoo, 2018). Durante la simbiosis el hongo mejora la captación de agua y nutrientes de la planta, transfiriendo fósforo (Pi) y otros nutrientes, y a cambio el hongo recibe carbono en forma de azúcares y lípidos procedentes de la fotosíntesis para completar su ciclo de vida. La simbiosis puede suponer un coste de hasta un 20% de los fotosintatos de la planta (Bago et al., 2000), por lo que la interacción se encuentra finamente regulada según las necesidades de ambos simbiontes. Aparte de los aspectos nutricionales, la simbiosis confiere a la planta otros beneficios como una mayor tolerancia a estreses abióticos, como salinidad y sequía, y bióticos, incrementando la resistencia a patógenos y plagas.

El establecimiento y funcionamiento de las micorrizas requiere de un alto grado de comunicación y coordinación entre el hongo MA y la planta hospedadora (López-Ráez et al., 2017; Pozo et al., 2015). Este diálogo molecular planta-hongo MA se inicia en la fase pre-simbiótica con la producción y exudación a la rizosfera de moléculas señal por parte de la planta. Estas señales son reconocidas por el hongo, estimulando la germinación de las esporas y el desarrollo de las hifas, facilitando la interacción entre ambos simbiontes (López-Ráez et al., 2017). Este proceso está altamente influenciado por las condiciones nutricionales, especialmente por la deficiencia de fósforo (Pi). Aquí, las estrigolactonas (SLs) juegan un papel fundamental, tanto en el control de las respuestas fisiológicas de la planta a la deficiencia de Pi (López-Ráez et al., 2017), como en su papel de moléculas señal en la rizosfera para el establecimiento de la simbiosis MA (Akiyama et al., 2005). Por otro lado, otra familia de metabolitos secundarios como son los flavonoides puede actuar también como moléculas señal

en la comunicación planta-microorganismo beneficioso (Hassan & Mathesius, 2012). Desde el punto de vista del hongo MA, estos también producen sus propias moléculas señal, conocidas como factores MYC, que desencadenan una reestructuración de la fisiología y del metabolismo de la raíz para favorecer la colonización. Sin embargo, la regulación de la biosíntesis de estas y otras posibles moléculas señal involucradas en el establecimiento de las simbiosis MA no está clara.

Más allá de la señalización pre-simbiótica, la extensión de la colonización de la raíz también está sujeta a control, donde el balance hormonal juega un papel clave. Puntos de control clave incluyen la atenuación de las respuestas defensivas de la planta tras el reconocimiento del simbionte, la regulación del flujo de fotosintatos hacia el hongo y la autorregulación de la simbiosis para evitar una excesiva colonización (Wang et al., 2018). De hecho, las condiciones ambientales y señales externas pueden afectar a la colonización. Los cambios en el balance hormonal y en las respuestas a hormonas por la simbiosis parecen estar implicados en la mejora de la tolerancia de la planta frente a diversos estreses. De hecho, las plantas micorrizadas muestran una mayor capacidad defensiva frente a distintos agresores relacionada con una capacidad potenciada de activar los mecanismos de defensa regulados por la ruta de los jasmonatos (JA).

Esta tesis se centra en el estudio de las rutas de señalización que regulan la micorrización, abordando desde la comunicación pre-simbiótica planta-hongo MA durante el establecimiento de la simbiosis, la extensión de la colonización, y el impacto de la simbiosis en los mecanismos de defensa de la planta, que llevan a un aumento de su resistencia frente a insectos herbívoros. Para lograr este objetivo general, **la Tesis Doctoral se ha dividido en 3 objetivos**. **El objetivo I** aborda el estudio de señales implicadas en la regulación a nivel molecular de la comunicación pre-simbiótica planta-hongo MA. En **el objetivo II**, se abordan los mecanismos de la regulación de la simbiosis bajo diferentes condiciones ambientales. Y finalmente, en **el objetivo III**, se estudia el efecto de la simbiosis en la regulación hormonal que da lugar a la resistencia inducida por micorrizas frente a insectos herbívoros.

Debido al importante papel de la simbiosis micorrícica en la mejora de la adquisición de nutrientes por la planta, fundamentalmente el Pi, es lógico que su establecimiento se encuentre estimulado en condiciones de deficiencia nutricional (Cosme & Wurst, 2013; Fusconi, 2014). En estas condiciones las plantas exudan a la rizosfera moléculas señal, como las SLs, que favorecen la interacción entre el hongo MA y las raíces de la planta hospedadora, favoreciendo la formación de la simbiosis (Akiyama et al., 2005; López-Ráez et al., 2017). Las SLs, además de

moléculas señal en la rizosfera, son fitohormonas que participan en la regulación de los cambios metabólicos y fisiológicos asociados a la deficiencia de Pi en plantas (Gamir et al., 2020; Waters et al., 2017). En **el Capítulo 1 del objetivo I** (Marro, Lidoy et al., 2022), se estudió la regulación de la biosíntesis y exudación de las SLs en diferentes condiciones nutricionales. Además del Pi, otro macronutriente esencial para el crecimiento y desarrollo de las plantas es el nitrógeno (N). Mediante el estudio de plantas de tomate crecidas bajo diferentes regímenes de Pi y nitrógeno (N), y tras analizar su impacto en el fenotipo de la planta y sus perfiles transcriptómico y metabólico, comprobamos que las plantas priorizan las respuestas a la limitación de N sobre la de Pi, y que, en esta regulación, las SLs juegan un papel clave. Para confirmar estos resultados, se analizaron las respuestas fisiológicas, transcripcionales y metabólicas en plantas deficientes en SLs cultivadas bajo diferentes regímenes de N y Pi, y en plantas tratadas con un pulso corto de 2'-*epi*-GR24, un análogo sintético de SLs. Estos experimentos mostraron que las SLs modulan la expresión de genes clave de las vías de señalización que regulan las respuestas a deficiencias tanto de Pi como de N, incluyendo los genes integradores de las respuestas N-Pi *PHO2* y *NIGT1/HHO*. Los resultados de este capítulo demuestran que las SLs juegan un papel clave como sensores durante las respuestas tempranas de las plantas a las deficiencias de N y de Pi, sirviendo de conectoras en la señalización cruzada N-Pi. Este trabajo nos ha permitido demostrar una nueva función de las SLs como fitohormonas.

Dado el papel promotor de la simbiosis MA de las SLs, su aplicación, junto a inoculantes micorrícicos, se ha propuesto para fomentar la simbiosis en sistemas agrícolas. Sin embargo, sintetizar o extraer SLs es un proceso costoso. Por ello, se realizó una búsqueda bibliográfica para encontrar otros posibles compuestos que pudieran estimular la simbiosis MA junto con las SLs. Observamos que en condiciones de deficiencia de Pi, la planta aumenta la producción y exudación de ciertos flavonoides a la rizosfera (Lidoy et al., en preparación). Estas moléculas son señales clave en el establecimiento de la simbiosis *Rhizobium*-leguminosa, que muestra numerosos paralelismos con las simbiosis MA, lo que sugirió un posible efecto de estos compuestos en el establecimiento de la simbiosis MA. De hecho, se ha propuesto que ciertos flavonoides tienen la capacidad de promover la simbiosis (Scervino et al., 2007), aunque los resultados parecen específicos del genotipo del hongo MA, del compuesto y de la dosis usada (Vierheilig et al., 1998). En **el Capítulo 2**, se estudió el papel específico de los flavonoides como compuestos de señalización en la simbiosis MA. Se analizó la capacidad de diferentes dosis de flavonoides pertenecientes a distintas subcategorías, tanto *in vitro* como *in planta*, de inducir la germinación de esporas y de estimular la colonización del hongo MA *Rhizophagus irregularis*.

Los resultados mostraron que los flavonoles tienen una mayor capacidad de estimulación de la simbiosis y revelan la importancia de la dosis utilizada.

Además de regular la "llamada" a los hongos MA, la planta regula el desarrollo de la simbiosis y la extensión de la colonización de la raíz dependiendo de las condiciones ambientales. En **el objetivo II**, se estudiaron los posibles mecanismos de regulación de los niveles de colonización bajo diferentes condiciones de estrés. En **el Capítulo 3** (Lidoy et. al, en preparación), se exploró cómo la activación en la planta de rutas de señalización de las respuestas de defensa frente a estrés afectan a la interacción con hongos MA. En este estudio, se evaluó la colonización de dos hongos MA diferentes, *Funneliformis mosseae* y *R. irregularis*. Las plantas fueron sometidas a condiciones de estrés salino o simulando situaciones de estrés biótico activando de rutas de defensa mediante la pulverización semanal de los tejidos aéreos con metil jasmonato, ácido abscísico y ácido salicílico. Se encontraron diferencias significativas en el nivel de colonización entre los dos hongos en función de los tratamientos aplicados y se exploraron los posibles mecanismos responsables de esas diferencias. Se hipotetizó que los efectos de los tratamientos en la colonización y las diferencias observadas entre *F. mosseae* y *R. irregularis* podrían deberse a: i) la señalización pre-simbiótica en la rizosfera, ii) el estado defensivo de la planta, iii) el aporte de fotosintatos (lípidos y carbohidratos) por parte de la planta, y iv) el control y autorregulación de la simbiosis. Mediante modelos lineales de los niveles de expresión de genes marcadores de las distintas vías se encontró que los cambios en niveles de colonización se correlacionaron con el intercambio de nutrientes entre partners, la regulación de la respuesta defensiva de la planta y con cambios en los mecanismos de control de la simbiosis y autoregulación. Centrándonos en el estrés por sal, la planta promovió el desarrollo del *F. mosseae*, que le aportó más beneficios nutricionales, en comparación con *R. irregularis*. Estos resultados apoyan la hipótesis de que la planta favorece activamente, mediante el aporte de carbono y la modulación de los mecanismos de control, el desarrollo del hongo AM que más beneficios le aporta.

La reorganización del metabolismo en la planta hospedadora por el establecimiento de la simbiosis puede desencadenar cambios en la respuesta de defensa frente a otros organismos, con frecuencia aumentando la resistencia de la planta frente a posibles diversos patógenos e insectos herbívoros. En **el objetivo III** abordamos los mecanismos moleculares involucrados en esta resistencia inducida por micorrizas frente a insectos herbívoros. Primero, analizamos la información existente sobre el impacto de los microorganismos asociados a plantas sobre interacciones con insectos. Existen pocos estudios a nivel molecular sobre los mecanismos involucrados en las interacciones a tres vías entre plantas, hongos MA e insectos. En **el Capítulo**

4 (Gruden, Lidoy et al., 2020), se analizó la literatura científica disponible al respecto. Se llevó a cabo un metaanálisis de los datos publicados y se analizó la existencia de posibles patrones comunes en las respuestas de las plantas teniendo en cuenta los grupos taxonómicos de los distintos organismos y de sus modos de vida. Concluimos que los mecanismos que se activan en las interacciones a dos vías, es decir, entre planta-microbio y planta-insecto, se activan también en las interacciones a tres vías entre microbio-planta-insecto. Además, la respuesta de la planta en las interacciones a tres vías se vuelve más compleja al cambiar la intensidad y el tiempo de respuesta y/o activarse respuestas adicionales. A pesar de la gran complejidad del sistema, nuestro análisis apuntó a que la interacción hormonal es el principal núcleo regulador en las interacciones tanto a dos como a tres vías. En concreto, la ruta de señalización de los jasmonatos juega un papel central en la integración de las respuestas, y que es de especial relevancia en las interacciones de la planta con microorganismos beneficiosos.

El Capítulo 4 muestra que la modulación de las respuestas en la planta por la interacción con microorganismos beneficiosos, incluidos los hongos MA puede afectar a su resistencia frente a plagas. De hecho, la micorrización puede llevar al precondicionamiento o "priming" del sistema inmunitario de las plantas, preparándolas para defenderse de manera más eficiente contra agresores, resultando en lo que se denomina Resistencia Inducida por Micorrizas (MIR) (Pozo et al., 2015). En **el Capítulo 5**, se estudiaron los mecanismos moleculares que gobiernan las interacciones a tres vías entre hongos MA, planta hospedadora e insectos herbívoros. Se observó que la simbiosis MA mejora la resistencia de las plantas de tomate contra dos herbívoros masticadores: el generalista *Spodoptera exigua* y el especialista *Manduca sexta*. El análisis del perfil transcriptómico por RNA-seq de la respuesta a herbivoría en plantas micorrizadas por *F. mosseae* y no micorrizadas reveló que la simbiosis tiene un impacto muy moderado en el transcriptoma de la hoja en ausencia de herbivoría. Sin embargo, en respuesta a herbivoría, las plantas micorrizadas mostraron cambios más pronunciados que las no micorrizadas, incluyendo una mayor activación de algunas de las respuestas de defensa reguladas por JA. Además, se encontró una regulación diferencial de la ruta del ET en las plantas micorrizadas, tanto en condiciones basales como en las sometidas a herbivoría. Mediante el uso de un modelo de redes alimentado con interacciones descritas en la bibliografía, se postuló que el ET podría modular la activación de las respuestas dependientes de JA. Para investigar el papel del ET en la regulación diferencial de las defensas asociadas a la MIR, se siguió una aproximación genética mediante el uso de líneas de tomate deficientes en la síntesis y percepción de ET. Estas líneas, a diferencia del genotipo silvestre, fueron incapaces de desarrollar MIR frente a ninguno de los dos herbívoros analizados (*S. exigua* y *M. sexta*). Posteriores análisis de expresión génica,

actividades enzimáticas y metabolómica dirigida nos permitieron concluir que la señalización de ET era necesaria para el *priming* de la biosíntesis de JA observado en plantas micorrizadas y para su mayor resistencia a herbivoría. Por tanto, en este capítulo confirmamos que la señalización de la ET es un elemento esencial en la compleja regulación hormonal subyacente a la MIR.

En resumen, el estudio llevado a cabo en la presente Tesis Doctoral muestra de forma holística la complejidad molecular de los procesos regulados en la simbiosis MA. Esta regulación tiene lugar desde la promoción del reclutamiento de hongos MA en condiciones de deficiencia nutricional, hasta el posterior control del establecimiento y mantenimiento de la simbiosis según las condiciones de crecimiento de la planta. Esta regulación está asociada a cambios hormonales que modulan las respuestas defensivas de la planta, haciéndola más resistente frente al ataque de insectos herbívoros.

Summary

There is a growing social awareness of the impact of agrochemicals on the environment and health, which has increased the demand for more sustainable and environmentally friendly agronomic alternatives. In fact, in recent years the use of beneficial microorganisms as inoculants that act as biofertilizers and bioprotection agents has increased considerably. Among these beneficial microorganisms, arbuscular mycorrhizal (AM) fungi are particularly important. These form mutualistic associations, called mycorrhizas, with more than 70% of vascular plants, including most species of agronomic interest (Barea et al., 2005; Brundrett & Tedersoo, 2018). During symbiosis the fungus enhances the plant's uptake of water and nutrients, transferring phosphorus (Pi) and other nutrients, and in return the fungus receives carbon in the form of sugars and lipids from photosynthesis to complete its life cycle. The symbiosis can cost up to 20% of the plant's photosynthates (Bago et al., 2000), so the interaction is finely regulated according to the needs of both symbionts. Besides nutritional aspects, the symbiosis confers other benefits to the plant as a greater tolerance to abiotic stresses, such as salinity and drought, and biotic stresses, increasing resistance to pathogens and pests.

The establishment and functioning of the mycorrhizal symbiosis requires a high degree of communication and coordination between the AM fungus and the host plant (López-Ráez et al., 2017; Pozo et al., 2015). This plant-AM fungus molecular dialogue starts in the pre-symbiotic phase with the production and exudation into the rhizosphere of signal molecules by the plant. These signals are recognized by the fungus, stimulating spore germination and hyphal development, facilitating the interaction between both symbionts (López-Ráez et al., 2017). This process is highly influenced by nutritional conditions, especially phosphorus (Pi) deficiency. Here, strigolactones (SLs) play a central role, both in the control of plant physiological responses to Pi deficiency (López-Ráez et al., 2017), and in their role as signal molecules in the rhizosphere for the establishment of the AM symbiosis (Akiyama et al., 2005). On the other hand, another family of secondary metabolites such as flavonoids can also act as signal molecules in beneficial plant-microorganism communication (Hassan & Mathesius, 2012). From the AM fungus point of view, the AM fungi also produce their own signal molecules, known as MYC factors, which trigger a restructuring of root physiology and metabolism to favor colonization. However, the

regulation of the biosynthesis of these and other possible signal molecules involved in the establishment of AM symbioses is unclear.

Beyond pre-symbiotic signaling, the extent of root colonization is also subject to control, in which hormonal balance plays a key role. Key control points include attenuation of plant defensive responses following symbiont recognition, regulation of photosynthate flow to the fungus, and autoregulation of the symbiosis to avoid excessive colonization (Wang et al., 2018). In fact, environmental conditions and external stimuli can affect colonization. Changes in hormone balance and hormone responses by the symbiosis seem to be involved in enhancing plant tolerance to several stresses. In fact, mycorrhizal plants show an increased defensive performance against different aggressors related to an enhanced ability to activate defense mechanisms regulated by the jasmonate pathway (JA).

This PhD thesis focuses on the study of the signaling pathways that regulate mycorrhization, addressing pre-symbiotic plant-AM fungus communication during the establishment of the symbiosis, the extent of colonization, and the impact of the symbiosis on plant defense mechanisms, leading to an increase in plant resistance to insect herbivores. To achieve this general objective, **the PhD Thesis has been divided into 3 objectives. The objective I** approaches the study of signals involved in the regulation at the molecular level of pre-symbiotic plant-AM fungus communication. **The objective II** addresses the mechanisms of symbiosis regulation under different environmental conditions. And finally, **the objective III**, studies the effect of symbiosis on the hormonal regulation that leads to mycorrhizal-induced resistance to insect herbivores.

Due to the important role of mycorrhizal symbiosis in enhancing plant nutrient acquisition, mainly Pi, it is logical that its establishment is stimulated under conditions of nutritional deficiency (Cosme & Wurst, 2013; Fusconi, 2014). Under these conditions, plants exude signal molecules, such as SLs, to the rhizosphere, which favor the interaction between the AM fungus and the host plant roots, favoring the formation of the AM symbiosis (Akiyama et al., 2005; López-Ráez et al., 2017). SLs, in addition to signal molecules in the rhizosphere, are phytohormones involved in the regulation of metabolic and physiological changes associated with Pi deficiency in plants (Gamir et al., 2020; Waters et al., 2017). **In Chapter 1 included in Objective I** (Marro, Lidoy et al., 2022), the regulation of biosynthesis and exudation of SLs under different nutritional conditions was studied. In addition to Pi, another essential macronutrient for plant growth and development is nitrogen (N). By studying tomato plants grown under different Pi and nitrogen (N) regimes, and after analyzing their impact on plant phenotype and

their transcriptomic and metabolic profiles, we found that plants prioritize responses to N limitation over Pi limitation, and that, in this regulation, SLs play a key role. To confirm these results, physiological, transcriptional and metabolic responses were analyzed in SLs-deficient plants grown under different N and Pi regimes, and in plants treated with a short pulse of 2'-epi-GR24, a synthetic analog of SLs. These experiments showed that SLs modulate the expression of key genes of signaling pathways that regulate responses to both Pi and N deficiencies, including the N-Pi response integrator genes PHO2 and NIGT1/HHO. The results of this chapter demonstrate that SLs play a key role as sensors during early plant responses to N and Pi deficiencies, serving as connectors in N-Pi cross-signaling. This work has allowed us to demonstrate a new function of SLs as phytohormones.

Due to the AM symbiosis-promoting role of SLs, their application, together with mycorrhizal inoculants, has been proposed to promote AM symbiosis in agricultural systems. However, synthesizing or extracting SLs is a costly process. Therefore, a literature search was conducted to find other possible compounds that could stimulate AM symbiosis together with SLs. We observed that under Pi deficiency conditions, the plant increases the production and exudation of certain flavonoids to the rhizosphere (Lidoy et al., in preparation). These molecules are key signals in the establishment of Rhizobium-legume symbiosis, which shows numerous parallels with AM symbioses, which suggested a possible effect of these compounds on AM symbiosis establishment. Indeed, it has been proposed that certain flavonoids have the ability to promote AM symbiosis (Scervino et al., 2007), although the results appear specific to the AM fungus genotype, the compound, and the dose used (Vierheilig et al., 1998). **In Chapter 2**, the specific role of flavonoids as signaling compounds in AM symbiosis was studied. The ability of different doses of flavonoids belonging to different subcategories, both in vitro and in planta, to induce spore germination and to stimulate colonization of the AM fungus *Rhizophagus irregularis* was analyzed. The results showed that flavonols have a greater capacity to stimulate symbiosis and reveal the importance of the dose used.

In addition to regulating the "call" to AM fungi, the plant regulates the development of the AM symbiosis and the extent of root colonization depending on environmental conditions. **In Objective II**, the possible mechanisms of regulation of colonization levels under different stress conditions were studied. **In Chapter 3** (Lidoy et. al, in preparation), we explored how the plant activation of signaling pathways responses upon stress affect the interaction with different AM fungi. In this study, colonization of two different AM fungi, *Funneliformis mosseae* and *R. irregularis*, was evaluated. Plants were subjected to salt stress conditions or simulating biotic stress by activating defense pathways through weekly spraying of shoot tissues with methyl

jasmonate, abscisic acid and salicylic acid. Significant differences in the colonization levels between the two fungi were found as a function of the treatments applied and the possible mechanisms responsible for these differences were explored. It was hypothesized that the effects of the treatments on colonization and the differences observed between *F. mosseae* and *R. irregularis* could be due to: i) pre-symbiotic signaling in the rhizosphere, ii) the defensive status of the plant, iii) photosynthate supply by the plant, and iv) control and autoregulation of the symbiosis. Using linear models of the expression levels of marker genes of the different pathways, changes in colonization levels correlated with the exchange of nutrients between partners, the regulation of the plant's defensive response and with changes in the specific mechanisms of control and autoregulation of the symbiosis. Focusing on salt stress, the plant promoted the development of *F. mosseae*, which provided more nutritional benefits, compared to *R. irregularis, whose colonization was restricted.* These results support the hypothesis that the plant actively favors, through carbon supply and control mechanisms the development of the AM fungus that provides more benefits.

The reorganization of metabolism in the host plant by the establishment of the symbiosis can trigger changes in the defense response against other organisms, often increasing the resistance of the plant against a variety of potential pathogens and insect herbivores. In **Objective III**, we address the molecular mechanisms involved in this mycorrhizal-induced resistance to insect herbivory. First, we analyze the existing information on the impact of plant-associated microorganisms on insect interactions. There are few studies at the molecular level on the mechanisms involved in three-way interactions between plants, AM fungi and insects. **In Chapter 4** (Gruden, Lidoy et al., 2020), the available scientific literature was reviewed. A meta-analysis of the published data was carried out and the existence of possible common patterns in plant responses was analyzed taking into account the taxonomic groups of the different organisms and their life modes. We conclude that the mechanisms that are activated in two-way interactions, plant-microbe and plant-insect, are also activated in three-way microbe-plant-insect interactions. In addition, the plant response in three-way interactions becomes more complex as the intensity and timing of the response changes and/or additional responses are activated. Despite the great complexity of the system, our analysis pointed to the hormone interaction as the main regulatory core in both two- and three-way interactions. In particular, the jasmonate signaling pathway plays a central role in the integration of responses, and that it is of particular relevance in plant interactions with beneficial microorganisms.

Chapter 4 shows that modulation of plant responses by interaction with beneficial microorganisms, including AM fungi, can affect plant resistance to pests. In fact, mycorrhizal

symbiosis can lead to preconditioning or "priming" of the immune system of plants, preparing them to defend themselves more efficiently against aggressors, resulting in what is called Mycorrhiza-Induced Resistance (MIR) (Pozo et al., 2015). **In Chapter 5**, the molecular mechanisms underlying the three-way interactions between AM fungi, host plant and insect herbivores were studied. AM symbiosis was observed to enhance tomato plant resistance against two chewing herbivores: the generalist *Spodoptera exigua* and the specialist *Manduca sexta*. RNA-seq transcriptomic profiling analysis of the response to herbivory in *F. mosseae* mycorrhizal and non-mycorrhizal plants revealed that the symbiosis has a very moderate impact on the leaf transcriptome in the absence of herbivory. However, in response to herbivory, mycorrhizal plants showed more pronounced changes than non-mycorrhizal plants, including increased activation of some of the JA-regulated defense responses. In addition, differential regulation of the ET pathway was found in mycorrhizal plants under both basal conditions and those subjected to herbivory. Using a network model fed with interactions described in the literature, it was postulated that ET could modulate the activation of JA-dependent responses. To elucidate the role of ET in the differential regulation of MIR-associated defenses, a genetic approach was followed using tomato lines deficient in ET synthesis and perception. These lines, unlike the wild-type genotype, were unable to develop MIR against either of the two herbivores tested (*S. exigua* and *M. sexta*). Subsequent analyses of gene expression, enzyme activities and targeted metabolomics allowed us to conclude that ET signaling was necessary for the priming of JA biosynthesis observed in mycorrhizal plants and for their increased resistance to herbivory. Thus, in this chapter we confirm that ET signaling is an essential element in the complex hormonal regulation underlying MIR.

In summary, the study carried out in this PhD Thesis shows in a holistic way the molecular complexity of the processes regulated in the AM symbiosis. This regulation takes place from the promotion of AM fungus recruitment under nutritional deficiency conditions, to the subsequent control of the establishment and maintenance of the AM symbiosis according to plant growth conditions. This regulation is associated with hormonal changes that modulate the plant's defensive responses, making it more resistant to attack by insect herbivores.

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Introduction

Introduction

Current intensive agriculture and bioinoculants

Current agriculture faces major challenges that could threaten its performance and sustainability (FAO, 2018). The world population continues to grow and it is estimated that it could increase to 8.5 billion in 2030, 9.7 billion in 2050 and 10.9 billion in 2100 (United Nations, 2019). With these prospects, the required nutritional requirements should be ensured for the growing population. It is estimated that world food production should increase by 70% by 2050 (FAO, 2018). In addition, the ecosystem is undergoing an abrupt change in conditions due to the climate crisis caused by anthropogenic activity, which may subject global food production to new environmental challenges (Bebber, 2015; Deutsch et al., 2018).

Intensive agriculture is sustained mainly by the abusive use of chemical fertilizers and pesticides. This abuse is related to the strong impact that the loss of productivity can have on the farmer's economy, the lack of effective alternatives and the lack of effective control of the use of agrochemicals. These agrochemicals are usually very stable in the environment leading to serious consequences in the ecosystem, such as eutrophication and contamination of aquifers (Evans et al., 2019), emission of greenhouse gases (Lynch et al., 2021) and contamination of soils with heavy metals (Atafar et al., 2008). Pathogens and pests cause important damage to their hosts, and up to 25% of global crop losses have been estimated in major crops (Savary et al., 2019; Savary & Willocquet, 2020). The abuse of pesticides can negatively affect the ecosystem as well as the farmers and consumers (Tilman et al., 2002). Among other reasons, this abusive use of agrochemicals has triggered social awareness of the need for a paradigm shift to find more sustainable and safer alternatives for the global ecosystem (Geiger et al., 2010).

Nowadays the paradigm is shifting, EU legislation on chemicals and pesticides is oriented to maximize the protection of human health and the environment and to avoid barriers for commercial trade. The EU supports new environmentally friendly agricultural techniques (EU Regulation 2018/848) and seeks to reduce eutrophication of aquifers (Council Directive 91/676/EEC, 1991). In this sense, the ‘European Green Deal’ aims at reducing at half the use of agrochemicals in the EU by 2030. Bioinoculants based on beneficial microorganisms can provide

multiple benefits to crops, offering a sustainable solution to traditional agriculture (Fig 1; Berg, 2009; Mącik et al., 2020). Beneficial microorganisms can enhance plant growth and development by providing water and essential mineral nutrients, as well as stimulating plant metabolism directly. On the other hand, some microbial inoculants have been shown to improve plant resilience to abiotic stresses such as salinity and drought (Berg, 2009); and to induce resistance to pests and pathogens, commonly through priming of plant defenses (Pieterse et al., 2014).

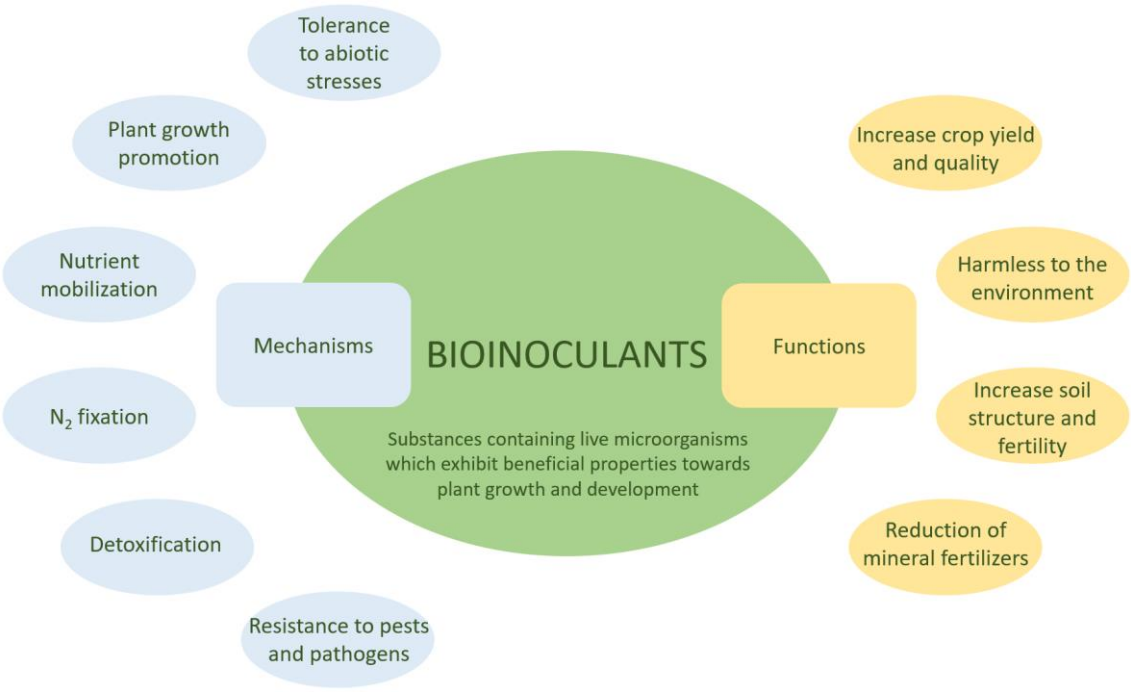

Figure 1 Overview of the benefits of bioinoculants. Adapted from Mącik et al. (2020).

Nitrogen (N) and phosphorus (Pi) are two of the most important macronutrients in agriculture. They are mainly supplied by synthetic fertilizers, which have a great impact on the ecosystem due to their production as well as on the residues they generate. Remarkably, two of the most widespread plant-microorganism symbioses can make a significant contribution of these nutrients acting as biofertilizers (Venturi & Keel, 2016). On the one hand, the *Rhizobium*-legume symbiosis can fix atmospheric N_2 supplying N to the host plant (Oldroyd et al., 2011). On the other hand, the arbuscular mycorrhizal (AM) symbiosis between AM fungi and the roots of most plants can greatly increase the nutrient uptake surface in the soil, as well as protection against several biotic and abiotic stresses (Jung et al., 2012; Latef et al., 2016). Because of all these beneficial properties, arbuscular mycorrhizae have enormous potential for their use as biostimulants in a more sustainable and balanced agriculture. Indeed, due to their benefits for

plants and agro- and ecosystems, AM fungi are already commercialized as biofertilizers and as bioprotection agents (M. Chen et al., 2018; Szczałba et al., 2019) by a growing number of companies in the agronomic and reforestation sector, for example: MycoStar and MycoSoil (Agrogenia Biotech, Spain), Mycogel (Kimitec, Spain) and Mycogrowth (Symborg, Spain).

Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhiza is the symbiosis between plant roots and AM fungi (Smith & Read, 2008). This interaction is the most ancient and widespread terrestrial symbiosis. It dates back over 450 million years and is believed to be responsible for the terrestrial colonization of plants (Field et al., 2015; Martin et al., 2017). Approximately, more than 70% of vascular plants can form this symbiosis (Brundrett & Tedersoo, 2018). AM fungi belong to the phylum Glomeromycota (Schüβler et al., 2001) with about 250 described species classified into 3 classes, 4 orders, 12 families and 33 genera belonging to this phylum (Bonfante & Desirò, 2015; Wijayawardene et al., 2018). AM fungi are obligate symbionts that require the formation of symbiosis with the roots of a host plant to complete their life cycle. The term "arbuscular" comes from the characteristic tree-like structures formed by these fungi in the root cortex cells (Fig 2). The highly branched hyphae of arbuscules maximize the surface area of contact with plant cells. They do not penetrate the cortex cell, but they are surrounded by an invagination of the plant cell membrane called the peri-arbuscular membrane (Parniske, 2008). It is in the arbuscules where the exchange of nutrients and metabolites takes place between the fungus and the plant. The fungus provides water and mineral nutrients while the plant provides the fungus with photosynthates (Bonfante & Genre, 2010).

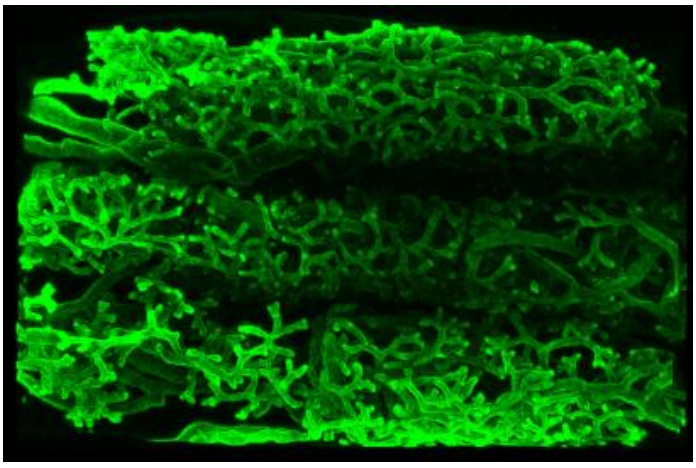

Figure 2 Three different AM arbuscules in the cortex cells. Arbuscules were stained with WGA-Alexa Fluor 488 and observed by confocal laser scanning microscopy. Image kindly provided by Ramona Schubert.

This symbiosis offers great benefits to the plant, but to a cost. It has been described that the plant can transfer up to 20% of photosynthates in the form of carbohydrates and lipids to the fungus (Bago et al., 2000; Keymer et al., 2017). AM fungi use these photosynthates to complete their life cycle and most store them in the form of lipids in balloon-shape intraradical structures called vesicles. Outside of the roots, AM fungi create an extensive network of extraradical hyphae that allows them to exploit soil mineral resources beyond the root depletion area. One of its main benefits is the contribution of phosphorus uptake. It is reported that symbiosis is promoted under conditions of Pi deficiency and inhibited under conditions of high fertilization (Carbonnel & Gutjahr, 2014). Several studies have shown that the mycorrhizal Pi contribution may suppose most part of the plant Pi uptake (Li et al., 2006; Smith & Smith, 2011). Pi is taken up by the extraradical mycelium and transformed into polyphosphates. Subsequently, it is transported to the arbuscules where it is hydrolyzed and translocated to the periarbuscular membrane (Ezawa & Saito, 2018; Hijikata et al., 2010). From there, it is taken up by arbuscule cell-specific Pi transporters, such as the PT4 (Balestrini et al., 2007). Apart from Pi, AM symbiosis can also provide the plant with N (Hodge, 2017). The extraradical mycelium can uptake N from inorganic ions (NO_3^- and NH_4^+) and organic compounds (Hodge & Storer, 2015). This N uptake is transformed into arginine and transported to the arbuscule. In the arbuscule, arginine is metabolized to NH_4^+ ions and translocated to the plant (Govindarajulu et al., 2005).

In addition to the nutritional nature, AM symbiosis can also affect plant growth and development, as well as resistance to stresses. The reorganization of the metabolism in the host plant by the establishment of the symbiosis can trigger changes in the resilience of the plant against abiotic stresses (Quiroga et al., 2017; Rivero et al., 2018; Sánchez-Romera et al., 2016), and can prime plant defenses against other organisms, often increasing plant resistance against a variety of potential pathogens and insect herbivores.

Signaling in the AM symbiosis development

The establishment of the AM symbiosis consists in two differentiated stages, the pre-symbiotic and the symbiotic stage. The pre-symbiotic stage includes the processes since the AM spore germinates until its penetration into the root of the host plant. The symbiotic stage includes the accommodation of the fungus within the root, the subsequent development of the arbuscules and the growth of the fungus both intra- and extra-radically.

Pre-symbiotic stage

AM spores germinate to form haploid coenocytic hyphae (Hijri & Sanders, 2005). When environmental conditions are suitable, these spores activate and germinate to establish symbiosis. If the fungus does not find a host plant, it is able to retract the cytoplasm and return to a dormant phase and germinate again later on (Bonfante & Genre, 2010). Although AM spores can germinate without the presence of a host root, the roots are able to exudate certain compounds that stimulate the metabolism of the fungus under favorable conditions (Besserer et al., 2006). Exuded metabolites can stimulate fungal metabolism inducing spore germination and hyphal growth and branching favoring root contact (Pinior et al., 1999). Under Pi deficiency, the conditions where AM symbiosis is promoted, plants exude strigolactones (SLs) into the rhizosphere, which favor the interaction between the AM fungus and the host plant roots (Akiyama et al., 2005; López-Ráez et al., 2017). In addition to SLs under Pi deficiency, the plant also increases the production and exudation of certain flavonoids into the rhizosphere (Lidoy et al., in preparation). Some flavonoids (isoflavones) are key signals in the establishment of *Rhizobium-legume* symbiosis, which shows numerous similarities with AM symbiosis, suggesting a possible effect of these compounds also in AM symbiosis establishment. Indeed, it has been shown that certain flavonoids can promote AM symbiosis (reviewed by Singla & Garg, 2017). Therefore, the plant would be able to modulate symbiosis recruitment by regulating signaling in the rhizosphere (Hassan & Mathesius, 2012; López-Ráez et al., 2017). These signal molecules initiate a molecular dialogue between the AM fungus and the host plant. By analogy to the *Rhizobium* Nod factors, in the presence of SLs, the fungus exudes Myc factors. Two types of molecules have been identified as Myc factors: short-chain chitin oligosaccharides (COs) and lipochitooligosaccharides (LCOs) with a variety of side-chain decorations (Genre et al., 2013; Maillet et al., 2011). These Myc factors are recognized by the plant through lysin-motif receptor-like kinases (LysM-RLKs) (Fliegmann et al., 2013; Kaku et al., 2006) that trigger a signaling cascade that reprograms the root to prepare it to accommodate the AM fungus (Camps et al., 2015; Genre et al., 2013). Once the hyphae contact the root, it differentiates into a swollen and highly branched hypha structure called hyphopodium that attaches to the root epidermis (Bonfante & Genre, 2010). Then, the symbiotic phase takes place.

Symbiotic stage

The plant guides the colonization of the AM fungus (Fig 3). Once the hyphopodium is formed, the epidermal cell forms what is called the pre-penetration apparatus (PPA). This PPA is a tunnel-shaped cell substructure crossing the vacuole that will guide the initial intraradical growth of the fungus (Genre et al., 2005). The establishment and development of the symbiosis

requires a high degree of coordination between the two partners (MacLean et al., 2017). During root colonization, a transcriptional reprogramming is activated in cells of the epidermis and the root cortex focused on transcriptional regulation, cell wall modification and modulation of the defensive response to accommodate the intraradical development of fungal structures (Gaude et al., 2012; Liu et al., 2003; Siciliano et al., 2007). From the hyphopodium, the hyphae grow intercellularly towards the inner cortex where they develop the arbuscules within the cells. The plant regulates the formation of the arbuscules through transcriptional reprogramming and hormonal signaling by activating GRAS transcription factors and through the involvement of gibberellins (MacLean et al., 2017; Pimprikar & Gutjahr, 2018).

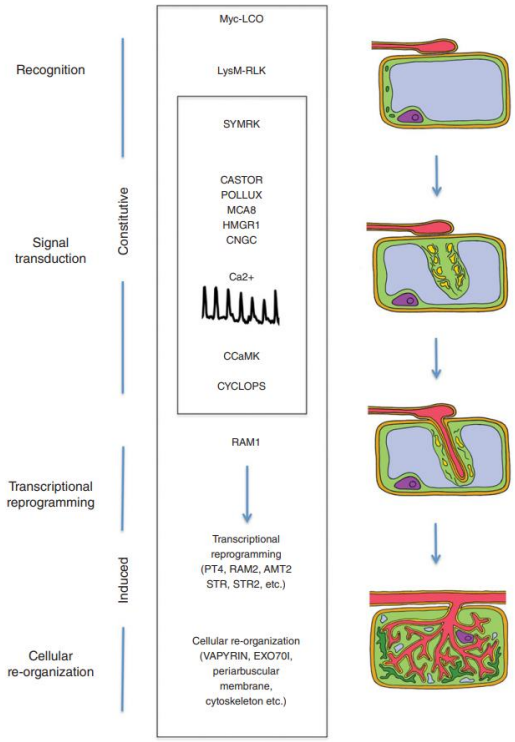

Figure 3 Molecular regulation of AM symbiosis. Regulatory mechanisms (left), molecular regulators (middle), and cellular stages (right) in AM development (Dursun et al., 2020).

AM Symbiosis regulation

The plant regulates the colonization at different levels in order to control the cost-benefit balance of the interaction, maintaining the mutualistic nature of the symbiosis (Vierheilig, 2004; C. Wang et al., 2018). This regulation occurs in all processes during symbiosis establishment. First, by regulation of rhizospheric signaling under nutritional stress conditions. Subsequently, mutual recognition leads to an attenuation of the plant's defensive response to

allow root colonization. Finally, in the well-established symbiosis the plant control fungal growth through the regulation of carbon flow and the activation of mycorrhizal autoregulation.

Environmental factors are important regulators of the AM symbiosis, being affected by innumerable factors, both biotic and abiotic (Hoeksema et al., 2010). Depending on the environmental context and nutrient availability, the plant regulates the symbiosis (Pozo et al., 2015). Under abiotic stress conditions, mainly nutrient deficiency, the plant can modulate the production of signal molecules. For example, under conditions of high Pi fertilization, where the plant no longer has a need for symbiosis, the plant represses the development of AM colonization (Breuillin et al., 2010; Menge et al., 1978). Under these conditions the plant reduces the pre-symbiotic dialogue through the reduction of the exudation of signal molecules to the rhizosphere such as SLs and flavonoids (Lidoy et al., in preparation; Yoneyama et al., 2012).

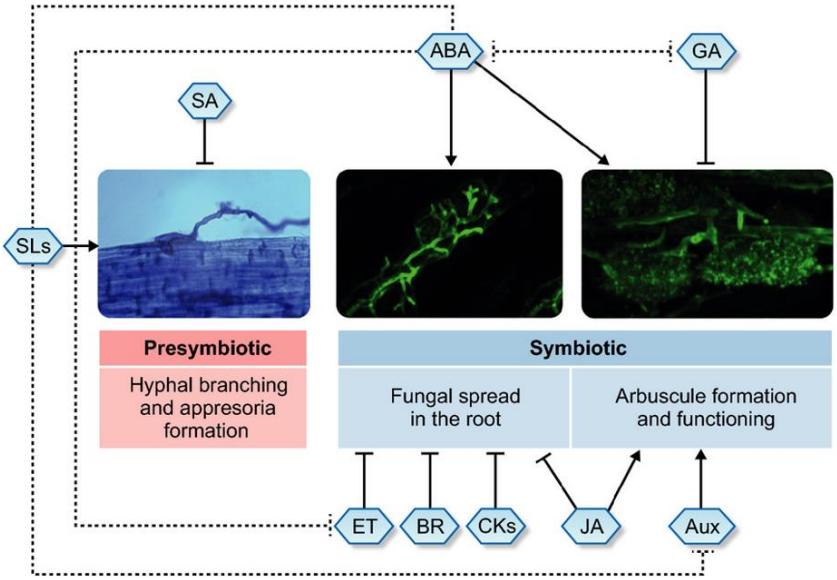

Figure 4 Phytohormone regulation of arbuscular mycorrhiza (AM) formation and functioning.The scheme summarizes the role of plant hormones in different stages of mycorrhiza development (presymbiotic, fungal hypha branching and appresoria formation;symbiotic, fungal colonization of the root cortex and arbuscule formation and functioning). Positive and negative effects are illustrated by arrows and blunt-ended bars,respectively, and dashed lines indicate interactions suggested to play a role in AM regulation. ABA, abscisic acid; Aux, auxins; BR, brassinosteroids; CKs, cytokinins; ET, ethylene; GA, gibberellins; JA, jasmonates; SA, salicylic acid; SLs, strigolactones (Pozo et al., 2015).

The attenuation of the defensive response to AM fungal recognition is essential for the establishment of symbiosis (García-Garrido & Ocampo, 2002; Siciliano et al., 2007). Using mutants, it has been shown that all plant hormones are involved, somehow, in the establishment and functioning of the symbiosis (Fig 4; Bedini et al., 2018; Liao et al., 2018; Pozo et al., 2015). Here, defensive hormones play a key role in regulating the plant's defensive response to the AM fungus (Pozo et al., 2015). On the other hand, the AM fungus actively

participates in the suppression of plant defenses by secreting peptide effectors (Kloppholz et al., 2011; Schmitz et al., 2019; Zeng et al., 2020). At more advanced stages, the symbiosis also maintains a strong impact on hormonal homeostasis to regulate colonization levels (Fernández et al., 2014; Gutjahr, 2014; López-Ráez et al., 2010; Pozo et al., 2015). Plants adjust its phenotype to the environmental context via a hormonal crosstalk. Therefore, the environmental impact on hormone levels will also have a direct impact on the interaction with the AM fungus (Pozo et al., 2015).

Due to the obligate biotrophic nature of AM fungi, the fungus is completely dependent on carbon input from the plant (Salmeron-Santiago et al., 2021). This extreme dependence makes the plant able to regulate the development of the fungus and mycorrhizal colonization. It has been described that the plant is able to select the fungus that offers the most nutritional input through carbon supply (Hammer et al., 2011; Kiers et al., 2011; Werner & Kiers, 2015). At the beginning, it was thought that the plant translocated to the fungus only sugars in the form of hexose as a carbon source (Parniske, 2008; Pfeffer et al., 1999). Subsequently, it was shown that AM fungi are not able to synthesize enough amounts of 16:0 fatty acids (Wewer et al., 2014) and that they need to be provided by the host plant (Keymer et al., 2017; Luginbuehl et al., 2017). Biosynthesis of these lipids occurs *in situ* in the arbusculated cells, activating during arbuscule cell formation a cohort of lipid biosynthesis and transport genes (Bravo et al., 2017; Gutjahr et al., 2012; Keymer et al., 2017; E. Wang et al., 2012).

The regulation of the symbiosis does not appear to be only a process of carbon sink towards the fungus, but it is regulated by more specific control processes (Vierheilig et al., 2008; Vierheilig, Garcia-Garrido, et al., 2000). Indeed, using split root systems, it was shown that colonization on one side of the system suppressed subsequent colonization on the other side, indicating a mechanism of mycorrhizal autoregulation (Meixner et al., 2005; Vierheilig, 2004; Vierheilig, Maier, et al., 2000). The arbuscules, due to their major role in the interaction between the two symbionts, undergo much of the autoregulation process, having a turnover of 2-7 days (Kobae et al., 2010). This process is used to control fungal growth and development according to the nutrient plant demand (C. Wang et al., 2018). The autoregulation of mycorrhiza shares a large part of mechanisms with the autoregulation of nodulation, although it is not yet fully understood (Catford et al., 2003; Foo et al., 2016).

Mycorrhiza in a multitrophic context

Under natural conditions plants interact simultaneously or sequentially with multiple organisms. In fact, they are surrounded and colonized by a multitude of microorganisms that can strongly influence plant performance and plant interactions with other organisms. The mycorrhiza influences the rhizosphere microbial communities and these also influence reciprocally the development of AM symbiosis. There is synergism between AM fungi and other beneficial microorganisms, such as phosphorus solubilizing bacteria (Barea et al., 2005). Interaction with soil beneficial microbes can also have an impact on plant interactions with other organisms, both local and systemically, generally conferring resistance against aggressors. Among the most important challenges, insect herbivores are a major threat to plants. Herbivory entails a great energetic cost to the plant, affecting nutritional resources and depleting photosynthetic capacity. While crop productivity has increased considerably since the green revolution, losses due to pests and pathogens have also increased despite the use of pesticides (Oerke & Dehne, 2004). The plant has developed resistance and/or tolerance mechanisms to reduce the negative effects of herbivory (Hanley et al., 2007; Mitchell et al., 2016). In basal conditions the plant has a wide set of expressed constitutive defenses. Among them, we can find physical barriers such as trichomes, spines and waxes, but also metabolites that are deleterious to the aggressor. Due to the high energetic cost of having all the defensive machinery constitutively activated, the plant has also developed inducible defensive mechanisms which are only activated upon herbivore attack (Bekaert et al., 2012). Very few defensive mechanisms are effective on their own, otherwise they would generate a high selective pressure to overcome this resistance by the insect (Janzen, 1980). The negative impact on the insect's performance is usually achieved synergistically by combining toxic, anti-digestive and repellent compounds (Broadway & Duffey, 1988; Duffey & Stout, 1996; Erb & Reymond, 2019). Terpenoids, phenolics and nitrogenous compounds, such as alkaloids and glucosinolates, are common toxic compounds. The antidigestive effect is achieved by inducing the synthesis of anti-nutritive proteins, such as protease inhibitors (PIs) that inhibit digestive proteases in the insect gut and reduce the digestibility of plant material (H. Chen et al., 2005; Green & Ryan, 1972). Plants also release volatiles that may repel the insect and/or in turn attract natural enemies of the attacker (Dicke, 2015). Most of these inducible defensive mechanisms against chewing insects are controlled at the transcriptional level by the jasmonate (JA) signaling pathway (Erb & Reymond, 2019; Wasternack & Hause, 2013). Moreover, it is modulated by a complex hormonal crosstalk that fine-tunes plant responses against the specific aggressor (Erb et al., 2012). Together with JA, salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) are the

most relevant phytohormones modulating plant defense responses against biotic stresses (Fig 5; Pieterse et al., 2012). Generally, SA-regulated defenses are effective against biotrophic pathogens, acting usually as antagonistic to JA signaling (Vos et al., 2013). JA regulates defenses against necrotrophic pathogens and chewing herbivores through two different branches co-regulated with ET and ABA. In Arabidopsis, ET acts as a positive regulator of the JA-dependent branch of the ERF1 transcription factor that is mainly effective against necrotrophic pathogens and in turn acts as an antagonist against the JA- and ABA-regulated MYC2 branch that regulates defensive responses against chewing herbivores (Verhage et al., 2011). Hormonal crosstalk provides an efficient regulatory system to shape and finetune the appropriate response to a particular stress.

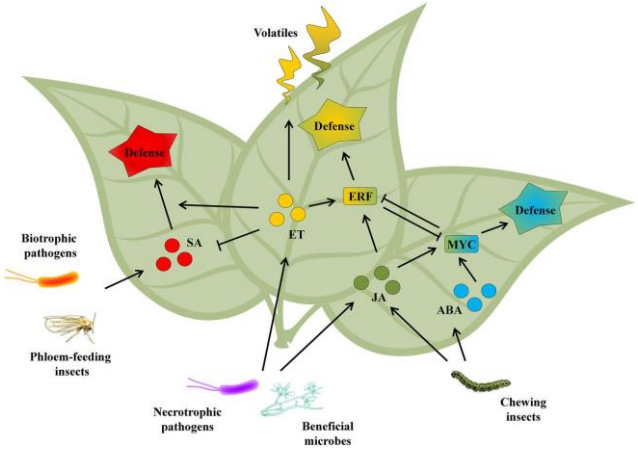

Figure 5 Simplified schematic representation of plant defense signaling networks involving the hormones ET, SA, JA, and ABA. Necrotrophic pathogen and beneficial microbes induce or prime ET- and JA-dependent signaling pathways, whereas chewing insects induce JA- and ABA-dependent signaling pathways. The ET- and ABA-regulated branches of the JA pathway are mutually antagonistic. ET alone or together with JA plays a role in volatile signaling. Arrows and end-blocked lines indicate positive and negative regulation, respectively (Broekgaarden et al., 2015).

In the case of AM symbiosis, the reorganization of metabolism in the host plant can trigger systemic changes in the defense response against other organisms, often increasing plant resistance to a variety of potential pathogens and insect herbivores, a mechanism known as Mycorrhizal Induced Resistance (MIR) (Pozo & Azcón-Aguilar, 2007). Beneficial effects of MIR have been demonstrated in the protection against a wide range of belowground aggressors such as soil-borne pathogens, nematodes or root-chewing insects (Currie et al., 2011; Jung et al., 2012; Olowe et al., 2018; Schouteden et al., 2015). This increased resistance does not occur only at the root level, but the priming of the plant immune system occurs also systemically, also protecting the plant against aboveground attackers. Nutrient supply experiments have revealed that MIR cannot be attributed only to improved nutritional status (Fritz et al., 2006; Pozo de la Hoz et al., 2021) and that the efficiency of MIR seems to be related to the lifestyle and feeding

guild of the aggressors (Pozo & Azcón-Aguilar, 2007). The spectrum of efficiency of MIR has been demonstrated against necrotrophic pathogens and leaf chewing insects (Campo et al., 2020; Fiorilli et al., 2018; Hartley & Gange, 2009; He et al., 2017; Jung et al., 2012; Lanfranco et al., 2011; Nair et al., 2015; Rivero et al., 2021; Roger et al., 2013; Sanchez-Bel et al., 2016; Sanmartín, Pastor, et al., 2020; Sanmartín, Sánchez-Bel, et al., 2020; Song et al., 2013, 2015; H. Wang et al., 2022). These organisms share susceptibility to JA-regulated defenses. Thus, it was proposed and later demonstrated experimentally using JA deficient mutants that potentiation or priming of JA-dependent defenses is a core mechanism involved in MIR (Jung et al., 2012; Mora-Romero et al., 2014; Song et al., 2013; H. Wang et al., 2022).

Defense priming

Priming is an adaptive strategy that enhances the defensive capacity of plants by potentiating the induction of defense mechanisms (Conrath et al., 2006; Martinez-Medina et al., 2016; Pastor et al., 2014). Different stimuli can trigger defense priming including beneficial microbes (ISR) (Choudhary et al., 2007; Pieterse et al., 2014). This preconditioning of the tissues for a more effective activation of defenses is associated to a reorganization of the plant at different levels: physiological, transcriptomic, proteomic, metabolic and even at the epigenetic level. This reorganization is long-lasting and can be maintained throughout the life cycle of the plant and has even been shown to be transmitted to the next generations. Interestingly, this systemic and long-lasting priming of defenses does not involve a large energetic cost for the plant (Martinez-Medina et al., 2016). Only when the plant encounters a subsequent challenge, the primed plant will respond more quickly and efficiently to the aggressor, thus enhancing resistance (Fig 6).

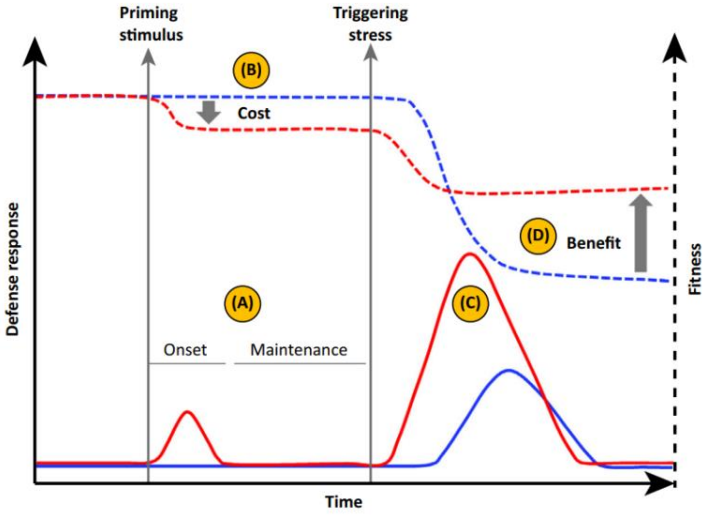

Figure 6 Scheme of the relation between defense responses (Solid Lines) and fitness (Dashed Lines) in primed (Red) versus unprimed (Blue) plants. Analysis of defense priming requires a set of steps encompassing both the assessment of plant defenses and the associated cost–benefit balance (A) Memory: two sequential environmental events are required for asserting memory in the absence of molecular markers: the priming stimulus and the triggering stress. During priming and in the primed state (before the triggering stress), plant defenses are expected to be only transiently and generally faintly induced. (B) Low fitness costs. (C) A more robust defense response. (D) Better performance. Therefore, priming enhances plant fitness in hostile environments (Martinez-Medina et al., 2016)

Several mechanisms have been proposed that may be mediating the defensive priming. Among them, changes at transcriptional level, in histone and chromatin compaction as well as in TF abundance could facilitate gene activation and epigenetic memory; increased levels of stress receptors that can facilitate greater perception of future challenges; increased accumulation of protein kinases that can participate in signaling transduction; the inactivation and reserve of defensive metabolites that can be activated upon challenge; a modulation of the hormonal balance that would allow modulation of large sets of defensive genes in a more bursty manner; and changes in primary metabolism that would allow mobilization of greater resources under immediate challenge. All these mechanisms are low-cost mechanisms for the plant that would be in congruence with low-cost systemic changes that would allow a more efficient response under a subsequent challenge (reviewed in Conrath et al., 2015; Mauch-Mani et al., 2017).

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Objectives

Objectives

GENERAL OBJECTIVE

To study the signaling involved in the regulation of the establishment and functioning of mycorrhizal symbiosis and its effect on the induction of induced resistance to insect herbivores.

SPECIFIC OBJECTIVES

1. To study the effect of plant nutritional conditions on the exudation of strigolactones, and to study its function in integrating plants' responses to nutritional deficiencies.

2. To determine the effect of flavonoids on AM spore germination and root colonization.

3. To study the molecular signaling in the regulation of mycorrhizal symbiosis under different environmental conditions.

4. To identify conserved patterns in molecular responses to 3-way between plants, microorganisms and insects through a meta-analysis of the scientific literature.

5. To study the impact of hormonal regulation in the mycorrhiza induced resistance against chewing herbivores.

Material & Methods

Material & Methods

Plant material

For the in planta bioassays *Solanum lycopersicum* L. was used as a model plant. As a general rule Moneymaker cultivar was used for our assays since it is one of the cultivars that is best characterized at the physiological, transcriptomic and metabolic levels. For assays with transgenic or mutant lines (Chap. 1 and Chap. 5) other genotypes were used because these deficient lines were generated in other cultivars. For Chap. 1, tomato cv. Craigella (LA3247) and its SL-deficient line SlCCD8-RNAi L09 (Kohlen et al., 2012) were used. For Chap. 5, tomato cv. UC82B and the ET-deficient mutant ACD (Klee et al., 1991) and ET-insensitive mutant never ripe (Nr) (Wilkinson et al., 1995). And for Chap. 2 tomato cv. Red Cherry (LA0337).

Seed surface sterilization and germination

Seeds are surface sterilized in 4% sodium hypochlorite (50% commercial bleach) for 10 min. Then seeds are washed with tap water and incubated in plastic trays containing sterile vermiculite at 25–27 °C, 16 h/8 h (day/night) and 65–70 % RH. Finally, plantlets are incubated for 10 days until transplant.

Arbuscular mycorrhizal fungal inoculum

In general, inocula based on raw inoculum was used for the mycorrhizal assays (Chap. 3 and Chap. 5). Isolates of *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler 2010 (DAOM 197198) and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (BEG12, International Bank of Glomeromycota, https://www.i-beg.eu/cultures/BEG12.htm) are continuously maintained in greenhouse conditions in a pot culture of Trifolium repens L. and Sorghum vulgare Pers. with a substrate consisting in vermiculite:sepiolite (1:1, v/v). Mycorrhizal treatments consist of plants inoculated with raw AMF inoculum containing colonized root fragments, spores and mycelia. Non-mycorrhizal plants receive a filtrate of the general microbial population of the AMF inoculum to homogenize microbial populations. For the spore based inoculum in Chap. 2, plants were inoculated with

spores of *R. irregularis* (MUCL 57021) produced by *in vitro* cultivation supplied by Koppert Biological Systems (The Netherlands).

Plant growth conditions

Plantlets were transplanted to 300-350 mL pots filled generally with sand:vermiculite (1:1, v/v). Plants were randomly distributed under greenhouse conditions (18–27 °C, 16 h/8 h (day/night), 30–70 % RH). The fertigation schedule included watering generally with Long Ashton nutrient solution (Hewitt, 1953) once or twice a week with the standard phosphorous concentration reduced to 25 %.

Mycorrhizal quantification – histochemical staining

A representative sample of the root system is collected at harvest. In general, the entire root system of a tomato plant grown under our conditions for 6 weeks can fit in a 50 mL Falcon. In case of a very bulky root system, to take a representative sample we should cut the root into three or four large fragments and collect alternate parts of these fragments. In the lab, root samples are cleared and digested in 10% KOH (w/v) for 2 days at RT (18 – 23°C). After that, root samples are rinsed thoroughly with tap water and acidified with 2% (v/v) acetic acid solution. The ink requires an acid medium to act properly. Then, fungal root structures are stained with a 5% (v/v) black ink (Lamy, Germany) and 2% acetic acid solution for 24 h at RT (Vierheilig et al., 2005). Finally, ink solution is washed with tap water. Used KOH and ink solution can be reused several times.

Mycorrhizal colonization is determined by grid-line intersection method (Giovannetti & Mosse, 1980) using a Nikon SMZ1000 stereomicroscope. This method evaluates the percentage of total root length colonized by the AMF. The root system is placed in a grid-line petri dish and spread out enough so that the roots are separated from each other as described in Garcia et al. (2020). To have a representative result, we have to count at least 200 root-gridline intersects per root sample.

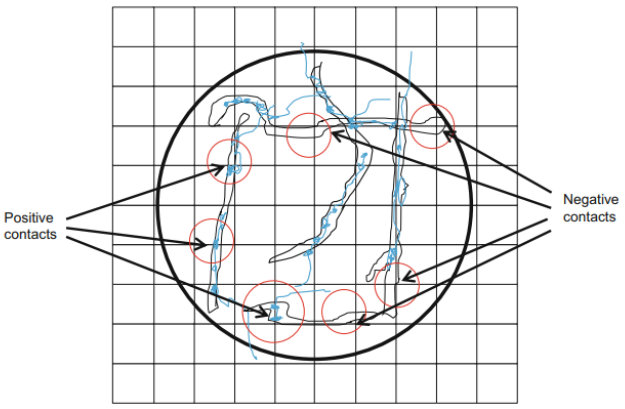

Figure 7 Scheme showing the gridline intersect method for quantification of AM fungal colonization in mycorrhizal roots. Obtained from Garcia et al. (2020) based on the method described by Giovannetti and Mosse (1980).

RNA extraction, cDNA synthesis and gene expression by qPCR

Plant tissue material is grinded in liquid nitrogen while maintaining the cold chain. 100 mg of the ground material is placed in a 2 mL eppendorf. 1 mL of TRIsure™ (Bioline, USA) is added under the fume hood and vortexed to homogenize the contents. Then 200 µL of chloroform is added, gently shaken and incubated for 3 min at RT. It is centrifuged for 15 min at 12000 g at 4ºC. The proteins and the cell wall remain in the interphase, the phenol remains in the lower phase. The upper phase containing the RNA is taken and transferred to a new 2 mL tube. After 500 µL of pure isopropanol is added to precipitate the nucleic acids. Mixing it by hand and incubating at minus 20°C for 20 min. Then it is centrifuged for 20 min at 12000 g at 4°C and all the supernatant is descarded. We have to be very careful not to remove the pellet. Then the pellet is washed with 200 µL of 75% ethanol. It is centrifuged for 5 min at 12000 g at 4ºC and the supernatant is discarded again. The pellet is let to dry with the lid open for about 30 min. The pellet is then resuspended with 50 µL of RNAse free water (miliQ). Finally the integrity of the RNA needs to be checked with electrophoresis.

This RNA is then treated with DNAse I to purify the RNA according to the manufacturer's instructions (NZYtech, Portugal). After the RNA is purified and concentrated using RNA Clean & Concentrator-5 column kit (Zymo Research, USA). First-strand cDNA is synthesized from 1 µg of purified total RNA using PrimeScript RT Master Mix (TaKara, Japan) according to the manufacturer's instructions.

Quantitative PCR reactions and relative quantification of specific mRNA levels have been performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Expression values have been normalized using the reference gene SlEF-1α (López-Ráez et al., 2010) encoding the tomato translation elongation factor-1α.

Determination of mineral nutrients (See Chapter 1 and 3)

Root exudate collection and purification of strigolactones (See Chapter 1)

Strigolactone analysis by LC-MS/MS (See Chapter 1)

***In vitro* spore germination of the AM fungus (See Chapter 2)**

RNA-seq transcriptional analysis (See Chapter 1)

Transcriptomic network analysis (See Chapter 1)

Ethylene emission quantification by Gas Chromatography (See Chapter 1)

LAP enzymatic activity (See Chapter 1)

Leaf phytohormonal metabolic profiling (See Chapter 1)

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Chapter 1

Strigolactones: new players in the nitrogen-phosphorus signalling interplay

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Strigolactones: new players in the nitrogen-phosphorus signalling interplay

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ABSTRACT

Nitrogen (N) and phosphorus (P) are among the most important macronutrients for plant growth and development, and the most widely used as fertilizers. Understanding how plants sense and respond to N and P deficiency is essential to optimize and reduce the use of chemical fertilizers. Strigolactones (SLs) are phytohormones acting as modulators and sensors of plant responses to P deficiency. In the present work, we assess the potential role of SLs in N starvation and in the N-P signalling interplay. Physiological, transcriptional and metabolic responses were analysed in wild-type and SL-deficient tomato plants grown under different P and N regimes, and in plants treated with a short-term pulse of the synthetic SL analogue *2'-epi*-GR24. The results evidence that plants prioritize N over P status by affecting SL biosynthesis. We also show that SLs modulate the expression of key regulatory genes of phosphate and nitrate signalling pathways, including the N-P integrators PHO2 and NIGT1/HHO. The results support a key role for SLs as sensors during early plant responses to both N and phosphate starvation and mediating the N-P signalling interplay, indicating that SLs are involved in more physiological processes than so far proposed.

INTRODUCTION

In a world with an increasing global population, one of the main challenges for modern agriculture is to enhance food production, while protecting the environment (Crist et al., 2017). Crops are constantly exposed to biotic and abiotic stresses which greatly impact their productivity, with nutrient deficiency being one of the most important limiting factors (Nair, 2019). Therefore, in order to face such drawback, intensive agriculture relies on a massive use of chemical fertilizers and pesticides to maintain high yield crop production (Majeed, 2018; Savci, 2012). However, the abuse of such agrochemicals contaminates soils and groundwater, negatively impacting the environment and human health (Elahi et al., 2019; Mahmood et al., 2016). Thus, there is a need to find more eco-friendly strategies to reduce agrochemicals input without compromising yield and food quality. In this sense, breeding of plants that are more efficient in the use of natural resources and able to perform better when grown under poor nutrient environments is a promising alternative (Qaim, 2020). Phosphorus (P), in the form of inorganic phosphate (Pi), and nitrogen (N), as nitrate and ammonium, are among the most important macronutrients for plant development, and their coordinated use is essential for optimal plant growth and yield (Hu & Chu, 2020; Oldroyd & Leyser,

2020). Nitrate, the preferred N source, tends to leach from the soil and Pi is relatively immobile; therefore plants can only use 30-40% of the N and less than 30% of the Pi sources applied as fertilizers, which results in both Pi and N deficiency in agricultural soils (Nasr Esfahani et al., 2021; Oldroyd & Leyser, 2020). Nowadays, the massive use of fertilizers is costly, and it is leading to an increased N and P leaching into the biosphere, with the consequent negative impact on the environment. Therefore, understanding how plants sense, signal and respond to Pi and N shortage is essential to optimize and reduce the use of chemical fertilizers, alleviating agricultural costs, and the excessive consumption of these non-renewable resources.

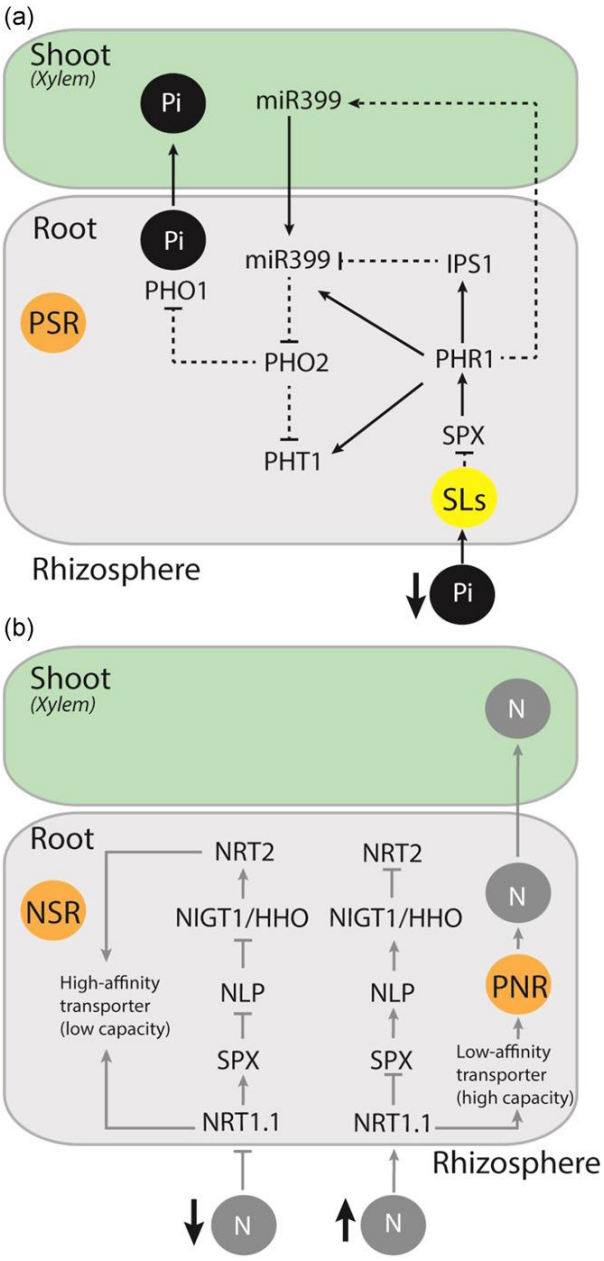

Figure 1 Schematic models of the core elements involved in the regulation of Pi and nitrate signalling pathways in plants. (a) under Pi deficiency, SLs biosynthesis is promoted. Through as yet unknown mechanism, SLs affect the complex SPX-PHR1, which becomes unstable releasing the master regulator PHR1. Then, PHR1 promotes the expression of Pi transporters from the PHT1 family in the roots, thus increasing Pi uptake. PHR1 also induces the expression of the microRNA miR399, which negatively regulates the repressor PHO2, and that of the non-protein coding gene IPS1. PHO2 downregulation prevents degradation of the Pi exporter PHO1, allowing Pi xylem loading and subsequent transport into the shoots. On the other hand, IPS1 can interact and block miR399 transcripts, preventing miR399-PHO2 binding and degradation of PHO2. Adapted from Puga et al. (2017). (b) N signalling pathway is mainly regulated by the transceptor NRT1.1. This sensor has the capacity to switch between low- and high-affinity depending on the external nitrate provision. Under optimal N conditions (N), NRT1.1 expression is induced activating the primary nitrate response (PNR) facilitating N transport to the shoots via xylem. Under these conditions, NRT1.1 interacts with specific SPX proteins, promoting its degradation and allowing the activation of the family of transcription factors NIN-LIKE PROTEIN (NLP), which are master regulators of nitrate responses. Subsequently, NLPs initiate transcriptional cascades by activating the expression of the GARP-type transcription factors NIGT1/HHO. NIGT1/HHOs act mainly as repressors, reducing the expression of the high-affinity transporters NRT2. Under nitrate deficiency, NRT1.1 is phosphorylated acting as a high-affinity (low capacity) transporter and triggering nitrate starvation responses (NSR). Under these conditions, NLPs become inactive, negatively affecting the expression of NIGT1/HHO repressors, which facilitates a slow activation of NRT2 transporters.

Nitrogen and Pi are also signalling molecules triggering downstream N and Pi responses, which are critical for plant adaptation to environments with variable nutrient availability (Raghothama, 2000). Therefore, sensing nutrient availability and signalling to coordinate appropriated responses is crucial for plant performance. To cope with P and N deficiency, plants have developed an array of adaptations that affects their growth and development, collectively known as Pi and N starvation responses (PSR and NSR, respectively). In Pi- and N-deficient environments, overall plant growth is reduced, but the root system is generally increased to favour nutrient foraging, thus increasing the root-to-shoot ratio (Hu & Chu, 2020; Oldroyd & Leyser, 2020). PSR requires a fine-tuned coordination of plant responses in which a number of genes and signalling molecules are involved (Fig. 1a). Here, the transcriptional activator PHOSPHATE STARVATION RESPONSE 1 (PHR1) plays a key role in the expression of most Pi starvation-induced genes (Bustos et al., 2010; Ham et al., 2018; Puga et al., 2017). Although PHR1 expression is not transcriptionally regulated, its activity is modulated by the plant Pi status, being negatively regulated by the SYG1/Pho81/XPR1 (SPX)-domain proteins. Under Pi limitation, the complex SPX-PHR1 becomes weak releasing PHR1, inducing the expression of high-affinity Pi transporters (PHTs) and facilitating Pi-acquisition (Fig. 1a). PHR1 also promotes the expression of the microRNA miR399 (Pant et al., 2008), which reduces the number of PHO2 transcripts, encoding a ubiquitin-conjugating E2 enzyme involved in protein degradation (Lin et al., 2008). Subsequently, down-regulation of PHO2 prevents the degradation of PHO1, a Pi transporter involved in Pi transport into the aerial tissues (T. Y. Liu et al., 2012). Therefore, miR399/PHO2 is an important component of the Pi signalling network operating downstream of PHR1 (Bari et al., 2006). PHR1 also promotes miR399 levels, it also induces the expression of IPS1, a non-protein coding gene involved in miR399 sequestration (Franco-Zorrilla et al., 2007) (Fig. 1a). Therefore, Pi acquisition and homeostasis is regulated by PHR1 and the triad ISP1-miR399-PHO2 (Franco-Zorrilla et al., 2007; Puga et al., 2017).

As for Pi, N signalling is also precisely fine-tuned, but this occurs through several interconnected signalling pathways (O'Brien et al., 2016). The primary nitrate response (PNR) corresponds to a rapid and nitrate-specific activation of sentinel genes, including the sensor NRT1.1 (Fig. 1b) (Maghiaoui et al., 2021; W. Wang et al., 2018). In Arabidopsis, this gene encodes a nitrate transporter with dual affinity (Ho et al., 2009; W. Wang et al., 2018). NRT1.1 has the capacity to switch between low- and high-affinity in response to external nitrate. At high concentrations, it works as a low-affinity (high capacity) transporter, triggering high-level nitrate responses and facilitating N transport to the aerial tissues. Under these conditions, the family of transcription

factors NINE-LIKE PROTEIN (NLP) is also activated. NLPs are master regulators of nitrate responses initiating transcriptional cascades as the induction of the NIGT1/HHO family, a group of G2-like GARP-type transcription factors. Then, NIGT1/HHOs repress the expression of the transporters of the family NRT2 (Hu & Chu, 2020). When environmental nitrate is limited, NRT1.1 is phosphorylated acting as a high-affinity (low capacity) transporter and triggering nitrate starvation responses (NSR) (Fig. 1b). Here, NLPs become inactive, negatively affecting the expression of NIGT1/HHO repressors, which facilitates a slow activation of high-affinity transporters NRT2, probably to increase nitrate uptake. In Arabidopsis, it has been shown that the expression of NRT2 transporters, especially NRT2.1 and NRT2.4, is regulated by the NIGT1/HHO repressors in a NRT1.1-dependent manner (Fig. 1b) (Kiba et al., 2018; Maeda et al., 2018; Medici et al., 2015). Some studies have suggested that NRT2.1 may also act as a sensor for root development, although its exact role in nitrate signalling is not yet clarified (W. Wang et al., 2018). Finally, the third pathway includes long-distance signals where cytokinin biosynthesis, C-terminally encoded peptides and glutaredoxins are involved (Ohkubo et al., 2017; Tabata et al., 2014).

Recent evidence shows that there is interplay between Pi and nitrate starvation signalling pathways, which is regulated at different levels (Hu & Chu, 2020; Medici et al., 2019; Ueda & Yanagisawa, 2019). It has been shown that NRT1.1 regulates the nitrate-activated PSR in a PHR1-dependent manner, and that PHO2 levels are reduced under Pi starvation in the presence of nitrate (Medici et al., 2019). Here, SPX proteins play a critical role as NRT1.1 can interact with specific SPX proteins promoting its degradation and allowing PHR1 activation. SPX proteins can also interact with NLPs. Therefore, the formation of the NRT1.1-SPX module allows NLP activation at high nitrate conditions (Hu & Chu, 2020). On the other hand, it was shown that nitrate uptake is reduced by Pi starvation via PHR1 (Maeda et al., 2018). An important role for AtNIGT1/HHOs in the integration of N-P plant responses has been also shown in Arabidopsis. The expression levels of *AtNIGT1/HHOs* are promoted by nitrate and by Pi starvation, but here only under high nitrate conditions, which is under control of both AtNLP7 and AtPHR1 (reviewed in (Ueda & Yanagisawa 2019; Hu & Chu 2020). Despite these recent findings, the regulatory mechanisms and compounds involved in the N-P signalling interplay are still poorly characterized.

Plant adaptation to nitrogen and Pi availability is also regulated by phytohormones. It is widely accepted that strigolactones (SLs) are an ancient and major class of endogenous plant growth regulators. They modulate, in coordination with other phytohormones, shoot branching, internode

elongation, root architecture, secondary growth, leaf senescence and reproductive development (Kohlen et al., 2012; Waters et al., 2017). Accordingly to their role as growth regulators, SL production is promoted by plants in response to Pi and N deficiency as adaptation to such stress conditions (López-Ráez et al., 2008; Yoneyama et al., 2012). In addition to act as phytohormones, SLs have a key role as chemical signals in the rhizosphere favouring plant association with beneficial microorganisms as arbuscular mycorrhizal fungi and rhizobia (Al-Babili & Bouwmeester, 2015; López-Ráez et al., 2017).

We have recently shown that SLs are early modulators of plant responses during Pi limitation, promoting the expression of key regulatory genes in the PSR and regulating metabolic changes to cope with Pi deficiency (Gamir et al., 2020). So far, the role of SLs in N starvation has not been investigated. In the present work, using tomato (*Solanum lycopersicum*) as a model, we assess the potential role of SLs as regulators of N starvation signalling. Moreover, we test whether they are also involved in the interplay between PSR and NSR. For that, we analyse the transcriptional and metabolic responses in wild-type and in the SL-deficient *SlCCD8*-RNAi L09 tomato plants grown under different Pi and nitrate regimes, and in plants treated with a short-term pulse of the synthetic SL analogue 2'-*epi*-GR24. We show that PSR is controlled by N status in tomato, and that SLs play a role in the regulation of the N-P interplay.

MATERIALS AND METHODS

Plant growth, conditions and treatments

Two independent experiments were performed in pot experiments. In experiment 1, tomato (*Solanum lycopersicum* L) cv. MoneyMaker plants were used. In experiment 3, tomato cv. Craigella (LA3247) and the SL-deficient line *SlCCD8*-RNAi L09 (Kohlen et al., 2012) were used. Seeds were surfaced-sterilized in 4% sodium hypochlorite for 10 min, washed with sterile demiwater and sown in trays containing sterile zeolite:sand (1:1) for germination at 25 ºC in darkness. Seedlings with two true leaves were transplanted individually into plastic pots (0.5 L) with a mixture of sterile zeolite and sand (1:1). The experimental design included two factors: P (2 levels: high [HP], 1.3 mM, and low [LP], 0.3 mM) and N (2 levels: [HN], 20 mM and [LN], 5 mM). Ten plants per treatment were grown. Plants were watered twice a week with 50 ml of the corresponding Hewitt nutrient solution (Hewitt, 1966), modified depending on the treatments as detailed in Table S1. Plants were grown

for six weeks under greenhouse conditions at 25/19 ºC with 16/8 h photoperiod and a relative humidity of 50-60%. Before harvest, root exudates from each plant were collected individually as described below. At harvest, shoots and roots were collected, weighed, snap-frozen in liquid nitrogen and kept at -80 ºC until analysis.

For the experiment in hydroponics (experiment 2), tomato (cv. MoneyMaker) seeds were surface sterilized in 4% sodium hypochlorite for 10 min, washed with sterile demiwater for 10 min, and germinated in a plate on moistened filter paper at 25 ºC in darkness. After two days, seeds were sown in 1.5 ml Eppendorf tubes filled with 0.5% Phytoagar and grown hydroponically in 3 L plastic containers with Hewitt nutrient solution (Hewitt, 1966) with 0.8 mM of Pi and constant aeration for 4 weeks. Growth conditions were 25/19 ºC with 16/8 h photoperiod and a relative humidity of 50-60%. Nutrient solution was replaced once a week. In this case, the experimental design included two factors: Pi (2 levels: with [+P] and without [-P, 0%]) and 2´-*epi*-GR24 (2 levels: with [GR24] and without [C]). After 4 weeks, half of the plants were transferred to nutrient solution without Pi (-P) and grown for an additional week. The other half was kept under the same Pi conditions as during the pre-cultivation (+P). Then, 10 nM of the active diasteroisomer 2'-*epi*-GR24 (a synthetic analogue of SLs) was applied to half of the plants of each treatment (GR24) in the nutrient solutions (+ and -Pi) for 1 h. After the treatment, plants were kept for 24 h with the corresponding nutrient solution (+ or -Pi) without 2'-*epi*-GR24 to allow them to respond to the treatment. Each of the four treatments comprised six replicates. Roots were collected, weighed, snap-frozen in liquid nitrogen and kept at -80 ºC until use.

Determination of mineral nutrients in leaves

Phosphorus and other element concentrations were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720-ES) after acid digestion of the samples. Total C and N content were determined using an Elemental Analyser (Leco Truspec CN), according to standard procedures. For the measurements, frozen shoots were ground into a fine powder and lyophilized. A 200 mg aliquot of dry tissue was used per sample. Six biological replicates per treatment were analysed.

Searching for tomato *SlNIGT1/HHO* genes

The family of the GARP-type transcription factors NIGT1/HHO has not been characterized in tomato. We searched the putative orthologue of the Arabidopsis AtNIGT1.4/HHO1 (also known as

HRS1) gene (At1g13300) in the tomato genome using BLAST on the platform Sol Genomics Network. A sequence with an open reading frame (ORF) of 1303 bp (Solyc05g009720), encoding a predicted 400 amino acids protein was found. The sequence showed a 74% identity with a 25% of query cover to AtNIGT1.2/HHO2 at nucleotide level and a 46% identity with an 84% of query cover at amino acid level. Specific primers for real-time quantitative PCR (qPCR) analysis of this gene were designed (Table S2).

RNA isolation and gene expression analysis by qPCR

RNA extraction and purification, synthesis of the corresponding cDNA and qPCR was performed as described in Gamir et al. (2020). Specific primers for genes involved in SL biosynthesis, and Pi and nitrate signalling pathways were used (Table S2). Six independent biological replicates were analysed per treatment. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak & Schmittgen, 2001). Expression values were normalized using the normalizer genes *SlEF*, encoding for the tomato elongation factor 1a, or *SlActin*, encoding for the tomato actin (Table S2).

Root exudate collection and purification of strigolactones

Root exudates were collected from each pot individually and used for further analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). For exudate collection, the substrate was rinsed with 1 L of tap water to remove the compounds accumulated during the plant growth. Subsequently, 50 mL of the corresponding Hewitt nutrient solution (Table S1) was added to each pot to reconstitute the treatments. Plants were kept for 48 h in the greenhouse and the 'fresh' exudates were collected individually by applying 1 L of tap water to each pot. The crude exudates were filtered through glass fiber filters by vacuum and concentrated and purified by solid phase extraction through Telos C18 (EC) SPE columns (Octadecyl 500 mg/3 mL) (Kinesis) using a SPE vacuum manifold (Supelco). For that, SPE columns were first pre-equilibrated with 5 mL of 100% acetone. Then, 1 L of each exudate solution was loaded onto the pre-equilibrated columns. Each column was washed with 5 mL of sterile demiwater, and the exudates were eluted with 5 mL of 100% acetone and collected in 10 mL amber tubes. Purified root exudates were stored at -80 ºC until use. Before LC-MS/MS analysis, the exudates were normalized to the same ratio of mL of exudate per g of root fresh weight.

Strigolactone analysis by LC-MS/MS

SL quantification was performed by LC-MS/MS as described by Rial et al. (2019). Samples were collected and purified as described above. Five µL of each sample were directly injected into the equipment. The samples were analysed on a Bruker EVOQ Triple Quadrupole Mass Spectrometer (Bruker), using an electrospray (ESI+) as ionization source.

Statistical analyses

All variables were subjected to analysis of variance (ANOVA) with Pi and N (experiments 1 and 3) or Pi and 2´-*epi*-GR24 (experiment 2) as the main factors including the interaction term. Data were checked for normality and homogeneity of variance before statistical analyses. Data from root fresh weight and Pi content were transformed using logarithms to remove the normality error. The statistical analysis was performed using the software Infostat (Di Rienzo et al., 2013) and its interface with the software R (R Core Team, 2009). The Tukey test ($p \leq 0.05$) was carried out when suited to compare means *a posteriori.*

RESULTS

P and N deficiency affect plant growth and development differentially

In order to determine the influence of P and N availability on plant growth, and how their individual deficiency affects the perception of the other, tomato plants were grown with different P-N combinations (experiment 1). As expected, plant growth was significantly reduced in plants grown under P and/or N deficiency as compared to those grown under 'optimal' control conditions (HPHN) (Fig. 2a). Shoot fresh weight was reduced by 62 and 73% under Pi (LPHN) or nitrate (HPLN) limitation, respectively. When both nutrients were deficient (LPLN), a reduction of 77% was detected (Fig. 2b). A similar pattern was observed in the roots. Hereto, a reduction of 36 and 46% was detected under Pi and nitrate deficiency, respectively, and 49% when both nutrients were limited as compared to 'optimal' conditions (Fig. 2b). Plants always performed better under high N conditions, independently of the Pi application, with both shoot and root weight being improved under these conditions (Figs. 2a and b). However, root-to-shoot ratio was higher under N deficiency (Fig. S1a). Root length also increased upon N deficiency, but not by Pi starvation (Fig. S1b). Leaves from plants grown under low Pi (LPHN) showed the characteristic dark green colour in the upper

surface (adaxial) and a purple tone on the lower surface (abaxial), as a consequence of the increase in anthocyanins; a phenotype almost absents when both nutrients were low (LPLN) (Figs. 2a and S2).

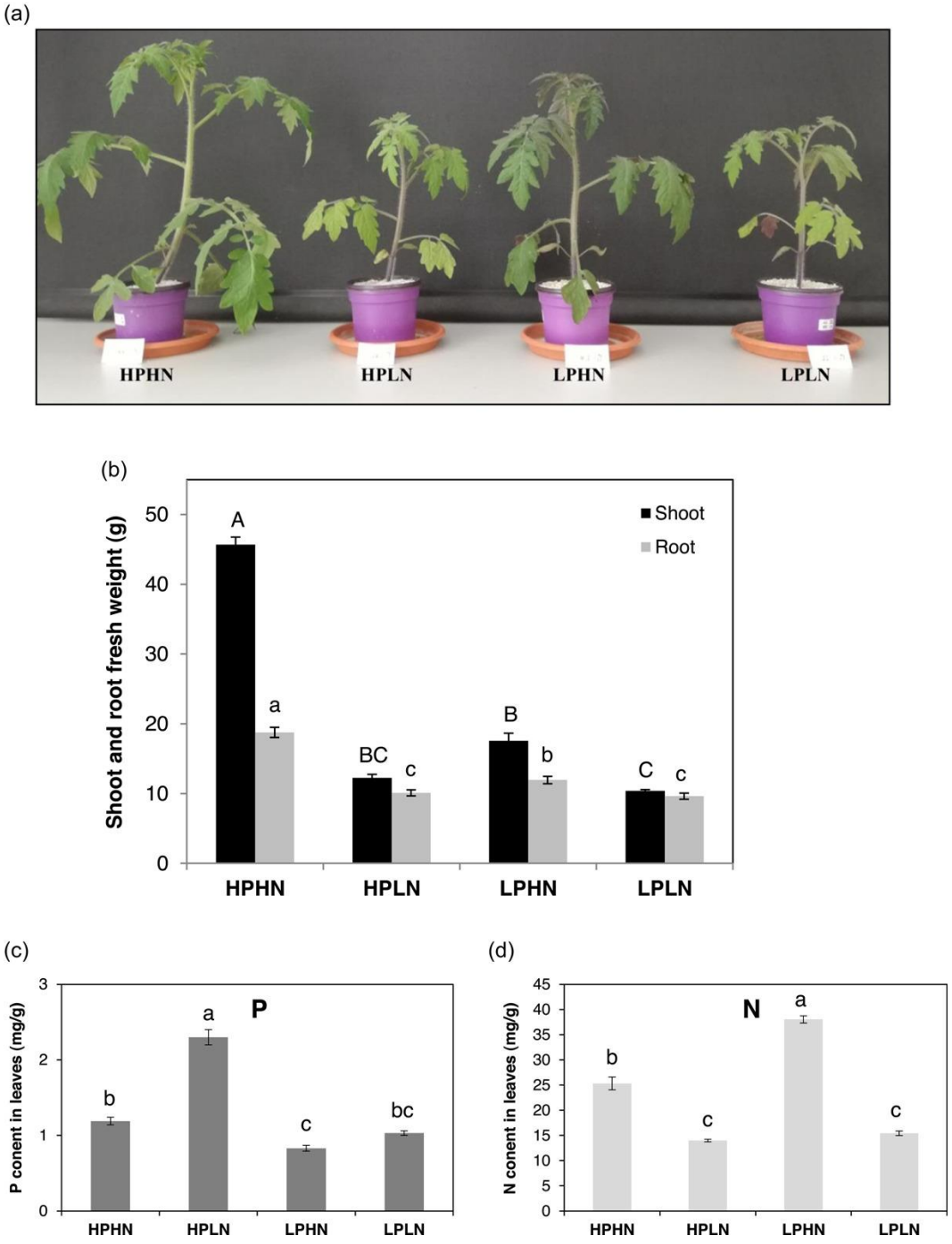

Figure 2 Effect of different Pi and nitrate regimes on tomato (cv. MoneyMaker) growth and performance. (a) Phenotypic comparison of 6-week old plants grown under optimal (control) Pi and nitrate conditions (HPHL), optimal Pi and limiting nitrate (HPLN), limiting Pi and optimal nitrate (LPHN) and deficiency of both nutrients (LPLN). Shoot and root fresh weight (b), phosphorus (P) (c) and nitrogen (N) (d) content in tomato leaves. Bars represent the means of ten (b) and six (c and d) independent replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$) according to the Tukey test.

As expected, P and N concentrations were reduced by 30% and 45% in leaves from plants subjected to low P and N, respectively (Figs. 2c and d). Conversely, the concentration of the two nutrients increased when the other nutrient was deficient. That is, P levels increased by 93% when grown under N deficiency and N levels increased by 50% under Pi limitation over the control growing conditions (HPHN) (Figs. 2c and d). The observed higher P and N concentration under low nitrate (HPLN) and Pi (LPHN) conditions, respectively, is likely related to the limitation of plant growth by deficiency of the other nutrient. This may lead to an increase of the concentration of a non-limiting nutrient in plant tissues, as has been reported in Medicago and pea (Bonneau et al., 2013; Nasr Esfahani et al., 2021). Indeed, there was a significant interaction of the two factors (P and N availability) on shoot and root biomass, as well as P and N content (Table S3). Carbon (C) is another primary element involved in plant growth and development, and it has been shown that the C:N ratio is a good indicator of N use efficiency (Zhang et al., 2020). Therefore, we also measured C levels in tomato leaves and calculated the corresponding C:N ratios for the different treatments (Table 1). Carbon content was homogeneous among the treatments except for LPHN, where a slight reduction was observed. The C:N ratio significantly increased in plants under low N, independently of the P status (HPLN and LPLN). Plants with a higher C:N ratio improved N use efficiency under N deficiency to ensure survival instead of growth (Zhang et al., 2020). In addition, other macro- and micro-nutrients were analysed by ICP-OES. In general, N deficiency reduced important macro and micronutrients as Fe, Cu and Na, while they slightly increased under Pi limitation (Table S4), supporting the idea that N limitation has a higher impact in plant growth than P.

	Nutrient content (mg/g)		
Treatment	**C**	**N**	**C:N**
HPHN	425.00 ± 1.98a	25.32 ± 1.27b	17.02 ± 0.96b
HPLN	426.67 ± 2.50a	14.00 ± 0.26c	30.31 ± 0.56a
LPHN	410.50 ± 2.45b	38.06 ± 0.70a	10.80 ± 0.18c
LPLN	420.83 ± 2.55a	15.42 ± 0.46c	27.42 ± 0.82a

Table 1 Carbon (C), nitrogen (N) levels and C:N ratios in tomato leaves from plants grown on different phosphorus (P) and nitrogen (N) combinations: high P and high N (HPHN), high P and low N (HPLN), low P and high N (LPHN) and low P and low N (LPLN). Values present the means of six independent replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$) according to the Tukey test.

Promotion of SL biosynthesis by Pi deficiency depends on N provision

SL biosynthesis is promoted by P and N deficiency (López-Ráez et al., 2008; Yoneyama et al., 2012). SLs are derived from carotenoids synthesized by the sequential action of several enzymes, such as the β–carotene isomerase (D27) and two carotenoid cleavage dioxygenases (CCD7 and CCD8) (Sun et al. 2014; Andreo-Jiménez et al. 2015; Waters et al. 2017). Here, we assess how different combinations of P and N levels affect SL biosynthesis in tomato. The expression of the biosynthetic genes - *SlD27*, *SlCCD7* and *SlCCD8* - were analysed by qPCR. Nitrate deficiency under optimal P provision (HPLN) significantly increased the expression of the three biosynthetic genes as compared to control conditions (HPHN). However, the highest induction for all genes was observed by P limitation under optimal N provision (LPHN) (Figs. 3a-c). Interestingly, this higher induction by P limitation was not observed under N deficiency (LPLN), suggesting that their expression seems to be ruled by the N status.

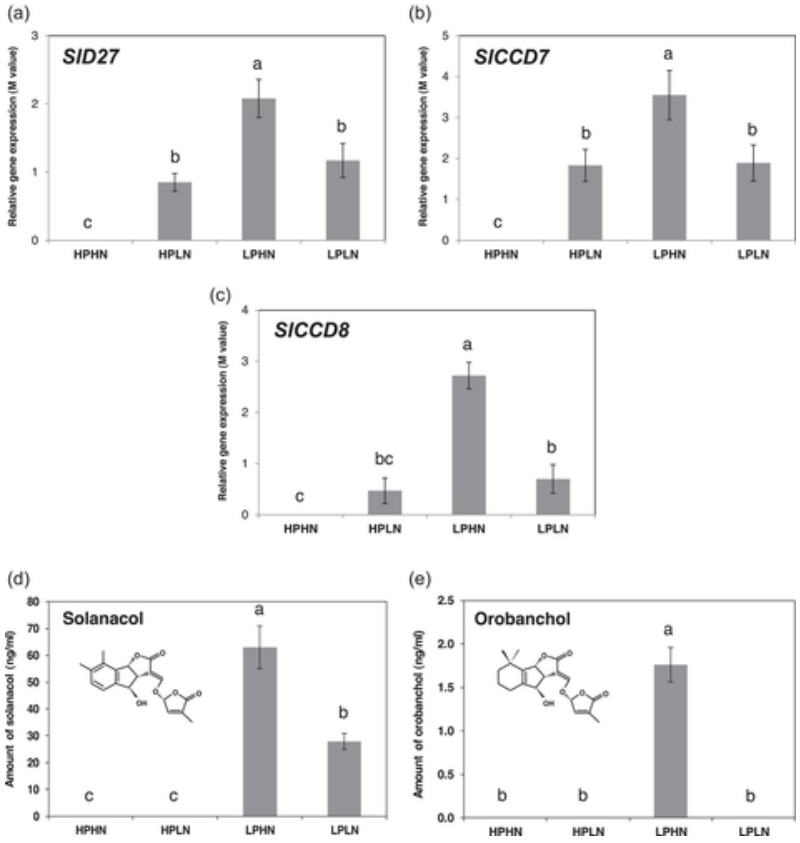

Figure 3 Effect of Pi and nitrate deficiency on SL biosynthesis. Tomato plants (cv. MoneyMaker) were grown under different nutrient regimes, as described in the legend to Figure 2. Gene expression analysis (M value) of the SL biosynthesis genes SlD27 (a), SlCCD7 (b) and SlCCD8 (c) in roots from 6-week old plants. Expression values were normalized using the housekeeping gene SlEF. M value (log2 ratio) is zero if there is no change; '+1' or '-1' indicate two-fold change induction or repression, respectively. Content of the SLs solanacol (d) and orobanchol (e) in root exudates. Bars represent the means of six independent replicates (±SE). Data not sharing a letter in common differ significantly (P < 0.05) according to the Tukey test.

A similar pattern was observed by the analytical quantification of the characterized tomato SLs solanacol and orobanchol (López-Ráez et al., 2008). None of the two SLs were detected in the root exudates under optimal nutrient conditions. Remarkably, they were also not detected under nitrate deficiency. The higher promotion of solanacol and orobanchol levels was detected by Pi deficiency under normal N conditions (LPHN) with solanacol levels being 35 times higher than those of orobanchol (Figs. 3d and e). When the availability of both nutrients was limited (LPLN), the promotion observed by Pi deficiency under normal N conditions was strongly reduced; solanacol levels were half of those in low Pi, and orobanchol fell below the detection limit (Figs. 3d and e).

Pi starvation signalling depends on plant's N status

The results on SLs biosynthesis and content support the proposed crosstalk between Pi and nitrate signalling pathways (Hu & Chu, 2020; Medici et al., 2019; Ueda & Yanagisawa, 2019). To further explore the mechanisms and the compounds regulating such interplay, we addressed the influence of nitrate levels on P-related signalling by analysing the expression of key genes regulating Pi starvation signalling, the triad ISP1-miR399-PHO2 (Fig. 1a). Transcript levels of *SlPHO2* were downregulated by Pi deficiency only under optimal N conditions (LPHN) (Fig. 4a). Remarkably, the

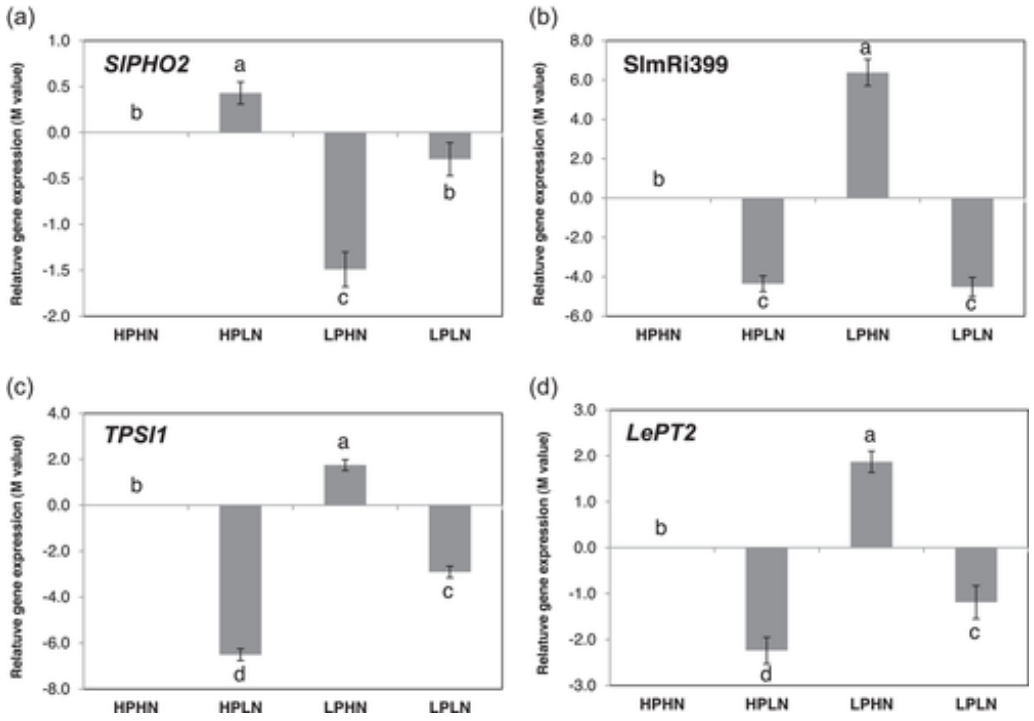

Figure 4 Expression analysis of genes associated to Pi signalling and homeostasis pathways. Effect of Pi and nitrate deficiency on the expression (M value) of genes encoding for the Pi signalling regulators SlPHO2 (a), SlmiR399 (b), LeTPSI1 (c) and the Pi transporter LePT2 (d) in tomato roots. Bars represent the means of six independent replicates (±SE). For data analysis, statistics and nutrient regimes see legends in Figures 2 and 3.

downregulation was suppressed when the two nutrients (Pi and nitrate) were limited (LPLN). Conversely, *SlPHO2* levels were slightly but significantly promoted by N deficiency under optimal Pi conditions (HPLN) (Fig. 4a). The opposite expression pattern was observed for SlmiR399 and *LeTPSI1*, the tomato homolog to IPS1 (C. M. Liu et al., 1997). Their transcript levels were increased by Pi limitation and reduced by N deficiency (Figs. 4b and c). In this case, when both nutrients were limited the expression of SlmiR399 and *LeTPSI1* was downregulated, being the N deficiency effect predominant over that shown by Pi deficiency. Strikingly, the same pattern was shown for the Pi transporter LePT2, which belongs to the PTH1 family of high-affinity Pi transporters and it is transcriptionally regulated by the plant Pi status (Gamir et al., 2020; Nagy et al., 2005). Indeed, its expression was induced by Pi limitation, but downregulated by N deficiency and when both nutrients were limiting (Fig. 4d).

The influence of nitrate and Pi supply in the expression of regulatory genes of the N signalling pathways (PNR and NSR) (Fig. 1b) was also investigated. So far, five genes encoding nitrate transporters belonging to the NRT1 and NRT2 families have been characterized in tomato (Albornoz et al., 2018). Two of them - LeNRT1.1 and LeNRT1.2 - belong to the NRT1 family, encoding for high-capacity and low-affinity nitrate transporters. The other three - LeNRT2.1, LeNRT2.2 and LeNRT2.3 - belong to the NRT2 family, encoding for low-capacity and high-affinity transporters. The expression of the high-capacity transporters *LeNRT1.1* and *LeNRT1.2* was repressed by N deficiency (HPLN) (Figs. 5a and b). However, the two genes showed a different regulation pattern under Pi limitation (LPHN): Transcript levels of *LeNRT1.1* decreased, while those of *LeNRT1.2* increased (Figs. 5a and b). When both Pi and N were deficient (LPLN), *LeNRT1.1* was similarly reduced, but *LeNRT1.2* was not detected. Low-capacity transporters (NRT2) also showed a differential expression pattern. Under Pi deficiency, the three genes *LeNRT2.1, LeNRT2.2* and *LeNRT2.3* were down-regulated as compared to the control conditions (Figs. 5c-e). However, no significant changes in the expression of *LeNRT2.1* and *LeNRT2.2* were detected under N deficiency, and only a slight reduction was observed when both nutrients were limiting (Figs. 5c and d). Conversely, the expression of *LeNRT2.3* increased under N limitation. Moreover, this induction was maintained when both nutrients were deficient (Fig. 5e).

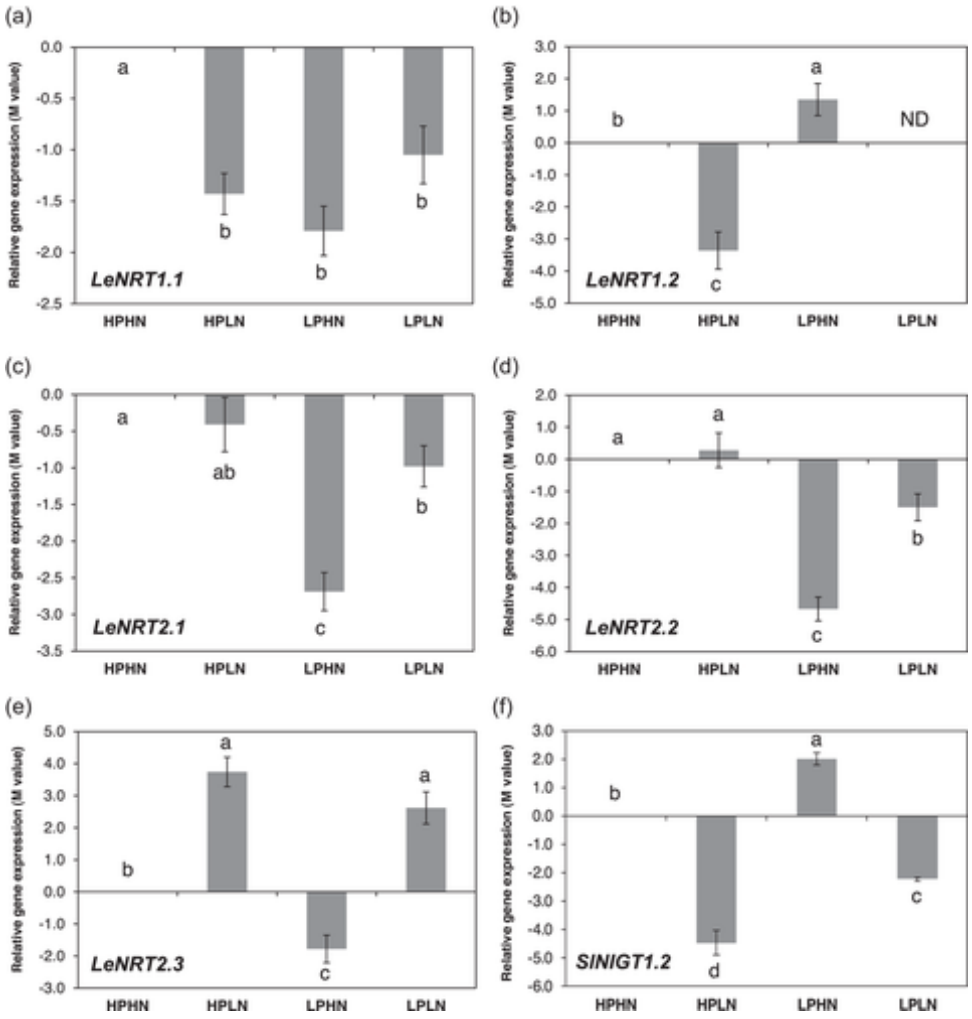

Figure 5 Expression analysis of genes associated to the nitrate signalling pathway. Effect of Pi and nitrate deficiency on the expression (M value) of the nitrate signalling pathway genes LeNRT1.1 (a), LeNRT1.2 (b), LeNRT2.1 (c), LeNRT2.2 (d), LeNRT2.3 (e) and SlNIGT1.2/HHO2 (f) in tomato roots. Bars represent the means of six independent replicates (±SE). For data analysis, statistics and nutrient regimes see legends in Figures 2 and 3.

The expression of the NRT2 transporters is regulated by the NIGT1/HHO repressors (Fig. 1b) (Kiba et al., 2018; Maeda et al., 2018; Medici et al., 2015), which play an important role in the N-P signalling interplay (Hu & Chu, 2020; Ueda & Yanagisawa, 2019; X. Wang et al., 2020). The expression of one of the putative tomato NIGT1/HHO genes, *SlNIGT1.2/HHO2* (Solyc05g009720), was analysed. Its transcript levels were repressed by N limitation (HPLN) and induced by Pi starvation (LPHN) as compared to control conditions (HPHN) (Fig. 5f). When both nutrients were deficient (LPLN), the expression of *SlNIGT1.2/HHO2* was also reduced, thus prevailing the N starvation effect. Overall, the gene expression data support the crosstalk between the PSR and NSR signalling pathways, and that plants generally prioritize the response to N starvation when both nutrients are limited.

Exogenous application of SLs affects Pi and N signalling

We show that SLs promotion under Pi limitation depends on N provision, and that Pi signalling is affected by the N status (Figs. 3 and 4). Previously, we demonstrated that SLs modulate PSR by transcriptionally regulating the regulatory genes ISP1-miR399-PHO2 (Gamir et al., 2020). Therefore, we aimed to assess whether SLs are also involved in NSR signalling and in the crosstalk between PSR and NSR. For that, we exogenously applied SLs in plants subjected to Pi deprivation and analysed the impact on NSR regulatory genes (Experiment 2). Plants were grown hydroponically under optimal Pi conditions (+P) or exposed to Pi shortage for the last week of growth (-P). Then, a 1h-pulse with 10 nM of the synthetic SL analogue 2'-*epi*-GR24 (orobanchol-type) was applied to half of the plants of each treatment (GR24). The expression of the five nitrate transporters described in tomato - *LeNRT1.1*, *LeNRT1.2*, *LeNRT2.1*, *LeNRT2.2* and *LeNRT2.3* - and *SlNIGT1.2/HHO2* was analysed by qPCR. As in experiment 1, the expression of *LeNRT1.1* was significantly reduced by Pi starvation, whereas that of *LeNRT1*.2 was induced (Figs. 6a and b). Under these conditions, the

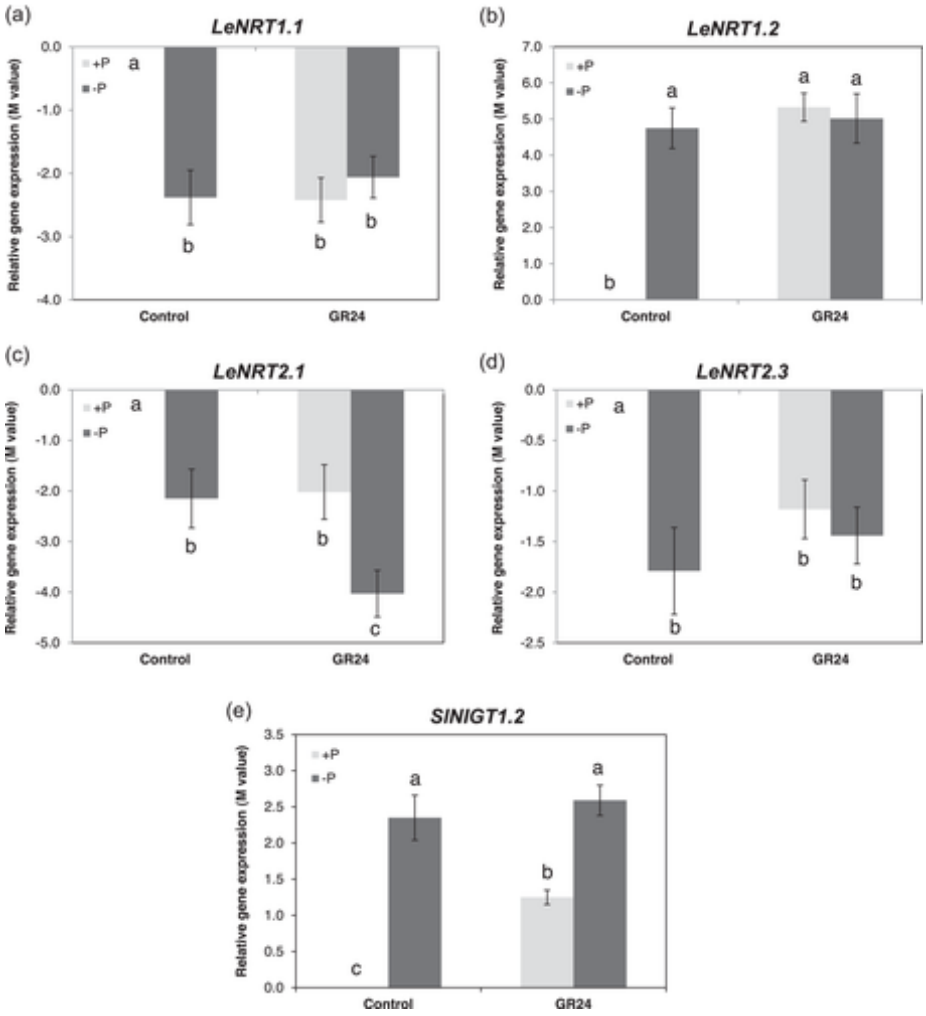

Figure 6 Effect of SLs on the expression of genes associated to the nitrate signalling pathway. Effect of the synthetic SL analogue 2'-epi-GR24 under normal (+P; grey bars) or deficient (−P; closed bars) Pi conditions in the expression (M value) of genes encoding for the nitrate signalling genes LeNRT1.1 (a), LeNRT1.2 (b), LeNRT2.1 (c), LeNRT2.3 (d) and SlNIGT1.2/HHO2 (e) in tomato roots. Plants were untreated (Control) or treated with 2'-epi-GR24 (GR24). Gene expression values were normalized using the housekeeping gene SlActin. Bars presents the means of five independent replicates (±SE). For statistics see legend in Figure 3.

expression of genes from the NRT2 family also showed the same pattern as in the previous experiment, except for *LeNRT2.2*, whose transcripts could not be detected upon 35 cycles of PCR. That is, *LeNRT2.1* and *LeNRT2.3* transcripts were reduced by Pi limitation (Figs. 6c and d). *SlNIGT1.2/HHO2* also showed the same pattern as in the previous experiment, being induced by Pi deficiency (Fig. 6e). Interestingly, the same expression pattern was observed for all the genes upon application of 2'-*epi*-GR24 under optimal Pi conditions (Fig. 6). Therefore, SLs mimic the effect of Pi starvation on N-related signalling in the absence of Pi limitation.

The SL-deficient line SlCCD8-RNAi L09 is partially altered in N signalling

To further assess the potential role of SLs as signals in N starvation and in the N-P signalling interplay, the response to P and N levels was compared in the SL-deficient line *SlCCD8*-RNAi L09 and the corresponding wild-type cv. Craigella (experiment 3). *SlCCD8*-RNAi L09 displays a 95% reduction in SL levels (Kohlen et al., 2012) (Fig. S3). The effect of nutrient availability on plant growth was similar in both genotypes. As in experiment 1 with MoneyMaker (Fig. 2), shoot and root fresh weights were reduced under P and/or N deficiency as compared to the 'optimal' conditions (HPHN) (Figs. S4a and

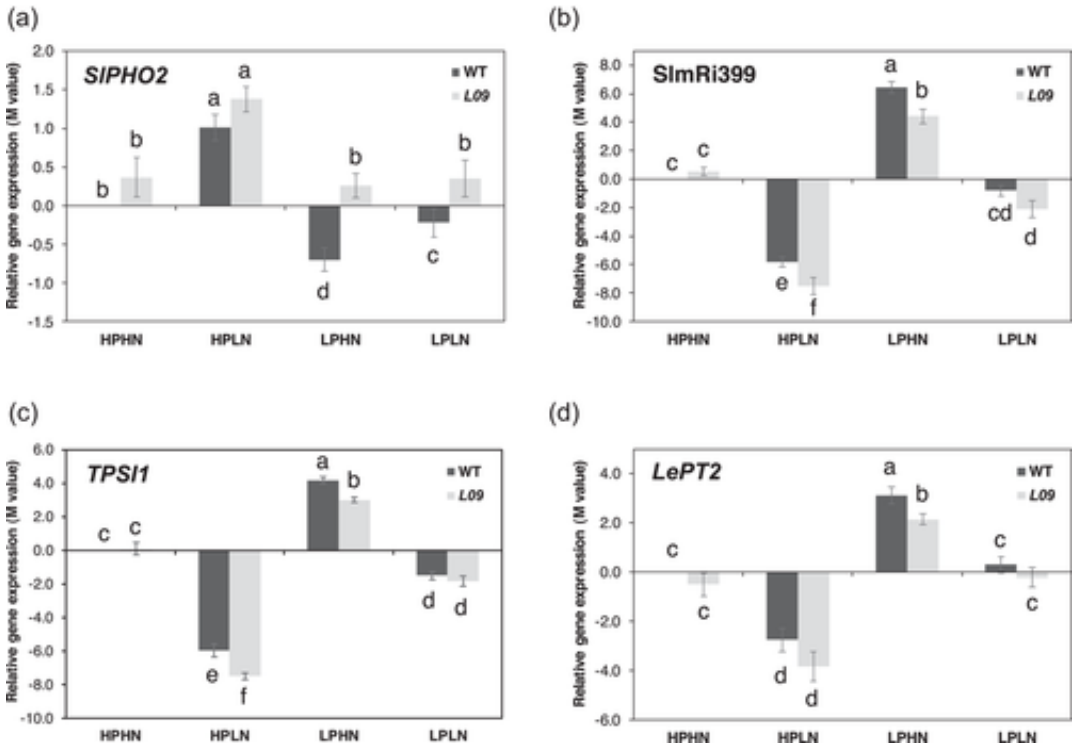

Figure 7 Expression analysis of genes associated to Pi signalling and homeostasis pathways in wild-type plants and in the SL-deficient line SlCCD8-RNAi L09. Effect of Pi and nitrate deficiency on the expression (M value) of genes encoding for the Pi signalling regulators SlPHO2 (a), SlmiR399 (b), LeTPSI1 (c) and the Pi transporter LePT2 (d) in tomato roots. Bars represent the means of six independent replicates (±SE). For data analysis, statistics and nutrient regimes see legends in Figure3

b). Remarkably, *SlCCD8*-RNAi L09 showed a higher root-to-shoot ratio than the wild-type in all the P-N combinations (Fig. S4c). The expression of the PSR signalling genes also showed a similar pattern to that observed previously for MoneyMaker (Fig. 4). The expression of *SlPHO2* was induced by N limitation and repressed by Pi deficiency in the wild type, a reduction that was diminished when both nutrients were limited (Fig. 7a). Interestingly, a different behaviour was observed for the SL-deficient line. *SlPHO2* expression was induced by N deficiency, as in the wild type; however, it did not respond to Pi starvation (Fig. 7a). The expression of the genes SlmiR399 and *LeTPSI1*, and *LePT2* also showed a similar pattern to that observed previously. Their transcript levels were reduced by N deficiency and promoted by Pi deprivation, being the induction abolished when both nutrients were limited (Figs. 7b-d). Remarkably, the effect by N and Pi deficiency was significantly lower in *SlCCD8*-RNAi L09 than in the wild-type, characterized by a decreased response to Pi deficiency (Figs. 7b-d).

As for PSR, the NSR regulatory genes also showed a similar pattern to that observed in experiment 1 with MoneyMaker (Figs. 5 and 8). *LeNRT1.1* and *LeNRT1.2* were repressed by N limitation, but Pi deficiency differentially affected their expression. Transcript levels of *LeNRT1.1* were decreased under Pi starvation, while those of *LeNRT1.2* were increased (Figs. 8a and b). When both nutrients were deficient, the expression of *LeNRT1.2* was down-regulated (Fig. 8b). Regarding the NRT2 genes, N deprivation induced the expression of *LeNRT2.1* and *LeNRT2.3*, while no changes were detected in *LeNRT2.2* (Figs. 8c-e). Conversely, Pi deficiency repressed the expression of the three genes, although this effect was reduced when both nutrients were limiting (Figs. 8c-e). The expression of *SlNIGT1.2/HHO2* was downregulated by N deficiency and induced by Pi starvation compared to control conditions (Fig. 8f). In the SL deficient line, the effects of N and Pi deficiency were generally in the same direction than in the wild-type, but significant differences were found between both genotypes, mainly under low N (Figs. 8b-f) and low Pi (Figs. 8a, b and e) conditions. Overall, the changes were less pronounced in *SlCCD8*-RNAi L09 than in the wild-type, supporting a role of SLs in the regulation of the key regulatory genes of NSR.

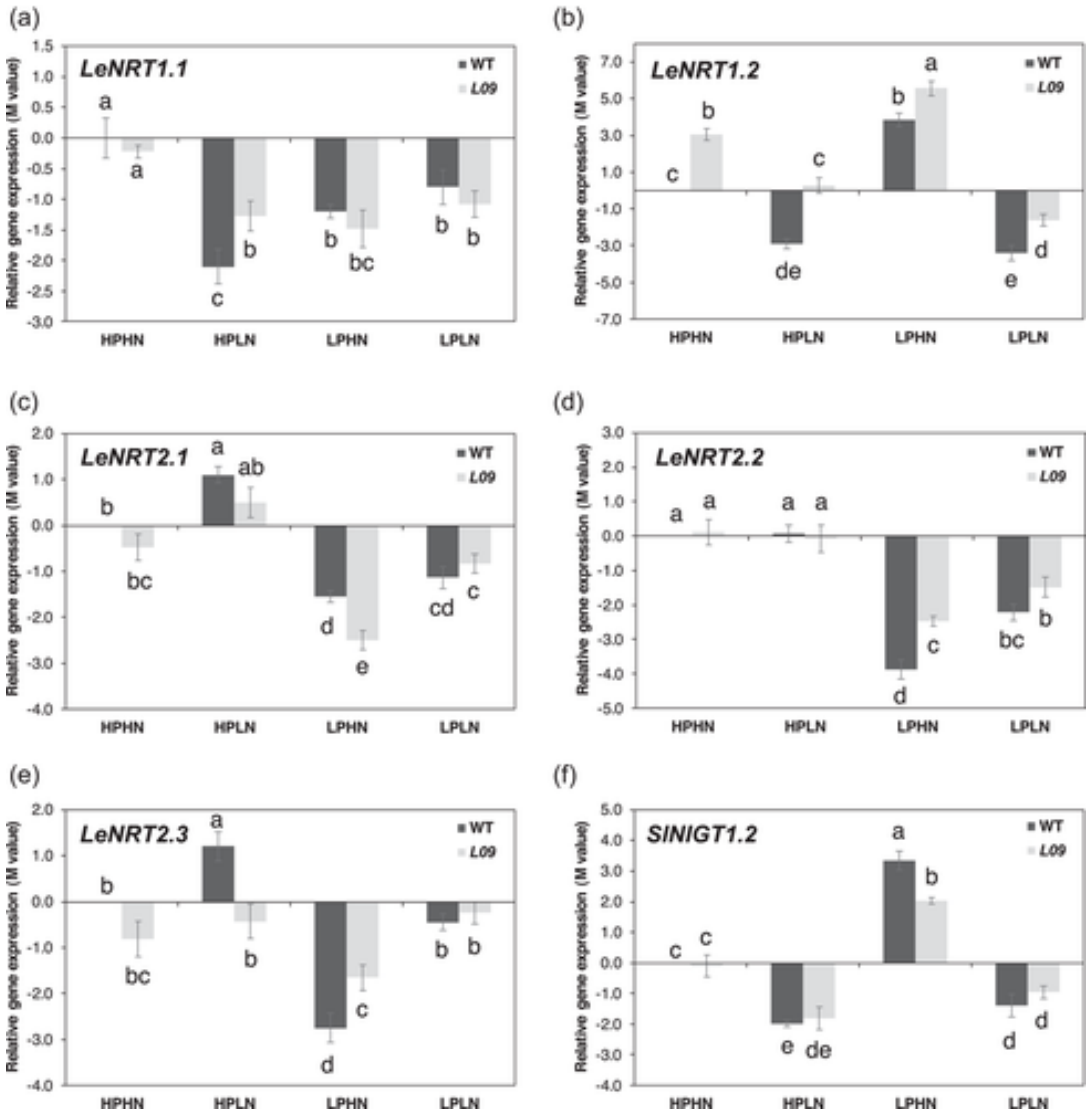

Figure 8 Effect of SLs on the expression of genes associated to the nitrate signalling pathway. Effect of the synthetic SL analogue 2'-epi-GR24 under normal (+P; grey bars) or deficient (−P; closed bars) Pi conditions in the expression (M value) of genes encoding for the nitrate signalling genes LeNRT1.1 (a), LeNRT1.2 (b), LeNRT2.1 (c), LeNRT2.3 (d) and SlNIGT1/HHO (e) in tomato roots. Plants were untreated (Control) or treated with 2'-epi-GR24 (GR24). Gene expression values were normalized using the housekeeping gene SlActin. Bars presents the means of five independent replicates (±SE). For statistics see legend in Figure 3.

DISCUSSION

Nitrogen as nitrate and/or ammonium and P in the form of Pi are the two most abundant macronutrients used by plants, being their coordinated use essential for optimal plant growth and maximal crop production (Hu & Chu, 2020; Nasr Esfahani et al., 2021; Oldroyd & Leyser, 2020).

Understanding how plants sense, signal and respond to N and Pi deficiency is crucial to optimize the use of these nutrients and reduce the need of fertilizers, alleviating agricultural costs and the excessive consumption of non-renewable resources. Under nutrient shortage, plants have the ability to optimize N and Pi uptake and use through a number of physiological adaptations. Thus, their overall growth is reduced, although the root system is usually expanded to facilitate nutrient foraging, increasing the root-to-shoot ratio (Hu & Chu, 2020; Oldroyd & Leyser, 2020). This is what we observed in this study with tomato plants grown under different nitrate and Pi regimes. The root-to-shoot ratio increased in plants subjected to nutrient deficiency, but the effect was stronger under N starvation. Plants grown under limiting N conditions performed worst in terms of growth and nutrient content independently of the Pi levels, suggesting that N status has a higher influence on plant growth and development than P status, as previously observed in Arabidopsis and rice (Medici et al., 2019).

SLs are phytohormones modulating plant growth under nutrient deficiency and stress conditions. It is well known that under nutrient limitation, mainly Pi, SLs modulate the coordinated development of roots and shoots to optimize nutrient uptake and use (Sun et al. 2014; Andreo-Jiménez et al. 2015; Waters et al. 2017; Santoro et al. 2021). Accordingly, SL biosynthesis is promoted by Pi starvation (López-Ráez et al., 2008; Yoneyama et al., 2012). In the present work, the highest promotion of the SLs solanacol and orobanchol was observed under Pi deficiency, but under sufficient N provision (LPHN). Under N deficiency, neither solanacol nor orobanchol were detected despite the induction of some biosynthetic genes. It might be that some SLs or SL-like compounds non-characterized so far were specifically promoted by nitrate deprivation. Interestingly, when both N and Pi were limiting, the increase in SLs triggered by Pi starvation was reduced (Fig. 3). A similar pattern was observed in alfalfa, where N deprivation supressed the promotion by P limitation of the SLs orobanchol and orobanchyl acetate (Peláez-Vico et al., 2016). These results suggest that N status influences SL levels, and that N deficiency has a negative impact in their induction by Pi starvation. Supporting this idea, no promotion of SLs was detected by N deficiency under sufficient Pi provision (Fig. 3), as previously reported in tomato and other plant species such as alfalfa and red clover (López-Ráez et al., 2008; Peláez-Vico et al., 2016; Yoneyama, Yoneyama, et al., 2007). A stimulatory effect of N starvation in SL biosynthesis has been reported in some plant species such as pea, sorghum and lettuce (Foo & Reid, 2013; Yoneyama et al., 2012; Yoneyama, Xie, et al., 2007). However, this effect was considerably weaker than that observed for Pi starvation. In line with this,

it was suggested that P but not N levels have a regulatory effect on SL biosynthesis (Yoneyama et al., 2012).

It is known that SLs act as sentinel molecules during Pi deficiency modulating the expression of key regulatory genes of PSR such as the triad IPS1-miR399-PHO2 in tomato and wheat (Fig. 1a) (Campos et al., 2018; Gamir et al., 2020). Here, we have confirmed the role of SLs in PSR signalling and addressed their involvement in NSR signalling. First, we have shown that SL biosynthesis and PSR signalling depend on plant N status. The expression of SlmiR399 and *LeTPSI1* was promoted under Pi deficiency, but their transcript levels were downregulated by N starvation, a repression that was also observed when both N and Pi were limiting. Remarkably, the effect by N and Pi deficiency in the SL-deficient line *SlCCD8*-RNAi L09 was significantly lower to that observed in the wild-type. The opposite pattern was observed for the PSR repressor *SlPHO2*, whose expression was repressed by Pi starvation and induced by N limitation in wild-type plants. The downregulation of *SlPHO2* by Pi deficiency was absent in the SL-deficient line, confirming a deficiency on the regulation of PSR in the SL-deficient line and supporting the role of SLs in this response. The repression of *SlPHO2* under Pi limitation was abolished when both nutrients were deficient (Fig. 4); showing again that the effect of N limitation overrules that of Pi limitation. An induction of *PHO2* levels by N deprivation has been previously shown in Arabidopsis (Medici et al., 2019). These authors also showed a de-repression of the PSR signalling genes in the *pho2* mutant, and proposed PHO2 as the integrator of the PSR and NSR signalling pathways (Medici et al., 2019). We found here that the expression of the triad LeTPSI1-miR399-PHO2 under the different P-N regimes agreed with that of SL levels, showing an interplay between the two signalling pathways, which depends on the plant's N status and where SLs seems to play a key role.

In agreement with this hypothesis, the expression of the N status sentinel genes NRT1, NRT2 and NIGT1/HHO was also regulated by SLs. The gene *LeNRT1.2* showed the same expression pattern as the PSR signalling genes SlmiR399 and *SlTPSI1* and correlated with that of SL levels (Figs. 3-8). *LeNRT1.2* was also induced by the exogenous application of the synthetic SL analogue 2'-*epi*-GR24 under optimal Pi conditions, mimicking the effect observed in Pi starvation and showing a SL dependency. LeNRT1.2 is homolog to the Arabidopsis nitrate transceptor (protein with transport and sensing function) AtNRT1.1, which triggers the PNR and NSR signalling pathways (Hu & Chu, 2020; Medici et al., 2019). This sensor shows a dual nitrate affinity depending on N availability (Ho et al., 2009; W. Wang et al., 2018). Here, a dual expression pattern was observed for the tomato

nitrate transporter *LeNRT1.2*, which could be associated to a dual nitrate affinity. Transcript levels of *LeNRT1.2* were increased under low Pi and optimal N conditions, suggesting a low affinity and high-capacity activity. Conversely, its expression was reduced by N deficiency under optimal Pi conditions, suggesting a high-affinity and low-capacity activity. This suggests that the tomato LeNRT1.2 could act as a nitrate transceptor during PNR similarly to AtNRT1.1 in Arabidopsis. Remarkably, the expression pattern of *LeNRT1.2* was opposite to that observed for the repressor *SlPHO2*. Since PHO2 integrates PSR and NSR signalling pathways under nutrient deficiency (Medici et al., 2019), and its expression is regulated by SL levels, we propose that SLs could modulate nitrate and Pi signalling through PHO2 by the regulation of the NRT1 sensors.

One of the mechanisms by which AtNRT1.1 modulates NSR signalling is through the regulation of some high-affinity transporters of the family NRT2, thus connecting PNR and NSR (Fig. 1b) (Maghiaoui et al., 2021; Medici et al., 2019). NRT2 transporters are involved in root nitrate influx, being their expression generally induced by N starvation (O'Brien et al., 2016). However, their transport capacity is low, so they are considered nitrate transceptors rather than transporters (Ho et al., 2009; Medici et al., 2015; O'Brien et al., 2016; W. Wang et al., 2018). Here, we show that the expression of the tomato *LeNRT2.3* was promoted by N deficiency but repressed by Pi starvation (Figs. 5 and 8). This expression pattern was opposite to that observed for SlmiR399 and *SlTPSI1*, and for SL levels. In addition, the repression under Pi deficiency was lower in *SlCCD8*-RNAi L09 than in the corresponding wild-type, suggesting a role for *LeNRT2.3* in both signalling pathways and the involvement of SLs in such regulation. Expression of *LeNRT1.2*, the proposed homologous to *AtNRT1.1*, was also opposite to that of *LeNRT2.3*. In Arabidopsis, the expression of *AtNRT2.1* is induced under N deficiency in an AtNRT1.1-dependent manner (Maghiaoui et al., 2021; Medici et al., 2019). Thus, the duo LeNRT1.2-LeNRT2.3 in tomato could play a similar role to AtNRT1.1-AtNRT2.1 in Arabidopsis, acting as nutrient sensors and connecting NSR and PSR signalling pathways through SL biosynthesis. Further studies are required to confirm this hypothesis.

Recently, the NIGT1/HHO family has been described as new player in the N-P signalling interplay. These transcriptions factors modulate Pi and nitrate uptake in order to maintain the P-N balance in plants. In Arabidopsis, the expression of NRT2 transceptors is regulated by AtNIGT1/HHO repressors in an AtNRT1.1-dependent manner (Fig. 1b) (Kiba et al., 2018; Maeda et al., 2018; Medici et al., 2015; X. Wang et al., 2020). In agreement with this, the expression of the putative tomato *SlNIGT1.2/HHO2* was repressed by N starvation. Therefore, it is tempting to speculate that this will

release the repression of *LeNRT2.3* to optimize N use under nitrate deficiency. Conversely, *SlNIGT1.2/HHO2* expression was induced by Pi starvation, which correlated with a repression of *LeNRT2.3*, probably to prioritize Pi uptake (Figs. 5 and 8). An induction of NIGT1/HHO genes under Pi deficiency was previously found in Arabidopsis and maize, coordinating Pi and nitrate uptake by targeting PHT1 Pi transporters and NRT1.1 (X. Wang et al., 2020). AtNIGT1/HHO can also target the Pi starvation signalling repressor AtPHO2 under Pi deficiency, activating Pi uptake and use (Kiba et al., 2018). Here in tomato, the induction of *SlNIGT1.2/HHO2* under Pi starvation also correlated with a reduction of *SlPHO2*, supporting a conserved regulatory mechanism across plant species. When Pi and N were limited, the expression of *SlNIGT1.2/HHO2* was also reduced, abolishing the induction by Pi starvation and indicating, once again, the priority for the plant of N over P status. The induction of *SlNIGT1.2/HHO2* by Pi deficiency was lower in *SlCCD8*-RNAi L09 compared to the wild-type, and it was induced by 2'-*epi*-GR24 under optimal Pi conditions, showing that its expression is regulated by SLs. The regulation by Pi starvation of all the analysed key elements in NSR was reduced in the SL-deficient line *SlCCD8*-RNAi L09 and mimicked under Pi sufficient conditions by exogenous SL application, confirming that SLs act as signals for Pi starvation. Since NIGT1/HHO transcription factors and NRT transceptors are important players integrating N-P signals, and their expression is regulated by endogenous SLs levels, we propose that SLs are key signals regulating the N-P interplay during fluctuating nutritional conditions.

Based on the present results, we propose an integrative model for the regulation of plant responses to nitrate and Pi deficiency (Fig 9). Under Pi deficiency and optimal N conditions, SL biosynthesis is induced. In the presence of SLs, PHR1 is released inducing the expression of miR399. In turn, miR399 reduces the levels of the repressor PHO2, activating the PSR pathway. SLs would also promote the expression of NRT1.1 (LeNRT1.2), either directly or in a PHO2-dependent manner, activating the PNR pathway. Subsequently, NRT1.1 and PHR1 would induce the expression of NIGT1/HHO repressors, blocking the expression of the high-affinity transporters/sensors NRT2 (LeNRT2.3) and inactivating the NSR pathway. A different scenario takes place under nitrate starvation, independently of Pi status, Here, SL biosynthesis is not promoted, just maintaining basal levels for normal plant growth. The absence of SLs gives rise to an up-regulation of PHO2, which blocks PSR signalling responses even when Pi levels are scarce. Low levels of SLs would also repress the expression of NRT1.1 (directly or through PHO2) inhibiting PNR, and those of NIGT1/HHOs. At the same time, this repression would allow the expression of the high-affinity NRT2 (LeNRT2.3) transceptors, activating NSR signalling.

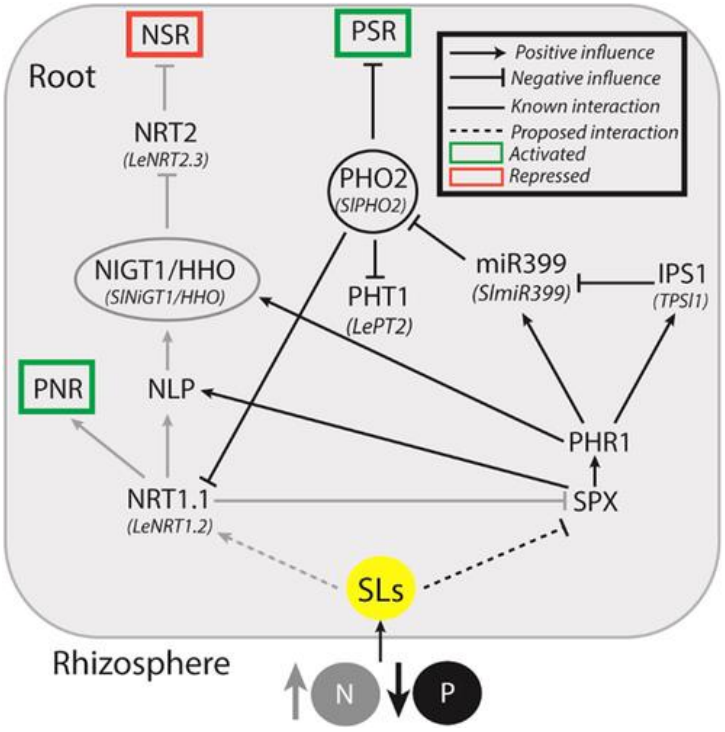

Figure 9 Proposed model for the regulation of plant responses to Pi and nitrogen deficiency and the potential role of SLs. Under Pi deficiency and optimal N conditions, SL biosynthesis is promoted, releasing PHR1 (via SPX degradation) and inducing the expression of miR399 and IPS1 (TPSI1). In turn, miR399 reduces the levels of the suppressor PHO2, activating the PSR pathway. Regarding N signalling, SLs would promote the expression of NRT1.1 (LeNRT1.2), either directly or in a PHO2-dependent manner, activating nitrate transport through the PNR pathway. At the same time, NRT1.1 (via NLPs) and PHR1 would induce the expression of the repressors NIGT1/HHOs, blocking the expression of the high-affinity transporters/sensors NRT2 (LeNRT2.3) and inactivating the NSR pathway.

Overall, our results provide evidences showing that SLs are early modulators of plant responses to Pi and nitrate starvation, acting as key signals in the N-P interplay. They modulate the expression of key regulatory genes of both signalling pathways and that of the N-P integrators such as the PHO2 and NIGT1/HHO repressors. The fact that the regulation of these genes is not completely abolished in SL-depleted plants indicates that other(s) regulatory mechanism(s), in addition to SLs, may also be involved in the N-P interplay. Further research is required to decipher these other mechanisms/molecules. We also show that plants prioritize responses to N over P limitation, N deficiency influencing strongly Pi starvation responses, probably through the regulation of SL biosynthesis. This knowledge will help to develop new strategies to optimize plant N and P uptake and usage, alleviating cost and reducing the excessive use of chemical fertilizers in agriculture.

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SUPPLEMENTAL MATERIAL

Figure S1. Analysis of different growth parameters in tomato plants subjected to different Pi and nitrate regimes. Calculation of shoot/root ratios (a) and root length (b). For nutrient regimes see legend in Figure 3. Bars represent the means of ten independent replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$) according to the Tukey test.

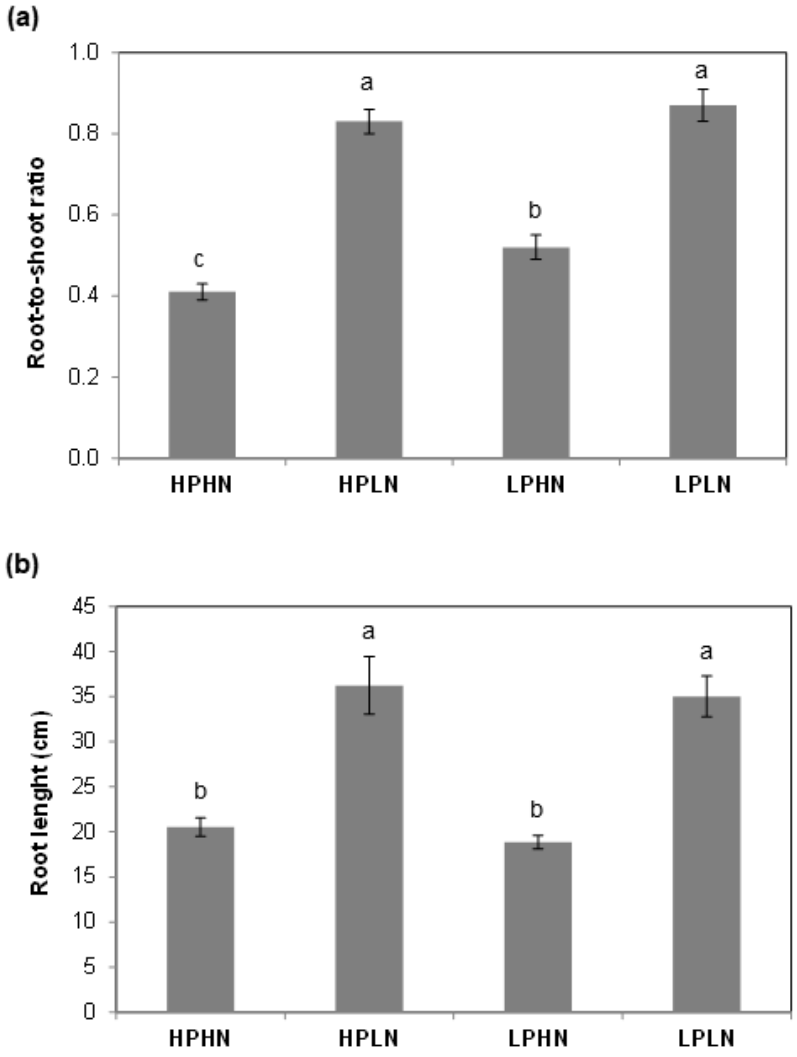

Figure S2. Picture showing the different anthocyanin accumulation in the upper face and in the underside of tomato leaves subject to different Pi and nitrate regimes. For the different nutrient regimes see legend in Figure 2.

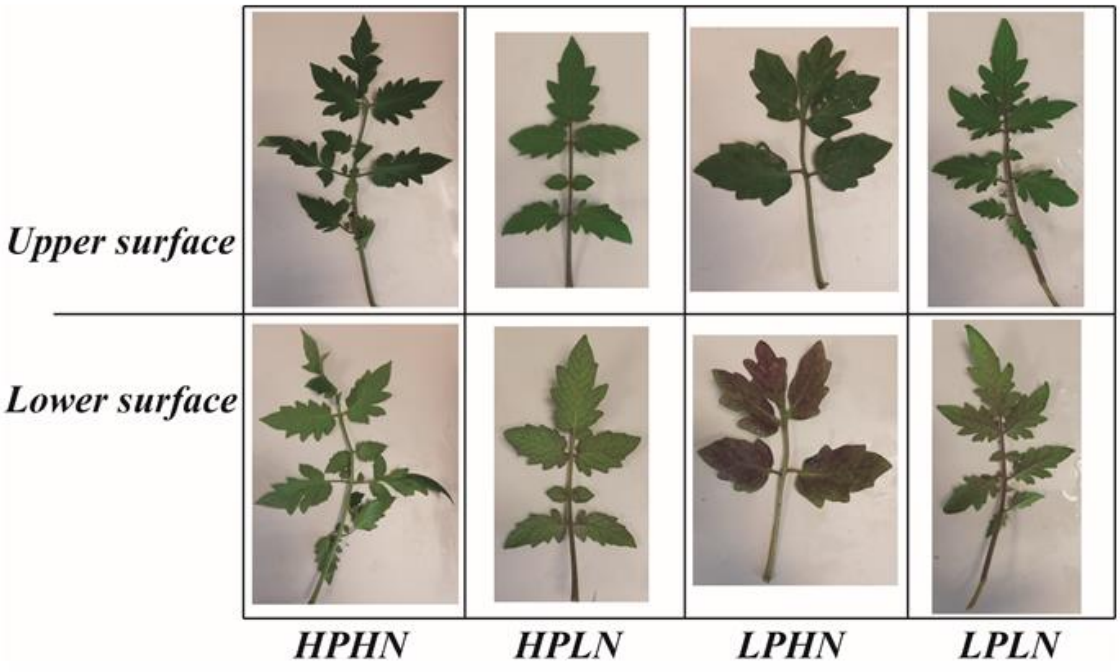

Figure S3. Effect of Pi and nitrate deficiency on SL biosynthesis. Tomato plants (wild-type cv. Craigella and the SL-deficient line *SlCCD8*-RNAi L09) were grown under different nutrient regimes, as described in the legend to Figure 2. Gene expression analysis (M value) of the SL biosynthesis genes *SlD27* (a), *SlCCD7* (b) and *SlCCD8* (c) in roots from 6-week old plants. Expression values were normalized using the housekeeping gene *SlEF*. M value (log2 ratio) is zero if there is no change; '+1' or '-1' indicate two-fold change induction or repression, respectively. Content of the SLs solanacol (d) and orobanchol (e) in root exudates. Bars represent the means of six independent replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$) according to the Tukey test.

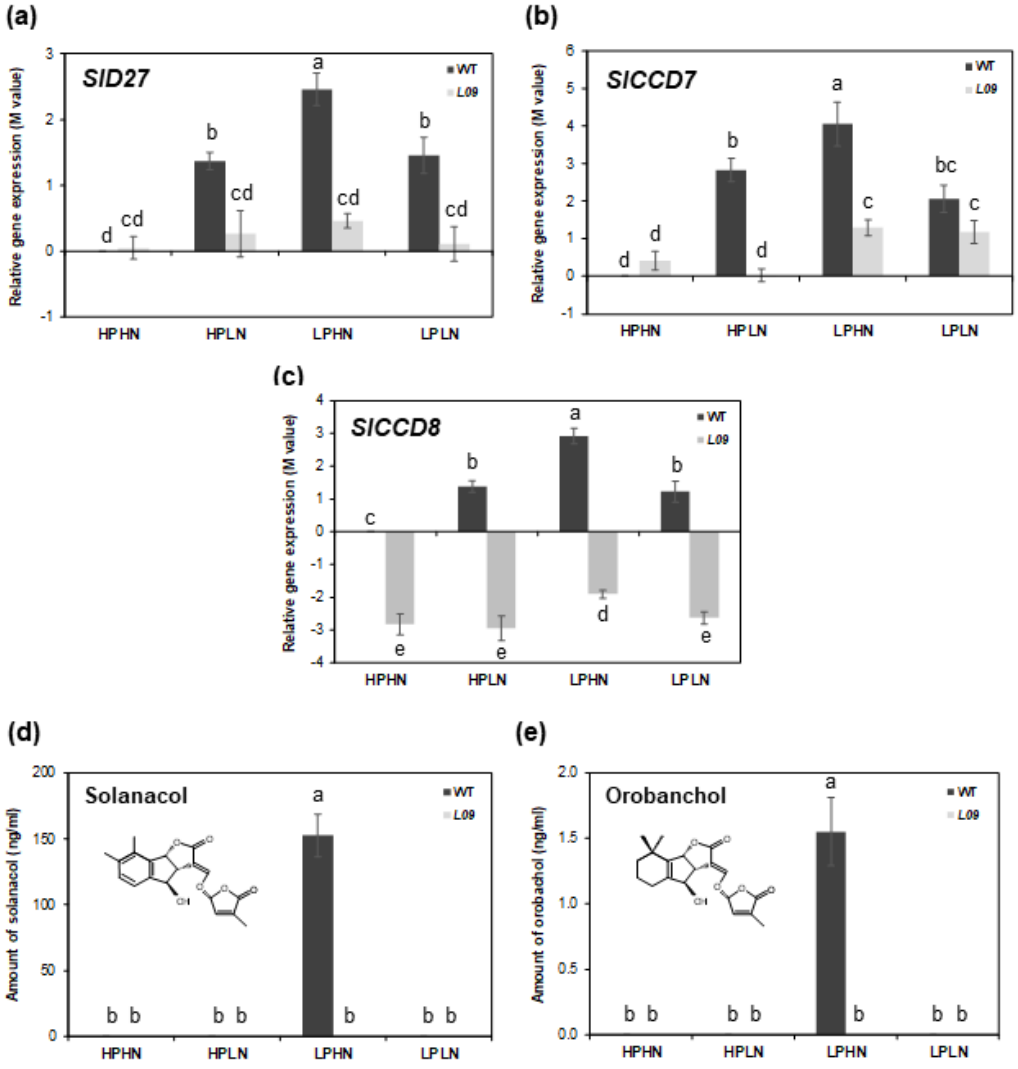

Figure S4. Effect of different Pi and nitrate regimes on tomato growth and performance in wild-type (cv. Craigella) plants and in the SL-deficient line *SlCCD8*-RNAi L09. Analysis of shoot and root fresh weight in wild-type (WT) plants (a) or in *SlCCD8*-RNAi L09 (L09) (b). Calculation of shoot/root ratios in wild-type (WT) or in *SlCCD8*-RNAi L09 (L09) (c). For the different nutrient regimes see legend in Figure 2. Bars represent the means of six independent replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$) according to the Tukey test.

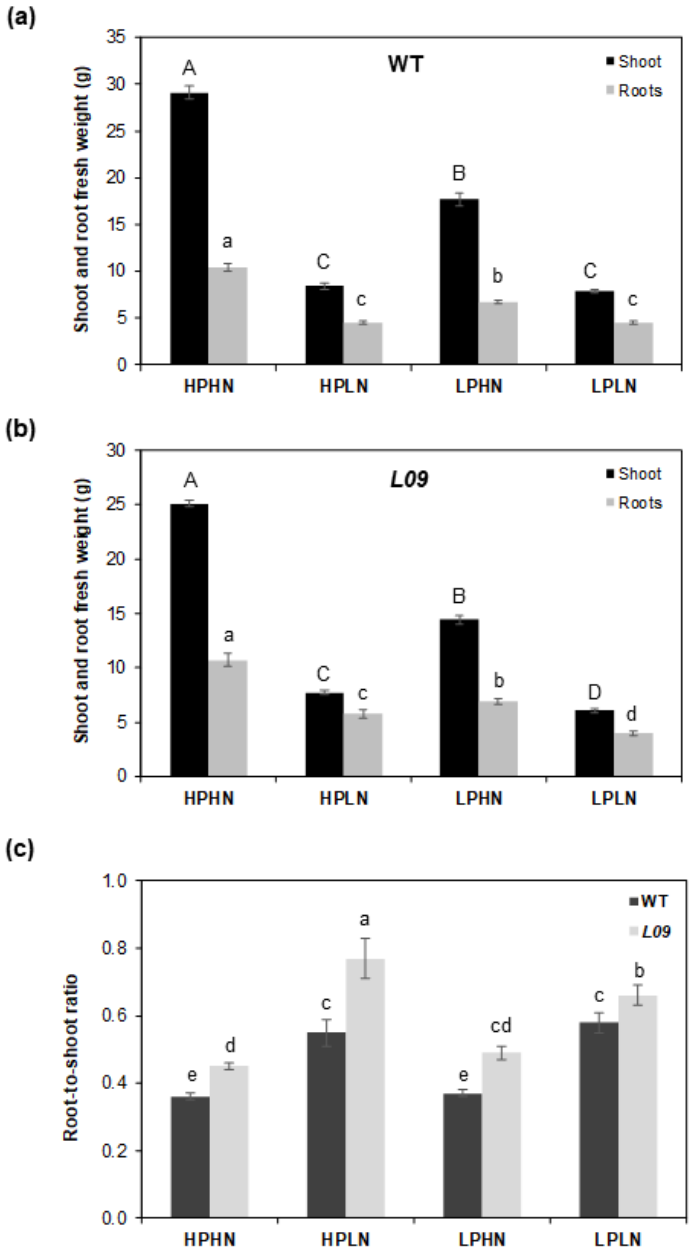

Table S1. Modified nutrient solutions (Hewitt, 1996) used for the different nutritional regimes. HPHN (high P and high N), HPLN (high P and low N), LPHN (low P and high N) and LPLN (low P and low N).

					HPHN		LPHN		HPLN		LPLN	
					100% Pi 100% N		25% Pi		25% N		25% Pi 25% N	
Compound	Mw	Stock sol. (g/L)	[mM]	Vol. stock solution	Vol. for 1L working sol.	[Final] (mM)	Vol. for 1L working sol.	[Final] (mM)	Vol. for 1L working sol.	[Final] (mM)	Vol. for 1L working sol.	[Final] (mM)
$MgSO_4 \cdot 7H_2O$	246.48	18.4	74.65	1L	20	1.4930	20	1.4930	20	1.4930	20	1.4930
Fe-EDTA	367.05	2.45	6.67	1L	10	0.0667	10	0.0667	10	0.0667	10	0.0667
$MnSO_4 \cdot H_2O$	169.02	1.35	7.99	0.25L	1	0.0080	1	0.0080	1	0.0080	1	0.0080
$CuSO_4 \cdot 5H_2O$	249.68	2.4	9.61	0.25L	0.1	0.0010	0.1	0.0010	0.1	0.0010	0.1	0.0010
$ZnSO_4 \cdot 7H_2O$	287.53	4.22	14.68	0.25L	0.1	0.0015	0.1	0.0015	0.1	0.0015	0.1	0.0015
H_3BO_3	61.83	18.6	300.82	0.25L	0.1	0.0301	0.1	0.0301	0.1	0.0301	0.1	0.0301
$Na_2MoO_4 \cdot 2H_2O$	241.95	0.35	1.45	0.25L	0.1	0.0001	0.1	0.0001	0.1	0.0001	0.1	0.0001
KNO_3	101.1	30.3	299.70	1L	10	2.9970	10	2.9970	**2.5**	0.7493	**2.5**	0.7493
$Ca(NO_3)_2 \cdot 4H_2O$	236.15	101.54	429.98	1L	20	8.5996	20	8.5996	**5**	2.1499	**5**	2.1499
$H_2NaPO_4 \cdot H_2O$	137.99	18.4	133.34	1L	10	1.3334	**2.5**	0.3334	10	1.3334	**2.5**	0.3334

Table S2. Primer sequences used in qPCR analysis.

Pathway	ID	Gene	Primers (5'-3')
SLs	AK323242	*SlD27*[1]	AGCCAAGAATTCGAGATCCC GGAGAAAGCCCACATACTGC
	GQ468556	*SlCCD7*[2]	AGCCAAGAATTCGAGATCCC GGAGAAAGCCCACATACTGC
	JF831532	*SlCCD8*[2]	CCAATTGCCTGTAATAGTTCC GCCTTCAACGACGAGTTCTC
Pi signalling	AF022874	*LePT2*[2]	AGTGGGAGCGTATGGGTTCTTA TTCCAAGTGCATTGATACAGCC
	T34808	*LeTPSI1*[2]	GAGGTGGCTCTCGTCGTTGAT TCTGCCTTATCCTTGAGATTGC
	NR_108003	*SlmiR399*[3]	ACACTCTATTGGCATGCAAC GCAACTCTCCTTTGGCATT
	Solyc02g078210	*SlPHO2*[3]	TCCAACTTTGCAGGACTCA CTTTGAATACTCTTTCGCACA
N signalling	X92853	*LeNRT1.1*[4]	TACTATTCAAGCTATGGGTGTTACG ATTTGTCCTCTTTCTTTTTTGTCCG
	X92852	*LeNRT1.2*[4]	TTTTAGGTGTTGAAGCTGTGGAGAG GCGATGTATAGGACCATGAGTTGTT
	AF092655	*LeNRT2.1*[5]	TTCCTGTTACATTTTGTCATTTCCC CAGATTCAAGACTATCCATTCCTCA
	AF092654	*LeNRT2.2*[5]	TCAAGGGAACGGAAGAACATTATTA GCTCATTGAACTAAAGATTGACGAT
	AY038800	*LeNRT2.3*[5]	AATGCATGGTGTTACTGGTAGAGAG CTAATAATAGGGACTAAAGGGGCT
	Solyc05g009720	SlNIGT1.2/HHO2[8]	ATCTGATTGGCTTAGATCTGT TGAAATGCTCCTCCACTTCC
Normalizer	X14449	*SlEF-1*[6]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC
	U60478	*SlActin2*[7]	TTGCTGACCGTATGAGCAAG GGACAATGGATGGACCAGAC

[1]Torres-Vera *et al.*, 2016; [2]Kohken *et al.*, 2012; [3]Gamir *et al.*, 2020; [4]Yao *et al.*, 2008; [5]Sánchez-Bel *et al.*, 2016; [6]Rotenberg *et al.*, 2006; [7]Yan *et al.*, 2013; [8]This work.

Table S3. Effect of different nutrient regimes (phosphate, Pi and nitrate, N) and their interaction on the different physiological parameters measured in this study, according to two-way ANOVA.

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

	Factors					
	Pi		Nitrate		Phosphorus*Nitrogen	
	F-value	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
Root fresh weight	108.35	***	239.6	***	51.3	***
Shoot fresh weight	315.4	***	1068.04	***	219.3	***
Nitrogen (mg/g)	84.9	***	488.5	***	54.4	***
Phosphorus (mg/g)	208.6	***	115.9	***	29.2	***

Table S4. Nutrient content in leaves from tomato plants grown on different phosphorus (P) and nitrate (N) combinations. For the different nutrient regimes see legend in Figure 2. Values represent the means of 6 independent replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$) according to the Tukey test.

	Nutrient content (mg/g)					
Treatment	**Ca**	**K**	**Mg**	**Na**	**S**	**Si**
HPHN	23.95 ± 0.96bc	19.53 ± 0.39a	2.58 ± 0.44b	0.21 ± 0.01a	5.53 ± 0.14a	0.64 ± 0.01ab
HPLN	19.31 ± 0.38c	11.33 ± 0.82b	2.27 ± 0.68b	0.11 ± 0.01b	4.20 ± 0.11b	0.53 ± 0.02b
LPHN	40.90 ± 1.22a	20.38 ± 0.70a	4.57 ± 0.16a	0.18 ± 0.01a	4.66 ± 0.13ab	0.65 ± 0.02ab
LPLN	20.52 ± 0.56c	14.00 ± 0.35b	2.51 ± 0.43b	0.12 ± 0.00b	4.10 ± 0.14b	0.63 ± 0.04ab

	Nutrient content (µg/g)					
Treatment	**Fe**	**Cu**	**Mn**	**Sr**	**Zn**	**Al**
HPHN	2.1 ± 0.02b	6.8 ± 0.42b	51 ± 3b	16.0 ± 1.0b	40 ± 4a	12.0 ± 1.0b
HPLN	1.7 ± 0.01c	3.7 ± 0.43c	33 ± 1c	13.0 ± 0.4b	23 ± 2c	6.0 ± 0.6c
LPHN	2.4 ± 0.02a	10.0 ± 0.41a	150 ± 8a	28.0 ± 1.0a	29 ± 1b	24.0 ± 3.0a
LPLN	1.7 ± 0.03c	4.0 ± 0.15c	40 ± 3c	15.0 ± 0.5b	27 ± 2bc	10.0 ± 0.7b

Chapter 2

Flavonoids promote *Rhizophagus irregularis* spore germination and tomato root colonization

Flavonoids promote *Rhizophagus irregularis* spore germination and tomato root colonization

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ABSTRACT

The use of arbuscular mycorrhizal (AM) fungi as bioinoculants in agricultural and natural ecosystems has great potential, being used as biostimulants, biofertilizers and bioprotection agents. However, despite all their potential benefits, the application of AM fungal inoculants in agriculture is still challenging because of the variability of the results when applied in production systems. Therefore, we need to find solutions to reduce such variability and promote symbiosis establishment to make them more efficient. In addition to strigolactones, flavonoids have been proposed to participate in the pre-symbiotic plant-AM fungus communication in the rhizosphere, although their involvement is still unclear. Here, we studied the specific role of flavonoids as signaling molecules in AM symbiosis. For that, both *in vitro* and *in planta* approaches were used to test the stimulatory effect on spore germination and AM symbiosis establishment of the AM fungus *Rhizophagus irregularis* of an array of different subclasses of flavonoids at 'physiological' doses. We show that the flavone chrysin and the flavonols quercetin and rutin were able to promote spore germination and AM symbiosis at low doses, confirming the role of these flavonoids as pre-symbiotic signaling molecules together with strigolactones. The results open the possibility of using these flavonoids in the formulation of mycorrhizal inoculants as promoters of the symbiosis, thus improving the efficiency of commercial inocula, and helping to implement their use in sustainable agriculture.

INTRODUCTION

The growing human population requires a considerable increase in food production, leading to overexploitation of natural resources (Godfray et al., 2010). Crop varieties with higher yields and greater resistance to environmental stresses and diseases are currently being developed. However, massive use of chemical fertilizers and pesticides is still required to provide essential nutrients and reduce disease damage in agricultural production systems. The use and abuse of these chemical products in agriculture are having a huge environmental impact, polluting soils and aquifers, contributing to climate change, and thus negatively affecting people, ecosystems and species worldwide (Evans et al., 2019; Lynch et al., 2021; Tilman et al., 2002). Therefore, there is an urgent need to find more sustainable and environmentally friendly alternatives to reduce the use of these harmful agrochemicals (Geiger et al., 2010).

One strategy that is gaining momentum worldwide is the use of beneficial microorganisms with biostimulant properties. These microorganisms establish symbiotic

associations with plants and significantly improve agroecosystems and crop production (Tkacz & Poole, 2015). Among these beneficial microorganisms stand out the arbuscular mycorrhizal (AM) fungi (belonging to the phylum *Glomeromycota*). AM fungi establish mutualistic associations with more than 70% of land plants, including most species with agronomic and industrial interest (cereals, vegetables, fruit trees, cotton, etc.), as well as ornamental and forest species (Barea et al., 2005; Brundrett & Tedersoo, 2018). This mutualistic association is known as AM symbiosis and is about 450 million years old (Heckman et al., 2001; Remy et al., 1994). It is characterized for the formation of specific structures within the roots of the host plant known as arbuscules (Parniske, 2008). In the arbuscules takes place the nutrient exchange between the fungus and the host plant (Bonfante & Genre, 2010). In addition to the arbuscules, the AM fungus develops a large network of hyphae, known as extraradical mycelium, which serves to explore larger areas of soil and constitutes the assimilative structure for mineral nutrients and water, thus functioning as pseudo roots (Parniske, 2008). The benefits of AM symbiosis in plant nutrition and health are well known (Barea et al., 2005; Wipf et al., 2019). In addition to a better nutrition, AM symbioses offer other benefits to the host plant including improved defense responses to pathogens and increased resilience to environmental stresses, such as drought and salinity (Pozo et al., 2015).

Despite the potential benefits of AM fungi, their application in agricultural settings remains a challenge due to the variability of results when applied to production systems, which hinders their commercialization and implementation (Tkacz & Poole, 2015). AM fungi are obligate biotrophs, so they depend on a host plant to develop and complete their life cycle (Parniske, 2008). This fact makes it difficult to implement the production of stable and homogeneous inoculants based on AM fungi. In addition, these inoculants are non-sterile with potential contamination problems. Therefore, most AM fungal products on the market are spore-based, which are easier to quantify and store, with higher homogeneity and lower risk of contamination. However, spore production is costly, and they take longer to colonize the roots. Moreover, the implementation of AM fungi as biostimulants at the agronomic and forestry level is being hindered by the variability of their effectiveness under field conditions. This variability resides mainly in three factors: a) the quality and effectiveness of the inoculants, b) the environmental conditions and c) the management techniques, especially chemical fertilization.

The establishment and proper functioning of AM symbiosis requires a high degree of communication and coordination between the AM fungus and the host plant (López-Ráez et al., 2017; Pozo et al., 2015). The molecular dialogue is initiated during the pre-symbiotic phase with the production and exudation into the rhizosphere of signaling molecules by the plant, primarily

strigolactones (SLs; López-Ráez et al., 2017). SLs are specifically recognized by the AM fungi present in the vicinity of the roots, stimulating spore germination, hyphal branching and exudation of fungal Myc-factors, thus facilitating the contact between the two partners and the establishment of the symbiosis (Akiyama et al., 2002; Besserer et al., 2006; Bonfante & Genre, 2010). SLs are derived from carotenoids and, due to their signaling role, their production by the plant occurs at very low amounts (on the order of pico- and nanomolar), and highly depends on the plant's nutritional status (López-Ráez et al., 2008; Marro et al., 2022; Yoneyama et al., 2012). In addition to signaling compounds in the rhizosphere, SLs are plant hormones related to plant responses to nutritional stresses, especially phosphate (Pi) deficiency (Gomez-Roldan et al., 2008; Umehara et al., 2008).

In addition to SLs, other plant-derived compounds such as flavonoids have been proposed to participate in the pre-symbiotic molecular dialogue in AM symbiosis (reviewed in (Hassan & Mathesius, 2012). However, their specific role and functioning is not entirely clear. Flavonoids comprise a large and diverse family of ubiquitous secondary metabolites, belonging to the phenylpropanoids. They play a diverse array of biological functions in plants, acting as antioxidants, pigments in flowers, fruits and vegetables, regulators of auxin transport, fertility, defense barriers against herbivores and microbial pathogens (phytoalexins), regulating root architecture and as signaling compounds in beneficial plant-microbe symbioses in the rhizosphere (Hassan & Mathesius, 2012). So far, more than 10,000 different flavonoids have been characterized. According to the chemical structure, they are subcategorized into different major groups, including flavonols, anthocyanins, flavones, isoflavonoids, flavanonols, flavanones, flavanols, and chalcones (Fig. 1) (Panche et al., 2016). Regarding their role as signaling molecules in the rhizosphere, the most studied and best-known function is that associated to the *Rhizobium*-legume symbiosis (Singla & Garg, 2017). This beneficial symbiosis is established between legumes and certain rhizobacteria, and it leads to the fixation of atmospheric nitrogen in an organelles called nodules, thus providing nitrogen to the host plant under nitrogen deficiency (Masson-Boivin & Sachs, 2018). The pre-symbiotic and symbiotic stages in the *Rhizobium*-legume symbiosis are similar to that of the AM symbiosis, indeed they share some of the components giving rise to the SYM pathway (de Bruijn, 2020; Mukherjee & Ané, 2011). Here, the molecular dialogue during the pre-symbiotic phase is initiated with the production and exudation into the rhizosphere of certain flavonoids (isoflavonoids; Fig. 1). Isoflavones are involved in the pre-symbiotic and symbiotic stages of the symbiosis, participating in the recruitment of compatible rhizobia by inducing or inhibiting bacterial Nod factors (Mandal et al., 2010; Shaw et al., 2006).

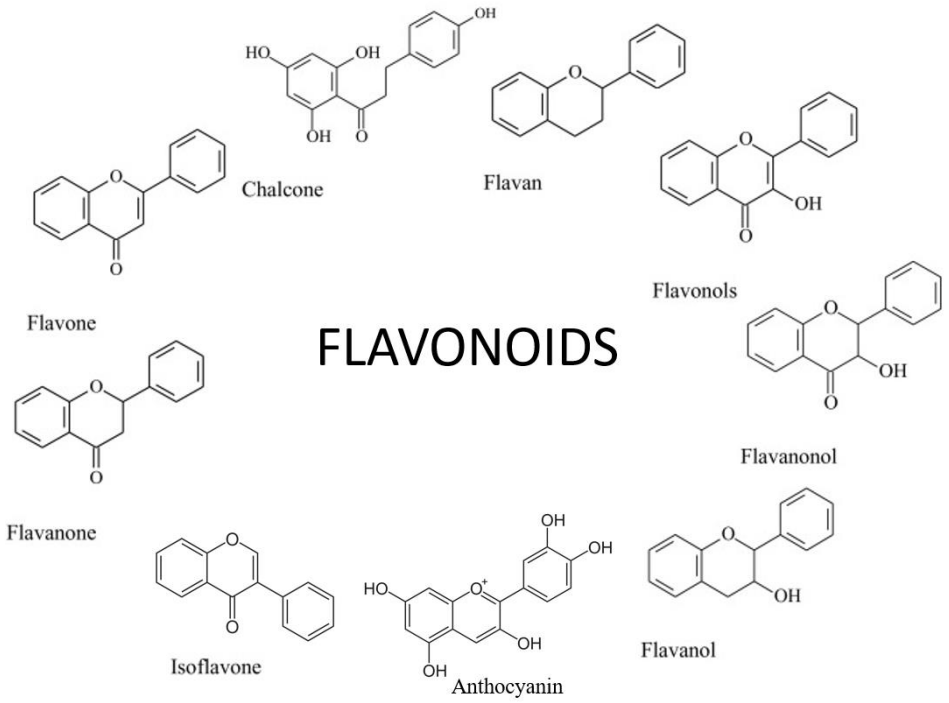

Figure 1 Schematic overview of the different groups of flavonoids according to their chemical structure.

At first, the role of flavonoids in AM symbiosis was questioned. Bécard and co-workers observed that maize mutants deficient in flavonoid biosynthesis showed a normal mycorrhizal colonization so proposed that they were not involved in the symbiosis (Bécard et al., 1995). Few years later, other authors showed that certain flavonoids presented activity either stimulating AM fungus spore germination or root colonization (Akiyama et al., 2002; Scervino et al., 2007; Steinkellner et al., 2007). However, the role of flavonoids in AM symbiosis is controversial as positive, negative or neutral results have been described (Vierheilig et al., 1998). Its role has been studied under very different experimental conditions, using different flavonoids, different concentrations and different partner genotypes (Singla & Garg, 2017; Vierheilig et al., 1998). However, their effect seems to be dependent on the AM fungus genotype and that different compounds may be involved at different stages of the AM symbiosis, controlling both positively and negatively pre-symbiotic signaling as well as symbiosis development and autoregulation (Scervino et al., 2005a, 2005b; Vierheilig & Piché, 2002 reviewed in Singla & Garg, 2017).

In the present study, we tried to shed light on the the specific role of flavonoids as signaling molecules in the AM symbiosis. Different flavonoids belonging to different subcategories and at different concentrations have been tested, both *in vitro* and *in planta*, for their capacity of inducing spore germination and stimulating colonization of *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler 2010 (Formerly known as

Glomus intraradices), the most widely used AM fungus in commercial products in the market. The results show that there is a class specificity and the importance of the dose used.

MATERIAL AND METHODS

In vitro germination of spores of the AM fungus *R. irregularis*

The experiments were carried out in 90 mm diameter Petri dishes with 35 ml of agar medium (2%) in deionized water under sterile conditions. The flavonoids used were the flavonols quercetin and rutin (Sigma-Aldrich, Germany), the flavone chrysin (Sigma-Aldrich, Germany), the isoflavone genistein (Sigma-Aldrich, Germany) and the pterocarpene medicarpin (kindly provided by Francisco Macías Lab). As positive control, the active enantiomer of the synthetic SL GR24 (2'-*epi*-GR24) (Scaffidi et al., 2014) was used. For the preparation of the different treatments, stock solutions (1 mM) were prepared by dissolving the different compounds in pure acetone. Prior the addition to the Petri dishes, all flavonoid dilutions were sterilized using 0.22 µm filters. Before preparation of the treatments serial dilutions in acetone were prepared for each compound. Then, in a laminar flow hood, 50 µl of the corresponding dilution was added per plate and spread homogeneously over the entire agar surface using a seeding loop. All treatments, including the controls, had a final concentration of acetone in the plate of 1‰. The plates were kept open for 1 h to allow absorption of the added compounds and the evaporation of the acetone. Subsequently, a solution with 15 axenic (surface sterilized) spores of the AM fungus *R. irregularis* [MUCL 57021; kindly supplied by Koppert Biological Systems (The Netherlands)] were added per plate. Then, the plates were sealed and incubated upside down at darkness at 25°C. Spore germination was evaluated daily. Due to the presence of multiple hyphae from the starter inoculum, germination was quantified by assessing the growth of new hyphae through the culture medium. Two independent experiments were performed with different concentrations of flavonoids, always ranging physiological concentrations. For the experiment 1, 5 independent replicates per treatment were used [5 plates with 15 spores per plate; therefore (75 spores per treatment)]. For the experiment 2, 7 replicates per treatment [7 plates with 15 spores per plate (105 spores per treatment)] were used.

AM colonization *in planta*

Tomato (*Solanum lycopersicum* L.) seeds of the genotypes Red Cherry (LA0337) were surface sterilized with 50% commercial bleach for 10 min and after washed thoroughly with tap

water. The seeds were then sown in sterilized vermiculite and incubated at 25–27°C, 16 h/8 h (day/night) and 65–70% RH in a climatic chamber. Ten-day-old seedlings were transplanted individually into 100 ml alveoli with sterile sand:vermiculite (1:1). Plants were inoculated with 675 spores of *R. irregularis* (MUCL 57021; Ri plants) supplied by Koppert Biological Systems (The Netherlands). As mycorrhizal control, a set of uninoculated plants was included (Nm plants). Ri plants were treated with quercetin, rutin, chrysin or genistein, at two different concentrations 0.01 and 0.1 µM. As a positive control, a treatment with the synthetic SL analogue 2'-*epi*-GR24 ($GR24^{4DO}$) was included. Negative controls were also included with plants untreated with any compound. For the application of the different compounds (flavonoids and 2'-*epi*-GR24), stock solutions (1 mM) were prepared by dissolving the different compounds in 100% acetone. Prior to their addition, the corresponding serial dilutions of the different compounds were prepared in Hewitt's nutrient solution (Hewitt, 1953), at a final acetone concentration of 1‰. To favor the symbiosis, modified Hewitt's solution was used, containing 25% of the standard phosphate levels. Plants were treated 2 times per week with the different compounds, using a volume of 10 ml. The control (untreated) treatments were also irrigated twice a week with 10 ml of 1‰ acetone. Ten independent replicates per treatment were used. Mycorrhizal levels were assessed 6 weeks after transplanting.

Quantification of mycorrhizal colonization

Quantification of mycorrhizal colonization was performed by histochemical staining as described in García et al. (2020). Roots were cleared and digested in a solution of 10% KOH (w/v) for 2 days at room temperature. The alkaline solution was washed thoroughly with tap water and acidified with a 2% (v/v) acetic acid solution. The fungal root structures were histochemically stained with a 5% (v/v) black ink (Lamy, Germany) and 2% acetic acid solution incubated at room temperature (Vierheilig et al., 2005). After 24 h the ink was washed with water and colonization was determined by the gridline intersection method (Giovannetti & Mosse, 1980), using a Nikon SMZ1000 stereomicroscope.

Statistics

Student's t-test was used to find significant differences between the means. Since the percentage of germination and mycorrhizal colonization did not have a normal distribution, the Bliss transformation was applied to the data before the analysis.

RESULTS

Effect of flavonoids on the germination of spores of *R. irregularis in vitro*

To deepen in the role of flavonoids as pre-symbiotic signals in AM symbiosis, the capacity of a series of flavonoids belonging to different subcategories of stimulating the germination of spores of the AM fungus *R. irregularis* was assessed *in vitro*. The flavonoids tested were: genistein (isoflavone), medicarpin (pterocarpene), chrysin (flavone), and the compounds present in tomato quercetin and rutin (flavonols) (Fig. 1). Different concentrations, ranging physiological levels, for the different compounds were used to evaluate both their effect and the most effective dose. Since most commercial products based on AM fungi use *R. irregularis* as biostimulant, spores of this fungus were used in our experiments. Two independent experiments were assessed:

Experiment 1

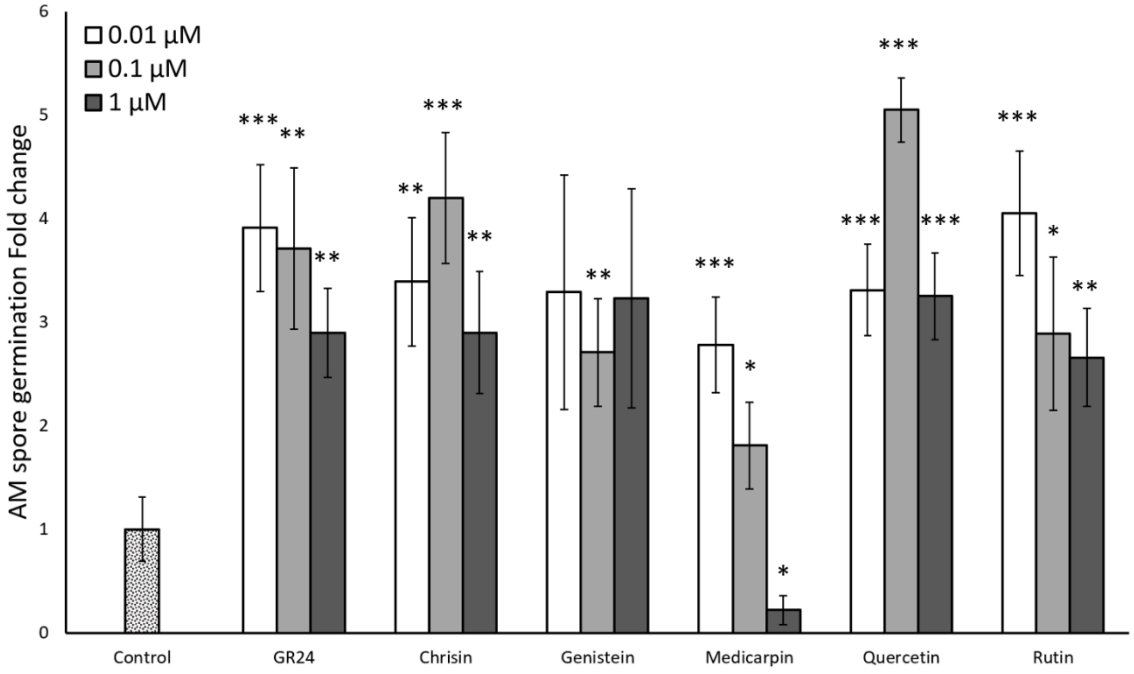

*Figure 2 Relative percentage of germination of spores of R. irregularis in vitro. Spores of R. irregularis were incubated for 10 days in Petri dishes with 2% Agar medium with three different concentrations of the flavonoids studied (0.01, 0.1 and 1 µM). The application of the synthetic strigolactone 2'-epi-GR24 was used as a positive control. The bars correspond to the mean of 5 independent replicates (15 spores/replicate) ± standard error. Statistical analysis was performed with t-test analysis between each treatment.compared with the control. * p<0.05, ** p<0.01, ***p<0.001.*

In a first assay, three different concentrations (0.01, 0.1 and 1 µM) of the different flavonoids were tested. As previously mentioned, SLs are well-known pre-symbiotic signals in AM symbiosis, having the ability to stimulate spore germination and hyphal branching of AM fungi (Akiyama et al., 2005; Besserer et al., 2006). Therefore, a treatment with an active enantiomer of the synthetic SL GR24 - 2'-*epi*-GR24 ($GR24^{4DO}$; Scaffidi et al., 2014) was included as a positive control. Spore germination was checked daily from the third day. Here, germination levels were quantified 10 days upon application. 2'-*epi*-GR24 induced spore germination at all three concentrations used, showing a slight decrease at the highest concentration (1 µM), indicating that the spores were viable and active (Fig. 2). The five flavonoids tested (genistein, medicarpin, chrysin, quercetin and rutin) also stimulated spore germination of *R. irregularis* significantly ($p < 0.01$) respect to the control. The isoflavone genistein induced about 2.5 times germination at all the concentrations tested (Fig. 2). Medicarpin application stimulated spore germination at low concentrations, 2.8- and 1.8-fold at 0.01 and 0.1 µM, respectively. Conversely, a significant inhibitory effect on spore germination was observed at the highest concentration (1 µM). For the flavone chrysin, the highest stimulation of germination was observed after application of 0.1 µM, with a 4.2-fold increase. The flavonol quercetin stimulated spore germination at the three tested concentrations. The highest induction of germination was observed at 0.1 µM, with a 5-fold stimulation. Upon application of 0.01 and 1 µM, about 3 times induction was observed (Fig. 2). Rutin, the other flavonol assessed, also induced germination at the three concentrations, being the highest stimulation observed at the lowest concentration (0.01 µM), with a 4.1-fold increase over the control. At higher concentrations (0.1 and 1 µM), the stimulation of germination were 2.9 and 2.7 fold, respectively (Fig. 2).

Experiment 2

To confirm the results observed, a second *in vitro* spore germination assay was carried out. Based on the previous experiment, in this second assay only the lower concentrations (0.01 and 0.1 µM) were used for the different compounds. In this assay, spore germination was faster than in the previous experiment. Thus, germination levels were quantified 5 days after application of the different flavonoids. As before, 2'-*epi*-GR24 induced spore germination at both concentrations used, indicating that the spores were viable and active (Fig. 3). In the case of the flavonoids, this time only the four compounds that showed the higher effect on germination in the previous assay (chrysin, genistein, quercetin and rutin) were tested. No effect of the flavone chrysin was detected at any of the concentration used (Fig. 3). In the case of the isoflavone genistein, both concentrations stimulated germination of the of *R. irregularis* spores. Application of 0.01 and 0.1 µM induced germination 2.7 ($p = 0.009$) and 2.3-fold ($p = 0.030$), respectively,

compared to the control (Fig. 3). These inductions were similar to that observed for the positive control 2'-*epi*-GR24 (Fig. 3). The flavonol quercetin stimulated spore germination about 2.5 times compared to the control at 0.1 µM (p = 0.006), while no stimulatory effect was observed at the lower concentration (0.01 µM) (Fig. 3). In the case of rutin, a 2.5-fold stimulation (p = 0.007) was observed at the lower concentration (0.01 µM), showing similar stimulation levels to those observed for 2'-*epi*-GR24 (Fig. 3). No significant effect was detected at 0.1 µM (Fig. 3). The results show that some of the flavonoids tested here, belonging to different subcategories, have the capacity of stimulate the germination of *R. irregularis* spores *in vitro* at low concentrations. Remarkably , the results also indicate that the dose used is important.

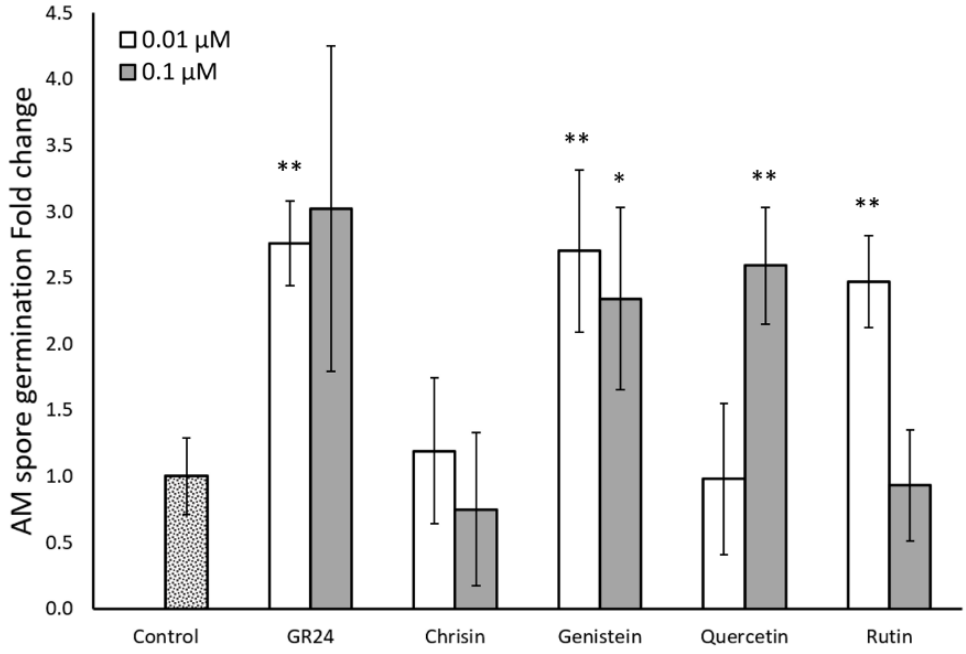

*Figure 3 Relative percentage of germination of spores of R. irregularis in vitro. Spores of R. irregularis were incubated for 5 days in Petri dishes with 2% Agar medium with two different concentrations of the flavonoids studied (0.01 and 0.1 µM). The application of the synthetic strigolactone 2'-epi-GR24 was used as a positive control. The bars correspond to the mean of 7 independent replicates (15 spores/replicate) ± standard error. Statistical analysis was performed with t-test analysis between each treatment.compared with the control. * p<0.05, ** p<0.01.*

Stimulatory effect of flavonoids of AM symbiosis in planta

Based on the results obtained *in vitro*, we next carried out an *in-planta* experiment to determine whether the increased spore germination rate induced by flavonoids resulted in a higher mycorrhizal root colonization. Tomato as a host plant and spores of the same *R. irregularis* strain used in the *in vitro* assays (MUCL 57021) were used. Application of 2'-*epi*-GR24 (positive control) highly (about 6 times) enhanced mycorrhizal colonization levels of *R. irregularis* at 0.01 and 0.1 µM (p < 0.001) compared to control plants (Fig. 4). Regarding the

flavonoid treatments, no effect in mycorrhization was observed upon application of the isoflavone genistein at any of the two concentrations applied. Conversely, a stimulatory effect was observed for the other three compounds tested compared to the control untreated plants. The flavone chrysin induced mycorrhizal colonization levels about 3 (p = 0.013) and 4 (p < 0.001) times at 0.01 and 0.1 µM, respectively, compared to the control (Fig. 4). The flavonol quercetin promoted mycorrhizal colonization more than 2 times after application of both 0.01 (p = 0.005) and 0.1 µM (p = 0.04) (Fig. 4). The other flavonol, rutin, increased mycorrhization about 3-fold (p = 0.039) upon application of 0.01 µM and about 2-fold at 0.1 µM, although this increase was not statistically significant (Fig. 4). The results show that the flavonoids chrysin, quercetin and rutin function as signaling molecules in the rhizosphere stimulating the establishment of AM symbiosis.

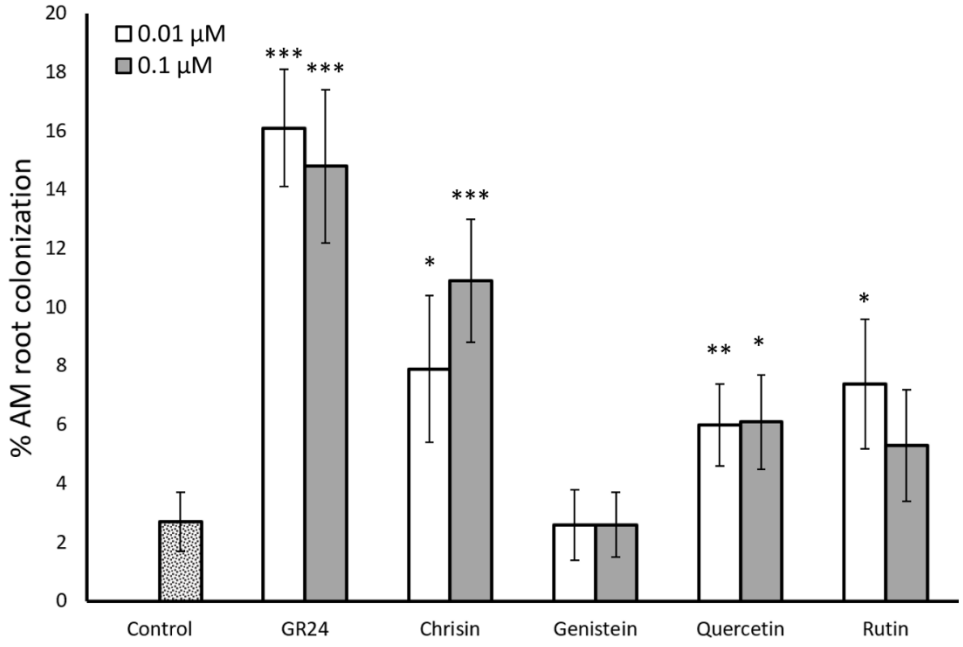

*Figure 4 R. irregularis root colonization levels of tomato plants. Plants were inoculated with 675 spores of R. irregularis and treated twice a week during 6 weeks with 10 mL of two different concentrations of the flavonoids studied (0.01 and 0.1 µM). The application of the synthetic strigolactone 2'-epi-GR24 was used as a positive control. The bars correspond to the mean of 10 independent replicates ± standard error. Statistical analysis was performed with t-test analysis between each treatment.compared with the control. * p<0.05, ** p<0.01, ***p<0.001.*

DISCUSSION

The application of AM fungal inoculants in agriculture is still challenging because of the variability of the results when applied in production systems (Duhamel & Vandenkoornhuyse, 2013; Tkacz & Poole, 2015). In this sense, a role of flavonoids in favoring AM symbiosis was

proposed, although their specific role and functioning here is still not clear (reviewed in Hassan & Mathesius, 2012). In the present study, we carried out both *in vitro* and *in vivo* assays to confirm their involvement in this beneficial symbiosis and to further investigate their specific function. Using *in vitro* assays, we showed that all the flavonoids tested -chrysin, genistein, medicarpin, quercetin and rutin-, belonging to different subclasses, stimulated AM fungal spore germination and hyphal growth at different 'physiological' doses (Figs. 2 and 3). Indeed, they showed a similar stimulatory germination activity as the synthetic SL analogue 2'-*epi*-GR24, indicating their high and specific activity. A role for the flavone chrysin in AM fungal spore germination and hyphal development was previously described, although with contradictory results. Firstly, an inhibitory effect of chrysin during the pre-symbiotic phase was shown in *Gi. margarita* (Bécard et al., 1992; Chabot et al., 1992). Conversely, an increase in the number of entry points and root colonization of this flavone was shown in *Gi. margarita*, in *Funneliformis mosseae* and in *R. irregularis* (Scervino et al., 2007). Therefore, it seems that fungal genotypes, experimental conditions and, probably, the different concentrations of the flavonoids tested could explain the divergences observed.

Our results were also consistent with the fact that certain flavonols, specially quercetin, was reported to stimulate spore germination and AM fungal hyphal growth of *Gi. margarita in vitro* (Bécard et al., 1992; Chabot et al., 1992; Poulin et al., 1997; Scervino et al., 2005b). A role of quercetin in stimulating spore germination and hyphal growth has been reported also for other AM fungi, such as *F. mosseae* (Kape et al., 1993), *R. irregularis* (Bécard et al., 1992; Poulin et al., 1997), *Claroideoglomus etunicatum* (Bécard et al., 1992; Tsai & Phillips, 1991), *G. macrocarpum* (Tsai & Phillips, 1991), *Gi. Rosea* (Scervino et al., 2005b) *and Gi. Gigantea* (Baptista & Siqueira, 1997). However, these effects were always studied at high concentrations (of the order of micromolar; Vierheilig et al., 1998). Conversely, no effect for the glycosylated derivative of quercetin, rutin, was previously described (Bécard et al., 1992; Chabot et al., 1992; Scervino et al., 2007), while we show here a stimulatory effect, especially at low doses (10 nM). Once again, the different concentrations of the flavonoids tested could explain the divergences observed, since the dose is critical for signaling molecules.

It seems clear that certain flavonoids can stimulate AM fungal development during the pre-symbiotic phase *in vitro*. However, an effect *in vitro* does not necessarily correlate with an increased mycorrhizal colonization in planta. Remarkably, we show here that the flavone chrysin, and the flavonols quercetin and rutin were also able to promote mycorrhizal root colonization *in planta* at low doses when AM fungal spores were used as inoculum (Fig. 4). Our results agree with previous results in different plant species. In tomato, the application of the

flavones chrysin and luteolin, and the flavonol morin increased root colonization in different AM fungi, while other flavonols such as rutin, kaempferol and isorhamnetin showed no effect (Scervino et al., 2007). Quercetin was found to be present in AM clover roots and shown to promote mycorrhizal colonization of *Gi. margarita* (Scervino et al., 2005a). Recently, quercetin has been related with the expansion of invasive plants, which showed increased levels of quercetin in root exudates and an enhanced mycorrhizal colonization than native plants. In addition, exogenous application of quercetin also increased AM fungal colonization of target plants (Pei et al., 2020; Tian et al., 2021). The results suggest that quercetin, and probably also its derivates such as rutin and other flavonoids, might act as a key signaling molecule in the rhizosphere promoting mycorrhizal colonization. In agreement with this hypothesis, Maloney and co-workers have recently proposed a possible role of flavonols, including quercetin, in the promotion of lateral root formation (Maloney et al., 2014), which are the preferred place for the AM fungus to colonize the roots of a host plant. Root exudates are an essential plant mechanism to interact with other (micro)organisms present in the rhizosphere, including AM fungi. A better understanding of the dynamics of flavonoid production and exudation during different environmental conditions and AM symbiosis would facilitate to find the optimal conditions for AM symbiosis establishment, especially when using spore-based inoculum.

In summary, we confirm here the role of certain flavonoids belonging to the subclass of flavonols in AM symbiosis, and show their relevance as signaling molecules during the pre-symbiotic phase promoting spore germination and hyphal development, and AM fungal colonization. The use of AM fungal-based biofertilizers is agriculture is demanding effective and efficient commercial inoculants mostly in seasonal crops. Therefore, the addition of selected flavonoids, such as quercetin, at low doses may have great benefits as accelerators of the pre-symbiotic, thus promoting symbiosis establishment and improving commercial products.

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Chapter 3

Regulation of mycorrhizal symbiosis by stress signaling in tomato

Regulation of mycorrhizal symbiosis by stress signaling in tomato

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ABSTRACT

The mutualistic symbiosis between plant roots and arbuscular mycorrhizal (AM) fungi is based in a balanced nutrient exchange between the both partners. Through the symbiosis, the host plant benefits from an improved nutrition and a better resistance/tolerance to multiple stresses. A functional symbiosis is fine-tuned regulated by different plant responses, mainly orchestrated by phytohormones. This allows plants to control fungal colonization extent according to its needs. According to this, environmental conditions or stress factors modulating phytohormone signaling may influence the symbiosis and plant fitness. Here, we explored how the activation of plant defense signaling following external stimuli affects the interaction with different AM fungal genotypes. Using tomato as a model plant, we compare *Funneliformis mosseae* and *Rhizophagus irregularis* in their ability to colonize roots when they were applied either independently or in combination under different stress conditions. Plants remained untreated or subjected to salt stress or to defense activation by systemic application of the main defense related hormones methyl jasmonate (MeJA), abscisic acid (ABA) and salicylic acid (SA). We found significant differences in the pattern of colonization between *F. mosseae* and *R. irregularis* depending on the type of stress. Changes in colonization levels correlated with differential regulation of plant defensive responses, nutrient exchange between partners, and changes in specific genes for symbiosis control and autoregulation. Focusing on salt stress, it impacted differently on the colonization by both AM fungi, in agreement with their benefits for the host. The colonization and functionality of the symbiosis with *F. mosseae* increased under salt stress, while colonization by *R. irregularis* was reduced. These changes correlated with differential regulation of lipids supplied to the fungus, plant defenses and symbiotic control in both interactions. Overall, the results support the idea that the benefits provided by the AM fungi influence plant control of colonization and carbon reward by the host plant.

INTRODUCTION

Fungi are important components of plant microbiota and fulfill multiple functions in plant health and ecosystems functioning (Pozo et al., 2021). Among plant-associated fungi, arbuscular mycorrhizal (AM) fungi are of special interest since they are widespread in very diverse environments and establish the most ancient plant-microbe symbiosis with more than 70% of land

plant species (Brundrett & Tedersoo, 2018; Genre et al., 2020). The AM symbiosis is a mutualistic association having important benefits for both partners, the plant and the fungus. It can improve plant nutrition, mainly increasing phosphorus (Pi), nitrogen and water uptake (Bonfante & Genre, 2010; Parniske, 2008; Xie et al., 2022). It can also enhance plant fitness by boosting plant resistance/tolerance against diverse stress conditions, including biotic and abiotic challenges (Lenoir et al., 2016; Parniske, 2008; Pozo et al., 2015; Ruiz-Lozano et al., 2018; Santander et al., 2017).

The term "arbuscular" comes from the characteristic highly branched tree-like structures formed by these AM fungi within the root cortical cells to increase the fungal-plant contact surface (Parniske, 2008). It is in the arbuscules where the exchange of nutrients between the two partners takes place. Soil Pi is taken up from soil by the fungal extraradical mycelium and translocated to the plant cell by the hyphae through the arbuscule. From there, it is delivered to the plant by specific plant Pi transporters, such as the PT4 (Balestrini et al., 2007; Ezawa & Saito, 2018; Hijikata et al., 2010). In return, the plant provides the fungal partner with photosynthates in the form of carbohydrates and lipids. Due to the obligate biotrophic nature of AM fungi, the fungus is completely dependent on this carbon input from the plant (Salmeron-Santiago et al., 2021). In fact, up to 20% of the carbon fixed by photosynthesis is directed to the fungus (Bago et al., 2000; Keymer et al., 2017). This extreme dependence makes the plant able to control fungal colonization according to the nutrient demand, growing conditions and/or fungal efficiency (Hammer et al., 2011; Kiers et al., 2011; Werner & Kiers, 2015). Remarkably, the regulation of the symbiosis is not a simple control process of the carbon sink towards the fungus, but it seems to be regulated by more specific control mechanisms at different levels (Ho-Plágaro & García-Garrido, 2022; MacLean et al., 2017; Vierheilig et al., 2000)

Two differentiated phases can be considered in the symbiosis establishment: the pre-symbiotic and the symbiotic stage. During the pre-symbiotic stage, a complex molecular dialogue occurs between the two partners in the rhizosphere before contact. This molecular communication starts when the plant exude through their roots signaling compounds into the rhizosphere, mainly strigolactones (SLs), to attract and activate the AM fungus and promote the symbiosis, specially under nutrient deficient conditions, acting as “cry for help” signals (Akiyama et al., 2005; Bouwmeester et al., 2007; López-Ráez et al., 2011, 2017). Among these compounds, strigolactones play an important role in this stage, inducing AM fungus spore germination, activating fungal metabolism and hyphal branching of germinating spores to promote the contact with the plant roots

(Besserer et al., 2006; Waters et al., 2017). On the other hand, the AM fungus releases Myc factors (short-chain chitin oligomers (COs) and lipochitooligosaccharides (LCOs)) that activate a set of genes belonging to the common symbiosis signaling pathway in the plant to facilitate fungal accommodation within the roots (Genre et al., 2013; MacLean et al., 2017; Maillet et al., 2011). The establishment and development of the symbiosis (symbiotic stage) also requires a high degree of coordination between the two partners (MacLean et al., 2017). During root colonization, a transcriptional reprogramming is activated in cells of the epidermis and the root cortex related to transcriptional regulation, cell wall modification and modulation of the defensive response to accommodate the fungus and control fungal development (López-Ráez et al., 2010b; Pimprikar & Gutjahr, 2018; Sugimura & Saito, 2017). The attenuation of the plant defensive response to AM fungal recognition is essential for the establishment of the symbiosis. In fact, the AM fungus actively promotes the suppression of plant defenses by secreting peptidic effectors (Kloppholz et al., 2011; Schmitz et al., 2019; Zeng et al., 2020). As in other symbioses, the recognition and establishment of a functional symbiosis require a very precise and fine-tuned regulation of plant responses, mainly orchestrated by phytohormones and other signaling molecules (Bedini et al., 2018; Martínez-Medina et al., 2019; Pozo et al., 2015). Indeed, almost all phytohormones studied to date are involved, to some extent, in the control of fungal colonization extension, arbuscular development and/or symbiosis functioning (Bedini et al., 2018; Ho-Plágaro & García-Garrido, 2022; Pozo et al., 2015). Phytohormones are molecular regulators that control many processes in the plant. They allow the integration of environmental and internal cues to generate specific responses modulating plant growth and development, defense regulation, and plant adaptation to different abiotic and biotic contexts, such as salt or drought stress or the interaction with beneficial or deleterious (micro)organisms (Bedini et al., 2018; Lenoir et al., 2016; Pieterse et al., 2012). Therefore, the environmental impact on hormone levels may have an impact on the plant interaction with AM fungi (Pozo et al., 2015). The most studied example is the effect of Pi availability. Under low Pi conditions there is a promotion on rhizosphere signaling and root transcriptional reprogramming giving rise to an increased symbiosis, while under high Pi conditions the symbiosis is repressed (Balzergue et al., 2011, 2013). However, whether other stresses may actively promote symbiosis establishment is still controversial (Aroca et al., 2013; López-Ráez, 2016).

It has been also shown that the plant has systemic control mechanisms to prevent excessive colonization, indicating a mechanism of mycorrhizal autoregulation. The autoregulation of mycorrhiza shares several mechanisms with the autoregulation of nodulation, although the precise

molecular mechanisms are not fully understood yet (Catford et al., 2003; Foo et al., 2016). Recently, a few genes involved in the autoregulation process, such as *CLV2* and CLE peptides, have been described (Ho-Plágaro & García-Garrido, 2022; Karlo et al., 2020; Müller et al., 2019; Wang et al., 2018). In addition to the regulation of the colonization rates (autoregulation), the plant also controls the arbuscule functionality and lifespan through the action of different transcription factors such as RAM1 and MYB1, among others, that regulate transcriptional programs related to nutrient exchange and regulation (Ho-Plágaro & García-Garrido, 2022). It has been proposed that some apocarotenoids (mycorradicin and α-ionols), known as the 'yellow pigment complex', are able to maintain the functionality of the AM symbiosis by regulating arbuscular turnover based on efficiency. These compounds are carotenoid-derived from the sequential action of two carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD1 (López-Ráez et al., 2015; Walter et al., 2015).

Some AM fungi are more efficient colonizers than others, and their benefits to the plant may also vary. In this sense, it has been proposed that some fungi are more efficient in improving plant nutrition, while others are better at enhancing stress tolerance (Marro et al., 2022). Thus, functional diversity has been described among AM fungi depending on specific properties (Rivero et al., 2018). Moreover, the changes in the host plant during the symbiosis establishment may vary depending on the colonizing fungus both in the absence of stress (Fernández et al., 2014; Rivero et al., 2015) and under stressful conditions (Rivero et al., 2018).

Due to the multiple benefits that AM symbiosis can provide for plants in agro- and eco-systems, bioinoculants based on AM fungi have already been commercialized as biofertilizers and bioprotection agents (Chen et al., 2018; Szczałba et al., 2019). However, the variability of the results under field conditions limits their use and potential applications nowadays. This variability is related to the high context dependency, as multiple environmental conditions may impact the symbiosis and its functionality (Hart et al., 2018; Holland et al., 2018; Kokkoris et al., 2019; Orine et al., 2022). Therefore, it is important to understand the effects of the environmental context on the plant-AM fungus interaction in order to improve the establishment of the symbiosis and their benefits, thus increasing its implementation in agricultural and ecological settings (Hartman & Tringe, 2019; Lenoir et al., 2016; Orine et al., 2022). To achieve these goals, we need to understand: i) how the symbiosis is regulated under different stresses, and ii) how different AM fungi may modulate the plant responses to stresses is essential to successfully implement and benefit from AM fungi in sustainable agriculture.

In the present study, we compare two different AM fungi, *Funneliformis mosseae* and *Rhizophagus irregularis*, alone or in combination, in their ability to colonize tomato roots under different stress conditions. We aim to explore how different 'environmental' conditions impact AM symbiosis establishment and functioning, including abiotic stress (salinity), or mimicking biotic stress. We achieved this by the activation of the main defense signaling pathways through the shoot exogenous application of the main defense-related hormones: abscisic acid (ABA), which is a central regulator of plant responses to osmotic stress and modulator of biotic stress responses; jasmonic acid (JA), that regulates responses to herbivorous insects and necrotrophic pathogens and salicylic acid (SA), which mainly orchestrates responses against biotrophic pathogens (Pieterse et al., 2012). We hypothesize that the plant regulates AM symbiosis and functionality depending on the environmental conditions, but its impact depends on the colonizing fungus. We also test whether inoculation with a combination of the two AM fungi leads to an improved symbiosis and enhanced benefits. For that purpose, we analyzed the expression of multiple marker genes associated with different signaling pathways to address their specific contribution to the differential impact on AM colonization. We specifically test for changes in: i) pre-symbiotic signaling, ii) the defensive status of the plant, iii) regulation of nutrient exchange and iv) the control and autoregulation of the symbiosis. Our results show that regulation of plant defenses and carbon supply (lipids and sugars) to the AM fungus are the most important contributors to the regulation of colonization in our system.

MATERIALS AND METHODS

Biological material and growing conditions

Isolates of *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler 2010 (DAOM 197198) and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (BEG12, International Bank of Glomeromycota) are continuously maintained in greenhouse pot cultures with *Trifolium repens* and *Sorghum vulgare*. The inoculum consisted of root fragments, mycelia and spores in a vermiculite-sepiolite (1:1, v/v) substrate. Tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were surface sterilized by immersion in 50% commercial bleach solution containing 0.02% (v/v) Tween20 for 10 min. Then, the seeds were rinsed thoroughly with sterile water and incubated for 14 days in sterile vermiculite at 25 ºC. Tomato plantlets were then transferred to 300 mL pots filled with sand, soil and vermiculite (1:1:1, v/v/v), supplemented or not

with mycorrhizal inoculum as described below. The soil was previously steam-sterilized (100 ºC, 1 h for 3 days consecutively), and the sand and vermiculite were autoclaved (121 ºC, 20 min). Four AMF treatments were performed: control plants without AM fungal inoculation (Non mycorrhizal, Nm); inoculated with *R. irregularis* (5% v/v) (Ri); with *F. mosseae* (5% v/v) (Fm); and with a mix of both, *R. irregularis* (5% v/v) and *F. mosseae* (5% v/v) (FmRi). Nm treatment received the same amount (5% v/v) of sterilized vermiculite-sepiolite as the other treatments. All plants received an aliquot of a filtrate (<20 µm) of the two AM fungi inoculum in order to homogenize the microbial populations present in the inoculum. Plants were grown in a glasshouse under controlled conditions (24 – 18 ºC; 16 : 8 h, light : dark) and watered when necessary with tap water and brought to field capacity once a week. Each week plants were watered with Long Ashton nutrient solution (Hewitt, 1953) with reduced phosphorus concentration (0.335 mM) to promote symbiosis establishment. Plants were harvested after 6 weeks of growth, and the fresh weight of shoots was determined. Root and shoot material were immediately frozen in liquid nitrogen and stored at −80 ºC. A homogeneous aliquot of each individual root system was taken for mycorrhizal assessment and quantification.

Phytohormone and salt treatments

The different treatments were applied since transplantation to act on mycorrhizal establishment of the symbiosis and maintained along the experiment. For salt stress treatment, plants were treated with 150 mM of NaCl solution two weeks after AM fungal inoculation. Any irrigation drainage from the substrate was carefully avoided in any subsequent irrigation. In order to test the effect of defense signaling activation and mimic both biotic and abiotic stresses, plants were treated with three different stress-related hormones aboveground. Prior to each phytohormone treatment the substrate and the pots were covered with a plastic to avoid any direct contact of the belowground tissues with the hormones. The treatments were applied by spray of the shoots until run off once a week from the second week upon AM fungal inoculation (in total four weeks treatment). The hormone treatments were: i) Abscisic acid (ABA) 50 µM; ii) Methyl jasmonate (JA) 50 µM; and iii) Salicylic acid (SA) 100 µM. Hormone stocks were prepared in ethanol and then diluted in sterile demi-water containing 0.02% (v/v) tween20 before application. For control treatment, plants were sprayed with a mock solution with the same ethanol concentration. Seven independent replicates were used for Nm, Fm and Ri treatments, and 11 replicates for FmRi treatment. Plants from the different treatments were distributed in the greenhouse following a full randomized design.

Mycorrhizal quantification

Mycorrhizal quantification was determined as described in Garcia et al. (2020) by root histochemical staining after clearing the roots in 10% KOH and staining the fungal structures with 5 % black ink in 2 % acetic acid solution (Vierheilig et al., 2005). Mycorrhizal colonization was determined following the gridline intersection method (Giovannetti & Mosse, 1980) using a Nikon SMZ1000 stereomicroscope. Quantification of AM fungal vesicles within the mycorrhizal roots was performed as described in Trouvelot et al. (1986).

Analysis of gene expression by qPCR

RNA extraction from roots, purification, synthesis of the corresponding cDNA and qPCR was performed as described in Gamir et al. (2020). Quantitative qPCR reactions and relative quantification of specific mRNA levels were performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak & Schmittgen, 2001), using the gene-specific primers described in the Table S1. Expression values were normalized using the normalizer gene SlEF-1α encoding the tomato translation elongation factor-1α (López-Ráez et al., 2010b). Four independent biological replicates per treatment were analyzed.

Determination of mineral nutrients in roots

Nutrient content in roots was measured at the Ionomic Laboratory of the Technical Services of the Estación Experimental del Zaidín (EEZ-CSIC) in Granada, Spain. Frozen roots were ground to a fine powder and lyophilized. Three or four biological replicates were analyzed for each treatment and results were normalized to dry weight. Element concentrations were analyzed after acid digestion of the samples (50 mg), by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720-ES). Full mineral nutrient data shown in Table S2.

Statistical analyses

For the mycorrhizal colonization and shoot fresh weight analyses, data were subjected to a two-way ANOVA and Tukey's HSD post hoc test (p <0.05) to compare between treatment levels (Factor levels: Chemical: Control, NaCl, ABA, JA, SA; Inoculation: Fm, Ri and FmRi; Nm was excluded from colonization analysis). Data were checked for normality and homogeneity of variance before statistical analyses. For a better fitting of models, mycorrhizal colonization was subjected to arcsine transformation of square rooted.

The effect of the experimental treatments on gene expression was analyzed by permutational analysis of variance (PERMANOVA, adonis function vegan R package, Oksanen, 2008). In this case, inoculation with *R. irregularis* and *F. mosseae* were treated as different variables in a crossing design (values 1/0). Due to genes were quantified in different runs of qPCR for the different chemical treatments, their expression values were normalized by calculating the standardized effect size of each gene expression relative to the non-mycorrhizal control without hormone treatment per run ($Value_{sample}$-$Mean_{control}$/$Desvest_{control}$). The matrix of gene expression was used as response variable in the PERMANOVA and chemical treatment, *R. irregularis* inoculation, *F. mosseae* inoculation and their interactions as explanatory factors (using euclidean distance as measure of dissimilarity and 999 permutations). To illustrate the found effects in PERMANOVA, the expression levels of selected marker genes of the main defense pathways were checked across treatment levels, and a principal components analysis (PCA) was arranged for the whole gene expression matrix and the distribution of experimental factors plotted against their first two axes. Similarly, to better reveal patterns for the colonization of each fungus separately, two PCAs were arranged only for the set of samples inoculated with either *R. irregularis* or *F. mosseae*.

The effect of gene expression on colonization was studied via linear modeling. Due to the lack of records for non-mycorrhizal treatment (i.e. no colonization), they were excluded from this analysis. In a first instance, the number of genes included in the analyses was reduced by variance inflation factor analysis (VIF) (vif function, car R package, Fox & Weisberg, 2011). Genes were removed progressively until any VIF value was below 5 from a model starting with every measured gene. The remaining set of genes was subjected to stepwise model selection where variables were added-removed until the model reached the lowest Akaike Information Criterion (AIC) value (ols_step_both_aic function, olsrr R package, Hebbali, 2020). A final model was built with the selected set of genes, i.e. the least number of genes that better explain mycorrhizal colonization. Correctness of model fitting was checked by using DHARMa R package (simulateResiduals function, Harti & Lohse, 2022).

Statistical analyses comparing two treatments were performed using Statgraphics with unpaired t-test analysis.

RESULTS

Activation of plant stress signaling differentially impacts mycorrhizal colonization by different AM fungi

The exogenous application of defense phytohormones - ABA, MeJA and SA -, mimicking plant stress had a low impact on plant growth. Salinity was the only treatment leading to a significant reduction of shoot biomass, evident in non-mycorrhizal plants and in plants colonized by *F. mosseae* and *R. irregularis* as compared with non-stressed (Control) plants. In mycorrhizal plants (Fm and Ri) this negative effect was lower, although only the combined inoculation with the two fungi (FmRi) completely suppressed the reduction in shoot biomass. Regarding the stress treatments, none of the hormonal treatments (ABA, JA and SA) had an effect on shoot biomass (Table 1). Mycorrhizal inoculation per se did not significantly affect plant biomass.

SFW (g)	Control	NaCl	ABA	JA	SA
Nm	10.0	8.0*	10.1	9.9	10.2
Fm	9.4	8.4*	10.3	9.4	10.0
Ri	9.5	8.5*	10.3	10.0	10.3
FmRi	9.7	9.6	10.4	10.0	10.4

Table 1 Effect of the different treatments on shoot fresh weight (SFW) of tomato plants. Plants inoculated with F. mosseae (Fm), R. irregularis (Ri) or a double inoculation of Fm and Ri (FmRi) were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 µM), methyl jasmonate (JA 50 µM) and salicylic acid (SA 100 µM). Data represents the means (n=7 for Nm, Fm, Ri; n=11 for FmRi).Data followed by (*) are significantly different to the Nm Control by HSD Tukey post hoc test (p<0.05).

AM colonization of tomato plants was well established after 6 weeks of inoculation in all mycorrhizal treatments, showing well-developed arbuscules, intraradical hyphae and vesicles. Absence of colonization was confirmed in non-inoculated (Nm) plants (data not shown). Mycorrhizal levels varied as response to the different AM fungal inoculants ($F_{2,110}$ = 5.44, P = 0.0056), the different types of stresses ($F_{4,110}$ = 3.91, P = 0.0052) and their combination ($F_{8,110}$ = 4.38, P = 0.0001), as colonization by *R. irregularis*, *F. mosseae* and their combination responded differently to the type of stress applied (Fig. 1). Under control conditions, the lowest colonization levels (14.8%) were found in plants inoculated with *F. mosseae* (Fm), while *R. irregularis* (Ri) and the combined AM fungal treatment (FmRi) showed greater colonization rates, 26.9 and 28.5%, respectively (Fig. 1).

Remarkably, the double inoculation with both AM fungi (FmRi), containing double the amount of inoculant, did not result in a higher mycorrhizal colonization. Salt stress significantly increased mycorrhizal colonization in Fm plants, while it was reduced in Ri plants as compared with colonization under control conditions. The colonization level in the combination (FmRi) was similar to Fm plants and did not differ from levels in the control treatment. ABA treatment only increased mycorrhization in the case of plants inoculated with *F. mosseae*, as observed under salt stress (Fig. 1). No effect for the other phytohormones applied (JA and SA) was detected in Fm nor Ri plants. However, all the three hormonal treatments - ABA, MeJA and SA - reduced mycorrhizal levels when the combination of the two AM fungi FmRi was used. Noteworthy, colonization seems to reach a maximum level, close to 30% of the root length regardless of the treatment (Fig. 1).

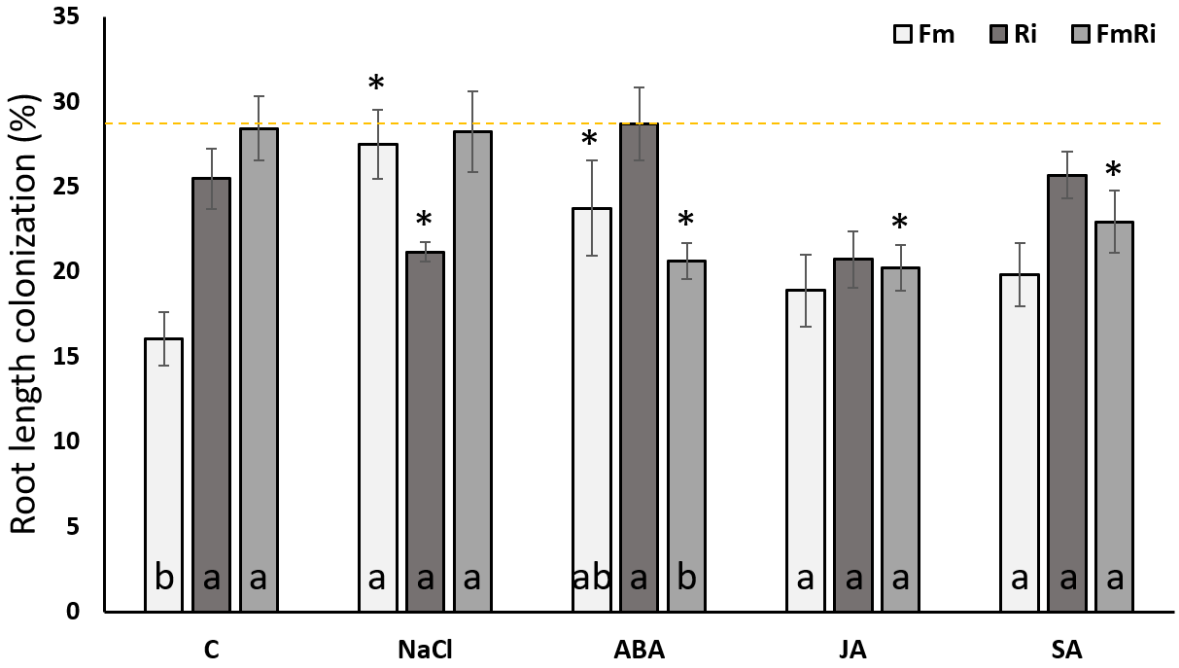

Figure 1. Mycorrhizal colonization in tomato roots. Plants inoculated with F. mosseae (Fm), R. irregularis (Ri) or a double inoculation of Fm and Ri (FmRi) were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 µM), methyl jasmonate (JA 50 µM) and salicylic acid (SA 100 µM). Data represents the means ± SEM (n=7 for Nm, Fm, Ri; n=11 for FmRi). Yellow dotted line: hypothetical threshold. Data from the different AMF within a given treatment not sharing a letter in common are significantly different according to the HSD Tukey post hoc test (p< 0.05). Columns marked by (*) denote significantly different colonization levels of the treatments as compared to the untreated controls for each AM fungus (t-test, p<0.05).

Shoot hormonal treatments and salinity impact defense signaling pathways belowground

We performed an extensive transcriptional analysis of multiple genes related to different functional categories, including hormone markers to test if the stress treatments actually activated those signaling pathways in roots. The expression of marker genes involved in pre-symbiotic signaling (strigolactone biosynthesis genes *D27*, *CCD7* and *CCD8*), defense responses (*PinII*, *LAPA*, *GluB*, *Pti5*, *PAL*, *P14C*, *PR1b1*) regulation of nutrient exchange between the symbiotic partners: coding for plant Pi transporters (*LePT4*), lipid biosynthesis and transport (*STR*, *FatM* and *DIS*) and carbohydrate metabolism and transport (*Lin6*, *SUT1*, *SUT4*, *SUS1*, *SUS3*) and control of the symbiosis (*RAM1*, *MYB1*; *Vapyring*, *CLE*, *CLV2*, *CCD1a*, *NSlegh*, *GH3.4* and *GH3.15*) was analyzed by qPCR. A PERMANOVA with all transcriptional data in the roots was performed and showed a significant effect of mycorrhizal inoculation and the stress treatments on the gene expression profiles (Table 2). Noteworthy, only the interaction of the stress treatments with *F. mosseae* inoculation was significant, while neither *R. irregularis* nor the double inoculation with the two AM fungi had a significant interaction with the stress treatments.

	Df	*F*	R^2	P
R. irregularis (Ri)	1	21.124	0.133	**0.001**
F. mosseae (Fm)	1	18.369	0.116	**0.001**
Stress treatment	4	9.092	0.229	**0.001**
Ri × Fm	1	13.868	0.087	**0.001**
Ri × Stress	4	0.955	0.024	0.470
Fm × Stress	4	1.938	0.049	**0.010**
Ri × Fm × Stress	3	0.875	0.017	0.586
Residuals	55		0.346	
Total	73		1.000	

Table 2. PERMANOVA analysis of the effect of mycorrhizal inoculants, the stress treatment and their interactions on the gene expression profile.

We assessed whether the different treatments lead to an activation of plant defense signaling pathways in the roots by checking the expression levels of selected marker genes of the main defense pathways (ABA, SA, JA). Salt stress strongly induced ABA metabolism. It induced more than 6 times the expression of *NCED1*, encoding for a nine 9-*cis*-epoxycarotenoid 1 -a key enzymatic step in ABA biosynthesis- (Thompson et al., 2000). Salt stress also induced the expression of *Le4*,

encoding an ABA-inducible dehydrin (Kahn et al., 1993), while it down-regulated the expression of an ABA-8'-hydroxylase (*ABA-hydrox*), involved in ABA catabolism (Nitsch et al., 2009). The results agree with a significant increase of ABA content in salt treated plants (Fig S1). Salt stress also induced SA-related pathogenesis related (PR) proteins as P14c and *Pr1b1*, while no significant changes were detected for marker genes associated with JA signaling (Table 3). Regarding the hormonal treatments applied systemically in the leaves, moderated changes were observed in roots. The periodic application of hormones in shoots did not alter endogenous root hormone levels in the roots (Fig. S2), but the hormonal signaling pathways were transcriptionally regulated (Fig. 2). ABA application reduced both the expression of *Le4* and *ABA-hydrox*. ABA treatment also triggered the expression of *LapA*, a JA-dependent peptidase co-regulated by ABA (Chao et al., 1999). MeJA application also significantly induced the expression of *LapA* (Table 3). Finally, SA treatment reduced the expression of the ABA markers *Le4* and *ABA-hydrox*, and that of *LoxD*, encoding a lipoxygenase D involved in JA biosynthesis (Wasternack & Song, 2017). These results agree with the well-known negative crosstalk between the JA-SA signaling pathways (van der Does et al., 2013; Wasternack & Song, 2017). The expression of another lipoxygenase (*LoxA*) was also regulated by SA treatment. *LoxA* encodes a lipoxygenase from the 9-LOX branch of oxylipins, largely root specific (Itoh et al., 2002), and generally antagonistic with the 13-LOX pathway responsible for JA biosynthesis. LOXA is involved in controlling the spread of the AM fungus within the roots and it is under the control of JA levels (León-Morcillo et al., 2012).

pathway	gene	C	NaCl	ABA	JA	SA
ABA	*NCED1*	1.00	**6.02**	0.88	1.95	1.18
	Le4	1.00	**4.17**	**0.50**	0.69	**0.43**
	ABA-hydrox	1.00	**0.21**	**0.38**	1.21	**0.19**
JA oxylipins	*LoxA*	1.00	2.20	1.24	2.34	**3.54**
	LoxD	1.00	1.86	1.35	0.99	**0.29**
	Jar1	1.00	1.32	**0.67**	**0.71**	**1.61**
	PinII	1.00	3.09	1.48	0.58	0.92
	LapA	1.00	1.90	3.81	**3.38**	1.40
SA	*PAL*	1.00	1.09	0.94	0.81	0.74
	P14c	1.00	3.45	1.96	0.80	1.81
	PR1b1	1.00	**11.73**	1.00	1.03	2.74

Table 3. Regulation of hormone marker gene expression by the different treatments. Plants were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 µM), methyl jasmonate (JA 50 µM) and salicylic acid (SA 100 µM). Data correspond to fold change in gene expression in treated non mycorrhizal plants as compared to the untreated control (n = 4). Color indicates fold changes over 2 fold (up; red) and below 0.5 (down; blue). Bold values indicate significantly different to the control (t-test, p<0.05).

Considering all genes analyzed, the PCA analysis also illustrates the impact of the stress treatments on root transcriptional profiling, and a good correlation of the hormone-related marker genes was observed with the treatments (Fig 2A). Noteworthy, the effect of mycorrhiza was clearly greater than the effect of the stress treatments for the genes analyzed, completely separating them from the non-mycorrhizal treatment -explaining 30% of the variance- (Table 2, Fig 2B). Interestingly, there was a common pattern associated with mycorrhization, as we observed a general mycorrhizal fingerprint where all mycorrhizal treatments clustered together (Fig. 2B). The full set of expression data including Nm and mycorrhizal plants are presented in Table S3.

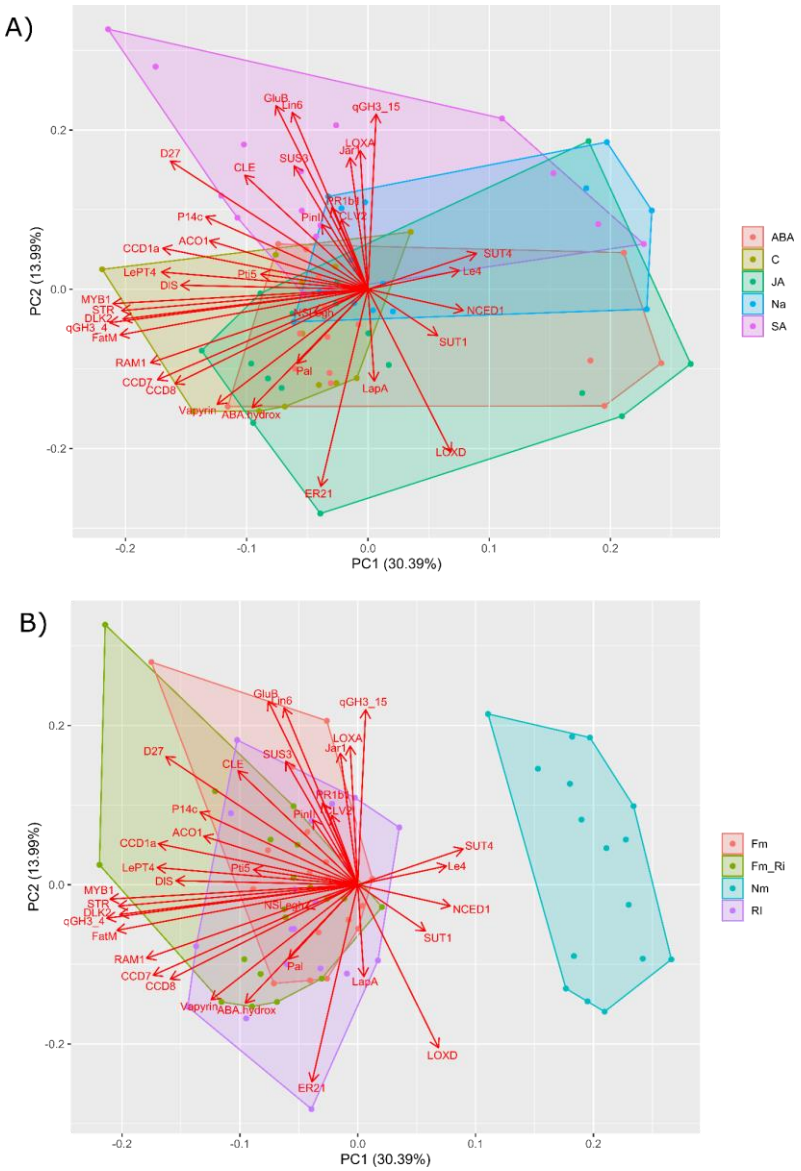

Figure 2. Impact of the treatments in gene expression in roots. PCA ordination of gene expression profiles, (A) upper panel shows the applied stress treatment and (B) lower panel show the mycorrhizal treatment. Gene data were transformed into standardized effect sizes and the PERMANOVA used Euclidean distance as measure of dissimilarity and 999 permutations. Plants from non-mycorrhizal (Nm) or mycorrhizal plants inoculated with F. mosseae (Fm), R. irregularis (Ri) or a combination of Fm and Ri (FmRi) were subjected to different treatments two weeks after inoculation: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 µM), methyl jasmonate (JA 50 µM) and salicylic acid (SA 100 µM).

Finally, a more detailed analysis focused on the impact of the stress treatments on root transcriptional profiling separately in *F. mosseae* and *R. irregularis* colonized plants, revealed that the salt stress and the SA treatments had the strongest impact on the transcriptional profiles (Fig. 3).

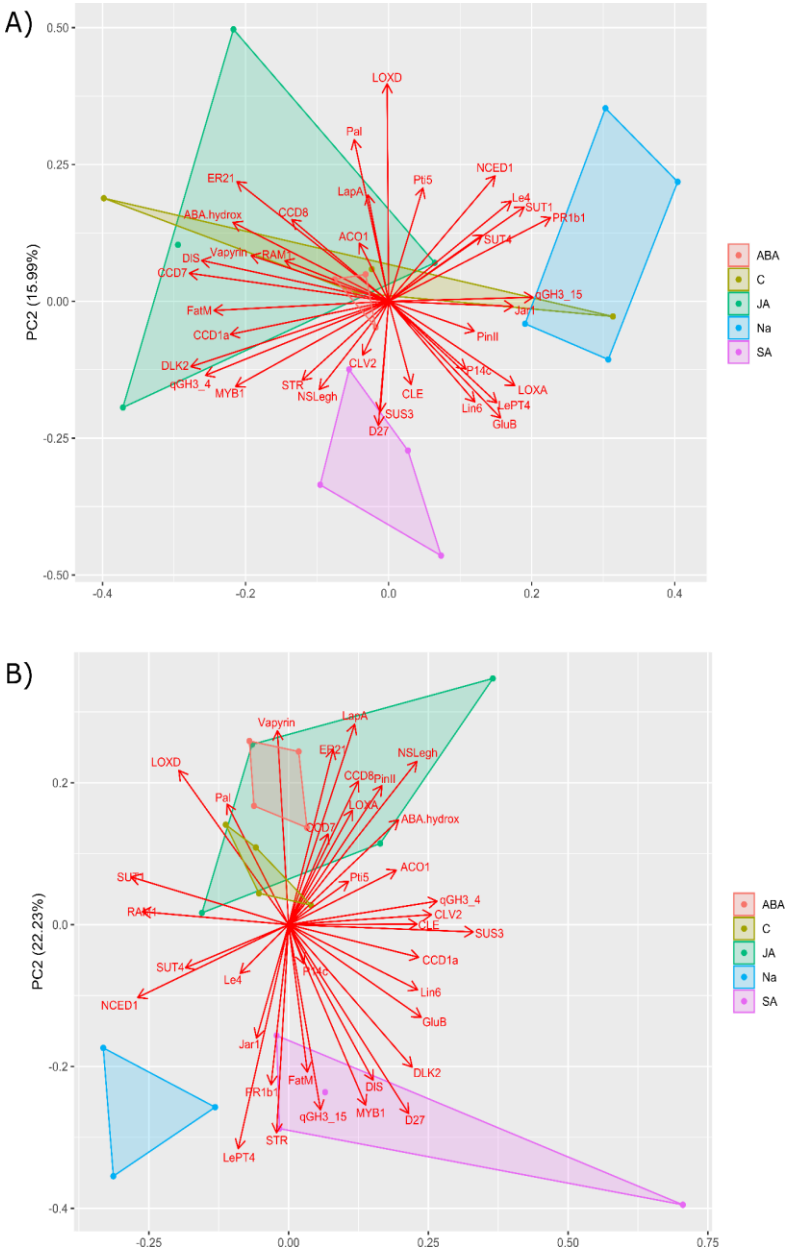

Figure 3. Impact of the treatments in root gene expression. PCA ordination of gene expression profile in plants colonized by R. irregularis (A) or F. mosseae (B). Plants from non-mycorrhizal (Nm) or mycorrhizal plants inoculated with F. mosseae (Fm), R. irregularis (Ri) were subjected to different treatments: control (C), application of 150 mM of NaCl (NaCl) or weekly treatment on shoot of abscisic acid (ABA 50 µM), methyl jasmonate (JA 50 µM) and salicylic acid (SA 100 µM).

Correlation of root gene expression profiles and mycorrhizal colonization

Once the effect of treatments in the roots and the transcriptional regulation of corresponding marker genes were confirmed, we explored the potential correlation between gene expression and mycorrhizal colonization levels**.** Aiming to identify those genes whose expression pattern better explained the variation in AM colonization, we arranged a stepwise model selection until a model showing the lowest AIC value was obtained. The final model, built with the least number of genes explaining the mycorrhizal colonization data, included six genes: *LePT4*, *FatM*, *SUS3*, *P14c*, *GluB* (Fig. 4A). *LePT4* and FatM showed a positive correlation with colonization. They are both involved in nutrient exchange between the AM fungus and the plant. *LePT4* encodes for a plant Pi transporter active in arbusculated cells and linked to the Pi uptake through mycorrhiza (Balestrini et al., 2007). *FatM* is involved in the supply of lipids from the host plant to the fungus (Bravo et al., 2017). On the other hand, a negative correlation was found between colonization and the gene encoding the sucrose synthase *SUS3*, related to carbohydrate metabolism, and with three genes related to plant defense, *PAL*, *P14c* and *GluB*, coding for a phenylalanine ammonia lyase, a basic PR1 protein and a basic b-1,3 glucanase, respectively (Gamir et al., 2017; Lefevere et al., 2020; van Kan et al., 1992). Thus, the analysis pointed to major correlation of the colonization levels with genes related to nutrient exchange and defense, but no changes in genes associated with the early signaling during the pre-symbiotic stage.

When a linear model was fitted, only *FatM*, *SUS3* and *P14c* significantly affected AM fungal colonization (Fig 4A). Although these genes were those better explaining colonization levels, they correlated also with other genes that, due to their co-variation, were not retained in the statistical selection. Plotting the whole correlation matrix (Fig. 4B) allowed to reveal sets of genes that significantly correlated with *FatM*, *SUS3* and *P14c* in explaining colonization levels. *FatM* correlated with *STR* and *DIS*, both involved in lipid transfer to the AM fungus (Keymer et al., 2017), and it also matched with *MYB1*, *DLK2*, *GH3.4*, *D27* and *CCD1a*, that are genes related to the control of the symbiosis (Fig. 4B) (Floss et al., 2017; Ho-Plágaro et al., 2021; López-Ráez et al., 2015; Walter et al., 2015). *SUS3* correlated with the genes associated with the autoregulation of AM symbiosis *CLE* and its receptor *CLV2* (Karlo et al., 2020; Müller et al., 2019; Wang et al., 2018). Finally, *P14c* matched with other defense-related genes, such as *GluB*, *Pti5*, *ACO1*, *PinII* and *LOXA*, and with the gene coding for the invertase LIN6 (Fig. 4B).

A

	Estimate	SS	$F_{1,51}$	P
LePT4	0.007	0.006	1.511	0.225
FatM	0.035	0.023	5.755	**0.020**
SUS3	-0.011	0.022	5.697	**0.021**
P14c	-0.027	0.020	5.103	**0.029**
GluB	0.019	0.009	2.395	0.128
PAL	-0.005	0.009	2.239	0.141
Residuals		0.201		

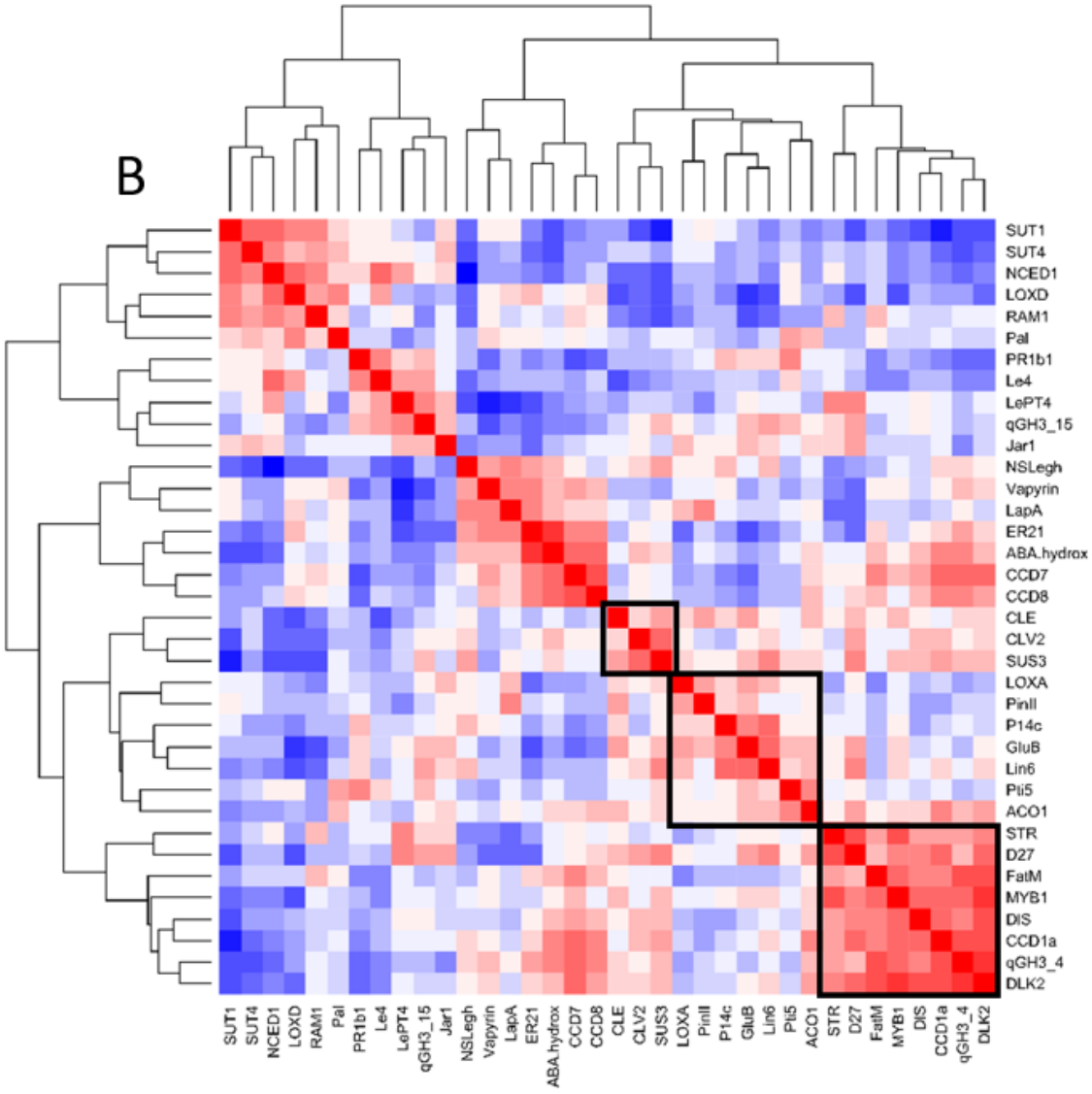

Figure 4. Correlation matrix of gene expression (A) Linear model showing the effect of expression of selected genes on arbuscular mycorrhizal fungal colonization in roots (arcsine transformed). Model estimate, SS – Sum of squares, F values and degrees of freedom (as subscript) and associated P values. (B) Correlations across gene expression. Red colors denote positive correlations, blue denotes negative correlations. Color intensity indicates the correlation strength.

Salt stress differentially impacts gene expression of mycorrhizal-related genes depending on the colonizing AM fungus

Since salt stress treatment had an opposite impact on *F. mosseae* and *R. irregularis*, we further analyze the effect of this treatment in more detail. Salinity increased *F. mosseae* colonization and repressed that of *R. irregularis*, while no effect when using the combination of the two AM fungi was observed (Fig. 1). We compared the expression profiles of the genes selected from the linear model as significantly affecting AM fungal colonization -*FatM*, *SUS3* and *P14c*-, and those correlating with these genes, according to the correlation matrix and according to their function. The different functional groups showed differential regulation patterns depending on the colonizing fungus. For instance, under salt stress, the genes related to lipid transfer (*FatM*, *DlS* and *STR*) were repressed in plants colonized by *R. irregularis*, while they did not change in *F. mosseae* colonized plants (Fig. 5). A similar pattern was found for *MYB1* and *DLK2*, associated with control of the symbiosis. In contrast, genes related to sugar metabolism (*SUS3*, *Lin6*) were repressed by salinity in plants inoculated with *F. mosseae* but induced in those colonized by *R. irregularis*. Regarding the genes associated with the autoregulation of the symbiosis *CLE* and *CLV2*, and those related with the biosynthesis of the 'yellow pigment' (arbuscule turnover) (D27 and CCD1a) were repressed in *F. mosseae* plants, whereas they did not change in *R. irregularis* plants. Finally, the defense-related genes were generally induced by *R. irregularis* upon salinity, while they did not change or were even partially repressed in *F. mosseae* plants. Interestingly, plants with double inoculation (FmRi) showed intermediate patterns, generally with less pronounced changes, to those corresponding with the individual inoculations.

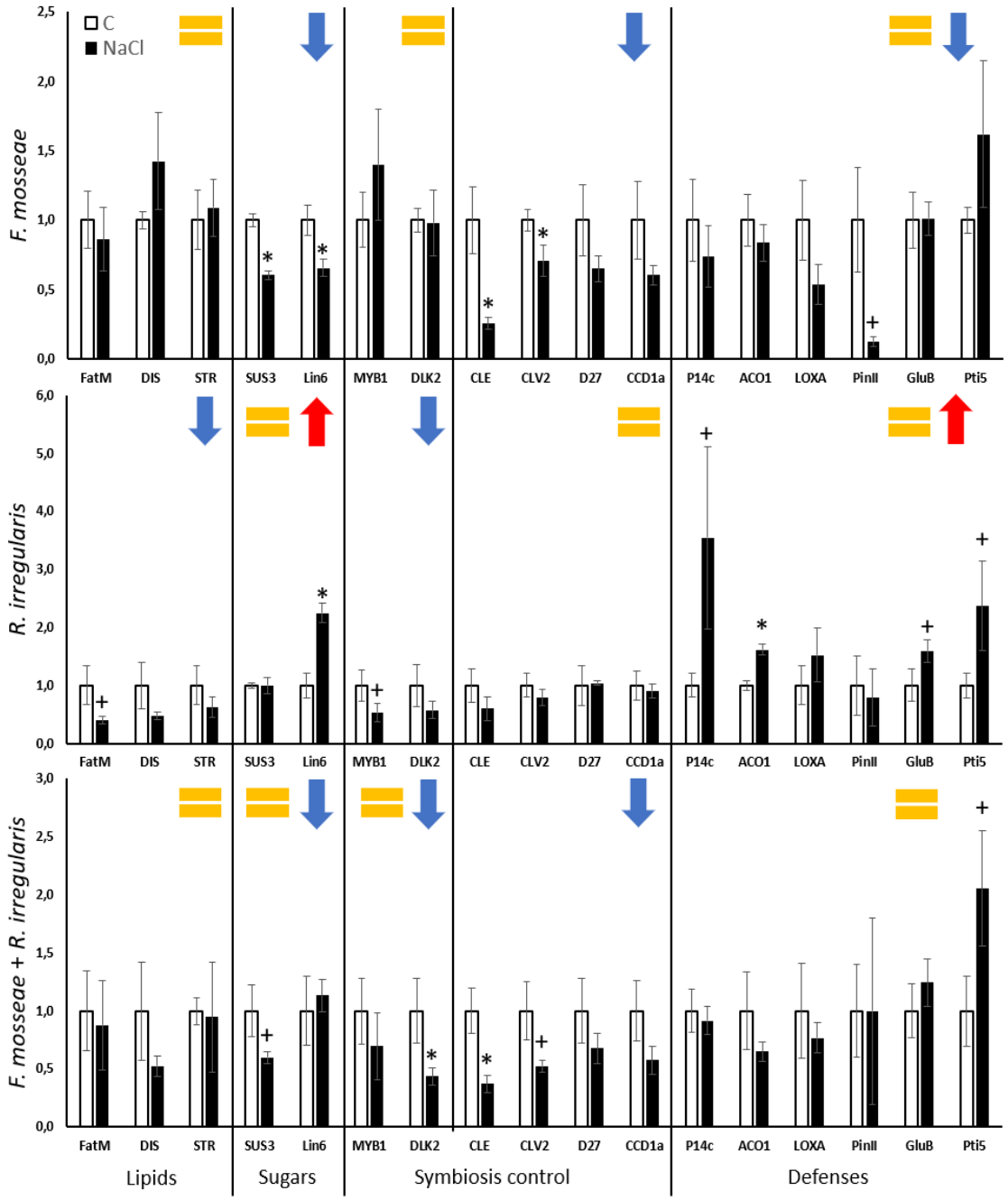

Figure 5. Impact of salt stress on gene expression in plants inoculated with the different AMF. Mycorrhizal plants inoculated with F. mosseae, R. irregularis or a combination of Fm and Ri (FmRi) were subjected to salt stress (150 mM of NaCl solution) (NaCl) or left untreated (C). Bars represent relative gene expression values in salt treated plants normalized to the values in the control treatment (set to 1) Expression values were normalized in each sample using the normalizer gene SlEF. Data shown are mean ± SEM of 4 independent biological replicates. Within each AM treatment bars marked by (*) are significantly different to their respective controls (t-test, p<0.05). Colored symbols represent the general trend (=, no changes; red arrow up, induction; blue arrow down, repression) in the regulation by salt for each given mycorrhizal treatment.

The impact of salt stress on symbiosis functionality depends on the colonizing AM fungus

Salt stress had an effect on P acquisition by the plant and the functionality of the symbiosis. In non-mycorrhizal plants, P content in roots was significantly reduced by salinity, similarly to that observed in plants colonized by *R. irregularis* (Fig. 6A). In contrast, plants colonized by *F. mosseae* alone or in combination with the two fungi did not show any effect of the stress in P levels. P uptake is a major benefit that the plant receives from the AM fungus, and the induction of plant Pi transporters in arbusculated cells is considered a hallmark of symbiosis functionality. We evaluated the expression of the mycorrhiza-specific Pi transporter *PT4* tomato gene (Balestrini et al., 2007; López-Ráez et al., 2015). When *LePT4* expression levels were normalized to the amount of AM fungus, we found that its expression was significantly induced under salt stress in plants colonized by *F. mosseae*, up to 4 times in Fm plants, and up to 6 in FmRi plants. Thus, the symbiosis seemed to be more efficient under stress conditions, as supported by the P concentration analysis (Fig. 6B). We also evaluated the abundance of fungal vesicles in the roots. Vesicles are reservoir structures from the fungus, usually rich in lipids. Remarkably, under salt stress the abundance of vesicles was reduced in plants inoculated with *R. irregularis*, with up to 2 or 3-fold reduction in roots colonized by Ri alone or in combination (FmRi), respectively (Fig. 6C). The reduced expression of genes related to lipid transfer in Ri colonized plants -not observed in Fm plants- may underlie this reduction in vesicles in plants colonized by *R. irregularis*.

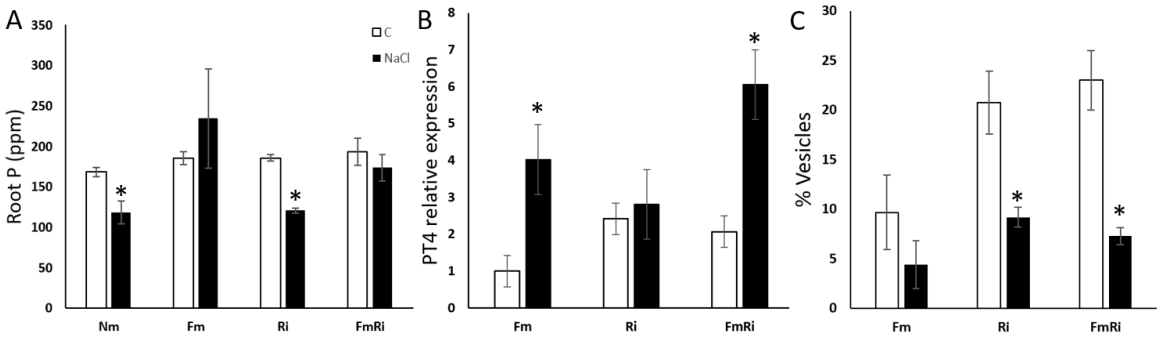

Figure 6. Impact of salt stress on P acquisition by the plant. Non-mycorrhizal (Nm) or mycorrhizal plants inoculated with F. mosseae (Fm), R. irregularis (Ri) or a combination of Fm and Ri (FmRi) were subjected to salt stress by adding 150 mM of NaCl solution (NaCl) or left untreated (C). (A) Phosphorus concentration in roots. (B) Relative expression of the plant phosphate transporter 4 (LePT4) normalized using the normalizer gene SlEF. (C) Abundance of fungal vesicles within the colonized areas. Data shown are mean ± SEM of 4 (A,B) or 5 (C) biological replicates. Columns noted with (*) are significantly different to their untreated controls (t-test, p<0.05).

DISCUSSION

The use of AM fungi as biostimulants in agricultural and ecological settings is an increasing strategy for sustainable plant management. However, despite the well characterized benefits of the symbiosis, their application is still challenging because of the variability of the results when applied into production systems (Duhamel & Vandenkoornhuyse, 2013; Tkacz & Poole, 2015). This variability mainly relies on the impact of environmental conditions on the development and functionality of the symbiosis, and on the functional diversity of the plant-fungal genotypes (Hart et al., 2018; Holland et al., 2018; Kokkoris et al., 2019; Orine et al., 2022). Here, we hypothesized that the plant is able to regulate the development and the extension of mycorrhizal colonization according to the plant needs and its environmental context, and that this effect varies depending on the fungal partner. We found significant differences in mycorrhizal colonization between two different AM fungi (*F. mosseae, R. irregularis*), that vary depending on the treatments applied. We explored if changes in colonization levels correlated with changes at the transcriptional level related to different plant signaling pathways. Interestingly, fungal colonization rates correlated with the modulation of the plant defensive responses, especially related to the SA-dependent pathway, changes related to carbon -lipids and sugars- supply from the plant to the fungus, and changes in the control and autoregulation of the symbiosis. Particularly, salt stress impacted differently *F. mosseae* and *R. irregularis*, promoting the colonization by the first one and restricting the latest. This differential regulation seems to be related to the benefits provided by each fungus, with *F. mosseae*, but not *R irregularis*, compensating the negative effect of salt stress on Pi acquisition. While the plant restricted lipid supply and enhanced defenses and symbiosis control in the interaction with *R. irregularis* under salt stress, defenses and symbiosis control were reduced and lipid supply maintained in the interaction with *F. mosseae*. These results support the Kiers' free market hypothesis, where greater benefits provided by the AM fungus is rewarded with a higher carbon input by the host plant.

It is known that the environmental context impacts organism homeostasis and may trigger systemic changes that modify its interactions with other organisms (Gruden et al., 2020). Phytohormones and their crosstalk play major roles in plant responses to the environmental context (Pozo et al., 2015). In the present work, we show that the exogenous application of stress related hormones to mimic stressful environments impacted mycorrhizal colonization. Our results support an active regulation of mycorrhizal levels by the plant, that maintains the colonization within defined

margins avoiding excessive colonization. This process is likely to be orchestrated by phytohormones: indeed, all studied phytohormones have an effect on the establishment and maintenance of AM symbiosis (Ho-Plágaro & García-Garrido, 2022; Pozo et al., 2015). However, promoting or repressing effects in mycorrhizal colonization for the same hormone have been sometimes reported, probably depending on the partners genotypes and the environmental/experimental conditions determining plant needs (Bedini et al., 2018; Pozo et al., 2015). This observation fits with our results showing that the impact of the stress conditions on colonization is dependent on the AMF genotype. The initial colonization levels seemed to be determinant, as the plant promoted colonization of the lower colonizer (*F. mosseae*) to reach the hypothetical maximum threshold, while it restricted the colonization by the most efficient colonizer *R. irregularis*. The hypothesis of a maximum threshold is supported by the fact that the double inoculation, containing the combination of both inocula did not reach higher levels than those achieved by *R. irregularis* alone. The results support that the colonization, once established, is well controlled in a delimited margin probably adjusted depending on the context, a process known as autoregulation (Wang et al., 2018). *R. irregularis* is a very good colonizer, usually reaching higher levels than other AMF, including *F. mosseae* (Liu et al., 2022; López-Ráez et al., 2010b). In fact, *R. irregularis* is usually the most abundant within roots in natural soils despite multiple AMF species being present in the soil (Varela-Cervero et al., 2015). The mechanisms underlying such success are under scrutiny, but several effectors with immunomodulatory properties have been described in *R. irregularis*, and to what extent are conserved among different AMF is yet to be explored (Zeng et al., 2018).

We aimed to investigate the mechanisms contributing to the regulation of the colonization under stress conditions. Following a transcriptomic approach with well characterized marker genes we analyzed the contribution of different signaling pathways to the changes in colonization observed in our system. The changes, both at the colonization and transcriptional levels, were more evident under salt treatment, probably as it really imposed a stress on the plant (as evidenced by the biomass reduction) and can affect the AM fungi directly or the symbiosis. Indeed, salinity affected the colonization by the two AM fungi differentially. Salt stress negatively impacted shoot biomass, and while the effect was less pronounced in mycorrhizal plants by *F. mosseae* and *R. irregularis,* only in the combined Fm-Ri inoculation the negative effect was abolished, pointing to a potential synergistic effect. The stress also led to a reduced P content in roots in Nm and Ri plants, but *F. mosseae* plants did not show such reduction. A protective effect of AM fungi against salinity has been shown in several plant species, such as lettuce, maize and tomato (Aroca et al., 2013;

Estrada et al., 2013; Rivero et al., 2018). The protection is usually related to enhanced plant tolerance by increasing water and nutrient uptake, photosynthesis capacity and a better ionic homeostasis (Evelin et al., 2019; Ruiz-Lozano et al., 2012). Indeed, it is described that the main benefit of AM symbiosis in salt stress is the mitigation of the reduced Pi uptake (Porcel et al., 2012). Here, while *F. mosseae* protected the plant against Pi depletion by salinity, no protection was observed in *R. irregularis* colonized plants. These changes correlated with the promotion of colonization by *F. mosseae* and with a reduced colonization by *R. irregularis*. We cannot exclude the effect of salinity on the fungus itself (Yamato et al., 2008) on the influence in mycorrhizal colonization. However, the active regulation by the plant promoting the most efficient AM symbiont *F. mosseae* and restricting the high demanding *R. irregularis* is likely.

We explored different potential mechanisms that may contribute to the effects observed through the analysis of marker genes related to pre-symbiotic signaling, plant defense mechanisms, control of nutrient exchange or specific control or autoregulation of the AM symbiosis. Rhizospheric signaling, mainly orchestrated by SLs, is involved in the *de novo* recruitment of mycorrhizal fungi and it is supposed to be stimulated under environmental stresses (Aroca et al., 2013; López-Ráez et al., 2008; Ruiz-Lozano et al., 2016). Analysis of genes involved in SL synthesis do not support a role of early signaling during the pre-symbiotic stage in the changes observed. Thus, we did not find support for a relevant "cry for help" under our experimental conditions. We initiated the stress treatments 2 weeks upon inoculation and maybe pre-symbiotic contact was already established, as it is reported that SLs do not play a main role in well-established symbioses (Al-Babili & Bouwmeester, 2015). In contrast, overall changes in the colonization levels correlated with the regulation of plant defenses, nutrient exchange and control of the symbiosis. Our global analysis points out that the tradeoff between nutrients (P) provided by the fungus and the carbon supplied by the plant plays a key role in the differential regulation of colonization. Under salt stress conditions, plants inoculated with *F. mosseae* showed higher mycorrhizal levels than under control conditions. Moreover, this enhanced colonization correlated also with a higher expression of the mycorrhiza-specific plant Pi transporter *PT4*. This transporter is required for symbiotic Pi uptake, being a well-documented marker of mycorrhizal levels and symbiosis functionality (Balestrini et al., 2007; Harrison et al., 2002). It has been shown that the host plant regulates carbon supply to the fungus based on the Pi input from the fungal partner (Helber et al., 2011), and that an enhanced lipid allocation towards the fungus promotes arbuscule formation (Feng et al., 2020). Colonization by *R. irregularis -not providing protection nor enhanced P uptake-* was reduced under these stress

conditions. The reduction correlated with lower expression of the genes involved in lipid synthesis and delivery to the AM fungus in the arbusculated cells, including *FatM* (encoding for an ACP-thioesterase) and DIS (disorganized arbuscules, encoding a β-keto-acyl ACP synthase I) and STR2 (Stunted Arbuscule2, encoding an heterodimeric Adenosine Triphosphate (ATP)-Binding Cassette (ABC) transporter) (Keymer et al., 2017). Remarkably, these genes were not inhibited by salt in the interaction with *F. mosseae*, supporting the idea of an active control of the plant over fungal colonization depending on the benefits obtained. Regarding the control of the arbuscule itself, salt reduced the expression of the negative regulator of arbuscle branching (DLK2) (Ho-Plágaro et al., 2021) and its senescence (MYB1) (Floss et al., 2017) in plants colonized by *R. irregularis* and the dual inoculation, likely promoting arbuscule formation to enhance symbiosis functionality.

Colonization levels by *F. mosseae* and *R. irregularis* also correlated with the modulation of plant defense responses, mostly associated with the SA, JA and ET-dependent signaling pathways. It is known that plants require a precise finetuning of their immune system in order to contain potential attackers while promoting mutualistic interactions, and AMF has to overcome plant defenses as well (Martínez-Medina et al., 2019; Plett & Martin, 2018; Zamioudis & Pieterse, 2012; Zipfel & Oldroyd, 2017). Under salt stress, a higher induction of defensive genes was observed in plants inoculated with *R. irregularis* as compared with those colonized by *F. mosseae*. This suggests that the plant is more actively trying to control this fungus, likely very demanding as it is a very good colonizer and displays a high number of vesicles -fungal energy reservoirs-. This enhanced defense response correlated with a reduction in mycorrhizal levels and vesicles abundance, indicating that the plant is indeed controlling colonization rates and nutrient flux to the fungus, likely favoring those AM fungi that are more efficient in nutrient supply. In agreement with this idea, a reduction in the expression of genes associated to symbiosis autoregulation - *CLE* and *CLV2* - was observed in plants inoculated with *F. mosseae* as compared with those colonized by *R. irregularis*. The same behavior was seen for the gene *CCD1a*, involved in the biosynthesis of mycorradicin and α-ionols that regulate the arbuscule lifespan (Walter et al., 2015). Taken together, our results support the idea that the tradeoff between mineral nutrients and carbon between the AM fungus and the host plant drives symbiotic levels and efficiency, and that it affects the modulation of plant defenses and the autoregulation of the symbiosis.

Overall, we show here that different AM fungi have different colonization strategies and that stress conditions may affect their interaction with the host plant. They also suggest that the

plant modulates the colonization extension according to the nutritional tradeoff, following the motto ‘more for the better’. Moreover, our results support the resilience of the mycorrhizal interactions, as despite the activation of different defensive pathways the system buffers the changes and overall, mycorrhizal colonization is maintained within a given range, likely balancing the interaction for mutual benefit. Finally, the results suggest that the combined AMF treatment was the more efficient in stress alleviation and seem to be more stable across treatments. This fact opens the possibility of using AM fungal consortia as commercial biostimulants in order to obtain improved benefits, especially in the ever-changing conditions found in nature and in the production systems.

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Figure S1. Root hormonal content expressed as ng*g^{-1}FW.

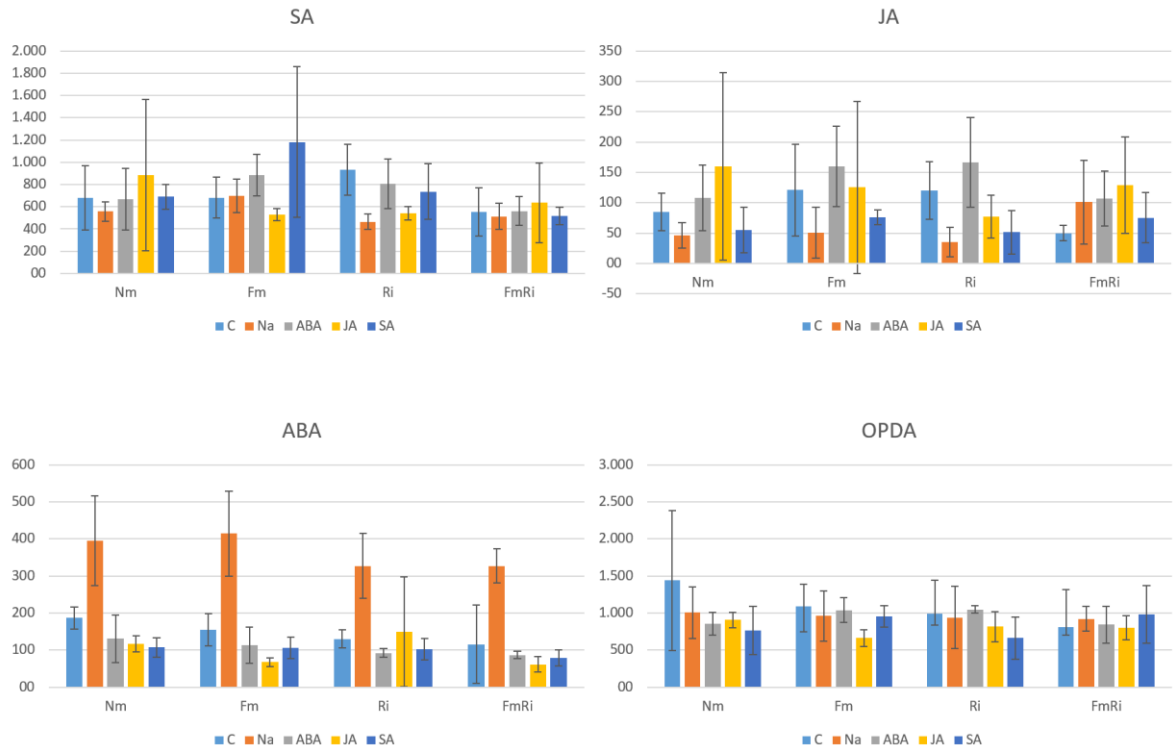


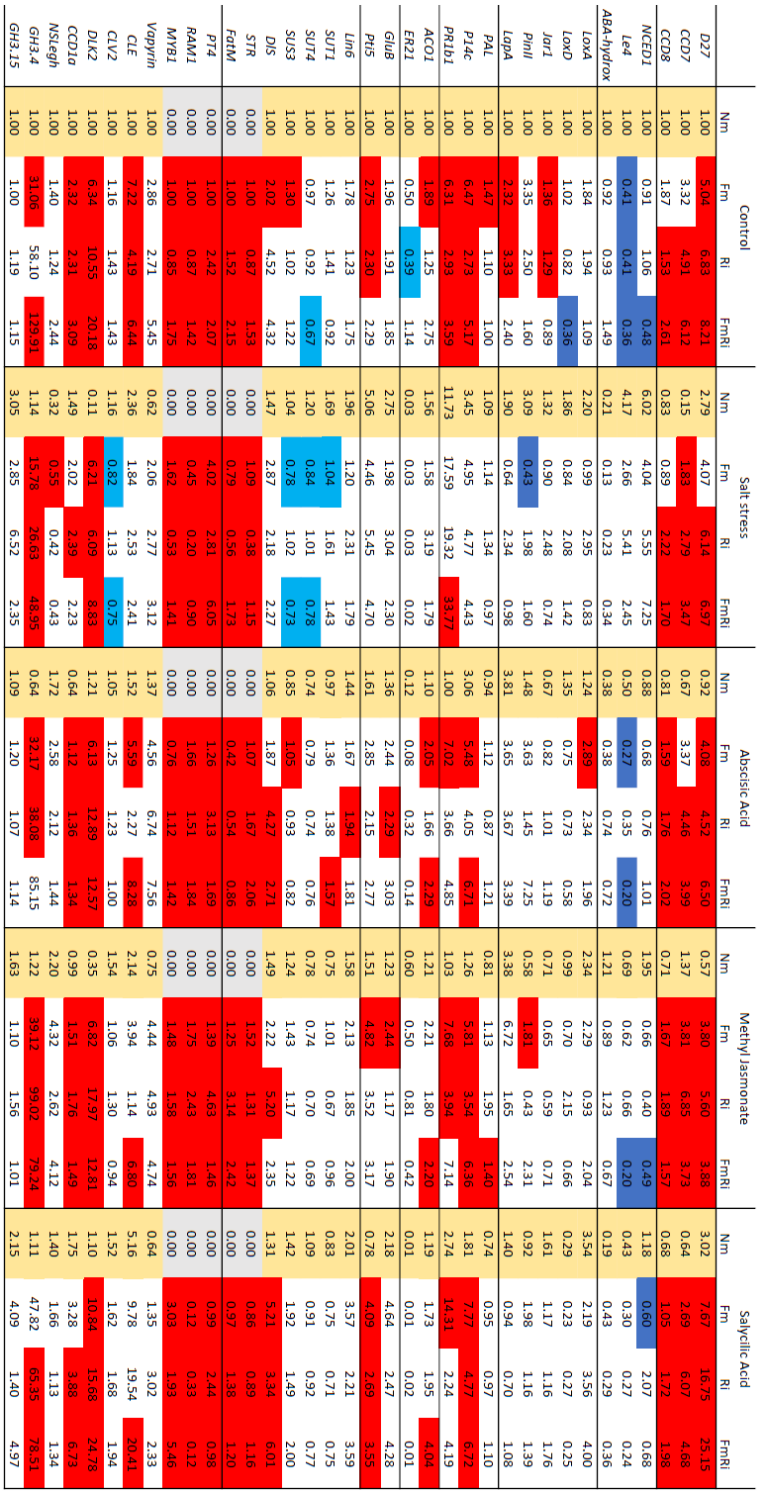
Table S1. Primers qPCR

Gene	Sequence F (5'-3')	Sequence R (5'-3')	Solyc	Reference
SlEF	GATTGGTGGTATTGGAACTGTC	AGCTTCGTGGTGCATCTC	Solyc06g009960	(Rotenberg et al., 2006)
D27	TTGGCTAGTTGGACCTTGTG	CAAAGTTTGGCACCATATTCA	Solyc09g065750	(Torres-Vera et al., 2016)
CCD7	AGCCAAGAATTCGAGATCCC	GGAGAAAGCCCACATACTGC	Solyc01g090660	(López-Ráez et al., 2010a)
CCD8	CAGGACAATGGCACATAGGT	GCGTCCGATTCGATTTG	Solyc08g066650	(López-Ráez et al., 2010a)
NCED1	ACCCACGAGTCCAGATTTC	GGTTCAAAAAGAGGGTTAGC	Solyc07g056570	(López-Ráez et al., 2010b)
Le4	ACTCAAGGCATGGGTACTGG	CCTTCTTTCTCCTCCCACCT	Solyc02g084850	(López-Ráez et al., 2010b)
ABA-hydrox	TGTCCAGGGAATGAACTTGC	CAATGGGACTGGGAATGGTC	Solyc04g078900	(López-Ráez et al., 2010a)
LOXA	GGTTACCTCCCAAATCGTCC	TGTTTGTAACTGCGCTGTG	Solyc08g014000	(López-Ráez et al., 2010b)
LOXD	GACTGGTCCAAGTTCACGATCC	ATGTGCTGCCAATATAAATGGTTCC	Solyc03g122340	(Uppalapati et al., 2005)
Jar1	CATTGAAACCATCTCCTTGA	TAAACTGCTTGCTGCTGTAAA	Solyc10g011660	(Scalschi et al., 2013)
Pin II	GAAAATCGTTAATTTATCCCAC	ACATACAAACTTTCCATCTTTA	Solyc01g095200	(Uppalapati et al., 2005)
LapA	ATCTCAGGTTTCCTGGTGGAAGGA	AGTTGCTATGGCAGAGGCAGAG	Solyc12g010020	(Yan et al., 2013)
PAL	CGTTATGCTCTCCGAACATC	GAAGTTGCCACCATGTAAGG	Solyc03g042560	(Martínez-Medina et al., 2013)
P14c	TATCTTAACGCTCACAATGCAG	GTTTTCACCGTAAGGTCCAC	Solyc01g106620	(Martínez-Medina et al., 2013)
PR1b1	CCAAGACTATCTTGCGGTTCA	CAGCTCTTGAGTTGGCATAGT	Solyc00g174340	(Bubici et al., 2017)
ACO1	AAGGGACTCCGCGCTCATA	CAAGTTGGTCACCAAGGTTAACC	Solyc07g049530	(Chersicola et al., 2017)
GluB	CCATCACAGGGTTCATTTAGG	CCATCCACTCTCTGACACAACT	Solyc01g059980	(Martínez-Medina et al., 2013)
Pti5	CGCGATTCGGCTAGACA	GCCTTAGCACCTCGCATTCT	Solyc02g077370	This work
Lin6	AGCACATTTATTCGCCTTCAAC	TTTGTGACGTGGCATAATAAGA	Solyc10g083290	(García Rodríguez, 2006)
SUT1	TTCCATAGCTGCTGGTGTTC	TACCAGAAATGGGTCCACAA	Solyc11g017010	(Hackel et al., 2006)
SUT4	TCTCCGCTGATATTGGATGG	GCAACATCGAGAAGCCAAAA	Solyc04g076960	(Sanmartín et al., 2020)
SUS1	GGATTGAAAGCCACGGAAAGG	ACCAGGCCTCAACGAATAGCA		(García Rodríguez, 2006)
SUS3	GGTTTCTGTCTGATTGTTATCC	ACAGAAGGGAAAAATGGCAAA	Solyc07g042550	(Goren et al., 2011)
STR	TAGTCCCAAGTTACATCAC	ACCATCTCCAAACCAAAG	Solyc01g097430	(Ho-Plágaro, 2018)
FatM	AGCCACAGGCCTTGTATTTG	TCCTCTTTGATGGCTTGCTTAC	Solyc05g008570	(Chialva et al., 2020)
DIS	AAATGAACGGGACAAAGTCG	GTTGGATGAAGCCATCCTGT	Solyc08g082620	(Chialva et al., 2020)
PT4	GAAGGGGAGCCATTTAATGTGG	ATCGCGGCTTGTTTAGCATTTC	Solyc06g051850	(Balestrini et al., 2007)
RAM1	CTCAGAATGTCAGAGGAAGAT	CCAGCAGCAGTATCAGAA	Solyc02g094340	(Ho-Plágaro, 2018)
Vapyrin	GAGAGTCTTTAATTGTTGAGC	TTAGCACCATTGAGTAAGAG	Solyc10g081500	(Ho-Plágaro, 2018)
CLV2	TGTTTCCAACTACTGGCGCT	TATCCAACGCGTCAAGCACT	Solyc04g056640	This work
DLK2	GGGAGTTGAAATTGCATTACCT	TAGTGAAATGGGCACCACAA	Solyc05g018413	(García-Garrido et al., 2010)
CCD1a	AAGCTTGAGAATTTCTGCA	GCCTGTGTAGTTCTCGTTGAT	Solyc01g087250	(López-Ráez et al., 2010a)
NSLegh	ATGCTGGTGAATGGGGTCTC	TCCCTCACCACAACCTTTCC	Solyc07g008240	(Martínez-Medina et al., 2019)
GH3.4	CTCCAGGGTGATTTCTGT	TTCTTTGGTCCACTGTCT	Solyc02g092820	(Liao et al., 2015)
GH3.15	GCACCCATTATTGAACTA	TCTTGGACTTATGATGAAGC		(Liao et al., 2015)

Table S2. Nutrient concentration in roots (ppm). Data shown are mean ± SEM of 4 biological replicates.

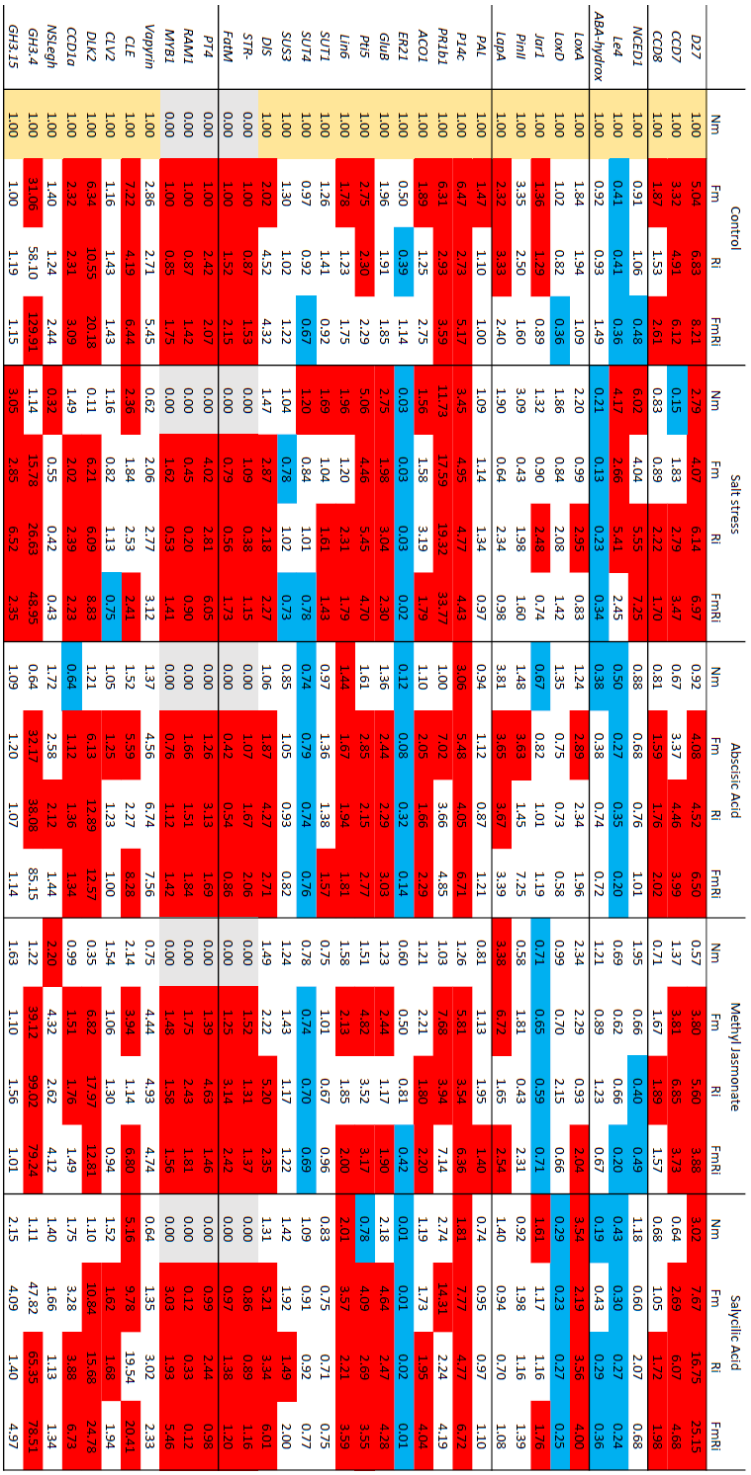
	Control				Salt stress			
Element	Nm	Fm	Ri	FmRi	Nm	Fm	Ri	FmRi
Al	14841.9±1554.5	19714±2754.5	19776.6±2239.9	22302.7±6789.8	14086.5±5514.5	19680.4±999.7	14877±4470.8	21614.3±7004.6
As	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Ca	8546.2±1812.3	11191.5±1254.1	10171.7±3290.7	11991.9±3742.3	8711±4007.5	10294.4±1641.4	41513±61777	19891.6±13972.8
Cd	1.4±0.1	1.5±0.2	1.6±0.3	1.6±0.2	1.3±0.3	1.7±0.2	1.4±0.1	1.7±0.3
Co	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Cr	9.5±1.9	13.4±1.8	11.2±3.1	14.7±4.7	45.3±22.8	15.3±3.2	22.4±8.2	21.2±16.8
Cu	61.2±8	67.3±8.1	71.6±13.5	75.9±19	44.9±8	60.5±12.8	60.2±21.4	87.8±17.7
Fe	9285.1±797.7	12216.4±1505.8	14607.9±5503.4	14097.1±4127.9	8009.6±2809.8	10782.6±2487.4	9077.9±2359.4	14043.5±3991.7
Hg	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
K	22125.4±740.6	22981.9±1757	24191.3±2438.7	24230.6±3688.1	15266.8±1786.6	18043.3±1190.3	14684.8±1329.3	16302.3±1682.1
Li	19.5±8.4	18.1±2	15.2±1.6	21.7±4.9	91.4±60.8	23.7±7.3	57.4±26.5	25±5.9
Mg	38661.1±2638.1	40156.4±2380.9	40120.3±3275.9	41724±4766.4	53306.9±5570.7	44490.8±5085.6	46995.4±10656.8	47409.5±7685.3
Mn	214.9±18.9	305.8±35.3	1225.5±1833.9	374.5±96.8	239.2±67.3	433±66.2	301.3±25.6	429.4±65.5
Mo	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Na	1147.2±278.4	1686.5±844.5	1785.7±925.6	1251.1±304.9	8300.4±1912.3	11564.1±1062.5	8111.1±717.7	8230.3±2264.4
Ni	10.5±1.8	13.7±2.4	16±4.9	15.7±5.3	26.4±9.2	12.9±3.6	15.8±3	30.6±31.7
P	168.3±11.6	185.4±15.3	185.8±7.9	193.2±34	118.6±28	234.7±106.2	120.7±5.1	173.7±32.4
Pb	11.4±2.6	16.7±2.5	14.9±2.8	20.1±5.8	7.9±1.4	12.5±1.2	8.8±0.1	15.9±5.1
S	4299.2±393.9	3756.7±228.2	4141.1±779.6	3741.8±860.9	2694.3±942.1	3299.6±522.5	2856.6±374.3	2721.8±950.6
Se	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Si	3602.5±322.1	3435.8±199.9	3876.9±545	3684.7±824.1	4459±574.1	6123.7±200.2	4367.8±528.4	3586.7±450.1
Sr	76±9.8	86.8±5.9	91±31.3	83.3±12.9	61.4±7.8	148.4±95.4	78.3±40.7	101.2±53.2
Ti	445.4±15.3	591.5±79.5	549.4±69.2	657±184.1	362±113.5	555.6±90.5	416.3±106.8	684.8±140.9
V	16.8±2.2	20.5±1.7	23.4±8.9	23.4±6.8	14.1±3.4	16±2.1	14.6±3.4	21.5±6.4

Table S3. Complete set of gene expression data. Data correspond to fold change in gene expression compared to the non-mycorrhizal untreated control (n = 4). Color indicates indicate significantly different to their own non-mycorrhizal treatment (t-test, p<0.05). Upregulation in red, downregulation in blue.



	Control			Salt stress			Abscisic Acid			Methyl Jasmonate			Salycilic Acid		
	Nm	Fm	Ri	FmRi	Nm	Fm	Ri	FmRi	Nm	Fm	Ri	FmRi	Nm	Fm	Ri	FmRi
D27	1.00	5.04	6.83	8.21	2.79	4.07	6.14	6.97	0.92	4.08	4.52	6.50	0.57	3.80	5.60	3.88	3.02	7.67	16.75	25.15
CCD7	1.00	3.32	4.91	6.12	0.15	1.83	2.79	3.47	0.67	3.37	4.46	3.99	1.37	3.81	6.85	3.73	0.64	2.69	6.07	4.68
CCD8	1.00	1.87	1.53	2.61	0.83	0.89	2.22	1.70	0.81	1.59	1.76	2.02	0.71	1.67	1.89	1.57	0.68	1.05	1.72	1.98
NCED1	1.00	0.91	1.06	0.48	6.02	4.04	5.55	7.25	0.88	0.68	0.76	1.01	1.95	0.66	0.40	0.49	1.18	0.60	2.07	0.68
Le4	1.00	0.41	0.41	0.36	4.17	2.66	5.41	2.45	0.50	0.27	0.35	0.20	0.69	0.62	0.66	0.20	0.43	0.30	0.27	0.24
ABA-hydrox	1.00	0.92	0.93	1.49	0.21	0.13	0.23	0.34	0.38	0.38	0.74	0.72	1.21	0.89	1.23	0.67	0.19	0.43	0.29	0.36
LoxA	1.00	1.84	1.94	1.09	2.20	0.99	2.95	0.83	1.24	2.89	2.34	1.96	2.34	2.29	0.93	2.04	3.54	2.19	3.56	4.00
LoxD	1.00	1.02	0.82	0.36	1.86	0.84	2.08	1.42	1.35	0.75	0.73	0.58	0.99	0.70	2.15	0.66	0.29	0.23	0.27	0.25
Jar1	1.00	1.36	1.29	0.89	1.32	0.90	2.48	0.74	0.67	0.82	1.01	1.19	0.71	0.65	0.59	0.71	1.61	1.17	1.16	1.76
PinII	1.00	3.35	2.50	1.60	3.09	0.43	1.98	1.60	1.48	3.63	1.45	7.25	0.58	1.81	0.43	2.31	0.92	1.98	1.16	1.39
LapA	1.00	2.32	3.33	2.40	1.90	0.64	2.34	0.98	3.81	3.65	3.67	3.39	3.38	6.72	1.65	2.54	1.40	0.94	0.70	1.08
PAL	1.00	1.47	1.10	1.00	1.09	1.14	1.34	0.97	0.94	1.12	0.87	1.21	0.81	1.13	1.95	1.40	0.74	0.95	0.97	1.10
PI4c	1.00	6.47	2.73	5.17	3.45	4.95	4.77	4.43	3.06	5.48	4.05	6.71	1.26	5.81	3.54	6.36	1.81	7.77	4.77	6.72
PR1b1	1.00	6.31	2.93	3.59	11.73	17.59	19.32	33.77	1.00	7.02	3.66	4.85	1.03	7.68	3.94	7.14	2.74	14.31	2.24	4.19
ACO1	1.00	1.89	1.25	2.75	1.56	1.58	3.19	1.79	1.10	2.05	1.66	2.29	1.21	2.21	1.80	2.20	1.19	1.73	1.95	4.04
ER21	1.00	0.50	0.39	1.14	0.03	0.03	0.03	0.02	0.12	0.08	0.32	0.14	0.60	0.50	0.81	0.42	0.01	0.01	0.02	0.01
GluB	1.00	1.96	1.91	1.85	2.75	1.98	3.04	2.30	1.36	2.44	2.29	3.03	1.23	2.44	1.17	1.90	2.18	4.64	2.47	4.28
Pti5	1.00	2.75	2.30	2.29	5.06	4.46	5.45	4.70	1.61	2.85	2.15	2.77	1.51	4.82	3.52	3.17	0.78	4.09	2.69	3.55
Lin6	1.00	1.78	1.23	1.75	1.96	1.20	2.31	1.79	1.44	1.67	1.94	1.81	1.58	2.13	1.85	2.00	2.01	3.57	2.21	3.59
SUT1	1.00	1.26	1.41	0.92	1.69	1.04	1.61	1.43	0.97	1.36	1.38	1.57	0.75	1.01	0.67	0.96	0.83	0.75	0.71	0.75
SUT4	1.00	0.97	0.92	0.67	1.20	0.84	1.01	0.78	0.74	0.79	0.74	0.76	0.78	0.74	0.70	0.69	1.09	0.91	0.92	0.77
SUS3	1.00	1.30	1.02	1.22	1.04	0.78	1.02	0.73	0.85	1.05	0.93	0.82	1.24	1.43	1.17	1.22	1.42	1.92	1.49	2.00
DIS	1.00	2.02	4.52	4.32	1.47	2.87	2.18	2.27	1.06	1.87	4.27	2.71	1.49	2.22	5.20	2.35	1.31	5.21	3.34	6.01
STR	1.00	1.00	0.87	1.53	0.00	1.09	0.38	1.15	0.00	1.07	1.67	2.06	0.00	1.52	1.31	1.37	0.00	0.86	0.89	1.16
FotM	0.00	1.00	1.52	2.15	0.00	0.79	0.56	1.73	0.00	0.42	0.54	0.86	0.00	1.25	3.14	2.42	0.00	0.97	1.38	1.20
PT4	0.00	1.00	2.42	2.07	0.00	4.02	2.81	6.05	0.00	1.26	3.13	1.69	0.00	1.39	4.63	1.46	0.00	0.99	2.44	0.98
RAM1	0.00	1.00	0.87	1.42	0.00	0.45	0.20	0.90	0.00	1.66	1.51	1.84	0.00	1.75	2.43	1.81	0.00	0.12	0.33	0.12
MYB1	0.00	1.00	0.85	1.75	0.00	1.62	0.53	1.41	0.00	0.76	1.12	1.42	0.00	1.48	1.58	1.56	0.00	3.03	1.93	5.46
Vapyrin	1.00	2.86	2.71	5.45	0.62	2.06	2.77	3.12	1.37	4.56	6.74	7.56	0.75	4.44	4.93	4.74	0.64	1.35	3.02	2.33
CLE	1.00	7.22	4.19	6.44	2.36	1.84	2.53	2.41	1.52	5.59	2.27	8.28	2.14	3.94	1.14	6.80	5.16	9.78	19.54	20.41
CLV2	1.00	1.16	1.43	1.43	1.16	0.82	1.13	0.75	1.05	1.25	1.23	1.00	1.54	1.06	1.30	0.94	1.52	1.62	1.68	1.94
DLK2	1.00	6.34	10.55	20.18	0.11	6.21	6.09	8.83	1.21	6.13	12.89	12.57	0.35	6.82	17.97	12.81	1.10	10.84	15.68	24.78
CCD1a	1.00	2.32	2.31	3.09	1.49	2.02	2.39	2.23	0.64	1.12	1.36	1.34	0.99	1.51	1.76	1.49	1.75	3.28	3.88	6.73
NSLegh	1.00	1.40	1.24	2.44	0.32	0.55	0.42	0.43	1.72	2.58	2.12	1.44	2.20	4.32	2.62	4.12	1.40	1.66	1.13	1.34
GH3-4	1.00	31.06	58.10	129.91	1.14	15.78	26.63	48.95	0.64	32.17	38.08	85.15	1.22	39.12	99.02	79.24	1.11	47.82	65.35	78.51
GH3.15	1.00	1.00	1.19	1.15	3.05	2.85	6.52	2.35	1.09	1.20	1.07	1.14	1.63	1.10	1.56	1.01	2.15	4.09	1.40	4.97

Table S3*bis*. Complete set of gene expression data. Data correspond to fold change in gene expression compared to the non-mycorrhizal untreated control (n = 4). Color indicates indicate significantly different to the non-mycorrhizal control (t-test, p<0.05). Upregulation in red, downregulation in blue.



Chapter 4

Ménage à trois: Unraveling the mechanisms regulating plant–microbe–arthropod interactions

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Ménage à trois: Unraveling the mechanisms regulating plant–microbe–arthropod interactions

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ABSTRACT

Plant–microbe–arthropod 3-way interactions have important implications for plant health. However, our poor understanding of the underlying regulatory mechanisms hampers their biotechnological applications. We searched for potential common patterns in plant responses regarding taxonomic groups or lifestyles. We found that most signaling modules regulating 2-way interactions also operate in 3-way interactions. Furthermore, the relative contribution of signaling modules to the final plant response cannot be directly inferred from 2-way interactions. Moreover, our analyses show that 3-way interactions often result in the activation of additional pathways, as well as in changes in the speed or intensity of defense activation. Thus, detailed, basic knowledge of plant–microbe–arthropod regulation will be essential for the design of environmentally friendly crop management strategies.

INTRODUCTION

Plants are central players in complex food webs with numerous organisms relying on the plant's resources. These plant-associated organisms, including microbes and arthropods, influence plant performance significantly, and determine the productivity in agro-ecosystems (Bagchi et al., 2014; Martínez-Medina et al., 2011; Saad et al., 2020; Wagg et al., 2011). Not surprisingly, research on plant–arthropod and plant–microbe interactions has become one of the central topics in plant biology.

Insects, representing the most species-rich group of arthropods, comprise around six million species, half of which are herbivorous (Schoonhoven et al., 2005). The diversity of pathogenic plant microbes is less characterized, but their threat to plants is equally renowned (Agrios, 2005). Besides parasitic interactions, plants establish mutualistic relationships with a plethora of organisms. Those include pollinators that are attracted to flower volatiles, natural enemies of attacking herbivores - such as predators and parasitoids that are attracted to volatiles (VOCs) emitted by herbivore-challenged tissues- and beneficial microbes (Pieterse & Dicke, 2007; Turlings & Erb, 2018) found in the rhizosphere and phyllosphere (Pieterse et al., 2014).

Plants often simultaneously or sequentially interact with both microbes and arthropods (Fig. 1). The response of plants to either of these threats can substantially change their suitability as a host plant for the other attacker (Pozo et al., 2020). For instance, plant-associated microbes can

change the quality of plants for herbivores by altering plant phenology, morphology, physiology and chemistry (Koricheva et al., 2009; Schädler & Ballhorn, 2017; Shikano et al., 2017; Tack & Dicke, 2013). Notably, beneficial microbes can improve plant health and induce resistance against a broad range of pathogens and pests (Jung et al., 2012; Mendes et al., 2013; Pieterse et al., 2014; Pineda et al., 2013) either directly as antagonists or indirectly by fine-tuning the plant immune system to prime plant defenses (Köhl et al., 2019; Selosse et al., 2014). Likewise, insect herbivores may impact plant-associated microbial communities by affecting the abundance, accessibility, suitability and chemistry of the host plant tissue for microbes (Biere & Goverse, 2016; Castagneyrol et al., 2018; M. De Vos, 2006). Such effects may even cascade up and down multiple trophic levels, impacting multitrophic webs in ecosystems (Rasmann et al., 2017; Tack & Dicke, 2013; Tao et al., 2017). Plant responses in 2-way interactions with either microbes or arthropods have been well characterized. These responses rely on the recognition of interacting organisms and specific activation of immune signaling and the related defense arsenal (Wilkinson et al., 2019). This arsenal is quite diverse, including physical barriers leading to cell wall reinforcement such as callose accumulation, and the production of repellent, toxic or digestibility reducing volatile and non-volatile compounds and lytic enzymes (Wilkinson et al., 2019). Plant immune signaling is regulated by small signaling molecules leading to a network of interconnected pathways, where the phytohormones jasmonic acid (JA) and salicylic acid (SA) have key regulatory roles (Pieterse et al., 2012). Other hormones such as ethylene (ET), abscisic acid (ABA), cytokinins (CK), auxins (AUX), and gibberellins (GA) can interact with the JA-SA-backbone in the orchestration of plant defenses (Robert-Seilaniantz et al., 2011; Tsuda & Somssich, 2015). The plant immune system is thus based on a highly flexible and complex signaling network. This flexibility allows plants to integrate multiple signals from their environment into an adaptive response that optimizes plant functions (Pozo et al., 2015). Only recently, studies have begun to unravel how such responses are regulated in more complex 3-way plant–microbe–arthropod (PMA) interactions (Pozo et al., 2020).

Here, we synthesize current information on plant-defense mechanisms driving PMA 3-way interactions to develop a conceptual model on the plant-signaling pathways mediating such tripartite interactions. We identified major regulatory modules and common mechanistic patterns guiding these complex interactions. In addition, we identify and discuss major bias sources and knowledge gaps and provide guidelines for future experiments.

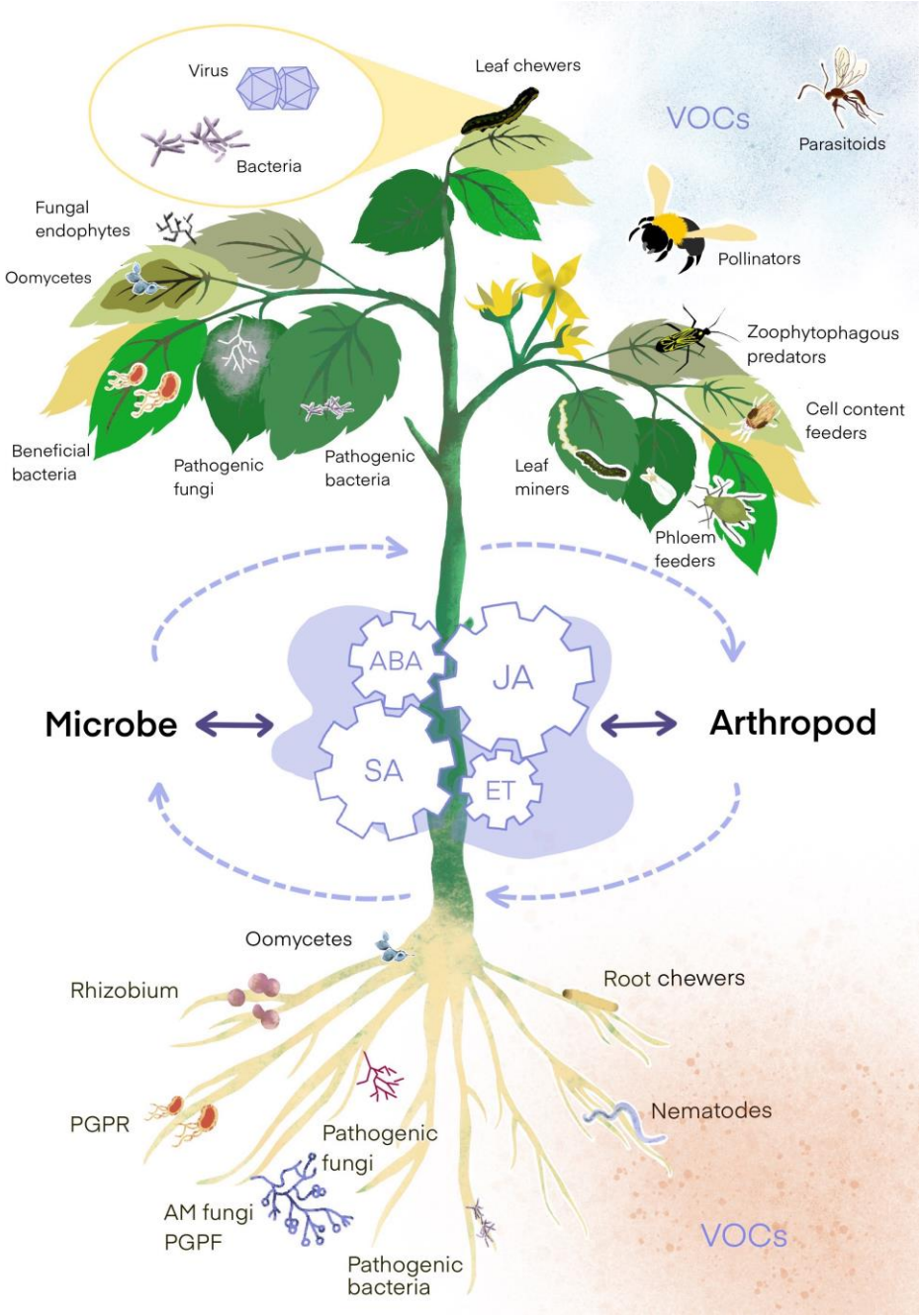

Figure 1 Illustration of multi-way interactions between plants, microbes and arthropods (PMA) and the main signaling pathways orchestrating the corresponding plant responses. Plants must fine-tune their molecular responses to the interaction with a plethora of organisms with different lifestyles. Microbes and arthropods interact and can alter each other's effects on plant health through their modulation of plant responses. Continuous arrows represent the 2-way interactions between the plant and the microbe or the arthropod. Discontinuous arrows represent the 3-way Plant-Microbe-Arthropod interactions. Major signaling pathways coordinating plant responses during 2-way and PMA interactions are represented in the figure: volatile organic compounds (VOCs), jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene signaling (ET). Major groups of arthropod and microbe lifestyles are illustrated by particular examples, microbes on the left side of the figure, arthropods on the right. PGPR: Plant growth promoting rhizobacteria; PGPF: Plant growth promoting fungi; AM fungi, arbuscular mycorrhizal fungi. The third trophic level is also represented by parasitoids and predators of arthropods. The insert (top left) represents arthropod-associated microbes impacting the arthropod interaction with the plant. Drawing by J. Lidoy, V. Lidoy and J. Lidoy.

MATERIAL AND METHODS

Building a mechanism database for PMA interactions

As a first step, we collected available information about the mechanisms activated in PMA interactions. Literature search was performed through the Web of Science database between 1980 and April 2019 using the query "(FAO_crop_list OR arabidopsis) AND (microbes OR bacteria OR virus OR pathogen OR fungi OR oomycete) AND (insect OR herbivore OR arthropod OR whitefly OR aphid) AND (gene expression OR transcript OR volatile OR chromatography OR proteomic OR metabolomic OR phytohormone) NOT effector)". The FAO_crop_list includes all crop genera listed in the World Programme for the Census of Agriculture 2020 (FAO, 2015). This search yielded 1352 references. Most publications on PMA interactions describe ecological aspects, mostly dealing with the organisms' performance in different systems or contexts. Since this review focuses on the molecular mechanisms regulating the interactions, reports lacking experimental evidence of the mechanisms operating in the PMA interactions were excluded. This resulted in 62 relevant references. Compiling publications on multiway interactions proved difficult, as the field lacks a specific category or ontology, limiting the exhaustiveness and precision of the search. Thus, we collected additional information through experts from the EU funded research network COST Action FA1405 "Using three-way interactions between plants, microbes and arthropods to enhance crop protection and production" (https://www.cost-camo.eu). We completed the search through snowballing and citation searching (Sayers, 2007), using citing articles and reference lists within relevant articles. This complementary search resulted in 29 additional manuscripts. Altogether, a final set of 90 manuscripts fulfilled our criteria and was analyzed in detail.

We structured the information as follows (Table S1):

1) general information about the publication (first author, title, DOI) and main findings
2) information about host plant
3) information about interacting microbe
4) information about interacting arthropod
5) information on the main signaling pathways regulated in the 2-way interaction of either microbe or arthropod with the plant, and those in the 3-way interaction
6) effect on plant performance in each 2-way and 3-way interaction
7) the methodology used for evaluating the plant response.

For each category, we created standard vocabulary and introduced up to three levels of ontology with the aim of obtaining biologically meaningful groups. Host plants, arthropods and microbes were first classified according to their taxa (species, family or higher taxonomic levels). At the highest level, arthropods were grouped according to their feeding mode and lifestyle, and microbes by their type of interaction with the plant in combination with their taxonomic classification at the kingdom level (e.g. pathogenic bacteria, plant growth promoting bacteria, mycorrhizal fungi, etc.). Molecular mechanisms were grouped into ontologies containing phytohormone signaling, primary metabolism and secondary metabolism at the highest level (all given in Table S1). In addition to the diversity of processes and pathways regulated, we also extracted information about the direction of the regulation, that is whether the process was induced (up-regulated) or repressed (down-regulated). This is important as the antagonism between pathways is key in fine-tuning plant responses (Shigenaga et al., 2017), such as the JA-SA negative crosstalk being a central module in regulating plant responses to biotic stress (Thaler et al., 2012).

The structured table was next processed in order to generate different visualizations. The Python language was used to load the data from the Excel table, preprocess, clean, filter and group lines and columns, and generate visualizations using several libraries and tools. Graphs were visualized using Graphviz visualization software (Gansner & North, 2000). Specifically, the *dot* program was used to produce layered layouts in the left-right orientation. Graphs were generated using a different hierarchical level for plants, microorganisms and arthropods, allowing data visualization at different levels of detail (Figs. S2 to S6- all graphs). Heatmap tables were computed and clustered on rows and columns using cosine distance and complete linkage. Finally, Euler diagrams were generated in order to compare the mechanisms triggered in plants of different families encountering beneficial or harmful microbes of the same taxonomic group and arthropods with the same feeding style.

Biases in methodological approaches

We reanalyzed microarray and RNA-seq transcriptomics studies from Solanaceae and Brasicaceae families (11 studies). To do this, we searched for normalized expression values with traceable gene identifiers in public transcriptomics repositories (GEO, SRA, ENA, ArrayExpress) and the publications' supplementary material, but we could retrieve these values only for two studies. Thus, instead of reanalyzing the datasets from normalized expression values, we compared the lists of differentially expressed genes which were available for three Arabidopsis and one potato

publications. We used Wilcoxon Sum Rank test implemented in MapMan (Usadel et al., 2005) to obtain the lists of regulated pathways and compared them with the pathways reported in the texts of the publications.

RESULTS AND DISCUSSION

Mechanism database for PMA interactions: Biases in studied biological systems

We generated a database compiling the available information on mechanisms shaping PMA interactions as described in Material and Methods. Briefly, we searched for relevant scientific articles in publicly available databases using specific keywords, consulting experts through the international network COST action FA1405, and through citations snowballing. The reference list was filtered to include only publications providing experimental evidence on the mechanisms, and the information was structured as shown in Table S1 and S2. When inspecting the overall data structure, we identified two major sources of bias. The first one is related to the taxonomic diversity of the interacting organisms, and the second one to the methodological approaches employed. Regarding the taxonomic bias, we found mechanistic studies of multiway interactions for plants belonging to 9 plant families. Most of the studies (64%) involved 2 families, Solanaceae (mostly *Nicotiana* and *Solanum* sp.) and Brassicaceae (mostly on *Arabidopsis* sp. and *Brassica* sp.), followed by studies involving Poaceae and Fabaceae. The microbes studied were grouped into three functional groups: (i) beneficial microbes, including plant growth promoting rhizobacteria (PGPR) or fungi (PGPF), and we considered AMF separately from other PGPF due to their very specific relationship with the plant and their high representation in the database (Parniske, 2008); (ii) pathogenic microbes (fungi, bacteria and viruses) and (iii) arthropod-associated microbes that influence the plant–arthropod interaction (Fig. 1). Beneficial microbes were most frequently studied in PMA interactions, with arbuscular mycorrhizal fungi (AMF) ranking first, followed by PGPR and other PGPF (mostly *Trichoderma* and *Piriformospora* -now *Serendipita*-). Remarkably, in some families (Fabaceae, Vitaceae, Plantaginaceae, Salicaceae and Fagaceae), AMF were almost the only microbes studied in a 3-way context. There is a clear focus on 3 insect orders, Lepidoptera ranking first, followed by Hemiptera and Coleoptera, mostly including herbivorous insects. A limited number of studies consider interactions with beneficial insects, e.g. predators/parasitoids of herbivorous

arthropods. Among beneficial arthropods, parasitoids were more frequently studied than predators (see Table S1).

Biases in methodological approaches

Some studies report conflicting information for the same set of interacting organisms. This can arise from differences in the experimental setup (see Box 1) or from the methodologies applied. To address the later source of bias, we compared the outcomes of studies using targeted and untargeted methodologies, since the analysis of untargeted methodologies is agnostic to the researchers' assumptions.

Box 1. Guidelines for experimental design: treatments, minimal standards for controls and information needed

Appropriate experimental setups are essential to reach robust conclusions on the molecular mechanisms governing PMA interactions. We highlight some key aspects to consider:

1. ***Appropriate controls***. To identify differential regulation in 3-way as compared to 2-way interactions it is crucial to determine the changes occurring in each individual interaction. The basal state of the host must also be determined. Thus, multiway studies should include four treatments: plants alone ('control'), plants interacting with the microbe (O1), with the arthropod (O2), and interacting with both (O1O2) (see Figure I).

Moreover, control treatments should be carefully considered for each experimental system to avoid misleading results derived from the inoculation method or accompanying microbes (Gryndler et al., 2018).

2. ***Order of challenge***. The order of the interactions is relevant for the final outcome and the operating mechanisms (Lazebnik et al., 2014). Therefore, interactions timing should be determined depending on specific research questions.

3. Age-*developmental stage of the interacting organisms* (Carella et al., 2015; Wilson et al., 2017). Plant responses are age/developmental stage-dependent (Carella et al., 2015; Kus et al., 2002; Wilson et al., 2017), hence the stage must be described for the plants under study. The arthropods developmental stage is also important. Arthropod instar, age as well as density should be clearly stated. Similarly, type and concentration of the microbe inocula and stage of the interaction should be taken into consideration.

4. ***Plant growth conditions***. Plant responses to biotic interactions are highly context-dependent. Environmental factors such as light quality and intensity, temperature, humidity, nutrient and water availability, can influence the interaction outcome. Hence, these parameters should be described in detail.

5. ***Plant organs/tissues to sample***. Plant organs act both in an autonomous and coordinated way in response to biotic stimuli. For example, root or shoot responses to the same stimuli may differ substantially. Responses can be local, can appear in distal, not-treated tissues (systemic responses) or both. Details regarding the anatomical and developmental characteristics of the plant material sampled for analysis may be crucial for the interpretation of the results.

6. ***Timing of sampling***. The plant integrates multiple signals leading to early and transient signaling events clearly differentiated from medium or late sustained responses. For example, dynamic changes in the hormone contents occur during infectious processes. Therefore, time-course experiments are very informative to understand plant responses in multi-way interactions.

7. *Quantifying responses*. The speed and intensity of plant defense responses are essential for their efficiency. In fact, defense priming seems to be a common mechanism. This can only be addressed through quantitative analysis comparing the intensity of the response in the 3-way *vs.* 2-way interactions.

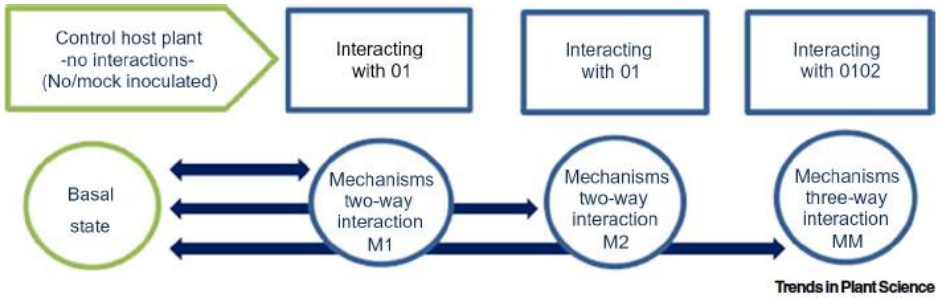

Figure I. Overview of optimal experimental design to study mechanisms shaping PMA interactions.

From the 86 publications analyzed, 34 used untargeted methodologies. Regarding metabolites detection, VOCs are generally analyzed through untargeted methodologies; while targeted methodologies are used for phytohormone analyses. Because of this, the comparison between targeted and untargeted analysis was not possible for these groups of metabolites. Thus, targeted and untargeted analyses were compared only for transcriptional data. Untargeted transcriptomic studies are not biased towards specific processes, while targeted ones usually focused on molecular markers of major stress-related pathways. Only Brassicaceae and Solanaceae families had enough numbers of mechanistic studies to merit an objective targeted-untargeted approach comparison. Contrary to our expectations, untargeted methods did not reveal additional processes. For example, more hormonal signaling pathways were reported in targeted than untargeted studies in Solanaceae (see Fig. S1). To test if all significant changes were reported in untargeted studies, we reanalyzed microarray and RNA-seq transcriptomics studies in these two families (three arabidopsis (*Arabidopsis thaliana*) and one potato (*Solanum tuberosum*) study). We used the Wilcoxon Sum Rank test implemented in MapMan (Usadel et al., 2005) to obtain the lists of regulated pathways and compared them with the pathways reported. Most of the pathways significantly altered according to our test were indeed highlighted in the publications (see Table S3). We conclude that, at the transcriptomics level, the major pathways triggered in 3-way interactions are already known and are well covered by targeted approaches allowing more precise quantifications. Thus, the transcriptomics methodology applied does not appear as a major source of bias regarding the pathways regulating PMA interactions. Nonetheless, the –omics approaches reveal new elements in those pathways and can shed light on the mechanisms governing their crosstalk (for example see (Petek et al., 2014)).

Can we predict mechanisms in multiway interactions based on 2-way interactions?

The overview of the mechanisms responding in 2- and 3-way interactions shows that the complexity of responses increases in the 3-way interactions, as a higher number of mechanisms is reported in 3-way compared to 2-way interactions in 56% of the studies (Fig. 2, Fig. 3, Figs S2 to S5). To compare the diversity of processes triggered in PMA interactions, we counted the overall number of pathways responding during the plant interaction with either the microbe or the arthropod alone or when exposed to both organisms. The diversity of responding pathways in the 3-way interactions increased 25% and 41% as compared to the plant responses to microbes and arthropods alone, respectively (Fig. 2). The regulation of JA, SA and ET signaling pathways, as well as the production of VOCs or glucosinolates, play a dominant role in 3-way interactions. Up-regulation of the JA signaling pathway, and to a lesser extent SA and ET pathways, are the most common molecular responses reported in the 3-way interactions (Fig. 2, Fig. 3). It is important to note that the diversity of processes regulated in 3-way interactions is not just the sum of processes regulated in each of the 2-way interactions. Processes not regulated in either 2-way interactions can respond when the plant is exposed to multiple interactors. For example, modulation of ET and AUX signaling was detected only in the 3-way potato-colorado potato beetle-potato virus Y interaction (*Solanum tuberosum*-*Leptinotarsa decemlineata*-*Potato virus Y*) (Petek et al., 2014). Additionally, the responses of the pathways triggered in 2-way interaction can also differ in their strength when plant is exposed to multiple organisms. For example, JA signaling is induced less when Arabidopsis is exposed to *Pieris rapae* and *Botrytis cinerea* as compared to *Pieris rapae* alone (I. A. Vos et al., 2015).

A noticeable node in the 2-way interactions is 'not determined' (ND) (Fig. 2, S2 and Table S1), illustrating the extent of missing 2-way controls in 3-way studies (see Box1). Out of 32 studies involving bacteria, 31% and 13% did not report the mechanism of the 2-way interaction with the microbe or the arthropod, respectively. Similarly, 22% of the studies involving fungi do not describe the mechanisms operating in the 2-way plant–fungus interaction. For viruses, 39% of the studies lack information about their interaction with the plant. This hinders the generation of predictive models on 3-way PMA interactions based on 2-way interaction studies.

The analysis of the available data revealed a stronger influence of the response to the insect than to the microbe in the 3-way interaction (Fig. 3, S4 and S6). To determine the influence of either the microbe or the insect in the 3-way interactions, we compared the overlap in the processes triggered in all possible combinations of insect feeding style, microbe type and plant family (26

combinations if we consider only the 4 most studied families). In 46% of the studied combinations, the overlap of processes triggered in 3-way interaction and the interaction with the insect only was higher than the overlap of processes triggered by the microbe alone. In contrast, the influence of the microbe was stronger in only 11% of the studied combinations (see Fig. S7).

Integrated analysis also shows that plant responses reported in 3-way interactions are rather different when considering harmful or beneficial microbes. Considering harmful microbes, SA signaling is almost exclusively induced in the case of viruses, while ROS, JA, VOCs, or secondary metabolites are also stimulated by fungal or bacterial pathogens. A more diverse set of signaling pathways is reported during interactions with beneficial microbes (Fig. 2, S2 and S6). Interestingly, induction of CK, GA, and AUX and down-regulation of ABA are only recorded in 3-way interactions with beneficial microbes, while induction of ABA and ROS are only recorded in entries from harmful interactions. These patterns seem to agree with plant growth related effects of beneficial microbes and activation of stress responses in deleterious interactions (Egamberdieva et al., 2017; Pieterse et al., 2012). The rest of mechanisms are induced by both beneficial and harmful interactions, but likely with different timings and intensity of response.

Beneficial microbes such as PGPRs and PGPFs not only promote growth but can also induce defense priming (Conrath et al., 2006; Martinez-Medina et al., 2016; Mauch-Mani et al., 2017; Pozo & Azcón-Aguilar, 2007; Selosse et al., 2014). For example, tomato plants colonized by the mycorrhizal fungi *Funneliformis mosseae* have been shown to be more resistant to chewing caterpillars through primed accumulation of JA and JA regulated defenses in response to the herbivore (He et al., 2017; Song et al., 2013). Defense priming was evaluated in 43% of the studies compiled here, and interestingly, priming was confirmed in almost 50% of those, highlighting its relevance in 3-way interactions (see online Tables S4 and S5). Most of the studies addressing priming deal with beneficial microbes (73%). Regarding arthropods, chewers were the most abundant category (65%). From the studies confirming priming, the most abundant combinations were AMF or PGPR with chewers (45%), followed by beneficial microbes–nematodes interactions (28%). Even in some studies not addressing priming, the data provided point to primed defenses in the 3-way interaction (for example see (Chiriboga M. et al., 2018; Sharma & Sharma, 2017)).

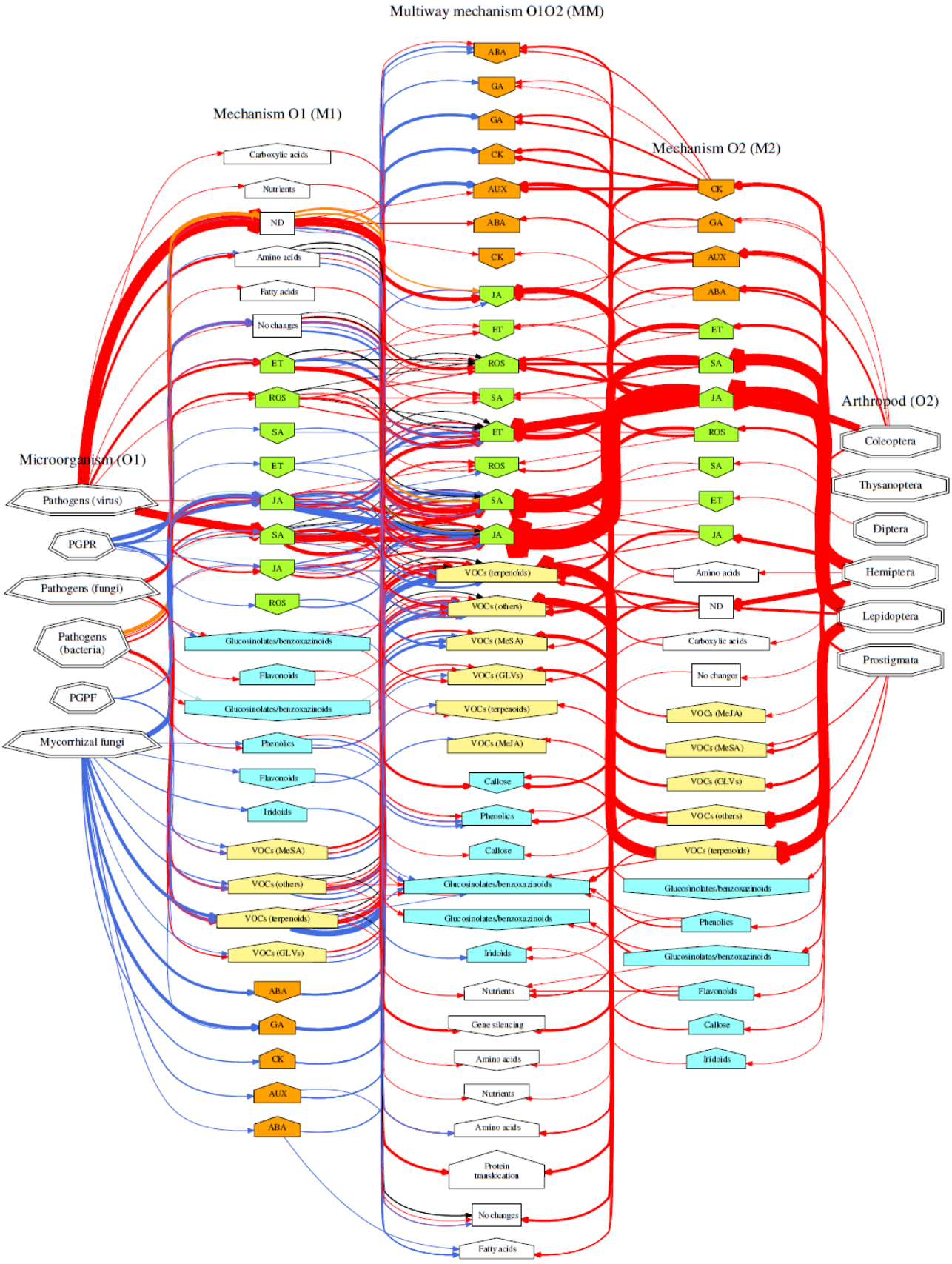
Multiway mechanism O1O2 (MM)
Mechanism O1 (M1)
Mechanism O2 (M2)
Microorganism (O1)
Arthropod (O2)
Pathogens (virus)
PGPR
Pathogens (fungi)
Pathogens (bacteria)
PGPF
Mycorrhizal fungi
Coleoptera
Thysanoptera
Diptera
Hemiptera
Lepidoptera
Prostigmata
Carboxylic acids
Nutrients
ND
Amino acids
Fatty acids
No changes
ET
ROS
SA
JA
Glucosinolates/benzoxazinoids
Flavonoids
Phenolics
Iridoids
VOCs (MeSA)
VOCs (others)
VOCs (terpenoids)
VOCs (GLVs)
ABA
GA
CK
AUX
Callose
Gene silencing
Protein translocation
VOCs (MeJA)
Trends in Plant Science

Figure 2 Overview of reported mechanisms responding in plants when exposed to different microbes (left), arthropods (right) or to a combination of both (centre). Heptagons, octagons and pentagons are used to represent microorganisms, arthropods and interaction mechanisms as graph nodes, respectively. On the left-hand side, the microbes included in the experiments are shown (O1), grouped according to their functional and higher taxonomy level, and linked with an arrow with the nodes representing the mechanisms that respond in the plant when exposed to the microorganism (M1). On the right side a similar representation is given for the taxonomically structured arthropods (O2) and the plant response to the infestation (M2). In the middle the nodes (MM) represent the mechanisms responding in plants when exposed to both organisms (O1O2). Colouring of the mechanism nodes reflects their function - yellow: VOCS; orange: growth related hormones; green: stress related hormones; light blue: secondary metabolism. O1O2 nodes are linked to both O1 and O2 nodes if the information is coming from the same experimental system. Blue and red color of the arrow indicates interaction with a beneficial or harmful organism for the plant, respectively. Light blue represents neutral effects and orange unknown effects to the plant (mostly insect symbionts that were not tested in direct interaction with the plant). The width of the arrow is proportional to the number of studies with the given observation. The up- and downregulation of the mechanism represented in a node is depicted by a shape pointing upward (upregulation) or downward (downregulation). ND - mechanisms not determined. For the full names of mechanism nodes see Supplementary Table 1.

We also considered studies (altogether 10) including arthropod-associated microbes (endosymbionts /entomopathogens) and plants (Insert in Fig. 1 and S5). They encompass Prostigmata and three insect orders (Coleoptera, Lepidoptera and Hemiptera) on the arthropods side, and 3 plant families (Solanaceae, Rosaceae and Poaceae). These interactions cannot be conclusively evaluated yet due to the insufficient number of studies, but again, phytohormone pathways (JA, CK and SA) are triggered in the 3-way interactions, with JA signaling being the pathway most consistently reported (see Fig. S5).

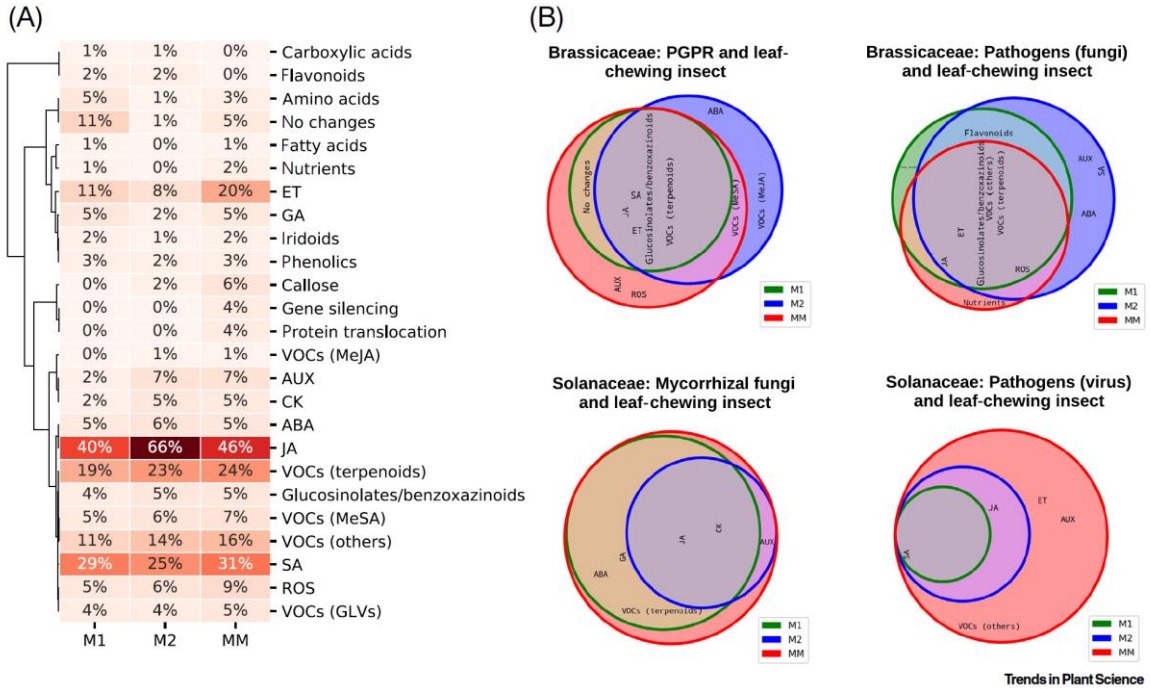

Figure 3 Mechanisms regulating plant responses when exposed to multiple organisms are more than the sum of the responses in 2-way interactions. (A) heatmap representing the frequency of a given mechanism in publications analyzing PMA interactions. Percentages of articles reporting changes in individual mechanisms during 2-way and 3-way interaction among the total number of articles compiled are given. The mechanisms are clustered using cosine distance and complete linkage. For heatmaps of individual families, check Supplementary Figure 4. (B) Comparison of mechanisms triggered when plants of different families are encountering leaf chewing insects and beneficial or harmful microbes of the same taxonomic group. Euler graphs were generated from information in the database (Supplementary Table 1). M1 – mechanisms triggered in plants interacting with the microbe, M2 – mechanisms triggered in plants interacting with the arthropod, MM – mechanisms triggered when plants interact with both organisms. The complete set of Euler diagrams is available as Figure S7.

Going towards systems biology

Studies of multiway interactions have mainly focused on the qualitative evaluation of a particular process in the plant response and the signaling pathway activated (the so-called 'signaling module'). The combination of signaling modules elicited in each specific interaction differs in composition, magnitude and timing (M. De Vos et al., 2005), leading to specific signal signatures that can eventually benefit either the plant or the attacker. Indeed, several studies noted that the same arrays of genes are activated in compatible and incompatible plant–microbe interactions, but that they differ in the timing and intensity of the response (Baebler et al., 2011, 2014; Coolen et al., 2016; Davila Olivas et al., 2017; Kim et al., 2014; Mur et al., 2006). This also occurs during 3-way interactions: For example, preinfestation of arabidopsis plants with larvae of *Pieris rapae* delays the induction of ET and SA signaling and ROS responses when the plant is infected with the fungus *Botrytis cinerea*. Additionally, the strength of the repression of GA signaling was higher (Coolen et al., 2016). Thus, data collected at only one time point, or lacking quantitative evaluation may be misleading. So far, a handful of experiments addressed the dynamics of plant responses quantitatively and with high time resolution, but only during the interaction with a single pathogen (Lewis et al., 2015), or triggering a specific signaling module (Hickman et al., 2017, 2019). The results of these studies revealed several waves of gene expression triggered even within a 24 h period, indicating the precise control of the response dynamics, likely shaping the specificity of the response. Thus, to disentangle the complexity of the immune signaling network it is crucial to understand the dynamics and quantitative properties of the system. However, most studies analyzed plant responses in multiway interactions at only onetime point. Consequently, the changes in network properties cannot be discerned from the existing datasets. The temporal aspect is receiving increasing attention now, so that more accurate estimations of network properties are expected in the coming years. Future experiments should be carefully designed to take into account, if possible, precise quantification and the time component, while considering limitations in both lab space and budget (see Box 1). Some new approaches may help, like that reported by La Manno and

coworkers (La Manno et al., 2018) for inferring dynamics of gene expression from single time point data by comparing the ratios of unspliced and spliced transcript counts.

A common approach to study mechanisms regulating interactions is the use of plant mutant/ genetically modified lines. Although such a reductionist approach is powerful in building base knowledge hypotheses and testing them, it is very difficult to discern gene function in a complex network solely from such studies. The behavior of the system may depend heavily on complex interactions between components within it (Hillmer et al., 2017; Westerhoff et al., 2009). Thus, we recommend combining different approaches to identify small differences relevant to the network responses. Ideally, they should include untargeted analysis, quantitative analysis of selected components and carefully designed functional experiments including mutant/genetically modified/edited lines.

Additionally, plant responses are coordinated at different levels, from cellular to tissue and organ responses. Different parts of the plant are usually exposed to different organisms, and while systemic responses in the plant are common, this responses in distal tissues differ from the local ones (Hilleary & Gilroy, 2018). This aspect was only partially covered in the studies performed so far.

Concluding remarks and future perspectives

Understanding plant responses to environmental factors and interactions with multiple organisms is crucial for biotechnological improvement of plant resilience, and consequently, to achieve efficient and sustainable crop management practices. Our synthetic review reveals that the information on the molecular mechanisms governing plant interactions with other organisms is still fragmented, and that further systematic studies are required to understand the regulation of plant responses. We found important literature biases regarding studied organisms and experimental designs, so that some mechanisms perhaps remain undiscovered. Taken altogether, drawing conclusions on the mechanisms involved in multiway interactions is more complex than expected. Nonetheless, our analysis points to phytohormone modules as major regulatory hubs in both 2-way and 3-way interactions, but the responses are fine-tuned in both timing and strength when plants are exposed to multiple interactors. Improved predictions will require systems biology approaches that merge mathematical modeling with experimental datasets encompassing the dynamics of the responses.

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SUPPLEMENTAL MATERIAL

Figure S1 Mechanisms responding in plants when exposed to the different interactions according to studies using targeted and untargeted analyses of transcriptional data.

Comparison of results in PMA interaction studies using targeted and untargeted transcriptomics. Heptagons, octagons and pentagons are used to represent microorganisms, arthropods and interaction mechanisms as graph nodes, respectively. On the left-hand side, the microbes included in the experiments are shown (O1), grouped according to their functional and higher taxonomy level, and linked with an arrow with the nodes of mechanisms that respond in the plant when exposed to the microorganism. On the right side a similar representation is given for the taxonomically structured arthropods (O2) and the plant response to the infestation. In the middle the nodes represent the mechanisms responding in plants when exposed to both organisms (O1O2). Colouring of the mechanism nodes reflects their function - yellow: VOCS; orange: growth related hormones; green: stress related hormones; light blue: secondary metabolism. O1O2 nodes are linked to both O1 and O2 nodes if the information is coming from the same experimental system. Blue and red color of the arrow indicates interaction with a beneficial or harmful organism for the plant, respectively. Light blue represents neutral effects and orange unknown effects to the plant (mostly insect symbionts that were not tested in direct interaction with the plant). The width of the arrow is proportional to the number of studies with the given observation. The up- and downregulation of the mechanism represented in a node is depicted by a shape pointing upward (upregulation) or downward (downregulation). ND - mechanisms not determined. For the full names of mechanism nodes see Supplemental Table S1.

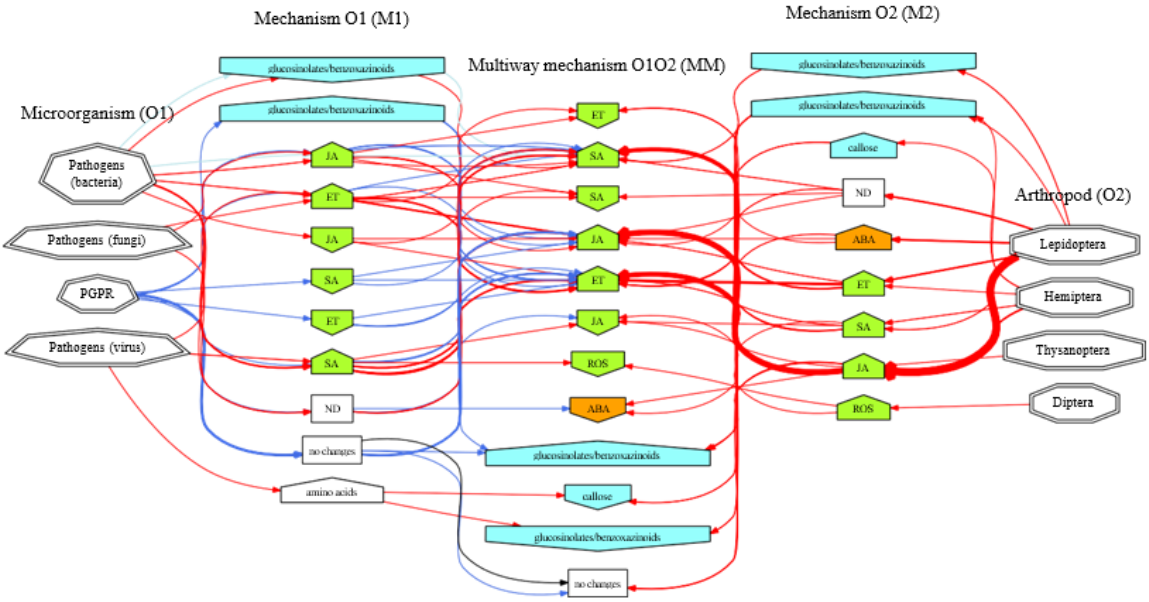

Figure S1.1: Brassicaceae: targeted methodologies

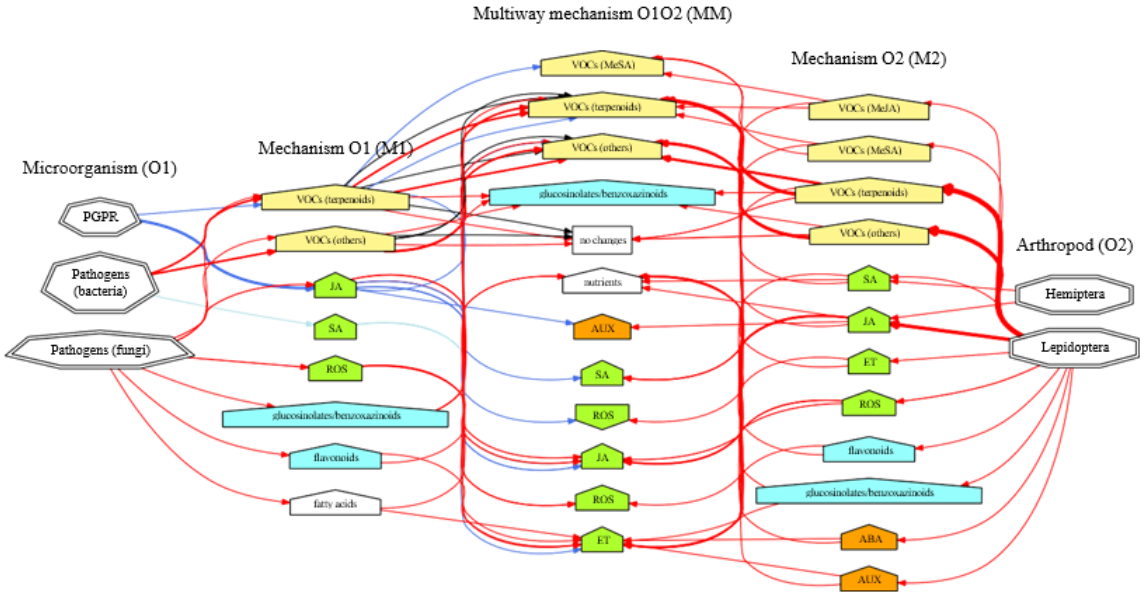

Figure S1.2: Brassicaceae: untargeted methodologies

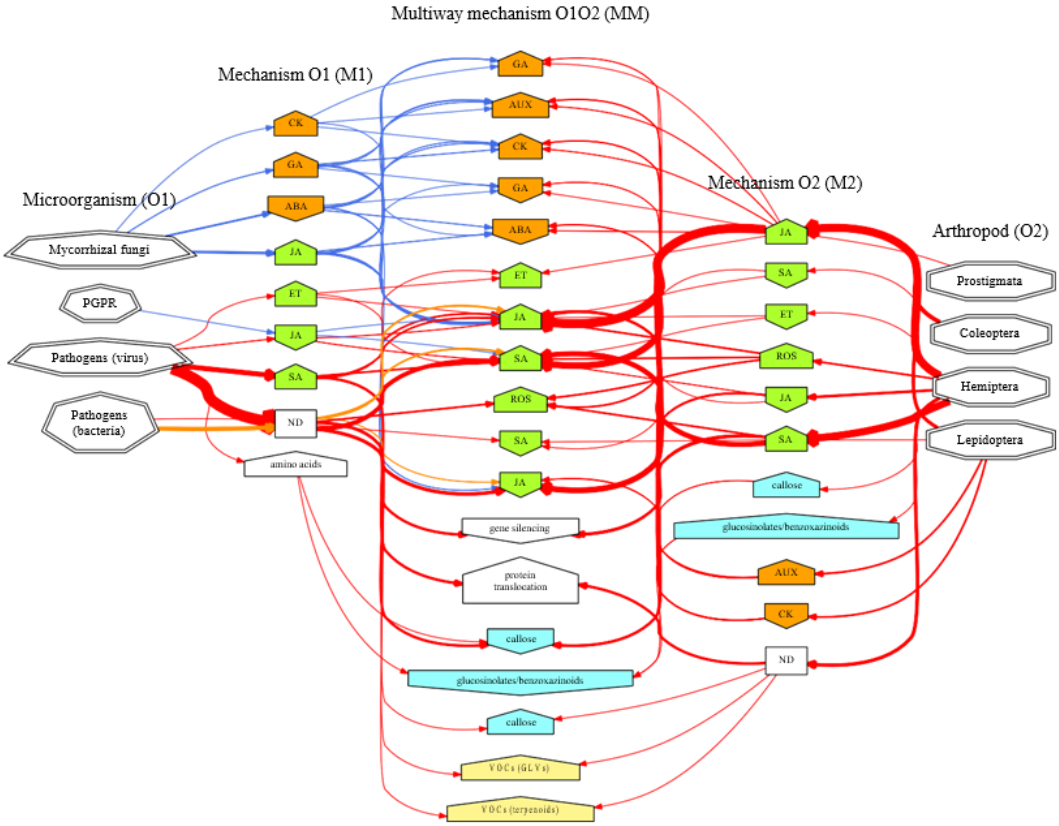

Figure S1.3: Solanaceae: targeted methodologies

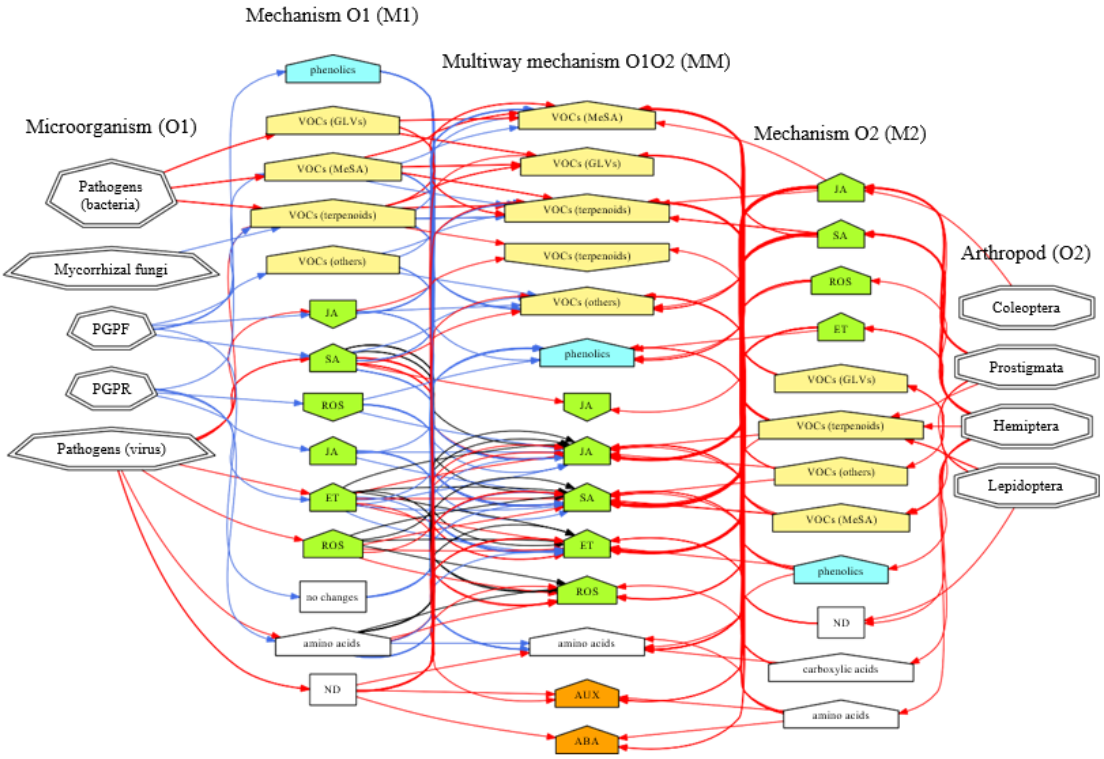

Figure S1.4: Solanaceae: untargeted methodologies

S2 Reported mechanisms responding in plant when exposed to microbes, arthropod or a combination of both compiled separately for each plant family

Heptagons, octagons and pentagons are used to represent microorganisms, arthropods and interaction mechanisms as graph nodes, respectively. On the left-hand side, the microbes included in the experiments are shown (O1), grouped according to their functional and higher taxonomy level, and linked with an arrow with the nodes of mechanisms that respond in the plant when exposed to the microorganism. On the right side a similar representation is given for the taxonomically structured arthropods (O2) and the plant response to the infestation. In the middle the nodes represent the mechanisms responding in plants when exposed to both organisms (O1O2). Colouring of the mechanism nodes reflects their function - yellow: VOCS; orange: growth related hormones; green: stress related hormones; light blue: secondary metabolism. O1O2 nodes are linked to both O1 and O2 nodes if the information is coming from the same experimental system. Blue and red color of the arrow indicates interaction with a beneficial or harmful organism for the plant, respectively. Light blue represents neutral effects and orange unknown effects to the plant (mostly insect symbionts that were not tested in direct interaction with the plant). The width of the arrow is proportional to the number of studies with the given observation. The up- and downregulation of the mechanism represented in a node is depicted by a shape pointing upward (upregulation) or downward (downregulation). ND - mechanisms not determined. For the full names of mechanism nodes see Supplemental Table S1.

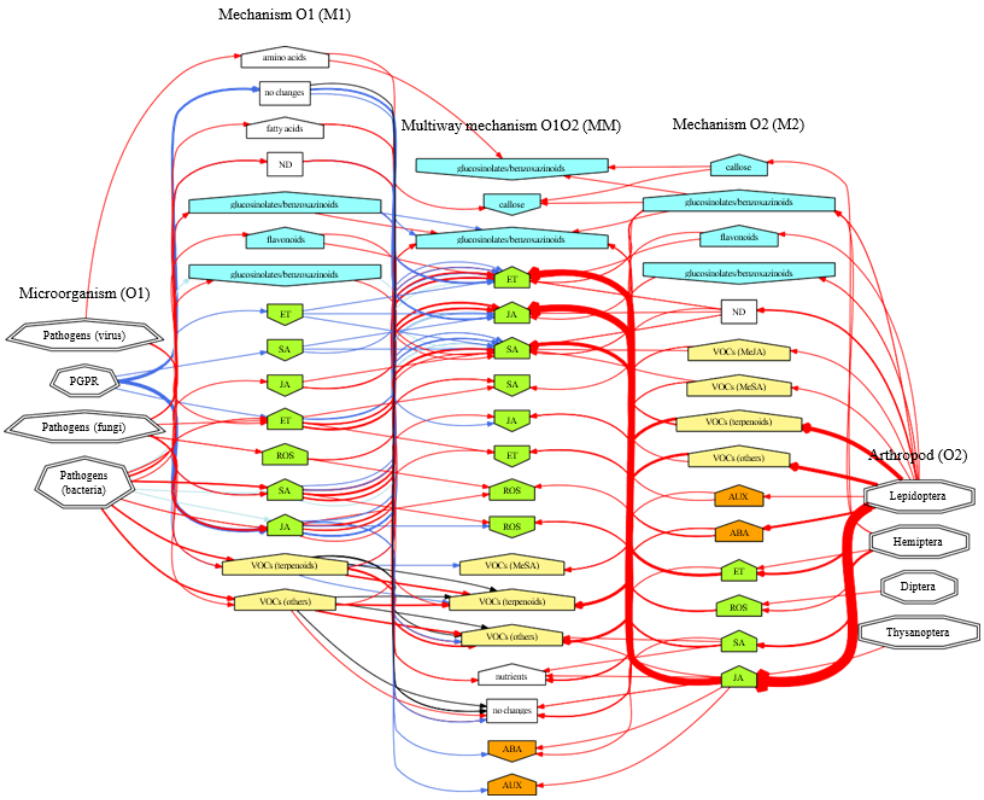

Figure S2.1: Brassicaceae

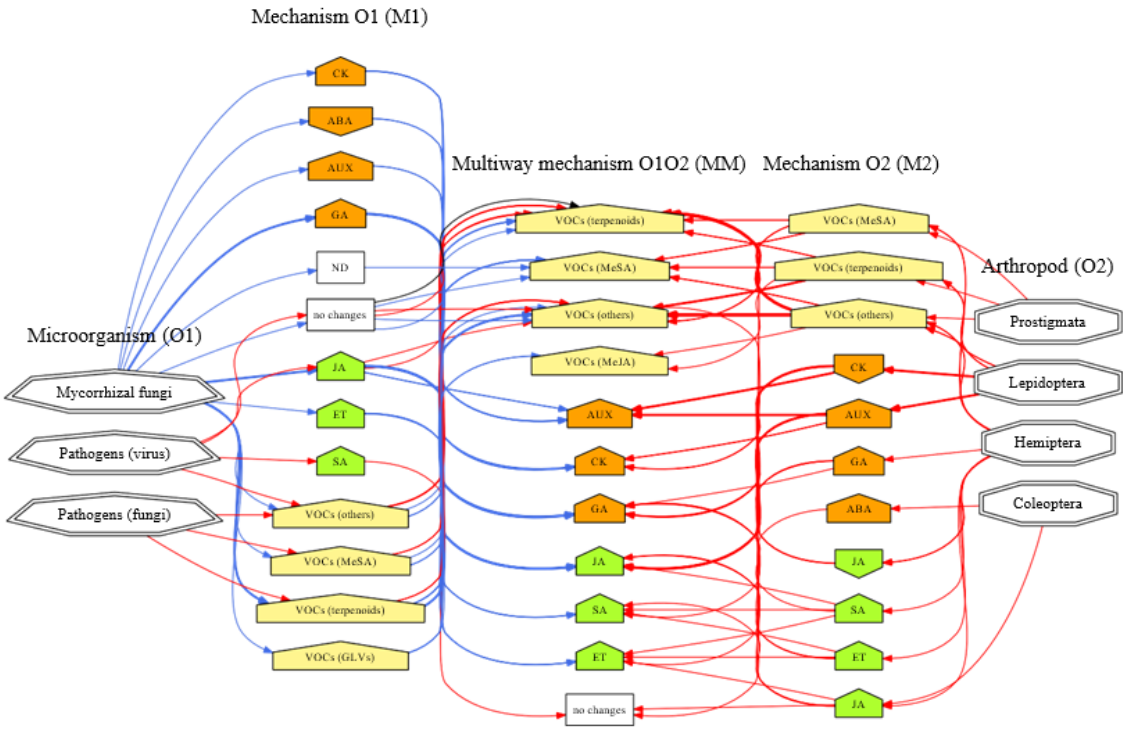

Figure S2.2: Fabaceae

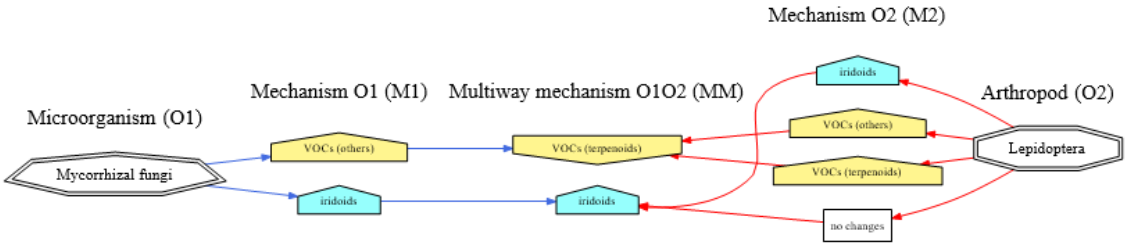

Figure S2.3: Plantaginaceae

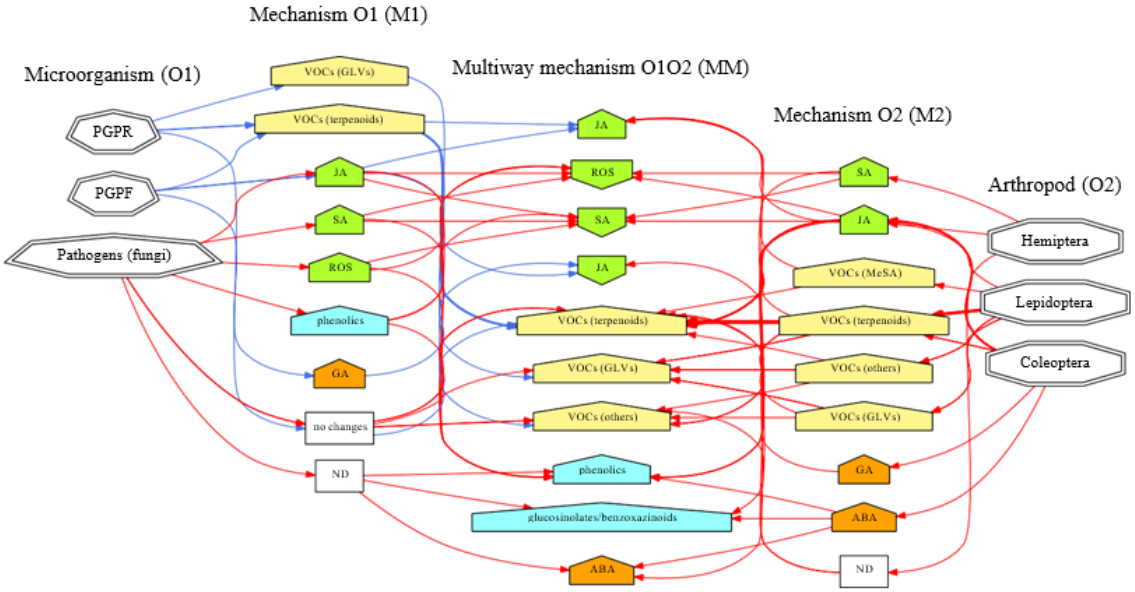

Figure S2.4: Poaceae

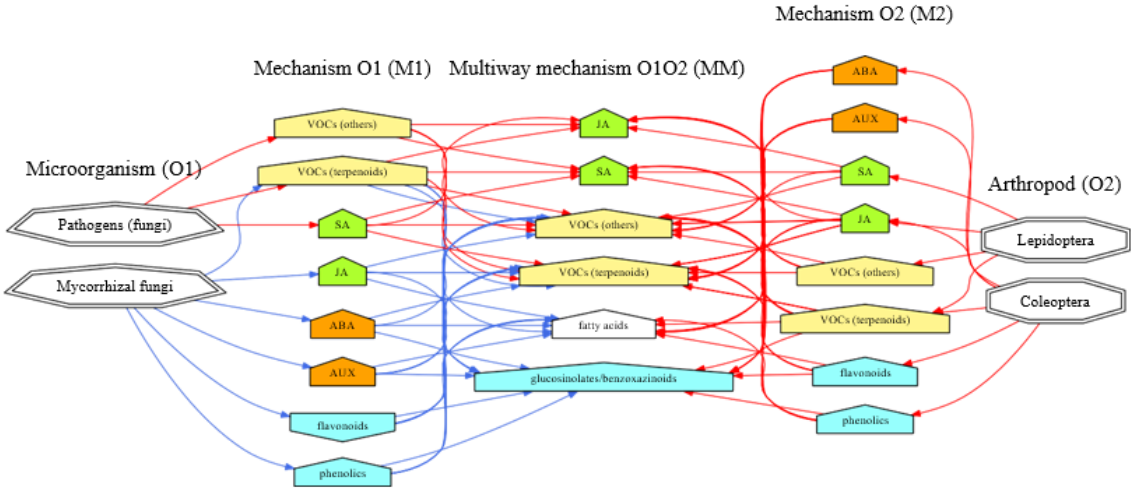

Figure S2.5: Salicaceae

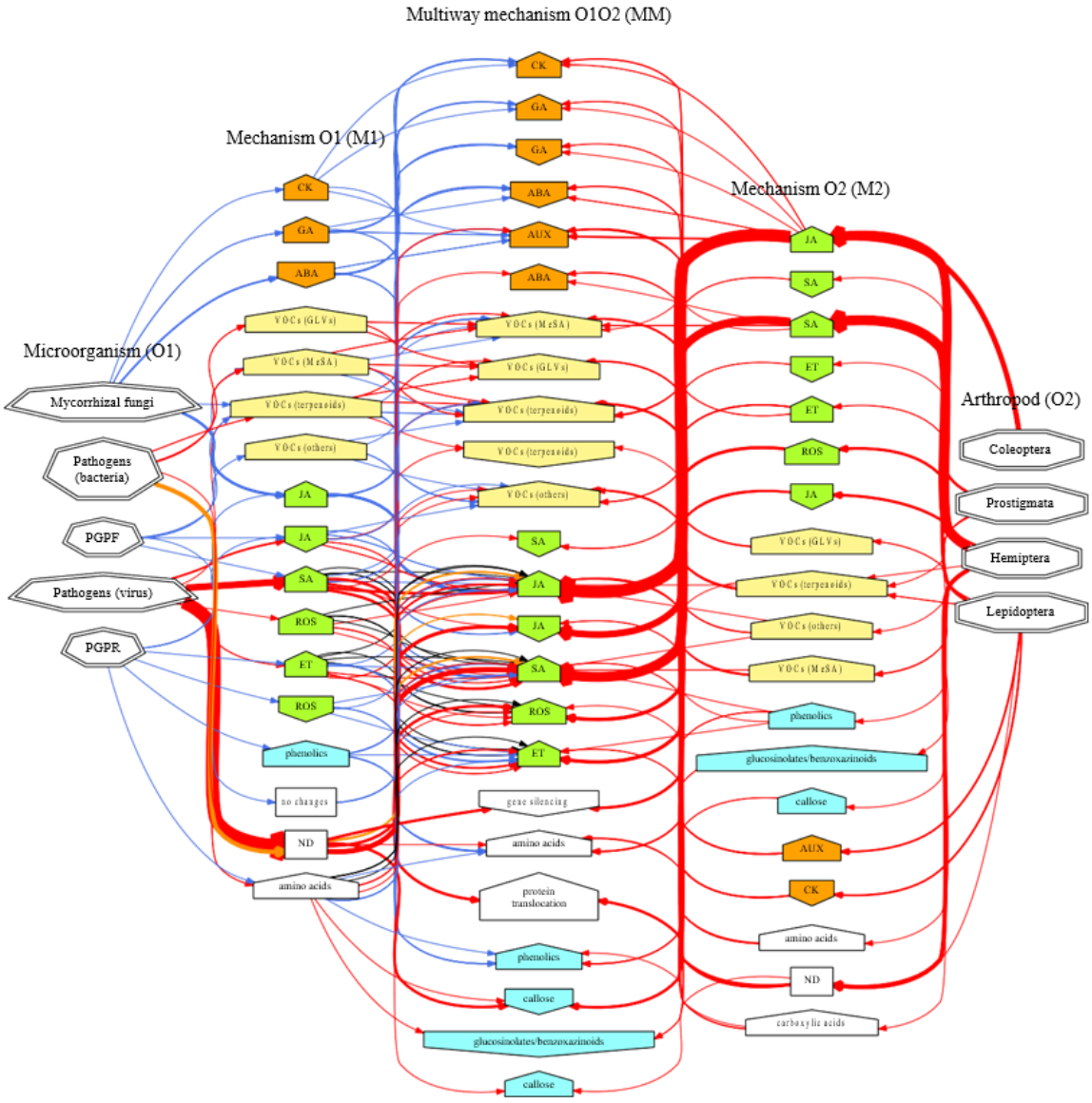

Figure S2.6: Solanaceae

S3 Overview of mechanisms responding in plants when exposed to microbes, arthropod or a combination of both group as they were detected in individual studies

Heptagons, octagons and rectangles are used to represent microorganisms, arthropods and interaction mechanisms as graph nodes, respectively. On the left-hand side, the microbes included in the experiments are shown (O1), grouped according to their functional and higher taxonomy level, and linked with an arrow with the nodes of mechanisms that respond in the plant when exposed to the microorganism. On the right side a similar representation is given for the taxonomically structured arthropods (O2) and the plant response to the infestation. In the middle the nodes represent the mechanisms responding in plants when exposed to both organisms (O1O2). Colouring of the mechanism nodes reflects their function - yellow: VOCS; orange: growth related hormones; green: stress related hormones; light blue: secondary metabolism. O1O2 nodes are linked to both O1 and O2 nodes if the information is coming from the same experimental system. Blue and red color of the arrow indicates interaction with a beneficial or harmful organism for the plant, respectively. Light blue represents neutral effects and orange unknown effects to the plant (mostly insect symbionts that were not tested in direct interaction with the plant). The width of the arrow is proportional to the number of studies with the given observation. ND - mechanisms not determined. For the full names of mechanism nodes see Supplemental Table S1.

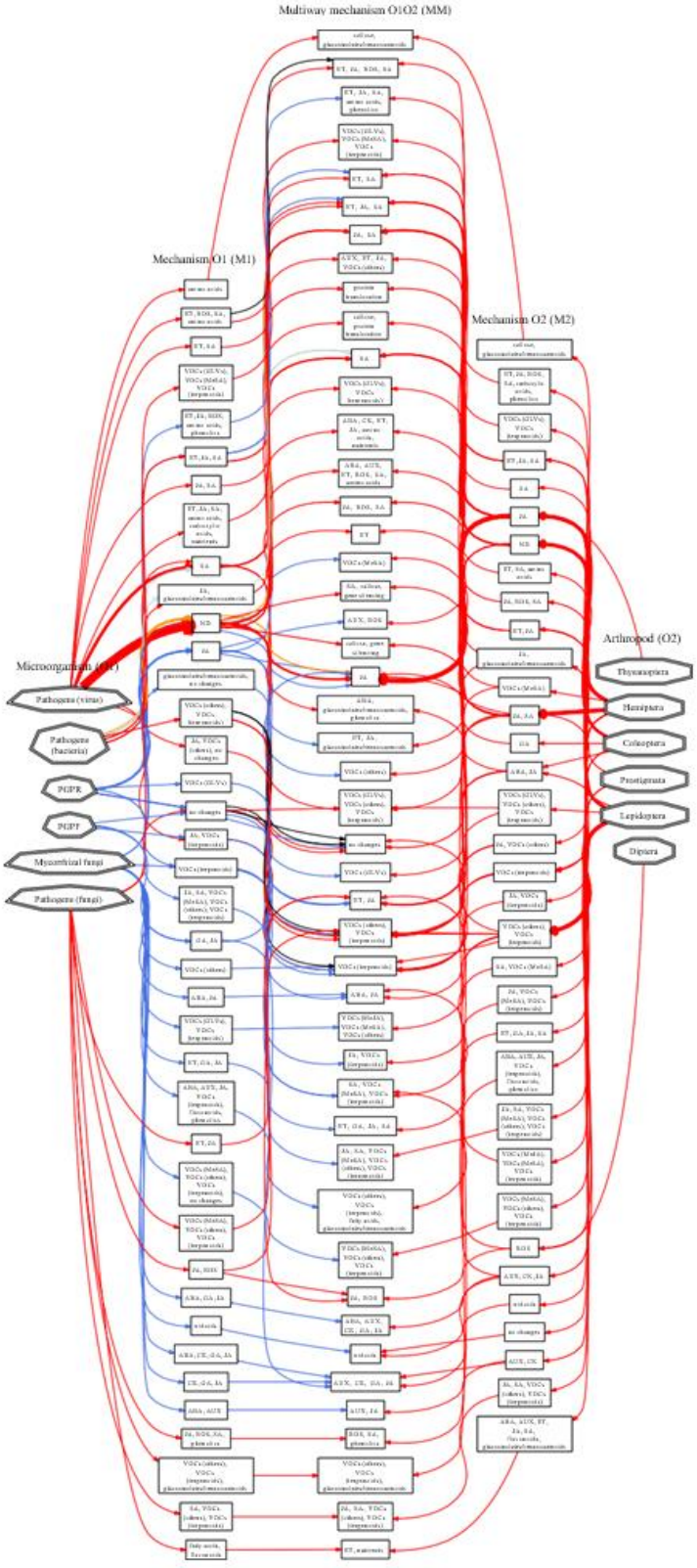

Figure S3.1: Brassicaceae, Fabaceae, Plantaginaceae, Poaceae, Salicaceae, and Solanaceae.

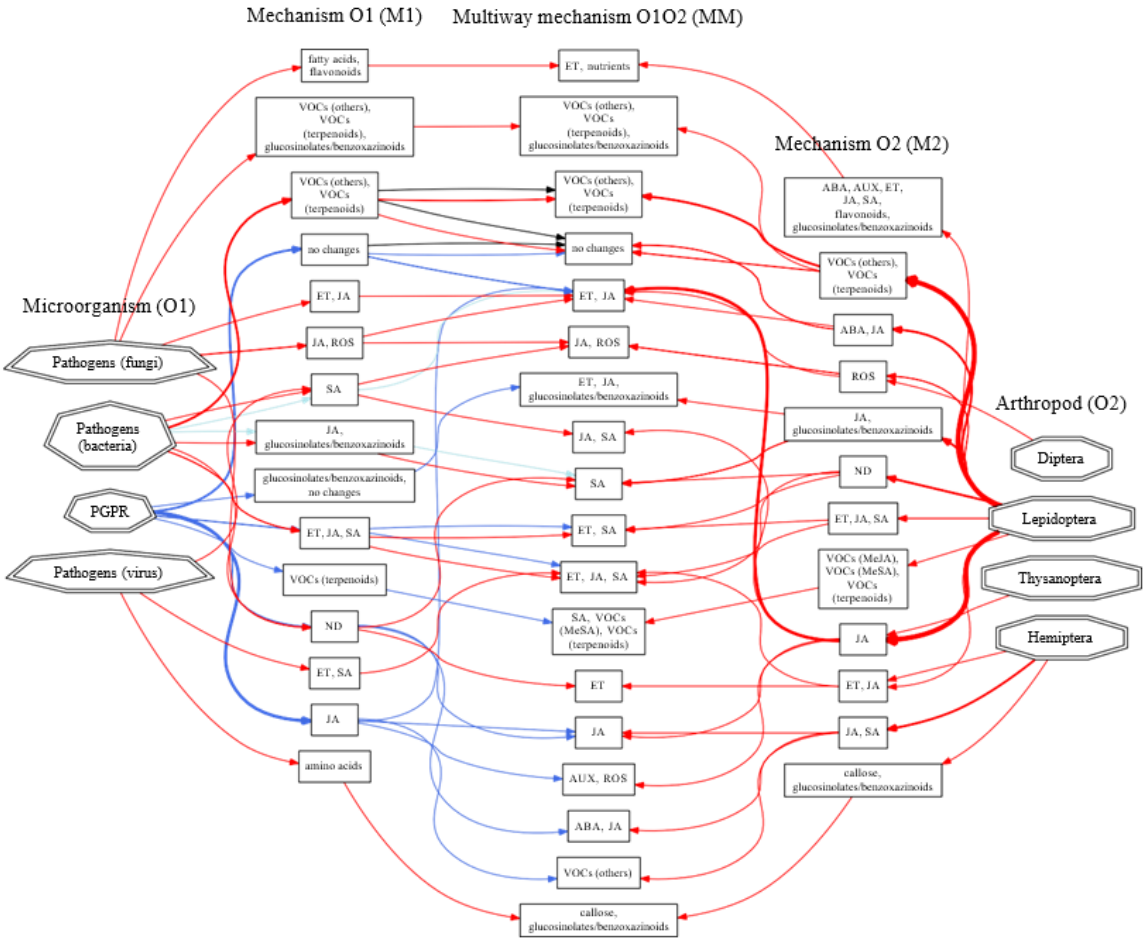

Figure S3.2: Brassicaceae

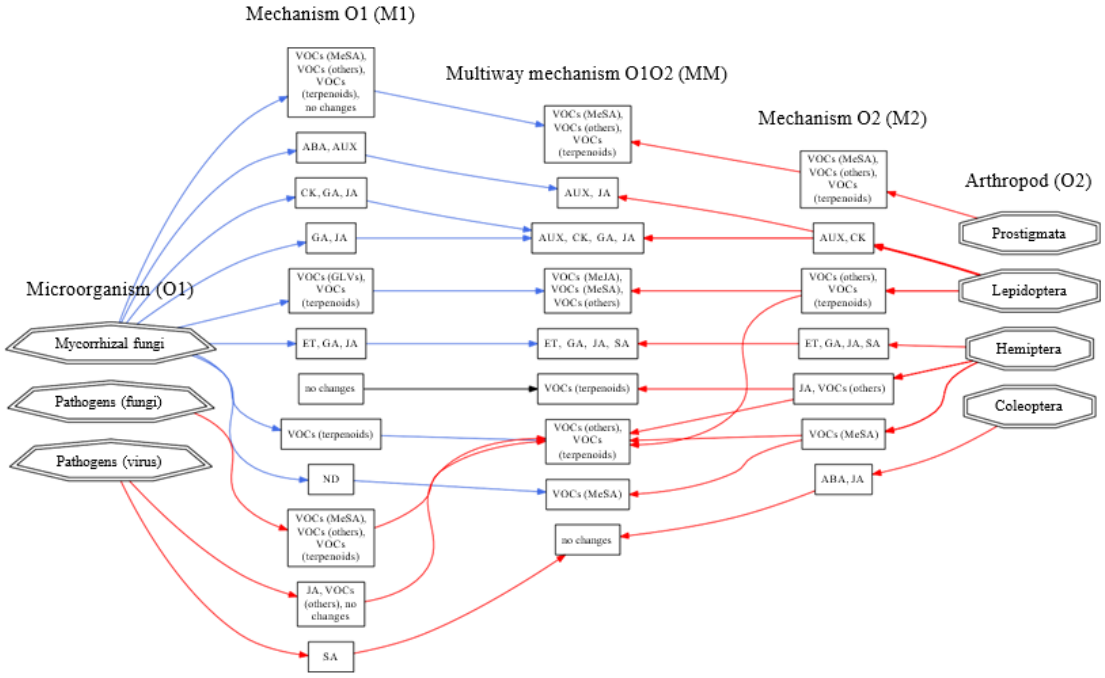

Figure S3.3: Fabaceae

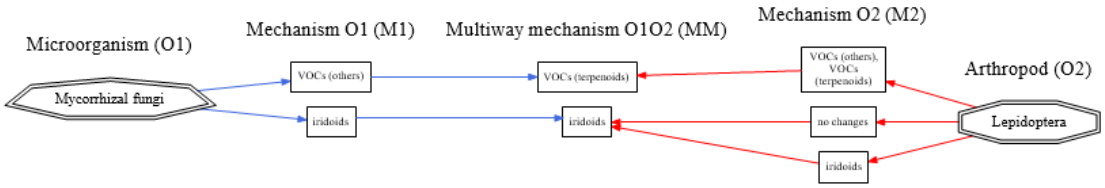

Figure S3.4: Plantaginaceae

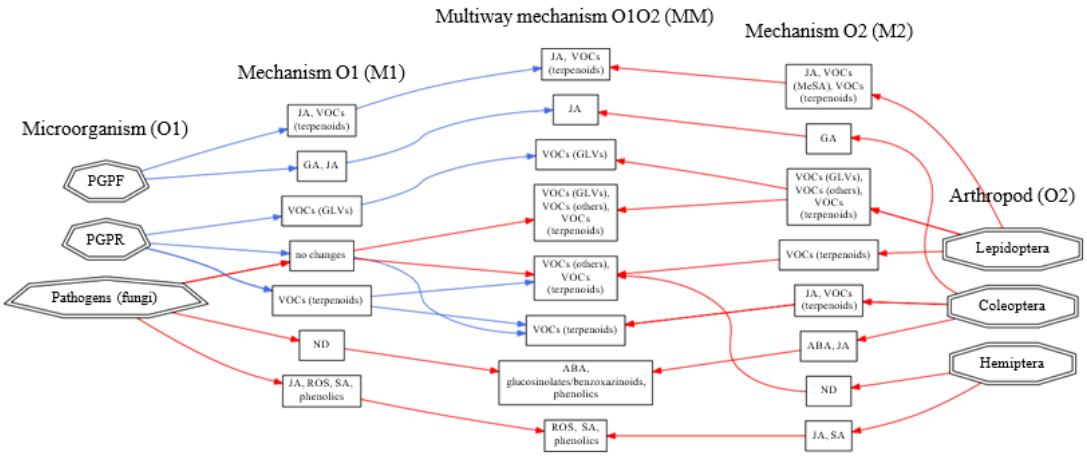

Figure S3.5: Poaceae

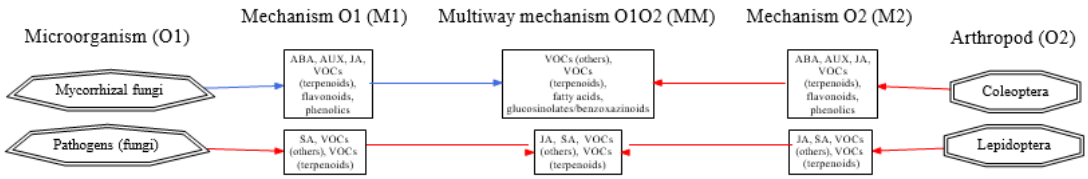

Figure S3.6: Salicaceae

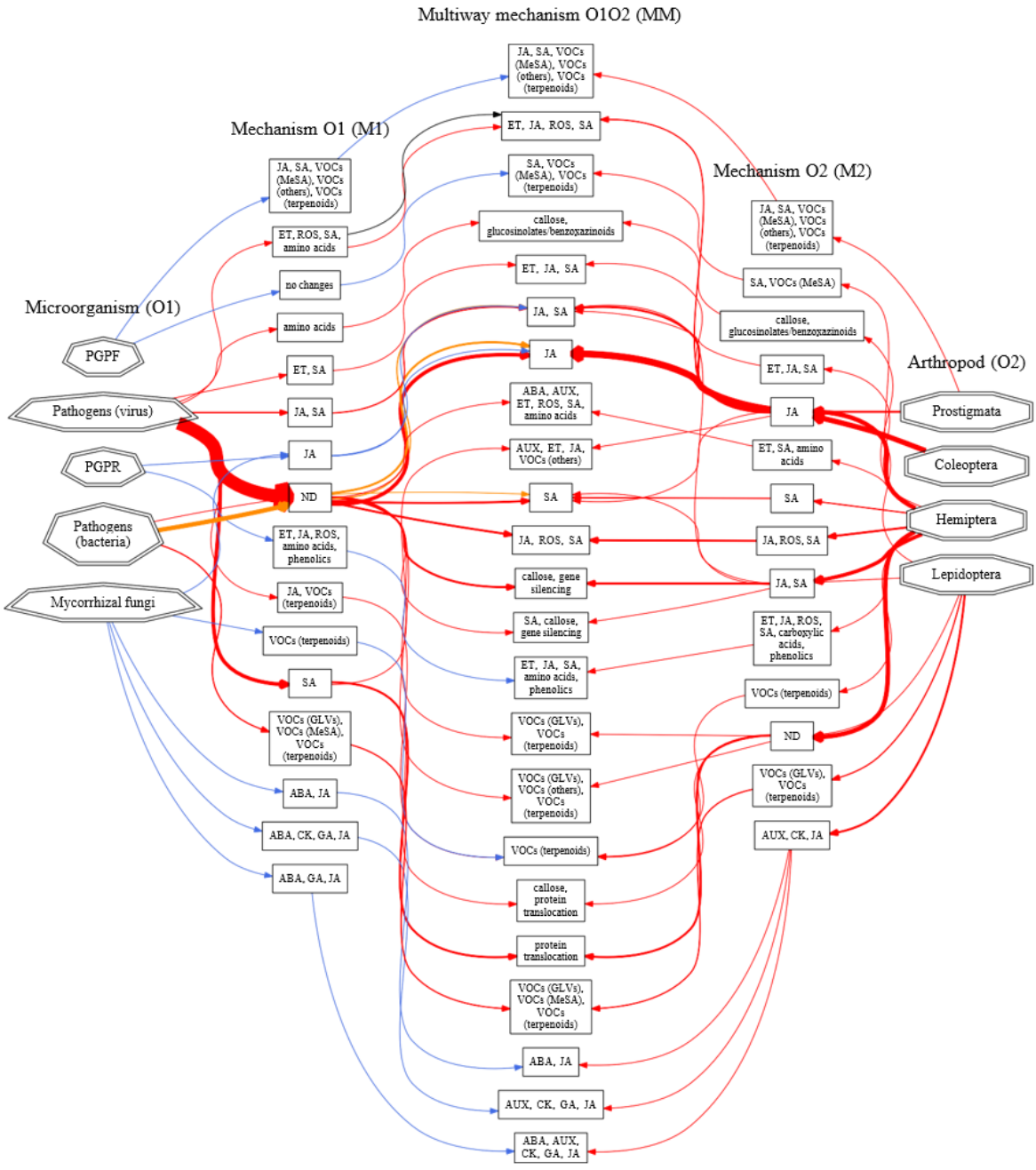

Figure S3.7: Solanaceae

S4 Frequency of individual mechanisms reported in publications de- scribing PMA interactions within selected families represented as a heatmap

The mechanisms are clustered using the cosine distance metric and the complete linkage method.

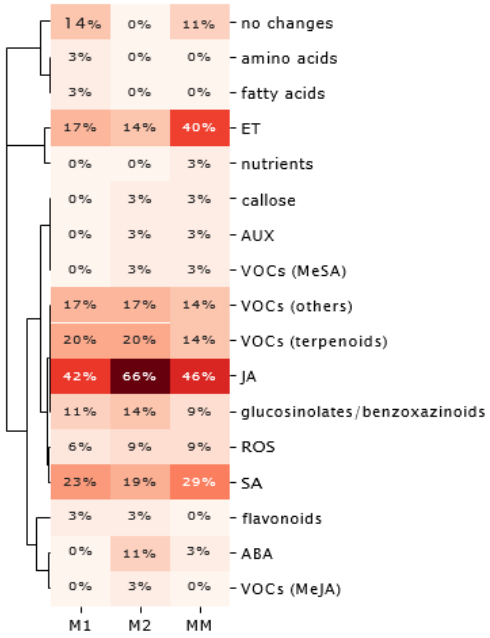

Figure S4.1: Brassicaceae

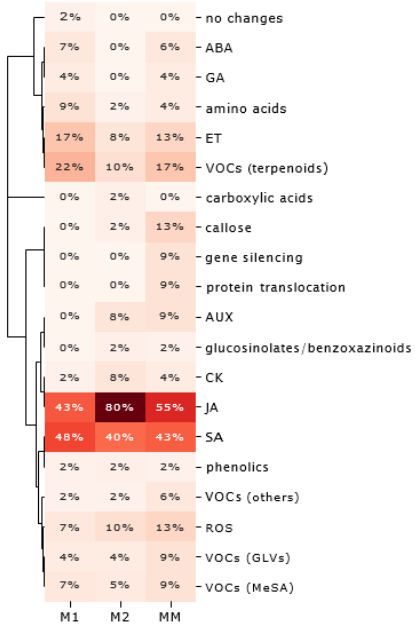

Figure S4.2: Solanaceae

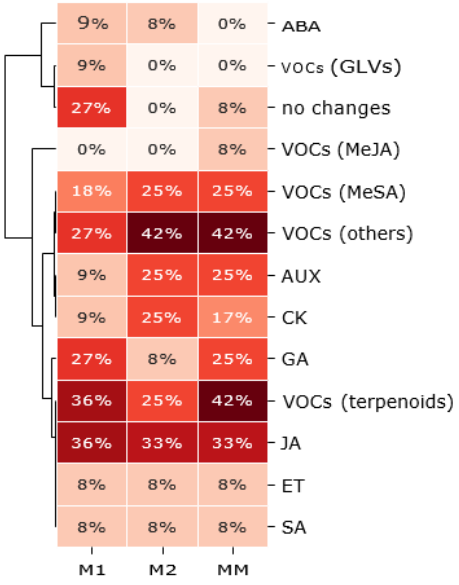

Figure S4.3: Fabaceae

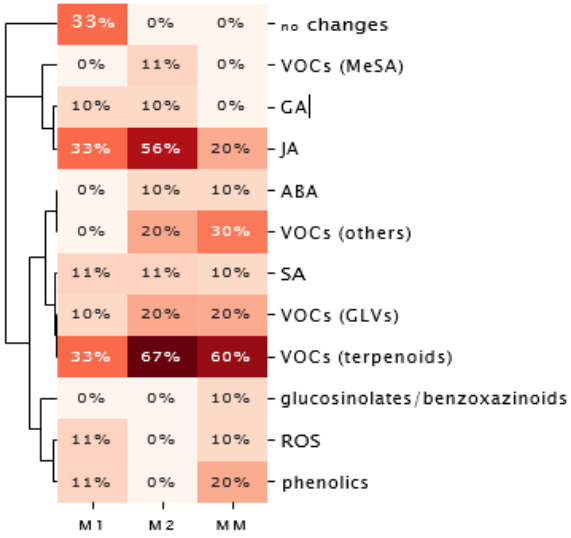

Figure S4.4: Poaceae

Figure S5. Mechanisms operating in PMA interactions involving arthropod- associated Microbes

Heptagons, octagons and rectangles are used to represent microorganisms, arthropods and interaction mechanisms as graph nodes, respectively. On the right-hand side, the insect microbes are linked with Arthropod hosts (O2) grouped according to their higher taxonomy level and linked with an arrow with the nodes of mechanisms that respond in the plant when exposed to the microorganism (Mechanism O1) or Arthropod (Mechanism O2). Both are linked to the nodes representing the mechanisms responding in plants when exposed to both organisms (Multiway mechanisms O1O2). Colouring of the mechanism nodes reflects their function - yellow: VOCS; orange: growth related hormones; green: stress related hormones; light blue: secondary metabolism. O1O2 nodes are linked to both O1 and O2 nodes if the information is coming from the same experimental system. Red color of the arrow indicates interaction with a harmful organism for the plant while orange color indicates unknown effects to the plant. The width of the arrow is proportional to the number of studies with the given observation. ND - mechanisms not determined. For the full names of mechanism nodes see Supplemental Table S1.

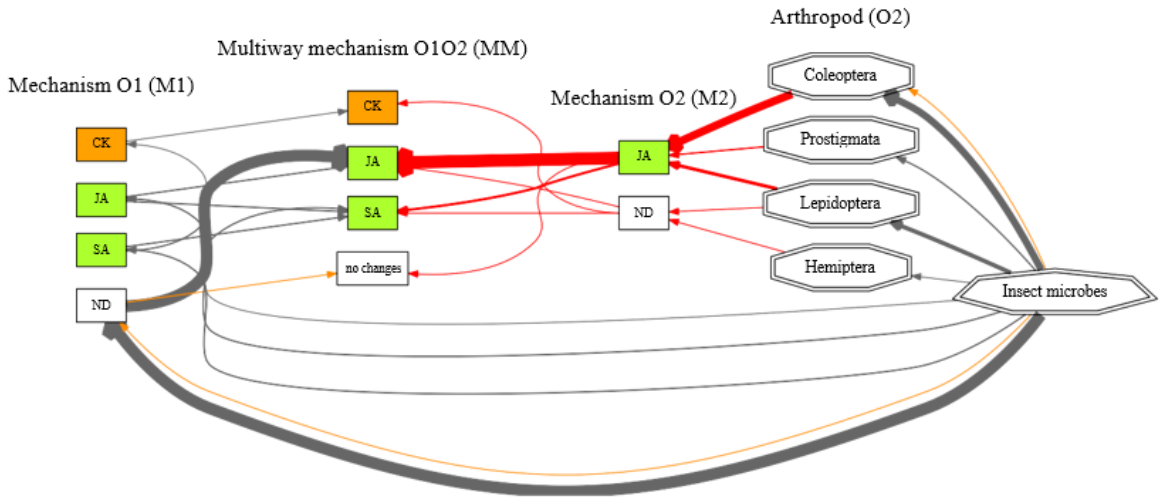

Figure S5.1: Poaceae, Rosaceae, and Solanaceae.

Figure S6. Comparison of mechanism triggered when plants of different fam- ilies encounter beneficial and harmful microbes of the same taxo- nomic group and arthropods with the same feeding style

The following Euler graphs were generated from the information present in the database (Supplemental Table S1). The legend is as follows:

- M1 - mechanisms triggered in plant in interaction with microbe,
- M2 - mechanisms triggered in plant in interaction with Arthropod, and
- MM - mechanisms triggered when plant is in interaction with both organisms.

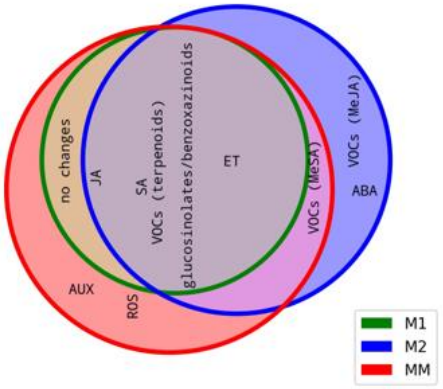

Figure S6.1: Brassicaceae: PGPR and leaf chewing insect

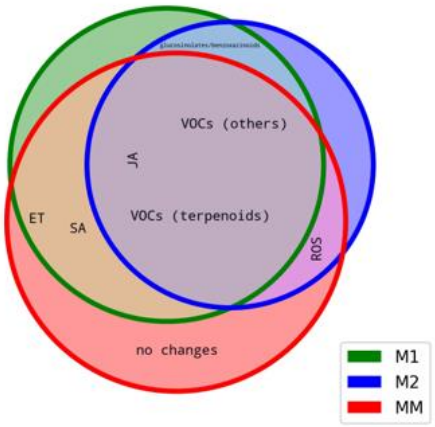

Figure S6.3: Brassicaceae: pathogens (bacteria) and leaf chewing insect

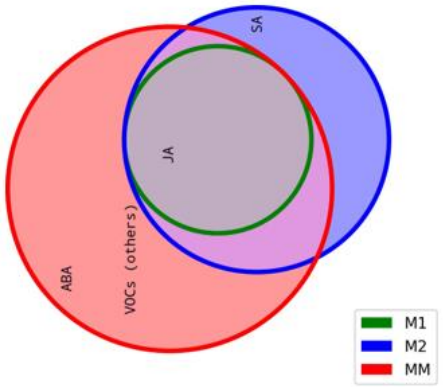

Figure S6.2: Brassicaceae: PGPR and phloem feeder

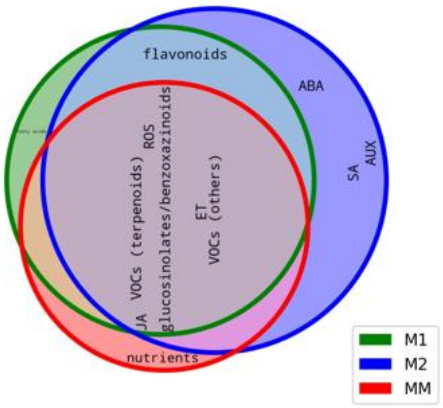

Figure S6.4: Brassicaceae: pathogens (fungi) and leaf chewing insect

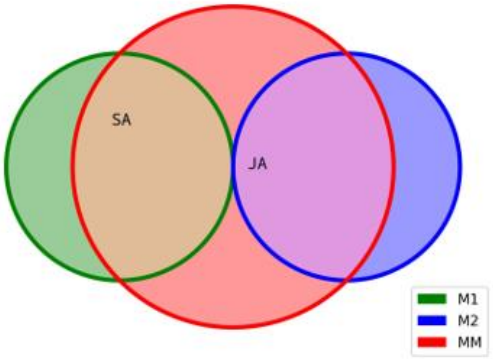

Figure S6.5: Brassicaceae: pathogens (virus) and cell content feeder

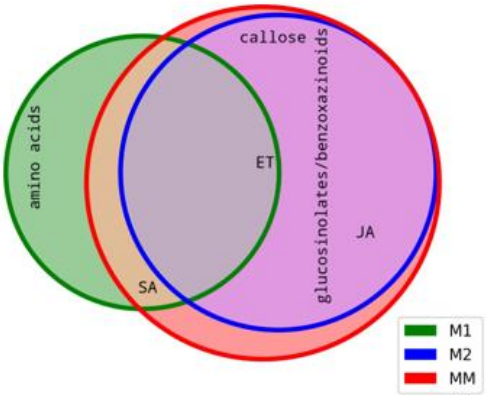

Figure S6.6: Brassicaceae: pathogens (virus) and phloem feeder

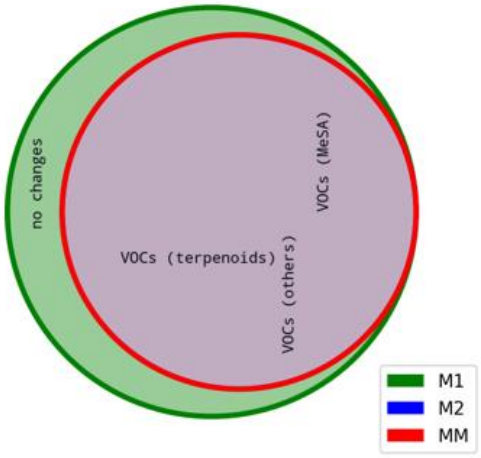

Figure S6.7: Fabaceae: mycorrhizal fungi and cell content feeder

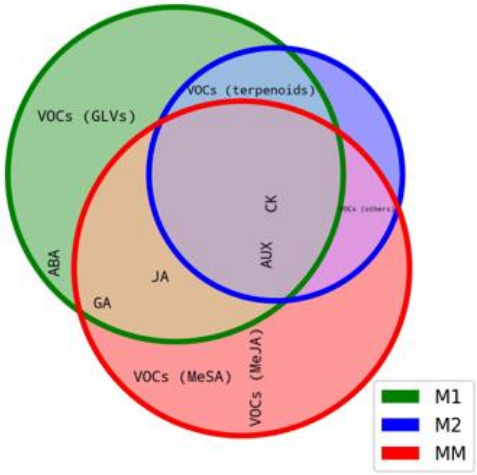

Figure S6.8: Fabaceae: mycorrhizal fungi and leaf chewing insect

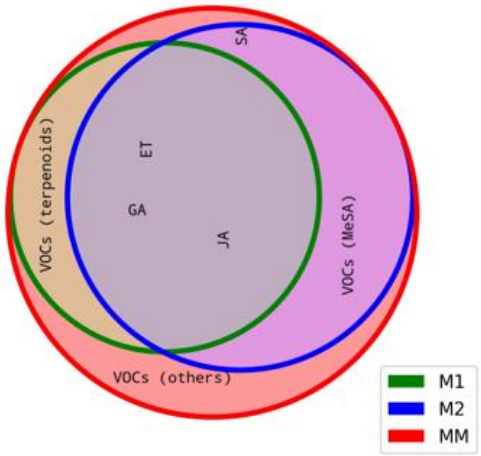

Figure S6.9: Fabaceae: mycorrhizal fungi and phloem feeder

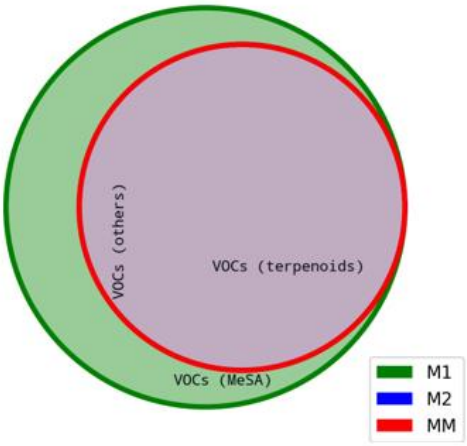

Figure S6.10: Fabaceae: pathogens (fungi) and leaf chewing insect

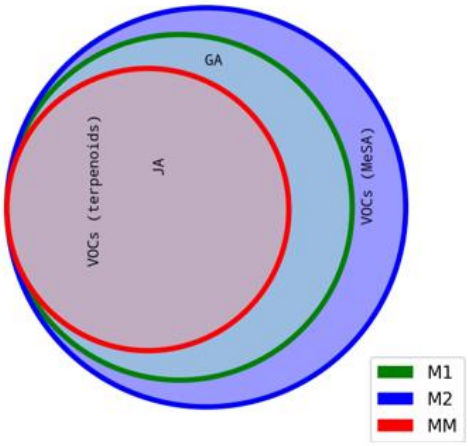

Figure S6.13: Poaceae: PGPF and leaf chewing insect

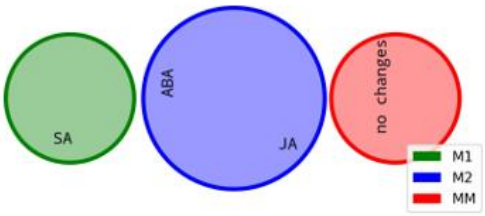

Figure S6.11: Fabaceae: pathogens (virus) and leaf chewing insect

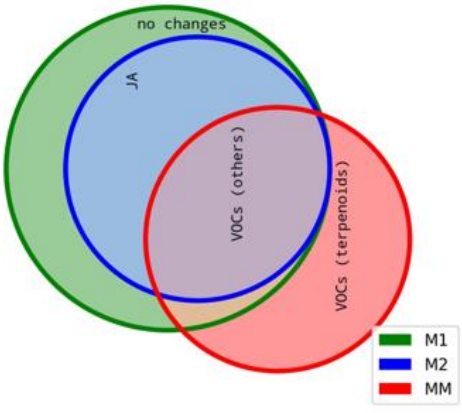

Figure S6.12: Fabaceae: pathogens (virus) and phloem feeder

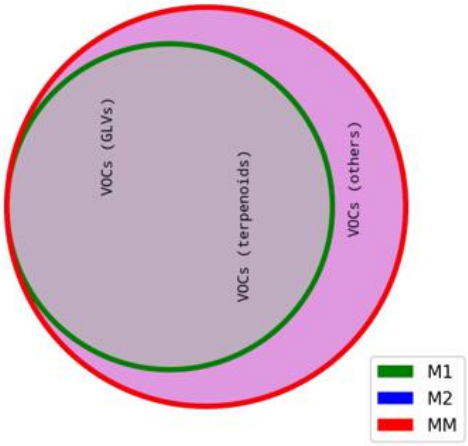

Figure S6.14: Poaceae: PGPR and leaf chewing insect

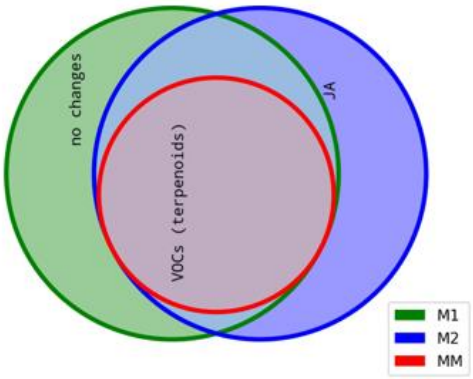

Figure S6.15: Poaceae: PGPR and root chewing insect

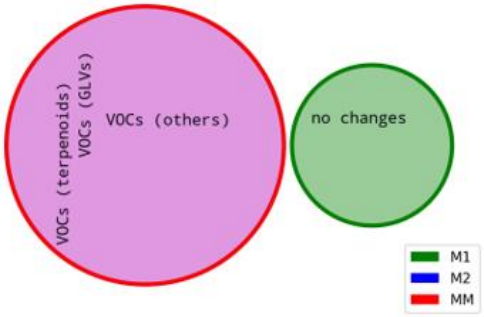

Figure S6.16: Poaceae: pathogens (fungi) and leaf chewing insect

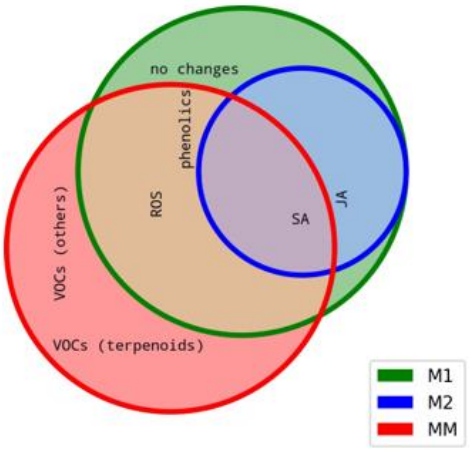

Figure S6.17: Poaceae: pathogens (fungi) and phloem feeder

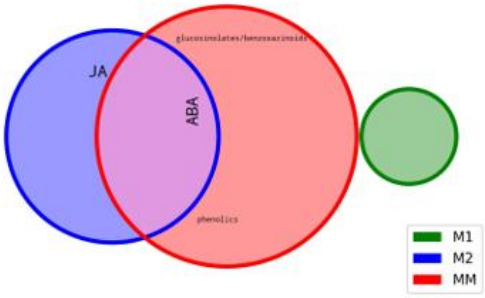

Figure S6.18: Poaceae: pathogens (fungi) and root chewing insect

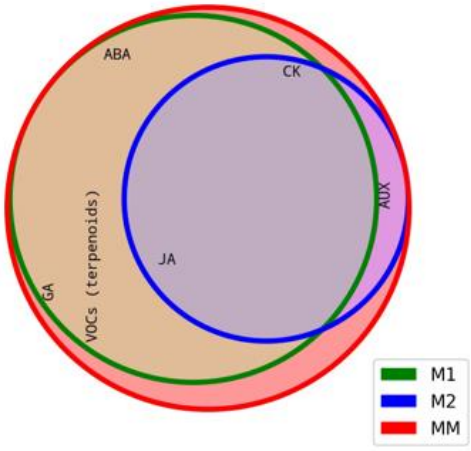

Figure S6.19: Solanaceae: mycorrhizal fungi and leaf chewing insect

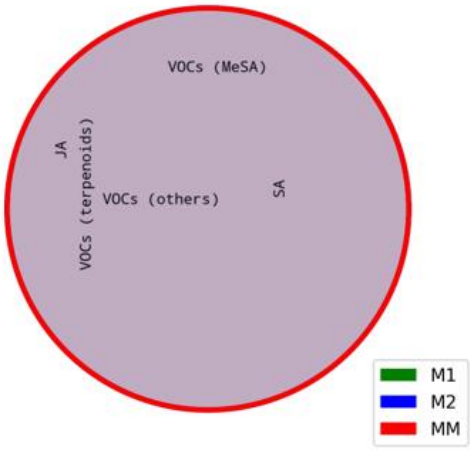

Figure S6.20: Solanaceae: PGPF and cell content feeder

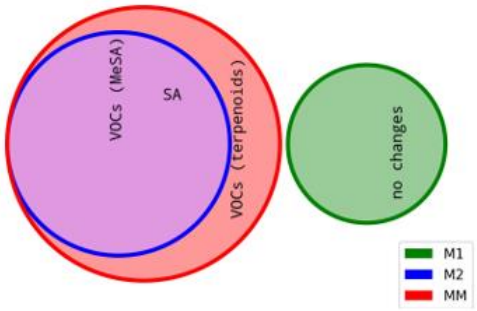

Figure S6.21: Solanaceae: PGPF and phloem feeder

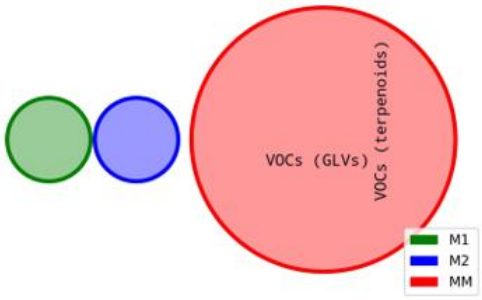

Figure S6.24: Solanaceae: pathogens (bacteria) and phloem—xylem feeder

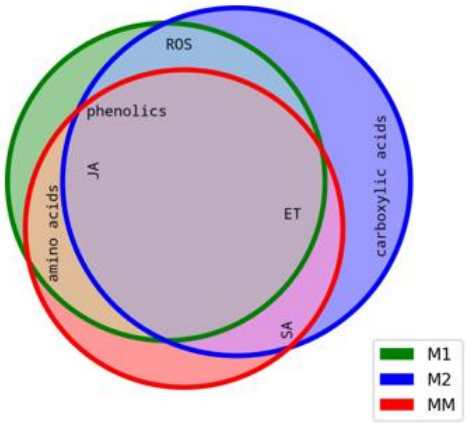

Figure S6.22: Solanaceae: PGPR and phloem feeder

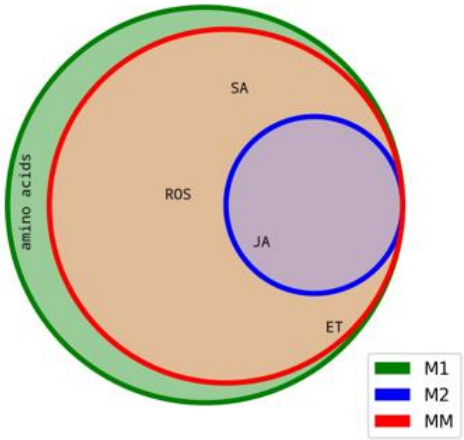

Figure S6.25: Solanaceae: pathogens (virus) and cell content feeder

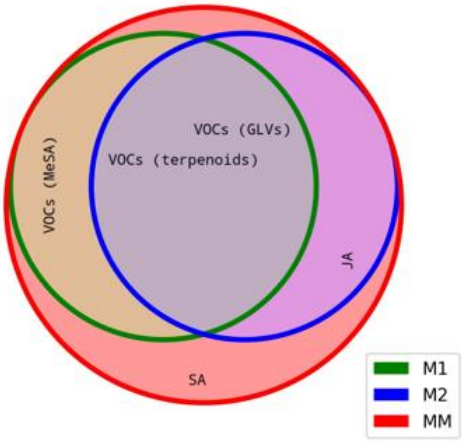

Figure S6.23: Solanaceae: pathogens (bacteria) and leaf chewing insect

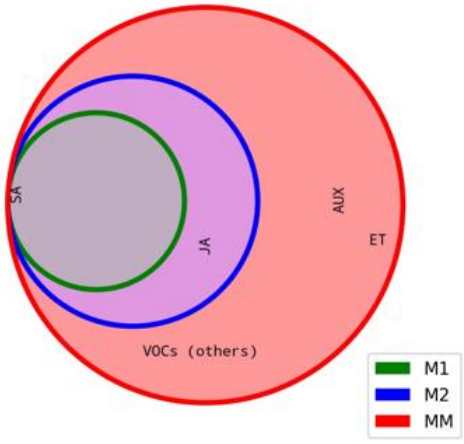

Figure S6.26: Solanaceae: pathogens (virus) and leaf chewing insect

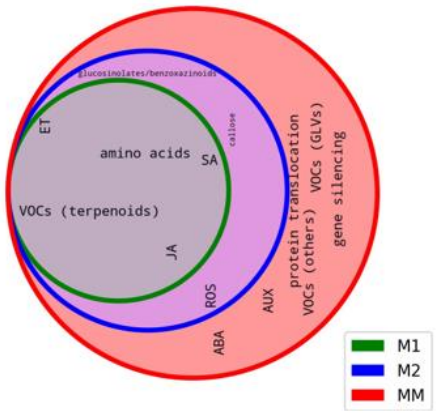

Figure S6.27: Solanaceae: pathogens (virus) and phloem feeder

Figure S7. Is the combination of microbe and arthropod with similar feeding styles important for the response in the plant?

Overview of the studies performed in 4 families (Solanaceae, Brassicaceae, Fabaceae, Poaceae) for which more than 5 studies were available. Studies were merged according to the combination of microbe and insect type (shown as nodes on the left side of the graph) and linked with mechanisms responding in the interacting plant when exposed to that combination (nodes on the right). Colouring of the mechanism nodes reflects their function - yellow: VOCS; orange: growth related hormones; green: stress related hormones; light blue: secondary metabolism. Blue and red color of the arrow indicates interaction with a beneficial or harmful organism for the plant, respectively. Light blue represents neutral effects. The width of the arrow is proportional to the number of studies with the given observation. The up- and downregulation of the mechanism represented in a node is depicted by a shape pointing upward (upregulation) or downward (downregulation). For the full names of mechanism nodes see Supplemental Table S1.

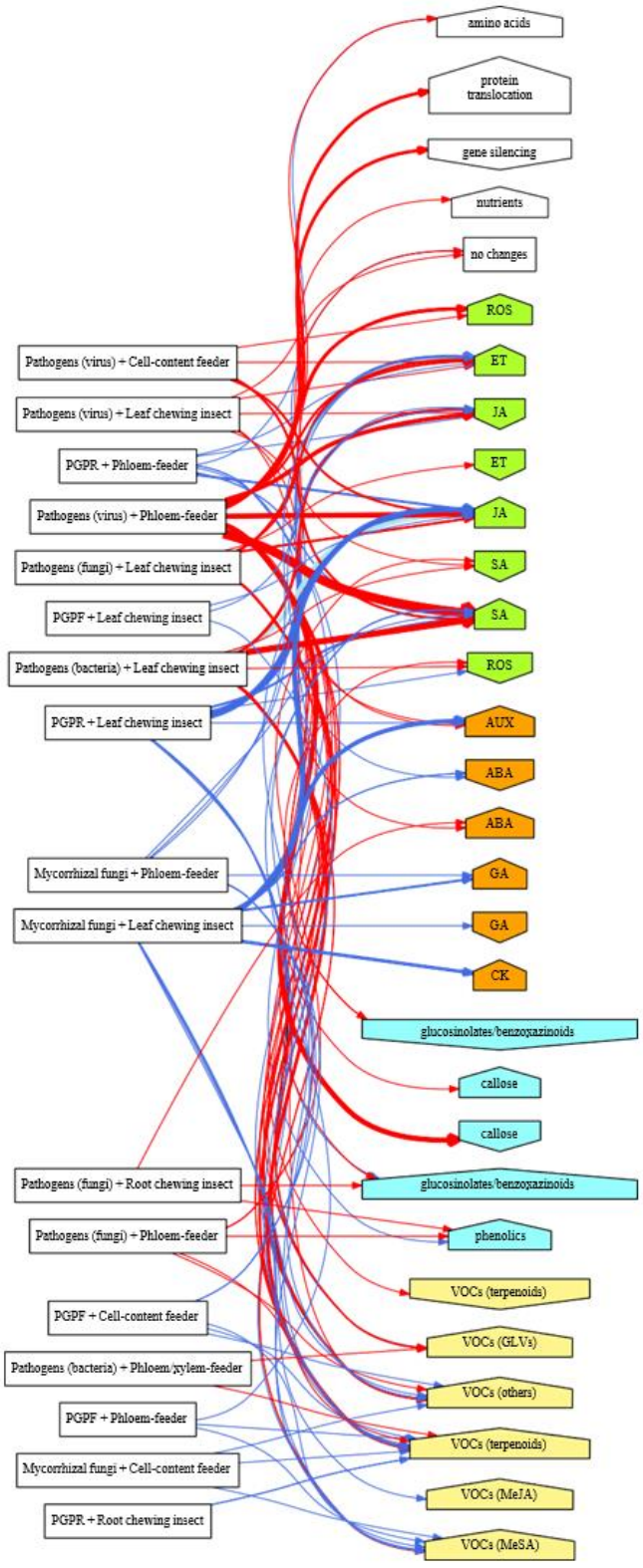

Figure S7.1: Poaceae, Brassicaceae, Fabaceae, and Solanaceae.

Chapter 5

Ethylene signaling is essential for mycorrhiza-induced resistance against chewing herbivores in tomato

Ethylene signaling is essential for mycorrhiza-induced resistance against chewing herbivores in tomato

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ABSTRACT

Root colonization by mutualistic mycorrhizal fungi can prime plant defenses, leading to Mycorrhiza-Induced Resistance (MIR) against different aggressors, including insect herbivores. Here we show that mycorrhizal fungi protect tomato plants against two chewing herbivores: the generalist *Spodoptera exigua* and the specialist *Manduca sexta,* and we explore the molecular mechanisms underlying such phenomena. Genome-wide transcriptional profiling comparing the three-way interactions between tomato, mycorrhizal fungi and the herbivores revealed that the root symbiosis had a very moderate impact on the leaf transcriptome in the absence of challenge, but remarkable changes appear in response to herbivory, including primed jasmonate-regulated defense responses. Among the basal changes, ethylene (ET) signaling was modulated by the mycorrhizal symbiosis, and further primed upon herbivory. Furthermore, mycorrhizal plants displayed elevated ET emission as confirmed by gas chromatography. Using network analysis, we show how ET signaling can modulate JA synthesis. We then used a genetic approach to functionally explore the role of ET signaling in the differential regulation of defenses associated with MIR. Tomato lines deficient in ET synthesis or perception were unable to develop MIR against any of the herbivores. Gene expression, enzymatic activity and targeted metabolomic analyses showed that ET signaling was required for the boosted JA biosynthesis in response to herbivory observed in mycorrhizal plants, and for the consequent priming of JA-dependent defenses. Thus, we demonstrate that ET signaling is an essential element in the hormone crosstalk underlying MIR against herbivores in tomato.

INTRODUCTION

Herbivory represents a significant loss of resources for the plant. Thus, the plant has to reorganize its primary metabolism while activating defensive mechanisms. In addition to the set of constitutive defenses, the plant triggers inducible defenses against in response to the attack by agresors. The plant induces the synthesis of defensive secondary metabolites that would negatively impact the attacker and releases volatiles to attract the aggressor's natural enemies. The negative impact on the insect's performance is usually achieved synergistically by combining toxic, anti-digestive and repellent compounds (Broadway & Duffey, 1988; Duffey & Stout, 1996; Erb & Reymond, 2019). The most relevant toxic metabolites are terpenoids, phenolics and nitrogenous compounds, such as alkaloids and glucosinolates. Herbivory also induces systemically the synthesis

of anti-nutritive proteins, as protease inhibitors (PIs) that bind to and inhibit digestive proteases in the insect gut (Green & Ryan, 1972); enzymes such as threonine deaminases (TD) or arginases which degrade within the gut the essential amino acids Thr or Arg respectively (Chen et al., 2005); or wound-induced polyphenol oxidases (PPOs) that impair the nutrient digestibility for the herbivore (Felton et al., 1989).

The jasmonate (JA) signaling pathway plays a central role in transcriptional reorganization and the induction of defenses against chewing herbivores (Erb & Reymond, 2019; Wasternack & Hause, 2013). Jasmonates are oxylipins synthesized from linolenic acid in the chloroplast membranes into 12-oxo-phytodienoic acid (OPDA). Subsequently OPDA is oxidized in the peroxisome to jasmonic acid, which can be further metabolized to various derivatives, including conjugates as jasmonoyl-L-isoleucine (JA-Ile) which binds to the CORONATINE INSENSITIVE1 (COI1) receptor that triggers the transcriptional regulation of the defense response (Yan et al., 2009). Other phytohormones contribute to shaping the defense response through crosstalk with other hormone signaling pathways, adjusting the plant response to the context perceived (Erb et al., 2012). Among them, ethylene (ET) is a gaseous phytohormone produced from the oxidation of its precursor, 1-Aminocyclopropane-1-Carboxylate (ACC) by ACC oxidases (ACO) (Yang & Hoffman, 1984). This phytohormone is involved in multiple processes from plant growth, development and defense, fruit ripening, senescence and fruit abscission and tolerance to diverse abiotic stresses (Dubois et al., 2018). It also contributes to the regulation of plant defense and interactions with other organisms through the modulation of JA dependent responses (Broekgaarden et al., 2015). Recently, several studies point that ET signaling could modulate JA biosynthesis upon herbivory (Chen et al., 2016; Hickman et al., 2017; Hu et al., 2021; Ma et al., 2020).

Under natural conditions plants interact simultaneously or sequentially with multiple organisms. In fact, they are surrounded and colonized by a multitude of microorganisms that can strongly influence plant performance and plant interactions with other organisms. Plant associated microbiota can alter plant responses to attackers above ground, being able to induce plant systemic resistance (ISR) to pathogens and pests (Pieterse et al., 2014; Pozo et al., 2021). Most of the molecular mechanisms that regulate two-way interactions between plants and arthropods are also activated in plant-microbe interactions. In three-way interactions between plants, microbes and insects, the same mechanisms are activated, but the response becomes more complex as additional

pathways are activated and the intensity and response time of the defenses may change (Gruden et al., 2020).

Over 70% of all vascular plants can naturally establish a mutualistic symbiosis with arbuscular mycorrhizal fungi (AMF; Brundrett & Tedersoo, 2018). The mycorrhizal symbiosis confers different benefits to the plant, from better mineral nutrition to better tolerance to biotic and abiotic stresses (Smith & Read, 2008), thus increasing plant resilience to cope with environmental challenges (Rivero et al., 2018). It can induce resistance to a broad range of pathogens and pests (Jung et al., 2012a; Rivero et al., 2021; Sanmartín et al., 2020; Song et al., 2013, 2015). Mycorrhiza can change plant phenology, morphology and chemistry affecting the feeding and subsequent tissue damage by the herbivore. It can enhance plant tolerance through the increase of plant primary metabolism and a reprograming of secondary metabolism (Rivero et al., 2021; Sanmartín et al., 2020). The enhanced resistance to pests in mycorrhizal plants (mycorrhiza-induced resistance, MIR); (Jung et al., 2012a; Pozo & Azcón-Aguilar, 2007) commonly rely on priming of plant defenses. Priming of plant defenses (or immune priming) is a cost efficient, adaptive defense strategy, in which preconditioned tissues are able to activate more efficiently plant immune responses upon challenge –usually leading to faster or stronger defense responses- (Conrath et al., 2006, 2015; Martinez-Medina et al., 2016; Mauch-Mani et al., 2017). Different experimental evidences point to a prominent role of JA signaling in the defense priming achieved in mycorrhizal plants (Jung et al., 2012a; Mora-Romero et al., 2014; Sanmartín et al., 2020; Song et al., 2013). However, little is known about the mechanisms regulating such priming of JA defenses.

The overall objective of this study was to understand the molecular mechanisms underlying MIR in response to chewing herbivores. We performed a genome-wide transcriptional profiling comparing the three-way interactions between tomato, mycorrhizal fungi and chewing herbivores of different specialization degrees. Transcriptional differences between non mycorrhizal and mycorrhizal plants were mostly found after herbivory, evidencing a primed response to the attack. The data pointed to a role of ET signaling in the differential regulation of defenses. We found enhanced ET production in mycorrhizal plants, both before and after insect attack, and network analysis revealed the potential connection of ET signaling with JA biosynthesis. Then, by using tomato lines deficient in ET signaling (synthesis and perception) we confirmed that differential ET signaling is essential for boosting the JA dependent defenses against chewing herbivores in mycorrhizal tomato, and therefore, it is a key component in MIR.

MATERIAL AND METHODS

Plants, mycorrhizal fungi and herbivore insects

Funneliformis mosseae (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (BEG12, International Bank of Glomeromycota, https://www.i-beg.eu/cultures/BEG12.htm) is continuously maintained in a pot culture of *Trifolium repens* L. and *Sorghum vulgare* Pers. in a substrate consisting of vermiculite:sepiolite (1:1, v/v) under greenhouse conditions. For all experiments, tomato seeds were surface sterilized in 4% sodium hypochlorite for 10 min, washed with water and incubated in plastic trays containing sterile vermiculite at 25–27 °C, 16 h/8 h (day/night) and 65–70 % RH. After 10 days, plantlets were transplanted to 350 mL pots filled with sand:vermiculite (1:1, v/v). Mycorrhizal treatments consisted of plants inoculated with 10 % (v/v) of raw *F. mosseae* inoculum containing colonized root fragments, spores and mycelia. Plants were randomly distributed under greenhouse conditions (25–27 °C, 16 h/8 h (day/night), 65–70 % RH). The fertigation schedule included watering with half-strength Hoagland solution (Hoagland & Arnon, 1938) once a week containing 25% of standard phosphorus (Pi). Generalist herbivore *S. exigua* Hübner (Lepidoptera: Noctuidae) eggs were obtained from the iDiv (Germany) for experiment 1, and from the University of Valencia (Spain) for experiment 2. *S. exigua* larvae were reared on artificial diet (Hoffman and Lawson, 1964) and maintained at 24 °C. Specialist herbivore, *M. sexta* L. (Lepidoptera: Sphingidae) eggs were obtained from the iDiv (Germany) for experiment 1 and from Universität Osnabrück (Germany) for experiment 2. Eggs were incubated at 26 °C and larvae were reared on detached tomato leaflets.

Experimental designs

1. Transcriptional profiling experiment

Surface sterilized tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were used for the transcriptional profiling experiment. The assay consisted in 6 treatments : Non-mycorrhizal control plants without herbivory (Nm), Non mycorrhizal plants challenged with *S. exigua* (Se), Non mycorrhizal plants challenged with *M. sexta* (Ms), *F. mosseae* inoculated control plants without herbivory (Fm), *F. mosseae* inoculated plants challenged with *S. exigua* (FmSe) and *F. mosseae* inoculated plants challenged with *M. sexta* (FmMs). Each treatment consisted of 6 independent plants as biological replicates. Mycorrhizal inoculation and growing conditions were as indicated above. After 5 weeks, a well-established mycorrhizal colonization was confirmed, and the herbivory

assays were performed. Three 3rd instar *S. exigua* larvae or two neonate *M. sexta* larvae were placed on the three apical leaflets of the third true leaf inside a clip cage (30 mm Ø). After 24 h, larvae were removed, and leaflets were frozen immediately in liquid nitrogen and stored at −80 ºC. The amount of damage was assessed to confirm herbivory and the damage between herbivores was similar.

2. Functional analysis of MIR in ethylene deficient lines

Seeds of *S. lycopersicum* wild-type UC82B, ET-deficient line ACD (Klee et al., 1991) and ET-insensitive mutant never ripe (Nr) (Wilkinson et al., 1995) were surface sterilized and germinated as described above. Seeds were kindly provided by Harry Klee (Florida, USA). After 8 weeks the herbivory assays were performed in mycorrhizal and non-mycorrhizal plants. Four 3rd instar *S. exigua* larvae or three neonate *M. sexta* larvae were placed on the third fully expanded leaf inside an entomological bag. Larval mortality and pupation were monitored every 48 h and *M. sexta* weight was determined at day 9 post infestation. For the *S. exigua* bioassay, each treatment consisted of seven independent plants, and four larvae were used per plant (a total of 28 larvae per treatment). For the *M. sexta* bioassay, ten independent plants were used per treatment, and three larvae were used per plant (30 larvae per treatment).

Mycorrhizal quantification – histochemical staining

As described in García et al. (2020) root samples were cleared and digested in 10% KOH (w/v) for 2 days at RT (18 – 23°C). After, root samples were rinsed thoroughly with tap water and acidified with 2% (v/v) acetic acid solution. Fungal root structures were stained with a 5% (v/v) black ink (Lamy, Germany) and 2% acetic acid solution for 24 h at RT (Vierheilig et al., 2005). Ink solution was washed with tap water. Mycorrhizal colonization was determined by the gridline intersection method (Giovannetti & Mosse, 1980) using a Nikon SMZ1000 stereomicroscope.

Ethylene emission quantification by Gas Chromatography

One detached leaflet of each tomato plant was placed into a 20 mL glass vial containing a sterile filter paper soaked in 200 µL of sterile distilled water to buffer the dehydration. The vials were left uncovered for 30 min to avoid the ethylene released as a result of detaching the leaf with the scalpel. After this time, for the herbivory treatments, we placed inside the vial one larvae of the corresponding herbivore and immediately after the vials were sealed. Vials were maintained at 23 °C under a 18 h photoperiod. 1 mL from each vial was withdrawn with a syringe and the area of the ethylene peak was analyzed in a gas chromatograph (Hewlett Packard 5890) with a flame-ionization

detector (FID). ET emission by the herbivores were determined to be negligible by analyzing vials containing only larvae.

RNA-seq transcriptional analysis

For RNA-seq analysis, three apical leaflets of the third true leaf contained within the clip cage were harvested and immediately flash frozen in liquid nitrogen. Three biological replicates per treatment were used, each consisting of pooled material from two plants. Samples were homogenized using pestle and mortar in liquid nitrogen. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's recommendations. The quality, quantity and size of extracted RNA was determined with a Bioanalyzer (Agilent, USA) and Nanodrop (ThermoFisher Scientific, USA). All samples had RIN > 8, A260/A280 > 1.8 and A260/A230 > 2. TruSeq stranded RNA-Seq library prep and paired-end sequencing on the Illumina NovaSeq 6000 platform was performed by Macrogen (S. Korea). Quality control of sequencing reads was performed using FastQC (Andrews et al., 2010). Sequences were mapped to the tomato genome version SL4.0 using STAR v2.7.2b (Dobin et al., 2013) and the ITAG 4.0 annotation (Hosmani et al., 2019). Differential expression analysis was performed in R using the DESeq2 package (v1.26.0; Love et al., 2014). Prior to statistical testing, genes not having at least 50 counts in at least three samples were excluded. Genes with DESeq2 FDR-adjusted p-value < 0.05 were considered significantly differentially expressed (DEG). Gene set enrichment analysis (GSEA) was performed with TMM normalized count values using the GSEA tool v4.0.3 (Subramanian et al., 2005) and gene sets based on GoMapMan (Ramšak et al., 2014) BINs. Gene sets with FDR-adjusted q-values < 0.05 were considered significantly enriched in up- or down-regulated genes. For easier visualization of the enriched gene sets, they were selected and organized in functional supergroups (Table S1).

Network analyses

Network analyses were performed on a specifically generated knowledge network of *S. lycopersicum*. First, the *Arabidopsis thaliana* large comprehensive knowledge network (Ramšak et al., 2018), containing high-quality relations (protein-protein binding, protein-DNA binding, miRNA targets) between Arabidopsis genes was translated to tomato using PLAZA orthologues (Proost et al., 2015). Additionally, tomato specific network of miRNA - transcript targets was generated using psRNATarget (Dai & Zhao, 2011) for tomato small RNAs present in miRBase (v22; Kozomara et al., 2019) and merged with the translated comprehensive knowledge network. A subnetwork with genes that were differentially expressed in at least one of the RNA-Seq pipeline contrasts (FDR

adjusted p-value <= 0.05) was extracted in the next step. Shortest path searches were performed using EIN3/EIL1 transcription factors as starting nodes (Solyc01g009170, Solyc01g096810, Solyc06g073720, Solyc06g073730) and genes related to JA synthesis for the end nodes (LOX: Solyc01g099190, Solyc03g006540, Solyc03g122340, Solyc05g014790, Solyc08g014000; AOC: Solyc02g085730; AOS: Solyc04g079730, Solyc10g007960, Solyc11g069800; OPR: Solyc07g007870, Solyc10g086220, Solyc11g013170). Network analyses were performed in R using the igraph package (v1.2.8; Csárdi & Nepusz, 2006) and results visualized in Cytoscape (Shannon et al., 2003).

Analysis of Gene Expression by qPCR

Total RNA was extracted from 100 mg of grinded leaves using TRIsure™ (Bioline, USA) and treated with DNase I (NZYtech, Portugal). After the RNA was purified and concentrated using RNA Clean & Concentrator-5 column kit (Zymo Research, USA). First-strand cDNA was synthesized from 1 μg of purified total RNA using PrimeScript RT Master Mix (TaKara, Japan) according to the manufacturer's instructions. Real-time quantitative PCR reactions and relative quantification of specific mRNA levels were performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and the gene-specific primers described in Table S2. Expression values were normalized using the reference gene SlEF-1α (López-Ráez et al., 2010) encoding the tomato translation elongation factor-1α. Six independent biological replicates per treatment were analyzed.

LAP enzymatic activity

Frozen leaf tissue was ground in liquid nitrogen into a fine powder. Ground tissue was mixed 1:18 (w:v) with protein extraction buffer (50 mM Tris-HCl [pH 8] and 0.5 mM $MnCl_2$). Samples were centrifuged at 14000 rpm for 10 min at 4 °C and the supernatant was collected. This process was repeated twice. For the LAP enzymatic activity, a stock solution of L-leucine-*p*-nitroanilide (LpNA; Sigma-Aldrich, Germany) was prepared in absolute ethanol. The reaction mixture contained 200 μL of 3 mM LpNA (in 50 mM Tris-HCl [pH 8] and 0.5 mM $MnCl_2$) and 40 μL of the sample protein supernatant. The reaction was incubated in a 96-well plate at 37 °C for 20 min. The absorbance was measured at 410 nm (Chao et al., 2000).

Hormonal analysis

Freeze dried plant material was used for hormonal analysis as described by Sánchez-Bel et al. (2018) with small changes. 30 mg of dry material was homogenized with 1 ml MeOH:H2O with

0.01% of HCOOH containing a pool of 1 µg.ml^{-1} of a mixture of internal standards of jasmonic-2,4,4d_3-(acetyl-2,2-d_2) acid (Sigma-Aldrich), own synthesized JA-Ile-d_6 and dhJA for 12-oxo-phytodienoic acid (OPDA) quantification. Samples were ground in cold and centrifuged at 15,000 rpm for 15'. The pH of the supernatant was reduced to 2.5–2.7 with acetic acid and the extraction was partitioned twice against diethyl ether. The organic phase was recovered and evaporated in a speedvac centrifuge. Samples were resuspended in 1 ml of H2O/MeOH (90:10) with 0.01% of HCOOH up to a final concentration of internal standards of 10 ng ml^{-1}. The chromatography was performed using a UPLC Kinetex C18 analytical column with a 5 µm particle size, 2.1 100 mm (Phenomenex). Samples were injected onto a Acquity ultraperformance liquid chromatography system (UPLC; Waters, Mildford, MA, USA), which was interfaced with a triple quadrupole mass spectrometer (TSD, Waters, Manchester, UK). Quantification was performed by using Masslynx 4.2 software.

Statistical analyses

Besides the methods and software for RNA-seq transcriptional analysis described above, statistical analyses were performed with unpaired t-test analysis using Statgraphics Plus 3.1. Comparison between treatments of larval mortality and pupation was performed using the Log-Rank test (Mantel-Cox) with the "Survival" and "survminer" packages in R. PCAs were performed using Metaboanalyst software.

RESULTS

Mycorrhizal symbiosis impacts the leaf transcriptomic response to herbivory

Mycorrhiza induced resistance has been shown to be effective against chewing insects (He et al., 2017; Roger et al., 2013; Song et al., 2013). In this regard, we previously showed that *F. mosseae* induced resistance in tomato against *S. exigua* (Rivero et al., 2021). To explore the molecular processes underlying the impact of *F. mosseae* colonization on insect performance, we compared the full transcriptional profile of leaves from non-mycorrhizal and mycorrhizal plants colonized by *F. mosseae* (Nm, Fm, respectively) without challenge, or subjected to herbivory by the generalist *S. exigua* (Se) and the specialist chewing herbivore *M. sexta* (Ms). Principal Component Analysis (PCA) was performed on the RNA-Seq data (Fig. 1A) with the first two principal components explaining 69.3% of the total variance. Herbivory had a strong impact on the transcriptome, with a

clear separation from non-herbivory treatments in the PCA plot. This separation was mostly explained by PC 1, which accounts for 57.5% of the total variance (Fig. 1A). In contrast to the effect of herbivory, mycorrhizal colonization itself causes a very moderate effect on the leaf transcriptome profile (Fig. 1A), as only 57 DEGs (FDR <0.05, Table S3) were detected in Fm plants as compared to the Nm controls. While the mycorrhizal symbiosis has a subtle impact under non-challenging conditions, the impact was stronger when the host plant was under herbivory (FmSe, FmMs, Fig. 1B), pointing to a differential plant response to the herbivore.

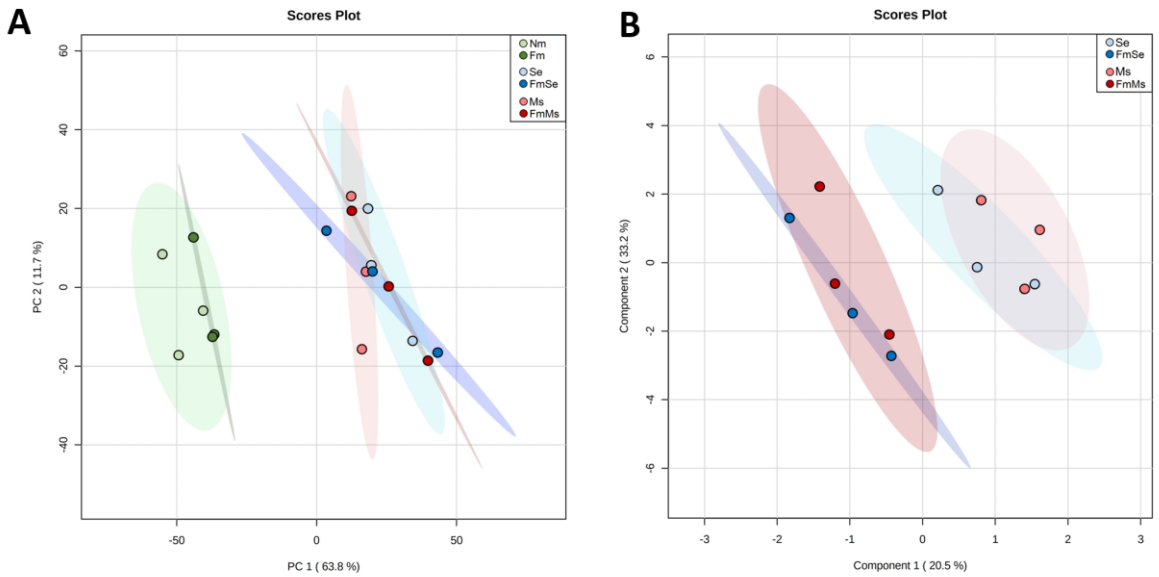


Figure 1 Overview of the impact of the different interactions on the transcriptome of tomato leaves. Tomato leaves from mycorrhizal (Fm) and non-mycorrhizal (Nm) plants were subjected to 24 h of S. exigua (Se, FmSe) or to M. sexta (Ms, FmMs) herbivory. (A) Overall transcriptomic PCA plot for all treatments. (B) Transcriptomic PLS-DA 2-D Scores plot between herbivory treatments in non-mycorrhizal (Se and Ms) and mycorrhizal (FmSe and FmMs) plants. The percentage of variance explained by the principal component are shown in brackets.

Mycorrhizal colonization affects key regulatory pathways and modulates plant transcriptional responses to herbivory

In the absence of herbivory, only few differentially expressed genes (DEGs) were found in mycorrhizal plants. However, gene set enrichment analysis (GSEA) revealed the modulation of several important cell processes (Fig. 2). Besides changes in cell division and structure (especially in “cell cycle” and “cell wall structure”) most changes were found in signaling related pathways (“sugar and nutrient signaling”, “receptor kinases signaling”), gene sets related to secondary metabolism (increase in “stress biotic receptors'' and “glycosyl and glucoroyl transferases”), and pathways related to the plant stress responses (“DNA chromatin structure”, “protein metabolism”, “RNA

regulation"). Hormone metabolism is also modulated by *F. mosseae,* as revealed by the enrichment of genes related to the ET and JA pathways. Remarkably, all these changes can be relevant for the regulation of stress responses and may mediate defense priming.

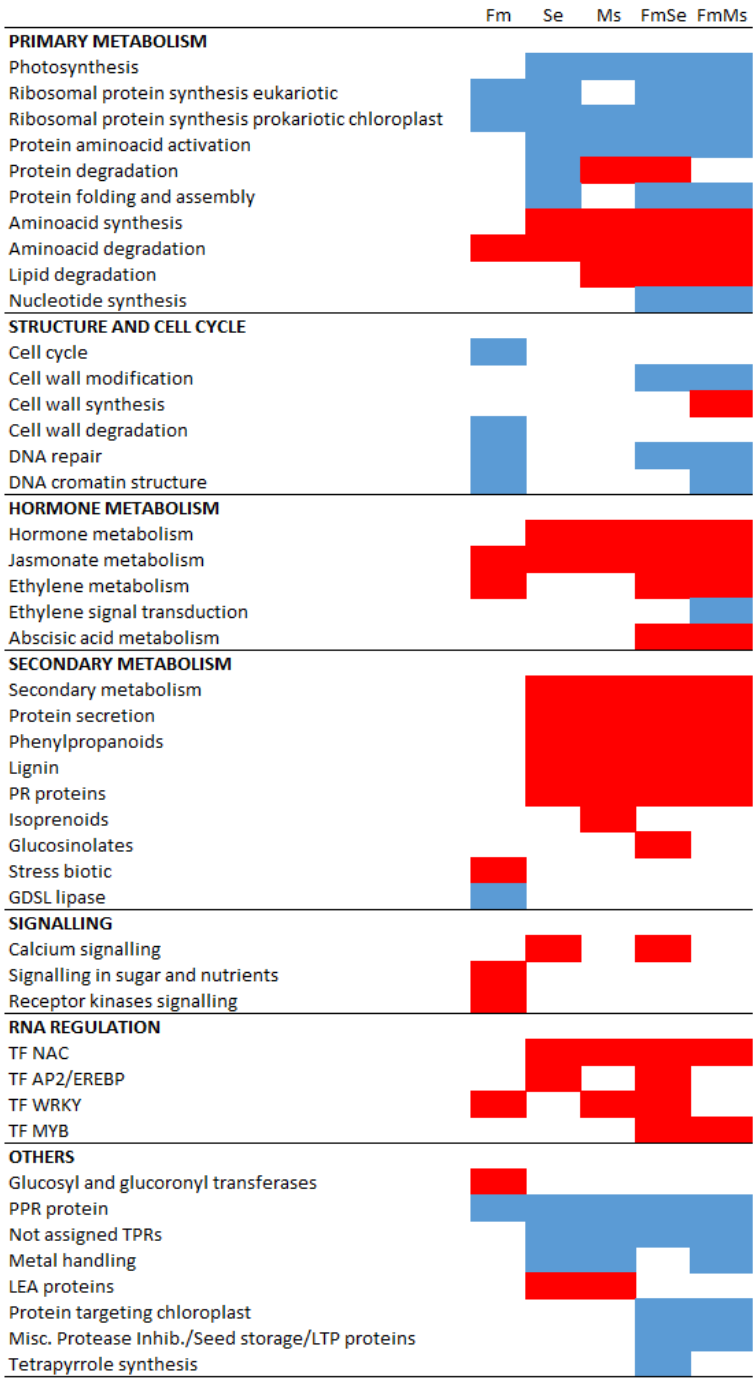


Figure 2 Mycorrhizal colonization affects key regulatory pathways and has a notorious impact on the transcriptomic changes of herbivory treatments. Tomato leaves from mycorrhizal (Fm) and non-mycorrhizal (Nm) plants were subjected to 24 h of S. exigua (Se and FmSe) or to M. sexta (Ms and FmMs) herbivory. Heatmap of changes on enriched gene sets in the different treatments as compared with their non-mycorrhizal control (Nm) from GSEA results. All treatments had three biological replicates each with a pool of two plants. Data represent enriched gene sets (GSEA,FDR<0.05). Blue and red cells indicates repression and induction of the gene set, respectively.

Regarding the responses to herbivory, the numbers of DEG differ in Nm plants challenged with *S. exigua* and *M. sexta* (Table S3), although the general response to both herbivores is conserved according to the GSEA, with most functional classes –related to both primary and secondary metabolism- regulated similarly (Fig. 2). Comparing responses to both herbivores (Se vs Ms), no DEGs were found between the generalist *S. exigua* and the specialist *M. sexta* treatments (Table S3). The two herbivores repress photosynthesis and impact synthesis and degradation of amino acids and protein metabolism by inhibiting protein synthesis. Both herbivores activate secondary metabolism, inducing the synthesis of phenylpropanoids and lignins as well as PR proteins (mainly proteinase inhibitors). Herbivory impacts hormonal metabolism, mostly by enrichment of the JA related gene set (Fig. 2). *M. sexta* has a lower impact on the primary metabolism, with less photosynthesis and protein synthesis related genes repressed than by *S. exigua* herbivory (Fig. S1). In mycorrhizal plants, the symbiosis intensifies this repression of the primary metabolism in response to herbivory (FmSe, FmMs, Fig. S3). As in non-mycorrhizal, mycorrhizal plants respond with an induction of the secondary metabolism and of JA-dependent PR proteins involved in defense against chewing herbivores (Fig. 2), including proteinase inhibitors and wound inducible carboxypeptidases (Table S4). Although the core of specific responses to herbivory are the same in mycorrhizal and non-mycorrhizal plants, new mechanisms are regulated in mycorrhizal plants after insect infestation. The most remarkable differences found in mycorrhizal plants in response to herbivory are related to hormonal metabolism: in addition to inducing the JA pathway, as in non-mycorrhizal plants, the symbiosis up-regulates ET and ABA metabolism (Fig. 2).

Mycorrhization primes JA-dependent defense response upon herbivory

The JA pathway is the major regulator of anti-herbivory defenses (Howe et al., 2018; Wasternack & Hause, 2013). To better characterize the defense responses differentially regulated in mycorrhizal plants, we specifically analyzed the transcriptional regulation of well-characterized JA-dependent anti-herbivory genes. As expected, these genes were induced by herbivory, but for most of them this induction was significantly higher in mycorrhizal plants than in non-mycorrhizal ones (Fig. 3A) confirming the mycorrhiza related priming of JA regulated defense responses upon herbivory. We explored whether mycorrhiza also primed JA biosynthesis. Remarkably, a small, yet significant induction of some JA biosynthesis genes was detected in mycorrhizal plants in non-challenged plants (Fig. 3B). Herbivory induces JA biosynthesis in both non mycorrhizal and

mycorrhizal plants, but the induction was stronger in mycorrhizal plants, although the primed induction was only significant upon *M. sexta* infestation.

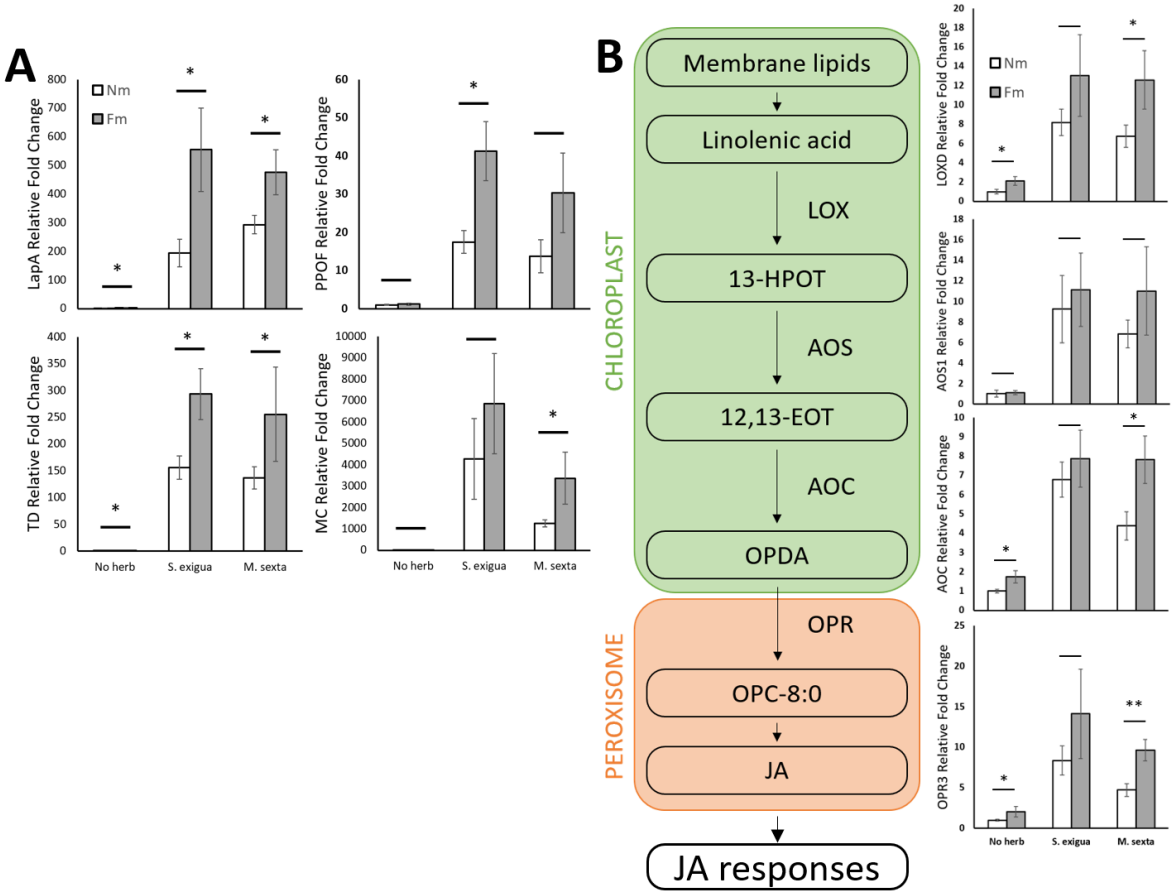
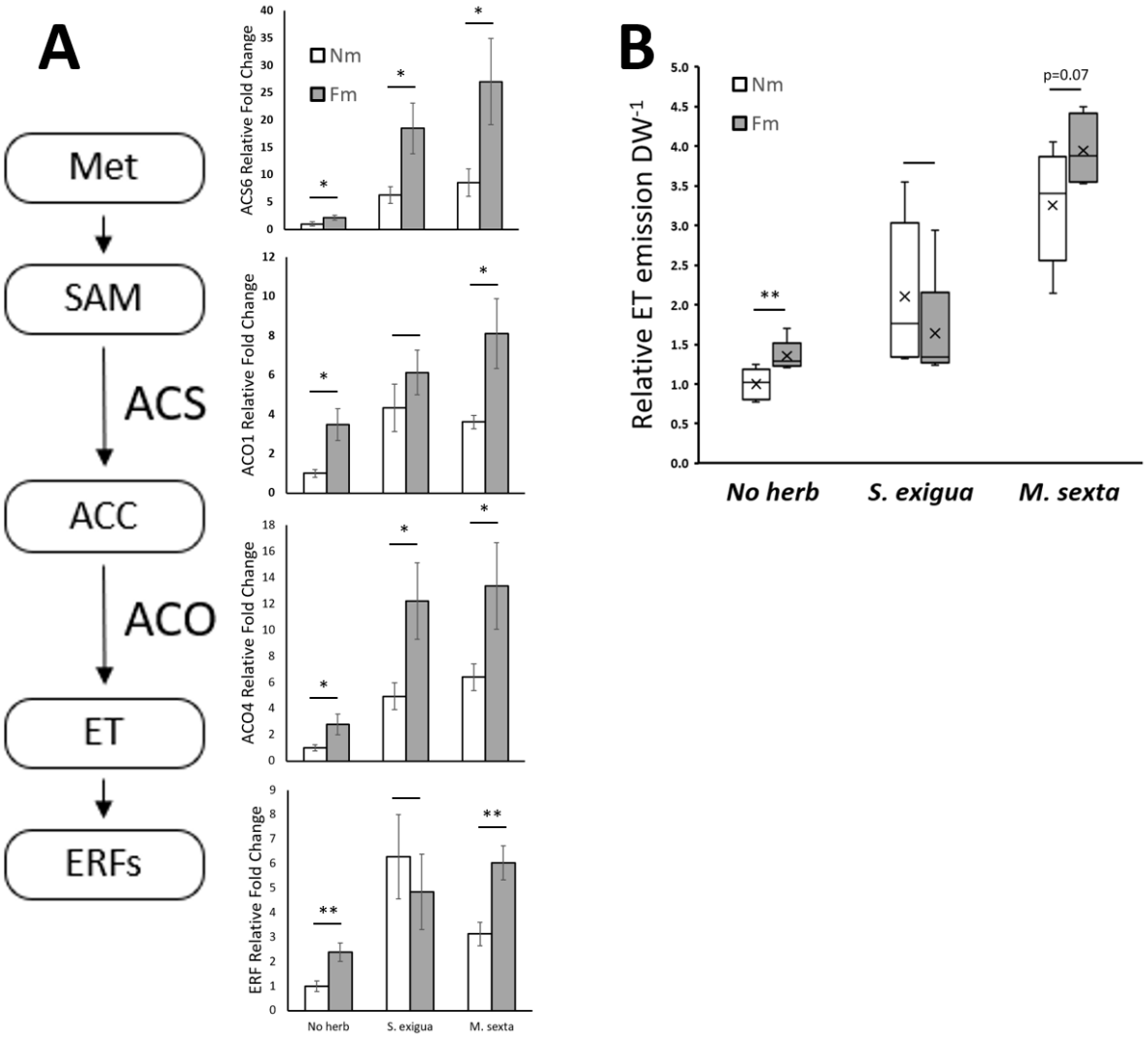


*Figure 3 JA dependent antiherbivory responses and JA biosynthesis upon herbivory are primed in AM plants. Tomato leaves from mycorrhizal (Fm) and non-mycorrhizal (Nm) plants were infested for 24 h with S. exigua (Se and FmSe) or M. sexta (Ms and FmMs) . (A) Relative expression of the JA dependent defense related marker genes: leucine aminopeptidase A (LapA, Solyc12g010020.1), polyphenol oxidase F (PPOf, Solyc08g074620.1), multicystatin (MC, Solyc00g071180.2) and threonine deaminase (TD, Solyc09g008670.2); and (B) Relative expression of JA biosynthetic pathway genes: lypooxigenase D (LOXD, Solyc03g122340.2), allene oxide synthase 1 (AOS1, Solyc04g079730.1), allene oxide cyclase (AOC, Solyc02g085730.2) and 12-oxophytodienoate reductase 3 (OPR3, Solyc07g007870.2). Expression values were normalized using the reference gene SlEF, which encodes for the tomato elongation factor-1a. Data shown are mean ± SEM of 6 biological replicates. Statistical analysis was performed with unpaired t-test analysis between each herbivory treatment. * p<0.05, ** p<0.01.*

Mycorrhization enhances ET metabolism and primes ET biosynthesis and signaling upon herbivory

Studies on the hormonal crosstalk between ET and JA (van Loon et al., 2006; von Dahl & Baldwin, 2007) suggests a complex regulatory function of ET in plant defenses. Since ET related genes were also among the differentially regulated gene sets in mycorrhizal plants, both in basal and herbivory conditions, we performed a targeted analysis on ET biosynthesis and signaling (Fig. 4A). Higher basal levels in mycorrhizal plants were confirmed for biosynthetic genes encoding the biosynthetic genes ACC synthase 6 (ACS6; Solyc08g008100.2) and ACC oxidases (ACO1 and ACOlike4; Solyc07g049530.2 and Solyc04g007980.2), responsible of the limiting step in ET biosynthesis, and for the ethylene responsive factor ERF (Solyc02g070040.1). These genes were upregulated in response to both herbivores, but the induction was primed in mycorrhizal plants (Fig. 4A). The primed response was stronger upon challenge with *M. sexta*, the system in which more mycorrhiza related changes were observed. To evaluate if the transcriptional activation of ET biosynthesis correlated with higher ET levels in mycorrhizal plants, we quantified ET emission by GC in a new set of plants. The analysis confirmed that leaves of *F. mosseae* plants emit significantly more ET than non-mycorrhizal plants in basal conditions (Fig. 4B). Herbivory treatments caused a clear increase in ET in both mycorrhizal and non-mycorrhizal plants. The levels were slightly higher in mycorrhizal plants in *M. sexta* infested leaves, although the increase was only marginally significant ($p<0.07$).

*Figure 4 Mycorrhiza primes the herbivory triggered biosynthesis of ethylene and response. (A) Relative expression of the ET biosynthesis genes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase 6 (ACS6, Solyc08g008100.2), ACC oxidase 1 (ACO1, Solyc07g049530.2) and ACC oxidase 4-like (ACO4-like, Solyc04g007980.2) and the ET responsive factor (ERF, Solyc02g070040.1). Tomato leaves from mycorrhizal (Fm) and non-mycorrhizal (Nm) plants were subjected to 24 h of S. exigua (Se and FmSe) or to M. sexta (Ms and FmMs) herbivory. (B) Boxplots show relative ET emission normalized to leaflet dry weight (DW). Single tomato leaflets of non-mycorrhizal plants (Nm) and mycorrhizal plants with F. mosseae (Fm) were challenged with S. exigua (Se and FmSe) or M. sexta (Ms or FmMs) for 18 h inside 20 mL glass vials. 1 mL of every sample was withdrawn from the vial and the area of the ethylene peak was analyzed in by gas chromatography. (A) Expression values were normalized using the reference gene SlEF, which encodes for the tomato elongation factor-1a. Data shown are mean ± SEM of 6 (A) or 5 (B) biological replicates. Outlier data points are defined as any value reaching past 1.5 times the interquartile range from either the lower or upper quartile. Statistical analysis was performed with unpaired t-test analysis between each herbivory treatment. + p<0.1, * p<0.05, ** p<0.01.*



Physical interaction network supports the connection of ethylene signaling with JA biosynthesis

To get further insights in the ET-JA signaling interactions in the differential response of mycorrhizal plants, gene expression results were plotted into a physical interaction network constructed by merging dispersed resources on metabolic pathways, protein-protein interactions, protein-DNA interactions and smallRNA-transcripts interactions (Ramšak et al., 2018). We next extracted a subnetwork of genes differentially expressed if comparing leaves of mycorrhizal and non-mycorrhizal plants and their direct interactors. This network was further explored by extraction of shortest paths between the nodes with function in ET signaling and the ones participating in JA synthesis (Fig. 5). The results show a broad activation of the JA and ET pathway genes in mycorrhizal plants without herbivory. In plants subjected to herbivory, the symbiosis has a differential impact

on these two pathways, pointing to a contribution of these pathways in the regulation of the plant responses to herbivory, and suggesting that the changes could be related to an interconnected regulation between both hormones.

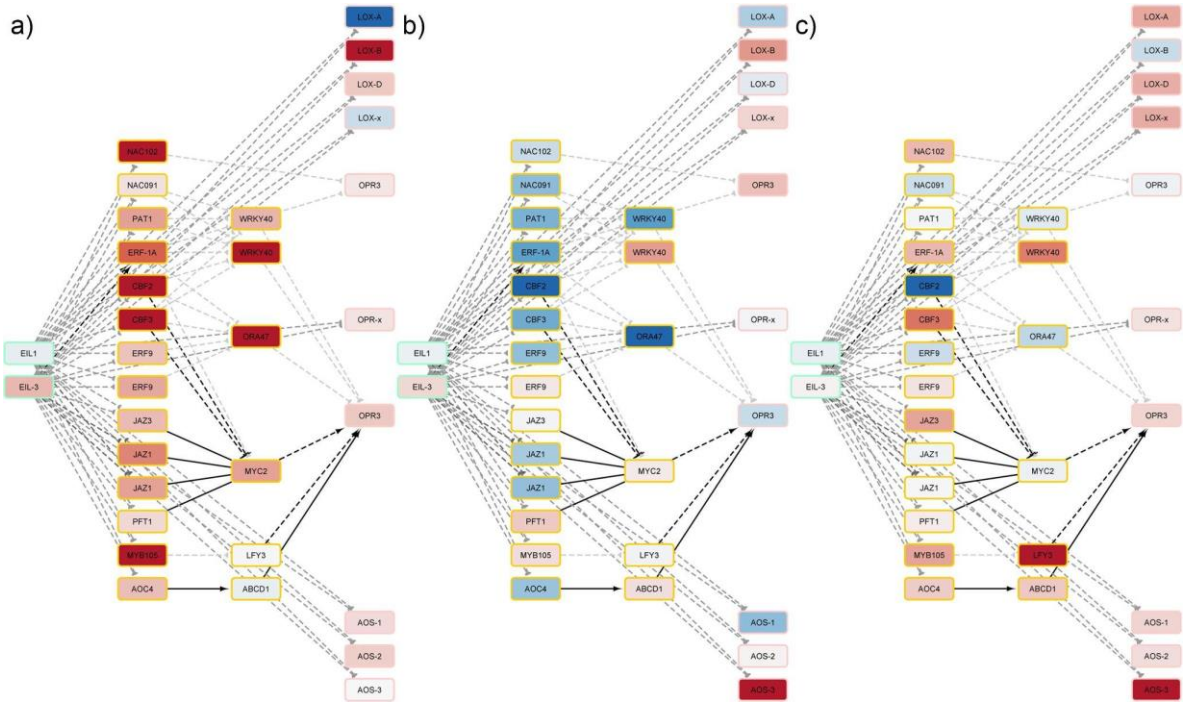


Figure 5 Mechanistic basis for mycorrhizal modulation of response to insect pests through ethylene signaling. Insight into the network of physical interactions between signaling network components is shown. Tomato responses to mycorrhiza without herbivory (a) and responses to either (b) M. sexta or (c) F. mosseae are shown. Nodes represent tomato protein coding genes and the node color shows regulation after the applied treatment (red = up-regulation, blue = down regulation). Connection type is shown as different line types (dashed = transcriptional regulation; solid = binding or synthesis), line arrows show the mode of action (arrow = activation; T = inhibition; half-circle = unknown).

Ethylene signaling is essential for MIR against herbivory

According to the identified physical link between ET signaling and JA synthesis, we hypothesized that the differential regulation of ET in mycorrhizal plants may contribute to MIR. To test such hypothesis we performed a new experiment using tomato lines impaired in ET production (ACD, expressing the *Pseudomonas* ACC deaminase that cleaves the ET precursor ACC; Klee et al., 1991) or ET perception (Nr: Never ripe, mutant in the ET receptor ETR3, Wilkinson et al., 1995), both in a UC82B background. We first confirmed that mycorrhizal colonization was well established: ET-deficient lines showed no significant differences in colonization compared with UC82B wt (Fig. S3A),

and there was no effect of the mycorrhizal treatment in the physiological development of the plant genotypes, so that any differential impact on herbivory would not be related to deficient mycorrhiza establishment or differential effects of the symbiosis on plant growth (Fig. S3B). We then confirmed the ET deficient phenotype of the lines regarding ET emission. ET is induced upon herbivory in the wild type (UC82B background), up to 2.5-fold after 3h of herbivory with *M. sexta* larvae (Fig. S4). As predicted, herbivore induced ET accumulation was significantly higher in mycorrhizal plants, confirming the primed accumulation of ET in response to herbivory. ET production was almost abolished in the ACD mutant, and while ET was still accumulated in response to herbivory in the ET insensitive mutant Nr, the primed response by mycorrhiza was lost (Fig. S4).

We hypothesized that primed ET production is essential for MIR. To test this hypothesis, we performed a new bioassay comparing the performance of both, the generalist and the specialist herbivores in mycorrhizal and non-mycorrhizal plants from all three backgrounds. In wild type (UC82B) plants, mycorrhizal colonization leads to a remarkable increase in mortality of *S. exigua* larvae as compared to non-mycorrhizal control plants. Remarkably, this increase in mortality in larvae feeding on mycorrhizal plants is lost in the ET deficient lines ACD and Nr (Fig. 6A). Differences were observed also in insect development: *S. exigua* larvae started pupating earlier when feeding on non-mycorrhizal plants as compared to mycorrhizal plants of the wild-type genotype, and significantly fewer larvae reached the pupal stage on mycorrhizal plants. In the ET deficient lines, no differences in pupation were observed between mycorrhizal and non-mycorrhizal plants. In the deficient lines, a reduction in the total number of pupae was observed, likely related to the remarkable increase in mortality from day 13 onwards. This mortality matches the initiation of larval pupation in the experiment (Fig. 6A). Intriguingly, many of these deaths were related to pupal cannibalism and malformations upon entering the pupal stage. The same experimental design was carried out with neonate larvae of *M. sexta*. It is reported that *M. sexta* mortality occurs mostly before reaching the L3 developmental stage. No effect on mortality was observed among the different treatments after 10 dpi due to the low mortality (Fig. S5). Thus, to estimate larval performance, we determined larval weights at day 9 post infestation. In the wild type UC82B, larval weight was significantly lower in mycorrhizal plants as compared to non-mycorrhizal plants (34% reduction). However, the effect of mycorrhizal colonization on larval weight disappeared in the ET deficient lines (Fig. 6B). These results suggest that ET signaling is required for MIR.

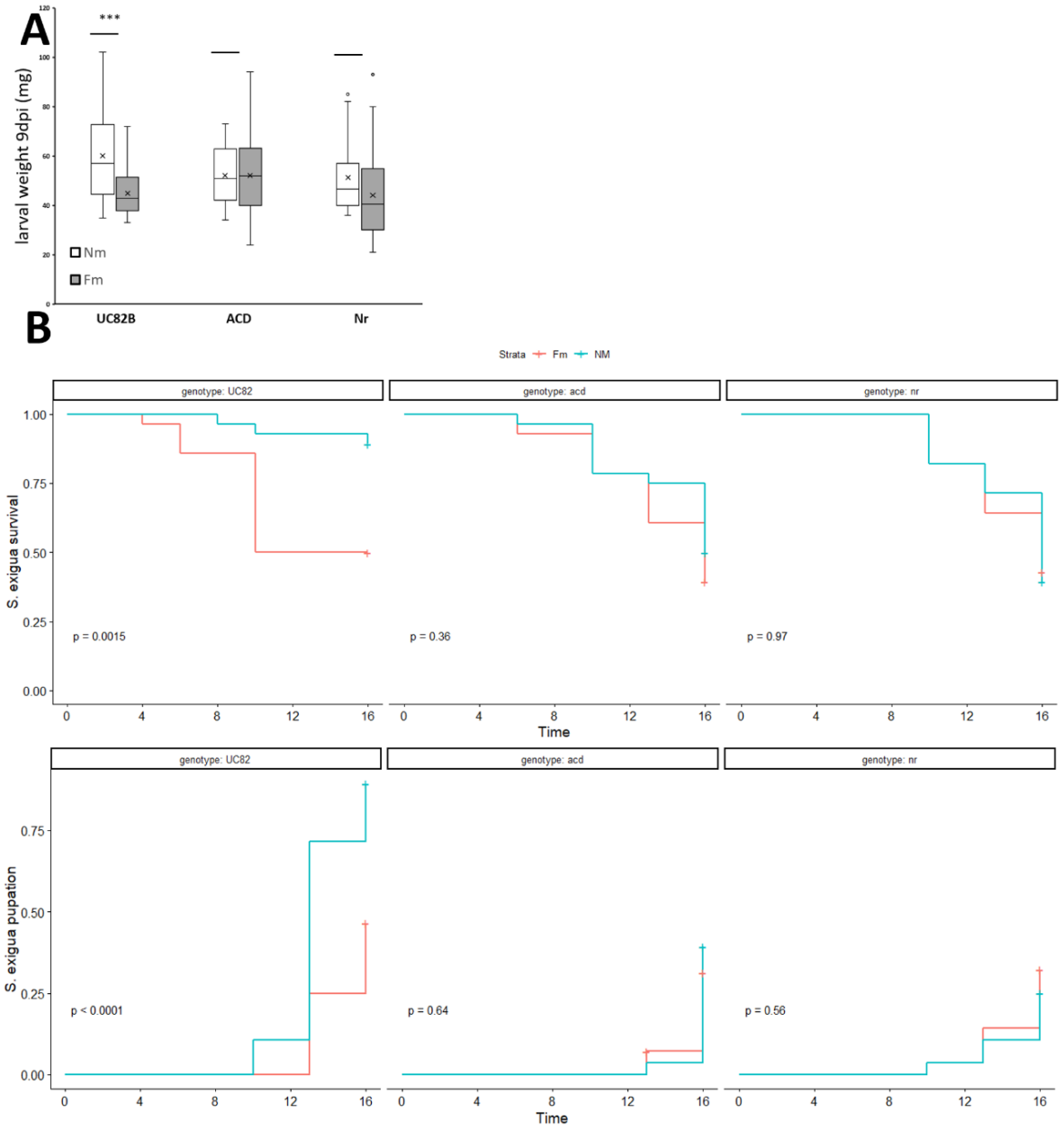


*Figure 6 ET deficient lines do not display MIR. Larval performance was monitored at (A) 9 dpi for M. sexta larvae and (B) every 2-3 days for S. exigua larvae on tomato plants of non-mycorrhizal plants (Nm) and mycorrhizal plants with F. mosseae (Fm) in a wild-type (UC82B) or ET deficient lines (ACD, Nr). We placed (A) 3 neonate M. sexta larvae and (B) 4 L2 S. exigua larvae on the plant first true leaf and let them feed inside an entomological bag of (A) 10 plants (n=30 larvae) and (B) 7 plants (n=28 larvae) per treatment. Before they had consumed the whole leaf, we moved them to the next consecutive leaf. (A) Outlier data points are defined as any value reaching past 1.5 times the interquartile range from either the lower or upper quartile. Statistical analysis was performed with (A) unpaired t-test analysis between each genotype and (B) differences between curves were estimated with a logrank (Mantel-Cox) test. *** p<0.001.*

Ethylene acts in MIR as a positive regulator of priming of JA biosynthesis

We hypothesized that the mechanism by which ET is required for mycorrhiza induced resistance is related to effects on the JA pathway. Because *M. sexta* treatment showed the greatest changes in ET metabolism compared with its non-mycorrhizal control (Fig 2, 4A and 4B) we decided to use this herbivore for follow up analysis of the molecular mechanisms underlying MIR. Using plants challenged with *M. sexta,* we addressed if the priming of defenses at the transcriptional level in mycorrhizal plants would be lost in the ET deficient lines. Leucyl aminopeptidase A (LapA), a key regulator of JA-related antiherbivory defenses (Fowler et al., 2009), showed a clear primed expression profile in mycorrhizal plants in the wildtype background, but the priming effect of mycorrhiza was lost in the ET deficient lines (Fig. 7A). The same result was found at the enzymatic activity level. LAP activity in M. sexta challenged leaves was higher in mycorrhizal plants than in non-mycorrhizal in the wild genotype, while this enhancement associated to mycorrhiza was lost in the deficient lines (Fig. 7B). Thus, we can confirm that ET is required for the primed activation of JA-dependent defenses. Finally, we aim to identify at what level is ET acting. For that, we studied the expression of genes involved in the 13-LOX pathway of JA biosynthesis (Fig. 7C). Mycorrhizal plants under *M. sexta* herbivory showed primed expression of LOXD and OPR3. AOS1 similarly showed also an increase in expression levels, although non-significant, whereas AOC expression was not altered by mycorrhiza. Remarkably, the influence of the symbiosis on gene expression was lost in the ET deficient lines. Noteworthy, in non-mycorrhizal plants, the ET deficient lines show higher expression of the JA biosynthetic genes than the UC82B wt, supporting the complex regulatory role of JA biosynthesis by ET. Finally, a targeted metabolic analysis of the leaves revealed higher levels of JA and JA isoleucine in wild-type mycorrhizal plants, confirming the primed JA accumulation in mycorrhizal plants, but this effect was also completely lost in the ET deficient lines (Fig. 7D). The results confirm that ET regulates the priming of the JA defensive pathway at the level of biosynthesis.

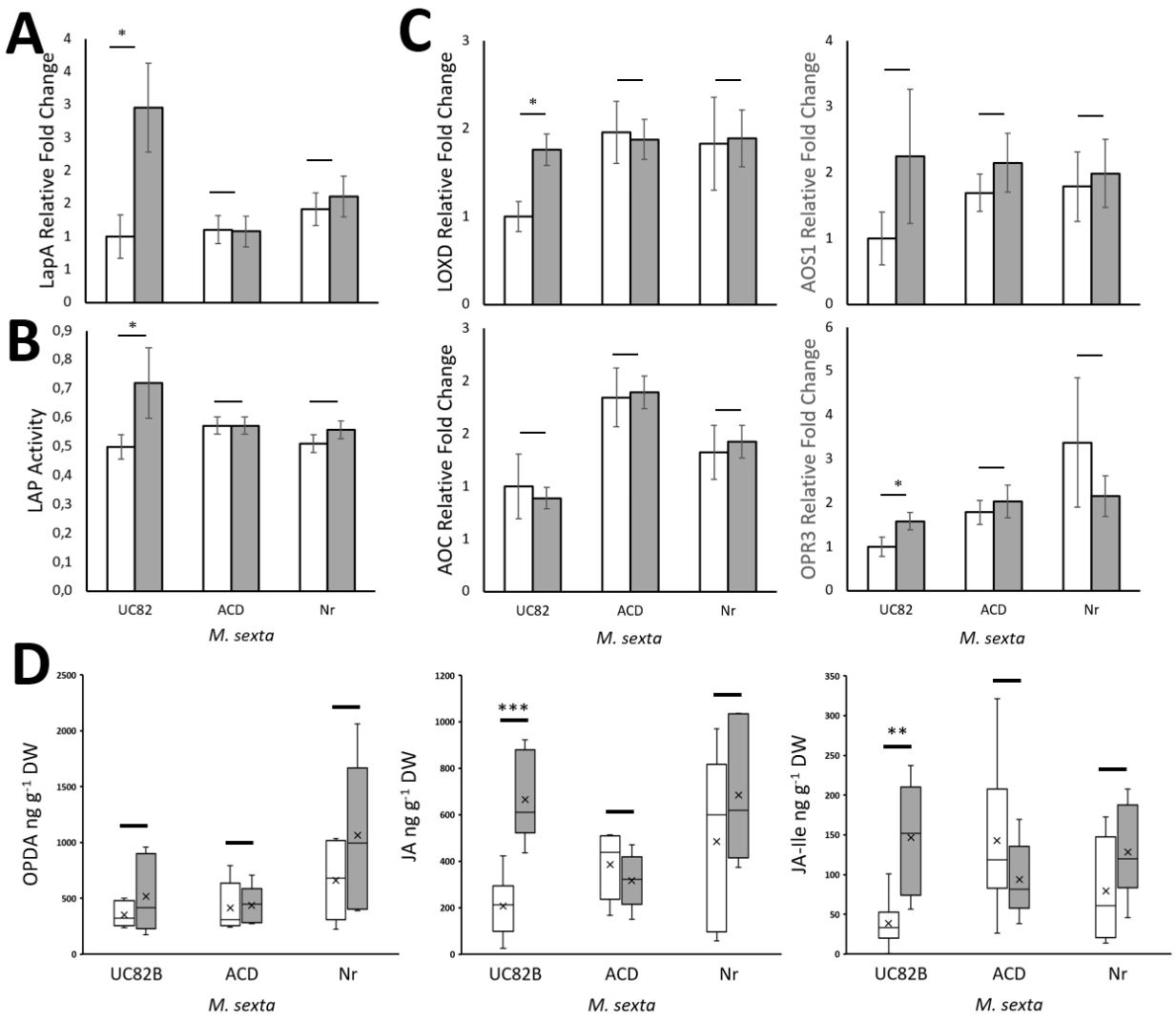


*Figure 7 ET is required for the primed JA burst in mycorrhizal tomato. Tomato plants of non-mycorrhizal plants (Nm) and mycorrhizal plants with F. mosseae (Fm) in the wild-type genotype (UC82B) or ET deficient lines (ACD, Nr) were subjected to herbivory. 3 M. sexta larvae were added per plant, and newly infested leaves were harvested 24h after infestation (10 plants per treatment, n= 30 larvae). (A) Relative expression of the JA dependent defense related marker gene leucine aminopeptidase A (LapA, Solyc12g010020.1). (B) Leucine aminopeptidase (LAP) enzymatic activity. (C) Relative expression of JA biosynthetic pathway genes: lypooxigenase D (LOXD, Solyc03g122340.2), allene oxide synthase 1 (AOS1, Solyc04g079730.1), allene oxide cyclase (AOC, Solyc02g085730.2) and 12-oxophytodienoate reductase 3 (OPR3, Solyc07g007870.2). (D) Levels of different JA metabolites (OPDA, JA and JA-Ile) in the challenged leaves. (A, B, C) Data represent mean ± SEM of 6 biological replicates. (A, C) Expression values were normalized using the reference gene SlEF, which encodes for the tomato elongation factor-1a. (D) Data are normalized to plant dry weight. Outlier data points are defined as any value reaching past 1.5 times the interquartile range from either the lower or upper quartile. Statistical analysis was performed with unpaired t-test analysis between each herbivory treatment. * p<0.05, ** p<0.01, *** p<0.001.*

DISCUSSION

Beneficial microorganisms such as AM fungi has the potential to induce a defensive primed state in the plant that enhance plant resistance to different aggressors. MIR has been demonstrated in different plant systems and mostly against necrotrophic pathogens and leaf-chewing insects (Hartley & Gange, 2008; He et al., 2017; Jung et al., 2012; Nair et al., 2015; Roger et al., 2013; Sanchez-Bel et al., 2016; Song et al., 2013). These aggressors are generally sensitive to JA regulated defense responses, so the spectrum of efficiency of MIR already pointed to the role of JA signaling in this phenomena. The use of JA deficient lines confirmed that JA is a central regulator of MIR (Mora-Romero et al., 2014; Song et al., 2013). However, the precise molecular mechanisms of defense priming in general, and in particular in mycorrhiza induced priming remain unknown. Defense priming implies a differential regulation of the plant's molecular defense mechanisms at multiple levels. Here we aimed to unravel the transcriptional reprograming in mycorrhizal plants in the absence or presence of herbivory and identify changes potentially related to defense priming and MIR. The results highlighted the key role of hormonal dependent signaling, specially ethylene and jasmonate in the differential capacity of mycorrhizal plants to respond to herbivore challenge. By combining transcriptomic, enzymatic and metabolomic analyses and herbivore bioassays with hormone deficient lines we were able to pinpoint ET signaling as a key element in MIR, as a regulatory element in the priming of JA dependent defenses.

In the absence of challenge, mycorrhizal colonization has a strong impact on root transcriptome and metabolome profiles, as already shown in tomato (López-Ráez et al., 2010; Rivero et al., 2015, 2018) but generate only minor changes in these profiles in leaves (Rivero et al., 2021). In our full transcriptome study, although few significant changes in expression of specific genes were identified, GSEA allowed us to discern that the symbiosis had a notable impact on diverse functional categories that could contribute to the primed state of the aboveground tissues in mycorrhizal plants. For example, we found that mycorrhizal symbiosis triggered changes related to transcriptional regulation, in histone and chromatin compaction as well as in the abundance of transcription factors and receptor kinases. Changes in these aspects have been proposed to underly the primed state in preconditioned plants (Conrath et al., 2015). Chromatin decompaction may facilitate gene activation and epigenetic memory of defensive responses and may contribute to ensure an adequate and timely response to different challenges. Elevated basal levels of key TF could also mediate changes in the transcriptional reprogramming associated with stress responses,

as shown for some TF mediating defense priming by beneficial microorganisms in Arabidopsis (Pescador et al., 2022; Pozo et al., 2008; van der Ent et al., 2008). Furthermore, mycorrhizal colonization induces glucosyltransferases that can conjugate nucleotide donor sugars to acceptors such as small molecules, hormones and flavonoids. These sugars confer different properties to the compounds: stability, transport, new functions and compartmentalization. This process may play a role in defense priming by allowing the accumulation of inactive defense-metabolite conjugates that upon challenge may quickly became functional Pastor et al. (2014). Another potential mechanism is the increase in levels of biotic stress receptors that would favor the perception of potential aggressors. An increase in the levels of PRRs and of protein kinases has been described also a mechanisms contributing to defense priming (Beckers et al., 2009; Tateda et al., 2014). Finally, changes in hormone metabolism and signaling may contribute, through hormonal crosstalk to the modulation of large sets of defensive genes upon challenge.

In contrast to the basal conditions, the transcriptional profile of mycorrhizal plants differed more from non-mycorrhizal plants under challenge by any of the herbivores. All these changes are consistent with the premise that defensive priming generates few basal changes in the organism, but in key regulatory aspects, and only in response to a challenge the defense response is potentiated (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017).

A more detailed analysis revealed that mycorrhizal plants display a stronger activation of JA-regulated antiherbivory responses. Both herbivores activated the JA signaling pathway, as it is the central pathway of resistance against chewing herbivores (Erb & Reymond, 2019; Wasternack & Hause, 2013). However, this induction was boosted in mycorrhizal plants, supporting the primed activation of this pathway in mycorrhizal plants. The transcriptomic analysis evidence a core of common responses of the plant to the different herbivores where the plant activates its secondary metabolism to defend itself against the aggressor while on the other hand it might reorganize its primary metabolism to better tolerate the herbivore. However, some differences in the activation of defenses and changes in primary metabolism between herbivores due to maybe their degree of specialization of the herbivore (Ali & Agrawal, 2012). Both herbivores in mycorrhizal plants strongly suppress the primary metabolism of the plant, while enhancing the plant's defensive response. The response to herbivory is still mediated by JA, although ABA and ET in mycorrhizal plants also appear to modulate the JA-dependent response. This agrees with previous data on Arabidopsis, were it was described that the ABA/JA synergy mediates an effective response to chewing insects (Verhage et

al., 2011) that is finetuned through antagonism with the ET-regulated pathway (Bodenhausen & Reymond, 2007; Verhage et al., 2011).

Our transcriptomic and metabolomic analyses demonstrate that under basal conditions mycorrhizal symbiosis induces ET synthesis, and that the increase in ET is potentiated upon herbivory. It has been extensively documented that herbivory damage induces ET emission in the plant (de Vos et al., 2005; Winz & Baldwin, 2001). ET is an important regulator of plant defenses. In fact, ET plays a complex modulatory role in the overall hormonal crosstalk shaping plant defense responses to specific challenges and determining the outcome of the interaction, through impact on secondary metabolism and defense protein synthesis (Broekgaarden et al., 2015). For example, in *Nicotiana attenuata* the antagonism exerted by ET on the JA-dependent pathway impairs nicotine synthesis against *M. sexta*-tolerant larvae (Onkokesung et al., 2010; von Dahl & Baldwin, 2007). On the other hand, ET/JA signaling is required for accumulating phenolamide levels in response to herbivory (Figon et al., 2021; Li et al., 2020; Li et al., 2018; Wang et al., 2020). Not surprisingly, attackers can modulate ET signaling in their favor. Paudel and Bede (2015) demonstrated in *Medicago truncatula*, that *S. exigua* manipulated ET signaling via elicitors in its saliva to antagonistically suppress the JA response to its advantage. Vogel et al. (2007) observed that chewing herbivores modulated the ET pathway to affect the hormonal crosstalk. Because of its release against herbivory and necrotrophic pathogens, and because it is targeted by aggressors to modulate hormonal crosstalk in their favor, we speculate that ET may have a dual role in the induction of defenses, depending on timing and doses factors, that could induce resistance or susceptibility to the aggressor.

Here we show that ET synthesis is modulated in mycorrhizal plants, and we confirmed *in planta* that differential regulation of ET production and signaling is essential for MIR against *S. exigua* and *M. sexta*. To our knowledge, this is the first study confirming the role of ET signaling in MIR. The relevance of the complex JA-ET interplay has been demonstrated in rhizobacteria-mediated ISR against necrotrophic pathogens (Pieterse et al., 2014; van Loon et al., 2006), and ET was also shown to participate in rhizobacteria- ISR against herbivores Pangesti et al. (2016). In their study they demonstrate the effect of WCS417r rhizobacterial-mediated JA/ET crosstalk in resistance against the herbivore *Mamestra brassicae* using mutants impaired in ET signaling. We show here that ET signaling is required for MIR in tomato through the regulation of the JA pathway. Using LapA as a marker of JA regulated antiherbivore defenses, gene expression and activity analyses revealed we illustrate the priming of these responses in mycorrhizal plants upon herbivory, and how this

priming is lost in ethylene-deficient mutants. Noteworthy, LapA acts as a chaperone regulating late JA and wound response (Fowler et al., 2009), so the implications of posttranscriptional modifications of altered levels of LapA remain to be explored. Furthermore, Transcriptomic and metabolomic analyses demonstrated that the ET effect was upstream to the JA dependent response, directly affecting JA biosynthesis. Our results show that the primed accumulation of JA and its bioactive conjugate JA-Ile in mycorrhizal plants is lost in ET-deficient lines. When analyzing JA, the biosynthetic pathway, we show that LOXD and OPR3, primed in mycorrhizal plants under herbivory, also lose their priming profile in the ET-deficient lines. The effect of ET on JA biosynthesis in response to wounding was modestly studied earlier. O'Donnell et al. (1996) demonstrated that inhibition of ET signaling partially inhibited JA synthesis. More recently, ORA47 an APETALA2/Ethylene-response factor (AP2/ERF) type transcription factor were shown to positively regulate the synthesis of the JA precursor OPDA by binding to the promoters of most of the JA biosynthetic genes (Chen et al., 2016; Hickman et al., 2017; Pauwels et al., 2008). In tomato, two ERFs are responsible for the quick transcriptional activation of JA synthesis by activating LOXD, AOC and OPR3 Hu et al. (2021), and in rice, the synergistic effect of the ET transcription factor EIL1 on LOX9 and JA biosynthesis was also shown (Ma et al., 2020). We have not seen significant changes in these transcription factors, probably they act on the early responses, while we have studied the late response. The biosynthesis of JA occurs rapidly after wounding and this is where we expect ET modulation to be having an effect. Taken all together we demonstrate here that ET signaling is directly involved in the priming of the JA signaling pathway induced by mycorrhizal symbiosis. The proposed regulatory model is presented in Fig. 8.

The molecular mechanisms of MIR are being addressed, and different mechanisms have been uncovered. The transcriptomic analysis points out that priming of induced defenses is likely mediated by a complex network of interconnected mechanisms in which hormone crosstalk play an essential role. Our study highlights that the AM symbiosis alters the complex plant hormonal crosstalk, and by modulating ET signaling boost JA biosynthesis and JA dependent defenses against herbivores. Understanding the mechanisms underlying MIR will pave the way to optimize the use of microbial inoculants for the induction of plant resistance, and therefore, for sustainable crop protection.

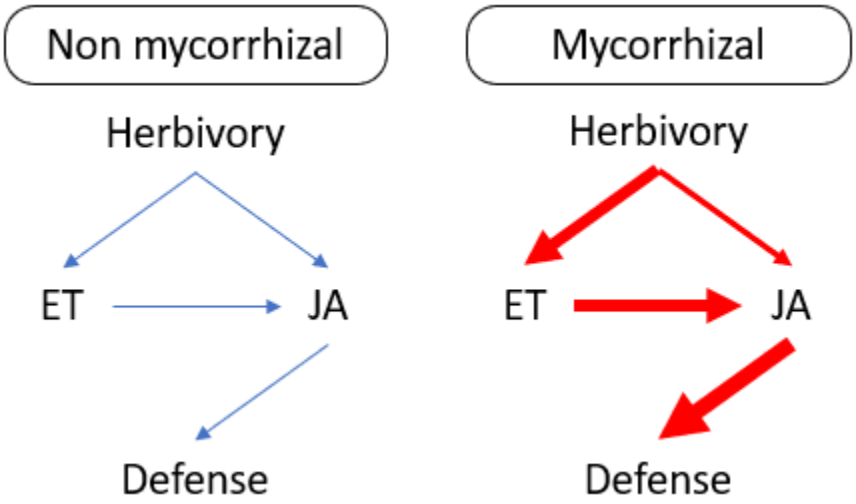


Figure 8 Proposed model for the role of ET signaling on priming of JA defense response during MIR. In non-mycorrhizal plants, herbivory induces JA-dependent defenses. This activation occurs through an induction of JA pathway biosynthesis, partially modulated by activation of the ET signaling pathway. Mycorrhizal plants exhibit minor basal changes in JA and ET metabolism. These changes potentiated upon herbivory lead to the priming of JA-regulated defensive responses to herbivory through a positive role of ET signaling on the herbivore induced JA burst.

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SUPPLEMENTAL MATERIAL

Figure S1. *M. sexta* has a lower impact on the primary metabolism compared with *S. exigua*. Tomato leaves from mycorrhizal (Fm) and non-mycorrhizal (Nm) plants were subjected to 24 h of *S. exigua* (Se and FmSe) or to *M. sexta* (Ms and FmMs) herbivory. Heatmap of *S. exigua* treatment changes on enriched gene sets compared with *M. sexta* treatment from organized GSEA results. All treatments had three biological replicates each with a pool of two plants. Data shown represent GSEA enriched gene sets with an FDR<0.05. Blue cells represent repression of the gene set.

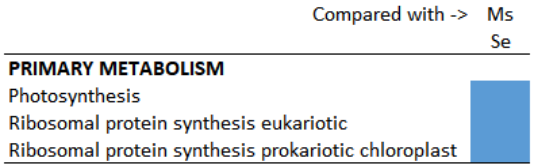

Figure S2. Mycorrhizal colonization deepens the primary metabolism repression upon herbivory and upon *M. sexta* boosts the secondary metabolism. Tomato leaves from mycorrhizal (Fm) and non-mycorrhizal (Nm) plants were subjected to 24 h of *S. exigua* (Se and FmSe) or to *M. sexta* (Ms and FmMs) herbivory. Heatmap of mycorrhizal herbivory treatment changes on enriched gene sets compared with their non-mycorrhizal herbivory from organized GSEA results. All treatments had three biological replicates each with a pool of two plants. Data shown represent GSEA enriched gene sets with an FDR<0.05. Blue cells represent repression of the gene set, red represents induction.

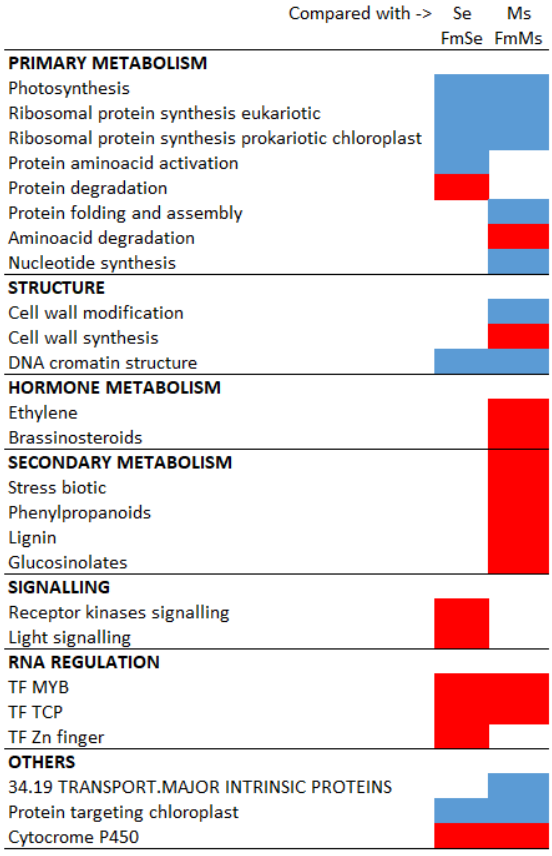

Figure S3. ET-deficient lines showed no differences in (A) AM colonization and (B) AM colonization doesn't change plant biomass after 8 weeks post inoculation. Tomato plants of non-mycorrhizal plants (Nm) and mycorrhizal plants inoculated with *F. mosseae* (Fm) in a wild-type (UC82B) or ET deficient lines (ACD, Nr). Mycorrhizal plants were inoculated with 10 % (v/v) of raw *F. mosseae* inoculum. Data shown as mean ± SEM of (A) 6 or (B) 10 biological replicates. Statistical analysis was performed with unpaired t-test analysis with each control treatment (A) UC82B wt and (B) non-mycorrhizal (Nm).

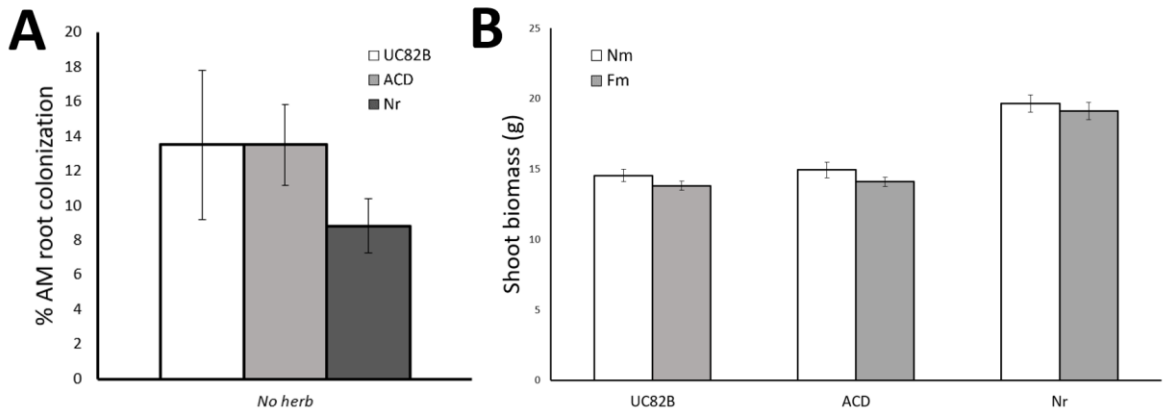

Figure S4. Mycorrhiza primed ET biosynthesis upon *M. sexta* herbivory is lost in ET deficient lines. Single tomato leaflets of non-mycorrhizal plants (Nm) and mycorrhizal plants inoculated with *F. mosseae* (Fm) in a wild-type (UC82B) or ET deficient lines (ACD, Nr) were incubated with *M. sexta* herbivory or without herbivory (No herb) for 3 h inside 20 mL glass vials. 1 mL of every sample was withdrawn from the vial and the area of the ethylene peak was analyzed in by gas chromatography. Boxplots of 5 biological replicates. Outlier data points are defined as any value reaching past 1.5 times the interquartile range from either the lower or upper quartile. Statistical analysis was performed with unpaired t-test analysis between each herbivory treatment. * $p<0.05$.

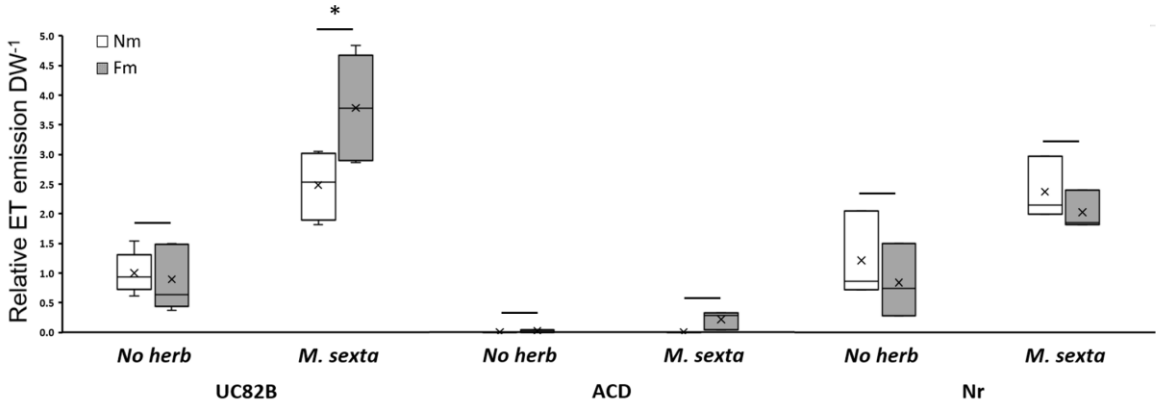

Figure S5. No effect on *M. sexta* mortality was observed among the genotypes. Larval performance was monitored at every 2-3 days on tomato plants of non-mycorrhizal plants (Nm) and mycorrhizal plants inoculated with *F. mosseae* (Fm) in a wild-type (UC82B) or ET deficient lines (ACD, Nr). We placed 3 neonate *M. sexta* larvae on the plant's first true leaf and let them feed inside an entomological bag of 10 plants (n=30 larvae) per treatment. Before they had consumed the whole leaf, we moved them to the next consecutive leaf. Differences between curves were estimated with a logrank (Mantel-Cox) test.

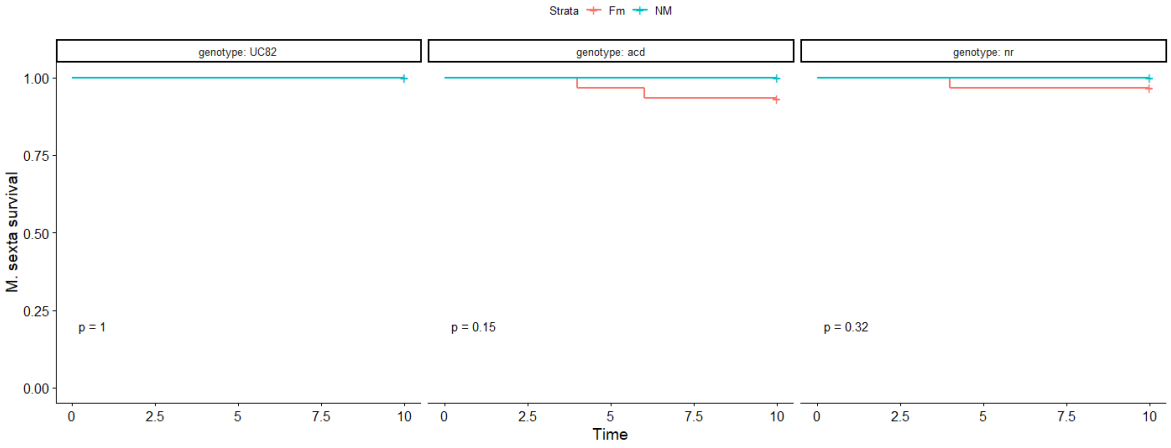

Table S1. GSEA Manually organized functional supergroups from enriched gene sets. Tomato leaves of non-mycorrhizal plants (Nm) and mycorrhizal plants inoculated with *F. mosseae* (Fm) were subjected to 24h of herbivory by the generalist insect *S. exigua* (Se and FmSe) and the specialist insect *M. sexta* (Ms and FmMs).

Functional supergroups	Enriched Gene Sets
Photosynthesis	1; 1.1; 1.1.1; 1.1.1.1; 1.1.1.2; 1.1.2; 1.1.2.2; 1.2; 1.3
Cell wall synthesis	10.1; 10.2; 10.2.1
Cell wall degradation	10.6.1
Cell wall modification	10.7
Lipid degradation	11.9; 11.9.2; 11.9.2.1
AA synthesis	13.1.1; 13.2; 13.2.3
Metal handling	15; 15.2
Secondary metabolism	16
Isoprenoids	16.1.2
Phenylpropanoids	16.2
Lignin	16.2.1
Glucosinolates	16.5.1; 16.5.1.1; 16.5.1.1.1
Hormone metabolism	17
Abscisic acid metabolism	17.1
Brassinosteroids metabolism	17.3.1
Ethylene metabolism	17.5.1
Ethylene metabolism	17.5.2

Jasmonate metabolism	17.7; 17.7.1
Tetrapyrrole synthesis	19
PR proteins	20.1.7
Stress biotic	20.1; 20.1.2; 20.1.2.1; 20.1.2.2
Stress abiotic heat	20.2.1
REDOX	21.4
Nucleotide synthesis	23.1
Glucosyl and glucoronyl transferases	26.2
P450	26.10
Misc. Protease Inhib./Seed storage/LTP proteins	26.21
GDSL lipase	26.28
TF MYB	27.3.25
TF NAC	27.3.27
TF TCP	27.3.29
TP AP2/EREBP	27.3.3
TF WRKY	27.3.32
TF AUX/IAA	27.3.40
TF Zn finger	27.3.7
TF Zn finger	27.3.8
DNA cromatin structure	28.1; 28.1.3; 28.1.3.2
DNA repair	28.2
Protein AA activation	29.1
Ribosomal protein synthesis prokariotic chloroplast	29.2.1.1; 29.2.1.1.1; 29.2.1.1.1.2
Ribosomal protein synthesis eukariotic	29.2.1.2; 29.2.1.2.1; 29.2.1.2.2
Protein targeting chloroplast	29.3.3
Protein secretion	29.3.4
Protein degradation	29.5.1; 29.5.3; 29.5.9
Protein folding and assembly	29.6; 29.8
Signalling in sugar and nutrients	30.1
Receptor kinases signalling	30.2; 30.2.3
Light signalling	30.11
Calcium signalling	30.3
Cell cycle	31.3
LEA proteins	33.2
Transport. Major intrinsic proteins	34.19
Not assigned TPRs	35.1.27
PPR protein	35.1.5

Table S2. Primers qPCR

Primer	Sequence	Solyc	Reference
SlEF-1-F	GATTGGTGGTATTGGAACTGTC	Solyc06g009960	Rotenberg et al., 2006
SlEF-1-R	AGCTTCGTGGTGCATCTC		
LOXD-F	GACTGGTCCAAGTTCACGATCC	Solyc03g122340	Uppalapati et al., 2005
LOXD-R	ATGTGCTGCCAATATAAATGGTTCC		
AOS1-F	CACCTGTTAAACAAGCGAAAC	Solyc04g079730	López-Ráez et al., 2010
AOS1-R	GACCTGGTGGCATGTTCGT		
AOC-F	GCACGAAGAAGAGAAGAAAGGAGAT	Solyc02g085730	Uppalapati et al., 2005
AOC-R	CGGTGACGGCTAGGTAAGTTTC		
OPR3-F	TTGGCTTAGCAGTTGTTGAAAG	Solyc07g007870	Uppalapati et al., 2005
OPR3-R	TACGTATCGTGGCTGTGTTACA		
PPOF-F	CGGAGTTTGCAGGGAGTTATAC	Solyc08g074620	Alba et al., 2015
PPOF-R	TTGATCTCCACACTTTCAATGG		
TD-F	AGCTCAAACACACGCGCTGGA	Solyc09g008670	Yan et al., 2013
TD-R	AACCCCCACCACCAACAGGT		
MC-F	GAGAATTTCAAGGAAGTTCAA	Solyc00g071180	Uppalapati et al., 2005
MC-R	GGCTTTATTTCACACAGAGATA		
LapA-F	ATCTCAGGTTTCCTGGTGGAAGGA	Solyc12g010020	Yan et al., 2013
LapA-R	AGTTGCTATGGCAGAGGCAGAG		
ACS6-F	GGGTTTCCTGGATTTAGGGT	Solyc08g008100	Ibort, 2017
ACS6-R	GGTACTCAGTGAAATAGTCGA		
ERF-F	GAGATCCTCTGGAGTCGAAAT	Solyc02g070040	Wang et al., 2020
ERF-R	ACTTGACTCTTCTTGCTGTAAT		
ACO1-F	AAGGGACTCCGCGCTCATA	Solyc07g049530	Chersicola et al., 2017
ACO1-R	CAAGTTGGTCACCAAGGTTAACC		
ACO4-like-F	CCCAGTTTCTTCATCCACTCA	Solyc04g007980	Satková et al., 2017
ACO4-like-R	AGAAAAGTCGACGACGGGTAT		

Table S3. RNA-seq transcriptional DEGs overview. Tomato leaves of non-mycorrhizal plants (Nm) and mycorrhizal plants inoculated with *F. mosseae* (Fm) were subjected to 24h of herbivory by the generalist insect *S. exigua* (Se and FmSe) and the specialist insect *M. sexta* (Ms and FmMs). Data shown represent DEGs with an FDR<0.05. Differential expression analysis was performed in R using the DESeq2 package.

	Down	Up
Fm vs Nm	25	32
Se vs Nm	2832	3340
Ms vs Nm	1978	2556
FmSe vs Nm	2733	2921
FmMs vs Nm	3292	3598
Se vs Ms	0	0
FmSe vs Fm	1801	2065
FmMs vs Fm	2243	2647
FmSe vs Se	0	0
FmMs vs Ms	12	22
FmSe vs FmMs	2	0

Table S4. Complete DEG table not available on this PhD Thesis

Discussion

Discussion

The use of bioinoculants in agriculture is gradually being implemented by farmers for a more sustainable future. They can provide multiple benefits, from enhanced uptake of nutrients and water to improved resistance/tolerance against biotic or abiotic stresses. However, bioinoculants show a high functional variability compared to synthetic fertilizers and pesticides due to their high context dependency. This PhD thesis focuses on the study of the impact of the context on the signaling pathways that regulate mycorrhizal symbioses. This is addressed in a holistic way, from the pre-symbiotic plant-AM fungus communication during the establishment of the symbiosis and the control of the extension of fungal colonization, to the impact of the symbiosis on the plant defense mechanisms at systemic level, which lead to an increased resistance against herbivorous insects.

Plants are sessile organisms and, therefore, highly dependent on the environmental context. As a consequence, they must reorganize their metabolism to adapt themselves to the ever-changing conditions. To do so, plants rely mainly on the hormonal balance that allows them to integrate environmental signals into physiological changes in an efficient and coordinated manner (Sparks et al., 2013). Phytohormones are metabolites that regulate a multitude of physiological processes such as growth, development, senescence, reproduction and defense responses. They are also involved in the plant's interaction with beneficial organisms such as AM fungi (Pozo et al., 2015). Indeed, all phytohormones studied to date are involved somehow in the recruitment, establishment or development of mycorrhizal symbiosis (Bedini et al., 2018; Ho-Plágaro & García-Garrido, 2022; Pozo et al., 2015). The establishment of a functional AM symbiosis includes two distinct phases: the pre-symbiotic phase and the symbiotic phase, and both phases are highly influenced by the environmental conditions.

Chapters 1 and 2 deal with the study of the signals involved in the regulation of the pre-symbiotic plant-AM fungus communication in the rhizosphere. This phase is strongly influenced by environmental conditions, especially nutritional deficiency, and involves a selective attraction of the AM fungus to the plant in response to a need as a "*cry for help"*. The most studied environmental condition for its impact on AM symbiosis is phosphorus (Pi) availability. Under Pi

deficiency, there is a promotion of rhizosphere signaling and root transcriptional reprogramming to promote the interaction with AM fungi, as the symbiosis can facilitate Pi acquisition. Remarkably, this effect is highly repressed under high Pi conditions (Balzergue et al., 2011, 2013). During this process, there is an induced biosynthesis and exudation of signaling compounds into the rhizosphere. Among these compounds, strigolactones (SLs) are known to play a key role, by inducing AM fungal spore germination, activating fungal metabolism and promoting hyphal branching of germinating spores to facilitate the contact with the plant root (Besserer et al., 2006; López-Ráez et al., 2017; Waters et al., 2017). SLs are also phytohormones playing a central role in regulating metabolic responses associated with Pi starvation responses (PSR) within the plant (Gamir et al., 2020; Waters et al., 2017). In addition to Pi, nitrogen (N) deficiency has been reported to have a stimulatory effect on SLs biosynthesis, and thus in AM symbiosis establishment, in pea, sorghum and lettuce, although this deficiency seems to be less critical that Pi starvation (Foo & Reid, 2013; Yoneyama, Xie, et al., 2007; Yoneyama et al., 2012).

In **Chapter 1,** the effect of Pi and N nutritional deficiencies on the impact on SLs biosynthesis and exudation was studied. For this purpose, plants were grown under different N and P regimes. By targeted metabolomics, we demonstrated that SL production and exudation occurred mainly under P deficiency. These results are in agreement with those previously reported in different plant species (López-Ráez et al., 2008; Peláez-Vico et al., 2016; Yoneyama et al., 2012; Yoneyama, Yoneyama, et al., 2007). Interestingly, we showed here that N starvation was able to abolish the induction observed under P limitation when plants were subjected to both deficiencies. The results show that plants prioritize responses to N over P deficiency by affecting SL biosynthesis. Calabrese et al. (2017) showed that N deficiency favored root transcriptional reprogramming to promote AM fungal development. Moreover, the usual repression of symbiosis under high P conditions is reduced by N deficiency (Javot et al., 2011; Nouri et al., 2014). These results suggest that there must be other plant-derived signaling molecules involved in the pre-symbiotic communication, besides SLs, as shown later in Chapter 2. We also showed that the levels of endogenous SLs correlated with the expression of PHO2 and NIGT1/HHO, key regulators involved in the integration of the two signaling pathways for Pi and N starvation responses (Kiba et al., 2018; Maeda et al., 2018; Medici et al., 2015). By using the SL-deficient line *SlCCD8*-RNAi L09 and exogenous application of the synthetic SL analog 2'-*epi*-GR24 SLs, we demonstrated for the first time that SLs are involved in the complex N-P signaling interplay. Based on this study, we were able to demonstrate a novel regulatory function of SLs in early plant responses to both N and Pi deficiencies that might indirectly affect the mycorrhizal symbiosis.

In **Chapter 2**, the role of new potential pre-symbiotic signals in AM symbiosis was assessed. In addition to SLs, other plant-derived compounds, such as flavonoids, have been proposed to participate in the pre-symbiotic molecular dialogue in AM symbiosis, although their specific roles remain unclear (reviewed in Hassan & Mathesius, 2012). Here, the specific role of flavonoids as signaling compounds in AM symbiosis was studied. We used the AM fungus *R. irregularis* since it is the model AM fungus, commercially available as an spore-based inoculant produced *in vitro*. We checked the ability of different flavonoids representing different groups to stimulate the spore germination and AM symbiosis establishment by using *in vitro* and *in planta* assays and different doses within a physiological range. We found out that the flavone chrysin and the flavonols quercetin and rutin were able to promote spore germination and AM symbiosis establishment at very low concentrations. The results are in agreement with previous reports on the role of these flavonoids in AM symbiosis (Scervino et al., 2007; Steinkellner et al., 2007), and we proposed their signaling role during the pre-symbiotic stage, together with SLs. A role for the flavonol quercetin in lateral root formation has been proposed (Chapman & Muday, 2021; Maloney et al., 2014), as previously proposed for SLs (Ruyter-Spira et al., 2011). Interestingly, lateral roots are the preferred places for AM symbiosis establishment. Therefore, both SLs and flavonoids seem to play a double role in AM symbiosis as signaling molecules for the fungus and plant regulators of root architecture. Based on these observations, it would be interesting to explore whether the production and exudation of flavonols are promoted by Pi and/or N starvation, which are the optimal conditions for mycorrhizal establishment. Our results open the possibility of using these compounds in the formulation of mycorrhizal inoculants as promoters of the symbiosis, thus improving the efficiency of commercial inocula.

In addition to the '*cry for help'* for AM fungi under Pi deficiency, the plant regulates the development of the symbiosis and the extent of root colonization depending on the environmental context (Pozo et al., 2015). In **Chapter 3**, we evaluated the mechanisms of the regulation of the AM symbiosis under different stress-related conditions. We used two of the most widespread and studied AM fungi, *R. irregularis* and *F. mosseae*, separated or in combination as a double inoculation with both AM fungi. Our starting hypothesis was that the plant can regulate colonization levels depending on its growing conditions or needs, and depending on the AMF. This regulation could be a consequence of the modulation of the pre-symbiotic signaling, as previously described (Chapters 1 and 2), or due to the regulation of the plant defense responses, the exchange of nutrients between the two partners, and the control and autoregulation of the symbiosis. In the absence of stress, the greatest colonization rates were achieved by R. *irregularis*, nearly twice that of *F. mosseae*, showing that it is a better

colonizer, as previously reported in tomato and other plants as rice (Liu et al., 2022; López-Ráez et al., 2010). It has been proposed that these differences in colonization between AM fungi could be due to a different attenuation/modulation of plant defense responses (Fernández et al., 2014). By activating different stress related signaling pathways, we observed that colonization levels of *R. irregularis* and *F. mosseae* varied depending on the type of the stress applied: stresses usually promoting colonization by *F. mosseae*, while repressing colonization by *R. irregularis*. However, mycorrhizal levels remained within a defined range in all cases, suggesting that there is a maximum, probably associated to an optimal nutritional tradeoff, which is controlled by the host plant through an autoregulation process. Indeed, our results showed that changes in colonization levels were explained by changes in the transcriptional regulation of defensive responses (mostly regulated by SA, JA and ET signaling). Remarkably, differences in colonization were also related to changes in genes associated with nutrient exchange (Pi, carbohydrates and lipids) between the partners, and in the control and autoregulation of the symbiosis. The expression of the genes LePT4, FatM, SUS3, P14c, GluB and PAL showed the most significant correlation with the mycorrhizal colonization data. Focusing on local stress in the roots, salinity negatively impacted plant performance, with a reduction in shoot biomass and in Pi content. Under these conditions, differences in the functionality of the symbiosis with *R. irregularis* and *F. mosseae* was observed. Colonization by *F. mosseae* was promoted by salt stress, and the symbiosis buffered the loss of Pi content by salt stress. Conversely, mycorrhization by *R. irregularis* was reduced under salt stress conditions and did not show any effect attenuating the negative effects on Pi levels. In this sense, it was shown that one of the major benefits of AM symbiosis against salt stress was a reduced loss of Pi uptake (Porcel et al., 2012). In the case of *R. irregularis*, the reduced colonization under salt stress correlated with a plant restriction of lipid supply to the fungus, an increase of defense responses and a greater control of the symbiosis. In contrast, the lipid supply was maintained *and defenses and symbiosis control attenuated* in the case of *F. mosseae* under salt stress. These results support the Kiers' free market hypothesis, where greater benefits provided by the AM fungus is rewarded with a higher carbon input by the host plant (Helber et al., 2011; Kiers et al., 2011; Werner & Kiers, 2015). Interestingly, the combined inoculation with the two AM fungi resulted in a synergistic effect achieving a higher protection of the plant from salinity.

It should be noted that plants in natural contexts are not isolated, or engaged in individual interactions, rather they interact with multiple (micro)organisms simultaneously or subsequently. These simultaneous interactions can impact in how the plant responds to other organisms (Pozo et al., 2020). A plethora of beneficial organisms are able to improve the plant

defensive capacity and induce systemic resistance (ISR) against potential aggressors (Gruden et al., 2020; Jung et al., 2012; Pieterse et al., 2014). Only few studies are available on the mechanisms involved in 3-way interactions between microbes, including AMF, plants and insects. In order to get some insights about these mechanisms, in **Chapter 4** we performed a literature meta-analysis comparing the molecular mechanisms governing plant simultaneous interactions with microbes and arthropods. The goal was to find out common patterns that could help us elucidate the influence of mycorrhiza on plant interaction with pests. Surprisingly, very little information was available in literature, suggesting that this is a relatively unexplored and emerging research field. Beneficial microbes were most frequently studied in these interactions, being AM symbiosis the most studied. Regarding the arthropods, the order Lepidoptera, which includes the species *S. exigua* and *M. sexta* used in Chapter 5, was ranked first. A database including the mechanisms activated in plants during 2-way interactions (plant-microbe or plant-arthropod) and those activated in the 3-way interactions between the three organisms was created. The biases regarding the model systems studied, the techniques used and the timing were spotted, and a series of guidelines for experimental designs were provided to maximize the information to be obtained in future research projects. The results evidenced that the plant response to 3-way interactions was much more complex than in the 2-way interactions and could not be directly predicted from 2-way interactions. This is consistent with the priming phenomena, as previous stimuli pre-condition the tissues for a more efficient activation of defenses against a second interactor (Conrath et al., 2006, 2015; Martinez-Medina et al., 2016; Mauch-Mani et al., 2017). Despite the great complexity in the regulation of 3-way interactions, our analysis highlights hormone crosstalk as the main regulatory core. More specifically, the JA signaling pathway -and to a lesser extent the ET and SA pathways-, are the most common molecular responses reported in the 3-way interactions.

Among the beneficial microbes with impact on pests, the mycorrhizal symbiosis is known to generally enhance plant resistance to certain pests and pathogens (Pozo et al., 2020). It has been shown that the molecular reprogramming that occurs during symbiosis establishment primes plant tissues for a more efficient activation of defenses against below and aboveground attackers, a phenomenon known as Mycorrhiza Induced Resistance (MIR) (Jung et al., 2012; Pozo & Azcón-Aguilar, 2007). MIR is mainly active against chewing insects and necrotrophic pathogens, and experimental evidences support that the protection is achieved through priming of JA.regulated of defense responses (Mora-Romero et al., 2014; Pozo & Azcón-Aguilar, 2007; Rivero et al., 2021; Sánchez-Bel et al., 2018; Song et al., 2013). However, despite

its relevance in natural systems and potential applications, the molecular mechanisms underlying priming and MIR are mostly unknown.

While mycorrhizal colonization has a strong local impact on root transcriptome and metabolome (López-Ráez et al., 2010; Rivero et al., 2015, 2018; Chapter 3) transcriptional and metabolic changes in leaves related to mycorrhiza are subtle in the absence of stress (Rivero et al., 2021, **Chapter 5)**. In fact, the full transcriptional profiling shown in **Chapter 5** revealed less than 100 genes differentially regulated between non-mycorrhizal and mycorrhizal plants in the absence of herbivory. Similarly, other RNA-seq analyses showed that transcriptome reprogramming affects a small group of genes with similar, and often low fold-change value in other plants (Baccelli et al., 2020). These subtle changes in leaves under basal conditions are able to lead to primed defense responses underlying MIR. In fact, although no major changes at the level of specific genes was observed in mycorrhizal plants, several key processes that could be mediating the defensive priming associated with MIR are slightly but consistently modulated in mycorrhizal plants. These include changes in transcriptomic regulation, biotic stress perception and modulation of secondary metabolism including changes in phytohormone metabolism.

In **Chapter 5**, we studied the impact of mycorrhizal symbiosis on the plant interaction with chewing herbivores, and analyzed the molecular mechanisms driving these 3-way interactions. *F. mosseae* was selected because comparative experiments conducted in our group showed that it showed greater benefits against biotic and abiotic stresses than *R. irregularis* in tomato plants (Jung et al., 2012; Pozo et al., 2002). These benefits could be derived by the greater impact on the plant root hormonal balance and metabolic profile of *F. mosseae* (Fernández et al., 2014; Rivero et al., 2015, 2018).

To explore the molecular mechanisms of MIR against chewing insects, we analyzed the transcriptional response of mycorrhizal plants to herbivory by two Lepidoptera, the generalist *S. exigu*a and the specialist *M. sexta*. Pests have been classified into two categories, specialist insects (which feed on a single species/plant family and they are adapted to their particular defense metabolites) and generalist insects (which are capable of feeding on a wide variety of species and families) (Ali & Agrawal, 2012). The general hypothesis is that generalist insects will trigger more the induction of plant defenses, while specialist insects would have developed strategies to minimize the activation of plant's defenses and overcome them, and may have developed a greater tolerance to the plant's defenses. In our system, the specialist herbivore avoided the repression of primary metabolism in non-mycorrhizal plants, thus ensuring a higher

nutrient flux. Surprisingly, in mycorrhizal plants the plant response became homogeneous between the two herbivores. As shown in **Chapter 4**, the hormonal pathway governing the interactions with beneficial microbes and herbivores was the JA pathway. Other hormones such as ABA and ET play a modulatory role in adjusting the JA-dependent defensive response. Strikingly, changes in the intensity of the response were also observed. Mycorrhizal plants primed JA-dependent defenses. Regarding ET, it was confirmed that mycorrhizal plants differentially modulated ET levels and that these in response to herbivory showed enhanced induction. ET is an important modulator of plant defenses, especially modulating JA-dependent defenses. The synergistic effect of ET with JA in defensive responses against necrotrophic pathogens has been mostly reported in arabidopsis, being antagonistic to the JA/ABA pathway that coordinates responses to chewing herbivores. However, it also plays a role in modulating responses to herbivory (Figon et al., 2021; D. Li et al., 2020; J. Li et al., 2018; Onkokesung et al., 2010; von Dahl & Baldwin, 2007; Wang et al., 2020). Thus, it appears that ET may have a dual role in the induction of defenses, depending on temporal and intensity factors, that could induce resistance or susceptibility to the aggressor. The enrichment of differentially regulated genes in the category related to ET prompted us to test their role in MIR. To our knowledge there is no study relating ET to MIR against herbivores. Only Pangesti et al. (2016) demonstrated the importance of ET in ISR against herbivores. In their study, they showed the effect of WCS417r rhizobacterial-mediated JA/ET crosstalk in resistance against the herbivore *Mamestra brassicae* using mutants impaired in ET signaling. Besides that work, ET had been described as essential for ISR, but only against pathogens (Pieterse et al., 2014; van Loon et al., 2006).

To explore the role of ET in the differential regulation of MIR-associated defenses, an analysis using tomato lines deficient in ET biosynthesis and perception was performed. These lines, unlike the wild-type genotype, were unable to develop MIR against either of the two herbivores tested (*S. exigua* and *M. sexta*). Based on these results, JA-dependent defensive markers, including gene expression and enzyme activities against herbivory were analyzed. The priming profile of LapA was lost in ET-deficient lines. Finally, from expression analysis of the JA biosynthesis pathway and targeted metabolomics, the effect of ET on MIR was linked to JA-dependent defensive priming by directly affecting the JA biosynthesis. The mechanisms involved in this process are still unknown. In this chapter, we confirm that ET signaling is an essential element in the complex hormonal regulation underlying MIR against insect herbivores.

In summary, this Doctoral Thesis presents an holistic analysis of the molecular complexity in the regulation of the AM symbiosis and its benefits for the plant. This regulation affects all the processes of the AM plant-fungus interaction, as well as in the interaction of these

with the ever-changing environment. The promotion of the recruitment of AM fungi in conditions of nutritional deficiency, the subsequent control of the establishment and maintenance of the symbiosis, according to the growth conditions of the plant, have been studied. Finally, we have proven that this regulation is associated with hormonal changes that modulate the defensive responses of the plant, making it more resistant to the attack by herbivorous insects, that is, increasing plant resilience.

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Conclusiones

Conclusions

Conclusiones

1. Las plantas de tomate priorizan las respuestas a las deficiencias de nitrógeno sobre las de fósforo a nivel fisiológico, transcriptómico y metabólico.

2. Las estrigolactonas actúan como moduladores durante las respuestas tempranas de la planta a las deficiencias de nitrógeno y fósforo, y median en la interacción de la señalización nitrógeno-fósforo, modulando la expresión de las vías de señalización de ambos nutrientes.

3. La aplicación exógena de flavonoides y estrigolactonas promueve la germinación de esporas y el establecimiento de la simbiosis del hongo micorrícico *R. irregularis* en función de la dosis aplicada. Entre los flavonoides probados, la flavona crisina y los flavonoles quercetina y rutina mostraron los mayores efectos estimuladores.

4. Bajo condiciones de estrés, la planta huésped regula los niveles de micorrización dependiendo del genotipo del hongo y del contexto ambiental, equilibrando la extensión de la simbiosis en beneficio mutuo a partir de la regulación del intercambio de nutrientes y de la respuesta defensiva y de autoregulación.

5. Considerando las interacciones planta-microbio-artrópodo, la mayoría de los módulos de señalización que regulan las interacciones a dos vías también operan en las interacciones a tres vías. En cambio, la complejidad de la respuesta de la planta aumenta, con cambios en la intensidad, el tiempo y/o la aparición de respuestas adicionales.

6. Las rutas de señalización hormonales y su interacción son el principal núcleo regulador en las interacciones a dos y tres vías. En particular, la ruta de señalización dependiente de jasmonato desempeña un papel central en la coordinación de las respuestas de las plantas.

7. La colonización por el hongo micorrícico *F. mosseae* mejora la resistencia del tomate frente a los herbívoros masticadores *Spodoptera exigua* y *Manduca sexta* mediante el *priming* de las defensas de la planta, incluyendo una mayor activación de las respuestas de defensa reguladas por jasmonatos.

8. Las plantas micorrizadas por *F. mosseae* mostraron una regulación diferencial de la vía del etileno, tanto en condiciones basales como de herbivoría. La señalización del etileno es esencial para el *priming* de la biosíntesis de jasmonato asociado a la micorriza tras la herbivoría y el consiguiente aumento de la resistencia.

Conclusions

1. Tomato plants prioritize responses to deficiencies in nitrogen over those in phosphorus at the physiological, transcriptomic and metabolic levels.

2. Strigolactones act as modulators during early plant responses to both nitrogen and phosphorus deficiencies and mediate the nitrogen-phosphorus signaling interplay by modulating the expression of phosphate and nitrate signalling pathways.

3. The exogenous application of flavonoids and strigolactones to the mycorrhizal fungus *R. irregularis* promotes spore germination and symbiosis establishment in a dose-dependent manner. Among the flavonoids tested, the flavone chrysin and the flavonols quercetin and rutin showed the greater stimulation effects.

4. Under stress conditions, the host plant regulates mycorrhizal levels depending on the fungal genotype and the environmental context, balancing the colonization extent for mutual benefit through the regulation of nutrient exchange, plant defenses and autoregulation responses

5. Considering plant-microbe-artropod interactions, most signaling modules regulating 2-way interactions also operate in 3-way interactions. However, the complexity of the plant response increases, with changes in intensity, timing and/or the occurrence of additional responses.

6. Hormone signaling pathways and their crosstalk are the main regulatory core in 2- and 3-way interactions. In particular, the jasmonate signaling pathway plays a central role in the coordination of plant responses.

7. Mycorrhizal colonization by *F. mosseae* enhances tomato resistance against the chewing herbivores *Spodoptera exigua* and *Manduca sexta* by priming plant defenses, including an increased activation of jasmonate-regulated defense responses.

8. Plants inoculated with *F. mosseae* showed differential regulation of the ethylene pathway under both basal and herbivory conditions. Ethylene signaling is essential for the mycorrhiza-associated priming of jasmonate biosynthesis upon herbivory and the consequent increased resistance.