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Metabolic reprogramming in arbuscular mycorrhiza symbiosis for enhanced resistance and tolerance against stresses

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TESIS DOCTORAL

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Programa de Doctorado en Biología Fundamental y de Sistemas

Reprogramación metabólica en simbiosis de micorrizas arbusculares para mejorar la resistencia y la tolerancia frente a estreses

Javier Rivero Bravo

Granada, 2019

Universidad de Granada

Facultad de Ciencias

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Memory presented to aspire to Doctor in Biology (with mention "International Doctor").

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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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El doctorando/*The doctoral cadidate* Javier Rivero Bravo y los directores de la tesis/*and the thesis supervisors* María José Pozo Jiménez y Víctor Flors Herrero

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de tesis y hasta donde nuestro conocimiento alcanza, en la relación del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Guarantee, by signing this doctoral thesis, that the work has been done by the doctoral candidate under the direction of the thesis supervisors and, as far as our knowledge reaches, in the performance of the work, the rights of other authors to be cited (when their results or publications have been used) have been respected.

Granada, 22 de abril de 2019

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A l@s Melliz@s de Riogordo que me lo han dado todo...

...y a David, que llega para quedarse

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SUMMARY / RESUMEN

Summary

Arbuscular mycorrhizal fungi (AMF) are microscopic soil organisms able to establish a mutualistic association with the roots of most vascular plants, known as arbuscular mycorrhiza (AM). This symbiosis has been of extreme importance for plants along the evolutionary process. For example, it is thought that they facilitated plants adaptation to land environment over 450 million years ago. Due to their biotrophic nature, AMF need the photosynthates from the host plant for a correct development and functionality of their symbiotic structures. In return, many benefits are provided to the host plant, as the well-studied enhanced mineral nutrition (Giovannetti *et al.*, 2017).

Plant advantages derived from the symbiosis go far beyond nutritional aspects. Mycorrhizal plants have shown a better tolerance and resistance against a broad range of environmental stresses caused by both abiotic (e.g. drought or salinity) and biotic (pests and pathogens) factors (Jung *et al.*, 2012; Ruiz-Lozano *et al.*, 2016). Mechanisms mediating AM-benefits are diverse and depend on the stress situation, and in most cases, are reported to be finely regulated by phytohormones (Pozo *et al.*, 2015). In addition, although AMF are generalists, a functional diversity has been described, with some fungi being more efficient than others in terms of benefits for a given host plant species or growth conditions. Thus, the final output of the symbiosis is determined by the plant-AMF genotypes and the imposed stress. Considering this complexity, greater effort is needed to unravel the mechanisms underlying this increased ability of the host plant to overcome adverse situations. This knowledge will contribute to promote the use of AM-inoculants as biostimulants and bioprotectors in agriculture as an environmentally-friendly alternative to the traditional crop management, highly dependent on chemical fertilizers and pesticides.

Nowadays, the study of metabolic rearrangement in plants subjected to stress is as a useful tool to understand the molecular mechanisms involved in stress tolerance. Plants display a considerable phenotypic (physiological and chemical) plasticity to adapt to deleterious conditions. Indeed, biosynthesis of certain secondary metabolites, such as toxins or osmoprotectant compounds, can be crucial for plant adaptation to stress growing conditions (Yang *et al.*, 2018). Therefore, those stimuli able to increase such metabolic

plasticity, will result in a better plant adaptation to the challenging environment (Gratani, 2014). In this regard, previous studies have reported how AM can induce an "alert state" in the host plant with minimal fitness costs, that leads to a faster and stronger activation of plant defenses upon aggressor attack, known as defense priming (Martínez-Medina *et al*., 2016; Mauch-Mani *et al*., 2017).

To better understand the changes in the plant metabolism that may coordinate the enhanced stress tolerance, we used an untargeted metabolomic approach through liquid chromatography tandem mass spectrometry (LC-MS). This technique has proved to be an excellent tool for the identification of a wide range of secondary metabolites differentially accumulated, providing valuable information about how plants alter their metabolism to respond adequately to different environmental stimuli (Tugizimana *et al.*, 2018).

Therefore, the present Doctoral Thesis focuses on the **study of the potential of mycorrhizal fungi to protect tomato plants against different stresses, exploring the metabolic rearrangement underlying such protection, with the aim of identifying metabolic pathways and compounds playing a key role in the increased tolerance/ resistance of AM-plants.**

To achieve this main objective, we performed a series of experiments studying the performance of tomato plants colonized by different AMF (*Funneliformis mosseae*, *Rhizoglomus irregulare* and *Claroideoglomus etunicatum*), under different growth conditions, including nonstress, moderate and severe abiotic stress (drought and salinity) and a biotic stress consisting on herbivory by the leaf chewing insect *Spodoptera exigua*. The experiments were complemented by untargeted metabolomic studies to characterize the plant response to the specific stress and explore any potential difference between mycorrhizal and nonmycorrhizal plants that may explain their improved resilience to overcome stress.

Firstly, we explored the changes in the metabolism of the host roots as a consequence of the symbiosis establishment. This fact is important, since it will allow us to differentiate between common and AMF-specific basal metabolic changes of those related to stress responses analyzed in the following experiments. This study is presented in the **Chapter 1** (Rivero *et al.*, 2015), where the metabolic profiles of roots colonized by *F. mosseae* or *R. irregulare* were compared with those from nonmycorrhizal plants. A strong metabolic

rearrangement was observed, where a shared core of overaccumulated metabolites was found in roots colonized by both AMF. Those compounds were mainly associated to defense related pathways including phenylpropanoids (lignins, lignans and phenylpropanoid-polyamine conjugates (PPCs)), oxylipins (responsible of synthesis of jasmonic acid (JA)) and benzylisoquinoline alkaloids (BIAs). In addition, besides this shared modulation, fungus-specific fingerprints were also found, which may be underlying the reported functional diversity among different AMF species.

Subsequently, **Chapter 2** compiles a series of experiments carried out under progressive levels of drought and salinity stresses (Rivero *et al.*, 2018). In this study, we included a *C. etunicatum* strain isolated from high-salinity and very dry soil in addition of the previously studied *F. mosseae* and *R. irregulare*. Inoculation with each tested AMF increased plant tolerance to drought and salinity, resulting the positive effect of the symbiosis strongest under the most severe stress conditions. Interestingly, *C. etunicatum*-colonized plants displayed the best performance upon salinity stress, being the most effective AMF excluding toxic Na^+ in aboveground tissues. This fact reinforces the concept of functional diversity among AMF species, while emphasizing the importance of inoculating with stress-adapted strains to maximize plant protection.

Remarkably, although both stresses had a similar impact in terms of biomass loss, only salinity produced a significant alteration in the secondary metabolism of the roots. The roots colonized by the salt-adapted *C. etunicatum* exclusively exhibited a differential accumulation of some metabolites likely related to stress, that were not detected in the other mycorrhizal plants (which could explain the strongest protection). However, since all AMF strains achieved a significant protection, and our interest is in identifying mycorrhiza mechanisms for stress protection, we focused our analysis on those metabolites with a common altered pattern in roots colonized by the three AMF. A B6 vitamer, flavanols, xanthones and lignans were found overaccumulated in all mycorrhizal plants only upon salinity stress (primed response), thus they were considered as good candidates in mediating AM-protection. In a subsequently assay, we confirmed the protective role of flavanol catechin and B6 vitamers against salt stress through exogenous application of the compounds to tomato plants. These results confirmed that the mycorrhizal symbiosis enhance the metabolic plasticity of the plants improving their ability to cope with abiotic stresses.

Finally, the possible modulation of the host metabolic response during Mycorrhiza Induced Resistance (MIR) against biotic stress has been assessed in **Chapter 3**. Previous studies reported how necrotrophic pathogens and leaf-chewing insects may be negatively impaired in their development when they feed on a mycorrhizal plant (Song *et al.*, 2013; Sánchez-Bel *et al.*, 2016). These studies evidenced the role of phytohormone jasmonic acid (JA) in mediating MIR. However, an overview of the metabolic routes modulated in leaves leading to MIR remain elusive. In our study model, MIR against herbivore attackers was evidenced. The larvae from the common pest *S. exigua* fed on *F. mosseae*-colonized tomato plants displayed higher mortality and delayed development than those fed on nonmycorrhizal plants.

The subsequent untargeted metabolomic study allowed to explore the metabolic reprogramming triggered in response to herbivory locally in damaged tissues, and systemically, in distal leaflets. The analysis revealed a set of compounds only overaccumulated in AM-plants in response to local damage. Among the metabolic alterations, it was noteworthy the presence of several primed compounds within three main families: alkaloids, fatty acids derivatives and phenylpropanoid-polyamine conjugates (PPCs). Because of their specific accumulation profile and reliable identification, we focused on PPCs, more specifically in feruloylputrescine (FP), since was previously suggested to play a role in plant defense against herbivory (Kaur *et al.*, 2010). Through pharmacological and genetic approaches, we confirmed that FP accumulation is JA-dependent, and we demonstrate that JA-signaling is required for the FP-priming triggered during MIR.

In summary, the study performed in this PhD Thesis demonstrate the protective ability of mycorrhizal fungi on plants of agronomic importance such as tomato, supporting their potential as biostimulants for sustainable agriculture. It also illustrates that the protection to not relate to a fixed, specific metabolic pathway. In contrast, the metabolic pathways altered and the metabolites showing a primed accumulation in mycorrhizal plants vary according to the stress conditions faced by the plant. Therefore, the mycorrhizal symbiosis enhances the metabolic plasticity of the plant allowing a more efficient activation of defenses. This major plasticity explains the broad character of the protection conferred by mycorrhiza, and thus, support their application as bioprotectors agents under multiple adverse conditions.

Resumen

Los hongos formadores de micorrizas arbusculares (AMF, del inglés "*Arbuscular Mycorrhizal fungi*") son organismos microscópicos del suelo capaces de establecer una asociación mutualista con las raíces de la mayoría de las plantas vasculares, conocida simbiosis micorrícica arbuscular (AM, del inglés "*arbuscular mycorrhiza*"). Esta simbiosis ha sido de suma importancia para las plantas a lo largo del proceso evolutivo. Por ejemplo, se cree que facilitaron la adaptación de las plantas al entorno terrestre hace más de 450 millones de años. Debido a su naturaleza biotrófica, los HMA necesita de los fotosintatos de su planta huésped para un mantener correcto desarrollo y funcionalidad de sus estructuras simbióticas. A cambio, proporcionan un gran número beneficios a la planta huésped, destacando la bien estudiada mejora de la nutrición mineral (Giovannetti *et al*., 2017).

Las ventajas para las plantas derivadas de la simbiosis van mucho más allá de los aspectos meramente nutricionales. Las plantas micorrizadas muestran una mejor tolerancia y resistencia frente a una amplia gama de estreses ambientales causados por factores abióticos (por ejemplo, sequía y salinidad) y bióticos (plagas y patógenos) (Jung *et al*., 2012; Ruiz-Lozano *et a*l., 2016). Los mecanismos que median dichos beneficios pueden ser diversos y dependientes del tipo de estrés, y en la mayoría de los casos, están finamente regulados por las fitohormonas (Pozo *et a*l., 2015). Además, aunque los AMF son generalistas, diversos estudios han descrito su diversidad funcional, mostrándose ciertos hongos más eficientes que otros en términos de beneficios aportados para una determinada especie de planta hospedadora o condiciones de crecimiento. Por lo tanto, el resultado final de la simbiosis se encuentra determinado por los genotipos del AMF, planta hospedadora y el estrés sufrido. Teniendo en cuenta esta complejidad, se necesita un mayor esfuerzo para dilucidar los mecanismos subyacentes a esta mayor capacidad de la planta huésped para superar condiciones adversas. Este conocimiento contribuirá a promover el uso agrícola de inoculantes micorrícicos como bioestimulantes y bioprotectores, representando una alternativa respetuosa con el medio ambiente frente al manejo tradicional de cultivos, altamente dependiente de los fertilizantes químicos y pesticidas.

Hoy en día, el estudio del reordenamiento metabólico que ocurre en plantas sometidas a estrés es una herramienta útil para comprender los mecanismos moleculares involucrados

en la tolerancia o resistencia frente a los mismos. Las plantas pueden mostrar una considerable plasticidad fenotípica (tanto fisiológica como química) para adaptarse a diferentes condiciones perjudiciales. De hecho, la biosíntesis de ciertos metabolitos secundarios, como toxinas u otros compuestos osmoprotectores, puede ser crucial para la adaptación de la planta en condiciones de crecimiento estresantes (Yang *et al*., 2018). Por lo tanto, aquellos estímulos capaces de aumentar dicha plasticidad metabólica producirán una mejor adaptación de la planta ante entornos desafiantes (Gratani, 2014). En este sentido, estudios previos han descrito cómo las AM pueden inducir un "estado de alerta" en la planta huésped, con un coste mínimos para su fitness, que finalmente lleva a una activación más rápida y fuerte de sus defensas tras una agresión, conocido como *priming* defensivo (Martínez-Medina *et al*., 2016; Mauch-Mani *et al*., 2017).

Para comprender mejor los cambios en el metabolismo de las plantas que pueden coordinar la mayor tolerancia al estrés, utilizamos un enfoque metabolómico no dirigido a través de cromatografía líquida en tándem con espectrometría de masas (LC-MS). Dicha técnica ha demostrado ser una excelente herramienta para el estudio e identificación de una amplia gama de metabolitos secundarios acumulados de manera diferencial, proporcionando información valiosa acerca de cómo las plantas alteran su metabolismo para responder adecuadamente a diferentes estímulos ambientales (Tugizimana *et al*., 2018).

Por todo ello, la presente Tesis Doctoral se centra en **el estudio del potencial de los hongos formadores de micorrizas arbusculares para proteger las plantas de tomate frente a distintos estreses, explorando la reorganización metabólica subyacente a dicha protección, con el objetivo de identificar rutas metabólicas y compuestos que desempeñen un papel clave en el aumento de la tolerancia y resistencia de las plantas de las plantas micorrizadas.**

Para lograr este objetivo principal, llevamos a cabo una serie de experimentos para estudiar el desempeño de plantas de tomate micorrizadas por diferentes AMF (*Funneliformis mosseae*, *Rhizoglomus irregulare* y *Claroideoglomus etunicatum*) durante distintas condiciones de crecimiento, que incluyen ausencia de estrés, estreses abióticos moderados y severos (sequía y salinidad), y estrés biótico consistente en herbivoría de por el insecto masticador de hojas *Spodoptera exigua*. Los experimentos se complementaron con estudios metabolómicos no dirigidos para caracterizar la respuesta de la planta frente a cada estrés específico y explorar cualquier potencial diferencia entre

las plantas en simbiosis y no micorrizadas que puedan explicar su mayor habilidad para superar el estrés.

En primer lugar, exploramos los cambios en el metabolismo de las raíces de la planta hospedadora como consecuencia del establecimiento de la simbiosis. Este hecho es importante, ya que nos permitirá diferenciar entre los cambios metabólicos basales comunes de los específicos para cada AMF, además de aquellos relacionados con las respuestas frente a cada estrés que se analizarán en los siguientes experimentos. Dicho estudio se presenta en el **Capítulo 1** (Rivero *et al*., 2015), donde comparamos los perfiles metabólicos de las raíces colonizadas por *F. mosseae* o *R. irregulare* con los de plantas no micorrizadas. En este trabajo encontramos una fuerte reorganización metabólica, observándose metabolitos comunes mayormente acumulados en las raíces colonizadas por ambos AMF. Esos compuestos pertenecían principalmente a rutas biosintéticas relacionadas con la defensa, incluyendo fenilpropanoides (ligninas, lignanos y conjugados de fenilpropanoides y poliaminas (PPCs)), oxilipinas (responsables de la síntesis del ácido jasmónico (JA)) y alcaloides bencilisoquinolínicos (BIAs). Además, más allá de dicha modulación similar, también observamos alteraciones específicas para cada hongo, que podrían ser las responsables a la diversidad funcional descrita entre diferentes especies de AMF.

Posteriormente, a lo largo del **Capítulo 2** realizamos una serie de experimentos sometiendo a las plantas de tomate a niveles progresivos de estrés por sequía y salinidad (Rivero *et al*., 2018). En dicho estudio, incluimos una cepa de *C. etunicatum* aislada de suelos expuestos a elevada salinidad, además de los AMF *F. mosseae* y *R. irregulare* estudiados en el capítulo previo. Todos los AMF estudiados incrementaron la tolerancia de la planta frente a ambos estreses, siendo el beneficio aportado por la simbiosis más fuerte cuanto más severas las condiciones. Curiosamente, las plantas colonizadas por *C. etunicatum* mostraron el mejor desempeño ante estrés salino, siendo el AMF más efectivo excluyendo el ión tóxico Na^+ de los tejidos de la parte aérea. Este hecho refuerza el concepto de diversidad funcional entre distintas especies de AMF, al tiempo que enfatiza la importancia de inocular con cepas adaptadas al estrés para maximizar la protección de las plantas.

Sorprendentemente, aunque ambos estreses tuvieron un impacto similar en cuanto a reducción de biomasa, solo la salinidad produjo una alteración significativa en el metabolismo secundario de las raíces. Las raíces colonizadas por el AMF adaptado a

salinidad *C. etunicatum* exhibieron una acumulación diferencial de algunos metabolitos, probablemente relacionados con el estrés, no detectados en las otras plantas micorrizadas (lo que podría explicar la mayor protección). Sin embargo, dado que todas las especies de AMF lograron una protección significativa, y nuestro interés está en identificar los mecanismos de micorrizas para la protección contra el estrés, centramos nuestro análisis en aquellos metabolitos con un patrón alterado común para los tres AMF estudiados. Una mayor acumulación de vitámeros B6, flavanoles, xantonas y lignanos en respuesta a salinidad fue observada en todas las plantas micorrizadas (respuesta *priming*), por lo que se consideraron buenos candidatos como mediadores de la protección en AM. En un ensayo posterior, confirmamos el papel protector del flavanol catequina y varios vitámeros B6 frente a estrés salino mediante su aplicación exógena en las plantas de tomate. Estos resultados confirmaron que la simbiosis micorrícica aumenta la plasticidad metabólica de las plantas, mejorando por ello su capacidad para hacer frente al estrés abiótico.

Finalmente, en el **Capítulo 3** evaluamos la posible modulación de la respuesta metabólica en la planta hospedadora durante la resistencia inducida por micorrizas (MIR, del inglés "*Mycorrhiza-Induced Resistance*") frente a estrés biótico. Se ha descrito en estudios previos cómo los patógenos necrotróficos y los insectos masticadores de hojas pueden verse perjudicados en su desarrollo cuando se alimentan de plantas micorrizadas (Song *et al*., 2013; Sánchez-Bel *et al*., 2016). Dichos trabajos evidencian el papel de la fitohormona ácido jasmónico (JA) en la mediación de MIR. Sin embargo, la visión general de las rutas metabólicas moduladas en tejidos aéreos que conducen a la MIR permanece aún desconocida. En nuestro modelo de estudio, se evidenció la MIR frente a estrés por herbivoría. Aquellas larvas de la plaga común *S. exigua* que se alimentaron en plantas de tomate colonizadas por *F. mosseae* mostraron una mayor mortalidad y retraso en su desarrollo que aquellas alimentadas con plantas no micorrizas.

El posterior estudio metabolómico no dirigido nos permitió explorar la reprogramación metabólica desencadenada en respuesta a la herbivoría en tejidos dañados (local) y en los foliolos adyacentes sin daños (sistémico). El análisis reveló un conjunto de compuestos sólo mayormente acumulados en las plantas micorrizadas tras respuesta al daño local. Entre las alteraciones metabólicas, cabe destacar la presencia tres familias principales de compuestos con varios metabolitos *primed*: alcaloides, derivados de ácidos grasos y conjugados de fenilpropanoides y poliaminas (PPCs). Debido a su perfil de acumulación

y su buena identificación, nos centramos en los PPCs, más específicamente en la feruloilestrescina (FP), ya que previos estudios habían sugerido que desempeñan un papel en la defensa de las plantas frente a herbivoría (Kaur *et al*., 2010). A través de aproximaciones farmacológicas y genéticas, confirmamos la acumulación de FP es dependiente de JA, y demostramos que la señalización por JA es necesaria para el *priming* de FP durante la MIR.

En resumen, el estudio llevado a cabo en la presente Tesis Doctoral demuestra la capacidad de los hongos micorrícicos para proteger a una planta de importancia agronómica como el tomate, lo que respalda su potencial uso como bioestimulantes para una agricultura sostenible. También ilustra que la protección proporcionada no se relaciona con una vía metabólica específica y fija. Al contrario, las vías metabólicas alteradas y los metabolitos mostrando un comportamiento *priming* en plantas micorrizadas pueden variar dependiendo de qué condiciones de estrés enfrenta la planta. Por lo tanto, la simbiosis micorrícica arbuscular aumenta la plasticidad metabólica de la planta, permitiéndole una activación más eficiente de las defensas. Esta mayor plasticidad explica el amplio rango de protección conferida por las micorrizas y, por lo tanto, respalda su aplicación como agentes bioprotectores en múltiples condiciones adversas.

INTRODUCTION

Introduction

1. The need for sustainable solutions for crop protection

The world's population is expected to grow to 9.73 billion by 2050 (FAO 2017), and the concomitant increase in food demand is one of the major challenges for the coming years. The required increase in crop production requires an optimal use of cultivable land and the optimization of agriculture practices. The surface dedicated to agricultural land has stabilized over the last 20 years at around 4.9 billion hectares, and an extraordinary increase is unlikely. Thus, developing different strategies to reach a more efficient agriculture, while minimizing its environmental impact is of outmost importance.

In the 1960s, the so called "Green Revolution" highly increased agricultural production worldwide, particularly in the developing world (Zeigler and Mohanty, 2010). This success was mainly due to the incorporation of high-yielding varieties achieved through extensive scientific breeding efforts, and to the use of chemical fertilizers and pesticides and advances in irrigation and mechanization technologies. However, this boom in crop production also resulted in several negative effects. A clear example has been the progressive soil degradation caused by the excessive use of fertilizers and pesticides, as the contamination of ground waters and salinization of aquifers, problems that are still ongoing nowadays (Gunnell *et al.*, 2007; Gomiero *et al.*, 2011). In addition, it is well known that the pollution generated from the use of pesticides may negatively affect beneficial insects and microbes, and once in the food chain, can seriously compromise the farmers and consumers health.

Accordingly, a major challenge nowadays is to accomplish a second green revolution that ensure increased food production and security, and environment protection. Towards this aim, it is required the optimization of environmentally-friendly and sustainable strategies for agriculture. Thus, new farming practices should improve the efficient use of nutrients and water and reducing pesticides without risking high yields. For that purpose, the exploitation of natural resources as optimization of the plant defense intrinsic mechanisms and the use of beneficial microbes improving plant nutrition and plant stress tolerance is receiving special attention. Indeed, there is an increased interest in the

development of efficient microbial inoculants providing crops with a large number of advantages, especially in adverse environments, without compromising the available resources (Dodd and Ruiz-Lozano, 2012). This is the frame for the research presented in this Doctoral Thesis. I aimed to explore the potential of different arbuscular mycorrhizal fungi (AMF) to enhance plant tolerance/resistance to diverse stressful growing conditions, focusing on the underlying mechanisms, with special emphasis on the changes at the metabolome level.

2. Plant phenotypic plasticity

In nature, plants constantly face a broad range of environmental challenges that seriously compromise their productivity. They need to detect, respond and adapt to the very diverse environmental conditions that can challenge their survival or disturb normal plant growth, including abiotic (drought, salinity, nutrient deficiency or excess, light intensity, etc.) and biotic (pathogens and pests) stress factors. In addition, nowadays some of these stresses have been worsen due to anthropogenic activities (e.g. climate change, land use, invasiveness, monocultures, etc.). To cope with these stresses, plants have developed a series of defense mechanisms that allow them to respond efficiently to the specific challenges.

Under stress situation or high environmental variability, plants must adjust their phenotype to these conditions to ensure their survival. This ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions is defined as phenotypic plasticity (Fusco and Minelli, 2010) (Figure 1). Phenotypic plasticity is achieved through epigenetic, transcriptional or post-transcriptional regulation entailing changes in anatomical traits and at the physiological, morphological or molecular levels (Nicotra *et al.*, 2010; Gratani, 2014).

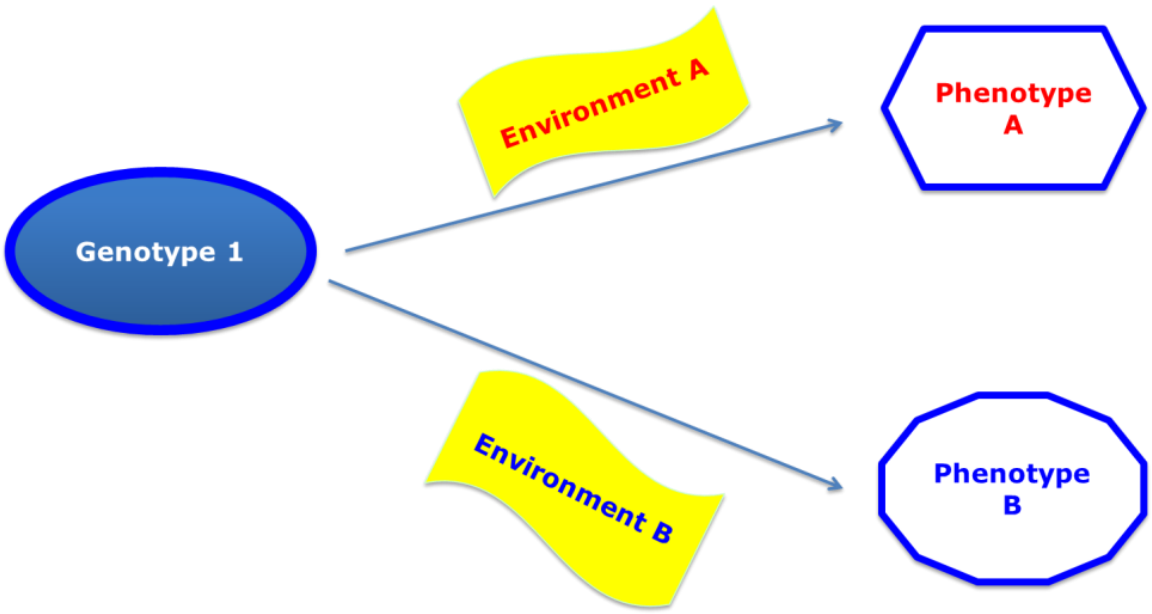

Figure 1 Schematic representation of phenotypic plasticity.

Plants able to show a remarkable phenotypic plasticity take advantage in extreme and heterogeneous environments, coordinating their responses to optimally balance growth and defense (Richter *et al.*, 2012). Moreover, due the better ability to adapt to different stress levels, phenotypic plasticity contributes to increase the range of environments in which plants can survive. In the specific case of plants finetune modulation of the synthesis, allocation and even function of these compounds according to the specific conditions, this ability is denominated as metabolic plasticity.

Indeed, plants can synthesize a broad range of active compounds with specialized roles to reduce damage by environmental challenges, for example by accumulating antioxidant and osmoprotectant compounds to face osmotic stress, as well as toxins in response to attacking pathogens or herbivores, or signals to attract beneficial organisms such as pollinators and symbionts.

It is noteworthy that this ability of plants is genetically controlled and heritable (Zhang *et al.*, 2013; Fortes and Gallusci, 2017), Therefore, together with natural selection, phenotypic plasticity is considered as the main mechanisms underlying plant species adaption to changing environmental conditions (Grenier *et al.*, 2016).

2.1. Signaling events in the regulation of plant stress responses

Plants perceive multiple cues from the environment that may indicate potential challenges. Perception of these cues alters the concentrations of several signaling compounds, including reactive oxygen and nitrogen species, calcium ions and phytohormones (Sparks *et al.*, 2013). They trigger signaling cascades regulating activators/repressors and transcription factors that can interact with each other, thus processing and integrating the multiple signals. This enables specific families of transcription factors to activate transcriptional responses. Interactions among key regulators mediate pathway crosstalk, so that some elements can act as molecular hubs integrating multiple signaling cascades, allowing the plant to shape the final response to the given conditions. Thus, signal generation and processing enables plants to flexibly respond and adapt their phenotype to their environmental context (Figure 2).

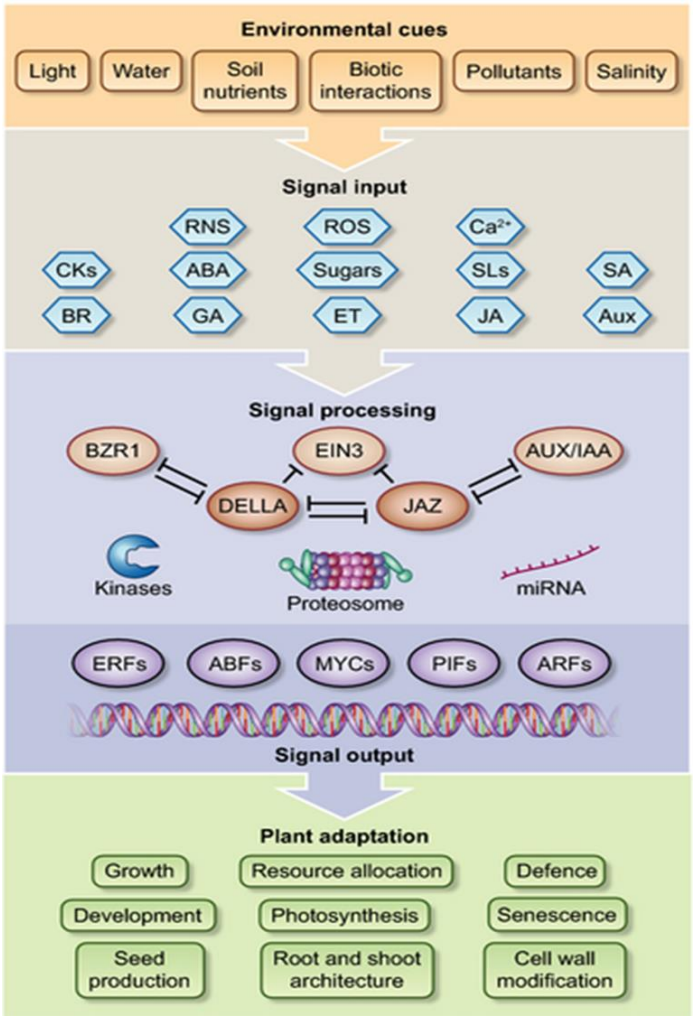

Figure 2 Integrative model describing how plants perceive and respond to environmental cues (Pozo *et al.*, 2015).

Focusing on phytohormones are defined as small molecules that act at very low concentrations, with a pivotal role regulating almost every developmental processes and defense responses in plants (Ciura and Kruk, 2018).

Traditionally, phytohormones were differentiated between regulators of developmental processes – auxins (AUX), gibberellins (GA), brassinosteroids (BR), cytokinins (CK) and strigolactones (SL) – and regulators of stress responses – salicylic acid (SA), jasmonic acid and its derivatives (JA), ethylene (ET) and abscisic acid (ABA). Although phytohormones can regulate specific processes, this division, even convenient in certain cases, is thoroughly artificial. In fact, they are not isolated pathways, but they interact extensively (Pieterse *et al.*, 2012; Murphy, 2015). Thus, the timing and relative concentration of the different hormones within the blend released upon a given challenge. Moreover, since plants are generally exposed to simultaneous multiple stresses of different nature, the hormonal crosstalk is essential to coordinate and prioritize the most needed plant defense and stress responses (Vos *et al.*, 2015). In fact, plant phytohormone signaling and their crosstalk implies that a plant response to one stress can affect how plant responds to another simultaneous or subsequent stressor (Rejeb *et al.*, 2014).

2.2. Role of secondary metabolism

More than 100 years ago, Kossel (1891) defined secondary metabolism as those metabolic pathways and molecule products (metabolites) of metabolism that are non-essential for the survival of the organism This mention comprises a large and very heterogenous number of metabolic routes and compounds present in higher plants and usually in a high structural diversity. Although their definition suggests that secondary metabolites (SMs) could be dispensable for plant life, they play important roles in many situations, including growth regulation, protection against stresses and other adaptations to environment, thus, being key components for plant survival.

In contrast, primary metabolism is related to these widespread compounds that are essential for the plant, can be found in all plant species and are usually in considerable amounts, for example amino acids, carbohydrates, fatty acids, carboxylic acids, etc. But despite such traditional definitions, the distinction between both primary and secondary metabolism is diffuse, and novel studies challenge such separation, showing that

metabolites from primary metabolism can play alternative defensive roles. For example, sugars and amino acids have been shown to play a role in the defense against pathogens (Ryan *et al.*, 2007; Pastor *et al.*, 2014).

Many secondary metabolites possess great relevance in determining how plants interact with environment, for example providing protection against both biotic and abiotic stresses (Ashraf *et al.*, 2018). Therefore, the ability to induce or not SMs in response to external stimulus is intimately linked to the phenotypic plasticity that a plant genotype is able to display under different environments (Nicotra *et al*., 2010).

Among SMs, we find compounds that although not playing major roles in normal growth under nonstressful conditions, they become essential for plants when they must respond efficiently to adverse environmental conditions (Yang *et al.*, 2018b). In addition, some SMs are present ubiquitously in all plants, while others are specific to some plant families as a consequence of natural selection in stressful environments.

Nonetheless, due to its considerable number and structural diversity, SMs can be classified in base of different criteria. Attending to their chemical structures and their biosynthetic pathways, we can classify SMs in three major groups: i) nitrogen-containing compounds such us alkaloids, cyanogenic glycosides and glucosinolates ii) phenolic compounds such as phenylpropanoids and flavonoids and iii) terpenes (isoprenoids) (Ashraf *et al.*, 2018).

2.3. Defense mechanisms against abiotic stress

Several studies indicate that abiotic stresses may impose up to 70% yield losses in crops (Kaur *et al*., 2008; Mantri *et al*., 2012). Among the most common abiotic stress factors are extreme temperatures and osmotic stresses derived from salinity and drought. It has been reported that about 45 million hectares are affected by salinity (Pitman and Lauchli, 2002; Munns and Tester, 2008), while drought, besides being a key limiting factor for agriculture, is being aggravated in the current context of climate change.

Drought and salinity stresses are often interconnected: an excess of salt in soils induce osmotic stress whereby plants face physiological drought (Pitman and Lauchli, 2002), and, therefore, both stresses may induce similar cellular damage such as oxidative stress or ionic and osmotic homeostasis imbalances.

In response to these stresses, plants react by a fine hormonal tuning. Among phytohormones, ABA (traditionally known as "stress hormone") has been the most studied as regulators of the plant response against abiotic stresses. In adverse conditions such as drought or high salinity, ABA levels increase resulting in the activation of genes related to defense strategies (Wani *et al*., 2016). ABA is known to regulate short-term responses as opening/closure of stomata, hence reducing water loss via transpiration, which is important in osmotic stress conditions for the maintenance of water balance (Sah *et al.*, 2016). In addition, ABA triggers the synthesis of antioxidant enzymes and osmoprotectants metabolites responsible of maintenance of the osmotic potential in plant cells (Pál *et al.*, 2018).

In the last years, the role of SA, JA and ET (traditionally associated to biotic stress responses) have also been studied as regulators of abiotic stress responses. For example, it has been reported that exogenous application of JA or its derivatives (mainly methyl-JA) ameliorate the negative effect of drought and salinity stress on plant fitness, for example avoiding root hydraulic conductivity inhibition caused by drought in *Phaseolus vulgaris* (Sánchez-Romera *et al.*, 2016). Therefore, the synergistic role in combination with ABA in osmotic stress signaling was reported (Riemann *et al.*, 2015).

Biosynthesis of SMs can also be of outmost importance on plant fitness and survival under certain extreme environments (Verma and Shukla, 2015). A common plant response to osmotic stress is the increase of compatible osmolites, known as osmoprotectants, whose accumulation in cells is crucial to balance the osmotic pressure to ensure an active water uptake form the soil to the roots. Two of the most studied osmoprotectants are the amino acid proline and glycine betaine (De la Torre-González *et al.*, 2018).

Similarly, biosynthesis of antioxidants is crucial for plant adaptation to stressful growing conditions since usually stress is associated to oxidative processes (Oh *et al.*, 2009). Antioxidants form a heterogeneous group, among which we can highlight phenolic compounds (as phenylpropanoids, lignins and lignans) (Rice-Evans *et al.*, 1997). A high expression of gene phenylalanine ammonia-lyase (*PAL*), first step in phenylpropanoid biosynthesis pathway, was found necessary for lettuce plants adaptation to stress environment (Oh *et al.*, 2009). In addition, we find flavonoids, whose antioxidant function in stressed plants is widely known (Agati *et al.*, 2012). Flavonoids have a key protective role in counter the stress-induced oxidative damage resulted by ROS

generation by both preventing its generation of and scavenging it once formed (Agati *et al.*, 2012; Fini *et al.*, 2011). Moreover, Yiu and collaborators (2011; 2012) demonstrated exogenous application of flavonol catechin increased flooding tolerance in tomato and fitness of pepper plants subjected to salinity stress. In addition, several vitamins (as ascorbic acid or α-tocopherol), have been reported role in plant protection against oxidative stress produced in response to various stresses with a damaging effect on cell membranes, organelles and other macromolecules (Mittler, 2002).

2.4. Defense mechanisms against biotic stress

Biotic stresses are also a major problem in agriculture, since they cause important losses in crops yield, being most of the damaged caused by microbial pathogens, weeds and insect pests. In order to face them, plants have developed different defensive strategies that can be classified in two main categories, constitutive and inducible (Mithöfer and Maffei, 2015). Plants have a sophisticated defense system that, as to the immune system of animals, can recognize certain molecules or signals to respond by activating an immune response adapted to the attacking organism. This fact is of extreme importance, since the plant must differentiate between negative interactions such as the attack of pathogens and pests, of those beneficial interactions. For this reason, plants have developed different strategies to recognize and either allow or repeal the integrating organisms. These strategies provide protection in case of parasites or benefits in the case of symbiotic organisms (Pieterse and Van Wees, 2015).

Constitutive defenses are always present, independent on the presence or absence of stress. They include physical barriers, such as trichomes, thorns, spines or waxes, providing mechanical protection to the plant surface. Moreover, constitutive defenses can also comprise production of antimicrobial compounds as the so-called phytoanticipins; toxic SMs such as alkaloids, phenols, anthocyanins, terpenoids or quinones that either kill or retard the development of aggressors, and they are normally stored as inactive forms (War *et al.*, 2012). Different plant families have specialized in developing toxic compounds against pathogens or insects (Speed *et al.*, 2015). These may be common (e.g. alkaloids, phenols, terpenes, cyanogenic glucosides…) or specific to a family (for example steroidal glycoalkaloids in Solanaceae or glucosinolates in Brassicaceae). But this constitutive synthesis and storage of toxic compounds can be costly since entails an

important redirection of resources from growth toward defense. Thus, in environments with medium to low risk of attack, defense mechanisms have evolved towards their inducibility (Bekaert *et al.*, 2012). These the toxic compounds synthetized *de novo* after stress are known as phytoalexins.

Therefore, inducible defenses are activated upon perception of the aggressor, and they are finely regulated by phytohormone-dependent signaling pathways. The phytohormones JA, SA, ABA and ET are the most studied in relation to plant responses against biotic factors (Pieterse *et al.*, 2012), although as mentioned above, other phytohormones could play regulatory roles through interaction with those major pathways. According to the aggressor lifestyle, one phytohormonal signaling pathway will prevail over the others in order to give a fine-tuned response to reduce harmful consequences.

In general, it is assumed that the activation of JA-signaling pathway is required for resistance against necrotrophic pathogens and chewing herbivores (Howe and Jander, 2008; Wasternack and Hause, 2013). JA coordinate two main JA-signaling pathways related to defense, which are co-regulated with others phytohormones and that are usually antagonistic. Defensive-signaling against necrotrophs pathogens are co-regulated with ET through the ETHYLENE RESPONSE FACTOR (ERF)-branch, while responses against herbivores is co-regulated with ABA through the MYC-branch (PRé 2008, Fernández-calvo 2011).

Focusing on the role of JA triggering defenses against herbivores, it highlights the induction of gene expression and subsequent accumulation of key metabolites able to reduce insect feeding through production of toxic and unpalatable compounds that harm insect development (Erb and Reymond, 2019). Among them, it highlights proteinase inhibitors (PI), antifeedant compounds that disrupt the enzymatic ability of the attacker resulting in an impaired digestion of plant material (Orozco-Cardenas *et al.*, 1993). Other examples of toxic defenses against herbivores regulated by JA are glucosinolates (sulfated metabolites essential for defense in Brassicaceae) (Schweizer *et al.*, 2013), benzoxazinoids (in Poaceae) (Dafoe *et al.*, 2011), and alkaloids as nicotine (in Solanaceae) (Wang *et al.*, 2015).

In the case of the SA-signaling pathway, it regulates defense responses effective mainly against biotrophic pathogens through the regulation of programmed cell death. SA signaling, through the activation of the master gen for SA-signaling transduction *NPR1*,

induces the expression of a group of genes coding for pathogenesis-related (PR) proteins, whose antimicrobial function negatively affects the pathogen development (van Loon *et al.*, 2006) and for several defensive secondary metabolites as monoterpenes (Riedlmeier *et al.*, 2017). Beside its role against biotrophic pathogens, SA-signaling has also been reported to be effective against phloem-feeders herbivores as aphids and whiteflies (Ali and Agrawal, 2012).

It is well documented that SA- and JA-dependent pathways are reciprocally antagonistic (Caarls *et al.*, 2015; Koornneef and Pieterse, 2008; Thaler *et al.*, 2012). It is remarkably that several pathogens can use this antagonism in their benefits; for example, necrotrophs can manipulate SA-signaling, with the subsequent descend in JA-related defenses, in order to promote their disease (Rahman *et al.*, 2012). It should be taken into account that This crosstalk can also mediate the cross impact of simultaneous interactions in the plant. As it is represented in Figure 3, the activation of a pathway by one organism can have a positive or negative impact on another one via crosstalk on the target signaling pathways (Lazebnik *et al.*, 2014)

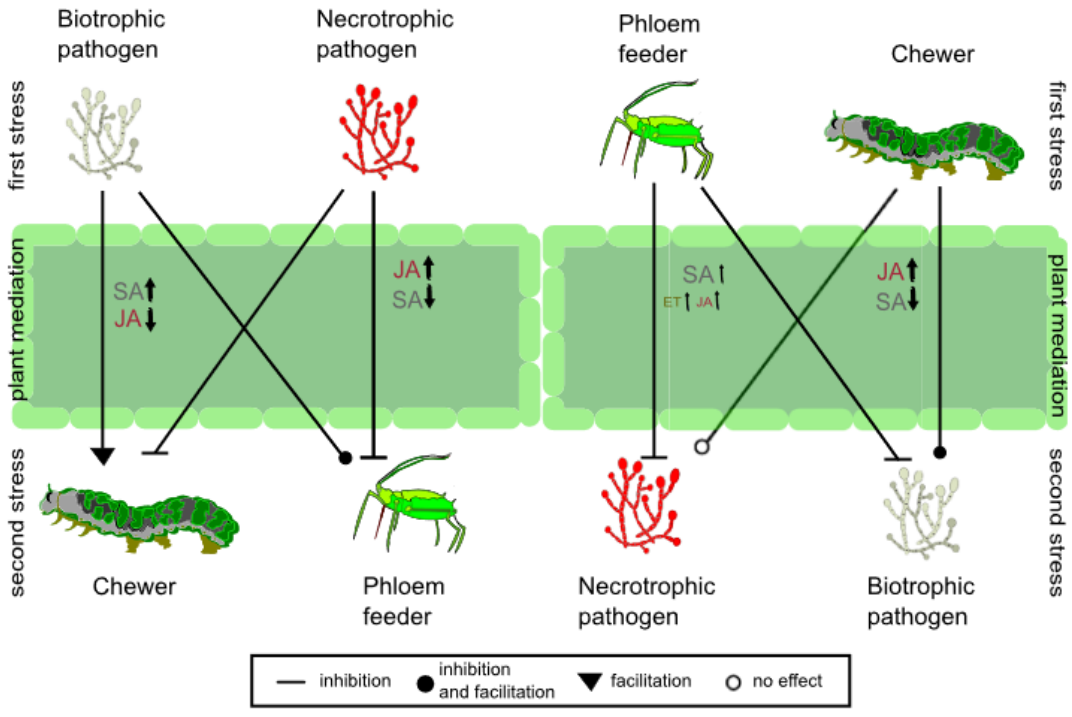

Figure 3 Overview of plant-mediated effects of pathogens on insects and of insects on pathogens of different trophic strategies or feeding modes (Lazebnik *et al.*, 2014).

In addition to these direct defenses, plants also can defense through indirect mechanism, as attracting the natural enemies of the attacking pests. Pioneer studies in this field showed that plants infested with spider mites can release a blend of volatiles that are more attractive to predatory mites compared to those released after mechanical wounding (Dicke and Sabelis, 1987). Subsequent studies confirmed the fact that after herbivore feeding, plants release of volatile organic compounds (VOCs) that can be recognized as cues for natural enemies, leading to an increased recruitment of pest´s predators and parasitoids with the clear benefit that this means for the attacked plant. These compounds, so-called as herbivore-induced plant volatiles (HIPVs), are defined as compounds that a plant does not synthesized unless it is damaged (synthesized *de novo*) or compounds that are also synthesized by undamaged plants but in larger amounts by damaged plants (Dicke and Baldwin, 2010). HIPVs can be released either at the damaged area or well from systemic tissues of wounded plants (Heil and Ton, 2008), and they mainly consist in terpenoids, fatty acid derivatives, phenylpropanoids and benzenoids (Mumm and Dicke, 2010).

An important group of HIPVs are the "green leaf volatiles" (GLVs), derivatives compounds from the oxylipin pathway (LOX) that are very quickly produced and released from damaged cell membranes (Dudareva et al., 2013). Another class of plant HIPVs comprises the terpenoids, a very diverse class of secondary metabolites. They are formed from two common C5 precursors, the isomers isopentenyl diphosphate and its isomer dimethylallyl diphosphate.

In the case of parasitoids, female wasps must recognize HIPVs to differentiate which kind of herbivore is producing plant-damage. Therefore, generalist parasitoids have to learn to distinguish among different HIPVs blends, while in the case of specialist this ability is considered innate (Turlings and Erb, 2018). In this sense, terpenoids are considered key HIPVs due their ubiquity and great variability among plant genotypes (Degenhardt *et al.*, 2009), while GLVs were found no to play an important role in parasitoid attraction. However, Allmann and collaborators demonstrated that plants under *Manduca sexta* feeding provide specific information to natural enemies through changes in the isomeric composition of emitted GLVs (Allmann and Baldwin, 2010), also resulting in a oviposition reduction by female *M. sexta* moths (Allmann *et al.*, 2013).

2.5. Induced resistance and defensive-priming

Induced resistance is an increase in basal immunity by which a plant can respond more efficiently to a challenge. This defensive status can be triggered through different stimuli, and it is usually based in the ability of the plant to retain some "stress memories" that make them to react more efficiently to a subsequent pathogen or insect attack (Pieterse and Van Wees, 2015). Depending on the type of stimulus acting as inducer and the mechanisms responsible for the resistance achieved, different terms are used for the induced resistance achieved. Therefore, the induced resistance can be differentiated into: i) systemic acquired resistance (SAR), when it is pathogen-induced, ii) herbivore-induced resistance (HIR) and iii) induced systemic resistance (ISR), when it is triggered by beneficial soil borne microbes (Pieterse *et al.*, 2014) (Figure 4). In addition, some chemicals (e.g. β-aminobutyric acid and b-amino-hexanoic) are also able to induce this state (Buswell *et al.*, 2018; Pastor *et al.*, 2014).

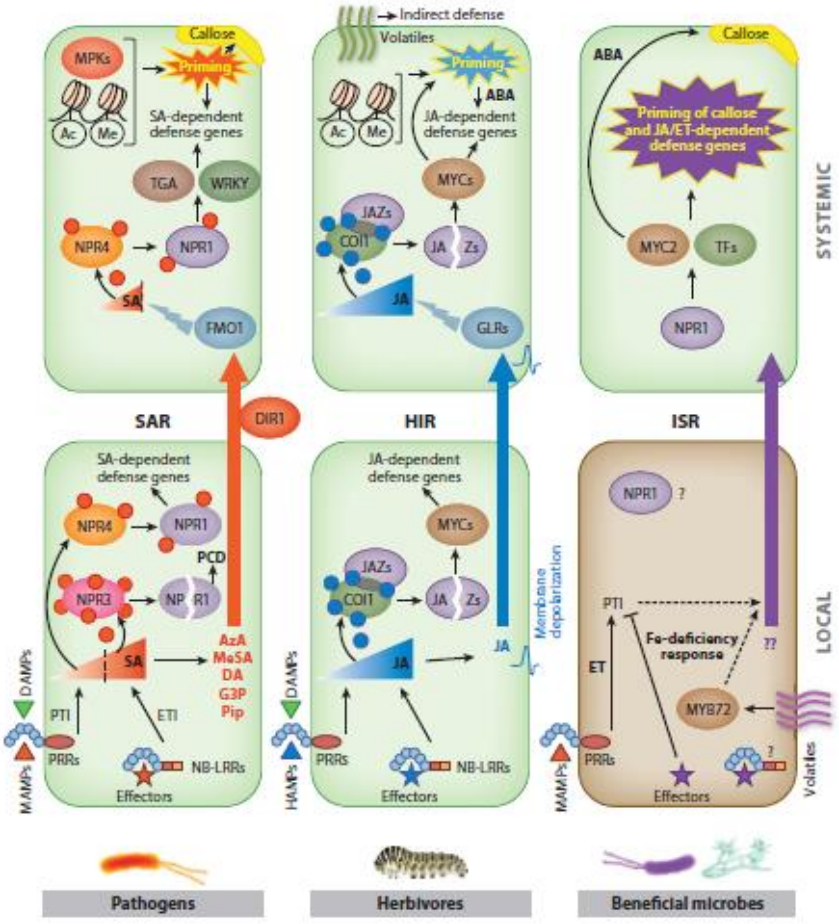

Figure 4 Schematic representation of molecular components and mechanisms involved in pathogen-induced systemic acquired resistance (SAR), herbivore-induced resistance (HIR), and induced systemic resistance (ISR) triggered by beneficial soil borne microbes. (Pieterse *et al.*, 2014).

In 1961, A. Frank Ross demonstrated that infection of TMV in tobacco plants leads the plant to be more resistant to subsequent attacks (Ross, 1961). This response, defined as systemic acquired resistance (SAR), is long-lasting and able to protect the plant against a wide range of pathogens such as fungi, bacteria or viruses (Fu and Dong, 2013; Vlot *et al.*, 2009). SAR is characterized by expression of a set of genes coding for PR proteins with antimicrobial activity (van Loon *et al.*, 2006). SAR activation is associated with elevated levels of phytohormone SA. This key role of SA has been confirmed through different experiments on transgenic lines deficient in the production of this phytohormone, where plants were unable to develop SAR response (Durrant and Dong, 2004).

Besides SAR, certain beneficial microbes from rhizospheric microbiome able to colonize plant-roots can confer a type of immunity similar to that induced by pathogens, denominated as induced systemic resistance (ISR) (Pieterse *et al.*, 2014). ISR response, like SAR, provides a systemic, durable, and broad-spectrum resistance that is effective against bacteria, fungi, viruses and insects. There are differences and similarities between SAR and ISR signaling pathways. While phytohormone SA is a major regulator of SAR, ISR activation by beneficial microbes is SA-independent and does not involve major changes in the activation of PR genes. In contrast, according to the studies in the model plant *Arabidopsis thaliana*, the ISR achieved upon interaction with beneficial microorganisms depends mainly on the hormonal pathways regulated by phytohormones JA and ET (Pieterse and Van Wees, 2015).

Defense priming is defined as the physiological state in which plants are pre-conditioned for the more efficient activation of defenses against environmental challenges h (Martinez-Medina *et al.*, 2016), and therefore, is considered as an intrinsic part of induced resistance (Mauch-Mani *et al.*, 2017). This is of greater importance in ISR since, as mentioned above, in general does not entail a basal activation of the defensive response.

Besides the priming stimuli from biotic origin (pathogens, arthropods and beneficial microbes) able to induce resistance via triggered immunity activation, abiotic cues and some chemicals can also act as warning stimulus signals and therefore trigger the establishment of priming (Avramova, 2019; Buswell *et al.*, 2018). This physiological state can be also constitutively displayed by the modification of some specific genes, generating priming mutants (as *edr1, ocp3, lin1* in Arabidopsis) with enhanced resistance against several pathogens (Gamir *et al.*, 2014). Thus, priming is accepted as an intrinsic

part of induced resistance, where the plant is strongly prepared to better deal with future stresses through a faster and/or stronger reaction upon subsequent challenge (Mauch-Mani *et al.*, 2017).

Recent studies have demonstrated that some stimuli induce direct changes in the plant and these changes are crucial for the enhanced defensive capacity. However, these changes have minimal fitness costs compared with directly induced defenses. Thus, priming is known for being a low-cost defensive response since plant´s defenses are not (or only slightly) activated in absence of stress. In addition, the primed state can be maintained during plant´s life and, due to changes at epigenetic levels, could be transmitted to future generations (Mauch-Mani *et al.*, 2017).

To explore the mechanisms underlying priming and the impact on both the defensive response and the plant fitness it is required to define different phases or stages in the process. The Figure 5 depicts a schematic representation of the distinct phases of priming in time according to Martínez-Medina and collaborators (2016).

Priming phase. During this stage, which takes place upon perception of the priming stimuli until the exposure to a challenging stress, different physiological, transcriptional, metabolic, and epigenetic changes may occur (Mauch-Mani *et al.*, 2017; Pastor *et al.*, 2014). These changes can be transient or maintained throughout the lifetime, and prepare the primed-plant for enhanced responsiveness when a challenge is present. In fact, recent studies suggest that defense priming can pass down generations, indicating an epigenetic component of transgenerational defense priming (Luna *et al.*, 2012; Rasmann *et al.*, 2012, Slaughther et al 2012). It should be noted that at the beginning of this stage and as consequence of the stimulus, a little defensive response can be transiently activated.

Postchallenge primed-state. Following stress primed plants show an enhanced responsiveness since they are conditioned for a faster and more efficient activation of defenses upon detection of potential stress situations. This fact can be explained since plants show enhanced signal transduction and defense responses. The underlying mechanism could be diverse because priming is a horizontal and multi-component mechanism (Mauch-Mani *et al.*, 2017). During this stage, the enhanced resistance or tolerance of the primed plants results in a better fitness after challenge, compensating the potential cost observed in the previous stage (Martínez-Medina *et al.*, 2016).

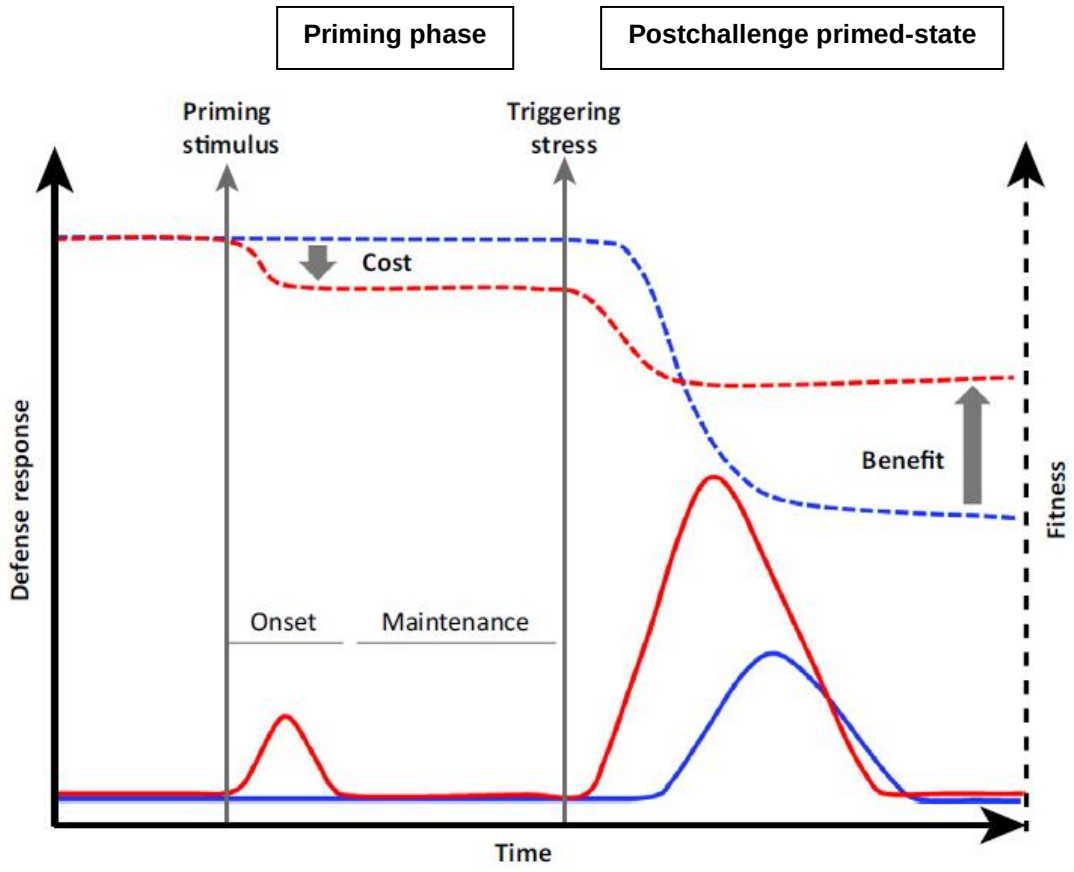

Figure 5 Scheme of the relation between defense responses (solid lines) and fitness (dashed lines) in primed (red) versus unprimed (blue) plants (Martinez-Medina *et al.*, 2016).

In addition, during these stages of priming, alterations in metabolic profiles are found compare with unprimed plants (Figure 6). Among them, the secondary metabolism reorganization has been shown to be crucial for triggering an effective primed defensive response to overcome different aggressions (Balmer *et al.*, 2015).

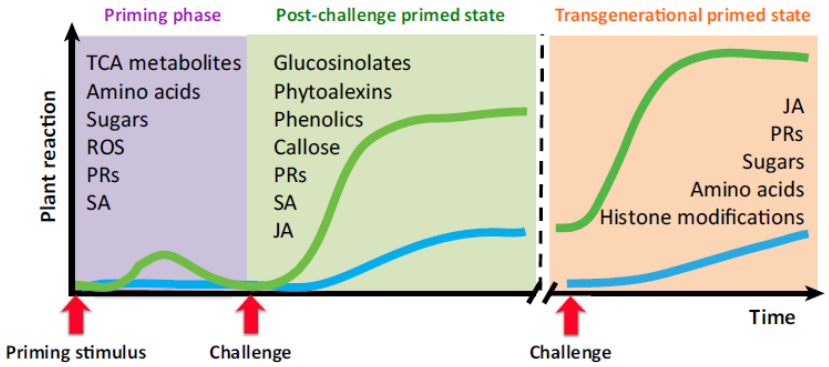

Figure 6 Primary and secondary metabolites, enzymes, hormones, and other molecules altered during different stages of priming (Balmer *et al.*, 2015).

3. Soil beneficial microorganisms: Arbuscular mycorrhizal fungi

Plants interact with a large number of organisms in their environment. Among them, multiple microorganisms live with the plants, and the term "plant microbiome" has received increasing attention in recent years, defined as the set of virus, prokaryotes, and eukaryotes, associated with distinct parts of the host plant (e.g. roots, leaves, shoots, flowers…) (Turner *et al.*, 2013). Focusing in the area of root influence (rhizosphere), which is considered one of the most energy-rich habitats on Earth (Bais *et al.*, 2006), plants can establish a great number of associations with a broad range of soil borne organism. From the point of view of plant fitness, these interactions could be deleterious (resulting in biotic stresses), or in other cases, positively influence both plant health and productivity (Lakshmanan *et al.*, 2014; Mendes *et al.*, 2013) (Figure 7). Therefore, plant must determine which kind of organism is facing to coordinate its response in each case (Hacquard *et al.*, 2016).

Among the organisms from the rhizosphere able stablish a beneficial association with plants roots we find growth-promoting rhizobacteria (PGPR) and fungi (PGPF), cyanobacteria or arbuscular mycorrhizal fungi (AMF). Due to the heterotrophic character of the plant associated-microbiota, host plants are able to reallocate up to 40% of their photosynthates into the rhizosphere (Bais *et al.*, 2006). On the other hand, the benefits provided from this organisms to their host plants may be diverse (Figure 7), being the most studied those related with the transfer of nutrients. Therefore, beneficial organisms can act as biofertilizers due to its capacity to facilitate mineral nutrients acquisition to the host plant through several natural processes. Among other mechanisms, associated microbiota can increase the solubility of iron through releasing siderophores into the rhizosphere (Crowley, 2006), or well increasing phosphorous solubilization through medium acidification (Khan *et al.*, 2013), that were inaccessible for the plant.

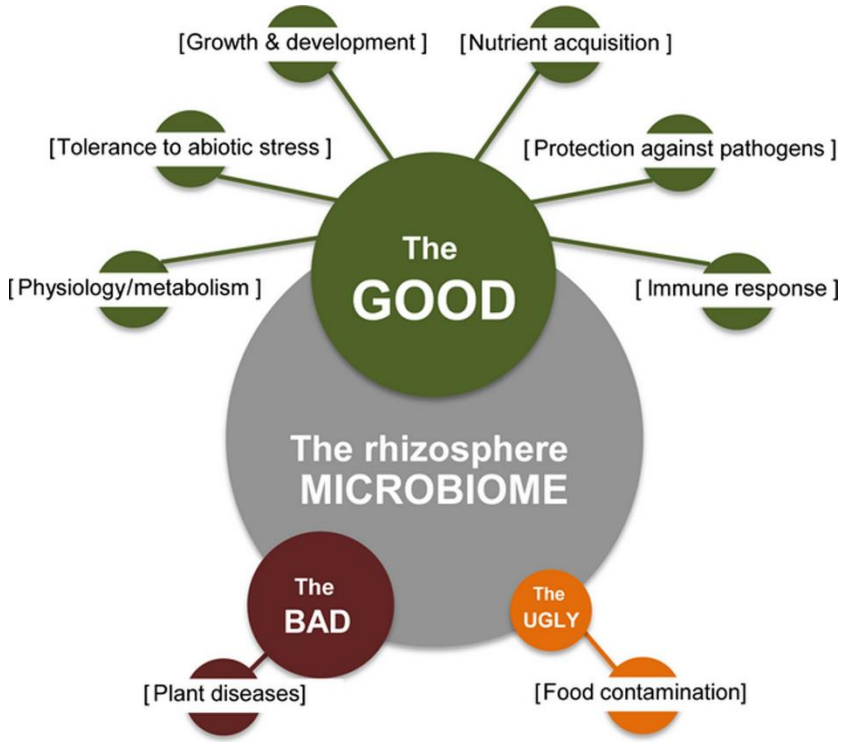

Figure 7 Schematic overview of the functions and impact of plant beneficial ('the good'), plant pathogenic ('the bad'), and human pathogenic microorganisms ('the ugly') on the host plant (Mendes *et al.*, 2013).

Besides this increased nutrient uptake, associations with soil beneficial microbes may result in enhanced plant fitness by promoting growth through the production of some phytohormone like compounds, or alleviating the effects of deleterious environmental factors. For example, beneficial-associated microorganism help plants to cope with abiotic stresses by synthesizing phytohormones as auxins, cytokinins or abscisic acid (Egamberdieva *et al.*, 2017). In addition, PGPRs and arbuscular mycorrhizas have been shown to facilitate the phytoremediation processes (Ferrol *et al.*, 2016; Xun *et al.*, 2015). Hence, increasing the use of beneficial soil-microorganisms would reduce the negative consequences of traditional fertilization as increase soil salinity, heavy metal accumulation or water eutrophication, having a key role to achieve more sustainable agriculture.

In addition, it has been previously described how plants associated with soil microorganisms showed a greater tolerance and resistance to both biotic and abiotic stresses (Beneduzi *et al.*, 2012; Lakshmanan *et al.*, 2014; Maffei *et al.*, 2014; Chitarra *et*

al., 2016; Ruiz-Lozano *et al.*, 2016; Quiroga *et al.*, 2017; Schädler and Ballhorn, 2017; Chialva *et al.*, 2018). Plants in association with soil beneficial biota can show an enhanced phenotypic plasticity (Goh *et al.*, 2013). This is particularly advantageous in changing environments where fine-tuned allocation of limited resources between growth and defense is critical for survival (Lakshmanan *et al.*, 2014; Pozo *et al.*, 2015). Due to this positive influence on plant communities soil biota is considered a key factor in ecosystem stability and resilience (Yang *et al.*, 2018).

3.1. The arbuscular mycorrhizal symbiosis

Among the beneficial microorganism able to interact with plants in the rhizosphere, mycorrhizal fungi stand out due to their great ecological relevance and widely distribution. These species can colonize plant´s roots establishing a mutualistic symbiosis known as mycorrhiza (from Greek *mikos*, fungi, and *rhiza*, root). Both symbionts are benefited, the fungi provide mineral nutrients and water to the host plant and, in return, the plant transfers carbohydrates resulting of photosynthesis to the fungi in the roots (Bonfante and Genre, 2010).

Approximately 90% of plant species are able to form this symbiosis, that is usually divided into two main categories, known as ecto- and endomycorrhizas, depending on whether the fungus colonizes the roots intercellularly or develops inside the host cells (Bonfante and Desirò, 2015). Endomycorrhizas in turn, can be grouped in three major types attending on morphological differentiation of root tissues and host plant lineages: ericoid mycorrhiza, orchid mycorrhizas and arbuscular mycorrhizas (van der Heijden *et al.*, 2015). Among them, arbuscular mycorrhizas are the most widespread and present greater importance from a beneficial point of view for the plant (Parniske, 2008).

Arbuscular mycorrhizas (AM) date back to the Ordovician, and are considered one of the most ancient and widely distributed symbiosis between fungi and land plants, and it is accepted that they played a key role in facilitating colonization of land by plants. They are formed by more than 70% of vascular plant species (Brundrett and Tedersoo, 2018) and arbuscular mycorrhizal fungi (AMF) species from the phylum Glomeromycota. Although their phylogeny and taxonomy have been constantly discussed and subjected to several changes (Schüßler *et al.*, 2001; Spatafora *et al.*, 2016; Wijayawardene *et al.*, 2018). A recent study assigned three classes, four orders, twelve families and 33 genera

to this phylum (Wijayawardene *et al.*, 2018), being currently represented by about 250 described species, although new AMF species are frequently described (Bonfante and Desirò, 2015).

The AMF are obligate biotrophs as they fully rely on plant the carbohydrates to complete their life cycle. It has been reported that up to 20% of photosynthetically fixed carbon may be received by the fungus (Bago *et al.*, 2000). In addition, it has been recently discovered that the host plant feeds the fungus not only with sugars but also with fatty acids (Keymer *et al.*, 2017). Once the AM have been established, the fungus develops an extended extraradical mycelium (ERM) able to act as a highly effective complementary radicular system. For example, the associated hyphal length density can reach more than 100 m per cm^{-3} of soil in prairie and pastures (Miller *et al.*, 1995), thus favoring the absorption of water and mineral nutrients (mainly phosphate and nitrogen) to the host plant.

The main characteristic of this symbiosis is the presence of arbuscules (from Latin, "*arbusculum*", meaning bush or little tree). These little tree-like subcellular invaginations are formed once the AMF colonizes the cortex cells of the roots and subsequently is surrounded by a plant-derived peri-arbuscular membrane, which prevents direct contact of the fungus with the plant cytoplasm (Parniske, 2008). It is in the arbuscule where the bidirectional nutrient exchange between symbionts takes place (Luginbuehl and Oldroyd, 2017). In addition, some AMF species also form other structures inside the roots known as vesicles. They are balloon-shaped structures organs acting as lipids storage of the fungus.

3.2. Arbuscular mycorrhiza life cycle

Establishment of the AM symbiosis is normally described in two steps: i) presymbiotic phase, which comprises from AMF spore germination until the fungal structures penetrates the host root, and ii) symbiotic phase, where fungus develops both intra- and extraradical mycelium and symbiosis structures, ending with the formation of new spores (Figure 8).

i) Presymbiotic phase

Germination. The AMF spores are in the soil as resistance structures, waiting for favorable conditions in their environment since their germination is influenced by abiotic and biotic factors (temperature, pH, light, humidity, etc. and microorganisms, root exudates, etc., respectively.). If a germinated spore does not find a possible host, it arrests its growth and retracts its cytoplasm back into the spore coming back into dormancy state, process that can be repeated up to 10 times (Bonfante and genre 2010).

Growth. After germination, a hypha develops from the germinative tube that will spread and branch in order to reach roots to colonize. This growth is erratic until reaching the rhizosphere of a host plant, where the perception of root exudates triggers hyphal branching towards the root. The most studied compounds with this function are strigolactones, a group of terpenoid lactones released into the rhizosphere by most plant roots (Lopez-Raez *et al.*, 2017). Reciprocally, the fungus produces signal molecules called myc factors that allow the recognition of the fungus by the plant, inducing the expression of host genes necessary for the establishment of the symbiosis. These *myc* factors are also able to stimulate the development of lateral roots favoring the contact between the symbionts. Finally, this process culminates in the formation of a swollen and highly branched hypha attached to the host plant root epidermis, called hyphopodium, which will initiate intraradical colonization.

Penetration. The fungus penetrates the host plant through the lateral younger roots (Tawaraya *et al.*, 2007). Unlike what is described for a large number of pathogens, penetration will never happen through damaged or wounded cells. After the contact, host cell undergoes drastic subcellular changes to facilitate the passage of fungal structures. An ephemeral structure composed of an aggregation of cytosol, cytoskeleton and organelles is formed. This structure, known as prepenetration apparatus, develops as cytoplasmic bridge across the cell, which is surrounded by endoplasmic reticulum, cytoskeleton and plasma membrane, guiding hyphal tip invagination and growth across the host cell (Genre and Bonfante, 2016; Pimprikar and Gutjahr, 2018).

ii) Symbiotic phase

Intracellular hyphal growth. The symbiotic phase initiates with the penetration hyphae advances growing towards the inner cortex. Depending on the type of growth of the hyphae, authors distinguish two colonization patterns: *Arum*-type, where hyphae mostly

growth intercellularly (most common), and *Paris*-type, where hyphae spread via intracellular passage of cortical cells (typical of tropical plants and much slower).

Arbuscules formation. Once the inner cortical cells have been reached, the intercellular hyphae branch laterally, penetrate the cell and divide in a dichotomous manner, forming the characteristic tree-like structures of this symbiosis, called arbuscules. Arbuscules are the site of nutrient exchange between the plant and the fungus and are therefore of crucial importance for the AM symbiosis. In each cell a single arbuscule is formed, continuously surrounded by a plant-derived membrane called the peri-arbuscular membrane**,** which prevents direct contact of the fungal hyphae with the plant cytoplasm and serves as the interface for nutrient transference between the symbionts (Luginbuehl and Oldroyd, 2017; Smith and Smith, 2011). Arbuscules are a very dynamic system, continuously generating and collapsing, with a relatively short life span of about two to eight days. After an arbuscule degradation, the cell recovers its initial morphology being capable of accepting again the formation of a new arbuscule (Kobae *et al.*, 2018; Walter *et al.*, 2010).

Vesicles formation. The hyphae can also form globular structures with a high lipid content, known as vesicles, whose main function is to serve as reserve organs for the fungus. The vesicles can be formed both intra- and extracellularly, and their number depends both on the species of AMF colonizing and on the conditions in which the symbiosis is found. In some AMF species, the vesicles can lead to the formation of spores inside the root, as in the case of *Rhizoglomus irregulare* (Smith and Read, 2008).

Extraradical mycelium development and spores formation. After the formation of the first arbuscules, the extant external hyphae strengthen and branch intensively until forming the so-called branched absorbing structures, whose main function is nutrient uptake (Bago *et al.*, 1998). This external mycelium can explore a volume of soil that otherwise would be inaccessible to the roots. Therefore, the host plant considerably increases the surface for absorption of nutrients and water. Finally, some of the extraradical hyphae will originate new spores, thus completing the life cycle of the fungus.

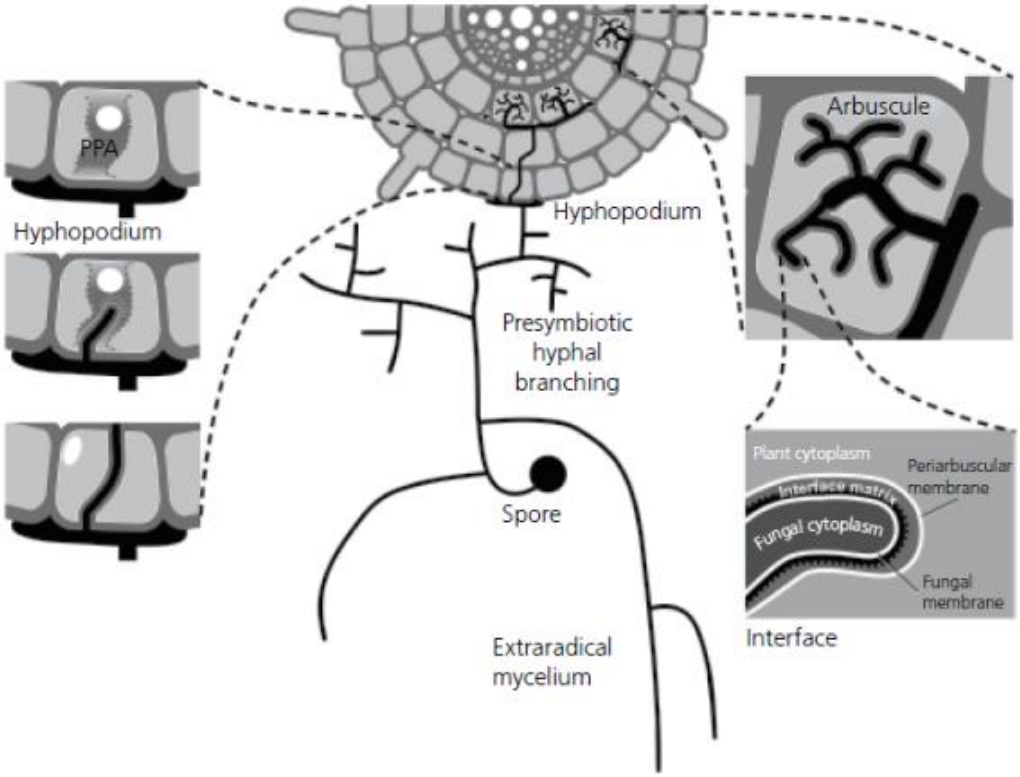

Figure 8 Steps in the establishment and major structural features of arbuscular mycorrhizas (Genre and Bonfante, 2016).

3.3. Regulation of the mycorrhizal symbiosis

The establishment, development and functioning of the AM symbiosis requires a high degree of coordination between both partners, based on a finely regulated molecular dialogue that integrate complex symbiotic programs (Kiers *et al.*, 2011). Upon recognition of the AMF in the rhizosphere, the plant must accommodate the fungus in the root tissue and control its spread through an important transcriptomic and metabolic rearrangement (Genre and Bonfante, 2016; Pimprikar and Gutjahr, 2018).

Regulation of the presymbiotic stage: during the molecular dialogue preceding root colonization, the so-called "Myc factors" from the fungus and the exudates released by the roots, give information to both partners about their reciprocal proximity. Myc factors are a mixture of lipochitooligosaccharides that act as diffusible signals known to be perceived by the plant and responsible of induce key changes in host cells that are necessary for mycorrhization (MacLean *et al.*, 2017). In the case of plants exudates, strigolactones (SLs) are carotenoid-derived phytohormones whose perception by AMF

since regulate crucial steps as spore germination, hyphal branching and efficient hyphopodium formation, thus increasing the chance of both partners to encounter (Kobae *et al.*, 2018; Lopez-Raez *et al.*, 2017).

It has been proposed that during the early phase of colonization, there is a transient and locally induction of SA (response against biotrophs), following of an active suppression that allows to continue with the colonization. Some studies support that this SA suppression could be due to secreting effector proteins by the AMF with the subsequent interference in the host's immune system (Kloppholz et al., 2011). In addition, several studies have focused in how AM establishment modulates SMs accumulation in roots. It has been reported that colonization of *Medicago truncatula* by *R. irregulare* induce an altered patter of SMs accumulation, with modifications in the levels of amino acids, fatty acids, apocarotenoids, flavonoids and carnitine-derivatives (Laparre *et al.*, 2014; Schliemann *et al.*, 2008).

In a well-established symbiosis, the development of AMF within the roots is mostly controlled by the host plant. In addition, the nutrient exchange between the symbiotic partners, is strongly regulated via transcriptional changes (Limpens and Geurts, 2018). It should be noted that this regulation of the symbiosis could be modulated under different environmental context, process regulated by phytohormones. For example, when the plant meets its own P requirements without the fungus, it avoids an excessive colonization through a repression of essential symbiotic genes, as those involved in SLs biosynthesis (Breuillin *et al.*, 2010; Lopez-Raez and Bouwmeester, 2008). On the contrary, different environmental conditions, as abiotic stresses drought or salinity, may increase the synthesis of SLs through overexpression of key genes in apocarotenoid biosynthesis pathway (López-Ráez, 2016).

Phytohormones interact to regulate the establishment and functioning of the AM symbiosis (Bedini *et al.*, 2018; Gutjahr, 2014; Liao *et al.*, 2018) and at the same time coordinate plants responses to the different environmental cues (Pozo *et al.*, 2015). (Figures 9 and 10) Interestingly, several works also showed in a well-established mycorrhizal symbiosis an altered content of several stress-related phytohormones (mainly JA, SA and ABA) (Fernandez *et al.*, 2014; Gutjahr, 2014; López-Ráez *et al.*, 2010; Pozo *et al.*, 2015).

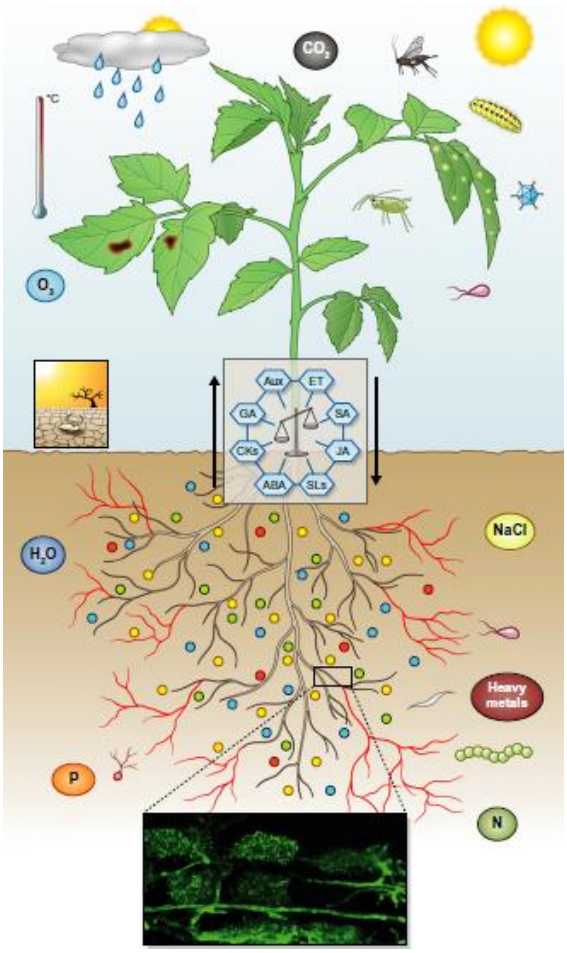

Figure 9 Phytohormones as mediators of the context dependence of mycorrhiza establishment and function (Pozo *et al.*, 2015)

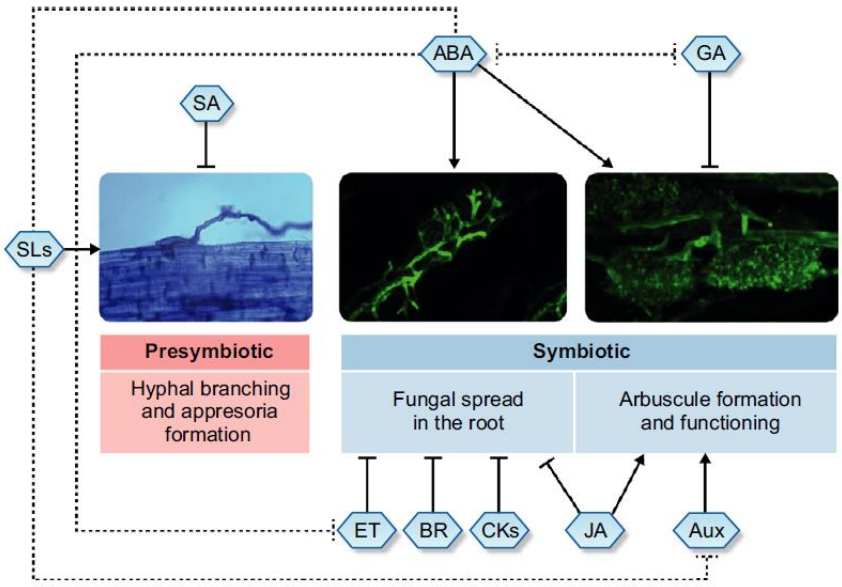

Figure 10 Phytohormone regulation of arbuscular mycorrhiza (AM) formation and functioning (Pozo *et al.*, 2015).

These altered phytohormonal levels have been found to be highly dependent on the genotype of the plant and fungal involved (Fernandez *et al.*, 2014). Fernández and collaborators observed different patterns of colonization between AMFs *R. irregulare* and *Funneliformis mosseae*, showing the first one higher colonization rates, but surprisingly the response phytohormonal alteration was lower in *R. irregulare* colonized-roots. They hypothesized that both situation may derive from a better ability of *R. irregulare* in "scape" of the control of the host plant (Fernández *et al.*, 2014).

Several authors have related this altered phytohormone homeostasis in mycorrhizal plants with their reinforced tolerance and resistance to stresses (Fernandez *et al.*, 2014; Jung *et al.*, 2012). Among these phytohormonal variations, the greatest accumulation of JA related compounds stands out due to its known role as a key stress-related regulator in plant-response against stresses.

3.4. Host plant benefits from arbuscular mycorrhizal symbiosis

Several benefits of the symbiosis have been described for the host plant. The fungus assists the host plant in the acquisition of water and mineral nutrients. This is extremely important for the uptake of inorganic phosphate, nitrogen and various microelements. under scarcity conditions. In addition to these nutritional improvements, the symbiosis enhances the plant ability to face stressful conditions, protects the host plant from different abiotic and biotic stress factors.

3.4.1. Improvement of mineral nutrition

The AM hyphal network can extend into areas that would not be accessible to roots of non-colonized plants. Due to the small hyphal size (diameter < 10 μm) they can penetrate into soil pores and cavities that the roots could not. Thus, the hyphae can absorb and transport water and nutrients from long distances, far from the depletion area of them around the roots, which is a great advantage for those mineral nutrients that diffuse slowly in the soil.

The best studied benefits of the symbiosis are related to the host plant P status. Plants can obtain Pi through a direct pathway by transporters in the root epidermal cells. Mycorrhizal

plants, in addition to this direct pathway can also use a mycorrhizal via, where Pi uptake occurs through the ERM. Several studies have shown that the mycorrhizal via may suppose the most part of P uptake, although the contribution of each of the pathways seems to vary highly according to the species involved (Li *et al.*, 2006; Smith *et al.*, 2011). Several Pi transporters have been identified and characterized in AMF, whose transcripts are predominantly expressed in ERM under low P availability (Benedetto *et al.*, 2005; Maldonado-Mendoza *et al.*, 2001). Subsequently, the Pi incorporated is transported is condensed into polyphosphate and transported to the arbuscules where it is finally hydrolyzed and translocated from the fungus to the plant cell cytosol (Ezawa and Saito, 2018; Hijikata *et al.*, 2010). Since Pi transport is crucial for the correct development of the symbiosis, the genes coding for these transporters are commonly used as markers of the functionality of the symbiosis, as *SlPT4* for *Solanum lycopersicum*, whose expression is restricted to arbusculated cells (Balestrini *et al.*, 2007).

In contrast to P acquisition, traditionally, the role of AM symbiosis in nitrogen (N) acquisition was neglected. However, more recent studies pointed out the importance of AM via in N uptake (Hodge, 2017). ERM can capture N from both inorganic ions (NO_3^- and NH_4^+) and organic compounds (small peptides or amino acids). In this sense, AM appears to contribute in higher degree to its host N acquisition when the source is the less mobile ion NH_4^+ (Hodge and Storer, 2015). As for Pi uptake, several studies have been characterized high-affinity ammonium transporters (López-Pedrosa *et al.*, 2006) and amino acid and nitrate transporters (Cappellazzo *et al.*, 2008; Tian *et al.*, 2010) in mycorrhizal mycelia. After the N uptake by ERM, this element is incorporated into arginine, the main long-distance transport molecule for N, and translocated into the arbuscule. Then, arginine is metabolized and resulting free NH_4^+ is then release into the periarbuscular space when it will be incorporated by the host plant (Govindarajulu *et al.*, 2005). Recently, it was shown that mycorrhizal plants can better respond to N depletion with a more efficient finetuning of the growth defense balance (Sánchez-Bel *et al.*, 2018).

This facilitation in the acquisition of nutrients may become essential for growth and survival in soils with severe deficiencies of these minerals, a situation that can be considered as abiotic stress. However, AM symbiosis not only helps the plant by favoring nutrient and water uptake, but also induces significant changes in multiple host traits, such as root architecture, growth rate, flowering and especially stress resistance (Chen *et al.*, 2018; Pozo *et al.*, 2015).

3.4.2. Improved abiotic stress tolerance in AM plants

The improved nutrition in mycorrhizal plants could be considered an indirect mechanism of defense since underlying enhance tolerance/resistance against stresses. In this sense, pioneering studies almost exclusively attributed host plant benefits to the improved nutritional status (Nelsen and Safir, 1982). However, subsequent experiments with nutritional supplements (as increased level of Pi) demonstrated that this better nutritional status was not the only mechanism promoting the increase in tolerance to stress (Fritz *et al.*, 2006; Poss *et al.*, 1985).

The fact that the plants colonized by AMF present a better development and fitness than non-mycorrhizal ones under different stressful conditions is widely accepted, with a lot of examples in literature (Bernardo *et al.*, 2019; Bitterlich *et al.*, 2018; Estrada *et al.*, 2013b; Quiroga *et al.*, 2018; Ruiz-Lozano *et al.*, 2016; Sánchez-Romera *et al.*, 2016; Santander *et al.*, 2017; Santander *et al.*, 2019), and the mechanisms underlying such protection may be diverse.

In the case of drought stress, the simplest mechanism is the facilitation of water supply via ERM from soils with low water potential (Santander *et al.*, 2017). In drought conditions, AM inoculation showed to modulate endogenous phytohormonal levels and upregulation of aquaporin gene expression, resulting in an increased tolerance by amelioration of root hydraulic conductivity inhibition (Barzana *et al.*, 2014; Quiroga *et al.*, 2018; Sánchez-Romera *et al.*, 2016).

ABA is the main phytohormone regulating plant responses against drought and salinity. In AM plants grown in the absence of stress, ABA levels remains unaltered or even reduced (Fernandez *et al.*, 2014; López-Ráez *et al.*, 2010). However, upon osmotic unfavorable conditions, levels of this phytohormone increase, which can be related with a primed response against such abiotic stresses (Aroca *et al.*, 2013). This ABA-primed response is of great importance for many processes, as for example the reported alteration in roots hydraulic properties allowing an increased water uptake during drought (Ruiz-Lozano et al., 2012). Also, in AM plants under stress an ABA dependent mechanism regulate the gene expression for the synthesis of aquaporins (Ruiz-Lozano *et al.*, 2009), water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient (Li *et al.*, 2014).

In addition, one of the main problems as consequence of osmotic stress, and especially in saline soils, is the mineral nutrient imbalanced in plant tissues. The antagonism of Na^+ towards K^+ produces cytotoxic effects on host plants, such as disruption of the enzymes structure or cell organelles damage, resulting in the inhibition of photosynthesis, respiration and protein synthesis. In this sense, several authors have reported that AM symbiosis can alleviate these deleterious effects through regulation of the expression of several genes encoding plant transporters involved in ion homeostasis, resulting in a reduction in the Na^+ ions in shoot tissues by its translocation to roots (Estrada *et al.*, 2013b; Porcel *et al.*, 2016; Santander *et al.*, 2019). Other boost-responses to deal with salinity and drought reported in AM plants is the strongest accumulation of SMs with an osmoprotectant role, as proline or glycine betaine (Bárzana *et al.*, 2015; Chun *et al.*, 2018; Santander *et al.*, 2019). Flavonoids, metabolites with protective antioxidant role, have been reported to be overaccumulated in AM plants subjected to water-related stresses (Abbaspour *et al.*, 2012). Moreover, a recent study reports AM-modulation during drought conditions on several groups of SMs, such as phenolic compounds, lipids and sugars (Bernardo *et al.*, 2019)

3.4.3. Induced resistance against biotic stress in mycorrhizal plants

Mycorrhizal plants have been also shown to be more resistant-tolerant to some biotic stress conditions. The first studies focused on the root, where several authors reported that the establishment of AM reduced the damage caused by different soil-borne pathogens as bacteria, fungi or nematodes, and phytophagous insects (Davis and Menge 1980, (Bärtschi *et al.*, 1981). However, the mechanisms were poorly understood, and in the case of fungal diseases, were mainly associated to a direct induction of some defense-related enzymes such as chitinases and glucanases (Pozo *et al.*, 1998) due to their potential to hydrolyze fungal cell wall polysaccharides. The potential of protection against pathogens was not limited to the roots colonized by the AMF. In split-root systems, the noncolonized roots from AM-plants displayed also enhanced resistance compared to nonmycorrhizal ones, suggesting that the induced resistance was systemically transmitted (Pozo *et al.*, 2002). Subsequent studies found that this protection could also be observed against certain aggressors in aboveground tissues of colonized plants (Pozo and Azcon-Aguilar, 2007; Sanchez-Bel *et al.*, 2016; Song *et al.*, 2015; Tao *et al.*, 2016).

During mycorrhiza establishment, to achieve a functional symbiosis it is necessary the modulation of plant defense responses. During the first stages of symbiosis there is a hormonal reprogramming contributing to changes in the transcriptional responses and in the secondary metabolism (Pozo *et al.*, 2015). This modulation can result in the activation of enhanced resistance capacity known as induced systemic resistance (Pieterse *et al.*, 2014) that stimulates the plant immune system towards an alert state, allowing a more efficient activation of defense mechanisms in response to future biotic challenges. Therefore, this specialized bioprotection achieved by mycorrhization has been denominated as mycorrhiza-induced resistance (MIR) (Pozo and Azcón-Aguilar, 2007), with increasing evidences suggesting that defense priming is behind MIR (Bruisson *et al.*, 2016; Gupta *et al.*, 2017; Jung *et al.*, 2012; Pieterse *et al.*, 2014; Pozo and Azcón-Aguilar, 2007; Sánchez-Bel *et al.*, 2016; Song *et al.*, 2015; Song *et al.*, 2013).

Different studies evidence a wide range of protection in AM plants against below-ground attackers such as soil-borne pathogens, nematodes or root-chewing insects (Currie *et al.*, 2011; Hao *et al.*, 2018; Jung *et al.*, 2012; Nair *et al.*, 2015; Olowe *et al.*, 2018; Pozo *et al.*, 2002; Schouteden *et al.*, 2015; Vos *et al.*, 2012). However, less reports of MIR against shoot herbivory can be found along literature compared to below-ground interactions. The outcome of the symbiosis in aerial parts is more variable and will depend on the lifestyle of the attacker (Jung et al., 2012; Pozo and Azcon-Aguilar, 2007; Singh and Giri, 2018) (Figure 11).

Regarding to viruses there is not much information. First studies in general described a higher susceptibility in AM plants (Daft and Okusanya, 1973; Shaul *et al.*, 1999). However, more recent reports shows controversial results, for example indicating a beneficial role of colonization in reduce virus severity symptoms in tomato plants infected by Tomato yellow leaf curl Sardinia virus (TYLCSV) (Maffei *et al.*, 2014), or increasing plant susceptibility against Tomato spotted wilt virus (TSWV) (Miozzi *et al.*, 2011). For biotrophic pathogens, is generally reported that can perform better on mycorrhizal plants (Jung *et al.*, 2012; Whipps, 2004). For example. it has been reported a higher susceptibility in AM against powdery mildew caused by *Erysiphe* spp. on barley and *Astragalus adsurgens* plants (Gernns et al., 2001; Liu et al., 2018).

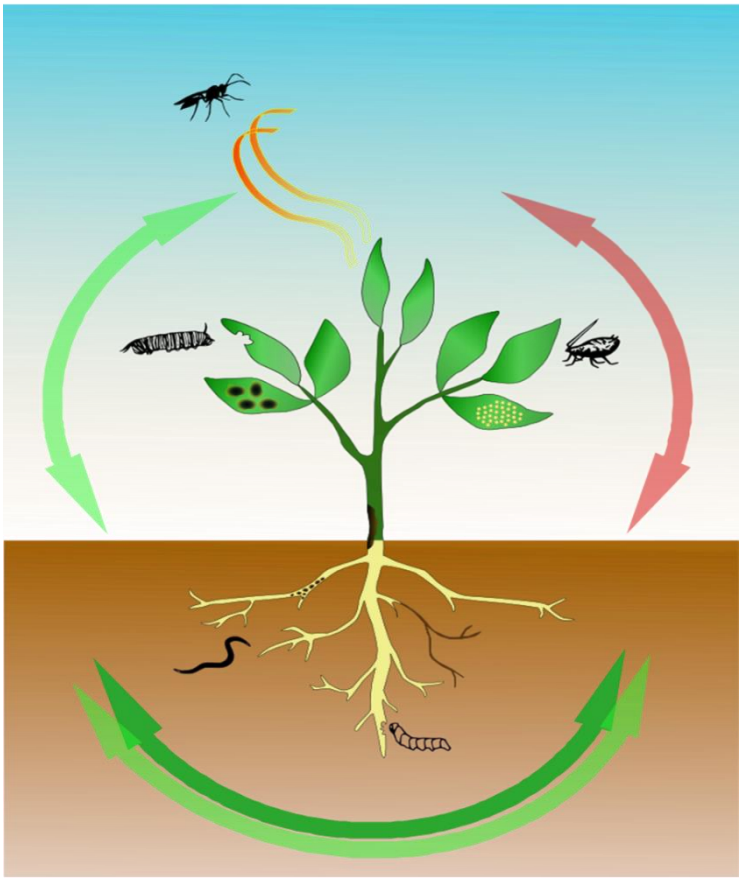

Figure 11 Effectiveness spectrum of mycorrhiza induced resistance (MIR). AM symbiosis confers protection against a wide range of root aggressors. Host protection is also effective in shoot tissues, mainly against necrotrophic pathogens and herbivores. On the other hand, biotrophic fungi, viruses and phloem-sucker insects in general develop better in mycorrhizal plants. In addition, AM plants also positively influence indirect defenses increasing the recruitment of natural enemies via modulation of volatiles emitted after herbivory. Green arrows indicate resistance, while red arrows indicate susceptibility in AM plants (Pozo and Azcón-Aguilar, 2007)

In contrast, the positive effect of AM symbiosis on plant resistance against necrotrophic shoot pathogens is widely reported, as for example against *Botrytis cinerea* (Bruisson *et al.*, 2016; Fiorilli *et al.*, 2011; Sánchez-Bel *et al.*, 2016), or *Alternaria* spp. (Fritz *et al.*, 2006; Nair *et al.*, 2015b; Song *et al.*, 2015). Interestingly, Sánchez-Bel and collaborators (2016) demonstrated an effective protection is context dependent, since transient nitrogen depletion perception by the plant interferes with MIR, since plant reprograms its defensive mechanisms towards prioritize abiotic stress tolerance.

Concerning the effects of mycorrhiza on plant-herbivore interactions, different outputs are reported, likely depending on many factors, such as the AMF, host plant and insect species interacting, and environmental factors (Pineda *et al.*, 2010b; Roger *et al.*, 2013). The effect and efficiency of MIR will vary depending on the degree of specialization of

the phytophagous herbivore, and the of its feeding guild. Generalist insects are usually reported as negatively affected by the presence of mycorrhizas, whilst specialists, which feed from one or only a small number of species (Ali and Agrawal, 2012), usually perform better on mycorrhizal plants, probably because of the improved nutritional quality of the host (Hartley and Gange, 2009; Jung *et al.*, 2012). For example, Minton and collaborators (2016) observed that despite AMF influences on defenses (increased proteinase inhibitor responses), caterpillar growth of specialist *Manduca sexta* in two Solanum species was unaffected, while the generalists *Spodoptera exigua* and *Helicoverpa armigera* showed an impaired development when feed on mycorrhizal plants (He *et al.*, 2017; Song *et al.*, 2013).

Attending on the feeding guild, phloem-sucker insects, such as aphids, whiteflies and other Hemiptera, showed a higher incidence in mycorrhizal plants (Jung et al., 2012). In the case of leaf chewers and miners they usually are negatively affected by mycorrhization (He *et al.*, 2017; Song *et al.*, 2013).

However, several reports differ from this general proposed model (Bernaola *et al.*, 2018; Tomczak *et al.*, 2016). In addition, several works observed that the beneficial is also dependent on the AMF specie inoculated, pointing out to certain functional diversity in inducing MIR. For example, He and collaborators (2017) found *F. mosseae* provides higher protection against *S. exigua* compared with AMFs *R. irregulare* and *Glomus versiforme*, and even different isolates from the same specie were reported to modulate plant resistance in a different-manner (Roger *et al.*, 2013). Therefore, improving our knowledge on the mechanisms underlying MIR is essential to unravel in which multitrophic models (host plant-AMF isolate-insect-environmental conditions) the protection will be effective. This fact will result in better predictive models, that will favour the application of AM in crops purpose (Jacott *et al.*, 2017).

Overall, we observe a general trend regarding the spectrum of MIR, whose protection role is mainly effective against necrotrophic pathogens and chewing leaf insects, curiously those organisms strongly impaired by JA-dependent defenses (Jung et al., 2012). In addition, and as commented in previous chapter, JA also mediates ISR by beneficial microbes (Pieterse *et al*., 2014), showing a remarkable primed response upon biotic challenge. It also reinforces this JA role in MIR the observations of altered transcriptomic profile in oxylipin pathway, which is responsible of its biosynthesis (Fernández et al., 2014; López-Ráez et al., 2010).

Altogether, these evidences suggest that JA-regulated plant defense mechanisms are key elements in the induction of resistance by arbuscular mycorrhizas (Jung *et al.*, 2012). In this sense, different authors confirmed the JA-priming in AM plants under biotic aggressions (He *et al.*, 2017; Sanchez-Bel *et al.*, 2016; Song *et al.*, 2015; Song *et al.*, 2013). This crucial role of JA is also supported by a recent study, where MIR against *Spodoptera littoralis* was more enhanced in JA-deficient mutants than on JA over-accumulating tomato plants, suggesting that the ability of increases JA by AM is crucial for the enhanced mycorrhizal defense (Formenti and Rasmann, 2019).

The effect of mycorrhizas can go beyond impacting direct defenses. A pioneer study from Guerrieri and collaborators showed that tomato plants colonized by *F. mosseae* attracted more the parasitoid *Aphidius ervi* (Guerrieri *et al*., 2004). Subsequent studies using *Phaseouls vulgaris* as model plant confirmed these patterns. AM plants infested with spidermite *Tetranychus urticae* showed a better fitness compared with noncolonized ones due to an enhanced preference of the predatory mite *Phytoseiulus persimilis* (Hoffman *et al.*, 2011a; Hoffmann *et al.*, 2011b). As mentioned, the arbuscular mycorrhizal symbiosis can modulate the host plant secondary metabolism, including effects on volatile compounds. In this sense, altered concentration and composition of terpenoids, important constituent of HIPVs, have been reported (Rapparini *et al.*, 2008; Sharma *et al.*, 2017). Moreover, emission of GLV (Z)-3-hexenyl acetate was higher in AM *plantago lanceolata* mycorrhizal plants in absence of stress (Fontana *et al.*, 2009), a compound with reported role in natural enemies attraction (Shiojiri *et al.*, 2006; Tahmasebi *et al.*, 2014).

Using the same model, terpenoids β-ocimene and β-caryophyllene were found over-emitted in mycorrhizal plants after *T. urticae* infestation, being related with higher attraction of predator at time *P. persimilis* (Schausberger et al., 2012).

Finally, the AM ability to recruit natural enemies may be modulated by other stresses. Higher incidence and development of aphids have been reported in symbiotic plants (Babikova *et al.*, 2014; Volpe *et al.*, 2018). However, the recruitment of parasitoid *Aphidius ervi* is also enhanced in *R. irregulare* colonized-plants, and even reinforced in water stress conditions, (Volpe *et al.*, 2018). The authors hypothesize that the observed effect is due to increased emission of Methyl salicylate in AM plants, a volatile compound previously shown to attract the parasitoid (Sasso *et al.*, 2007).

4. Metabolomics in plant resistance research

4.1. Untargeted metabolomics

Metabolomics comprises the different biochemical approaches that allow the detection of low molecular weight compounds substrates and products of metabolism, namely metabolites (typically between 50-1000 Da), in a given organ, tissue or cell type (Fiehn, 2002). This -omic includes several techniques. Among them, liquid or gas chromatography coupled with mass spectrometry (LC/MS, GC/MS) and nuclear magnetic resonance spectroscopy (NMR) are the most used techniques (Cajka and Fiehn, 2016; Kim *et al.*, 2011). Focusing on chromatographic tools coupled with mass spectrometry, this tool has shown to provide high sensitivity, reproducibility and versatility in this field. The chromatography allows to separate the mixtures of metabolites in complex matrices such as plant extracts. Subsequently, following generation of ions in the ionization source, the mass spectrometer determines the mass-to-charge ratios (m/z). Depending whether metabolomic analyses are focused on measuring the identity of a predefined set of metabolites or not, we differentiate between targeted or untargeted metabolomic (Johnson *et al.*, 2016). Both techniques are extremely helpful in the study of metabolic imbalance associated to defense responses. Targeted studies focused on the analysis of a few well-characterized metabolites to determine plant responses. Hence, targeted studies have been used for hormonal analysis, or quantification f amino acid content, specific vitamins, flavonoids, or plant toxic compounds in plant tissues.

These targeted analyses focused on the study of previously known groups of metabolites, thereby missing much of the complete chemical picture. Conversely, untargeted approaches are used to determine metabolites of different structural and chemical characteristics that belong to different metabolic pathways. This is especially important for plant secondary metabolites identification and quantification. Unfortunately, a limitation of this full profiling studies is the absence of commercially available standards for most of the compounds, therefore additional structural and *in silico* studies have to be used to complement the analysis (De Vos *et al.*, 2007). The signals obtained (potential metabolites) in an standard analysis could be over thousands, thus providing an important holistic overview on the functioning of the metabolic reprogramming in specific experimental conditions.

Recently, Peters and collaborators have defined the term “Eco-Metabolomics”, as the field inside metabolomic that comprises the study of bioactive compounds that allows understand the molecular mechanism underlying species interactions with the environment (Peters *et al.*, 2018). In plant research, metabolomic approaches are widely used for elucidating secondary metabolic changes involves in plant phenotypic adaptations to environmental changes or responses to other organisms, such as competitors, pathogens, pest or beneficial symbionts (Arbona *et al.*, 2013; Brunetti *et al.*, 2013; Hong *et al.*, 2016; Peters *et al.*, 2018).

While it is true that metabolomics provides of key information about the metabolic changes driving plant responses to their surrounding environment, other complementary -omics techniques are necessary in order to obtain a better understanding of the metabolic regulatory networks and their role in plant responses to abiotic and biotic stress.

4.2. Metabolomics in plant-priming research

Metabolomic is downstream of other –omics such as transcriptomic and proteomic, thus is the closest to diverse crucial biological processes determining plants ecological interactions. Untargeted metabolic approaches have been applied to unravel both plant responses to deal with abiotic and biotic stresses (Balmer *et al.*, 2013; Nakabayashi and Saito, 2015; Obata and Fernie, 2012; Rodziewicz *et al.*, 2014; Tenenboim and Brotman, 2016). The increase of knowledge about the main pathways and metabolites mediating tolerance/resistance in hostile environment give us valuable information of plant defensive mechanisms.

Different reports have described that plants can reach a primed phase after a chemical application of known primed-state inducers, as β-aminobutyric acid or hexanoic acid, being an important tool to identify metabolites underlying induced resistance. For example, exogenous application of β-aminobutyric acid application was shown to trigger metabolic reorganization in plants to anticipate against future attack (Pastor *et al.*, 2014). Hexanoic acid and azelaic acid (natural signaling compound) were compared in their ability to reorganize for enhance plant defense in tobacco cells, finding several common patterns such as increased accumulation in phenylpropanoids-polyamine conjugates (PPCs) (Djami-Tchatchou *et al.*, 2017). Evaluate the observed primed-metabolites during defense priming phase in their potentially anti-stress role can be of extreme importance in

order to develop future strategies well enhancing these key metabolites synthesis or well through their exogenous application. In Arabidopsis plants, priming induced by β-aminobutyric acid treatment showed higher accumulation of indole-3-carboxylic acid and hypoxanthine, whose subsequent exogenous application showed protection against the necrotrophic fungi *Plectosphaerella cucumerina* (Gamir *et al.*, 2014).

For this reason, a branch of metabolomics studies has been focused on discovering compounds belonging to "priming-fingerprint" underlying processes of plant displaying induced resistance *al.*, 2014; Tugizimana *et al.*, 2018). Then, metabolomic could be understood as a part, together with transcriptomic and proteomics, of techniques aimed to unravel the different faces of priming, that has been named as "prime-ome" (Balmer *et al.*, 2015).

It should be noted that untargeted metabolomic allows us to identify potential key compounds mediating induced resistance against a broad number of aggressor. However, the fact that a compound is overaccumulated after stress does not mean that it plays an active role in response to it, since it may be the result of a collateral reorganization of the metabolism. For this reason, it is essential to evaluate the activity of the potential metabolites in mediating mitigation against stress. For example, a metabolomic study showed a high accumulation in the metabolite 1-methyltryptophan in tomato plants under attack by *B. cinerea* and *Pseudomonas syringae*. Subsequent root application of this compound demonstrated its ability in protecting tomato plants against the pathogens (Camañes *et al.*, 2015)

Several studies have been focused on identifying the metabolic changes associated to ISR. For example, Arabidopsis associated with the rhizobacterium *Pseudomonas fluorescens* show an enhanced resistance against several bacterial pathogens and the insect pest *Spodoptera exigua*. A metabolomic study in this model pointed to a prominent role of glucosinolates and camalexin in plant enhanced resistance (van de Mortel *et al.*, 2012). Other study suggested the key role of phenylacetic acid in mediating ISR *against fusarium* wilt disease in tomato plants associated with beneficial rhizospheric bacterium *Bacillus fortis* (Akram et al., 2016).

4.3. Metabolomics in AM research

Metabolomics approaches have been used to decipher the soil dialogue in the rhizosphere between plant roots with both beneficial and deleterious organism (Oburger and Schmidt,

2016; van Dam and Bouwmeester, 2016). In this sense, several studies have focused in unraveling the metabolic reprogramming observed in plant roots associated with soil beneficial organisms, such as in the legume–rhizobia symbiosis (Zhang *et al.*, 2012).

Some studies have explored the metabolic changes associated to mycorrhizas. For example, changes associated to the formation of mycorrhizas in poplar were reported (Kaling *et al.*, 2018; Tschaplinski *et al.*, 2014).

Regarding to the arbuscular mycorrhizal symbiosis, one of the main limitations for such studies is the obligate biotrophy of the fungi. Thus, the experimental design is crucial to understand changes compared with non-mycorrhizal plants. One of the first reports of an untargeted metabolomic study was performed on *M. truncatula* roots colonized by AMF *R. irregulare* (Schliemann et al., 2008). This study showed an increase in isoflavonoids, apocarotenoids and primarily fatty acids levels. A subsequent study performed by Laparre and collaborators (2014), also found a high number of metabolites overaccumulated in mycorrhizal roots, highlighting propionyl- and butyryl-carnitines compounds. Saia and collaborators analyzed the metabolic rearrangement in roots of durum wheat plants colonized by a commercial consortium of different AMF species, observing decreased accumulation of high number of metabolites in all metabolic pathways, especially amino acids and saturated fatty acids (Saia *et al.*, 2015).

Some studies have addressed the impact of other microorganisms in the plant-AMF interaction. Interestingly, co-inoculation with PGPR did not result in these low accumulations of these compounds. In other essay, modulation of root exudation in wheat associated with beneficial fungi *Trichoderma atroviride* or *R. irregulare* were compared (Lucini *et al.*, 2019). They found a distinctive metabolic modulation in function of the associated organism. These studies point out a fingerprint associated to arbuscular mycorrhizae in the modulation of secondary metabolism compared with other soil-beneficial organisms.

Less studies deal with metabolic changes in above-ground tissues of AM plants (Schweiger and Muller, 2015). A recent study compared the metabolomes from roots and shoot tissues in *Senecio jacobea* plants in symbiosis with *R. irregulare* (Hill *et al.*, 2018). Authors found significant changes in the root metabolome, mainly in previously reported apocarotenoids as blumenols and several pyrrolizidine alkaloids, whose potential role as anti-herbivore defense was discussed. Interestingly, in this study an altered shoot

metabolome by mycorrhizal establishment was no observed. Metabolic profiling in shoots of mycorrhizal *Lotus japonicus* plants suggested a negative impact on metabolites connected to central catabolic and amino acid metabolism (Fester *et al.*, 2011). These changes were found mainly in sink leaves, while the source leaves and flowers showed an increase in stress related metabolites. *Nicotiana attenuata* transgenic lines impaired in their ability to establish AM symbiosis showed a lower fitness and higher accumulation in foliar amino acids when they are in competition with AM-colonized plants (Wang *et al.*, 2018b). It was suggested that amino acid overaccumulation is related with the worst capacity in P uptake of noncolonized plants.

In a multi-species study comparing three species from Plantaginaceae family, *M. truncatula* (Fabaceae) and monocot *Poa annua* (Poaceae) significant increases in phosphorus were reported, whereas leaf metabolomes were only marginally altered (Schweiger *et al.*, 2014). Among the plant species analyzed, M. *truncatula* exhibited the highest modulation with approx. 15% of its metabolic features altered by the AMF colonization, while the Plantaginaceae species showed less than 6% and finally the monocot *P. annua* with 1.7%. However, a common fingerprint in the metabolomic rearrangement can be found within the dicotyledonous species, highlighting the decreased accumulation of several organic acids from the citric acid cycle. Finally, through a combination of untargeted and targeted metabolomics, Wang and collaborators recently discovered several blumenols derivatives (hydroxy- and carboxyblumenol C-glucoside) only accumulated in leaves from six mono- and dicotyledonous crop and model plants colonized by AMF *R. irregulare* (Wang *et al.*, 2018a). They demonstrated that these metabolites are transported from roots to shoots, being their accumulation positively correlated with AM percentage of colonization. Thus, authors showed that blumenols could be used as robust markers of AM symbiosis in the shoots

Considering that AM plants exhibit an enhanced ability to cope with both abiotic and biotic stresses, it is very interesting to evaluate the metabolic profile alteration upon stress. Besides the basal metabolic changes as consequence of AM establishment, this should allow the discovery of key metabolic pathways and compounds underlying the enhanced stress tolerance by mycorrhizal fungi. Several studies have focused in that field. For example, durum and bread wheat plants colonized by *F. mosseae* were subjected to drought, showing a better fitness, for example through an better water use efficiency, than noncolonized ones, and the authors observed that AM establishment altered root

secondary metabolome (Bernardo *et al.*, 2019). Several lipids from the cholesterol biosynthetic pathway were low accumulated in AM roots, suggesting they may be supplied to the AMF. Additionally, alkaloids and flavonoid showed lower levels in AM plants, and this was discussed as a consequence of the better plant fitness found in mycorrhizal plants.

Focusing on MIR against biotic stresses, several studies focused in unraveling the transcriptomic changes underlying enhanced defense (Song *et al.*, 2015; Song *et al.*, 2013; Wang *et al.*, 2018b), normally highlighting in the most cases the main role of JA-priming in MIR (He *et al.*, 2017; Nair *et al.*, 2015; Song *et al.*, 2015; Song *et al.*, 2013). Regarding metabolomic studies, most of them perform targeted approaches, quantifying main phytohormones and compounds with known role anti-stress, such as alkaloids or iridoid glycosides (Andrade *et al.*, 2013; Fontana *et al.*, 2009; Tomczak *et al.*, 2016). Interestingly, Sánchez-Bel and collaborators described the shoot metabolome in AM-tomato plants after 72h post infection with *B. cinerea* and its modulation by nitrogen starving perception (Sánchez-Bel *et al.*, 2018; Sanchez-Bel *et al.*, 2016). They reported overaccumulation of phenolic acids, amino acids, indoles and several intermediates in the oxylipin pathway (responsible of JA biosynthesis), relating this alteration with the observed protection. However, the analysis of changes only due to the AM establishment (priming phase) is missing.

INTEREST OF STUDY AND AIMS

Interest of the Study and Aims

Plants are continuously exposed to a broad range of challenges that can compromise their development and productivity. During the last decades, the human beings have has tried to avoid yield losses derived from environmental stresses mainly through an excessive use of fertilizers, pesticides, or irrigation water. However, these common practices have had undesirable effects as soil degradation, water and environmental pollution, especially by the abuse of pesticides. In addition, pesticides can have a negative impact on non-target organisms such as beneficial microbes and insects and a real threat to human health.

In the current scenario of social awareness of those risks, and considering the needs for environment protection and food security while maintaining high productivity in agriculture, environmentally friendly and sustainable strategies for agriculture are demanded. In this sense, the use of natural resources as soil beneficial microbes as inoculants is a promising strategy to improve crop health, especially in adverse environments.

Among these beneficial microorganisms, the widespread arbuscular mycorrhizal fungi (AMF) are receiving special attention. The present Doctoral Thesis focusses on the arbuscular mycorrhizal symbiosis (AM) that can be established between these fungi and the roots of most terrestrial plants. This beneficial partnership is reported to provide the host plant several advantages as better mineral nutrition and water acquisition and stress resistance.

Despite the reported potential benefits of the symbiosis provided to the host plant, the underlying mechanisms remain poorly explored. Previous studies propose that the protection conferred by the mycorrhizal symbiosis does not imply a direct activation of plant defenses, but instead, it induces a physiological state that results in an improved capacity to respond to stress, known as defense priming. However, a thorough study on the molecular mechanisms has not been performed.

In this PhD Thesis, we undertake an untargeted metabolomic approach to identify metabolic pathways and compounds with a potential role in AM protection against stress using tomato as a model system. Untargeted metabolomic approaches have been proved

to be an excellent tool in plant research, providing an overview of the plant secondary metabolism reprogramming under stress, and allowing the identification of potential key metabolites in plant protection.

Basic knowledge on the mechanisms underlying enhanced stress tolerance achieved through beneficial interactions with AMF is essential to understand their full potential and improve their biotechnological applications in agriculture.

Therefore, the overall aim of this PhD Thesis is ***to study the potential of different arbuscular mycorrhizal fungi to alleviate stress in tomato plants, and to explore the potential contribution of the metabolic reprogramming occurring in mycorrhizal plants growing under adverse conditions, in order to elucidate key metabolites underlying the enhanced resistance/tolerance against diverse stresses.***

To achieve the main objective, the following **specific objectives** were defined and addressed:

1. To describe the metabolic rearrangement occurring in roots upon colonization by two widely studied arbuscular mycorrhizal fungi *Funneliformis mosseae* and *Rhizoglomus irregulare* (Chapter 1).

2. To compare the ability of different mycorrhizal fungi (*F. mosseae*, *R. irregulare* and *Claroideoglomus etunicatum*) to alleviate the negative effect of abiotic stress conditions in tomato plants, and to identify the mechanisms underlying the enhanced tolerance, with special emphasis on change in metabolic profiles (Chapter 2).

3. To determine the effect of *F. mosseae* on tomato interaction with the phytophagous insect *Spodoptera exigua*, in order to evaluate the occurrence of mycorrhiza induce resistance and to identify key metabolites mediating such impact (Chapter

GENERAL MATERIALS & METHODS

General Materials & Methods

In this section I will address the most general aspects that are repeated throughout this doctoral thesis. Nevertheless, in some cases it would be required to go to specific materials and methods from different Chapters in order to obtain a more detailed information.

1. Biological material

1.1. Tomato

For our experiments *Solanum lycopersicum* cv. Moneymaker was used.

In Chapter 3 it was also used the jasmonic acid-signaling defective mutant (*jasmonic acid–insensitive1*; *jai1*), with its corresponding genetic background as control (cv. Castlemart).

1.2. Arbuscular mycorrhizal fungi (AMF) spp.

As obligate symbionts, AMF must be continuously maintained in an open-pot culture of *Trifolium repens* L. mixed with *Sorghum vulgare* Pers. (Steud.) Millsp. & Chase plants in a greenhouse. The final inocula consist of substrate (vermiculite/sepiolite, 1:1), spores, mycelia, and infected root fragments from those cultures.

In the present Doctoral Thesis, the following AMF spp. have been used:

- *Rhizoglomus irregulare* (Ri, formerly known as *Rhizophagus irregularis* or *Glomus intrarradices*) DAOM 197198
- *Funneliformis mosseae* (Fm, formerly known as *Glomus mosseae*) BEG 112.
- *Claroideoglomus etunicatum* (Ce, EEZ163), AMF isolated from a highly salinized soil from Cabo de Gata Natural Park (Almería, Spain), by Estrada *et al*. (2013b).

2. Seed surface sterilization and germination

Tomato seeds were surface-disinfected by immersion in 4% NaHClO (10 min) containing 0.02% (v/v) Tween 20®, followed by at least 3 rinsed thoroughly with sterile water (10 min each) to remove any trace of chemicals.

After surface disinfection, tomato seed were placed in an open container with sterile vermiculite at 25°C and incubated for 7-10 days under controlled conditions in the greenhouse. Then, small tomato seedlings were transplanted to pots and AMF-inoculated or not according to the treatment.

3. Growing substrates

Soil, sand and vermiculate were the substrate used for growing. Previously to the tomato transplanting, sand and vermiculate substrates were separately autoclaved (120°C, 20 min) before mixing them. In the case of soil, it was steam-sterilized (100ºC, 1 h for 3 d consecutively), and then mixed with autoclaved sand: vermiculite.

4. Greenhouse and growing conditions

Plants were grown within a greenhouse under controlled climatic conditions:

- Temperature 18-24°C
- Relative humidity 60-70%
- Photoperiod 16/8 light/dark respectively

Plants were randomly distributed, and moved each two days in order to avoid possible environmental imbalances (e.g. excessive sun, shade or wind flow).

Plants were watered intercalating tab water with Long Ashton nutrient solution (Hewitt, 1966) to supply them the nutrients, but containing a reduced concentration of standard phosphorus (25% P), in order to prevent mycorrhizal inhibition due to an excess of P.

5. AM inoculation

At the same time than plantlets were transferred to pots containing the sterile substrate, mycorrhizal treatments were inoculated by adding 10% (v/v) *of* AMF inoculum. Uninoculated control plants received a 3 ml aliquot of a filtrate (< 20 μm) of each AM inoculum, in order to provide the general microbial population but free of AMF propagules.

6. AM staining

The non-vital ink histochemical staining of AM structures was performed according to adapted protocol from published by Vierheilig *et al*., 2005. First of all, just after the harvest, roots were washed and subsequently incubated in 10% potassium hydroxide (KOH) in 15 ml falcon tubes and kept overnight at room temperature. This remove the cell root cytoplasm and most of the nuclei, and roots become very clear with the vascular cylinder distinctly visible. Subsequently, KOH is removed and roots were rinsed several times with tap water. Later, roots were incubated in acetic acid (CH_3COOH) during 5-10 min in order to acidify them. After this, acetic acid is removed and now roots are incubated with staining solution (5% commercial ink and 2% acetic acid) and kept in room temperature overnight. In this case, we used commercial black ink (*Sheaffer Skrip jet black®),* although another brand can be also used. Finally, ink solution is removed (can be used for future stains after being filtered) and roots were washed again with distilled water, being now ready for its mycorrhizal estimation.

The stained roots can be left at 4 degrees in distilled water for several months, although if they wish to preserve them for longer it is advisable to keep them in 100% glycerol.

7. AM estimation of colonization

The extent of mycorrhizal colonization (expressed as percentage of total root length colonized by the AMF) was calculated according to the gridline intersection method (Giovannetti & Mosse, 1980) using stereomicroscope under bright field conditions. The methodology of counting of colonized-roots is summarized in **Figure 1**. The root sample was distributed in a grid-line Petri dish and then observed under stereomicroscope.

Vertical and horizontal gridlines were scanned and the presence or absence of colonization was recorded at each point where the roots intersected a line. At least 200 root-gridline intersects were recorded per root sample.

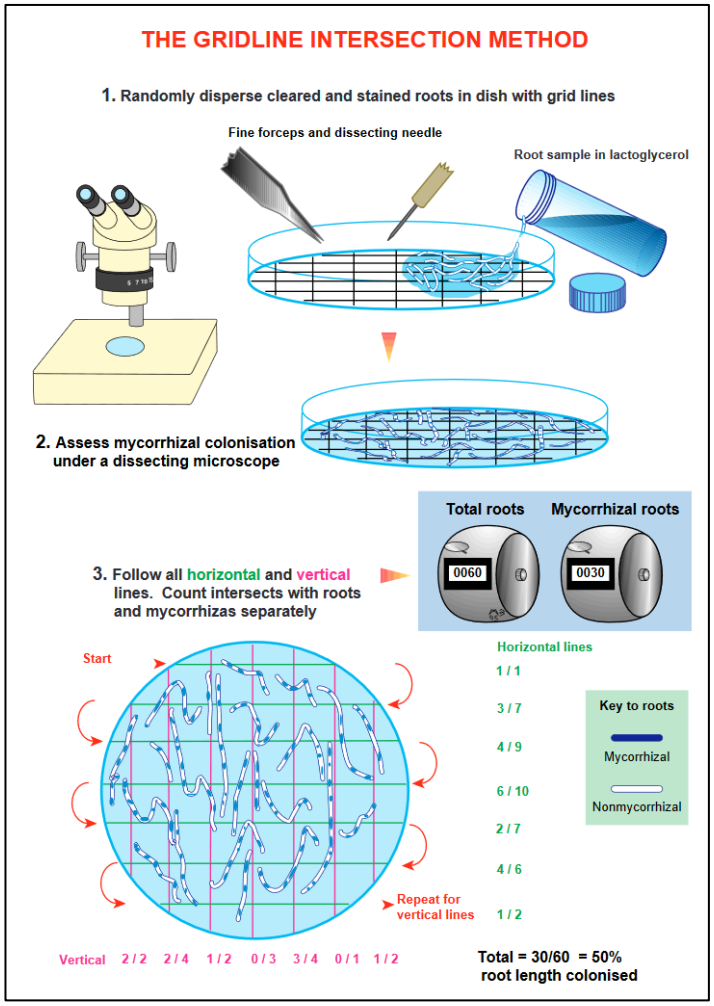

Figure 1 The gridline intersection method of Giovanetti and Mosse (1980) illustrated by Brundrett *et al*. (1996).

8. Determination of mineral nutrients in plant tissues

Nutrient content was measured at the Ionomic Laboratory of Technical Services of the Centro de Edafología y Biología Aplicada del Segura (CEBAS, CSIC), Murcia, Spain. Element concentrations were analyzed after acid digestion of the samples, by inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP 6500 Duo Thermo). Total C and N contents were determined using an Elemental Analyzer (Leco Truspec CN, St Joseph, MI, USA) according to standard procedures.

9. RNA extraction and gene expression quantification

Total RNA from tomato leaves was extracted and treated with DNase enzyme using different protocols (see specific Chapter). Subsequently, the RNA was purified through a column using the RNA Clean & Concentrator-5 kit (Zymo Research), and stored at -80°C until use. RNA integrity and quality were evaluated by gel electrophoresis as well as measuring 260/230 and 260/280 ratios in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The first-strand cDNA was synthesized with 1 µg of purified total RNA using the different *reverse transcriptase* enzyme kits. All kits were used according to the manufacturer's suggested protocols.

Gene expression was studied by RT-qPCR using SYBR® *Premix Ex Taq* ™ (TaKaRa) and an iCycler 5 device (Bio-Rad) according to the manufacturer's suggested protocols.

Each 10 µl reaction contained:

5 µl of 2x SYBR® *Premix Ex Taq* ™ (TaKaRa)
0.125 µl of each primer pair
3.75 µl of RNase-free H_20
1 µl of a dilution 1:10 of synthetized cDNA.

The RT-qPCR protocol consisted on:

	40 s at 95°C (initial denaturation)
x35	5 s at 95ºC (cyclic denaturation)
	30 s at 58ºC (Fluorescence measurement)

After the last RT-qPCR cycle, amplification procedure was checked through a Melt curve protocol ranging 60-95ºC.

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 housekeeping gene *SlEF-1α* (López-Ráez *et al*., 2010), which encodes for the tomato elongation factor-1α. The functionality of AM symbiosis was quantified using the marker

gene *LePT4*, which encodes a mycorrhiza-inducible phosphate transporter expressed in arbusculated cells (Balestrini *et al*., 2007). Nucleotide sequences of the primers used were:

SlEF-1α-F 5′-GATTGGTGGTATTGGAACTGTC-3′
SlEF-1α-R 5′-AGCTTCGTGGTGCATCTC-3′

LePT4-F 5′-GAAGGGGAGCCATTTAATGTGG-3′
LePT4-R 5′-ATCGCGGCTTGTTTAGCATTTC-3′

SLPI-II-F 5'-GAAAATCGTTAATTTATCCCAC-3′
SLPI-II-R 5'-ACATACAAACTTTCCATCTTTA-3'

10. Liquid chromatography and electrospray ionization (LC-ESI) mass spectrometry

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

Freeze-dried root or leaf tissues (50 mg) were homogenized on ice in 1 ml of MeOH:H_2O (10:90) containing 0.01% of HCOOH. The homogenate was centrifuged at 15000 g for 15 min at 4°C. The supernatant was recovered and filtered through 0.2 μm cellulose filters (Regenerated Cellulose Filter, 0.20 μm, 13 mm Ø, pk/100; Teknokroma, St Cugat, Spain). Subsequently, 20 μl aliquot of the filtered supernatant was injected into an Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Mildford, MA, USA), which was interfaced with a hybrid quadrupole time-of-flight equipment (Q-TOF-MS Premier). Analytes were eluted with an aqueous methanol gradient containing 0.01% HCOOH. Solvent gradients and further chromatographic conditions were performed as previously described (Agut *et al*., 2014). A solution of leucine enkephalin at a concentration of 2 ppm in CH_3CN:H_2O (50:50) with 0.1% HCOOH was simultaneously introduced into the Q-TOF instrument via the lock-spray needle for accurate mass-to-charge ratio (m/z) determinations. The biological replicates were randomly injected. The LC separation was performed with a C18 analytical column, 5 μm particle size, 2.1 mm × 100 mm.

To precisely identify metabolites in a full-scan analysis, a LC–ESI Q-TOF MS, a dedicated library was generated using chemical standards. Standards for hormones and

their derivatives, amino acids, indolic compounds, phenols, flavonoids and vitamins (up to 95 compounds) were prepared at a final concentration of 100 ppb in a composite solution and injected through the LC in both positive and negative electro-spray ionization (ESI+; ESI−) modes (**Table 1**). The compounds from this library were characterized at the level of retention time, exact mass and spectrum fragmentation (Schymanski *et al*., 2014). Thus, it allows to identify compounds by matching exact mass and retention time between standard and experimental samples.

For those compounds that were not represented in the internal library, the signals obtained in the untargeted metabolomic analysis were confirmed by contrasting the fragmentation spectrum in the Massbank, Metlin or Human Metabolome databases (www.massbank.jp; www.masspec.scripps.edu; www.hmdb.ca). To identify compounds, at least two of the following three criteria were needed: matching the exact mass; matching the retention time between the standard and experimental samples; and contrasting the obtained fragmentation spectrum with those available databases.

10.2. Full scan data analysis

Data were acquired in centroid mode and subsequently transformed into cdf files using the Databridge from MassLynx 4.1 software (MassLynx 4.1, Waters). Chromatographic signals were processed using the software R for statistical purposes. Signals from ESI^+ and ESI^- were processed separately. Peak peaking, grouping and signal corrections were performed using the XCMS algorithm (Smith *et al*., 2006). Metabolite amounts were analyzed on the basis of normalized peak area units relative to the dry weight. Statistical analysis was performed using the MarVis Suit 2.0 software tool for clustering and visualization of metabolic biomarkers (Kaever *et al*., 2015). The Kruskal–Wallis test ($P < 0.05$) was applied to analyze the metabolomic differences between treatments. Adduct and isotope correction were also performed by using associated software packages MarVis Filter and MarVis Cluster.

To determine a global behavior of the signals, principal component analyses (PCA), sparse partial least-squares discriminant analysis (sPLSDA) and heat map plots were generated. More information is referred in each specific Chapter.

11. Statistical analyses

Besides the methods and software for metabolomic analysis already described, all statistical analyses (two-way and single ANOVAs and post hoc tests applied when appropriate, as indicated in the corresponding figure legends) were conducted using Statgraphics Plus 3.1 (Rockville, MD, USA), 'R' software v.2.9.2 (R Development Core Team) and the XCMS package. Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively. When data did not meet any of the assumptions of ANOVA, square-root transformations were applied.

Table 1. HPLC-QTOF-MS library of standards compounds used for identification of metabolites based on exact mass and retention time matching.

Compound	Exact mass	Ion mass	Ionization mode	RT (min)
Salicylic acid	138.032	137.024	ESI-	3.58
Salicylic acid glucoside (SAG)	300.085	299.077	ESI-	2.54
Salicylic acid glucose ester (SGE)	300.085	299.077	ESI-	2.74
Salicylhydroxamic acid	153.043	152.034	ESI -	2.25
p-Aminobenzoic acid	137.048	138.05	ESI+	1.71
Jasmonic acid	210.126	209.118	ESI-	5.71
Jasmonate-Isoleucin	323.210	322.202	ESI-	6.40
N-[(-)-Jasmonoyl]-Methionine (JAMet)	341.16	340.15	ESI-	6.15
Jasmonoyl-L-phenylalanine	357.194	356.186	ESI-	6.49
Jasmonoyl-L-valine (JAVal)	309.194	308.186	ESI-	6.22
OPDA	292.204	291.196	ESI-	6.96
Abscisic acid	264.136	263.128	ESI-	6.73
ABA-Glucoside	426.189	425.181	ESI-	5.35
5-Hydroxyindole-3-acetic acid	191.058	192.066	ESI+	2.64
Indole-3-acetamide	174.079	175.087	ESI+	3.22
N-(3-indoleylacetyl)-L-alanine	246.100	247.108	ESI+	4.03
Indole-3-carboxaldehyde	145.053	146.061	ESI+	4.05
Methyl indole-acetate	189.079	190.087	ESI+	5.31
Indole-3-acetyl-Isoleucine	288.147	289.155	ESI+	5.87

Compound	Exact mass	Ion mass	Ionization mode	RT (min)
Indole-3-acetyl-L-phenylalanine	322.130	323.139	ESI+	5.97
Indole-3-carboxaldehyde	145.053	144.045/146.061	ESI-/+	4.05
Indole-3-acetonitrile	156.060	157.077	ESI+	4.59
Indole-3-acetic acid-L-aspartic acid	290.090	289.082	ESI-	3.51
Indole-3-pyruvic acid	203.058	202.050/204.066	ESI-/+	0.46
I3CA methyl ester	175.060	176.071	ESI+	5.44
Indole-3-acetyl-L-tryptophan	361.142	360.135/362.151	ESI-/+	5.83
Gibberellic acid	346.141	345.142	ESI -	4.17
Alanine	89.048	90.056	ESI+	0.43
Arginine	174.112	175.12	ESI+	0.37
Asparagine	132.054	133.061	ESI+	0.46
Aspartic acid	133.038	132.03	ESI-	0.43
Cysteine	121.020	122.028	ESI+	0.45
Glycine	75.032	76.040	ESI+	0.43
Glutamine	146.069	147.077	ESI+	0.43
Glutamic acid	147.053	148.061	ESI+	0.43
Histidine	155.07	156.077	ESI+	0.37
Isoleucine	131.095	132.102	ESI+	0.57
Leucine	131.095	132.102	ESI+	0.57
Lysine	146.106	147.113	ESI+	0.43
Methionine	149.051	150.059	ESI+	0.48
Phenylalanine	165.079	166.087	ESI+	1.00
Proline	115.063	116.071	ESI+	0.41
Serine	105.043	106.05	ESI+	0.41
Threonine	119.058	120.066	ESI+	0.43
Tryptophan	204.090	205.098	ESI+	1.72
Tyrosine	181.074	182.082	ESI+	0.53
Valine	117.079	118.087	ESI+	0.43
Ferulic acid	194.058	193.05	ESI-	4.07
Cinamic acid	148.052	147.045	ESI-	5.32
Indole acetic acid	175.063	174.055	ESI-	4.25
Caffeic acid	180.042	179.035	ESI-	3.10
Chlorogenic acid	354.095	353.087	ESI-	2.95

Compound	Exact mass	Ion mass	Ionization mode	RT (min)
Scopoletin	192.042	193.04	ESI+	3.93
Sinapic acid	224.069	223.06	ESI -	4.22
Pyruvic acid	88.016	87.008	ESI -	0.48
Fumaric acid	116.011	115.016	ESI -	0.61
Malic acid	134.021	133.014	ESI -	0.46
Ketoglutaric acid	146.021	145.013	ESI -	0.51
Maleic acid	116.011	115.000	ESI -	0.61
Ascorbic acid (C)	176.032	177.06	ESI+	4.09
Thiamine (B1)	265.110	265.11	ESI+	0.43
Riboflavin (B2)	376.138	377.146	ESI+	3.76
Pyridoxal 5-phosphate (B6)	247.025	248.03	ESI+	0.79
Folic acid (B9)	441.139	440.13	ESI -	3.35
Quercetin	302.043	301.03	ESI -	5.81
Naringenin	272.069	271.064	ESI -	5.63
Hesperetin	302.079	303.08	ESI+	5.75
Galacturonic acid	194.042	193.034	ESI -	0.45
Pipecolic acid	129.079	128.071	ESI -	0.43
Adipic acid	146.141	145.050	ESI -	2.14
2-Aminoadipic acid	161.069	160.061	ESI -	0.43
6-Benzylaminopurine	225.101	226.108	ESI+	4.67
Kinetin	215.081	216.08	ESI+	4.47
Vanillin	152.047	153.05	ESI+	3.42
Camalexin	200.041	201.049	ESI+	5.24
Nicotinic Acid	123.032	124.024	ESI+	0.56
AMP	347.063	346.05	ESI -	0.53
ADP	427.029	428.03	ESI+	0.5
ATP	506.996	508.003	ESI+	4.11
FAD	785.157	786.160	ESI+	3.5
NADH	665.120	664.101	ESI -	1.38
Quercetin	302.043	301.03	ESI -	5.81
Naringenin	272.069	271.064	ESI -	5.63
Hesperetin	302.079	303.08	ESI+	5.75
Apigenin	270.053	269.052/271.076	ESI-/+	7.9
Neohesperidin	610.190	609.182	ESI-	6.4

Compound	Exact mass	Ion mass	Ionization mode	RT (min)
(+) - Catechin	290.079	289.070	ESI-	3.99
(-) Epicatechin	290.079	289.740	ESI-	2.96
(-)-Epigallocatechin	290.079	305.068/307.089	ESI-/+	2.96
Naringin	580.179	579.169	ESI-	6.11
Quercitrin	448.101	447.093	ESI-	6.69
Kaempferol	286.048	285.040	ESI-	4.99
Cis-jasmone	164.240	165.255	ESI+	8.001
Coumarine	146.037	147.059	ESI+	5.07
Shikimic acid	174.053	173.045	ESI-	0.48

CHAPTER 1: METABOLIC TRANSITION IN MYCORRHIZAL TOMATO ROOTS

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Chapter 1: Metabolic transition in mycorrhizal tomato roots

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Abstract

Beneficial plant–microorganism interactions are widespread in nature. Among them, the symbiosis between plant roots and arbuscular mycorrhizal fungi (AMF) is of major importance, commonly improving host nutrition and tolerance against environmental and biotic challenges. Metabolic changes were observed in a well-established symbiosis between tomato and two common AMF: *Rhizoglomus irregulare* and *Funneliformis mosseae*. Principal component analysis of metabolites, determined by untargeted liquid chromatography–mass spectrometry, showed a strong metabolic rearrangement in mycorrhizal roots. There was generally a negative impact of mycorrhizal symbiosis on amino acid content, mainly on those involved in the biosynthesis of phenylpropanoids. On the other hand, many intermediaries in amino acid and sugar metabolism and the oxylipin pathway were among the compounds accumulating more in mycorrhizal roots. The metabolic reprogramming also affected other pathways in the secondary metabolism, mainly phenyl alcohols (lignins and lignans) and vitamins. The results showed that source metabolites of these pathways decreased in mycorrhizal roots, whilst the products derived from α-linolenic and amino acids presented higher concentrations in AMF-colonized roots. Mycorrhization therefore increased the flux into those pathways. Venn-diagram

analysis showed that there are many induced signals shared by both mycorrhizal interactions, pointing to general mycorrhiza-associated changes in the tomato metabolome. Moreover, fungus-specific fingerprints were also found, suggesting that specific molecular alterations may underlie the reported functional diversity of the symbiosis. Since most positively regulated pathways were related to stress response mechanisms, their potential contribution to improved host stress tolerance is discussed.

Introduction

Beneficial organisms are common in the rhizosphere and they provide important ecosystem services (Philippot *et al*., 2013). They can greatly contribute to plant performance by improving nutrition, stress tolerance and plant phenotypic plasticity, an important advantage in heterogeneous environments where precise allocation of limited resources between growth and stress resistance is critical for survival (Pozo *et al*., 2015). Among these beneficial organisms, soil-borne fungi from the phylum Glomeromycota, known as arbuscular mycorrhizal fungi (AMF) deserve special attention. They are able to establish the most ancient and widespread plant–fungal symbiosis, known as arbuscular mycorrhizas (AM), with more than 80% of all terrestrial plant species (Smith and Read, 2008). AMF are obligate biotrophs, and it is assumed that the host plant allocates photosynthates to the fungus for the formation, maintenance, and function of mycorrhizal structures (Bago *et al*., 2000). In return, the AMF improve plant acquisition of water and mineral nutrients. This symbiosis is extremely important for the uptake of inorganic phosphate, but also contributes to the uptake of nitrogen (N) and various trace elements (Smith and Smith, 2011; Hodge and Storer, 2015). Besides plant nutrition, the symbiosis impacts the plant's ability to overcome biotic and abiotic stresses, commonly improving host tolerance to unfavorable environmental conditions and resistance to pathogens (Gianinazzi *et al*., 2010; Jung et al., 2012; Ruiz- Lozano *et al*., 2012; Selosse *et al*., 2014). The establishment and maintenance of the association requires a high degree of coordination between both partners, and bidirectional (plant and fungal) control assures a fair trade of resources between the symbionts (Kiers *et al*., 2011). Indeed, a precise regulation of host hormone levels has been proposed as a central mechanism in the regulation of the interaction (Pozo *et al*., 2015).

Although there is no strict partner specificity in AM, the outcome of AM interactions depends on the interacting partners and the environmental conditions (Walder *et al*., 2012; Smith and Smith, 2015). Actually, there is evidence for "functional diversity" occurring as plant and fungal genotypes determine the benefits of the interaction; some combinations being more efficient than others in terms of nutrition and/or stress resistance (Feddermann *et al*., 2010; Jung *et al*., 2012; Mensah *et al*., 2015; Smith and Smith, 2015). For example, colonization of roots by *Funneliformis mosseae* or *Rhizoglomus irregulare*, the two widespread AMF used in this study, resulted in different levels of bioprotection against Phytophthora root rot in tomato or Fusarium wilt in melon, and to drought stress in lettuce (Pozo *et al*., 2002; Marulanda *et al*., 2003; Porcel *et al*., 2006; Martínez-Medina *et al*., 2009).

Different studies have tried to unravel the molecular mechanisms regulating AM and their impact on plant fitness, most of them focused on differential gene expression and protein profiles (Liu *et al*., 2007; Guether *et al*., 2009; López-Ráez *et al*., 2010; Abdallah *et al*., 2014). A consistent output of "omic" studies on AM has been the observation that mycorrhizal colonization significantly impacts host gene expression and metabolomic profiles (Salvioli and Bonfante, 2013). Metabolomics is a valuable technology which provides comprehensive quantitative profiling of metabolites in biological systems; and liquid or gas chromatography coupled with mass spectrometry (LC–MS or GC–MS) are widely used analytical tools for such untargeted metabolomic studies (De Vos *et al*., 2007). The first metabolomic studies in AM interactions were targeted analyses focusing on a few well-characterized metabolites to monitor plant responses (Stumpe *et al*., 2005; Sawada *et al*., 2009; López-Ráez *et al*., 2010), however, a whole picture of the impact of AM on the general metabolic profile was missing. Nowadays, metabolomic untargeted approaches allow the separation and detection of a wide range of metabolites, such as amino acids, fatty acids, organic acids, sugar phosphates, nucleotides, and glycoside derivatives, providing a global fingerprint about the quantitative and qualitative changes in the secondary metabolism of the host plant (De Vos *et al*., 2007). The LC–MS technique is highly sensitive for the detection of key molecules in the phenotypic mechanisms underlying organism responses to abiotic or biotic interactions (Sardans *et al*., 2011; Gamir *et al*., 2012). Indeed, untargeted metabolomic studies have revealed the importance of metabolic reprogramming as a determinant in other plant–microbe

symbioses, e.g., legume–rhizobia associations (Zhang *et al*., 2012) and ectomycorrhizas in poplar (Tschaplinski *et al*., 2014).

Few studies have addressed the metabolome reprogramming associated with AM and knowledge concerning metabolomic transitions in mycorrhizal plants remains restricted to a few recent studies. Concerning changes in mycorrhizal roots, research has been limited to legumes, in particular to the model plant *Medicago truncatula* and to one AMF strain, *R. irregulare* (Schliemann *et al*., 2008; Laparre *et al*., 2014). The studies have revealed an impact of *R. irregulare* colonization on primary and secondary metabolism, mainly on amino acids (glutamic acid, aspartic acid, and asparagine), fatty acids (palmitic and oleic acids), apocarotenoids (cyclohexanone and mycorradicin derivatives), and isoflavonoids (daidzein, ononin, and malonylononin). Remarkably, the impact of the symbiosis on the host metabolism extends to aboveground tissues and can vary with environmental conditions (Fester *et al*., 2011). Moreover, recent multispecies metabolomic analysis of leaves from *R. irregulare*-colonized plants showed a common core of mycorrhiza-related and highly accumulated metabolites shared by dicotyledonous and monocotyledonous plants, although they also evidenced many species-specific responses (Schweiger *et al*., 2014).

This study aims to decipher the impact of a well-established mycorrhizal association with two widespread and well characterized AMF (*F. mosseae* and *R. irregulare*) in the root metabolic profile of a non-legume, tomato (*Solanum lycopersicum*), where the benefits of AM have been shown to be agronomically relevant in terms of stress resistance and fruit quality (Pozo *et al*., 2002; Fritz *et al*., 2006; Aroca *et al*., 2008; Gianinazzi *et al*., 2010; Bárzana *et al*., 2012; Giovannetti *et al*., 2012; Zouari *et al*., 2014). LC–MS revealed important changes in the metabolome of mycorrhizal tomato roots and we discuss the potential relevance of these changes in host fitness.

Materials and Methods

Plant Material and AMF Inoculation

Arbuscular mycorrhizal fungi isolates of *R. irregulare* (BEG 121; formerly *Glomus intraradices*) and *F. mosseae* (BEG12; formerly *Glomus mosseae*) from the International

Bank of Glomeromycota (http://www.i-beg.eu) are continuously maintained in an open-pot culture of *Trifolium repens* L. mixed with *Sorghum vulgare* Pers. (Steud.) Millsp. and Chase plants in a greenhouse. The inocula consist of substrate (vermiculite/sepiolite, 1:1), spores, mycelia, and infected root fragments from those cultures. Tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were surface disinfected by immersion in 4% NaHClO (10 min) containing 0.02% (v/v) Tween 20®, rinsed thoroughly with sterile water and incubated for 3 days in an open container with sterile vermiculite at 25°C in darkness. Plantlets were transferred to 1 L pots containing a sterile sand:soil (1:1) mixture. Pots for mycorrhizal treatments were inoculated by adding 10% (v/v) *F. mosseae* or *R. irregulare* inoculum. Uninoculated control plants received the same amount of autoclaved mycorrhizal inoculum together with a 3 ml aliquot of a filtrate (<20 μm) of both AM inocula, in order to provide the general microbial population but free of AMF propagules.

A total of ten plants were used for each treatment. Plants were randomly distributed and grown in a greenhouse at 24/16°C with a 16/8 h diurnal photoperiod and 70% humidity. Plants were watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus (P) concentration, and water was supplied daily to maintain the substrate at 100% field capacity, as reported in El-Mesbahi *et al*. (2012). Plants were harvested after 8 weeks, the fresh weight of shoots and roots was determined, and the material immediately frozen in liquid N and stored at -80°C. An aliquot of each individual root system was reserved for mycorrhizal quantification.

Determination of Mycorrhizal Colonization

Mycorrhizal colonization was estimated after clearing washed roots in 10% KOH and subsequent staining of fungal structures with 5% ink in 2% acetic acid (Vierheilig *et al*., 2005). The extent of mycorrhizal colonization (expressed as percentage of total root length colonized by the AMF) was calculated according to the gridline intersection method (Giovannetti and Mosse, 1980) using a Nikon Eclipse 50i microscope and bright field conditions.

Phosphorus, Carbon, and Nitrogen Content

Total P, carbon (C), and N content in the roots was measured at the Ionomic Laboratory of Technical Services of the *Centro de Edafología y Biología Agraria del Segura* (CSIC),

Murcia, Spain. Three biological replicates, each consisting of a pool of roots from three independent plants (nine plants in total), were analyzed for each treatment. Frozen roots were ground to a fine powder and lyophilized. P concentrations were analyzed after an acid digestion of the samples, by inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP 6500 DUO THERMO). Total C and N contents were determined using an Elemental Analyzer (LECO TRUSPEC CN) according to standard procedures.

Analysis of Gene Expression by RT-qPCR

Total RNA from tomato roots was extracted and treated with DNase using the Direct-zol RNA MiniPrep kit (Zymo Research). Subsequently, the RNA was purified through a column using the RNA Clean and Concentrator-5 kit (Zymo Research), and stored at -80°C until use. The first-strand cDNA was synthesized with 1 μg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad). Four independent biological replicates were analyzed per treatment. All kits were used according to the manufacturer's suggested protocols.

Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak and Schmittgen, 2001). Expression values were normalized using the housekeeping gene *SlEF-1α* (López-Ráez *et al*., 2010), which encodes for the tomato elongation factor-1α. The functionality of AM symbiosis was quantified using the marker gene *LePT4*, which encodes a mycorrhiza-inducible phosphate transporter expressed in arbusculated cells (Balestrini *et al*., 2007). Nucleotide sequences of the primers used were:

SlEF-1α-F 5′-GATTGGTGGTATTGGAACTGTC-3′,
SlEF-1α-R 5′-AGCTTCGTGGTGCATCTC-3′;

LePT4-F 5′-GAAGGGGAGCCATTTAATGTGG-3′,
LePT4-R 5′-ATCGCGGCTTGTTTAGCATTTC-3′.

Reagents and Standards

All standards, including amino acids, salicylic acid, phenols, IAA, 5-Hydroxyindole-3-acetic acid, Indole-3-acetamide, *N*-(3-indoley lacetyl)-L-alanine, Indole-3-

carboxaldehyde, Methyl indole-acetate, jasmonic acid (JA), abscisic acid, salicylic acid glucoside ester, OPDA, carboxylic acids, and sugars were purchased from SIGMA (Barcelona, Spain). Methanol (HPLC grade) was obtained in SIGMA (Barcelona, Spain), formic acid and NaOH were obtained from J.T Baker (Deventer, Holland). Indole-3-carboxylic acid and 1,4-diaminobutane were obtained from VWR (Barcelona, Spain).

Liquid Chromatography and ESI Mass Spectrometry

LC–ESI Full Scan Mass Spectrometry (Q-TOF Instrument)

Freeze-dried roots (50 mg) were homogenized on ice in 1 ml of MeOH:H_2O (10:90) containing 0.01% of HCOOH. The homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was recovered and filtered through 0.2 μm cellulose filters (Regenerated Cellulose Filter, 0.20 μm, 13 mm D. pk/100; Teknokroma). A 20 μl aliquot of was injected in the HPLC. The full metabolomic profiling was performed using an Acquity UPLC system (Waters, Mildford, MA, USA) interfaced to hybrid quadrupole time-of-flight (QTOF MS Premier). Analytes were eluted with an aqueous methanol gradient containing 0.01% HCOOH. Three biological replicates, each consisting of a pool of three independent plants (nine plants in total), were randomly injected in duplicate for every treatment. The LC separation was performed with an HPLC SunFire C18 analytical column, 5 μm particle size, 2.1 mm × 100 mm (Waters). Solvent gradients and further chromatographic conditions were performed as previously described (Gamir *et al*., 2012; Agut *et al*., 2014). The LC–ESI Q-TOF MS library of plant compounds was used for a straight identification in full-scan analysis. Standards for phenols, indolic compounds, amino acids, hormones and their derivatives (up to 93 compounds) were prepared (100 ppb) in a composite solution (**Table S2**). The standard solution was injected through the HPLC in both positive and negative electro-spray ionization (ESI^+; ESI^-) to identify compounds by matching exact mass and retention time between standard and experimental samples.

Full Scan Data Analysis

Data were acquired in centroid mode and subsequently transformed into cdf files using the Databridge from MassLynx 4.1 software (MassLynx 4.1, Waters). Chromatographic signals were processed using the software R for statistical purposes. Signals from ESI^+ and ESI^- were processed separately. Peak peaking, grouping and signal corrections were

performed using the XCMS algorithm (Smith *et al*., 2006). Metabolite amounts were analyzed on the basis of normalized peak area units relative to the dry weight. The Kruskal–Wallis test ($P < 0.05$) was applied to analyze the metabolomic differences between treatments. To determine a global behavior of the signals, principal component analyses (PCA) plots were generated using the Multibase 2015 algorithm. Statistical and heat map analysis were performed using the MarVis Suit 2.0 software tool for clustering and visualization of metabolic biomarkers (Kaever *et al*., 2014). Adduct, isotope correction, clustering, and color heat map visualization were also performed by using associated software packages MarVis Filter and MarVis Cluster.

Statistical Analyses

All statistical analyses (ANOVA, post hoc, and t-test) were conducted using Statgraphics Plus 3.1 (Rockville, MD, USA), "R" software version 2.9.2 (R Development Core Team)2 and the XCMS package.

Results

Root colonization by *F. mosseae* and *R. irregulare* and physiological status of the plant

Plants were harvested 8 weeks after inoculation with the mycorrhizal fungi. Root and shoot fresh weights were determined and mycorrhizal colonization, P, C, and N content in the roots were analyzed (**Fig. 1 and Table S1**). Staining of fungal structures with in the roots showed that the mycorrhizal symbiosis was well established in both inoculation treatments, with abundant fungal colonization of the root cortex and well-formed arbuscules. Vesicles, the fungal storage structures, were more abundant in the roots with the most effective colonizer (*R. irregulare*), as has been described in previous studies (López-Ráez *et al*., 2010; **Fig. 1A, B**). Absence of fungal structures was confirmed in roots of the nonmycorrhizal controls, and the extent of root length colonized by *F. mosseae* or *R. irregulare* differed significantly (**Fig. 1C**, $P < 0.01$). The functionality of the symbiosis was assessed by analyzing the expression of the tomato gene *LePT4*, which encodes a phosphate transporter induced in arbuscule containing cells, where most of the

nutrients exchange takes place; it is therefore used as marker of a functional symbiosis. A very strong induction of *LePT4* expression was detected in mycorrhizal roots, reaching similar levels in the interaction with both fungi (**Fig. 1D**).

The symbiosis did not have a significant effect on shoot or root biomass under our experimental conditions (**Table S1**). However, both AM treatments enhanced the P content in roots compared to those from nonmycorrhizal controls (**Fig. 1E**). Similarly, N content was higher in mycorrhizal roots (**Table S1**), while total carbon content in the roots remained unaltered. Accordingly, the C/N ratio showed a significant reduction in mycorrhizal roots (**Fig. 1F**).

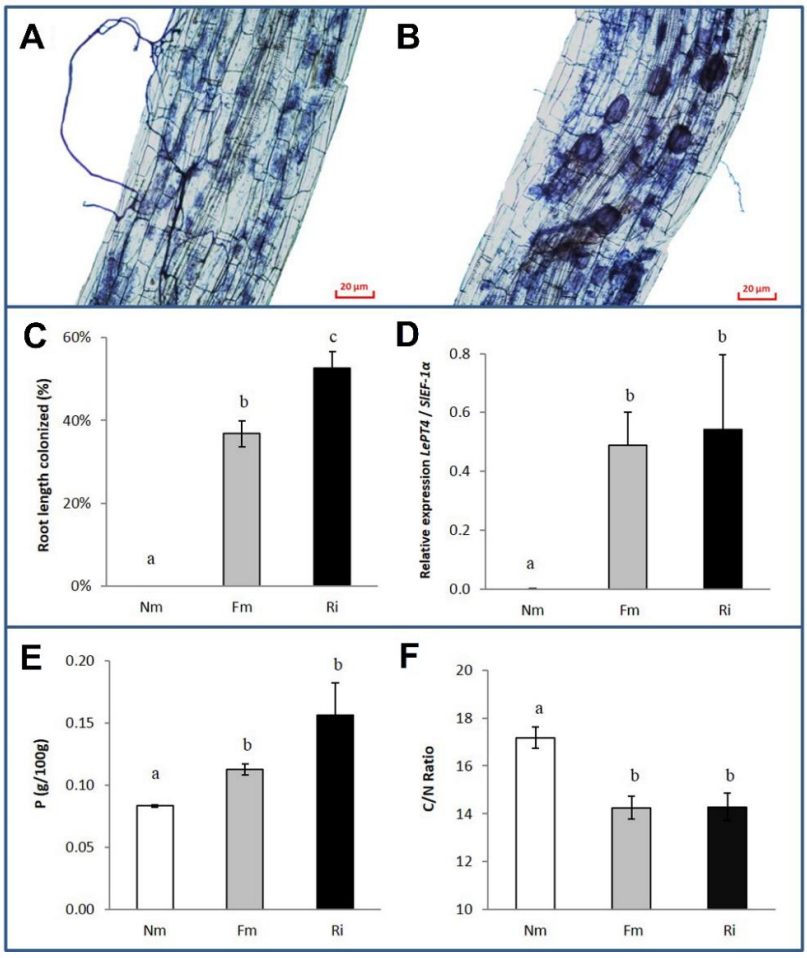


Fig.1 Fungal colonization and nutrient content in nonmycorrhizal (Nm) and mycorrhizal tomato roots colonized by either *Funneliformis mosseae* (Fm) or *Rhizoglomus irregulare* (Ri), 8 weeks after inoculation. (A) Ink-staining of fungal structures in Fm and **(B)** Ri colonized roots. **(C)** Percentage of root length colonized by the mycorrhizal fungi. Data represent the means of 10 independent biological replicates ± SE. **(D)** Expression levels of the tomato gene *LePT4* normalized to the housekeeping tomato gene *SlEF-1α*. Data represent the means of four independent biological replicates ± SE. **(E)** Root P content and **(F)** C/N root content ratio. Data represent the means of three independent replicates each consisting of a pool of roots from three independent biological replicates ± SE. Data not sharing a common letter differ significantly ($P < 0.05$) according to the Newman–Keuls test.

AMF colonization has a strong impact on the metabolic profile of host roots

We analyzed the reprogramming of the tomato root metabolism associated with well-established symbiosis with each AMF. Following the chromatographic analysis, a bioinformatic processing of the detected signals was performed, and cluster and functional pathway analyses were performed in order to obtain plausible biological information of such metabolic reprogramming.

Untargeted metabolomic analysis of root extracts via HPLC coupled with a quadrupole time-of-flight mass spectrometer revealed a total of 1407 signals in ESI^- mode and 1860 signals in ESI^+ mode. A supervised principal component analysis of these signals ($P <$ 0.1, 847 and 1029 signals in ESI^- and ESI^+ mode, respectively) showed a clearly separated behavior between roots colonized by *F. mosseae* (Fm) or by *R. irregulare* (Ri) and nonmycorrhizal roots (Nm), (**Fig. 2A**). According to the two main components, no overlap was observed between the mycorrhizal and Nm groups in any of the ESI modes. It is noteworthy that ESI− showed a similar behavior between signals detected for Ri and Fm. Hierarchical cluster analysis of the different groups confirmed previous observations: signals from roots colonized by both AMF clustered closely compared to those from Nm roots (**Fig. S1A**).

The clusters corresponding to compounds with the most contrasting accumulation patterns across different treatments were selected from the heatmap analysis (**Fig. 2B**) for detailed analysis. The clusters of selected signals were analyzed separately (**Table S2**). Firstly, the number of over-accumulated compounds specific to Nm, Fm, and Ri was subtracted from the heatmap. Secondly, those signals that were highly accumulated in two of the experimental conditions (such as Nm + Fm, Nm + Ri, or Fm + Ri) were also isolated for subsequent Venn-diagram and pathway analysis (**Fig. S1B and Table S2**). The selection, including 1876 signals, contained 300 differentially accumulated in mycorrhizal roots, as illustrated in the Venn diagram (**Fig. S1B**). These signals are of interest as they may correspond to compounds relevant for the known benefits of the mycorrhizal interaction, including improved host stress resistance. These metabolites can be generated as a plant response to the AMF colonization or by the AMF themselves. Despite the core of compounds highly accumulated in both AM roots, there are many specific signals only triggered either by Fm (85signals) or Ri (35 signals), (**Fig. S1B**). Interestingly, the metabolic impact of Fm is stronger than that of Ri. These results suggest

that tomato plants retain common responses to different AMF, as they are conserved in both interactions, but there is, in addition, a set of responses that may be specific to particular interactions. In order to understand the biological meaning of this metabolic transition, we classified the signals contained in the selected clusters from the heat map analysis and Venn diagram (**Fig. S1B**) into a pathway ontology using the MarVis Pathway 2.0 (Kaever *et al*., 2014) linked to the KEGG *Solanum lycopersicum* database (**Table S2**). We particularly focused on those signals that were strongly reduced in both mycorrhizal treatments (Cluster 1), signals highly accumulated in both mycorrhizal interactions (Cluster 2), signals exclusively accumulated in Fm colonized roots (Cluster 3), and finally, signals exclusively accumulated in Ri colonized roots (Cluster 4). Clearly, the major impact of AM on plant metabolism takes place in the primary metabolism, mainly in the amino acid and sugar metabolism (including many hits among the tricarboxylic and other carboxylic acids) but also in some specific secondary metabolites, such as phenolic alcohol derivatives, vitamins, and plant hormones, particularly oxylipins and cytokinins (**Table S2**). Both fungi impacted the 13-LOX oxylipin pathway, with multiple hits in the linoleic and α-linolenic acid metabolism. Remarkably, a clearly overrepresented category in mycorrhiza-enriched compounds is that of metabolites related to ATP-binding cassette (ABC) transporters. ABC transporters are largely expressed in roots and mediate the transport of many secondary metabolites with signaling and defensive functions (Yazaki, 2006).

A closer look at the pathways containing signals with lower levels in mycorrhizal roots (Cluster 1) revealed that other compounds from the same pathways are strongly overrepresented in AM. This suggests that the reduction of these compounds in mycorrhizal roots is a consequence of the metabolic flux along the pathways that reduces substrates of a given reaction accumulating the product compounds. Regarding the specific signals, Fm had a stronger impact on amino acids, sugars, and phenolics compared with Ri.

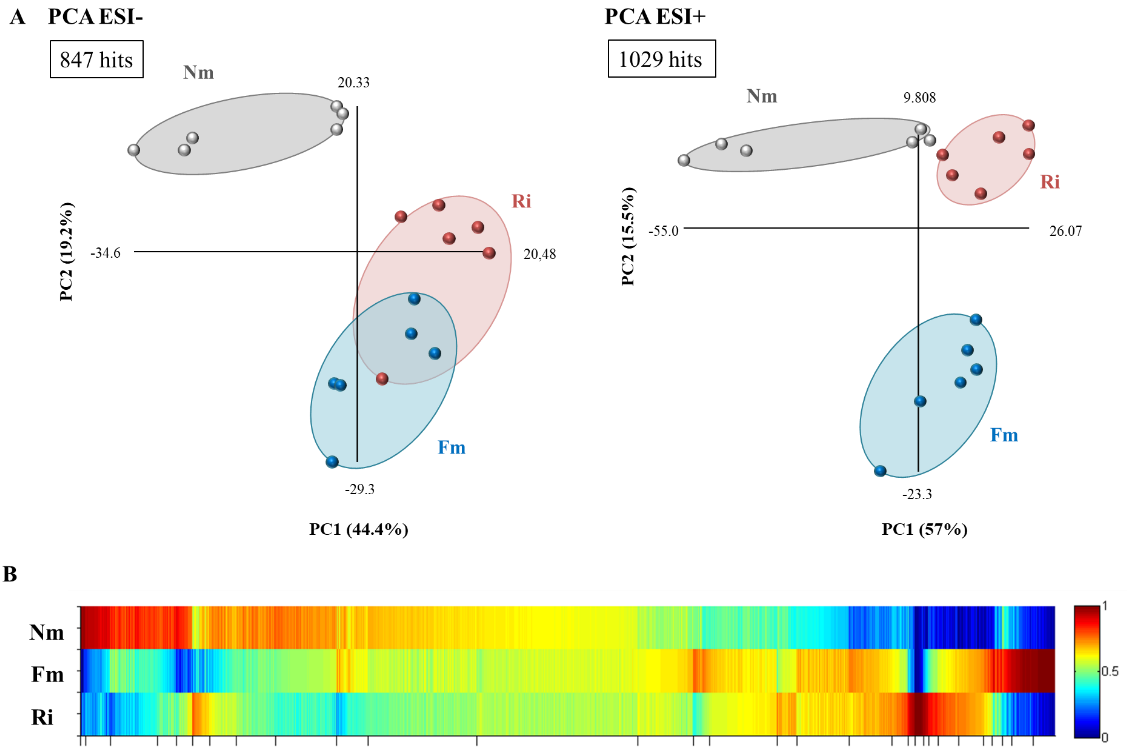


Fig. 2 Overview of metabolite behavior in nonmycorrhizal (Nm) and mycorrhizal roots interpreted using principal component and heat map analysis. (A) Analysis of major sources of variability of ESI^- and ESI^+ signals obtained from a untargeted analysis by HPLC–QTOF MS monitoring metabolomic changes in roots colonized by *F. mosseae* (Fm) or *R. irregulare* (Ri). Data points represent six replicates per treatment injected randomly into the HPLC-QTOF MS. The identified signals corresponding to different treatments were compared using the non-parametric Kruskal–Wallis test, and only data with a $P < 0.1$ between groups were used for a supervised analysis. **(B) Heat map of the metabolite profiling**, generated with MarVis Filter and Cluster packages, following a Kruskal–Wallys test ($P < 0.05$) by combining positive and negative electrospray ionization analysis. Each color band represents a single compound detected in Nm, Fm, and Ri, whose accumulation is indicated for each treatment by the indicated color scale ranging from high (red) to low (blue) accumulation. The concentration of the metabolites was determined in all samples by normalizing the chromatographic pick area for each compound with the dry weight of the corresponding sample.

Impact of the arbuscular mycorrhizal symbiosis on amino acid metabolism

One of the major pathways altered in mycorrhizal roots was the metabolism of the amino acids (**Fig. 3 and Table S2**). Phe, Tyr, Tryp, and Leu/Ile were consistently less concentrated in mycorrhizal roots colonized by both AMF. Contrastingly, many metabolites derived from Cys, Lys, Ala, Gln, Phe, Tyr, and Trp were highly accumulated in mycorrhizal roots (**Table S2**). The higher concentration of amino acid derived metabolites in AM would explain the lower concentration of free amino acids as metabolic sources (**Fig. 3**). It is noteworthy that Phe and Tyr are the main amino acids that generate phenolic acids and their derivatives, highly accumulated in AM roots. This

observation suggests a very likely circulation of the basic amino acids into more complex secondary metabolites, which are indeed found in higher concentrations in the symbiotic roots. On the other hand, glutamate (Glu) and aspartate (Asp) were found in higher levels in both mycorrhizal roots. As their active role in the incorporation of N in AM plants is well reported (Govindarajulu *et al*., 2005; Schliemann *et al*., 2008), their higher levels are consistent with the increase in N observed in both mycorrhizal treatments (**Fig. 1B**). Finally, some AMF-dependent regulation of the amino acids was also observed. For example, His and Met accumulated at higher levels in Fm, whilst they were hardly present in Nm and Ri.

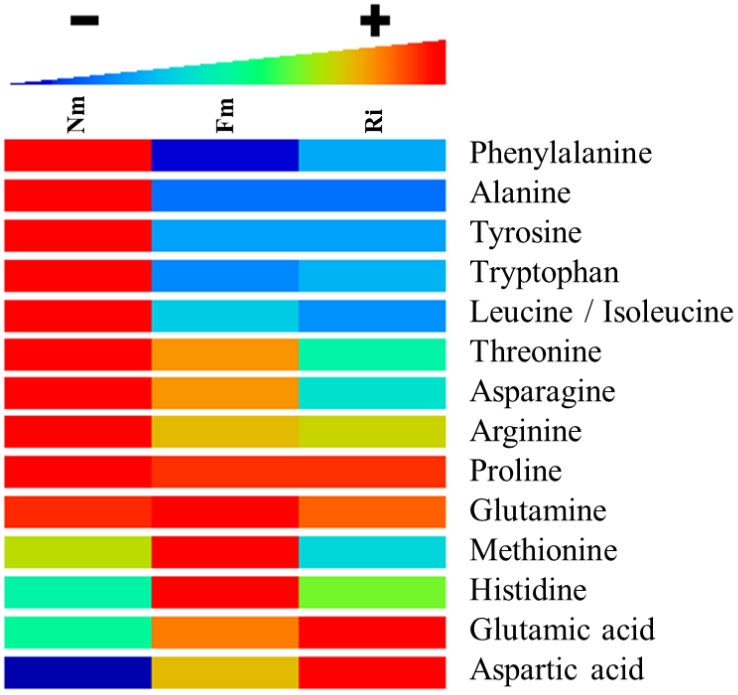


Fig. 3 Heat map analysis of amino acid content in nonmycorrhizal (Nm), *F. mosseae*-colonized (Fm) and *R. irregulare*-colonized roots (Ri). Samples for analysis were collected 8 weeks after fungal inoculation. Data points represent six biological replicates injected randomly into the HPLC–QTOF MS. Color scale represents the variation in the accumulation of the amino acids, from high (red) to low (blue) contents. Signals corresponding to different treatments were compared using the non-parametric Kruskal–Wallis test, only data with a $P < 0.1$ (between groups) were used for a supervised analysis. Values are relative to the sample dry weight and normalized to the lowest amount.

Impact of the arbuscular mycorrhizal symbiosis on amino Acid derived compounds: phenolic alcohol derivatives, benzylisoquinolines, and conjugated polyamines

The phenyl-alcohol metabolism was also strongly affected in AM. This metabolism includes deamination of Phe and Tyr by the Phenylalanine ammonia lyase enzyme (Cochrane *et al*., 2004), after which a set of phenolic acids are converted into aldehydes and alcohols by successive reductions. The phenolic alcohols are precursors of important cell wall components such as monolignans and lignins. In addition, coumaryl and coniferyl alcohols can be converted into more complex flavonoids with cell protective functions (Wang *et al*., 2013). As described above, the identified upstream compounds of this pathway, Phe and Tyr, were found in lower levels in mycorrhizal roots (**Fig. 3 and 4**), while the content of other intermediary compounds, such as ferulic acid, coumaryl alcohol, and coniferyl alcohol, was higher in the colonized roots (**Fig. 4**). Additionally, other tentatively identified monolignans (400.152, 362.173, 354.110, and 398.137 m/z) were also more concentrated in mycorrhizal roots (**Fig. 4**). These observations suggest that AMF stimulates a reorganization of specific cell wall components.

Other amino acid derived compounds related to defense were also found in higher levels in mycorrhizal roots. Plants often produce alkaloids to defend themselves against pests, diseases, and other external biological stimuli. Several mass signals identified as benzylisoquinoline alkaloids (BIAs), such as 271.107; 369.126; 332.112; and 273.124 m/z, were found in high quantities in AMF colonized roots (**Fig. S2**). Higher accumulation of several polyamines and their conjugates were also found, some putatively identified as spermidine (145.112 m/z), tricaffeoylspermidine (631.274 m/z), and triferuloylspermidine (673.242 m/z), (**Fig. S2**).

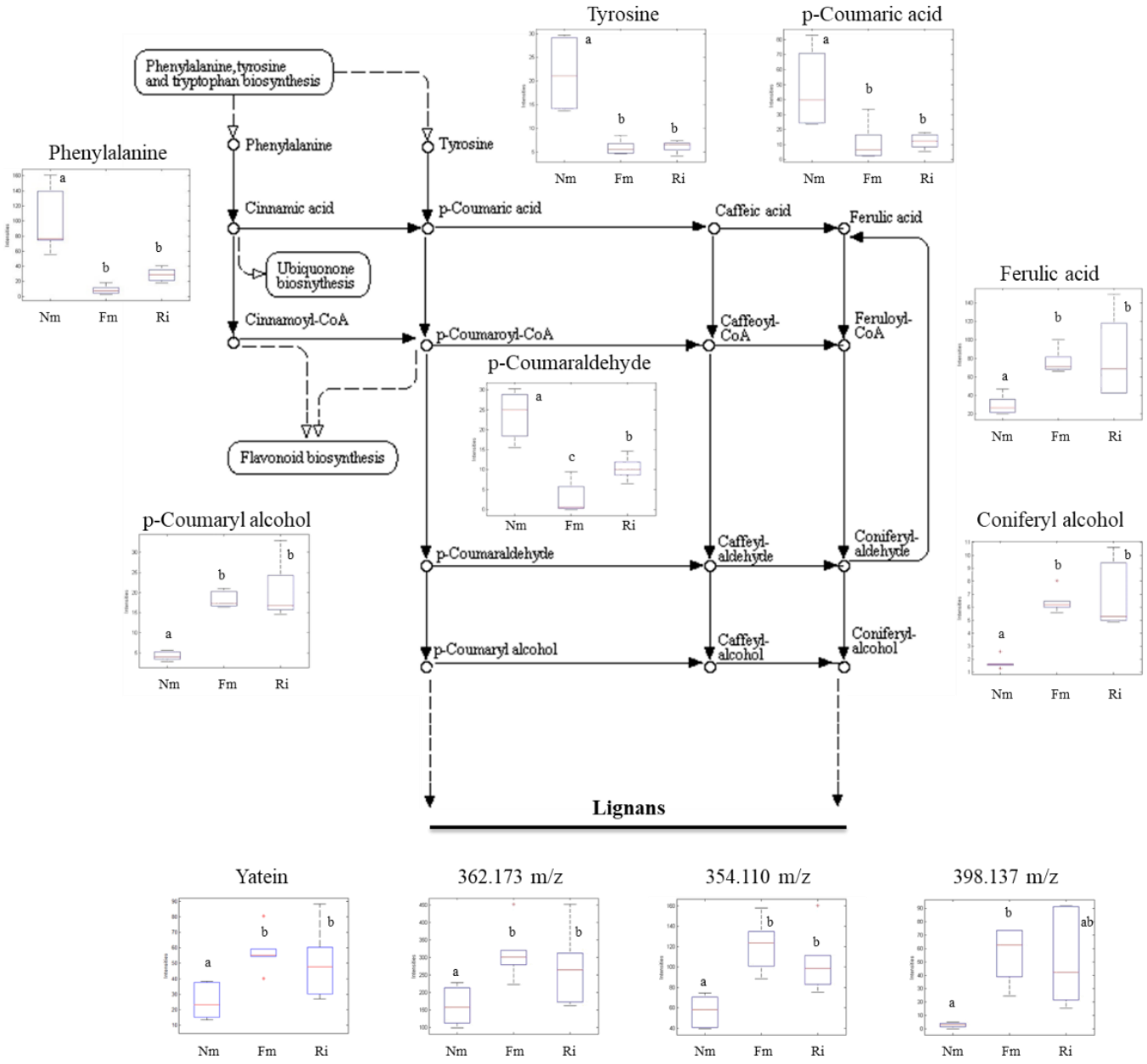


Fig. 4 Profile of selected metabolites from the phenylpropanoid, lignin, and lignan biosynthetic pathways. Nonmycorrhizal roots (Nm), and roots colonized by *F. mosseae* (Fm) and *R. irregulare* (Ri) were processed for relative quantification analysis by HPLC–QTOF MS. The metabolite concentration in each sample was determined by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. For those compounds matching two or more identification criteria (exact mass, positive fragmentation spectrum, or chemical standards) names are assigned. Those compounds only tentatively identified are assigned only by an m/z ratio. Dotted arrows mean multiple metabolic steps, straight arrows mean single steps. Data in the same plot not sharing a common letter differ significantly ($P < 0.05$) according to the Newman–Keuls test.

Impact of arbuscular mycorrhizal symbiosis on the oxylipin pathway

The untargeted metabolomic analysis revealed α-linolenic acid derivatives as major metabolic targets for mycorrhizal symbiosis. Among the 45 signals related to the oxylipin pathways altered in mycorrhizal roots (**Table S2**), 11 compounds were fully identified by either exact mass or fragmentation spectrum (**Fig. 5**), and they corresponded to the 13-LOX branch of the oxylipin pathway. This branch leads to the biosynthesis of the phytohormone JA and derivatives, known to be altered in AM in different plant species (Wasternack and Hause, 2013; Fernández *et al*., 2014). With the exception of α-linolenic acid, the source metabolite, the compounds identified in this pathway showed higher levels in mycorrhizal roots (**Fig. 5**). Most of them showed higher concentrations in roots colonized by both AMF, although to different levels depending on the particular compound and the colonizing fungi. Remarkably, the levels of the bioactive forms of JA methyl-JA (Me-JA) and JA-Ile conjugates were accumulated in significantly higher levels only in the Fm roots.

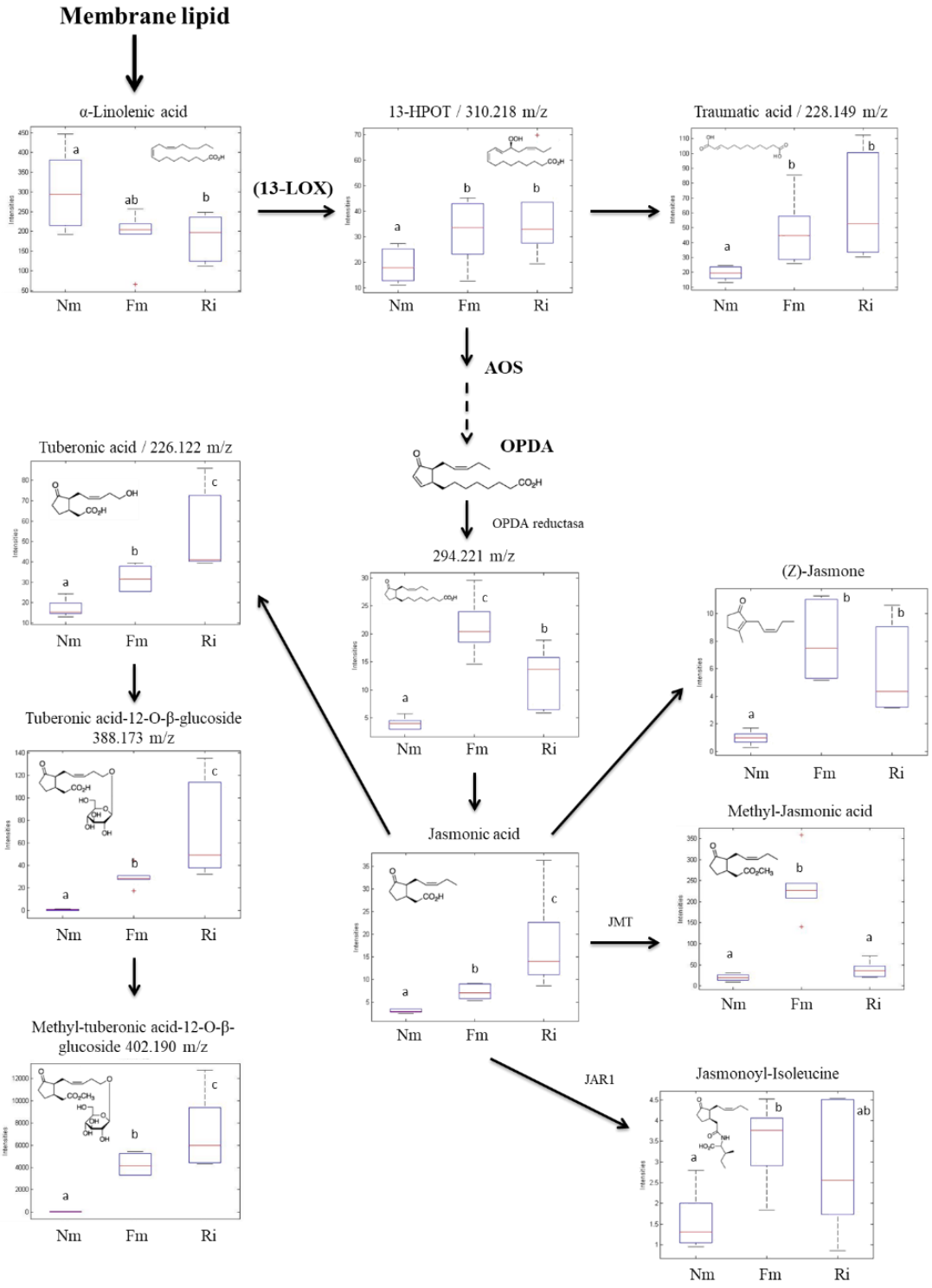


Fig. 5 Accumulation profile of compounds related to the 13-LOX oxylipin pathway. Nonmycorrhizal (Nm), *F. mosseae* (Fm) and *R. irregulare* (Ri) colonized roots were processed for relative quantification analysis by HPLC–QTOF MS. Concentration of metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. For those compounds matching two or more identification criteria (exact mass, positive fragmentation spectrum, or chemical standards) names are assigned. Those compounds tentatively identified are assigned by an m/z ratio together with their putative names. Dotted arrows mean multiple metabolic steps, straight arrows mean single steps. Data in the same plot not sharing a common letter differ significantly ($P < 0.05$) according to the Newman–Keuls test.

Discussion

Several studies have detailed the transcriptional reprogramming in the host plant during interaction with AMF, not only transiently during the establishment of the symbiosis, but also in the maintenance of the symbiosis (Hohnjec *et al*., 2005; Liu *et al*., 2007; Guether *et al*., 2009). While there is evidence for these mycorrhiza associated transcriptional changes in multiple plant families, the metabolic impact on host roots have been only monitored in a few plant species, mainly legumes (Schliemann *et al*., 2008; Laparre *et al*., 2014). Our research presents a complete metabolomic analysis in roots of a relevant crop, tomato, in symbiotic interaction with two different AMF: *F. mosseae* and *R. irregulare*, both known to increase tomato resistance against biotic and abiotic stresses and yield (Pozo *et al*., 2002; Fritz *et al*., 2006; Aroca *et al*., 2008; Gianinazzi *et al*., 2010; Bárzana *et al*., 2012; Vos *et al*., 2012). These fungi are among the most studied and widely distributed AMF in agricultural and ecological settings. *R. irregulare* is the most commonly used AMF in commercial inoculants, and is widely used as a model organism in AM research, because it is readily grown using *in vitro* cultivation in monoxenic conditions, and its genome is now available (Declerck *et al*., 2005; Tisserant *et al*., 2013). In contrast, *F. mosseae* cannot be cultivated in monoxenic cultures but is usually very efficient in increasing host resistance to pests and pathogens (Jung *et al*., 2012).

Increases in P levels in the host following root colonization by AMF is one of the major and most reported benefits of mycorrhizal interactions, although the increase depends on the partners involved and the experimental conditions (Smith and Smith, 2015). However, AM are not always associated to increased vegetative biomass (Smith and Smith, 2011), and improved stress tolerance has been proposed as another major benefit of the symbiosis (Gianinazzi *et al*., 2010; Selosse *et al*., 2014). In our experimental system, both *F. mosseae* and *R. irregulare* colonization increased the total P levels in tomato roots (cv. Moneymaker), whilst shoot and root biomass, root/shoot ratio and total carbon content were not significantly altered. Interestingly, total N was significantly higher in mycorrhizal tomato roots, and consequently the C/N ratio was reduced.

Our untargeted metabolome analysis confirmed, following restrictive statistical analyses, that the metabolome of mycorrhizal tomato roots is significantly different from that of nonmycorrhizal tomatoes. Pioneering work by Schliemann *et al*. (2008), and later Laparre

et al. (2014) showed that there are clear differences in the development and symbiosis-dependent primary and secondary metabolism of *M. truncatula* roots colonized by the AMF *R. irregulare*.

A large number of signals related to sugar and carboxylic acid metabolism showed elevated levels in mycorrhizal roots. This suggests that primary sugar metabolism was activated by the symbiosis as has been shown in multiple mycorrhizal systems (Bago *et al*., 2000; Zouari *et al*., 2014) probably related to an increase in the host photosynthesis to increase C-fixation (Kaschuk *et al*., 2009). Total C content, however, remained unaltered in mycorrhizal roots, probably due to the fact that part of the host-derived C is taken by the fungal partner to maintain the mycelial network (Bago *et al*., 2000). Indeed, transcriptional regulation of carbohydrate related genes and activation of sugar transporters are reported in AM (Bago *et al*., 2000; Doidy *et al*., 2012). Another notable target of mycorrhizal metabolic reprogramming corresponds to amino acid metabolism, which is one of the pathways with more hits amongst the AM-related differential signals (**Table S2).** A strong reduction in the accumulation of several amino acids (Trp, Tyr, Phe, Ala, Leu) was observed, probably because of their function as source compounds of amino acid-derived secondary metabolites (**Fig. 3**). For example, Phe and Tyr are precursors of the phenylpropanoid pathway, and several intermediaries of this pathway were found to be highly accumulated in the mycorrhizal roots. Both amino acids were found in low levels in mycorrhizal tomato roots, and the data suggest that most phenolic derivatives may have been redirected to the formation of lignans and lignins. An increase in lignans has been previously reported in other root-beneficial fungus interaction involving *Piriformospora indica* (Baldi *et al*., 2010). Regarding the lignins, it has been shown that mycorrhizal colonization can increase the lignin content of the root cell walls (Ziedan *et al*., 2011), and cell-wall lignification is one of the proposed mechanisms restricting penetration by phytopathogenic fungi in mycorrhizal roots (Jung *et al*., 2012). Remarkably, a similar reduction of amino acid content was observed in aboveground tissues of mycorrhizal *L. japonica* (Fester *et al*., 2011), and *Arabidopsis* plants treated with the defense-priming agent β-amino butyric acid showed a lower content of all amino acids except Glu (Pastor *et al*., 2014). Thus, it is tempting to speculate that the reduction of basic and aromatic amino acids is a common response to defense-priming stimuli. However, this putative relationship requires further experimental confirmation.

Despite an overall reduction in most amino acids, our study revealed a higher accumulation in the mycorrhizal roots of Glu and Asp (**Fig. 3**), important amino acids in uptake of N by AMF extra-radical mycelium (Govindarajulu *et al*., 2005). The elevated N levels observed in AM, together with the elevated levels of these amino acids, also reported in other mycorrhizal systems (Schliemann *et al*., 2008) suggest that N uptake and assimilation is stimulated in mycorrhizal tomato roots. An impact of AMF colonization on enzymes catalyzing the biosynthesis of N rich compounds such as alkaloids has been also described (Zeng *et al*., 2013). Our metabolomic analysis showed that both BIAs and conjugated polyamines, all with defense-related functions in plants, are also over accumulated in mycorrhizal compared to nonmycorrhizal roots.

One of the clear targets of root reprogramming in mycorrhizal roots was the oxylipin pathway. All the metabolites of the 13-LOX branch identified either through the fragmentation spectrum, exact mass or using standards, were more concentrated in mycorrhizal roots, including several bioactive forms of the phytohormone JA. This may explain the reduced levels of the source metabolite α-linolenic acid in AM. The induction of most of the intermediates of the pathway is consistently reproduced in both mycorrhizal root systems, although the relative levels of the different compounds differ according to the AMF. The alteration of multiple metabolites in the pathway, known to be precisely regulated by inter conversion among them (Wasternack and Hause, 2013) and differential accumulation in response to the particular AMF, support their involvement in fine-tuning of metabolic reprogramming in response to particular growth conditions, the partners involved and the symbiotic stage reached (Wasternack and Hause, 2013; Fernández et al., 2014). Elevated levels of the JA-related volatile compounds Me-JA and CIS-jasmone, both with known roles in defense against biotic stresses, are reported here for the first time in mycorrhizal roots. Moreover, Me-JA is also known to be involved in the plant response to abiotic stresses like drought or salinity (Fahad *et al*., 2015). The exact role of JA and its derivatives in the control of AM remains controversial, as exogenous application of the hormone provides contrasting results, and JA deficient mutants have relatively subtle mycorrhizal phenotypes that seem to depend largely on the host plant species (Wasternack and Hause, 2013). In some plant systems LOX-silencing does not significantly affect AMF colonization, therefore it has been proposed that activation of JA-signaling is a downstream event triggered by this symbiosis (Wasternack and Hause, 2013). In fact, *PvLOX2*-silencing in common bean roots is reported to have no effect on

mycorrhiza establishment, whilst it does impair mycorrhiza-induced resistance (Mora-Romero *et al*., 2014). Thus, the results suggest that AM functioning implies a precise regulation of the oxylipin pathway that may contribute to improving stress resistance in mycorrhizal plants. It is noteworthy that the genes coding for alkaloid biosynthetic enzymes are JA-inducible (Mishra *et al*., 2013; Wasternack and Hause, 2013). Additionally, phenylpropanoid-polyamine conjugated (PPCs), other N rich compounds related to defense responses, were found in elevated levels in the mycorrhizal roots and are also described to be under JA regulation in several plants, including tomato (Kaur *et al*., 2010).

Although the metabolic pathways altered by the mycorrhizal symbiosis were common to both *F. mosseae* and *R. irregulare* interactions, many compounds showed specific responses to one of the interactions. These results suggest, therefore, a fine-tuned developmental regulation of these pathways in an AMF-dependent manner. In terms of fitness costs, we have observed no reduction in plant biomass despite the extensive metabolic changes encountered in AM roots. AMF-induced resistance against soil pathogens in tomato roots seems to be related to cost-efficient defense regulation mechanisms (Pozo *et al*., 2002; Steinkellner *et al*., 2012; Vos *et al.*, 2012; Selosse *et al*., 2014). There is experimental evidence of the role of the JA-signaling pathway in priming of plant defenses by mycorrhizas (Song *et al*., 2013). The contribution of other particular metabolic pathways altered in mycorrhizal roots to the enhanced host stress resistance–tolerance remains to be experimentally demonstrated.

To sum up, following a untargeted full metabolomic approach, the metabolic transition in roots colonized by two widely distributed AMF, *F. mosseae* and *R. irregulare*, was successfully characterized. An important reprogramming of some major metabolic pathways in both mycorrhizal interactions was observed, pointing to common responses associated to AM, although there were also some AMF-specific responses. Many of the changes are related to plant defense mechanisms, and may underlay the well-known effects of the interaction on plant-stress tolerance. The identification of differentially regulated pathways in this study is instrumental to functional studies aiming to reveal the mechanistic basis of AM benefits, such as the improved resistance–tolerance to biotic and abiotic stresses. Moreover, these studies can pave the way to improving the biotechnological applications of AMF in agricultural settings and in the production of

plant secondary metabolites with medicinal or nutritional properties (Gianinazzi *et al*., 2010; Pedone-Bonfim *et al*., 2015).

Supplementary Data

Treatment	FW Shoot (g)	FW Root (g)	Root : Shoot	Ntotal (g/100g)	Ctotal (g/100g)	C/N
Nm	10.27 ± 1.57 a	4.68 ± 0,66 a	0.48 ± 0.03 a	1.91 ± 0.06 a	32.69 ± 0.35 a	17.17 ± 0.45 a
Fm	11.80 ± 0.54 a	6.42 ± 0,43 a	0.54 ± 0.02 a	2.14 ± 0.03 b	30.50 ± 1.21 a	14.26 ± 0.47 b
Ri	10.56 ± 0.62 a	5.53 ± 0,48 a	0.52 ± 0.02 a	2.23 ± 0.22 ab	31.75 ± 2.82 a	14.29 ± 0.58 b

Table S1 Shoot and root fresh weight, nitrogen (N) and carbon (C) content, and C/N ratio of nonmycorrhizal (Nm) and mycorrhizal tomato plants eight weeks after inoculation with *F. mosseae* (Fm) or *R. irregulare* (Ri). Fresh weight data represent the means of ten independent biological replicates ± SE. P, N and C content and C/N ratio represent the means of three independent biological replicates each consisting of a pool of roots from three independent plants ± SE. Data in the same column not sharing a common letter differ significantly ($P < 0.05$) according to Student-Newman-Keuls test.

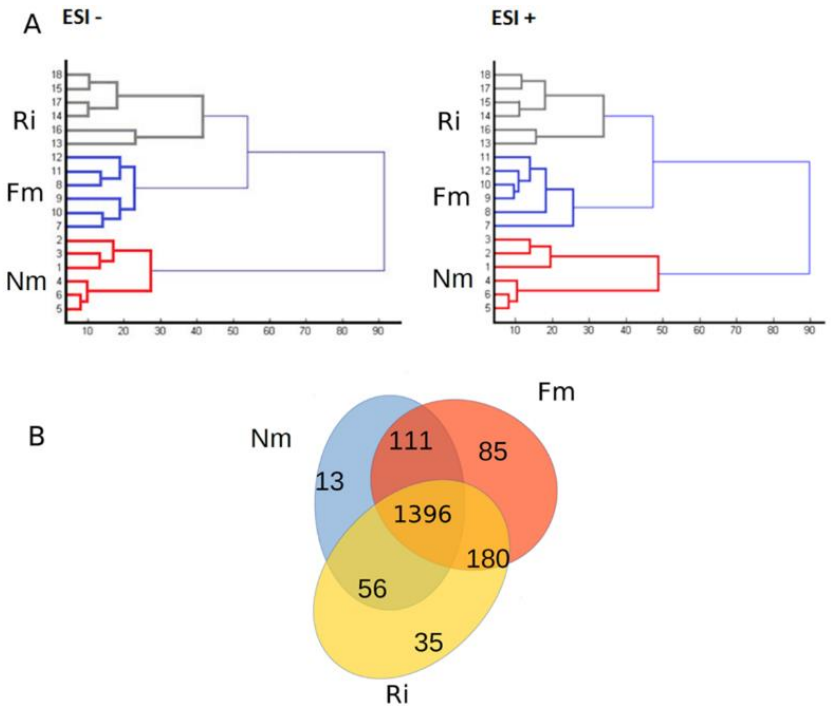
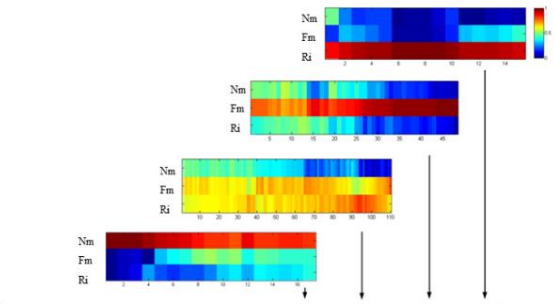


Fig. S1 A) Hierarchical clustering of both ESI^+ and ESI^- compounds found in nonmycorrhizal (Nm), *F. mosseae* (Fm) and *R. irregulare* (Ri) colonized roots. Samples were collected 8 weeks after inoculation with Fm or Ri. For each treatment, six replicates were injected randomly into the HPLC-QTOF-MS. The signals corresponding to different treatments were compared using the non-parametric Kruskal-Wallis test, and only data with a $p<0.1$ between groups was used for a supervised analysis. The clustering was performed using the package MarVis Filter from the software MarVis 2.0. **B) Venn diagram** of combined ESI^+ and ESI^- compounds obtained from the selected clusters represented in the Fig. 2 and table S2.

Numbers inside the shared coloured region represents the number of shared differentially accumulated compounds either by Nm-Fm, Nm-Ri or Fm-Ri.



	Solanum lycopersicum (tomato)	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4
	ABC transporters -	-	14	3	1
Aminoacid metabolism	Alanine; aspartate and glutamate metabolism -	-	7	1	2
	Amino acids	-	3	1	1
	Amino sugar and nucleotide sugar metabolism -	-	10	5	1
	Biosynthesis of amino acids -	-	16	9	2
	Cysteine and methionine metabolism -	-	10	1	1
	Aminoacyl-tRNA biosynthesis -	-	3	1	1
	Lysine biosynthesis -	-	4	1	1
	Lysine degradation -	-	4	4	-
	Glycine; serine and threonine metabolism -	-	8	3	2
	Histidine metabolism -	-	5	6	1
	Tryptophan metabolism -	-	12	8	5
	Tyrosine metabolism -	-	7	3	1
	Valine; leucine and isoleucine biosynthesis -	-	5	3	-
	Valine; leucine and isoleucine degradation -	-	2	4	-
	Phenylalanine metabolism -	-	8	-	-
	beta-Alanine metabolism -	-	6	2	1
	Arginine and proline metabolism -	-	12	5	1
Oxylipins	Linoleic acid metabolism -	-	2	-	16
	alpha-Linolenic acid metabolism -	-	9	1	-
	Fatty acid degradation -	-	2	-	-
	Arachidonic acid metabolism -	-	14	-	1
Sugar metabolism	Carbon metabolism -	-	9	6	1
	Citrate cycle (TCA cycle) -	-	4	-	-
	Fructose and mannose metabolism -	-	6	-	-
	2-Oxocarboxylic acid metabolism -	-	21	8	6
	Galactose metabolism -	-	8	1	-
	Pentose and glucuronate interconversions -	-	14	6	-
	Pentose phosphate pathway -	-	5	4	-
	Glycolysis / Gluconeogenesis -	-	3	-	-
	Pyruvate metabolism -	-	6	-	-
	Starch and sucrose metabolism -	-	6	-	-
	Glyoxylate and dicarboxylate metabolism -	-	10	3	-
Phenolic alcohols and derivatives	Isoquinoline alkaloid biosynthesis -	2	17	4	-
	Lignans	-	14	4	1
	Phenylpropanoid biosynthesis -	-	7	2	1
	Flavone and flavonol biosynthesis -	-	3	1	-
	Flavonoid biosynthesis -	-	5	1	2
	Stilbenoid; diarylheptanoid and gingerol biosynthesis	-	3	2	2
	Limonene and pinene degradation -	-	16		
	Monoterpenoid biosynthesis -	-	14	2	7
	Ubiquinone and other terpenoid-quinone biosynthesis -	-	6	2	1
	Diterpenoid biosynthesis -	6	8	3	-
	Purine metabolism -	-	12	5	2
	Pyrimidine metabolism -	-	12	6	-
	Nicotinate and nicotinamide metabolism -	-	5	3	1
	Tropane; piperidine and pyridine alkaloid biosynthesis -	-	10	4	1
Vitamins	Thiamine metabolism -	-	3	-	-
	Riboflavin metabolism -	-	3	2	-
	Vitamin B6 metabolism -	-	7	1	-
	Pantothenate and CoA biosynthesis -	1	8	2	1
	Folate biosynthesis -	1	4	1	4
	Plant hormone signal transduction -	-	2	-	-
	Zeatin biosynthesis -	-	6	1	1
	Porphyrin and chlorophyll metabolism -	-	9	2	-
	Propanoate metabolism -	-	3	1	-
	Butanoate metabolism -	-	6	2	-
	Carotenoid biosynthesis -	1	2	-	-
	Glucosinolate biosynthesis -	-	10	6	5
	Glutathione metabolism -	-	3	-	-
	Glycerolipid metabolism –	-	5	5	-
	Inositol phosphate metabolism -	-	2	-	-
	Ascorbate and aldarate metabolism -	-	7	7	-

Table S2 Identified pathways and compounds found in selected clusters. Signals corresponding to different treatments were compared using the non-parametric Kruskal-Wallis test, and only data with a $p<0.1$ between groups were used for a supervised analysis. Quantitative values are relative to the sample dry weight and normalized to the lowest amount, and are represented following a colour scale ranging from blue (low) to red (high) accumulation. The signals have been selected following a criteria of maximum colour differences between treatments. Cluster 1 includes signals from the heatmap overrepresented in nonmycorrhizal roots (Nm), Cluster 2 includes signals from the heatmap overrepresented at the same time in both *F. mosseae* (Fm) and *R. irregulare* (Ri) colonized roots, Cluster 3 includes signals from the heatmap overrepresented only in Fm colonized roots and Cluster 4 includes signals from the heatmap overrepresented only in Ri colonized roots. The table was built grouping the number of hits provided by the package MarVis Pathway (MarVis 2.0) that were organized by pathways inside each cluster.

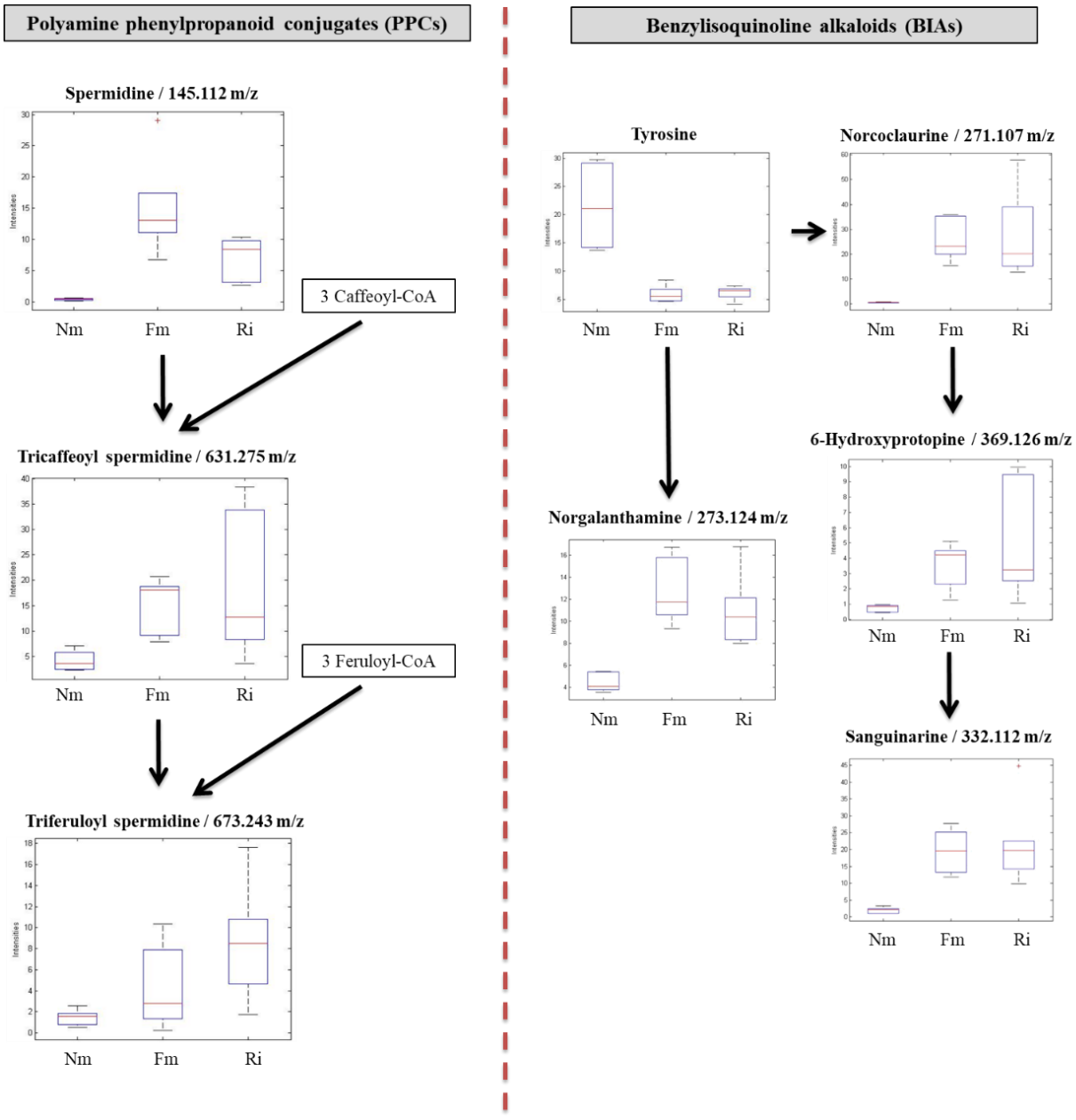


Fig. S2 Polyamine phenylpropanoid conjugates (PPCs) and benzylisoquinoline alkaloids (BIAs) differentially accumulated in nonmycorrhizal (Nm), *F. mosseae* (Fm) and *R. irregulare* (Ri) colonized roots. For each treatment, six replicates were injected randomly into the HPLC-QTOF MS. Compounds tentatively identified were assigned by a m/z ratio together with their putative names.

Table S3 HPLC-QTOF-MS library of standards compounds used for identification of metabolites based on exact mass and retention time matching.

Compound	Exact mass	Ion mass	Ionization mode	Retention time (min)
Salicylic Acid	138.032	137.024	ESI-	12.2
Cinamic Acid	148.052	147.045	ESI-	10.5
Indole acetic acid	175.063	174.055	ESI+/ESI-	6.3
Caffeic Acid	180.042	179.035	ESI-	4
Jasmonic Acid	210.126	209.118	ESI-	11.1
Abscisic Acid	264.136	263.128	ESI-	9.6
Jasmonate-Isoleucin	323.21	322.202	ESI-	13.4
Ferulic Acid	194.058	193.05	ESI-	5.6
OPDA	292.204	291.196	ESI-	13
SAG	300.085	299.077	ESI-	4.1
SGE	300.085	299.077	ESI-	4.1
Chlorogenic acid	354.095	353.087	ESI-	4.4
ABA-Glucoside	426.189	425.181	ESI-	4.9
Camalexin	200.041	201.049	ESI+	11.8
Glycine	750.32	760.398	ESI+	Not retained
Alanine	890.477	900.555	ESI+	0.8
Arginine	174.112	175.12	ESI+	0.5
Asparagine	132.054	133.061	ESI+	0.8
Aspartic Acid	133.038	132.03	ESI-	1.4
Cysteine	121.02	122.028	ESI+	0.8
Glutamine	146.069	147.077	ESI+	0.8
Glutamic acid	147.053	148.061	ESI+	0.8
Histidine	155.07	156.077	ESI+	0.6
Isoleucine	131.095	132.102	ESI+	1.4
Leucine	131.095	132.102	ESI+	1.4
Lysine	146.106	147.113	ESI+	0.6
Methionine	149.051	150.059	ESI+	1.1
Phenylalanine	165.079	166.087	ESI+	2.1
Proline	115.063	116.071	ESI+	0.8
Serine	105.043	106.05	ESI+	0.8
Threonine	119.058	120.066	ESI+	0.8
Tryptophan	204.09	205.098	ESI+	2.6
Tyrosine	181.074	182.082	ESI+	1.2
Valine	117.079	118.087	ESI+	0.9
5-Hydroxyindole-3-acetic acid	191.058	192.066	ESI+	3.4
Indole-3-acetamide	174.079	175.087	ESI+	4.1

N-(3-indoleylacetyl)-L-alanine	246.1	247.108	ESI+	8.1
Compound	**Exact mass**	**Ion mass**	**Ionization mode**	**Retention time (min)**
Indole-3-carboxaldehyde	145.053	146.061	ESI+	5.5
Methyl indole-acetate	189.079	190.087	ESI+	10.3
N-[(-)-Jasmonoyl]-Methionine (JAMet)	341.16	340.15	ESI-	12.7
Jasmonoyl-L-phenylalanine	357.194	356.186	ESI-	13.3
Jasmonoyl-L-valine (JAVal)	309.194	308.186	ESI-	12.5
Indole-3-acetyl-Isoleucine	288.147	289.155	ESI+	12.1
Indole-3-acetyl-L-phenylalanine	322.13	323.139	ESI+	12.6
Indole-3-carboxaldehyde	145.053	144.0449/146.0606	ESI-/+	5.3
Indole-3-acetonitrile	156.06	157.077	ESI+	-
Indole-3-acetic acid-L-aspartic acid	290.09	289.082	ESI-	9.8
Indole-3-pyruvic acid	203.058	202.0504/204.0661	ESI-/+	-
I3CA methyl ester	175.06	176.071	ESI+	4
Indole-3-acetyl-L-tryptophan	361.142	360.1348/362.1505	ESI-/+	11.9
Adipic Ac	146.141	145.05	ESI -	3
Ketoglutaric Ac	146.021	145.013	ESI -	--
Galacturonic Ac,	194.042	193.034	ESI -	1.5
Malic Ac,	134.021	133.014	ESI -	2.1
Folic Ac, (B9)	441.139	440.13	ESI -	4.3?
Fumaric Ac,	116.011	115.016	ESI -	-
Gibberellic Ac,	346.141	345.142	ESI -	5.5
Ascorbic Ac, (C)	176.032	177.06	ESI+	11.5
Maleic Ac,	116.011	115	ESI -	-
Nicotinic Ac,	123.032	124.024	ESI+	1.6
p-Aminobenzoic Ac,	137.048	138.05	ESI+	2.7
Pyruvic Ac,	880.16	87.008	ESI -	4.14?
Salicylhydroxamic Ac,	153.043	152.034	ESI -	3.3
Sinapic Ac,	224.069	223.06	ESI -	4.9
Pipecolic Ac,	129.079	128.071	ESI -	0.9
2-Aminoadipic Ac,	161.069	160.061	ESI -	0.9
AMP	347.063	346.05	ESI -	2.6
ADP	427.029	428.03	ESI+	

ATP	506.996	505.987	ESI -	
ATP		508.003	ESI+	
Compound	**Exact mass**	**Ion mass**	**Ionization mode**	**Retention time (min)**
FAD	785.157	786.16	ESI+	-
NADH	665.12	664.101	ESI -	--
Riboflavin (B2)	376.138	377.146,00	ESI+	3.9
Pyridoxal 5-phosphate (B6)	247.025	248.03	ESI+	4.1
Vanillin	152.047	153.05	ESI+	4.2
Quercetin	302.043	301.03	ESI -	10.9
Naringenin	272.069	271.064	ESI -	10.7
Scopoletin	192.042	193.04	ESI+	4.5
Hesperetin	302.079	303.08	ESI+	10.8
6-Benzylaminopurine	225.101	226.108	ESI+	6.2
Kinetin	215.081	216.08	ESI+	4.2
Thiamine (B1)	265.11	265.11	ESI+	0.5
Zeatin (not in the library)	219.112	220.119	ESI+	2.44 in real sample

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CHAPTER 2: ROOT METABOLIC PLASTICITY UNDERLIES FUNCTIONAL DIVERSITY IN MYCORRHIZA-ENHANCED STRESS TOLERANCE IN TOMATO

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Chapter 2: Root metabolic plasticity underlies functional diversity in mycorrhiza-enhanced stress tolerance in tomato

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Abstract

Arbuscular mycorrhizal (AM) symbioses can improve plant tolerance to multiple stresses. We compared three AM fungi (AMF) from different genera, one of them isolated from a dry and saline environment, in terms of their ability to increase tomato tolerance to moderate or severe drought or salt stress. Plant physiological parameters and metabolic profiles were compared in order to find the molecular mechanisms underlying plant protection against stress.

- Mycorrhizal growth response was determined, and ultrahigh-performance LC-MS was used to compare the metabolic profile of plants under the different treatments.

- All AMF increased plant tolerance to stress, and the positive effects of the symbiosis were correlated with the severity of the stress. The AMF isolated from the stressful environment was the most effective in improving plant tolerance to salt stress. Differentially accumulated compounds were identified and the antistress properties of some of them were confirmed.

- We demonstrate that AM symbioses increase plant metabolic plasticity to cope with stress. Some responses were common to all AMF tested, while others were specifically related to particular isolates. Important metabolism reprograming was evidenced upon salt stress, and we identified metabolic pathways and compounds differentially accumulated in mycorrhizas that may underlie their enhanced tolerance to stress.

Introduction

In the current scenario of accelerated climate change, drought and salinity are considered the abiotic factors with major impact on plant development and productivity, causing serious agricultural yield losses. Moreover, they are expected to become more severe in large areas of the planet (Sheffield *et al*., 2012; Trenberth *et al*., 2014). In plants, both stresses induce morphological, physiological, biochemical and molecular alterations, directly or indirectly impacting multiple plant processes. Plant responses to these situations are complex and, upon perception of the stress, sophisticated signaling cascades shape the appropriate defense responses (Cominelli *et al*., 2013; Albacete *et al*., 2014; Golldack *et al*., 2014). These responses include mechanisms to protect plant cells by inducing an array of stress-responsive genes and proteins, including antioxidant systems to counteract the production of reactive oxygen species (Gill and Tuteja, 2010). Drought and salinity are often interconnected: an excess of salt generally induces physiological drought in plants (Pitman and Lauchli, 2002), and can, therefore, induce similar cellular damage. However, despite the similarities derived from the osmotic stress, salinity causes an additional stress by Na^+ toxicity and consequent K^+ deficiency as it competes with K^+ uptake (Estrada et al., 2013a). Therefore, while some plant responses may be common (e.g. the accumulation of osmolytes such as proline, soluble carbohydrates or glycine betaine), others are specific for each stress and its severity. For example, under drought stress, plants promote root growth to improve water uptake from the surrounding soil, while under salt stress water uptake is reduced to limit Na^+ absorption.

In addition to the plant intrinsic mechanisms for stress tolerance, associations with soil beneficial microbes may result in enhanced plant fitness by alleviating the effects of deleterious factors. This is particularly advantageous in changing environments where fine-tuned allocation of limited resources between growth and defense is critical for survival (Goh *et al*., 2013; Lakshmanan *et al*., 2014; Pozo *et al*., 2015). The mechanisms

underlying the benefits provided by the associated microbiota are diverse. Some are direct (e.g. by increasing nutrient availability in the soil or antagonizing pathogens), or indirect through priming of plant defense mechanisms or increasing plant phenotypic plasticity in a broad range of traits towards improved resistance or tolerance to stress situations (Luo *et al*., 2009; Goh *et al*., 2013; Selosse *et al*., 2014; Pozo *et al*., 2015).

Among these beneficial organisms, soilborne fungi from the phylum Glomeromycota, known as arbuscular mycorrhizal fungi (AMF), have received special attention because of their ubiquity and notorious contribution to plant health. They are able to establish the most ancient and widespread plant–microbe symbiosis, known as arbuscular mycorrhizas, present in c. 80% of all terrestrial plant species and considered a key step in their evolution (Smith and Read, 2008). AMF are obligate biotrophs, and the host plant allocates photosynthates to the fungus for the development, maintenance and function of mycorrhizal structures (Bago *et al*., 2000). In return, the fungus assists the host plant in the acquisition of water and mineral nutrients, becoming extremely important for the uptake of inorganic phosphate and various micronutrients (Smith and Smith, 2011; Hodge and Storer, 2015). However, AM symbiosis not only helps the plant, by favoring nutrient and water uptake, but also induces significant changes in multiple host traits, such as root architecture, growth rate, flowering and especially stress resistance (Pozo *et al*., 2015). Indeed, previous studies showed a strong hormonal and metabolic rearrangement in mycorrhizal roots and shoots of multiple plant species, including tomato (López-Ráez *et al*., 2010; Schweiger *et al*., 2014; Rivero *et al*., 2015; Schweiger and Müller, 2015; Hill *et al*., 2018), that may underlie the increased plant's ability to overcome unfavorable conditions (Gianinazzi *et al*., 2010; Jung *et al*., 2012; Ruiz-Lozano *et al*., 2012; Selosse *et al*., 2014). Enhanced antioxidant systems, higher water-use efficiency and faster activation of defense-related enzymes are among the stress tolerance responses boosted by AMF (Nadeem *et al*., 2014). Metabolic plasticity has been also related to the enhanced responsive capacity of AM plants when dealing with combined stresses such as nutrient depletion and simultaneous pathogenic infections (Sánchez-Bel *et al*., 2016).

Attending to the reported benefits, AMF inoculations appear as a valuable tool for boosting the plant capacity to adapt their phenotype to challenging conditions, that is, increasing its phenotypic plasticity to cope with adverse environmental stresses (Nadeem *et al*., 2014; Abdel Latef *et al*., 2016). It has been reported that under drought or salinity stress, AM plants can perform better than nonmycorrhizal (Nm) ones through facilitation

of water uptake, increase in photosynthesis ability and improved ion homeostasis (Estrada *et al*., 2013a; Augé *et al*., 2014; Porcel *et al*., 2015; Ruiz-Lozano *et al*., 2016; Sánchez-Romera *et al*., 2016). It is noteworthy that some recent studies showed functional diversity among different AMF strains in relation to their ability to induce tolerance, resulting in different degrees of protection (Chitarra *et al*., 2016). Indeed, some studies point out a better plant performance under abiotic stress when they are inoculated with AMF isolated from soils conditioned by the same abiotic stress (Estrada *et al*., 2013a,b). In fact, native plants thrive in various abiotically stressed environments thanks to AMF that have coevolved with them and are essential for their adaptation to stressed conditions (Rodríguez and Redman, 2008).

In order to develop future strategies aimed at alleviating the negative effects of multiple stresses in a changing environment, understanding the plant defense responses in these situations is crucial. Regarding the use of AMF, a better knowledge of how AM symbiosis increases plant tolerance against diverse stresses is fundamental. Untargeted metabolomics approaches have proved to be an excellent tool to provide a global view of plant responses under stress (Sardans *et al*., 2011; Gamir *et al*., 2012; Rodziewicz *et al*., 2014). In particular, the LC-MS technique is highly sensitive, allowing for the detection of key molecules in the phenotypic adaptation responses.

Materials and Methods

Plant and fungal materials and growing conditions

Isolates of *Funneliformis mosseae* (BEG12; formerly *Glomus mosseae*) and *Rhizoglomus irregulare* (DAOM 197198 formerly *Rhizophagus irregularis*) (Young, 2015), widely distributed and used worldwide as inoculants, were obtained from the International Bank of Glomeromycota (http://www.i-beg.eu), while *Claroideoglomus etunicatum* (EEZ163) was isolated from a highly salinized soil from Cabo de Gata Natural Park (Almería, Spain), one of Europe's most arid ecosystems, by Estrada *et al*. (2013b).

All AMF were continuously maintained in open-pot cultures of *Trifolium repens* mixed with Sorghum vulgare plants in a glasshouse. The inocula were obtained on a vermiculite-sepiolite substrate, and contained infected root fragments, mycelia and spores, from the corresponding fungus.

Tomato seeds (*Solanum lycopersicum* cultivar Moneymaker) were surface-disinfected by immersion in 4% NaHClO (10 min) containing 0.02% (v/v) Tween20, rinsed thoroughly with sterile water and incubated for 7 d in an open container with sterile vermiculite at 25°C. Tomato plantlets were then transferred to pots containing a sterile soil: sand: vermiculite mixture (1:1:1, v/v). The soil was steam-sterilized (100ºC, 1 h for 3 d consecutively) and the sand and vermiculite were autoclaved (120°C, 20 min) before mixing. Plants were subjected to the different treatments described in the following and pots were randomly distributed and grown in a glasshouse at 24:18°C with a 16 : 8 h, light : dark regime.

Experimental design

Two different experiments were carried out, both following a full factorial design with two factors. **Experiment 1** (the 'mycorrhizal experiment') tested the effect of AMF inoculation on plant tolerance to drought and salt stress. Thus, factors were mycorrhizal treatments, with four levels (nonmycorrhiza, and inoculation with *F. mosseae, R. irregulare* or *C. etunicatum*) and the stress factor, with five levels (nonstress, moderate drought, severe drought, moderate salinity, severe salinity). In this experiment shoot and root fresh weight (FW), mycorrhizal colonization, mycorrhizal growth response (MGR), nutrient content, metabolomics profiles and electrical conductivity of the substrate were analyzed. In **Experiment 2** (the 'chemical treatment experiment'), the two factors were chemical treatments, with five levels (control, catechin, pyridoxic acid, pyridoxamine dihydrochloride and pyridoxal hydrochloride) and the stress treatments, with three levels (nonstress, moderate salt stress, severe salt stress). In this experiment root and shoot FW, relative water content (RWC) and photosystem II (PSII) efficiency were determined.

Mycorrhizal inoculation and quantification

For the mycorrhizal experiment, plants were grown in 750 ml pots. Mycorrhizal treatments were performed by adding to the growing substrate 10% (v/v) of *F. mosseae* (Fm), *R. irregulare* (Ri) or *C. etunicatum* (Ce) inoculum. Control plants received the same proportion of the inoculum substrate without mycorrhizal propagules, and all plants received an aliquot of a filtrate (< 20 µm) of the mix of the three AMF inocula, in order to homogenize microbial populations but free from AMF propagules.

Mycorrhizal colonization was estimated after clearing washed roots in KOH (10%) and after subsequent staining of fungal structures with 5% ink in 2% acetic acid (Vierheilig *et al*., 2005). The extent of mycorrhizal colonization (expressed as percentage of total root length colonized by the AMF) was calculated according to the gridline intersection method (Giovannetti and Mosse, 1980) using a Nikon Eclipse 50i microscope and brightfield conditions.

Stress treatments

After transplanting, plants were watered three times a week to field capacity during the first 4 wk. One of those weekly waterings was performed with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration.

Thereafter, three different treatments were applied during a further 4 wk: nonstress (Ns), watering conditions were as in the previous weeks; salinity (Sal), watering was performed as before but 75 or 150 mM NaCl solution was used instead of water for moderate or severe salt stress, respectively; drought (Dro), plants were also watered three times a week, but only until reaching 75% or 50% field capacity for moderate or severe drought stress, respectively. Stress conditions were selected following previous studies (Porcel *et al*., 2015; Ruiz-Lozano *et al*., 2016) and their deleterious effects in the plant confirmed in preliminary experiments. A total of 20 treatments (four fungal treatments and five stress conditions) were performed, and at least six independent biological replicates were used for each treatment. In the case of mycorrhizal treatments, two additional plants were added to confirm AM symbiosis establishment before the application of the stress treatments.

Plants were harvested after 8 wk of growth. The FW of shoots and roots was determined, and the material was immediately frozen in liquid nitrogen and stored at -80 °C until use for metabolomic analysis. An aliquot of each individual root system was reserved for mycorrhizal quantification and the MGR was calculated as the biomass ratio between mycorrhizal (Fm, Ri or Ce) and Nm plants, as previously described (Zheng *et al*., 2015).

Chemical treatment experiment

A follow-up experiment was performed to test the potential contribution to plant stress tolerance of some of the differentially accumulated compounds identified in the previous

experiment. The experiment followed a full factorial design with two factors: chemical treatment, with five different levels, and salt stress, with three levels (Ns, moderate (75 mM NaCl) and severe (150 mM NaCl)). Seven plants were used for each of the 15 distinct combinations of treatments, with a total of 105 plants. The chemicals applied were selected according to their accumulation profile in the metabolome analysis from the previous experiment and their commercial availability: catechin (CAT) (C1788; Sigma-Aldrich), pyridoxic acid (PA) (P9630; Sigma-Aldrich), pyridoxamine dihydrochloride (PM) (P9380; Sigma-Aldrich) and pyridoxal hydrochloride (PL) (271748; Sigma-Aldrich). Tomato plantlets were transferred to 450 ml pots containing a sterile sand: vermiculite mixture (1:1, v/v). and watered three times a week with Long Ashton nutrient solution. After 4 wk of growth, the salt stress treatments (0, 75 and 150 mM NaCl) were initiated and maintained for 15 d. Chemical treatments were performed twice, the first 2 d before starting the salt treatment, and the second after 1 wk of salt treatment. The application of the chemicals was carried out by watering the plants with a 50 ml water solution containing 0.001% methanol and 2 mM CAT, or 100 µM of PA, PM or PL. For the control treatment (C), plants were irrigated with the same amount of water-methanol solution than the other treatments. Plants were harvested after 15 d of NaCl stress, the FW of shoots and roots was determined, and a leaflet was reserved for RWC determination.

Leaf relative water content and PSII efficiency determination

The efficiency of PSII was measured in plants from the chemical treatment experiment using a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a noninvasive assessment of plant photosynthetic performance by measuring Chl fluorescence. FluorPen quantifies the quantum yield of PSII as the ratio between the variable fluorescence in the light-adapted state (FV') and the maximum fluorescence in the light-adapted state (FM'), according to Oxborough and Baker (1997). An actinic light intensity of 1000 µmol (photons) m^{-2} min^{-1} was used. Measurements were taken in the second youngest leaf of all plants after 7 d of NaCl salinity stress.

Leaf RWC was determined on the same day of the harvest as follows: a leaflet from five different plants per treatment was weighed immediately (FW) and then immersed in a closed tube with a wet piece of cotton tissue and incubated for 24 h at 4°C in darkness.

After that, the piece of leaf was weighed again (turgid weight (TW)) and dried in an oven (75°C) for 2 d (DW). RWC was calculated as [(FW - DW) / (TW - DW)] x 100.

Determination of mineral nutrients in plant tissues

Nutrient content in roots and shoots was measured at the Ionomic Laboratory of the Technical Services of the Centro de Edafología y Biología Aplicada del Segura (CEBAS, CSIC), Murcia, Spain. Four biological replicates were analyzed from each treatment from the mycorrhizal experiment. Frozen roots were ground to a fine powder and lyophilized, while shoot material was obtained by grinding dry leaves. Element concentrations were analyzed after acid digestion of the samples, by inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP 6500 Duo Thermo). Total C and N contents were determined using an Elemental Analyzer (Leco Truspec CN, St Joseph, MI, USA) according to standard procedures.

Liquid chromatography and electrospray ionization (LC-ESI) mass spectrometry

LC-ESI full scan mass spectrometry (Q-TOF instrument). Freeze-dried roots (50 mg) from the nonstress and moderate stress treatments in the mycorrhizal experiment were homogenized on ice in 1 ml of MeOH:H_2O (10:90) containing 0.01% of HCOOH. The homogenate was centrifuged at 15 000 g for 15 min at 4°C. The supernatant was recovered and filtered through 0.2 µm cellulose filters (Regenerated Cellulose Filter, 0.2 µm, 13 mmD pk/100; Teknokroma, St Cugat, Spain). An aliquot of 20 µl was injected into an Acquity ultraperformance LC system (UPLC) (Waters, Milford, MA, USA), which was interfaced with a hybrid quadrupole time-of-flight instrument (Q-TOF-MS Premier). Analytes were eluted with an aqueous methanol gradient containing 0.01% HCOOH. Six biological replicates were randomly injected for every treatment. LC separation was performed using an UPLC Kinetex C18 analytical column with a 5 µm particle size, 2.1 x 100 mm (Phenomenex, Madrid, Spain). Solvent gradients and further chromatographic conditions were performed as previously described (Agut *et al*., 2014). A solution of leucine enkephalin at a concentration of 2 ppm in CH_3CN:H_2O (50:50) with 0.1% HCOOH was simultaneously introduced into the Q-TOF instrument via the lock-spray needle for accurate mass-to-charge ratio (m/z) determinations. The LC-ESI-Q-TOF-MS library of plant compounds was used for a straight identification in full-scan analysis. Standards for phenols, indolic compounds, amino acids, hormones and their

derivatives (up to 84 compounds) were prepared (100 ppb) in a composite solution (Rivero *et al*., 2015) and injected through the UPLC in both positive and negative ESI (ESI^+; ESI^-) modes. To identify compounds, at least two of the following three criteria were needed: matching the exact mass; matching the retention time between the standard and experimental samples; and contrasting the obtained fragmentation spectrum with those available in the Massbank, Metlin and/or Human Metabolome databases (www.massbank.jp; www.masspec.scripps.edu; www.hmdb.ca).

Full scan data analysis. Data were acquired in centroid mode and subsequently transformed into .cdf files using the Databridge from MASSLYNX 4.1 software (MassLynx 4.1; Waters). Chromatographic signals were processed using the software R for statistical purposes. Signals from positive and negative ESI (ESI^+; ESI^-) were processed separately. Peak peaking, grouping and signal corrections were performed using the XCMS algorithm (Smith *et al*., 2006). Metabolite amounts were analyzed on the basis of normalized peak area units relative to the DW. The Kruskal–Wallis test ($P < 0.05$) was applied to analyze the metabolomic differences between treatments. To determine the global behavior of the signals, data obtained from ESI^+ were normalized by median, transformed by cube root and scaled by the pareto method, and subsequently both sparse partial least-squares discriminant analysis (sPLSDA) and heat-map plots were generated using METABOANALYST, a comprehensive web-based package for a range of metabolomics applications (Xia and Wishart, 2016). Statistical and heat-map analyses were performed using the MARVIS SUIT 2.0 software tool for clustering and visualization of metabolic biomarkers (Kaever *et al*., 2015). Adduct, isotope correction and clustering were also performed by using the associated software packages MARVIS FILTER and MARVIS CLUSTER.

Statistical analyses

Besides the methods and software for metabolomic analysis already described, all statistical analyses (two-way ANOVAs and post hoc tests applied when appropriate, as indicated in the corresponding figure legends) were conducted using STATGRAPHICS PLUS 3.1 (Rockville, MD, USA), 'R' software v.2.9.2 (R Development Core Team) and the XCMS package. Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively. When data did not meet any of the assumptions of ANOVA, square-root transformations were applied. For the mycorrhizal

experiment, AMF inoculation and stress treatment were used as factors, while chemical application and stress treatments were the two factors used in the ANOVA for the chemical treatment experiment. ANOVA tables are included as Supporting Information (**Tables S3–S6**).

Results

Mycorrhizal growth response increases with stress severity both drought and salt stresses had a negative effect on plant biomass in all treatments. As expected, the weight loss was higher at the severe stress levels (**Table S1**). In Nm plants, shoot biomass was reduced by 38% in moderate and by 81% in severe drought stress, whereas salinity was reduced 47% and 81% after moderate and severe salt stress, respectively (**Tables S1, S2**). All AMF tested efficiently colonized tomato roots, and the impact of the stress was remarkably lower in all mycorrhizal plants. Therefore, both mycorrhiza and stress factors significantly affected shoot and root growth, and the interaction between them was also significant (**Table S3**). The growth improvement by AMF was more noticeable under the most severe drought conditions (**Fig. 1a; Table S1**). All AMF enhanced plant growth during the stress imposed by NaCl, mostly at the severe stress level in aboveground tissues. Interestingly, the MGR in Ce plants under both salinity levels was significantly higher than in Fm and Ri plants. It is notable that, regarding root biomass, only Ce-colonized plants showed a better MGR under salt stress, and increased the root biomass 3.8-fold compared with Nm plants in the most restrictive conditions for growth (150 mM NaCl) (**Fig. 1b; Tables S1, S3, S4**).

As plants subjected to abiotic stresses are usually affected in their nutrient uptake capability, we analyzed the nutrient content of the plants from the different treatments. A supervised analysis (sPLSDA) of the data showed a strong impact of the stresses on nutrient accumulation in roots (**Fig. S1**). Changes in nutrient content differed with the type and severity of the stress. Remarkably, although some differences were observed in the nutrient profiles of mycorrhizal and Nm plants under nonstress conditions, differences were bigger in plants growing under both severe drought and salt stress conditions (**Fig. S1b**). Among the microelements, Na^+ is the main one responsible for the toxic effects of salt stress. As expected, Na^+ concentration increased upon NaCl stress in shoots and roots in both Nm and mycorrhizal plants (**Fig. 2**). However, AMF-colonized roots presented

lower concentrations of this ion compared with Nm ones under both moderate and severe salt stress conditions. As for the plant biomass, both mycorrhizal and stress factors significantly affected Na^+ concentration in shoots and roots, although the interaction between factors was only significant in shoots (**Table S5**). It is noteworthy that Ce was the most efficient in reducing Na^+ concentration in leaves. Thus, tomato plants colonized by Ce, the salt-adapted AMF, displayed the best performance under salt stress, excluding Na^+ in both below- and above-ground tissues (**Fig. 2**).

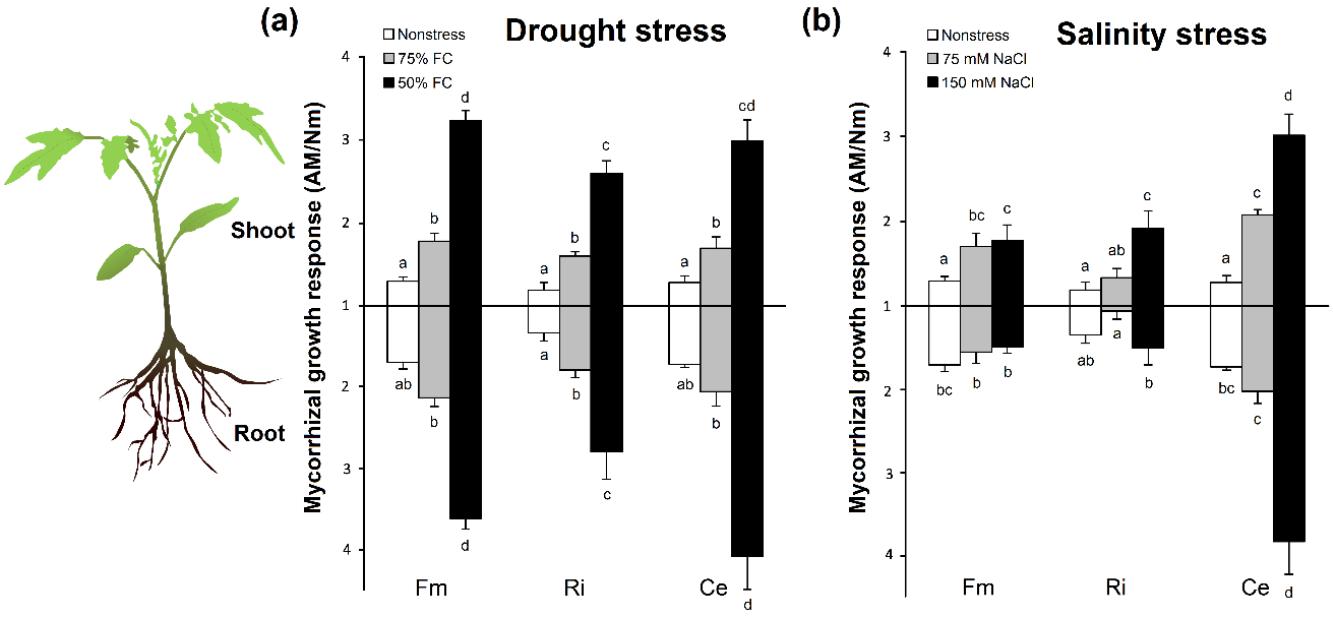


Fig. 1 Mycorrhizal growth response (MGR) of *Solanum lycopersicum* plants colonized by different arbuscular mycorrhizal fungi (AMF) under different stress conditions. Four-week-old nonmycorrhizal tomato plants (Nm) or plants colonized by the AMF *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) or *Claroideoglomus etunicatum* (Ce) were subjected to different abiotic stress conditions during four additional weeks (8 wk in total). MGR from shoot or root tissues (indicated above or below the line) was calculated as the ratio between biomass of mycorrhizal (Fm, Ri, Ce) and nonmycorrhizal plants (Nm). White bars, nonstress growing conditions (well-watered, no salt); gray bars, moderate stress conditions; black bars, severe stress conditions**. (a) Drought stress:** 75% FC, moderate drought stress (plants watered to 75% field capacity); 50% FC, severe drought conditions (plants watered to 50% field capacity). **(b) Salinity stress**: plants were watered with 75mM NaCl (moderate salinity) or 150mM NaCl (severe salinity). Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Data are expressed as means ± SEM (n = 6). Means within shoot or root not sharing a letter in common differ significantly according to Fisher's least significant difference post hoc test ($P < 0.05$).

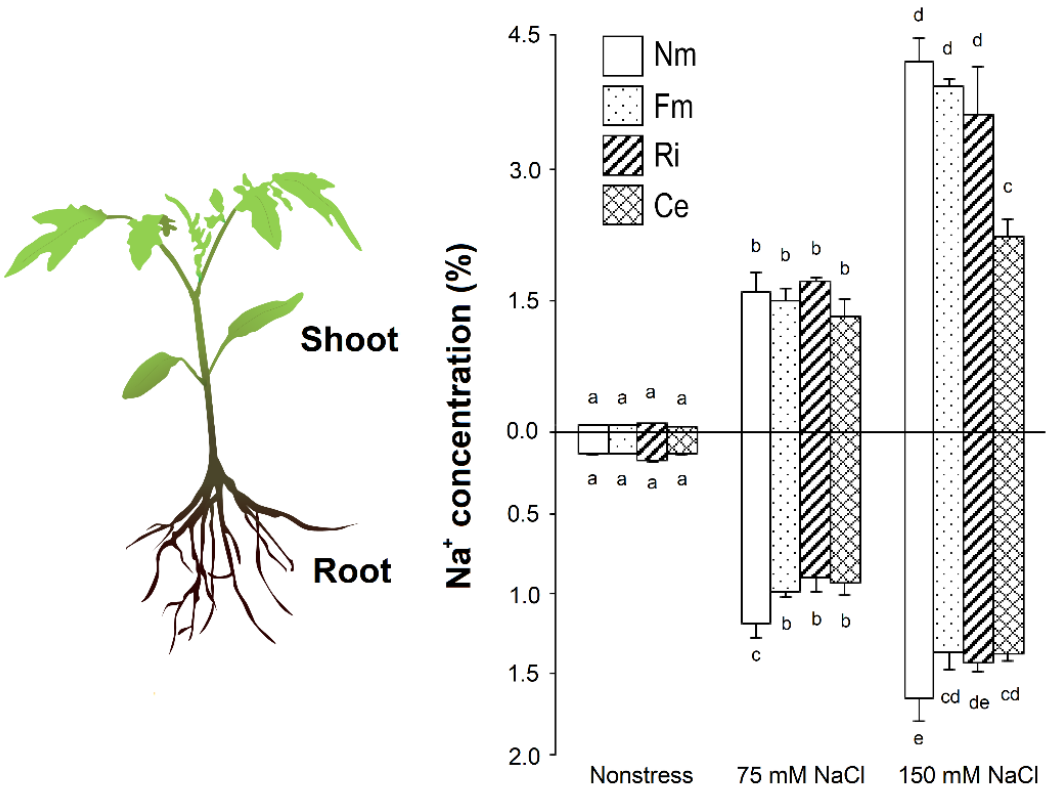


Fig. 2 Na^+ concentration in shoot and root tissues of *Solanum lycopersicum* plants colonized by the arbuscular mycorrhizal fungi *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) and *Claroideoglomus etunicatum* (Ce) compared with nonmycorrhizal plants (Nm), grown under saline conditions. Four-week-old plants were subjected to moderate or severe salt stress (75 or 150 mM NaCl, respectively) or grown under nonstress conditions during 4 wk (8 wk in total). Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Data are expressed as means ± SEM (n = 4). Means within shoot or root not sharing a letter in common differ significantly according to the Fisher's least significant difference test ($P < 0.05$)

Metabolic reprograming depends on the challenging stress and the colonizing AMF

Untargeted metabolomic analysis was performed in roots of plants subjected to moderate stress. Three-dimensional supervised analysis (3D-sPLSDA) of the root metabolic profiles showed that mycorrhizal establishment had a significant impact both in the presence and absence of stress (**Fig. 3**). The first important observation is the reduced impact of *F. mosseae* and *R. irregulare* in comparison to *C. etunicatum*. The 3D-sPLSDA showed a strong separation induced by *C. etunicatum* while the others, although separated, remain closer to Nm plants. Differences were also evident regarding the applied stress. A reduced metabolic reprograming was observed in response to drought stress, since within each mycorrhizal treatment, the metabolic profile in well-watered vs

drought-stressed plants grouped together (Nm/NmDro, Fm/FmDro, Ri/RiDro, Ce/CeDro) (**Fig. 3**). By contrast, salt stress had a strong impact in all Nm and AMF colonized plants. It is noteworthy that Fm and Ri plants showed overlapping metabolic responses upon salt-stress, with profiles strongly separated from those in Nm salt stressed plants. The strongest changes were observed in Ce-colonized plants following salt stress as they showed a very different metabolic rearrangement, grouping separately from any other experimental condition in the 3D plot (**Fig. 3**).

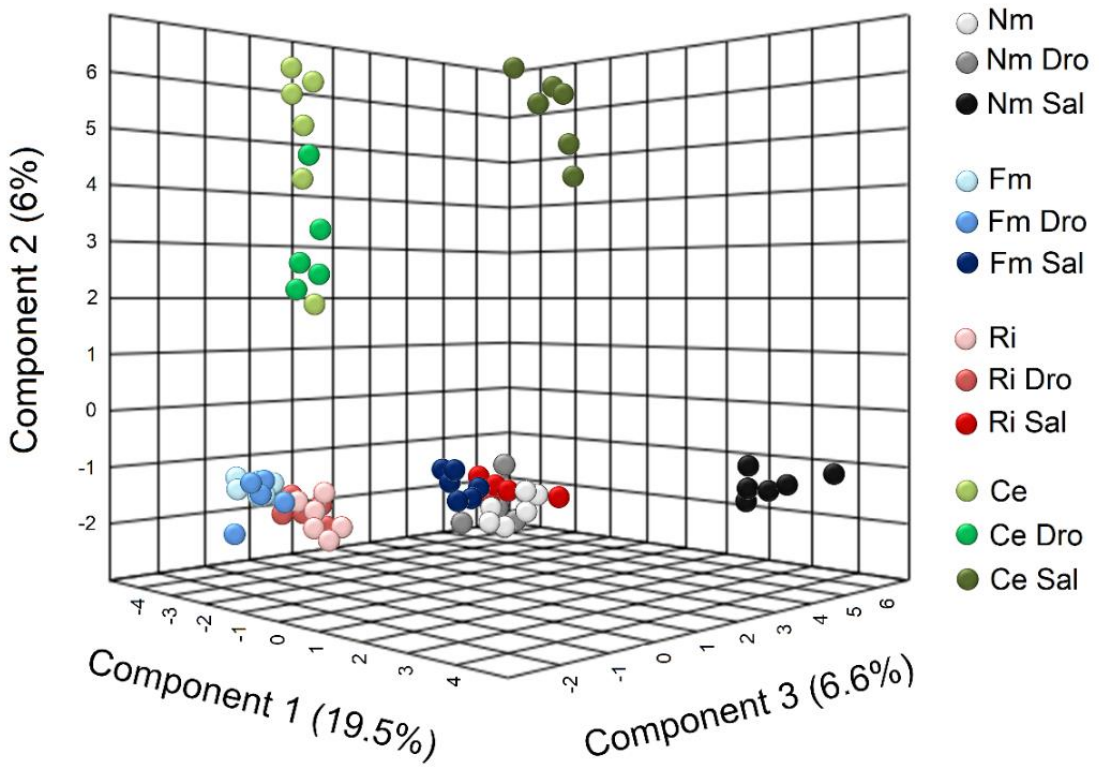


Fig. 3 Overview of the metabolomic reprogramming in mycorrhizal roots grown under different abiotic stress conditions. Four-week-old tomato plants were subjected to different abiotic stress conditions (nonstress, drought and salinity) during four additional weeks (8 wk in total). A supervised three-dimensional sparse partial least squares discriminant analysis (sPLSDA) representation of the major sources of variability from positive electrospray ionization signals was obtained from a nontargeted metabolic analysis. An ultrahigh-performance liquid chromatography interfaced with a quadrupole time-of-flight mass spectrometer was performed to monitor metabolomic changes. Signal intensity was determined in all samples after normalizing the chromatographic area for each compound to the DW of the sample. All registered signals were compared using nonparametric Kruskal–Wallis test, and only data with differences between groups significant at $P < 0.05$ were used for the supervised analysis (n = 6). White-gray-black dots, nonmycorrhizal plants (Nm); blue scale dots, *Funneliformis mosseae*-colonized plants (Fm); red scale dots, *Rhizoglomus irregulare*-colonized plants (Ri); green scale dots, *Claroideoglomus etunicatum*-colonized plants (Ce). Different stresses are represented by scale color, lighter for nonstress conditions, medium or dark scale for 75% of field capacity (drought; Dro) or 75mM NaCl (salinity; Sal), respectively.

Heat-map analysis of the top 100 compounds displaying the strongest changes in accumulation confirmed that mycorrhizal colonization has an important impact on root metabolism even in the absence of stress (**Fig. 4**). To follow up, we focused our attention on those metabolites with higher accumulation in AM plants as they are likely candidates to contribute to the stress tolerance. Among the differentially accumulated metabolites, we focused on four different types of clusters (**Fig. 4**). First, there was a group of compounds markedly accumulated in AM plants, regardless of the stress and the colonizing AMF. This cluster therefore includes common mycorrhiza-associated metabolites and was classified as cluster I. Despite these AM common changes, there are also differences between the different fungal treatments. Fm and Ce showed specific accumulation patterns, while the impact on root secondary metabolism is lower upon colonization by Ri as it is not showing any clear cluster of differentially accumulated metabolites. Thus, we identified a group of compounds highly accumulated in Fm under all experimental conditions (cluster II), and similarly, Ce-overrepresented compounds were classified as cluster III, some of them showing a further increase in Ce plants upon salt treatment. Finally, we grouped as cluster IV those compounds accumulated in response to salt stress in all mycorrhizal treatments.

Subsequently a detailed study of the signals within each cluster was carried out. Considering that all three fungi promoted plant growth in either the absence or presence of stress, some of the metabolites included in cluster I may contribute to growth promotion and/or stress tolerance. Lignans, oxylipins, derivatives of the mevalonate pathway and three amino acids (glutamic acid, histidine and cysteine) were found among the compounds identified in this cluster (**Fig. 5**). Remarkably, amino acids such as Glu and His, oxylipins and lignans were previously described to accumulate in roots colonized by *R. irregulare* and *F. mosseae* (Rivero *et al*., 2015). In this study, we could also identify the geranylgeranyl diphosphate (GGPP), a key metabolite of the mevalonate pathway strongly accumulated in AM roots. Probably, as a consequence, several carotenoid and gibberellin intermediates downstream of GGPP were also strongly accumulated in AM roots. It is noteworthy that compounds in the carotenoid pathway, showing elevated concentrations in all mycorrhizal roots, reached the highest accumulation in Ce plants (**Fig. 5**).

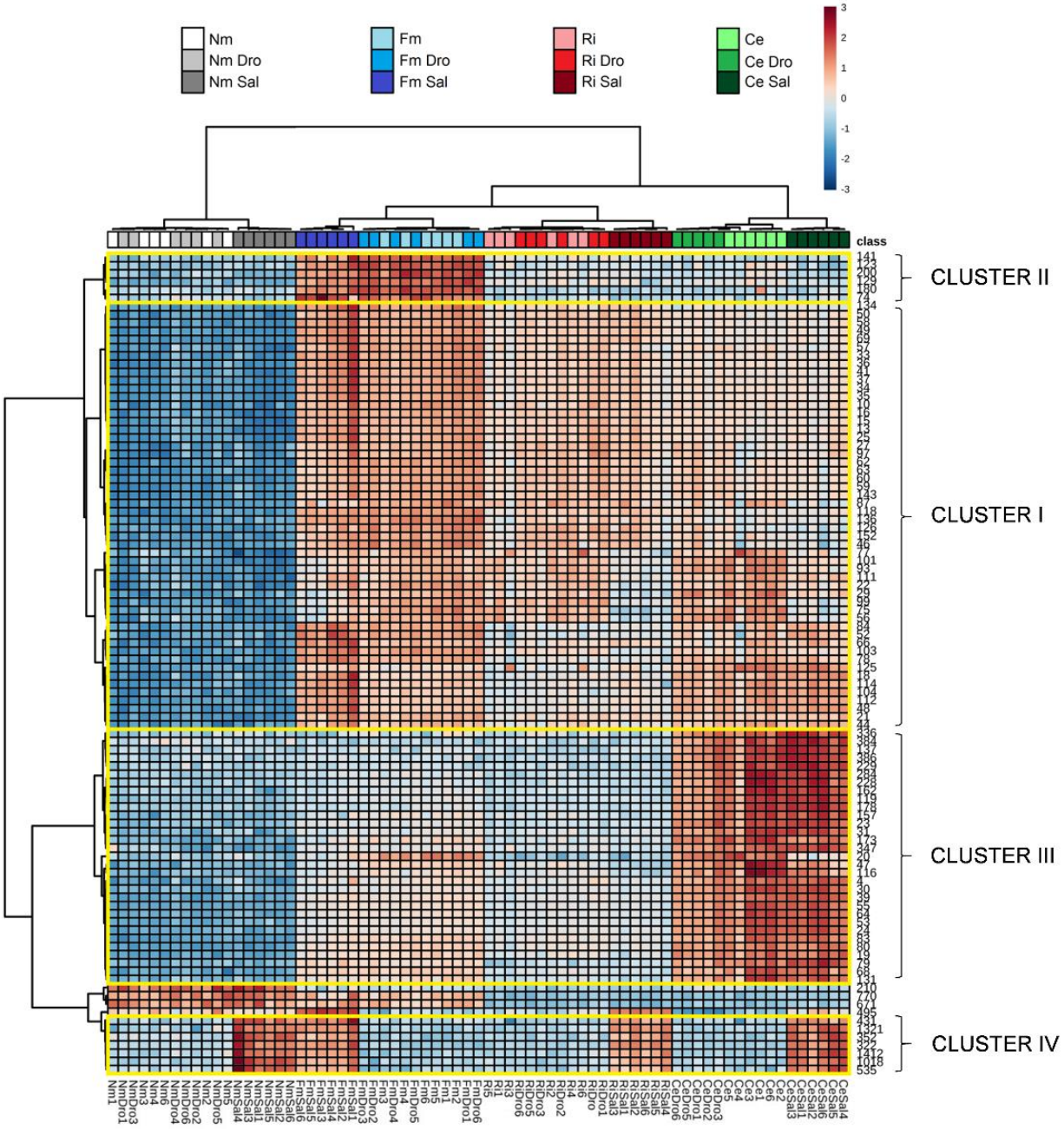


Fig. 4 Heat-map and clustering of differentially accumulated root metabolites from nonmycorrhizal and mycorrhizal plants grown under different abiotic stress conditions. Four-week-old tomato plants were subjected to different abiotic stress conditions (nonstress, drought and salinity) during four additional weeks (8 wk in total). Data acquisition from a nontargeted metabolic analysis was as described in Fig. 3. From all positive ionized acquired signals, the 100 signals showing the strongest differences (according to ANOVA) among treatments are represented. In the above axis, mycorrhizal treatments are represented as follows: white-gray-black bars, nonmycorrhizal plants (Nm); blue scale bars, *Funneliformis mosseae*-colonized plants (Fm); red scale bars, *Rhizoglomus irregulare*-colonized plants (Ri); green scale bars, *Claroideoglomus etunicatum*-colonized plants (Ce). Different abiotic stresses are represented by scale color, lighter for nonstress conditions, medium or dark scale for 75% of field capacity (drought; Dro) or 75 mM NaCl (salinity; Sal), respectively. Based on the different metabolic accumulation patterns observed, four groups were selected. Cluster I includes compounds overaccumulated in all mycorrhizal roots, regardless of the growing conditions. Clusters II and III include compounds overaccumulated only in Fm- or Ce-colonized roots, respectively. Cluster IV include compounds highly accumulated upon salt stress.

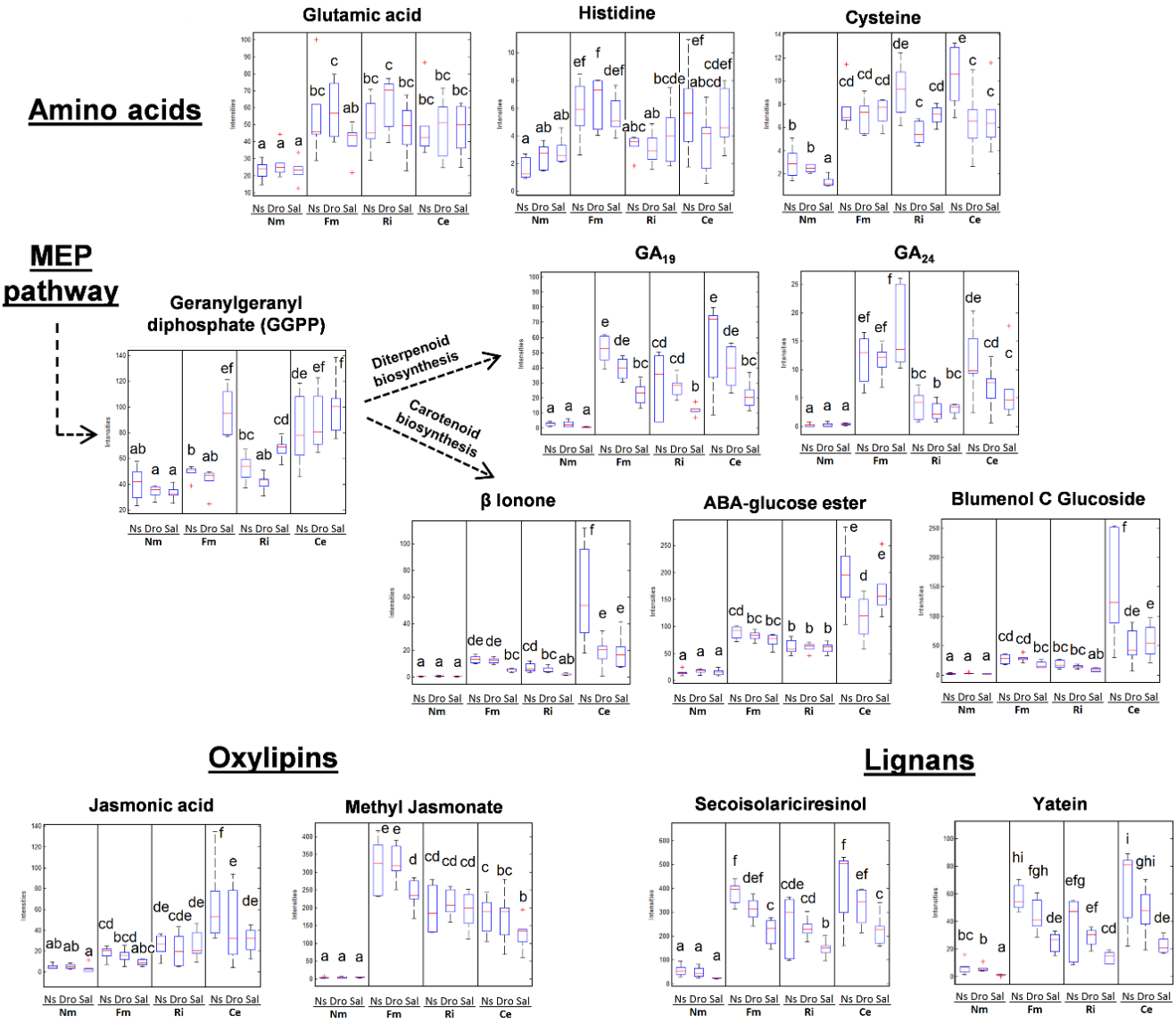


Fig. 5 Boxplots of selected metabolites with high basal accumulation in mycorrhizal roots compared with nonmycorrhizal roots (Nm). Four-week-old Nm and mycorrhizal tomato plants were subjected to different abiotic stress conditions, nonstress (Ns), drought (75% of field capacity; Dro) and salinity (75 mM NaCl; Sal), during four additional weeks (8 wk in total). Different arbuscular mycorrhizal fungi were used: *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) and *Claroideoglomus etunicatum* (Ce). Data acquisition from a nontargeted metabolic analysis was as described in the Fig. 3 legend. Boxes represent the interquartile range, red lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range, and red crosses show outliers. Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Data not sharing a letter in common differ significantly according to the Fisher's least significant difference test ($P < 0.05$, n = 6).

Regarding AMF-specific changes, the number of metabolites differentially accumulated in Fm (cluster II) is lower than those with high accumulation in Ce (cluster III) (**Fig. 4**). Cluster III includes compounds with higher concentrations in Ce plants regardless of the stress (**Fig. S2**), or some with high accumulation upon salt stress only in these plants (**Fig. 6**). Among the metabolites showing a higher accumulation in Ce regardless of the stress

we found compounds mainly related to the Chl and porfirin metabolism, such as biliverdin, (3Z)-phycocyanobilin, coproporphyrinogen and pyropheophorbide (**Fig. S2**). These observations suggest that *C. etunicatum* could help the plant to maintain an elevated photosynthetic activity even under stress conditions. We therefore focused on compounds with primed accumulation upon salt stress in Ce plants, as they are candidates to mediate the increased salt tolerance and growth stimulation induced by *C. etunicatum*. Accurate identification by exact mass and fragmentation spectrum revealed tomatidine, solasodine, solanocardinol and 2,3-acetoxysolanodulcidine, all of them belonging to the family of steroideal glycoalkaloids (SGAs).

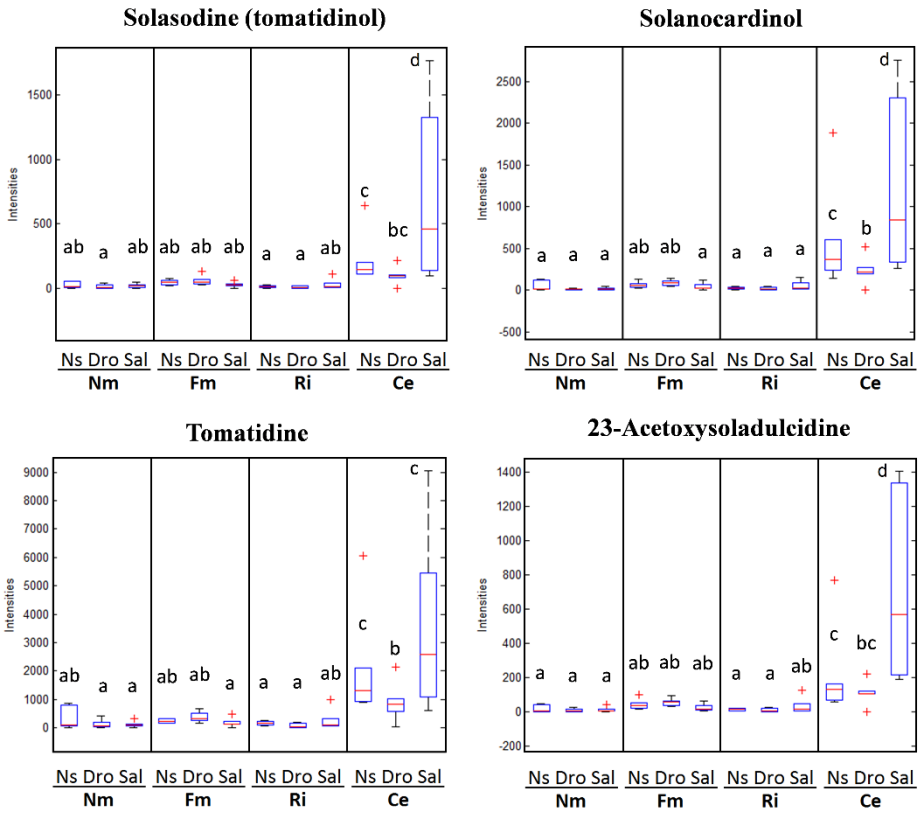


Fig. 6 Steroideal glycoalkaloids (SGAs) display a priming profile upon salt stress in roots colonized by *Claroideoglomus etunicatum* (Ce). The treatments were nonmycorrhizal plants (Nm) and plants colonized by *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) and *Claroideoglomus etunicatum* (Ce). Four-week-old Nm and AM tomato plants were subjected to different abiotic stress conditions, nonstress (Ns), drought (75% of field capacity; Dro) and salinity (75mM NaCl; Sal), during four additional weeks (8 wk in total). Data acquisition from a nontargeted metabolic analysis was as described in the Fig. 3 legend. Boxes represent the interquartile range, red lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range, and red crosses show outliers. Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Data not sharing a letter in common differ significantly according to the Fisher's least significant difference test ($P < 0.05$, n = 6).

Mycorrhiza-related priming fingerprint underlying salt stress tolerance

In relation to the compounds that accumulate specifically under salt stress (cluster IV), osmoregulators such as proline were found (**Fig. S3**). Their accumulation pattern, showing basal levels under drought stress but high under salinity conditions, confirm how salt stress has a stronger impact on the metabolome than does drought, probably because of the combination of osmotic and toxic stress associated with salt. A more detailed analysis of the cluster of salt-accumulated compounds revealed that some of them presented a greater accumulation in response to salt in AMF-colonized plants than in Nm plants, that is, they showed a primed accumulation profile (**Fig. 7**). As all AMF conferred salt stress tolerance to some extent, we hypothesize that these compounds with primed accumulation in mycorrhizas are potentially related to the enhanced tolerance to salt stress presented by all mycorrhizal plants. Accordingly, we focused on compounds that hardly change in Nm-stressed plants but were strongly accumulated upon salt stress in roots colonized by any of the studied AMF (AM-primed metabolites). A detailed analysis showed the regulation of biosynthetic pathways for compounds described to participate in stress tolerance and defense priming, such as, for instance, B6 vitamers (**Fig. 7**). Indeed, pyridoxamine 5-phosphate, significantly reduced in all AM plants upon salt stress, is the precursor of 4-pyridoxate, which is primed in salt-stressed AM plants (Fig. 7). Other compounds such as the flavanol CAT or the xanthone dulciol C, which may be transformed into catechin-glucoside and dulciol B/A/Garcinone E, respectively, show primed profiles. Among other interesting compounds, the lignan liriodendrin also showed a primed accumulation, whereas its known precursor, syringin, showed a strong decline in mycorrhizal plants.

To test whether the identified AM-primed compounds play a role in host stress tolerance, we selected some of those commercially available as CAT and several B6 vitamers (PA, PM and PL) and tested their ability to protect tomato plants through exogenous application in a new experiment (**Fig. 8; Table S6**). All B6 vitamers promoted plant growth in the absence of stress, whereas only PM improved root and shoot growth under salt stress conditions (**Fig. 8a**). Moreover, all treated plants had higher leaf RWC under severe stress conditions (**Fig. 8b**). In addition, all the tested compounds improved PSII efficiency under salt stress (**Fig. 8c**). PSII efficiency dropped with increasing severity of the salt stress in Nm plants, but all chemical treatments ameliorated this stress effect, especially under the most severe conditions.

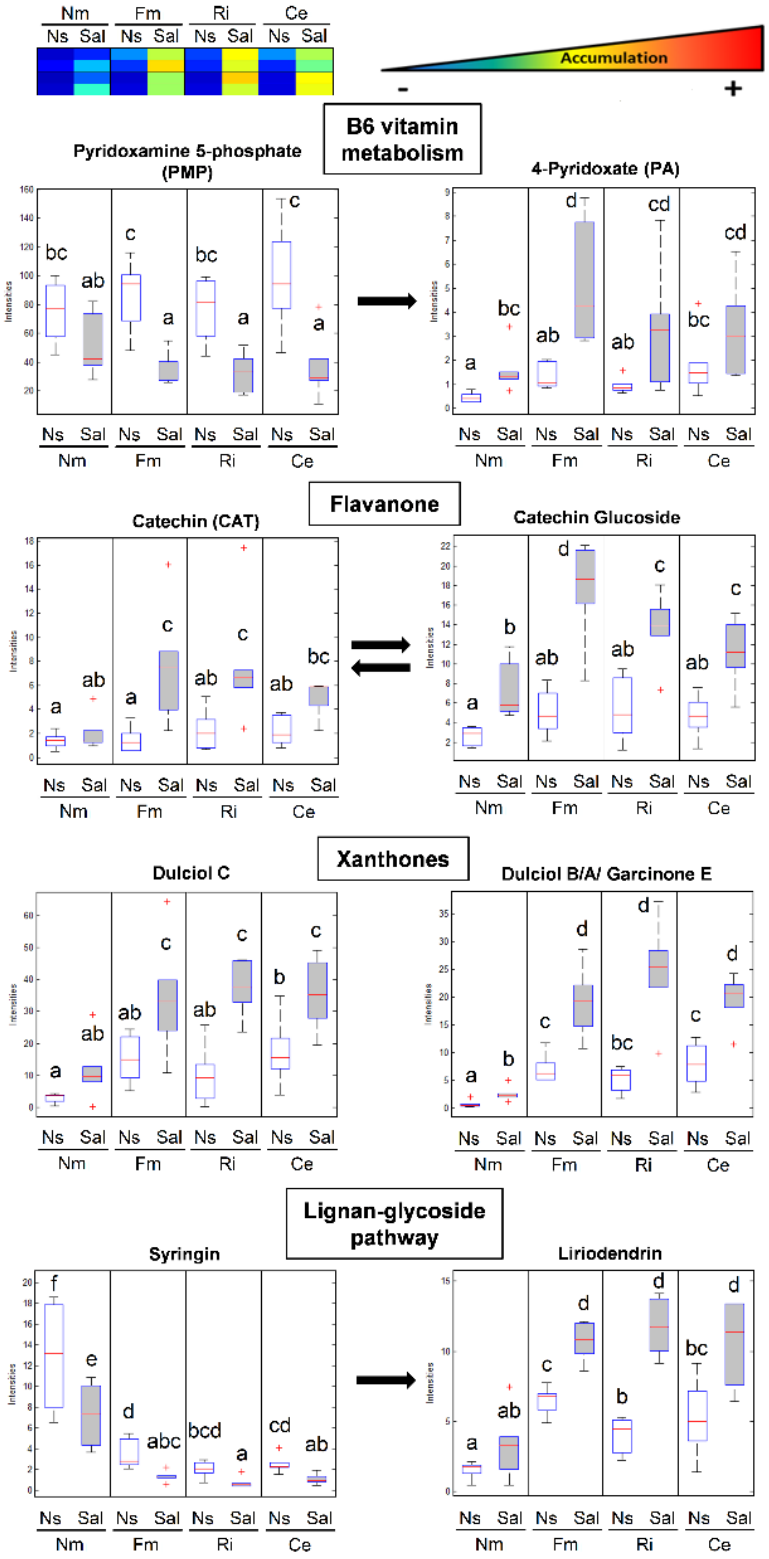


Fig. 7 Compounds with a primed accumulation pattern after salt stress in mycorrhizal roots colonized by all the tested arbuscular mycorrhizal fungi. Four-week-old tomato plants, nonmycorrhizal (Nm) or colonized by *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) or *Claroideoglomus etunicatum* (Ce), were subjected to salinity stress or nonstress conditions. When available, the precursor is shown and the graphs are connected by arrows. White bars, accumulation in plants grown under nonstress conditions (Ns); gray bars, accumulation in plants grown under moderate salt stress condition (Sal, 75 mM NaCl). Data acquisition from a nontargeted metabolic analysis was as described in the Fig. 3 legend. Boxes represent the interquartile range, red lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range, and red crosses show outliers. Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Data not sharing a letter in common differ significantly according to the Fisher's least significant difference test ($P < 0.05$, n = 6).

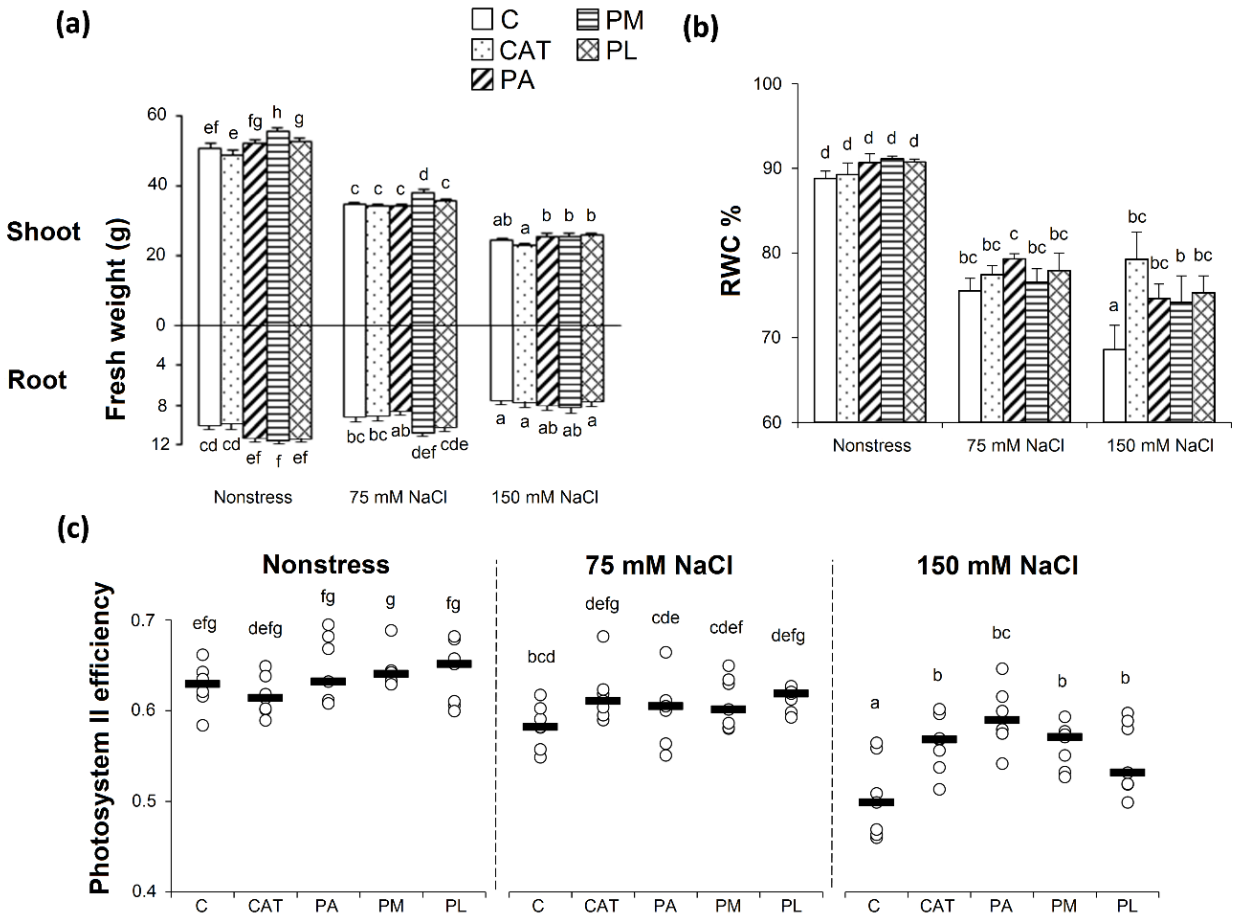


Fig. 8 Effect of the exogenous application of catechin and B6 vitamers on the physiological parameters of *Solanum lycopersicum*. Plants were grown under nonstress conditions (Ns) or under two values of NaCl salinity: moderate (75 mM NaCl) and severe (150 mM NaCl). Control plants were mock-treated (white bar) and the rest were treated twice by irrigation with a 50 ml aqueous solution of catechin (dotted bars, CAT, 2 mM), PA (diagonal-lined bars, 100 µM), pyridoxal hydrochloride (horizontal bars, PL, 100 µM) and pyridoxamine dihydrochloride (rhombus bars, PM, 100 µM). **(a) Shoot and root FW** (n = 7); **(b) leaf relative water content (RWC)** (n = 5). (a, b) Data are means ± SEM. **(c) Efficiency of photosystem II** after 7 d of NaCl salinity stress (n = 7); white dots represent each measure and black lines the medians. Two-way factorial ANOVA (using chemical treatment and NaCl stress as factors) were performed in all cases. Data not sharing a letter in common differ significantly according to the Fisher's least significant difference test ($P < 0.05$).

Discussion

It has previously been found, in different plant systems, that AM symbiosis can enhance tolerance to abiotic-related stresses, including drought and salinity (Aroca *et al.*, 2013; Ruiz-Lozano *et al.*, 2016). However, few studies have compared protection by different AMF strains under both stresses at different intensities. In the present study, we compared three different AMF (*F. mosseae*, *R. irregulare* and *C. etunicatum*) in terms of their ability

to induce host plant protection against drought or NaCl salinity. Furthermore, we investigated their impact on the host stress-induced reorganization of the metabolome in order to identify key metabolites involved in the mycorrhiza-related protection against osmotic stresses.

Under nonstress conditions, *F. mosseae* and *C. etunicatum* slightly increased root and shoot biomass. However, under drought conditions, all AMF-colonized plants showed higher biomass above and below ground than did Nm plants, and the growth promotion in mycorrhiza (MGR) was positively correlated with the degree of stress. In the context of water scarcity, an increase in root biomass may be crucial for plant survival as it allows them to explore more soil volume for water acquisition. As the MGR clearly increased with stress severity, our results support the concept of the mycorrhizal symbiosis as a 'health insurance', where the symbiosis benefits become more obvious under stressful conditions. This concept provides clues as to why the symbiosis is so widespread and conserved even in plant species where growth promotion by mycorrhizas under nonstress conditions is not common.

When plants were subjected to NaCl salinity, the MGR was again more evident upon the most severe conditions. However, despite the significant increase promoted by all AMF, *C. etunicatum* showed a much greater ability to promote growth under salt stress, particularly root growth. In fact, it was the only AMF that increased root growth under severe salinity stress. Under salinity conditions, all plants showed accumulation of Na^+ in their tissues, whose toxic effect has been widely reported (Estrada *et al*., 2013a). Remarkably, the concentration of Na^+ in roots was reduced in mycorrhizal treatments, while the accumulation of Na^+ in leaves was significantly lower in plants colonized by *C. etunicatum*. It is notable that *C. etunicatum* was the only salt-adapted AMF in our study, as it was isolated from a high-salinity soil in a very dry area (Estrada *et al*., 2013a). Therefore, our results, in agreement with those from Estrada et al., show not only functional diversity among AMF species, but also the importance of stress environments as source of stress-tolerant AMF strains.

In order to identify the potential mechanisms underlying the enhanced stress tolerance, the metabolic profile of tomato roots subjected to moderate drought or salinity stress was compared with that of plants under the nonstress condition. Although both stresses resulted in a strong reduction of root biomass (38% and 47% drought and salinity,

respectively), only salinity drastically altered their metabolite profiles. These results highlight the different strategies of plants in coping with both types of stress. While both drought and salinity result in osmotic stress, salinity also implies Na^+ toxicity effects. Thus, under drought, plant responses seem more oriented towards modifying the root system and physiological processes, such as hydraulic properties, to improve water uptake (Sánchez-Romera *et al*., 2016; Bitterlich *et al*., 2018). However, under salt stress, accumulation of osmoprotectant and antioxidant metabolites as proline or trigonelline was observed, while the concentrations of these compounds remained unaffected under drought stress.

We found a strong reorganization of the metabolic profiles of roots as a consequence of mycorrhiza establishment even in the absence of stress. Some amino acids, lignans, oxylipins and carotenoids were found to accumulate in AMF-colonized roots, extending previous observations for *F. mosseae* and *R. irregulare* (Rivero *et al*., 2015) to a fungal strain from a different family, *C. etunicatum* from the Claroideoglomeraceae. It should be noted that some metabolites detected in this and other studies analyzing mycorrhizal roots (Rivero *et al*., 2015; Saia *et al*., 2015; Hill *et al*., 2018) may be of a fungal origin. Some of the metabolites accumulating in mycorrhizal roots could be involved in enhanced tolerance of stress, as has been reported for cysteine or oxylipins (Okazaki and Saito, 2014; Genisel *et al*., 2015). Oxylipins such as the phytohormone jasmonic acid (JA) and derivatives reduced the negative effect of drought and salinity stress on plant fitness when exogenously applied (Riemann *et al*., 2015). In our study, AM colonization resulted in accumulation of JA, being the highest JA concentration found in roots colonized by *C. etunicatum*, the more efficient bioprotector against salt stress. Besides oxylipins, several metabolites from the carotenoid pathway (ABA glucosyl ester (ABA-GE), b-ionone and blumenol C glucoside) were also accumulated in AM roots, particularly in those colonized by *C. etunicatum*. The carotenoid pathway is known to be involved in several processes associated with plant stress adaptation (Havaux, 2014). For example, ABA regulation of stomata closure is key for drought tolerance to reduce water losses. Although ABA has not been detected in our analysis, we found higher concentrations of ABA-GE in mycorrhizal roots, a reservoir for the rapid production of active ABA (Xu *et al*., 2014). Higher ABA-GE concentration has been reported in xylem sap after drought or salt stress, and its role as a root-shoot signaling molecule has been argued (Sauter *et al*., 2002; Goodger and Schachtman, 2010).

The comparison of the metabolic profiles also highlighted changes specifically associated with the colonization by each AMF, *C. etunicatum* and *R. irregulare* triggering the higher and lower number of changes, respectively. In previous studies, we also found a lower impact of *R. irregulare* in root secondary metabolism when compared with *F. mosseae* in different plant species (Fernández *et al*., 2014; Rivero *et al*., 2015), suggesting a higher capacity of *R. irregulare* to evade plant responses to the fungal colonization. Among the compounds enriched in Ce plants, we found components from the porphyrin pathway, including biliverdin. Biliverdin is an antioxidant molecule also found to promote lateral root formation (Balestrasse *et al*., 2005; Yen Hsu *et al*., 2012), and other porphyrin metabolites have been related to drought tolerance (Phung *et al*., 2011). Some compounds, in addition to higher basal concentrations in Ce plants, showed a further increase upon salt stress. Among them, the presence of SGAs was noteworthy. These compounds, produced in more than 350 plant species, seem especially relevant in the Solanaceae. Although there is not much information relating these compounds to plant stress responses, their pharmaceutical, antimicrobial and insecticidal properties have been reported (Patel *et al*., 2013; Chowanski *et al*., 2016). Moreover, higher accumulation of solasodine (the most studied SGA) was reported after salt stress or methyl jasmonic acid (MeJA) elicitation in different Solanum species (Shilpha *et al*., 2015). Further research is needed to determine whether the higher carotenoid and porphyrin contents, together with the primed accumulation of SGAs, may underlie the higher protection against salt stress provided by *C. etunicatum.*

It should be noted that, despite the higher protection achieved by colonization with *C. etunicatum*, all mycorrhizal plants were more resistant to salinity. Therefore, we hypothesized that some protective compounds may be differentially accumulated in all mycorrhizal plants. Defense priming has been shown to mediate mycorrhiza protection against biotic stress (Jung *et al*., 2012; Sánchez-Bel *et al*., 2016). Since priming has been described as an effective defense strategy against abiotic and biotic stresses (Mauch-Mani *et al*., 2017), we looked for salt-induced compounds with a primed accumulation pattern in all AMF treatments. A remarkable priming fingerprint was found in all mycorrhizal roots subjected to salt stress. Among the mycorrhiza-primed compounds we found xanthones. They have been previously reported to be induced in root cultures by chitosan (Brasili *et al*., 2016), a major component of most fungal cell walls, including AMF. They are a group of bioactive polyphenols with reported antioxidant, anti-inflammatory,

antimicrobial and cytotoxic activities (Zubricka *et al*., 2015). However, while their protective effect against biotic stress has been studied (Franklin *et al*., 2009), their possible role in inducing tolerance against abiotic stresses remains unknown. Also of interest is the accumulation of some glycosylated lignans, a heterogeneous class of pharmacologically active compounds (Teponno *et al*., 2016). Among the primed lignans identified is liriodendrin, reported to display a strong antioxidant activity (Li *et al*., 2015). It is noteworthy that the liriodendrin precursor syringing shows a clear reduction in AM plants under salt stress, supporting an enhanced biosynthesis of this compound in mycorrhizal roots.

Other compounds with primed pattern after salinity stress in AM plants are the flavonoids CAT and its glycosylated derivative. Flavonoids are reported to have plant protective properties through strong antioxidant activity (Agati *et al*., 2012), and elevated concentrations of flavonoids have been described in AM roots (Schliemann *et al*., 2008; Mechri *et al*., 2015; Mollavali *et al*., 2016). A potential contribution of CAT to plant protection against abiotic stress is also supported by reports of the increased salinity or flooding tolerance achieved after exogenous application of CAT to sweet pepper and tomato plants (Yiu *et al*., 2011, 2012).

Pyridoxic acid also showed a clear primed response in mycorrhizal plants. PA is a catabolic product of the vitamin B6 pathway. Concomitantly, we found the opposite pattern – marked reduction in mycorrhizal roots upon salt stress – in one of the vitamers of this pathway, pyridoxamine 5-phosphate, supporting the idea that this route is turned on by salt stress, and further enhanced in mycorrhizal plants. The implication of this pathway in salt stress tolerance is well established: Arabidopsis *SALT OVERLY SENSITIVE 4* (*SOS4*) encodes a pyridoxal kinase that is involved in the biosynthesis of pyridoxal-5-phosphate, another active form of vitamin B6 (Shi *et al*., 2002). While SOS4 mutants are hypersensitive to NaCl, exogenous application of pyridoxine partially restores the mutant ability to grow under salt conditions. The B6 vitamers' protective role against biotic stress has also been reported (Zhang *et al*., 2015). The modulation in mycorrhizal plants of B6 vitamer accumulation upon infection with the foliar pathogen *Botrytis cinerea* (Sánchez-Bel *et al*., 2016) is remarkable, as vitB6 itself can induce defense priming against this pathogen (Zhang *et al*., 2014). All these results suggest a central role of B6 vitamers in the defense priming associated with the AM symbiosis that deserves further investigation.

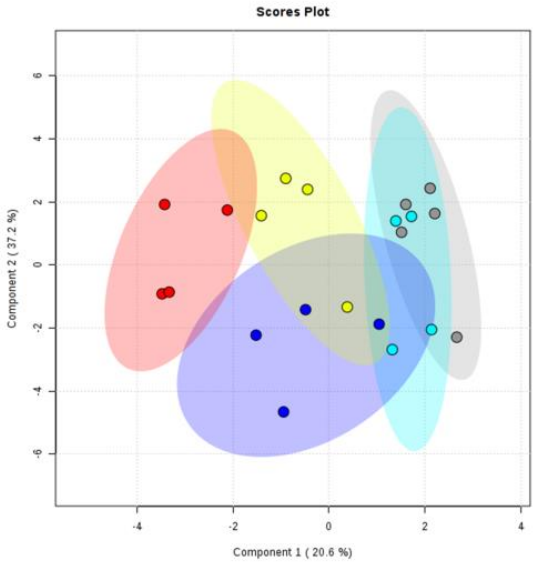
According to this experimental evidence, catechin and B6 vitamers, among others, are excellent candidates to mediate AMF protection against salt stress. We aimed to test whether these compounds are actually able to protect tomato plants against severe salt stress. All the studied compounds had a positive impact on at least one of the parameters analyzed related to plant fitness, including shoot or root growth, relative water content or photosystem efficiency. Here, it is worth noting that the protective effect of mycorrhizal establishment was higher than that upon application of the purified compounds. In fact, the mycorrhizal effect would be equivalent to treatments with a blend of compounds. It is important to point out that the compounds primed in mycorrhizal roots depend on the stress imposed, illustrating the plastic character of the priming phenomena. Thus, mycorrhizal colonization is a more versatile strategy for plant protection than is application of purified compounds or traditional breeding for specific traits or lines hyperaccumulating particular compounds.

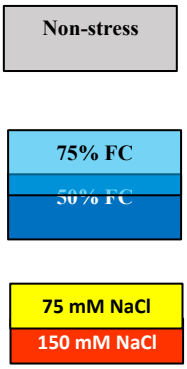
In conclusion, by using tomato plants and different AMF we provide evidence of mycorrhizal alleviation of the negative effects of drought and salinity. Our results support the idea that the benefits of AM symbioses for plant fitness increase with adversity, and illustrate functional diversity among different AMF isolates, highlighting the importance of stressful environments as sources of stress-adapted microorganisms with higher protective properties. The untargeted metabolomic analysis performed pinpointed some compounds with primed accumulation in mycorrhizal plants, probably mediating the enhanced tolerance observed. The differences in the metabolic profiles depended on the stress faced, thus revealing that mycorrhizal symbiosis enhances the phenotypic/metabolic plasticity of the plant to cope with particular stresses. Exploring the functional diversity among different AMF isolates and the metabolic reprograming associated with their positive impact on plant tolerance to multiple stresses will pave the way for the optimized application of beneficial microorganisms in sustainable agriculture, potentially counteracting the effects of climate change.

Supplementary Data

(a)

B (mg/Kg)	
Ca (g/100g)	Na (g/100g)
Co (mg/Kg)	Ni (mg/Kg)
Cr (mg/Kg)	P (g/100g)
Cu (mg/Kg)	S (g/100g)
Fe (mg/Kg)	Sr (mg/Kg)
K (g/100g)	V (mg/Kg)
Li (mg/Kg)	Zn (mg/Kg)
Mg (g/100g)	N (mg/Kg)
Mn (mg/Kg)	C (g/100g)
Mo (mg/Kg)	N (g/100g)





(b)

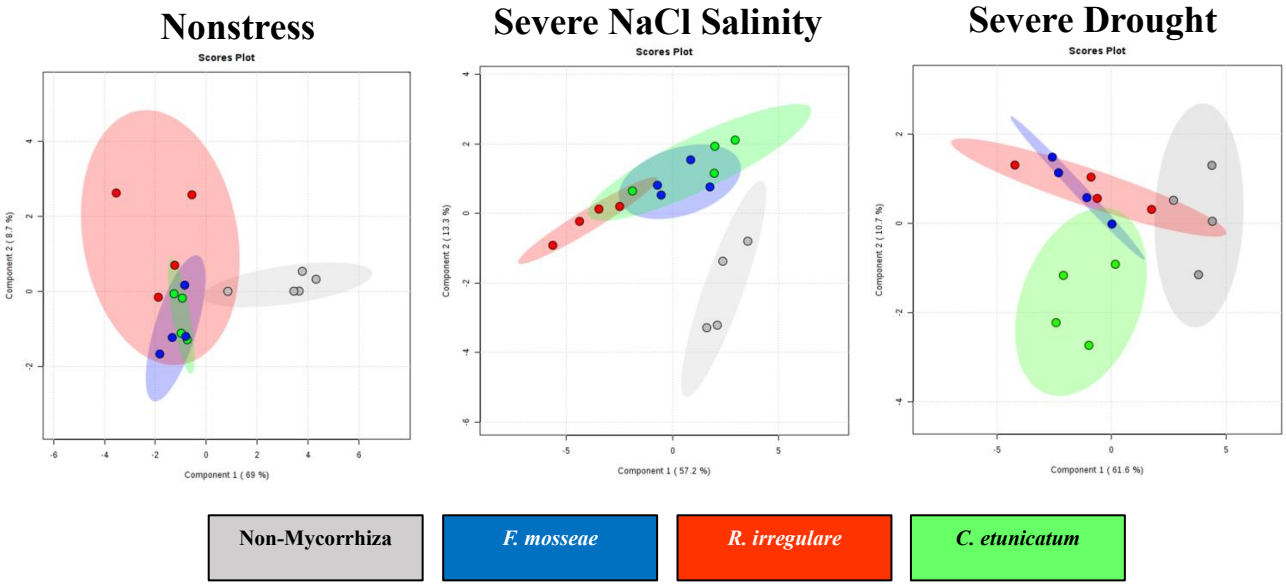
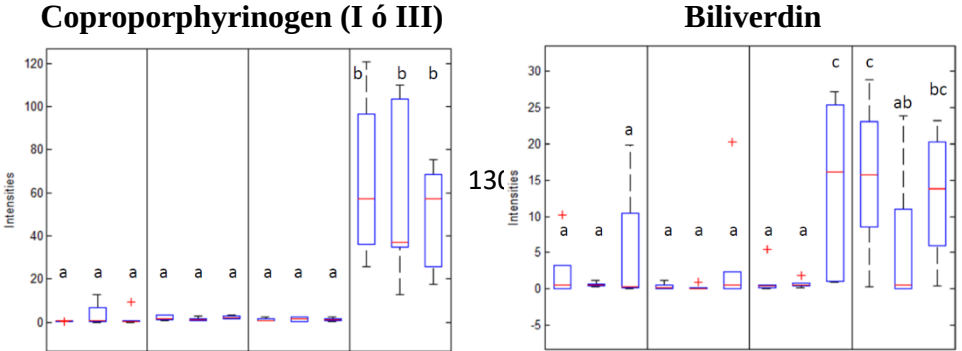


Fig. S1 Overview of nutrient reorganization patterns in *Solanum lycopersicum* roots subjected to abiotic stress conditions (drought or NaCl salinity) in plants colonized or not by different arbuscular mycorrhizal fungi (AMF). Tomato plants were grown for four weeks and then subjected to different abiotic stress conditions for four weeks more (eight weeks in total). Sparse partial least squares discriminant analysis (sPLSDA) was performed with de data obtained for the main 21 nutrients. **a)** Nutrient accumulation patterns in non-colonized (Nm) tomato roots subjected, or non (Ns), to drought (moderate -75%- or severe -50 %- of field capacity), or salinity (moderate -75 mM- or severe -150 mM- NaCl) stresses. **b)** Nutrient accumulation in roots of nonmycorrhizal plants (Nm) or colonized by the AMF *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) or *Claroideoglomus etunicatum* (Ce), subjected to abiotic stress conditions.



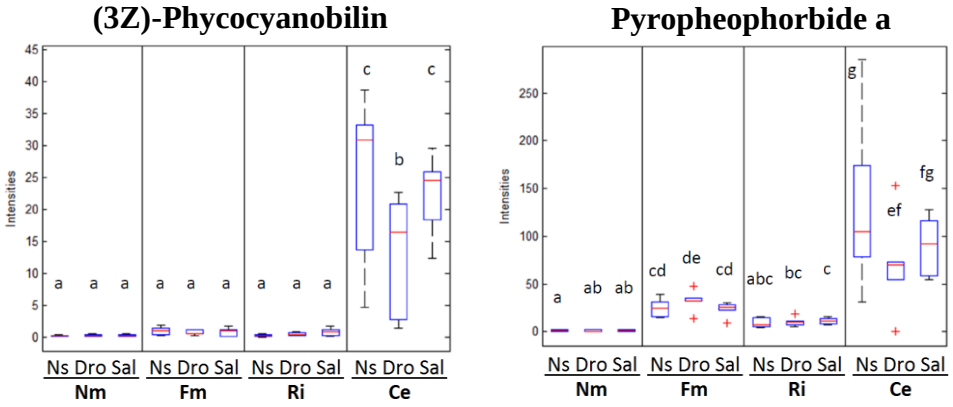


Fig. S2 Boxplots of selected metabolites from the porphyrin family in *Solanum lycopersicum* roots, showing higher accumulation in plants colonized by *Claroideoglomus etunicatum* (Ce). Other treatments were nonmycorrhizal plants (Nm) and plants colonized by *Funneliformis mosseae* (Fm) or *Rhizoglomus irregulare* (Ri). Tomato plants were grown for four weeks and then subjected to different abiotic stress conditions: nonstress (Ns), drought (75% of field capacity; Dro) and salinity (75 mM NaCl; Sal) for four weeks more (eight weeks in total). Data acquisition from a untargeted metabolic analysis as described in Fig. 3 legend. Boxes represent the interquartile range, red lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and red crosses show outliers. Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Bars not sharing a letter in common differ significantly according to the Fisher's LSD test ($P < 0.05$, n = 6)

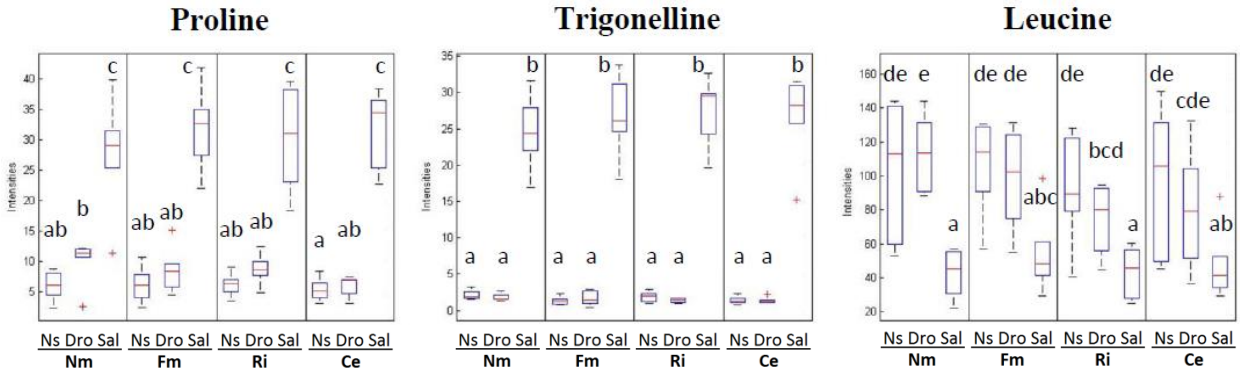


Fig. S3 Boxplots of selected metabolites in *Solanum lycopersicum* roots showing the strong effect of salinity on their accumulation patterns. Other treatments were nonmycorrhizal plants (Nm) and plants colonized by *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) or *Claroidoeglomus etunicatum* (Ce). Tomato plants were grown for four weeks and then subjected to different abiotic stress conditions: nonstress (Ns), drought (75% of field capacity; Dro) and salinity (75 mM NaCl; Sal) for four weeks more (eight weeks in total). Data acquisition from a untargeted metabolic analysis as described in Fig. 3 legend. Boxes represent the interquartile range, red lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and red crosses show outliers. Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Bars not sharing a letter in common differ significantly according to the Fisher's LSD test ($P < 0.05$, n = 6).

Condition	Severity	Treatment	FW Shoot (g)	FW root (g)	Shoot : Root	Electrical conductivity (ms/m)	Root length colonized (%)
Non-stress		*Non-mycorrhizal*	9.66 ± 0.94 f	4.86 ± 1.05 de	1.91 ± 0.06 ef	0.91 ± 0.02 a	
		F. mosseae	12.20 ± 0.31 gh	8.28 ± 0.39 h	1.52 ± 0.05 abc	1.24 ± 0.15 a	59.83 ± 0.60 f
		R. irregulare	10.22 ± 0.65 f	6.53 ± 0.49 fg	1.76 ± 0.02 bcde	1.30 ± 0.04 a	51.33 ± 10.43 def
		C. etunicatum	12.48 ± 0.51 h	8.40 ± 0.19 h	1.47 ± 0.07 ab	1.01 ± 0.07 a	48.83 ± 1.88 cdef
Drought stress	**75% FC**	*Non-mycorrhizal*	6.00 ± 0.82 cd	3.28 ± 0.55 bc	1.87 ± 0.08 de	1.29 ± 0.27 a	
		F. mosseae	10.68 ± 0.55 fg	7.02 ± 0.35 g	1.54 ± 0.10 abcd	0.97 ± 0.03 a	42.03 ± 3.16 cde
		R. irregulare	9.60 ± 0.32 f	5.90 ± 0.30 efg	1.64 ± 0.08 bcde	1.51 ± 0.23 a	36.08 ± 2.88 bc
		C. etunicatum	10.17 ± 0.82 f	6.77 ± 0.59 fg	1.51 ± 0.02 ab	0.75 ± 0.13 a	36.00 ± 3.75 bc
	50% FC	*Non-mycorrhizal*	1.78 ± 0.29 a	1.08 ± 0.17 a	1.65 ± 0.14 bcde	1.47 ± 0.34 a	
		F. mosseae	5.77 ± 0.21 cd	3.92 ± 0.14 bcd	1.47 ± 0.01 ab	1.36 ± 0.32 a	30.25 ± 2.29 b
		R. irregulare	4.63 ± 0.27 bc	3.03 ± 0.36 b	1.63 ± 0.23 bcde	2.07 ± 0.19 a	40.00 ± 2.89 bcd
		C. etunicatum	5.33 ± 0.45 bcd	4.42 ± 0.44 cd	1.24 ± 0.10 a	1.11 ± 0.14 a	16.17 ± 2.24 a
Salinity stress	**75 mM NaCl**	*Non-mycorrhizal*	5.08 ± 0.95 bcd	2.87 ± 0.71 b	1.94 ± 0.15 ef	9.25 ± 0.81 b	
		F. mosseae	8.68 ± 0.78 ef	4.44 ± 0.36 cd	1.96 ± 0.05 ef	7.61 ± 0.73 b	59.67 ± 5.07 f
		R. irregulare	6.80 ± 0.55 de	3.03 ± 0.28 b	2.26 ± 0.07 fg	8.49 ± 0.53 b	52.50 ± 5.07 def
		C. etunicatum	10.57 ± 0.31 fg	5.80 ± 0.41 ef	1.86 ± 0.11 cde	8.81 ± 0.93 b	48.00 ± 3.28 cdef
	150 mM NaCl	*Non-mycorrhizal*	1.87 ± 0.19 a	0.77 ± 0.07 a	2.43 ± 0.11 g	22.80 ± 2.67 e	
		F. mosseae	3.32 ± 0.34 ab	1.14 ± 0.06 a	2.95 ± 0.33 h	17.30 ± 0.16 d	52.83 ± 0.73 def
		R. irregulare	3.58 ± 0.39 ab	1.15 ± 0.16 a	3.19 ± 0.18 h	13.87 ± 1.13 c	53.50 ± 8.52 ef
		C. etunicatum	5.63 ± 0.46 cd	2.93 ± 0.30 b	1.96 ± 0.16 ef	15.57 ± 0.07 cd	49.33 ± 5.63 cdef

Table S1 Shoot and root fresh weight (Fw), shoot:root ratio, substrate electrical conductivity at the end of the experiment and percentage of root length colonized by arbuscular mycorrhizal fungi (AMF) of *Solanum lycopersicum* plants grown under different abiotic stress conditions. Nonmycorrhizal plants (Nm) or plants colonized by the AMF *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) or *Claroideoglomus etunicatum* (Ce), were grown for four weeks and then subjected to different abiotic stress conditions: nonstress (Ns), drought (moderate -75%- or severe -50 %- of field capacity), or salinity (moderate -75 mM- or severe -150 mM- NaCl) for four additional weeks. Two-way factorial ANOVA (using AMF species and stress treatment as factors) were performed. Data are expressed as mean ± SEM (n=6). Means not sharing a letter in common differ significantly according to the Fisher's LSD post hoc test ($P < 0.05$).

(a)

	% Growth inhibition compared to non-stress conditions			
	Drought stress		Salinity stress	
	75% FC	50% FC	75 mM NaCl	150 mM NaCl
	Shoot fresh weight			
Non-mycorrhizal	38%	81%	47%	81%
F. mosseae	12%	52%	29%	73%
R. irregulare	6%	55%	33%	65%
C. etunicatum	18%	58%	15%	55%
	Root fresh weight			
Non-mycorrhizal	32%	77%	40%	84%
F. mosseae	15%	53%	47%	86%
R. irregulare	10%	54%	54%	82%
C. etunicatum	19%	48%	31%	65%
	Total fresh weight			
Non-mycorrhizal	35%	80%	44%	82%
F. mosseae	15%	53%	37%	79%
R. irregulare	14%	57%	46%	74%
C. etunicatum	18%	53%	21%	59%

(b)

	% Growth promotion compared to Non-mycorrhizal plants				
	Non-stress	Drought stress		Salinity stress	
		75% FC	50% FC	75 mM NaCl	150 mM NaCl
	Shoot fresh weight				
F. mosseae	26%	78%	222%	71%	78%
R. irregulare	5%	60%	156%	33%	91%
C. etunicatum	29%	70%	194%	108%	201%
	Root fresh weight				
F. mosseae	70%	112%	255%	52%	48%
R. irregulare	34%	79%	173%	3%	49%
C. etunicatum	73%	106%	300%	100%	281%
	Total fresh weight				
F. mosseae	45%	90%	234%	64%	70%
R. irregulare	26%	67%	166%	23%	80%
C. etunicatum	45%	83%	238%	105%	226%

Table S2 Shoot, root and total fresh weight percentage of a) inhibition due to the applied stress compared to nonstress conditions, and b) promotion due to the colonization with different AMF species compared to nonmycorrhizal plants. Nonmycorrhizal plants (Nm) or plants colonized by the AMF *Funneliformis mosseae* (Fm), *Rhizoglomus irregulare* (Ri) or *Claroideoglomus etunicatum* (Ce), were grown for four weeks and then subjected to different abiotic stress conditions: nonstress (Ns), drought (moderate -75%- or severe -50%- of field capacity), or salinity (moderate -75 mM- or severe -150 mM- NaCl) for four additional weeks.

Analysis of Variance for Shoot Biomass- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	13,802	3	4,601	48,060	**0,000**
B:Stress	46,947	4	11,737	122,600	**0,000**
INTERACTION					
AB	2,706	12	0,226	2,360	**0,009**
RESIDUAL	11,679	122	0,096		
TOTAL (CORRECTED)	74,323	141			

All F-ratios are based on the residual mean square error

Analysis of Variance for Root Biomass- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	11,565	3	3,855	57,530	**0,000**
B:Stress	29,313	4	7,328	109,370	**0,000**
INTERACTION					
AB	1,858	12	0,155	2,310	**0,012**
RESIDUAL	6,499	97	0,067		
TOTAL (CORRECTED)	49,980	116			

All F-ratios are based on the residual mean square error

Analysis of Variance for Shoot:Root-Ratio- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	0,489	3	0,163	14,970	**0,000**
B:Stress	2,267	4	0,567	52,010	**0,000**
INTERACTION					
AB	0,395	12	0,033	3,020	**0,001**
RESIDUAL	1,057	97	0,011		
TOTAL (CORRECTED)	4,183	116			

All F-ratios are based on the residual mean square error

Analysis of Variance for Electrical Conductivity- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	30,009	3	10,003	5,940	**0,002**
B:Stress	2445,490	4	611,374	363,270	**0,000**
INTERACTION					
AB	111,896	12	9,325	5,540	**0,000**
RESIDUAL	67,318	40	1,683		
TOTAL (CORRECTED)	2654,720	59			

All F-ratios are based on the residual mean square error

Analysis of Variance for Percentage of Mycorrhization- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	726,737	2	363,368	5,590	**0,008**
B:Stress	4823,310	4	1205,830	18,560	**0,000**
INTERACTION					
AB	702,953	8	87,869	1,350	0,251
RESIDUAL	2273,620	35	64,961		
TOTAL (CORRECTED)	8558,330	49			

All F-ratios are based on the residual mean square error

Table S3 ANOVA tables corresponding to the analysis of data in Table S1.

Analysis of Variance for Dro Shoot MGR- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	0,097	2	0,049	4,180	0,022
B:Drought stress	3,300	2	1,650	141,710	0,000
INTERACTION					
AB	0,034	4	0,009	0,740	0,571
RESIDUAL	0,524	45	0,012		
TOTAL (CORRECTED)	3,956	53			

All F-ratios are based on the residual mean square error

Analysis of Variance for Dro Root MGR- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	0,419	2	0,209	9,440	0,000
B:Drought stress	3,460	2	1,730	77,980	0,000
INTERACTION					
AB	0,108	4	0,027	1,210	0,319
RESIDUAL	0,998	45	0,022		
TOTAL (CORRECTED)	4,985	53			

All F-ratios are based on the residual mean square error

Analysis of Variance for Sal Shoot MGR- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	0,519	2	0,259	15,890	0,000
B:Salt Stress	1,124	2	0,562	34,460	0,000
INTERACTION					
AB	0,308	4	0,077	4,720	0,003
RESIDUAL	0,701	43	0,016		
TOTAL (CORRECTED)	2,707	51			

All F-ratios are based on the residual mean square error

Analysis of Variance for Sal Root MGR- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	1,752	2	0,876	46,650	0,000
B:Salt Stress	0,533	2	0,267	14,190	0,000
INTERACTION					
AB	0,903	4	0,226	12,010	0,000
RESIDUAL	0,808	43	0,019		
TOTAL (CORRECTED)	4,061	51			

All F-ratios are based on the residual mean square error

Table S4 ANOVA tables corresponding to the data included in Fig. 1.

Analysis of Variance for Na+ Shoot Concentration- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	0,476	3	0,159	10,480	0,000
B:Salt Stress	20,860	2	10,430	689,120	0,000
INTERACTION					
AB	0,364	6	0,061	4,000	0,004
RESIDUAL	0,560	37	0,015		
TOTAL (CORRECTED)	22,343	48			

All F-ratios are based on the residual mean square error

Analysis of Variance for Na+ root Concentration- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Mycorrhizal inoculation	0,057	3	0,019	4,870	0,006
B:Salt Stress	6,331	2	3,165	818,000	0,000
INTERACTION					
AB	0,043	6	0,007	1,850	0,116
RESIDUAL	0,143	37	0,004		
TOTAL (CORRECTED)	6,604	48			

All F-ratios are based on the residual mean square error

Table S5 ANOVA tables corresponding to the data included in Fig. 2.

Analysis of Variance for Shoot Biomass- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Chemical	233.177	4	58.294	15.060	**0.000**
B:Salt Stress	13178.800	2	6589.420	1702.690	**0.000**
INTERACTION					
AB	60.087	8	7.511	1.940	0.063
RESIDUAL	348.300	90	3.870		
TOTAL (CORRECTED)	13820.400	104			

All F-ratios are based on the residual mean square error

Analysis of Variance for Root Biomass- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Chemical	25.685	4	6.421	5.200	**0.001**
B:Salt Stress	162.380	2	81.190	65.780	**0.000**
INTERACTION					
AB	17.318	8	2.165	1.750	0.097
RESIDUAL	111.089	90	1.234		
TOTAL (CORRECTED)	316.472	104			

All F-ratios are based on the residual mean square error

Analysis of Variance for RWC- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Chemical	160.448	4	40.112	2.800	**0.035**
B:Salt Stress	3246.140	2	1623.070	113.380	**0.000**
INTERACTION					
AB	160.989	8	20.124	1.410	0.215
RESIDUAL	787.312	55	14.315		
TOTAL (CORRECTED)	4343.020	69			

All F-ratios are based on the residual mean square error

Analysis of Variance for Photoefficiency- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Chemical	0.018	4	0.004	4.180	**0.004**
B:Salt Stress	0.128	2	0.064	59.910	**0.000**
INTERACTION					
AB	0.014	8	0.002	1.640	0.124
RESIDUAL	0.096	90	0.001		
TOTAL (CORRECTED)	0.255	104			

All F-ratios are based on the residual mean square error

Table S6 ANOVA tables corresponding to data in Fig. 8.

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CHAPTER 3: MYCORRHIZAL SYMBIOSIS ENHANCES HERBIVORE MORTALITY AND PRIMES LOCAL ACCUMULATION OF DEFENSIVE COMPOUNDS IN RESPONSE TO HERBIVORY IN TOMATO

In preparation

Chapter 3: Mycorrhizal symbiosis enhances herbivore mortality and primes local accumulation of defensive compounds in response to herbivory in tomato

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Abstract

Plant association with arbuscular mycorrhizal fungi (AMF) can increase their ability to overcome multiple stresses, but the impact on plant interaction with herbivorous insects is controversial. Here we show higher mortality of the leaf-chewer *Spodoptera exigua* when fed on tomato plants colonized by the AMF *Funneliformis mosseae*, confirming Mycorrhiza Induced Resistance (MIR) in this system. An untargeted metabolic study through UPLC-MS showed that the symbiosis had a very limited impact on the leaf metabolome in the absence of stress, but significantly modulate the response to herbivory in the attacked areas. A cluster of overaccumulated metabolites was identified in mycorrhizal plants in those leaflets damaged by *S. exigua* feeding, while unwounded, distal areas respond similarly to those in nonmycorrhizal plants. The primed-compounds were mostly related to alkaloids, fatty acid derivatives and phenylpropanoids-polyamine conjugates (PPCs). Primed accumulation of the PPC feruloylputrescine was confirmed

by targeted analysis, and pharmacological and genetic approaches showed that its production is regulated by Jasmonic acid signaling pathway. Thus, our results evidenced for the first time the AM impact on metabolic reprograming upon herbivory that leads to primed accumulation of defensive compounds and discuss their potential function in MIR.

Introduction

Plants are dynamic systems, able to interact with a broad range of organisms, that may result in a deleterious or beneficial output. Among the many positive interaction with soil-borne organisms, the establishment of arbuscular mycorrhizas (AM) deserve special attention. Due to the biotrophic nature of AMF, host plants must provide them of photosynthates and lipids for the development, maintenance and function of mycorrhizal structures (Bago *et al.*, 2000; Keymer *et al.*, 2017). In return, the AMF assists the host plant in the acquisition of water and mineral nutrients, becoming extremely important for the uptake of inorganic phosphate and various micronutrients (Smith and Smith, 2011; Hodge and Storer, 2015). However, these benefits are not only restricted to a better mineral and nutrimental status, but also AM colonized plants exhibit an enhanced ability to deal with environmental adverse condition, such as harmful abiotic and biotic stresses.

The establishment and maintenance of AM symbiosis involves a high degree of coordination between both partners based on a finely regulated molecular dialogue that comprises host transcriptional and metabolic changes (Kiers *et al.*, 2011; Pimprikar and Gutjahr, 2018). In this regard, oxylipin pathway, which is responsible of phytohormone jasmonic acid (JA) biosynthesis, have been found positively stimulated/activated in roots of mycorrhizal plants (López-Ráez *et al.*, 2010; Fernández *et al.*, 2014; Rivero *et al.*, 2015). This increases in JA were related with the enforced capacity of AM plants to face biotic attacks, since it is well known the key role of jasmonates in coordinating plant responses to this type of stress.

Association of plants with certain beneficial microbes, among them AMF, can stimulate plant's immune system reaching an "alert" state denominated as induced systemic resistance (ISR) (Choudhary *et al.*, 2007; Pieterse *et al.*, 2014). ISR entails a great

advantage for the plants, since it triggers faster and more efficient responses to biotic challenges. Moreover, it can be transmitted to distal parts from the roots where AMF colonization takes place (Pieterse *et al.*, 2014).

One of the intrinsic part of ISR is priming, defined as the physiological state in which plants are conditioned for the superactivation of defenses against environmental challenges (Martínez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017). In addition, priming is known for being a low-cost defensive response since plant´s defenses are not (or only slightly) activated in absence of stress, but they are strongly prepared to be triggered upon subsequent challenge in a faster and/or stronger manner (Mauch-Mani *et al.*, 2017).

The specific ISR stimulated by the AMF establishment was redefined as "mycorrhiza induced resistance" (MIR) (Pozo and Azcon-Aguilar, 2007; Pozo *et al.*, 2013). Several reports demonstrate the functionality of MIR protecting against a wide range of below-ground aggressors such as soil-borne pathogens, nematodes or root-chewing insects. However, in aboveground tissues this range of MIR efficiency is under evaluation since it may depend on the life-style of aggressor. In general, while biotrophic pathogens are benefited by mycorrhizal establishment, the growth and survival of necrotrophs are impaired in AM plants (Jung *et al.*, 2012). In the case of insects, MIR is effective depending on the degree of specialization and the feeding guild of the herbivore. In this sense, generalist herbivores are strongly impaired by MIR (Jung *et al.*, 2012; Roger *et al.*, 2013). Whereas, specialists are supposed to be less sensitive to the protection triggered by MIR, since they are adapted to the host plant defenses. In addition, phloem and cell content feeders are also less sensitive to MIR because they trigger a low response in the host, while leaf-chewers show high susceptibility to this enhanced defense in AM plants (Hartley and Gange, 2009; Jung *et al.*, 2012).

MIR as a specialized ISR is finely regulated by jasmonates (JA) and ethylene (Et) (Pangesti *et al.*, 2016; Pozo *et al.*, 2015). JA-signaling has been widely reported as key regulator of defensive responses against necrotrophic pathogens and leaf-chewing herbivores. For example, proteinase inhibitors (PI), antifeedant compounds that can negatively impact herbivores development, are known to be induced after herbivory in a JA-dependent manner (Orozco-Cardenas *et al.*, 1993). Moreover, JA regulates the biosynthesis of toxic secondary metabolites as pyrrolizidine alkaloids, sesquiterpenoids

or glucosinolates (Erb and Reymond, 2019). Interestingly, these organisms are the most affected by MIR in aboveground tissues. This fact, together with the up-regulation of oxylipin pathway in AM-plants, suggest that JA-priming may be the main mechanism coordinating MIR.

This hypothesis is supported by need of an intact JA perception and signaling for an effective MIR. Tomato plants colonized by the AMF *Funneliformis mosseae* displayed enhanced resistance in response to *Alternaria solani* and the generalist leaf-chewer *Helicoverpa armigera*, showing overexpression of several genes of the oxylipin pathway and a JA-primed accumulation (Song *et al.*, 2013; Song *et al.*, 2015). When mutants and transgenic lines impaired in JA perception or signaling were used, MIR was abolished, confirming the key role of this phytohormone in this model. MIR was reported to be effective against *S. exigua* in peanut and tomato by priming JA-dependent defenses (He *et al.*, 2017). Among the different AMF tested *F. mosseae*-colonized plants showed the highest rate of protection against the caterpillar. In addition, a recent study performed by Formenti and Rasmann (2019) confirmed that the increasing in JA as consequence of mycorrhizal symbiosis is crucial for the enhanced protection.

Higher expression of wound-response genes codifying for serine proteinase inhibitors (*PI-I* and *PI-II*) have been reported in mycorrhizal-plants under herbivory (Song *et al.*, 2013). In addition, JA exogenous application in *Solanum dulcamara* plants resulted in higher activity of proteinase inhibitor activity only in mycorrhizal plants compared with noncolonized ones (Minton *et al.*, 2016). Note that the changes triggered during MIR are not restricted to JA-dependent defenses, AM plants also display increases in toxic secondary metabolites such as alkaloids o or iridoid glycosides (Andrade *et al*., 2013; Tomczak *et al*., 2016). However, most of these works are focused on targeted metabolic analysis towards known compounds, so a global overview of metabolic-reprogramming in AM-plants in response to herbivores remains unknown.

Untargeted metabolomic approaches showed high accumulation of isoflavonoids and blumenols in roots of *Medicago truncatula* colonized by *Rhizoglomus irregulare* (Schliemann *et al.*, 2008; Laparre *et al.*, 2014). In a similar study performed in roots of durum wheat colonized by different AMF species, authors observed decreased accumulation of high number of metabolites in all metabolic pathways, especially amino

acids and saturated fatty acids (Saia *et al.*, 2015). The tomato roots metabolomic rearrangement was studied in well-established symbiosis with *R. irregulare* and *F. mosseae* (Rivero *et al.*, 2015). Both AMF showed some similarities in the metabolic alteration (e.g. upregulation of oxylipins and phenylpropanoids biosynthetic pathways), but specific modulation depending of the colonizing-fungi was observed. These observations reinforce the previously reported functional diversity among different AMF (Feddermann *et al.*, 2010; López-Ráez *et al.*, 2010; He *et al.*, 2017; Rivero *et al.*, 2018).

To study global metabolic transition in AM-plants exposed to stress, it is crucial to identify potential primed-compound that can be mediating acquired benefits (Tugizimana *et al.*, 2018). Thus, an untargeted metabolomic study on tomato roots colonized by different AMF allowed to identify several primed-metabolites involves in increased tolerance against salinity stress, such as B6 vitamers or flavonoid catechin (Rivero *et al.*, 2018). Another recent study during drought reports AM-modulation stress on several groups of secondary metabolites, such as phenolic compounds, lipids and sugars (Bernardo *et al*., 2019). However, data from nonstress condition are required for determine the primed-pattern of this compounds since it is not possible discriminate whether accumulation is consequence of the imposed stress or due to the AM establishment.

Fewer and more variable reports have been published focusing on aboveground metabolic changes in AM-plants (Schweiger and Muller, 2015). First study on mycorrhizal *Lotus japonicus* plants observed lower accumulation of several amino acids (Fester *et al*., 2011). Moreover, plant fertilization resulted in significant major metabolic changes compared with AM establishment. In other study was observed that the modulation of leaf metabolome by AM is strongly dependent on the plant species (Schweiger *et al.*, 2014). While *M. truncatula* shoot had approx. 15% of its metabolic features altered, in monocotyledonous *Poa annua* only was the 1.7%. In addition to plant species, the metabolic rearrangement in the shoot or the root of AM plants is also different. In fact, Se*necio jacobea* plants colonized by *R. irregulare* showed significant changes in the roots such as previously reported blumenols and several pyrrolizidine alkaloids, while in shoot any alteration was described (Hill *et al*., 2018). Using a combination of untargeted and targeted metabolomics, have been recently described for the first time a group of markers blumenol-derivates that seems to be exclusively accumulated in shoot as a consequence

of the mycorrhization, independently of the plant species or the imposed stress (Wang *et al.*, 2018).

Less information is available about the metabolic rearrangement in shoots of AM plants following biotic challenges. Overaccumulation of phenolic acids, amino acids, indoles and several intermediates in the oxylipin pathway (responsible of JA biosynthesis) were observed in leaves of tomato AM plants upon *B. cinerea* infection, what suggest that metabolic changes in the leaves also contributes to the final output of MIR (Sánchez-Bel *et al.*, 2016).

In the present study, we investigate whether *Funneliformis mosseae* colonization in tomato plants is an effective strategy to control the common pest *Spodoptera exigua*. Following an untargeted metabolomic approach, we focus on identify primed compounds in different damaged leaf tissues that potentially may be underlying the enhanced ability of AM to cope with this type of biotic stress.

Materials and Methods

Plant and fungal materials and growing conditions

The arbuscular mycorrhizal fungi (AMF) *Funneliformis mosseae BEG12* (*F. mosseae*, formerly *Glomus mosseae*, International Bank of Glomeromycota, https://www.i-beg.eu/cultures/BEG12.htm), was maintained in an open-pot culture of *Trifolium repens* L. mixed with *Sorghum vulgare* Pers.(Steud.) Millsp. & Chase plants in a greenhouse. The inoculum consisted of substrate (vermiculite/sepiolite,1:1), spores, mycelia and infected root fragments from those cultures. Tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were surface disinfected by immersion in 4% NaHClO (10min) containing 0.02% (v/v) Tween20 ®, rinsed thoroughly with sterile water and incubated for seven days in an open container with sterile vermiculite at 25◦C. Tomato plantlets were transferred to 250 ml pots containing a sterile sand:vermiculite (1:1) mixture. Pots for mycorrhizal treatments were inoculated by adding 10% (v/v) *F. mosseae* inoculum. Uninoculated plants received an aliquot of a filtrate (< 20 µm) from *F. mosseae* inoculum in order to provide the general microbial population but free of AMF propagules.

A total of ten plants were used for each treatment, randomly distributed and grown in a greenhouse at 24/16 ◦C with a 16/8 h diurnal photoperiod and 70% humidity. Plants were watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus (P). Plants were harvested after 8 weeks, the fresh weight of shoots and roots was determined, and the leaf material immediately frozen in liquid N and stored at −80ºC. An aliquot of each individual root system was reserved for mycorrhizal quantification.

Mycorrhizal colonization determination

Mycorrhizal colonization was estimated after clearing washed roots in KOH (10 %) and subsequent staining of fungal structures with 5% ink in 2% acetic acid (Vierheilig *et al*., 2005). The extent of mycorrhizal colonization (expressed as percentage of total root length colonized by the AMF) was calculated according to the gridline intersection method (Giovannetti and Mosse, 1980) using a Nikon Eclipse 50i microscope and brightfield conditions.

Insect rearing and herbivore performance set-up in tomato plants

Eggs from *S. exigua* (Lepidoptera: Noctuidae) were kindly provided by Dr. S. Herrero's lab (ERI-BIOTECMED, Universitat de Valencia, Spain). After hutching, larvae were reared on artificial diet (Greene *et al.*, 1976) at 25±3ºC with 70±5% relative humidity and a 16/8 h diurnal photoperiod. Once they reached second instar, two larvae were placed in a leaflet from the third true leaf of each six week-old tomato plant, using clip-cages to limit the feeding area and avoid their scape. Clip-cages were moved into new leaflets each two days to make sure they always had food available. Infestation was maintained for two weeks. To monitor insect development, larvae biomass and mortality were annotated every day. Dead larvae were replaced by new ones in order to keep the same stimulus in the plant. Finally, eight weeks-old plants with two weeks of herbivory infestation were harvested. Leaflets from noninfested plants (Control, -), leaflets from infested plants where *S. exigua* directly fed (Local, +L) and leaflets contiguous to those with direct damage (Systemic, +S) were separately harvested and immediately frozen in liquid nitrogen and stored at -80 °C until use for molecular analysis (**Fig. S1**).

Liquid chromatography and electro-spray ionization mass spectrometry

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

Freeze-dried leaves (50 mg) were homogenized on ice in 1 ml of MeOH:H_2O (10:90) containing 0.01% of HCOOH. The homogenate was centrifuged at 15000 g for 15 min at 4ºC and the supernatant was recovered and filtered through 0.2 µm cellulose filters (Regenerated Cellulose Filter, 0.20 µm, 13 mmD. pk/100; Teknokroma, St Cugat, Spain). Subsequently, 20 µl of the filtered supernatant were injected into an Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Mildford, MA, USA), which was interfaced with a hybrid quadrupole time-of-flight equipment (Q-TOF-MS Premier). Analytes were eluted with an aqueous methanol gradient containing 0.01% HCOOH. Solvent gradients and further chromatographic conditions were performed as previously described (Agut et al., 2014). Five biological replicates were randomly injected for every treatment. LC separation was performed using an UPLC Kinetex C18 analytical column with a 5 µm particle size, 2.1 x 100 mm (Phenomenex). To accurately identify the signals detected, a second fragmentation function was introduced into the TOF analyzer. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Agut *et al*., 2014; Gamir *et al*., 2014).

To precisely identify metabolites, a library of plant metabolites was generated using chemical standards. The compounds from this library were characterized at the level of retention time, exact mass and spectrum fragmentation (Schymanski *et al*., 2014). Up to 84 compounds were prepared at a final concentration of 100 ppb in a composite solution (Rivero *et al*., 2015) and injected through the UPLC in both positive and negative electro-spray ionization (ESI^+; ESI^-) modes. For those compounds that were not represented in the internal library, the signals obtained in the untargeted metabolomic analysis were confirmed by contrasting the fragmentation spectrum in the Massbank, Metlin or Human Metabolome databases (www.massbank.jp; www.masspec.scripps.edu; www.hmdb.ca).

Full scan data analysis

Data were acquired in centroid mode and subsequently transformed into .cdf files using the Databridge from MassLynx 4.1 software (MassLynx 4.1, Waters). Chromatographic signals were processed using the software R for statistical purposes. Signals from positive

and negative electrospray ionization (ESI^+; ESI^-) were processed separately. Peak peaking, grouping and signal corrections were performed using the XCMS algorithm (Smith *et al*., 2006). Metabolite amounts were analyzed based on normalized peak area units relative to the dry weight. Adduct, isotope correction, and Kruskal–Wallis test ($P < 0.05$) were performed by using the MarVis Suit 2.0 software tool (Kaever *et al*., 2015). To determine a global behavior of the signals, data obtained from positive and negative ESI were combined using MarVis Suit 2.0 software. Subsequently, data were normalized by sum, transformed by cube root and scaled by pareto method in order to obtain the sparse partial least squares discriminant analysis (sPLSDA) and heat-map plots, which were generated using MetaboAnalyst software (www.metaboanalyst.ca), a comprehensive Web-based package for a range of metabolomics applications (Xia and Wishart, 2016).

Feruloylputrescine targeted identification and quantification

Thirty milligrams of freeze dried plant leaves were extracted with a aqueous solution of MeOH:H_2O (10:90) containing 0.01% of HCOOH. The content of the tube was vortexed and left at 4°C in order to rehydrate the plant sample. Five glass beads (2 mm Ø) were added and the extraction was performed in a mixer mill at a frequency of 30 Hz for 1 minutes. The samples were centrifuged at 15000 g for 15', and the supernatant was recovered and filtered through a 0.2 µm of regenerated cellulose filters. For the analysis an external calibration curve of feruloylputrescine that ranged from 1 to 200 ppb was used. The neutral mass loss 265.1>177.1 was used following a ESI^+ for quantification in MS/MS spectrometry. A second extraction was conducted, and the supernatant was joined to the previous one. The chromatographic separation was carried out by injection of 20 µl on an UPLC Kinetex C18 analytical column with a 5 µm particle size, 2.1 x 100 mm (Phenomenex). The quantification of feruloyl putrescine was done in an Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Mildford, MA, USA) interfaced with a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK).

Experiments for feruloylputrescine regulation analysis

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker) were grown in greenhouse as previously described. After two weeks, plants leaves were weekly sprayed with 50 µM

methyljasmonic acid (MeJA) or 50 µM abscisic acid (ABA) in order to "mimic" constant herbivory conditions for three weeks more. Shoots were harvested and 50 mg of freeze-dried leaves were collected for feruloylputrescine targeted quantification as described above.

For the second experiment, two tomato genotypes were used: a JA-signaling defective mutant (*jasmonic acid–insensitive1*; *jai1*) and its corresponding wild-type (Castlemart; CM). As *jai1* lines are infertile, they are maintained as heterozygous plants, so that *jai1* recessive homozygous plants are selected by PCR as described by Li *et al*., 2004. Plants were inoculated or not with *F. mosseae*, grown and infested with *S. exigua* as described above. Eight week-old plants with (subjected to *S. exigua* herbivory for two weeks) were harvested and 50 mg of freeze-dried leaves were collected for feruloylputrescine quantification.

Feruloylputrescine direct effect on *S. exigua* bioassay

S. exigua larvae were reared on artificial diet (AD) until they become third instar. Sixteen newly molted third instar larvae continued feeding on AD, whereas other sixteen were transferred into AD supplemented with 100 µg/ml of feruloylputrescine (AD+FP). Their growth was monitored daily during the next days. Every 24h, the fresh biomass of larva was determined, and the state of development (different instars and pupae) and mortality were also annotated. The bioassay was stopped 8 days post treatment since most of the larvae had begun to pupate. The bioassay was repeated twice.

Statistical analyses

Besides the methods and software for metabolomic analysis described above, all statistical analyses (two-way ANOVAs and post hoc tests applied when appropriate, as indicated in the corresponding figure legends) were conducted using Statgraphics Plus 3.1 (Rockville, MD, USA), "R" software version 2.9.2 (RDevelopmentCoreTeam) and the XCMS package. Statistical analysis of larvae survival was performed using the Kaplan–Meier estimator, a non-parametric statistic used to estimate the survival function from the monitored lifetime data (Kaplan and Meier, 1958).

Results

Arbuscular mycorrhizal symbiosis increases mortality of *S. exigua* larvae

In order to determine whether root colonization by *Funneliformis mosseae* affects *S. exigua* performance on tomato plants, we infested tomato plants displaying a well-established symbiosis. The percentage of root colonization by the mycorrhizal fungus was approx. 13% by the end of the experiment (**Fig. S2a**). The symbiosis had no effect on plant growth (**Fig. S2b**). This is of interest for studies on defense mechanisms by avoiding the potential effects of improved plant growth on the insect performance. AM establishment clearly was detrimental to the development of *Spodoptera exigua*. Larvae fed on *F. mosseae* colonized tomato plants (Fm) showed a higher mortality than those feeding in nonmycorrhizal plants (Nm). In fact, 50 % of the larvae were dead by day 10 in Fm plants, while the mortality was only 15% for larvae on Nm plants (**Fig. 1a**). The application of the nonparametric Kaplan Meier estimator of the survival function for the larvae population confirmed that survival was significantly reduced in mycorrhizal plants (**Fig. S2c**). Despite the differences in the gain of weight of the survivors between Fm and Nm plants was not statistically significant, there was a clear trend showing a reduced larval gain weight in Fm plants (**Fig. S2d**). In addition, at 11 days following infestation, the 17% of larvae fed on Nm plants started to pupae, while in mycorrhizal tomatoes none of the larvae reached this developmental stage (**Fig. 1b**).

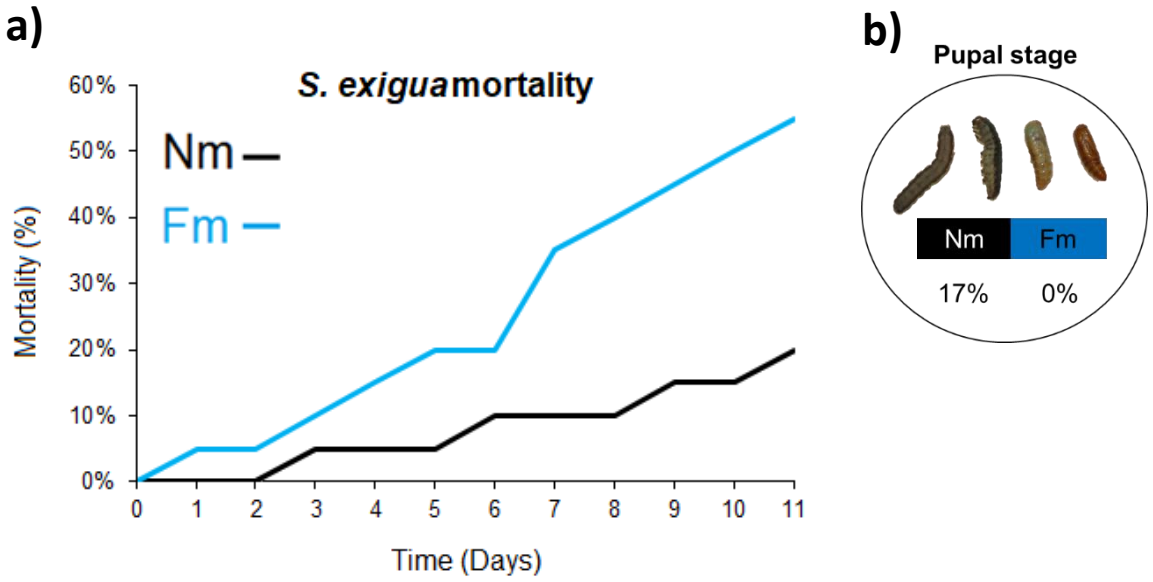


Fig. 1 Performance of *Spodoptera exigua* larvae fed on nonmycorrhizal plants (Nm, black line) or in plants colonized by AMF *Funneliformis mosseae* (Fm, blue line) a) Percentage of mortality observed and b) percentage of individuals reaching pupal stage. Six weeks old tomato plants colonized or not colonized by *F. mosseae* were infested with second instar *S. exigua* larvae (two per plant) using clip cages, and mortality recorded daily. After 11 days, larvae reaching pupal stage were also annotated. (n =20).

S. exigua herbivory, but not the mycorrhizal symbiosis _per se_, had an important impact on the leaf metabolome.

We hypothesized that reduced larval survival rate and performance in Fm plants compared with noncolonized ones is a result of changes in the defense response of the plant related to the mycorrhizal symbiosis. To test this possibility, we compared the plant response of Nm and Fm colonized plants subjected (+) or not (-) to *S. exigua* feeding during 14 days through an untargeted metabolomic analysis. To have a more precise overview of the metabolic regulation upon herbivory, we harvested the leaflets were the larvae fed separately from the other unwounded leaflets of the same leaves. Thus, herbivory related changes were explored in local wounded leaflets (+L) or in unwounded leaflets (systemic response, +S) as shown in **Fig. S1**. A total of 1132 signals (potential compounds) were registered, and subsequent statistical analysis showed 200 signals with significantly different accumulation in at least one of the different treatments ($P < 0.05$). A representation of a supervised analysis (sPLSDA) of these 200 signals revealed that, as expected, herbivory had a strong impact on plant metabolism (**Fig. 2a**). According to the two main components contributing to data variation, the differences were more pronounced locally at the feeding sites than systemically in leaflets that were not exposed to direct herbivory, since the group of these signals are more distant compared to undamaged leaves (**Fig. 2a**). These observations were further confirmed by a heatmap analysis (**Fig. 2b**). This analysis clearly showed the rearrangement of the leaf metabolomic profile following herbivory. Remarkably, in the absence of stress (Nm- vs. Fm-), AM symbiosis had very low impact on the foliar metabolic profiles since only one hit out of 1132 detected displayed altered accumulation in mycorrhizal plants (**Fig. S3ab**). Although still not fully characterized, exact mass identification coincides with 11-carboxyblumenol C-9-O-Glc, a metabolite recently reported as shoot marker of AM symbiosis (Wang *et al.*, 2018).

Funneliformis mosseae colonization primes the accumulation of specific metabolites in locally damaged leaves in response to *S. exigua* herbivory

According to the differences observed in larval mortality, we focused our search on potential differences in response to herbivory between mycorrhizal and nonmycorrhizal plants. Few changes in the metabolite profiles were observed between Nm and Fm in the systemic response to herbivory (Nm+S vs. Fm+S), as confirmed by the overlap in both sPLSDA and heatmap plots (**Fig. 2ab**). In contrast, the local responses to herbivory were modulated as illustrated the clear separation between the Nm+L and Fm+L samples in the sPLSDA (**Fig. 2a**). The heatmap analysis confirmed these clearly separated profiles, while uninfested Nm and Fm and systemic Nm+S and Fm+S clustered together. The distinct pattern of metabolites accumulation in Fm+L revealed a stronger accumulation of a set of compounds resembling a priming profile (highlighted by a black square **Fig. 2b**). Coherently with the primed character of this cluster of compounds, the differences were restricted to Fm+L leaves, whereas these metabolites were not modulated by the presence of AM symbiosis in the absence of herbivory. Intriguingly, those compounds seem to be accumulated in a similar way in both Nm and Fm plants in systemic tissues.

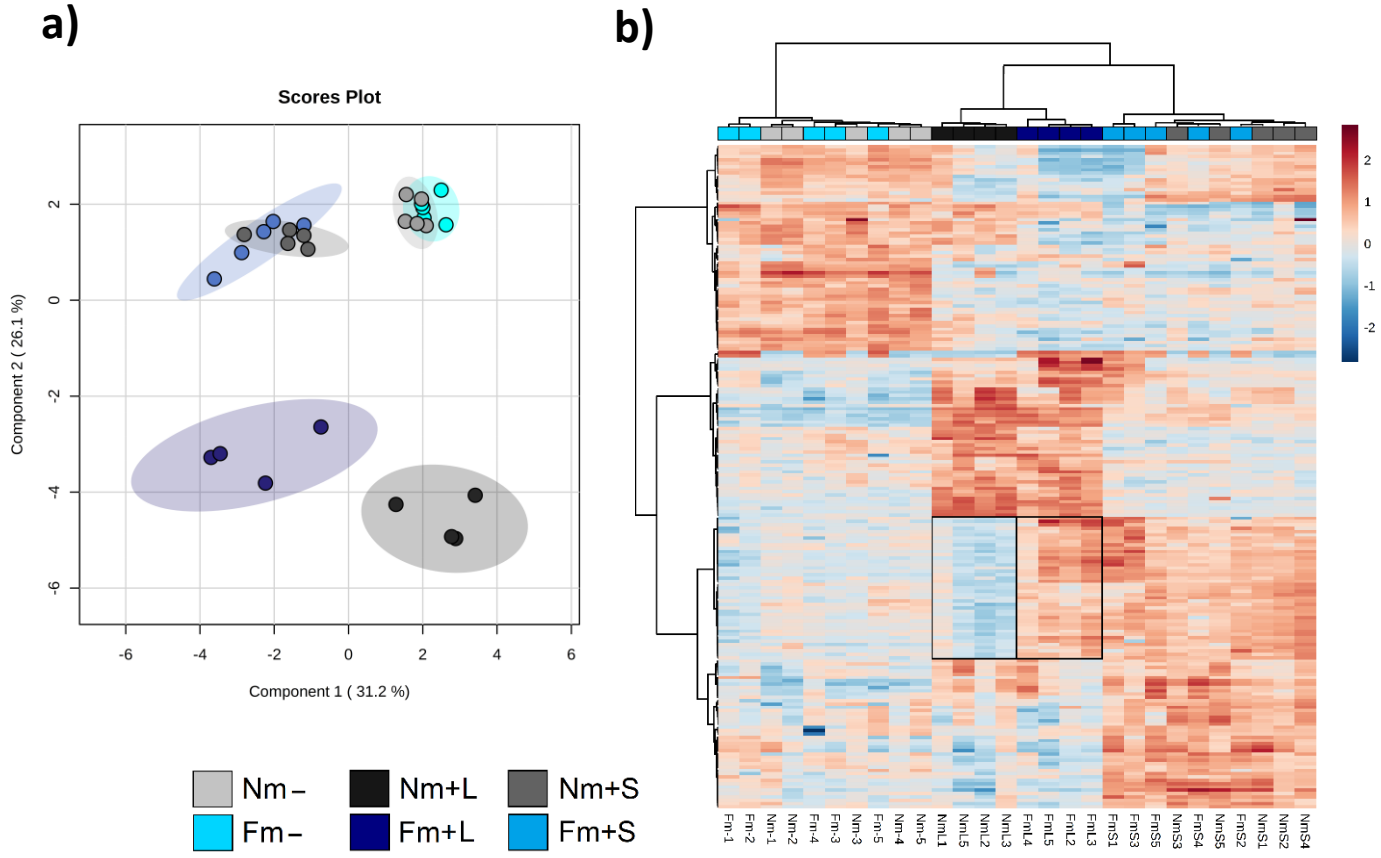


Fig. 2 Overview of the metabolomic reprogramming in tomato leaves subjected to *Spodoptera exigua* feeding for 15 days. Six weeks old tomato plants nonmycorrhizal (Nm) or colonized by the arbuscular mycorrhizal fungi *Funneliformis mosseae* (Fm) were infested with S. *exigua* second instar larvae, and leaves were harvested after 2 weeks of systemic herbivory. Lyophilized leaf material from control noninfested plants (-), infested leaflets (local response to herbivory, +L) and nondamaged leaflet from infested plants (systemic response to herbivory, +S) were analyzed through ultra-high performance liquid chromatography interfaced with a quadrupole time-of-flight mass spectrometer (UPLC-Q-TOF-MS) in order to monitor metabolomic changes. Signals intensity was determined in all samples after normalizing the chromatographic area for each compound to the dry weight of the sample. All 1132 registered signals from combination of both positive and negative electrospray ionization (ESI) were compared using nonparametric Kruskal–Wallis test, and only data with significant differences between groups at $P < 0.05$ were used for the supervised analysis (n = 5; n = 4 for local), obtaining 200 signals. Grey and blue colour scales represent Nm and Fm treatments respectively, and for each case, lighter colour represents nonstress, darkest colour represent changes in infected, damaged tissue (L) medium colour represent changes in systemic responses to herbivory (S). **a) Supervised three-dimensional sparse partial least squares discriminant analysis (sPLSDA) representation of the major sources of variability. b) Heat-map and clustering representation of the 200 signals showing differences among treatments. Cluster of metabolites showing an over-accumulation pattern upon herbivory in L tissue only in Fm plants (priming pattern) in highlighted.**

Characterization of the metabolic pathways differentially regulated in response to herbivory

Following untargeted metabolomic analysis, we identified the signals of interest by exact m/z or/and individual spectrum match with databases. Only those signals whose accumulation differed in response to herbivory were classified into the major metabolic pathways. For such classification we used the *S. lycopersicum* Kegg database, and subsequently we represented the percentage of differentially regulated metabolites in each of these categories for each condition **(Fig. 3).** This classification illustrates that the local response to *S. exigua* herbivory resulted in altered accumulation of compounds of multiple pathways, but mostly phenolics compounds (21%), terpenoids (14.9%), carbohydrates (10.8%), and flavonoids (10.8%). In distal leaflets, the systemic response resulted in activation of those pathways too, but a higher alteration in amino acids and alkaloids was found. Conversely, in mycorrhizal plants, these metabolic patterns of response were different. In local damaged leaflets the major differences were found in the percentage of metabolites altered within the fatty acid metabolism (more than 3-fold), and alkaloids (2-fold) when compared with the local response in Nm plants. In contrast, the proportion of herbivore modulated compounds related to sugar and flavonoid metabolism was lower in Fm plants. Regarding the systemic responses, the proportion of alkaloids and metabolites related to nucleotide metabolism was almost double in Fm than in Nm plants.

Thus, the results illustrate that mycorrhization implies a stronger activation mostly of compounds related to fatty acid metabolism and alkaloids.

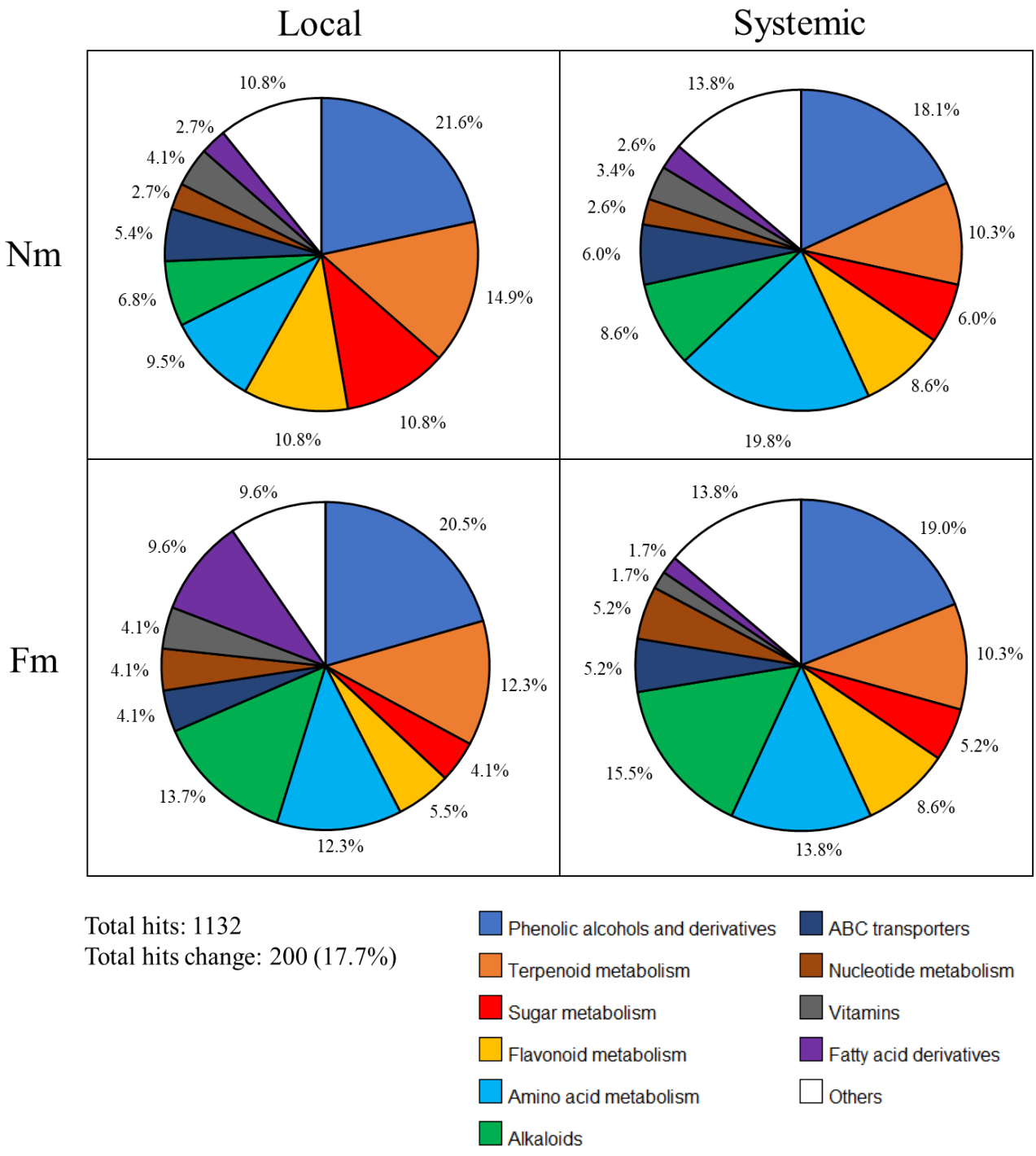


Fig. 3 Composition of altered metabolic pathways from tomato plants leaves under *S. exigua* herbivory. Six weeks old tomato plants nonmycorrhizal (Nm) or colonized by the arbuscular mycorrhizal fungi *Funneliformis mosseae* (Fm) were infested with S. *exigua* second instar larvae, and leaves were harvested after 2 weeks of systemic herbivory. After untargeted metabolomic analysis through ultra-high UPLC-Q-TOF-MS (described in Fig. 2 legend), obtained signals were compared using nonparametric Kruskal Wallis test ($P < 0.05$), and only data with differences between herbivory treatments (local or systemic) and nonstress conditions were used for the supervised analysis (n = 5; n = 4 for local). The resulting hits were identified by exact m/z or/and spectra coincidence and subsequently grouped into the different *S. lycopersicum* metabolic pathways from Kegg databases included in software MarVis 2.0. Finally, the pie charts with the percentage of main metabolic pathways altered in each treatment were represented.

Mycorrhiza-primed metabolites in local response to *S. exigua* herbivory

As described above, the most remarkable difference between the response to herbivory in Nm and Fm plants is the cluster of compounds displaying a primed accumulation pattern in local tissues (**Fig. 2b, highlighted cluster**). This suggests a likely role of these compounds and their pathways in the mycorrhiza-induced resistance (MIR). Identification through exact mass and/or fragmentation revealed that among the metabolites within this primed-cluster, we found alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates (PPCs). The alkaloids, including physostigmine, huperzine A and cotinine, appeared overaccumulated locally after caterpillar feeding only in in Fm plants, remaining unaltered locally in Nm plants. Interestingly, the priming profile of these compounds was only observed in local tissues, since they were accumulated in systemic leaves upon herbivory in both Nm and Fm plants (**Fig. 4a**).

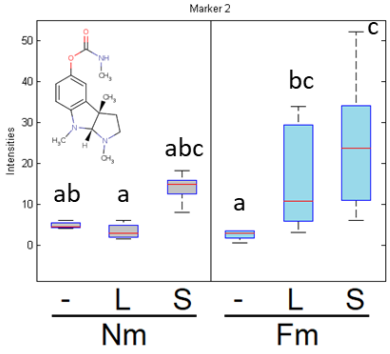
Among the fatty acid derivatives showing a primed accumulation profile in Fm leaves we found azelaic acid and 4-oxododecanedioic acid. These compounds, unlike is the accumulation pattern described for alkaloids, were not altered at all in nonmycorrhizal plants, and only marginally in systemic leaves of mycorrhizal plants (**Fig. 4b**).

Finally, a group of **phenylpropanoids-polyamine conjugates (PPCs)**, also known as hydroxycinnamic amides or phenolamides (**Fig. 4c**), formed by condensation of phenolic acids with amines such as polyamines and arylamines were found to be primed in local leaves of Fm plants. This was the case for feruloylputrescine (FP) and feruloylagmatine that showed significantly higher accumulation levels only in Fm local leaves in response to the herbivory. In contrast, other compounds in this group, as tricoumaroylspermidine and feruloyl-2-hydroxyputrescine, besides the local accumulation in response to herbivory in Fm plants, were also accumulated in systemic tissues regardless the mycorrhizal status of the plant.

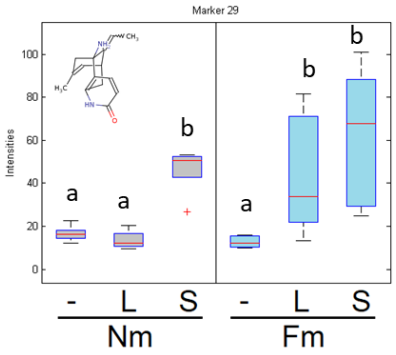
Since PPCs have been reported to have an effect on herbivores, and considering that feruloylputrescine is commercially available, we selected this compound for further analysis. We optimized a targeted quantification of this phenol-conjugate to confirm the data and we further studied its regulation as a model of a priming compound to unravel the mechanisms underlying the MIR in the studied tomato-*F. mosseae*-*S. exigua* multitrophic interaction.

a) Alkaloids (ESI+)

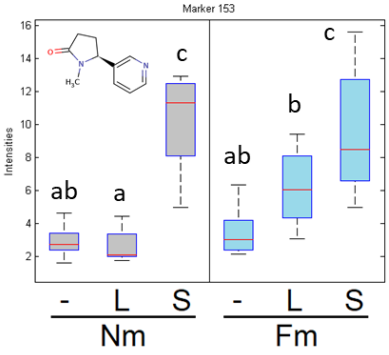
Physostigmine



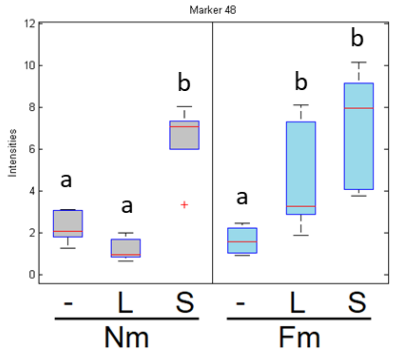
Huperzine A



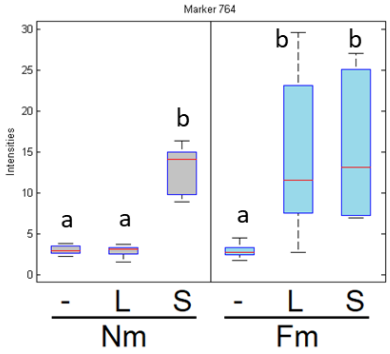
Cotinine



m/z 333.167

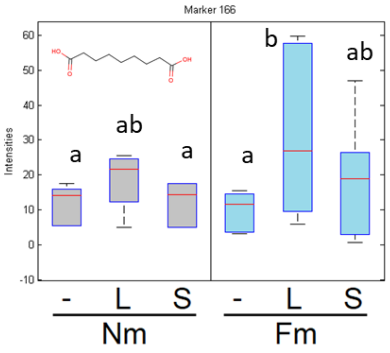


m/z 279.132

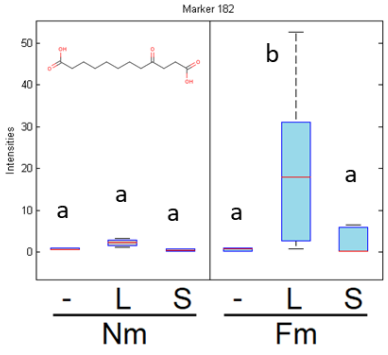


b) Fatty acids derivatives (ESI-)

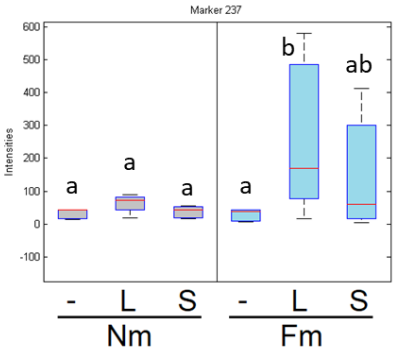
Azelaic acid



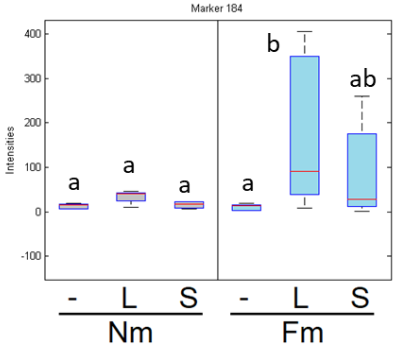
4-Oxododecanedioic acid



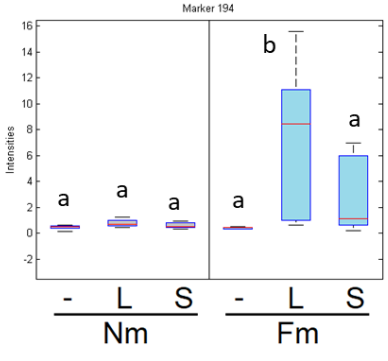
m/z 328.227



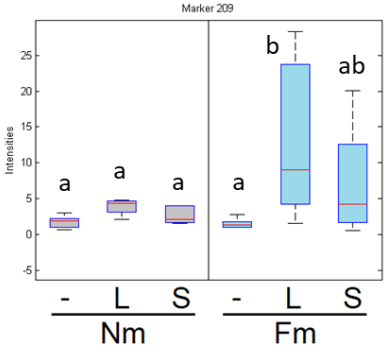
m/z 330.242



m/z 364.203



m/z 262.1433



c) Phenylpropanoids-polyamine conjugates (ESI+)

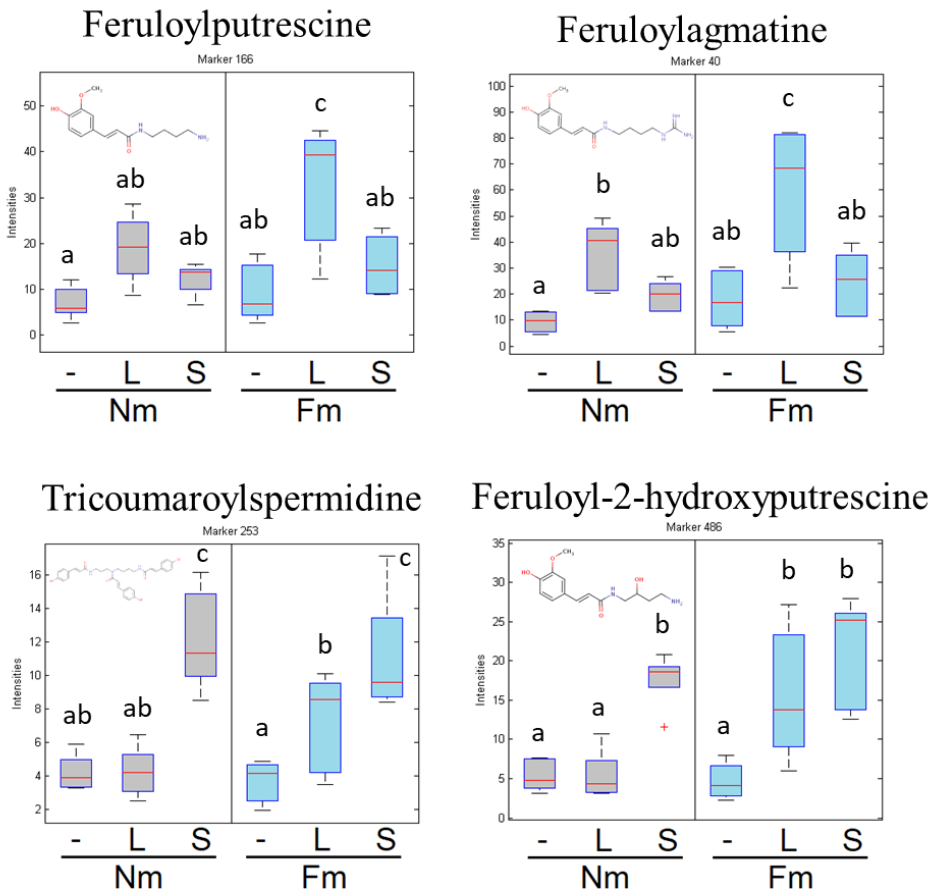


Fig. 4 Boxplots of selected metabolites with a primed accumulation pattern in response to local herbivory (L). Six weeks old tomato plants, previously colonized by arbuscular mycorrhizal fungi (AMF) *Funneliformis mosseae* **(Fm)** or not (nonmycorrhizal, **Nm**), were infested with second instar larvae of S. *exigua*, and maintaining the infestation for two weeks more. Leaves from plants in absence of stress, or well with local **(L)** or systemic **(S)** damage by *S. exigua* feeding were analyzed through a untargeted metabolic assay in order to monitor metabolic changes (data acquisition as described in Fig. 2 legend). Grey colour represents accumulation in Nm plants and blue colour represents accumulation in Fm colonized plants. Boxes represent the interquartile range, thick red-lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and red-crosses show outliers. Putative metabolites were classified into three main metabolic groups, **a)** alkaloids family, **b)** fatty acids derivatives family and **c)** phenylpropanoid-polyamine conjugates (PPCs) family. Two-way factorial ANOVA (using Fm colonization and herbivory treatments as factors) were performed. Data not sharing a letter in common differ significantly according to the Fisher's LSD test ($P < 0.05$, n = 6).

Feruloylputrescine priming in mycorrhizal plants is jasmonic acid-dependent

To confirm the identification of FP and aiming to a more accurate quantification of its levels in plants, we developed a quantitative targeted method using LC-MS using the commercially available product as a standard. Once characterized the chromatographic and spectrometric parameters, primed accumulation of FP in leaves of Fm plants upon herbivory was further confirmed by using a UPLC coupled to a triple quadrupole mass spectrometer (**Fig. 5a**).

With the aim to explore the regulation of FP primed accumulation, a new assay mimicking *S. exigua* herbivory was performed. Tomato plants were weekly sprayed with the two phytohormones described as major regulators of plant responses to chewing insects, jasmonic acid (JA, in the form of methyl JA (MeJA)) and abscisic acid (ABA) (**Fig. 5b**). While the ABA application did not increase FP levels significantly, MeJA treatment clearly increased its concentration, revealing that JA is a positive regulator of the phenol-conjugate FP. To confirm the relevance of JA-dependent pathway in FP priming, a new assay using JA signaling mutants was performed. Two genotypes: wild-type (WT, cv. Castlemart) plant and the JA signaling mutant (*jai1*), were used to confirm whether mycorrhizal priming of FP accumulation is dependent of JA signaling (**Fig. 6**). The *jai1* mutant was impaired in the Fm-induced priming of FP suggesting the relevance of JA mediating MIR-triggered metabolic responses. To make sure that the different genotypes had JA-dependent defensive response, a qPCR with the wound-response gene encoding for serine proteinase inhibitor II (*PI-II*) was performed (**Fig. S4**). It was observed that the response to herbivory was fully compromised independently of the mycorrhization. Surprisingly, when the genotype was Castlemart the symbiosis with *F. mosseae* was enough to trigger an increase in the level of FP in the absence of wounding what differs from the cv. Moneymaker. Despite the elevated levels of FP in Fm plats, the herbivory further induced the accumulation of FP, confirming that Fm colonization primes FP accumulation in different tomato cultivars. *S. exigua* feeding increased the FP levels in both Nm and Fm plants also in the *jai1* genotype, however priming was impaired in JA defective mutants supporting the fact that an intact JA-dependent pathway is required for mycorrhiza related FP priming, but not for the basal response to herbivory.

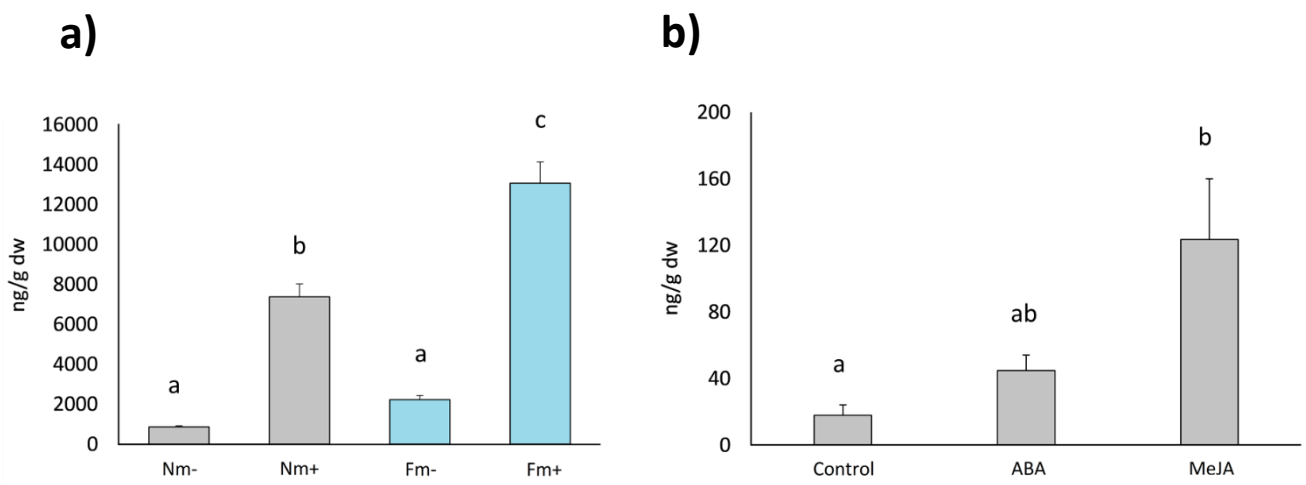


Fig. 5 Feruloylputrescine (FP) quantification in tomato plants leaves. Two assays using *S. Lycopersicum* var Moneymaker plants were performed. At the end of each assay, leaves samples were lyophilized and subsequently analyzed by combination of liquid chromatography (LC) with a triple quadrupole detector (TQD) *in a precursor ion scanning mode*. Grey-bars represents FP concentration in nonmycorrhizal plants (Nm) and blue-bars represents concentration in Funneliformis mosseae (Fm) colonized plants. **a)** Total FP confirmation and quantification in local-infested leaves (represent as +) from tomato plants where untargeted metabolic analysis were performed. Two-way factorial ANOVA (using Fm colonization and herbivory treatments as factors) was performed. Data not sharing a letter in common differ significantly according to the Fisher's LSD test ($P < 0.05$, n = 4). **b)** FP quantification in tomato plants weekly sprayed with the main reported hormones involved in plant defensive responses to chewing insects. Plants were treated with 50 µM of abscisic acid (ABA) or methyl-jasmonic acid (MeJA) once a week for three weeks, the last one just 24h before harvest. One-way factorial ANOVA was performed. Data not sharing a letter in common differ significantly according to the Fisher's LSD test ($P < 0.05$, n = 4).

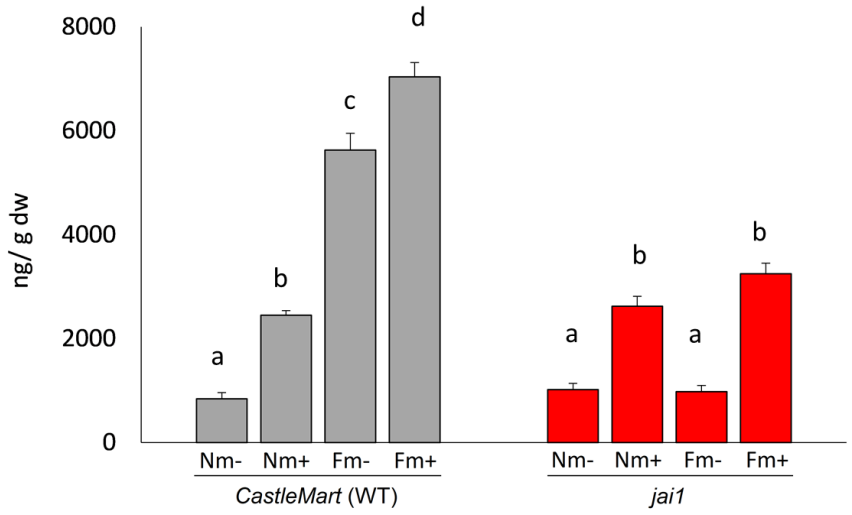


Fig. 6 Feruloylputrescine (FP) quantification in leaves of wild-type (WT, cv. Castlemart, grey bars) and JA-signaling mutant *jai1 (red bars)* of tomato plants. Six weeks old tomato plants, previously colonized by arbuscular mycorrhizal fungi (AMF) *Funneliformis mosseae* **(Fm)** or not (nonmycorrhizal, **Nm**), were infested with second instar larvae of *S. exigua* (represented as +), and maintaining the infestation for two weeks more. Two-way factorial ANOVA (using Fm colonization and herbivory treatments as factors) were performed for each genotype. Data not sharing a letter in common differ significantly according to the Fisher's LSD test ($P < 0.05$, n = 5).

Feruloylputrescine direct effect on *S. exigua* fitness

Higher accumulation of FP in response to local herbivory suggests a possible role of this compound in the enhanced caterpillar mortality observed. To test its effects, we performed a bioassay where third instar *S. exigua* larvae were fed on artificial diet supplemented or not with 100 µg/mL of FP. Larval weight (**Fig. 7a**), weight and survival (**Fig. 7b**) were monitored every 24h for 8 days. Along the time-course experiment neither larval weight nor survival were affected by FP. Thus, we found no evidence for a direct toxic effect of FP on *S. exigua* development.

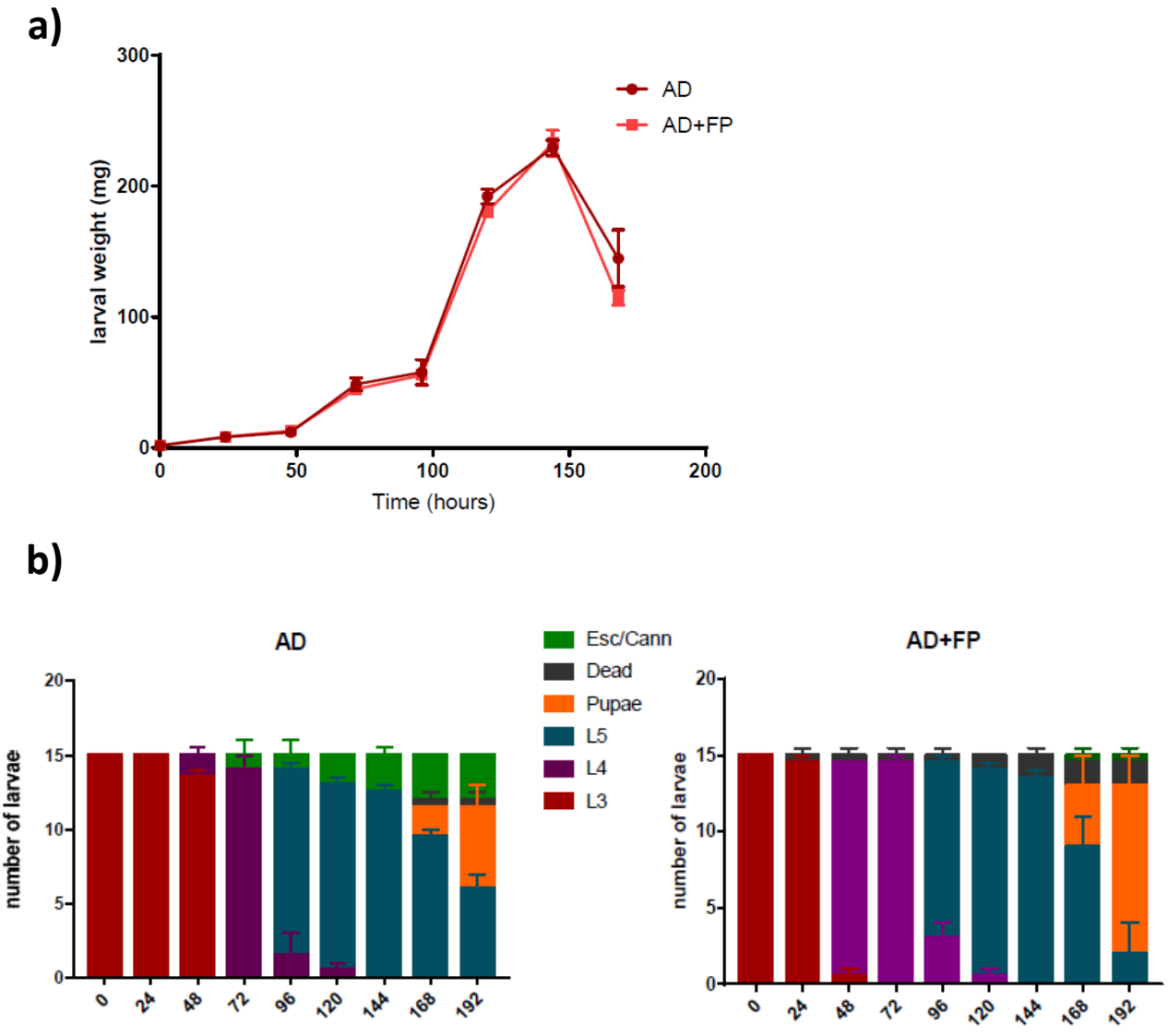


Fig. 7 Feruloylputrescine (FP) activity on *Spodoptera exigua* development. Third instar S. exigua larvae were fed with artificial diet alone (AD) or well supplemented with 100 µg/ml of FP (AD+FP). Different variables were recorded every 24h for 8 days. **a)** Larval weight of *S. exigua*. **b)** Number of larvae of *S. exigua* reached each development instar, pupae or dead. Significant differences were not found by the application of FP in any of the measured parameters, according to Student t-test ($P < 0.05$, n = 16).

Discussion

In the present study, we demonstrated that tomato root colonization by the arbuscular mycorrhizal fungi (AMF) *F. mosseae* impact the host interaction with the generalist pest *S. exigua*. Larvae feeding in these mycorrhizal plants showed a significant increased mortality and delayed development, thus being the plant more efficient in containing the herbivore. Although the protective role of AM against pathogens and the molecular mechanism underlying have been studied, very few papers reported on the influence of arbuscular mycorrhizal (AM) plants against chewing leaf insect (He *et al.*, 2017; Minton *et al.*, 2016; Song *et al.*, 2013). Mycorrhizal plants have been reported to be more resistant against *Helicoverpa armigera* that displayed a reduced larval weight gain after 72 h of feeding (Song *et al.*, 2013). Notably, despite the current lack of knowledge of the mechanisms regulating mycorrhiza-induced resistance (MIR), it is acknowledged that AM plants display an optimal tuning of their defense and notably this improved resistance against *Spodoptera litura* was shown to be transferred through common mycorrhizal networks (Song *et al.*, 2014).

However, the effectiveness of MIR against caterpillar pests must be considered carefully since there are several experimental elements that may result into a different output interaction. For example, specialist *Manduca sexta* growth was unaffected after feeding on two mycorrhizal Solanum Spp, even if defenses were enhanced by AM establishment (Minton *et al.*, 2016). This fact could be due to their specialist behavior, which allow them to avoid plant defense (Jung *et al.*, 2012). Moreover, although the strongest defensive capacity has been studied in AM plants through phytohormonal measurements or well quantify accumulation of previously reported metabolite with toxic effect, there are no studies been carried out analyzing the possible modulation of host plant metabolism in MIR.

Here, by using untargeted metabolomics analysis we attempted to profile the metabolic tuning of a symbiont when interacting with an herbivore at the local and systemic levels. Note that although in previous metabolomic studies the AMF colonization had a strong impact in the roots in absence of stress (Rivero *et al.*, 2015), this modulation is not observed in the leaves at the same conditions. The bidimensional sPLSDA analysis showed that local and systemic responses upon herbivory are rather different and

segregate between them and compared with control leaves. The general trend is similar in mycorrhizal plants, and the impact of the mycorrhizal treatment in the metabolomic fingerprint becomes only obvious in the local leaves. Following a bioinformatic processing of the signals, the heatmap analysis showed that herbivory is an important criterion for clustering, since the metabolites of infested leaves cluster closer compared with uninfested. However, the AMF colonization does not separate in systemic or in noninfested leaves, but clearly cluster separately when local leaves are studied. In this case, the heatmap exhibits a cluster of compounds with a clear priming accumulation profile locally upon herbivory, where several metabolites accumulate to a higher level in Fm plants than in noncolonized ones.

Phenylpropanoids, terpenoids, flavonoids, sugars and amino acid metabolic pathways were the more altered routes in secondary metabolism in local responses to leaf tissue damage by *S. exigua* attack in both Nm and Fm plants. In systemic tissues, the same metabolic pathways were regulated in response to herbivory, but the proportion of the amino acids and alkaloids was higher. This suggests a likely preparation of systemic leaves for a subsequent attack. In mycorrhizal plants, the increase in alkaloids in both local and systemic damaged leaves seems more pronounced. The insecticidal properties of these compounds may be underlying the observed protection. In addition, it is remarkable the 3-fold higher accumulation of compounds from fatty acids metabolism in local infested leaves compared with the noncolonized plants. This group could be also of great importance for plant defenses since comprises among other to JA precursors from oxylipins biosynthetic pathway, which modulation is reported in roots of AM-plants (Fernández *et al.*, 2014; Rivero *et al.*, 2015; Rivero *et al.*, 2018).

We focused our study on those compounds showing a priming profile in the local response of mycorrhizal plants to herbivory, as they may be related with the enhanced mortality of the larvae when feeding in these plants. The three main families of compounds within this cluster were alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates (PPCs). Alkaloids are well characterized group of compounds with toxic effect against herbivory (Mithöfer and Maffei, 2015). Beside Fm induction in local damaged leaves, found alkaloids also showed high accumulation in systemic leaves, suggesting a higher mobility of these compounds that may contribute to improved defensive responses against subsequent infestations in distal tissues. Among the ones

found, cotinine, a common metabolite from catabolism of nicotine was observed (Saremba *et al.*, 2018). In addition, two acetylcholinesterase inhibitors were found: physostigmine (also known as eserine) and huperzine A (Luo *et al.*, 2010; Rattan, 2010).

Different studies have addressed the involvement of fatty acids as chemical signals triggering defenses during negative interactions (Lim *et al.*, 2017). In our experiment we found, there was a higher accumulation of azelaic acid in Fm plants locally upon herbivory. Azaleic acid has been proposed to be a component of plant systemic immunity regulating the priming of plant defenses (Jung *et al.*, 2009). Interestingly, azelaic acid exogenous application in tobacco cells were found to primed genes involved in the systemic acquired resistance, exhibiting an enhanced synthesis of PPCs among other compounds (Djami-Tchatchou *et al.*, 2017), suggesting a role of azelaic acid in priming of this family of secondary metabolites. For 4-oxododecanoic acid there is no reports linking it with increases plant defense, and for the rest of fatty acids derivatives further research is needed to fully identify these metabolites and characterize their role in MIR.

PPCs (also known as phenolamides) are a diverse class of secondary metabolites present ubiquitously in plants. In our conditions, both tricoumaroylspermidine and feruloyl-2-hydroxyputrescine metabolites showed a similar patter than alkaloids, with primed behavior in Fm plants in local damaged leaves and high accumulation in the systemic response in both Nm and Fm plants. Conversely, feruloylagmatine and feruloylputrescine (FP) only were overaccumulated locally in the herbivory damaged plants in Fm, but their levels remained unaltered in distal tissues. These compounds were suggested to play an important mostly on plant development and defense (Edreva *et al.*, 2007; Macoy *et al.*, 2015), especially after challenge with pathogens (López-Gresa *et al.*, 2011; Yogendra *et al.*, 2015) or virus (López-Gresa *et al.*, 2016). Regarding to PPCs role in anti-herbivore defense, it was reported an enhanced growth of *S. litoralis* that fed on *Nicotiana* mutant plants lacking PPCs. Additionally, *Nicotiana* plants sprayed with caffeoylputrescine (CP) reduced *Manduca sexta* larval growth (Kaur *et al.*, 2010), suggesting that PPCs are important players in plant defense against leaf chewers.

Therefore, in the present work we performed a fully identification of FP through a targeted chromatographic analysis comparation with the pure chemical standard, where its primed-accumulation pattern in the local response to herbivory was confirmed. Follow

up analysis were performed to elucidate the phytohormonal regulation of FP accumulation in response to herbivory. For such purpose, we followed both pharmacological and genetic approaches. In previous experiments with tobacco, authors already suggested that PPCs may be under control of JA (Gális *et al.*, 2006). We demonstrated that exogenous MeJA treatments triggered FP accumulation in tomato plants. Additionally, we found that the JA signaling deficient mutant *jai1* were impaired in FP priming in mycorrhizal plants upon infestation. Thus, our results support that JA participates in the fine tuning of PPCs during mycorrhiza related priming. Note that mycorrhizal *jai1* plants were still functional accumulating significantly higher amounts of FP in response to herbivory what means that JA is mostly regulating a hypersensitization of the plant to over-accumulate FP but not its basal responses to caterpillars. Interestingly, in our study we confirmed that the JA implication in MIR is mediated by defense priming (Jung *et al.*, 2012; Song *et al.*, 2013; Martínez-Medina *et al.*, 2016). The JA responsive marker *PI-II*, was mostly induced following herbivory with no fitness costs for the plant. Furthermore, this *PI-II* priming was abolished in the signaling *jai1* mutants, what confirmed that JA priming was mediating MIR as it was reported before with other mycorrhiza-insect interactions (Song *et al.*, 2013).

Finally, we performed to unravel whether FP has a direct deleterious activity on *S. exigua* development through it addition to artificial diet. In this sense, previous reports showed controversial results. Leaf chewing insects S*podoptera Mauritia* and *Parnara guttata* fed on a sugar solution containing a 15% of FP or CP were unaffected in their development compared with sugar diet alone (Alamgir *et al.*, 2016). However, the brown planthopper *Nilaparvata lugens* showed a higher mortality. In addition, previous oviposition by *Spodoptera exigua* moths on *Nicotiana* plants triggered higher larvae mortality and primed CP accumulation after application of oral secretions (Bandoly *et al.*, 2015). Another experiment performed with specialist *M. sexta* also elicited similar plant response, although these oviposition-primed changes did not affect subsequent larval development (Bandoly *et al.*, 2016). In our experimental conditions, we did not observe any alteration in growth parameters or mortality compared with control treatments, although further studies are needed to address its possible indirect role mediating the synthesis of toxic compounds for insect development.

In conclusion, our results indicate that *F. mosseae* colonization is and effective strategy to increases tomato plant resistance against *S. exigua.* The study also indicates that the symbiosis establishment modulates host metabolism in response to herbivory, mainly through a primed accumulation of fatty acids derivatives, alkaloids and PPCs. Metabolite FP is fully identified and its strongest accumulation in Fm colonized upon herbivory requires JA signaling,

Supplementary Data

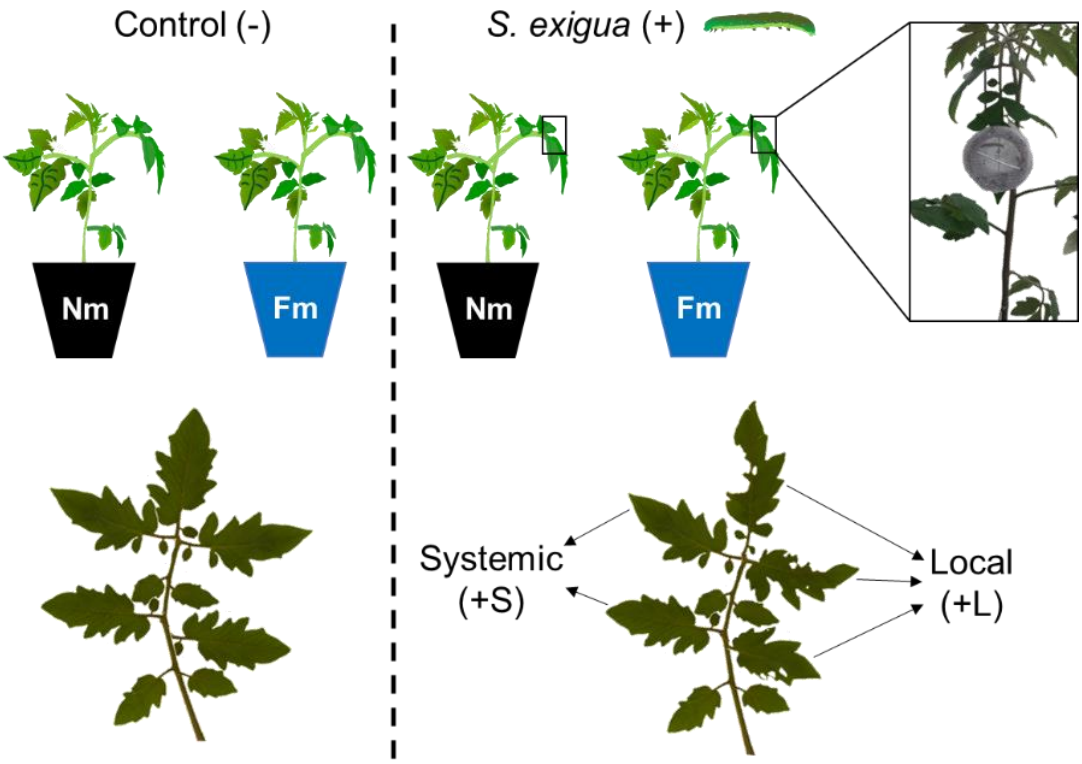


Fig. S1 Experimental design and harvested shoot material in *F. mosseae* colonized (Fm) or nonmycorrhizal (Nm) tomatoes, infested (+) or not (-) with *S. exigua*. Two larvae were placed into a clip-cage per plant. Clip-cages were moved every day into new leaflets, for two weeks. At the end of the assay, shoot material was collected from each treatment, differentiating among leaflets from no stressed plants (-), leaflets with damage from *S. exigua* feeding (local, +L) and those leaflets without damage but contiguous to where *S. exigua* fed (systemic, +S)

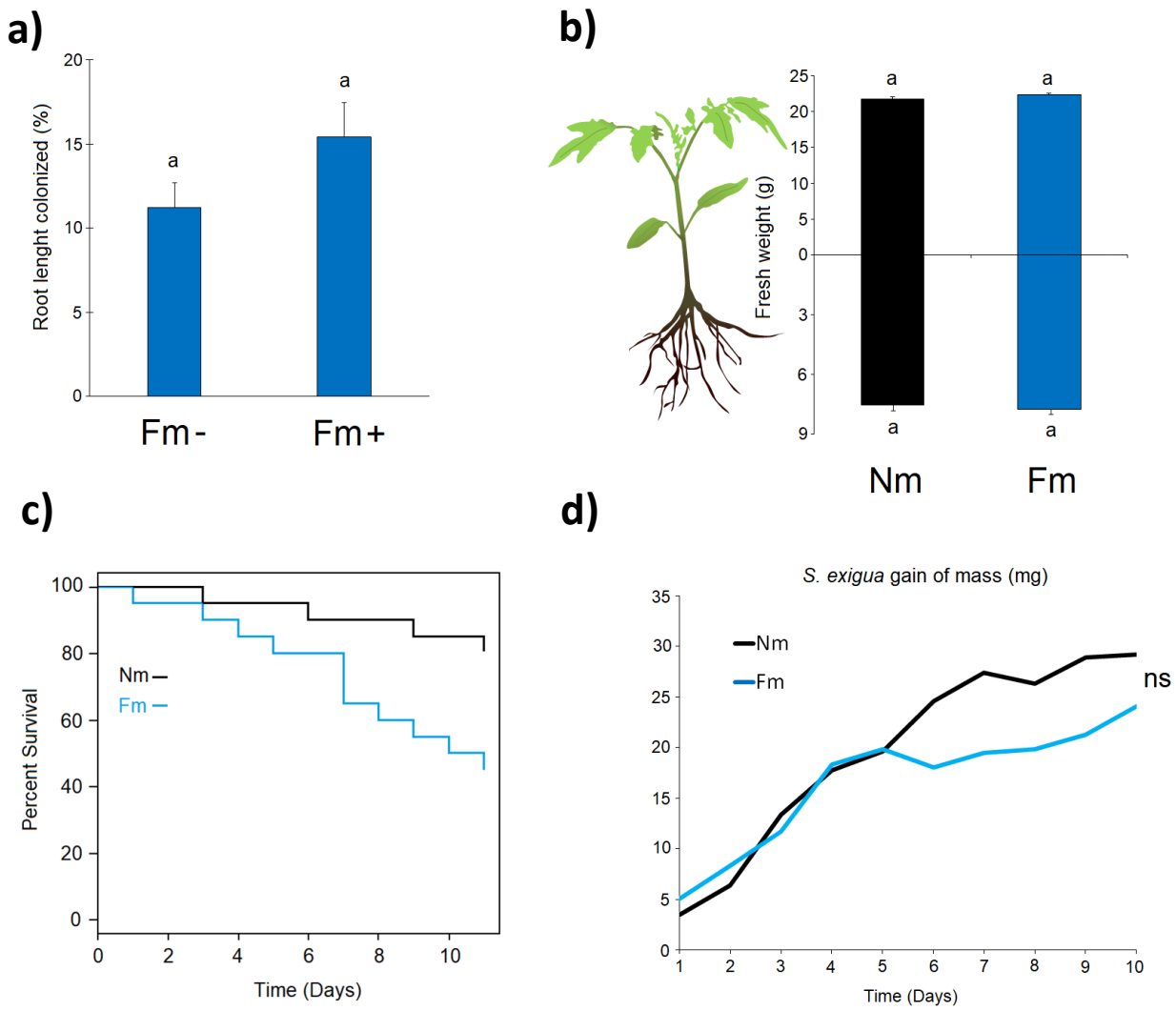


Fig. S2 Physiological parameters measured. a) Percentage of root length colonized by *F. mosseae* in 8 weeks-old tomato plants grown in absence of stress (Fm-) or subjected to 2 weeks herbivory by *S. exigu*a (Fm+). (n=10). **b)** Shoot and root fresh weight of 8 weeks-old of nonmycorrhizal (Nm, black bars) or AMF *F. mosseae* colonized tomato plants (Fm, blue bars) (n=10). **c)** The Kaplan–Meier survival analysis test showed significant differences between treatments according to Chi-squared test (n =20, $P < 0.05$). **d)** Weight gain of *Spodoptera exigua* larvae fed on six weeks old tomato plants colonized by AMF *F. mosseae* (Fm, blue line) or in absence of symbiosis (Nm, black line). Larvae biomass was counted every day until day 10. Only biomass of survivors was recorded. (n=10). In **a) b) d)** means not sharing a letter in common differ significantly according to the Fisher's LSD post hoc test ($P < 0.05$).

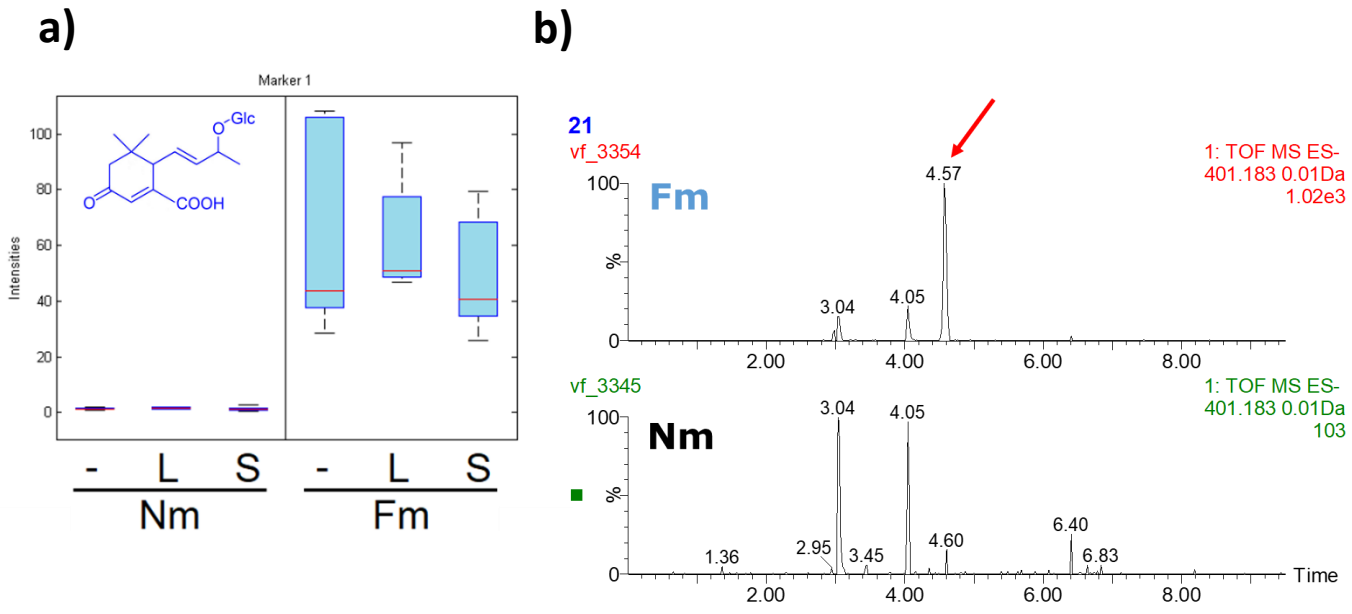


Fig. S3 Single signal more accumulated in *F. mosseae* colonized tomato plants in nonherbivory condition. a) Boxplot representation of the putative metabolite with an exact m/z 402.190. **b)** ESI-chromatogram of filtered ion mass 401.183, observing the peak at 4.57 min only in Fm plants.

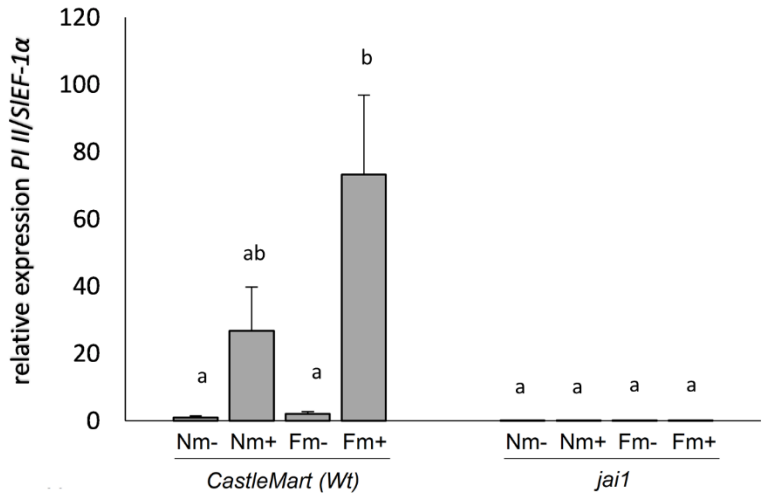


Fig. S4 Gene expression of proteinase inhibitor II (*PI-II*) in leaves of tomato wild-type (Castlemart) and JA-signaling mutant *jai1*, in nonmycorrhizal (Nm) or colonized by *F. mosseae* (Fm) conditions, and infested during 2 weeks by *S. exigua* (+) or in absence of stress (-). Data are expressed as mean ± SEM (n=3). Means not sharing a letter in common differ significantly according to the Fisher's LSD post hoc test ($P < 0.05$).

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GENERAL DISCUSSION

General Discussion

The purpose of this section is to integrate the results to providing an overview of the work done and describe the future perspectives of this line of research.

Beyond the nutritional improvement on the host plant, the protection granted by AM symbiosis to face different stress conditions is widely accepted. In this area, first studies mainly focused on reporting these benefits, so the underlying mechanisms remained mostly unknown. On the other hand, in those studies where these mechanisms were addressed, generally only both phytohormones and metabolites with a known defensive role were addressed through targeted studies.

Nowadays, the development of new holistic technologies of study (-omics) allow us to study from a general perspective the different alterations of the host plant that are underlying these benefits. In this sense, metabolomic is downstream of other -omics as transcriptomic and proteomic, and therefore, is closest to the diverse crucial biological functions for the bioprotection processes. Untargeted metabolomic approaches and specially LC-MS, are today a useful tool able to detect and identify thousands of metabolites, and how they are modulated by different growth conditions and during plants negative or positive interaction with other organisms.

In the present PhD Thesis we have shown the potential of different AMF to protect the host plant against stresses from different origins. Subsequently, we have analyzed the modulation of the metabolic response of mycorrhizal plants when facing those adverse conditions, with the aim of identifying biosynthetic pathways and specific metabolites boosted in AM plants that could explain their enhanced capacity to overcome stresses.

Functional diversity among AMF species

Evidences of functional diversity among different AMF species have been reported regarding their ability to protect the colonized-plant (Estrada *et al.*, 2013a; Estrada *et al.*, 2013b; He *et al.*, 2017). These differences have been found even among different isolates from the same AMF species specie (Roger *et al.*, 2013). Thus, as far as possible, it is

necessary to design experimental set up with distinct AMF in order to differentiate the distinct AMF in order to differentiate the general benefits of this symbioses and the underlying mechanisms, that could be considered as universal for these organisms, from those specific to a given AMF species. It is for this reason that we carried out tests with different AMF, in both nonstressful (**Chapter** 1, *F. mosseae* and *R. irregulare*) and under moderate and severe drought and salinity stress conditions (**Chapter 2**, *F. mosseae*, *R. irregulare* and *C. etunicatum*). *F. mosseae* and *R. irregulare* were selected because they are widely found in natural soils and are normally used as model AMF in experimental studies, which have reported valuable information. In addition, they are the most commonly used AMF to elaborate commercial inoculates. In the case of *C. etunicatum*, isolated from a high salinity area of southern Spain, previous studies from Estrada and collaborators have described how its colonization conferred higher protection to salinity to host plants compared with another AMF tested (Estrada *et al.*, 2013a; Estrada *et al.*, 2013b). However, there were no evidences of the possible metabolic reorganization upon stress and its possible role on the observed enhanced tolerance. In contrast, during biotic stress tests (Chapter 3) it was not possible to use distinct AMF species due to the methodology and experimental design, since the management of the insect and the monitoring of its performance makes experimental development more complex. Thus, we selected *F. mosseae* since previous experiments in our group showed higher benefits against biotic stress in tomato plants colonized by *F. mosseae* compared with *R. irregulare* in most cases (Pozo et al., 2002, Jung et al., Lidoy *et al*., unpublished). Moreover, this higher protection conferred by *F. mosseae* colonization has been reported in other studies including other plant species (He *et al.*, 2017; Pozo *et al.*, 2002).

For all these considerations, the design of commercial inoculants based on a consortia of AMF covering a wide range of beneficial properties is receiving special attention since they can complement each other in their provided advantages. This fact can be of outmost importance because in real farming conditions usually there is no single stress factors, but along the crop growing periods different stresses may affect the plant, sometimes even simultaneously.

However, the rational design of such consortia requires a deep basic knowledge on the mechanisms underlying mycorrhiza induced stress tolerance and the properties of individual AMF strains and their impact in the host plant. Therefore, we address the study

of the metabolic alterations in the host plant associated to the AM symbiosis in both control and stressful conditions.

Root metabolic reorganization by AM in the absence of stress:

AM symbiosis requires a high degree of coordination between both partners based on a finely regulated molecular dialogue that integrate complex symbiotic programs, comprising a transcriptomic and metabolic rearrangement in host roots (Genre and Bonfante, 2016; López-Ráez *et al.*, 2010; Pimprikar and Gutjahr, 2018; Schliemann *et al.*, 2008). In **Chapters 1** and **2**, we demonstrate that root-colonization by AMF entails two class of patterns in root metabolic reprogramming, i) common metabolic alterations for the AMF tested and ii) fungal specific modulation.

Among the common core of changes, we observed a negative impact of mycorrhizal symbiosis on amino acid content. This pattern could be explained for the upregulation of downstream metabolomic pathways. This is clear with the amino acids phenylalanine and tyrosine, main sources of the phenylpropanoids pathway, which presented many metabolites more accumulated in mycorrhizal plants (especially lignins and lignans). Conversely, those amino acids involved in nitrogen incorporation, as glutamate and aspartate, were found overaccumulated in AM roots, which can explain their described lower carbon/nitrogen ratio. Moreover, other groups of metabolites as several vitamins, carotenoids and gibberellin intermediates were also more accumulated by colonization for all the AMF.

Following with the common alterations in the root metabolome, the biosynthetic pathways of benzylisoquinolines, alkaloids and oxylipins, clearly related with defensive responses, were also higher in AM plants compared with nonmycorrhizal. Specifically, the oxylipins metabolic route is extremely important since, besides including multiple other bioactive molecules, it is in charge of jasmonic acid (JA) production through the 13-LOX pathway, which is thought to regulate AM establishment at different levels, such as controlling fungal spread within the roots and favoring arbuscule formation and functioning (Pozo *et al.*, 2015).

However as commented above, some specific metabolites were differently regulated by each AMF. The overview of the metabolic reprogramming provided by principal components analysis and heatmap plots showed how the more aggressive colonizer *R. irregularis* resulted in less alterations in the root metabolome than *F. mosseae*. These results agree with those obtained through targeted analysis by Fernández and collaborators (2014), since they found less changes in defense-related hormones triggered by *R. irregularis* than by *F. mosseae.* This stronger ability to colonize in *R. irregulare* together with the lower metabolic changes triggered in comparation with Fm, may suggest a lower control by the plant over this fungi. Several studies have reported the secretion by *R. irregulare* of effectors able to suppress host plant defensive mechanisms (Kloppholz *et al.*, 2011; Voß *et al.*, 2018). In view of the observed results, we hypothesize a greater potential of *R. irregulare* for the synthesis of efficient effectors, although studies on the effector repertoire from other AMF as *F. mosseae* are required to test such hypothesis.

The bigger impact of Fm in the plant metabolome, especially regarding defense related compounds and JA derivatives may underly the reported higher potential for biotic stress protection. As mentioned above, in both experiments from **Chapter 1** and **2**, methyl jasmonic acid (MeJA) was found accumulated to a higher level in Fm roots. MeJA is considered as an active signal in JA responses (Wasternack and Hause, 2013). Therefore, it could be interesting to deep in the relation between basal overaccumulation of this compounds with the highest ability of the mycorrhizal plant to overcome deleterious organisms susceptible to JA-related defenses, as necrotrophic pathogens or leaf chewing insects (Jung *et al.*, 2012). Thus, our results support the hypothesis that the degree of protection provided is influenced by the greater or lesser control exercised by the host plant.

addition, the AMF that conferred the highest protection against salt stress, *C. etunicatum*, also displayed the highest metabolic changes in tomato roots during nonstress conditions. It is noteworthy that this AMF was isolated from a very dry and saline area, so that it has likely evolve mechanisms to cope with such stress, and also that lead to the increase tolerance in the plant, since as obligate biotroph its own survival depends on the survival of the plant.

In summary, studying the modifications in the metabolome of AM plants during nonstress conditions is not only important to decipher the molecular mechanisms regulating the maintenance and functioning of the symbiosis, but it is also very useful since it allows us to decipher between this inherent basal modulation from that taking place in response to stress. In this sense, those metabolites whose accumulation are triggered by the symbiosis in adverse conditions to higher levels than in nonmycorrhizal plants, but were not modulated by the mycorrhiza in the absence of stress, are considered primed-compounds and good candidates in mediating the increased protection in AM plants.

AM establishment increases the phenotypic plasticity of the host plant improving its ability to overcome different stresses

Under drought and salinity stress conditions (Chapter 2), plants colonized with all the tested AMF showed better fitness than those nonmycorrhizal. Moreover, in general the benefits of the symbiosis were more pronounced under the most severe stress conditions, which support the hypothesis that the greater the challenge, the greater the benefits that these organisms can bring to their host plans. Although all AMF showed a similar pattern in promoting root and shoot biomass during drought, *C. etunicatum* clearly performed best promoting the growth in both tissues under salinity stress. This greater protection was also reflected in the fact that it was the only AMF that lowered the concentration of Na^+ ion (toxic in excessive amounts) accumulated in the aboveground tissues of the host.

Once known the metabolomic associated to the mycorrhizal status, and confirmed the effective protection against the described stresses, we carried out the study and identification of those potential compounds that may underly the enhance stress tolerance. For this purpose, we selected the moderate level of stress (75% of field capacity and 75 mM NaCl, for drought and salinity respectively) since after the severe treatments, plants presented great damage (especially those not colonized) that may result in artifacts, reflecting more changes related to tissue damage than those related to active responses to stress.

The results showed that while the reorganization of the metabolism by the symbiosis in response to salinity was clear, surprisingly, there were no significant changes in the case of drought. In this case, although the protection was evident in all mycorrhizal plants, we

could not identify any biosynthetic pathway or key metabolite mediating this increased tolerance. This result contrasts with the recent study published by Bernardo and collaborators (2019), where in two wheat cultivars colonized by *F. mosseae* they observed altered metabolic profiles in response to drought. However, this work lacks the proper controls, then missing the metabolomic changes under nonstressful conditions, so we do not know if these changes are caused because of the symbiosis establishment regardless the stress or if they are indeed induced by stress. Thus, our results suggest that the greater tolerance achieved in mycorrhizal plants upon drought stress is due to other mechanisms beyond the impact on the host secondary metabolism. Several mechanisms have been well documented as mediators of drought resistance in mycorrhizal plants, as the better efficiency in the uptake and use of water, for example through higher hydraulic conductivity an enhanced induction of aquaporins (Chitarra *et al.*, 2016; Quiroga *et al.*, 2018; Sánchez-Romera *et al.*, 2016).

On the contrary, salinity resulted in a strong metabolic reorganization in AM plants. Since all mycorrhizal plants showed some degree of protection, we focused on those compounds showing a stronger accumulation in all AMF treatments when compared with nonmycorrhizal plants, it should be noted that the most protective AMF *C. etunicatum* triggered specific metabolomic patterns both in the absence and induced after salinity. Although we did not delve into the possible role of these compounds as our main objective was to decipher common mechanisms for AM symbiosis, exploring the function of these compounds will be of great interest for follow up research projects.

Among the general cluster of metabolites showing a priming profile, we found a lignan-glycoside, two xanthones, flavanol catechin and its glucoside derivate, and evidence of upregulation of the B6 vitamers pathway. But this primed-accumulation profile does not explain by itself whether these compounds have an active role in salt stress alleviation. For such purpose, we selected metabolites represented in the primed-cluster of B6 vitamers and catechin because their protective role against abiotic stress have been previously suggested and their commercial availability (Shi *et al.*, 2002; Yiu *et al.*, 2011; Yiu *et al.*, 2012). Their exogenous application through watering clearly enhanced plant fitness under salt stress conditions, in terms of biomass, percentage of relative water content in leaves and photosystem II efficiency, confirming their role underlaying the enhanced host tolerance to overcome salinity stress.

Besides the effect of the AM symbiosis in roots, it is known that the symbiosis can have also a protective effect on aboveground tissues, as evidenced by systemic protection against some biotic stresses, for example reducing necrotrophic leaf pathogens and herbivory. In this thesis we explored the aboveground changes in the context of plant resistance to herbivory, using the generalist leaf chewing insect *Spodoptera exigua* as a model. As described above, we selected *F. mosseae* due to previous observations showing its strongest ability to protect the plant to different biotic stressors compared with another AMF (He *et al.*, 2017; Pozo *et al.*, 2002). In our experimental system Fm efficiently reduced *S. exigua* development and survival, confirming its ability to trigger MIR against this herbivorous insect. Then, we focused our analysis to explore the metabolic changes that may underlie such protective effect.

Modulation of the plant metabolome in aboveground tissues by the AM symbiosis is minimal during non-stress conditions

Less studied than in roots is the interaction that the mycorrhizal symbiosis may have on the metabolism of aboveground tissues in the host plant. In contrast to the high impact of the symbiosis in the root metabolome described in the previous chapters, we report in **Chapter 3** that, in our experimental conditions, only one of the 1132 signals registered showed altered accumulation in *F. mosseae* colonized plants. This metabolite coincides with the exact mass of a blumenol derivative, that has recently been reported as a potential marker of aerial part mycorrhization due to its consistent overaccumulation in different mycorrhizal plants (Wang *et al.*, 2018). This minimal modulation in the leaves metabolome is in agreement with the previous study performed by (Hill *et al.*, 2018). In other study, a more significant modulation was observed, being strongly dependent of the plant species, ranging the percentage of altered metabolites from only 1.7 % in *Poa annua* to 15% in Plantaginaceae species (Schweiger *et al.*, 2014). Conversely, in our experiment when biotic stress occurred, there was a clear reorganization of the metabolite profiles in response to stress. Therefore, this absence of basal changes reinforces the idea of arbuscular mycorrhizas as a low-cost defense, since it only involves metabolic changes when the situation requires it.

F. mosseae **colonization impinge on *S. exigua* fitness and modulates local metabolic defensive responses in damaged leaflets**

In order to evaluate the mycorrhiza induced resistance (MIR) in tomato plants in symbiosis with *F. mosseae* against a biotic stress, plants were infested with larvae from the common pest *S. exigua*. Host plant protection by mycorrhiza was confirmed, since larvae fed on *F. mosseae* colonized tomato plants showed a higher mortality and delayed development compared with those feeding on nonmycorrhizal plants. We hypothesized that the observed negative impact of *F. mosseae* colonization on the insect could be related to primed plant defense responses.

Under *S. exigua* herbivory mycorrhizal plants exhibited an increased expression of JA-responsive marker gene *PI-II*, which *codes for* the antifeedant proteinase inhibitor II, well documented to impair larval digestion. This fact supports the proposed model of JA-as a central regulator during MIR. Attending to the plant metabolome in aboveground tissues, we discover that the rearrangement associated to the plant response upon herbivory is clearly different locally, at the place of damage, than at the systemic level, in unwounded leaflets close to the damage ones. Thus, we analyzed separately the local and systemic response. This result agrees with those from *Catharanthus roseus* plants, where different transcriptomic and metabolomic responses to herbivory were found when comparing local damaged leaves with systemic ones (Dugé de Bernonville *et al.*, 2017). The local response to *S. exigua* herbivory resulted in altered accumulation of phenolics compounds, terpenoids, carbohydrates and flavonoids. Meanwhile, the systemic response altered these pathways too, but a higher alteration in the amino acids and alkaloids groups was observed.

Overview representations evidenced that only in those leaflets directly damaged by *S. exigua* the defensive metabolic rearrangement was modulated by *F. mosseae* establishment. This modulation mainly comprised three families of primed-compounds: alkaloids, fatty acid derivatives and phenylpropanoids-polyamine conjugates (PPCs). The toxic effect of alkaloids against herbivores has been widely reported (Mithöfer and Maffei, 2015). Among this group of compounds, we identified the metabolite cotinine (product of nicotine catabolism) and two acetylcholinesterase inhibitors: physostigmine (also known as eserine) and huperzine A. Regarding to fatty acids derivatives azelaic acid is highlighted, whose role regulating the priming of plant defenses has been already

proposed (Jung *et al.*, 2009), and 4-oxododecanoic. Regarding to PPCs, their role in anti-herbivore defenses has been reported (Kaur *et al.*, 2010). We selected one of the metabolites belonging to this group, feruloylputrescine (FP), to investigate in more detail both the hormonal regulation underlying its primed-regulation pattern and its possible effect on *S. exigua*.

Further research with pharmacological and genetic analysis using tomato mutants impaired in JA signaling allowed us to confirm that FP is positively regulated by JA, and that the overaccumulation in *F. mosseae* colonized plants was indeed JA-dependent. This is in agreement with the well stablished role of JA as the main phytohormone regulating plant responses against herbivores. As a next step, and considering the controversial information about the possible role of PPCs in plant defense against herbivory, we focused on unraveling whether FP may underly MIR in our model. We addressed a potential direct toxic effect of FP on the larval development, but we could not find any impairment on *S. exigua* larvae development after they fed on artificial diet supplemented with FP, suggesting that there is no direct toxic effect on the insect. This result agrees with the observations by Alamgir and collaborator (2016), as they did not find any alteration in two leaf chewing insects (S*podoptera Mauritia* and *Parnara guttate)* fed with similar FP supplemented meal. However, in other studies the deleterious effect on herbivores fitness is suggested (Bandoly *et al.*, 2016; Bandoly *et al.*, 2015; Kaur *et al.*, 2010). Thus, further research is needed to accept or totally discard the possible direct effect of PPCs on insect's development, as well as its indirect role mediating the synthesis of other toxic compounds.

Mycorrhizal protection against stresses: more than the sum of the parts

Altogether, the results obtained in the present Doctoral Thesis, raise a main conclusion: the greatest metabolic plasticity exhibited by mycorrhizal plants seems to underlie the protection conferred by the symbiosis. In addition, this greater plasticity cannot be assumed by a single group of compounds, but depending on the stress suffered the mycorrhizal symbiosis will have greater capacity to induce the synthesis of those that play a role in facing stress. In addition, it must be taken into account that other mechanisms can exert a positive action on the fitness of the plant, as in drought stress,

where the protection exhibited cannot be attributed to the modulation of the metabolism, but will be mediated by other mechanisms such as a better efficiency in water uptake.

The growth promotion effect of the AM symbiosis, although widely accepted, as one of the major advantages of the symbiosis, is not always evident. The effect on growth are strongly dependent in the plant genotype and the abiotic context, and sometimes symbiosis can even result in a lower growth of the host plant. Focusing on the better capacity to cope with stresses, some studies have suggested that this protection is determined exclusively by a best nutritional status of the host plant. However, several studies have shown otherwise. For this reason, it is useful not to observe significant effects on plant growth in mycorrhizal plants when addressing stress studies, since otherwise nutritional aspects may mask defensive aspects. Indeed, in our experiments there was no significant effect on biomass in the absence of stress, as seen in Chapters 1 and 3. Although in Chapter 2 there was a small growth promotion in the absence of stress, it was minimal compared to the effect observed under stressful conditions. Therefore, our research supports the role of the symbiosis as a "life insurance", that is, the benefits brought to the plant become more visible under stressful conditions. Thus, the use of AM inoculants should be reconsidered in those very controlled cultivation crops where the nutritional inputs are high may not have obvious benefits if no major problems arise, but will clearly be manifested if they undergo a stress phase, for example a drought period or they suffer a pathogen or pest attack.

Therefore, the increased tolerance and resistance derived from the establishment of the symbiosis is multifaceted, and will depend on the type of host plant, the stress the plant is facing, the colonizing fungus and the environment that surrounds them.

Future work

- During the abiotic stresses experiments, as our main objective was to decipher general metabolic mechanisms, the role of several metabolites with an interesting accumulation profile in interaction with specific AMF remained unexplored. Of special interest is *C. etunicatum*, that in our study provided the highest stress tolerance in the host plant. Compounds from porphyrin metabolic pathway were clearly

overaccumulated in plants colonized by this AMF under nonstress condition. This group have been previously related to drought tolerance (Phung *et al*., 2011), and among them, biliverdin is one of the most studied, with a reported role in lateral root formation (Balestrasse *et al*., 2005; Yen Hsu *et al.*, 2012). In any case, this group would be involved in a baseline tolerance to salinity, since stress does not induce accumulation. Even more interesting is the possible role of steroidal glycoalkaloids (SGAs), because their exclusive primed-profile under salinity stress in *C. etunicatum* makes us think that this induction can play an active role in abiotic stress alleviation. It is noteworthy that SGAs have been more studied for their antimicrobial and insecticidal properties (Chowanski *et al*., 2016), which also makes interesting to study the protective role of *C. etunicatum against* biotic stress in future experiments.

- In general, we have considered that the compounds found in mycorrhizal roots have, in a major part, a plant origin, but this is not necessarily true. For some metabolites, their accumulation in nonmycorrhizal roots is under the detection limit, while in AM roots are strongly accumulated, and there is no match with the experimental exact mass in the plant metabolic databases. Altogether, these facts suggest that they could have a fungal origin. Unfortunately, the databases on fungal metabolites are limited, and specially in the case of AMF that are extremely poorly studied at this level. Therefore, it would be interesting to perform metabolomic studies strictly on the mycelium, both extra and intraradical, to develop AMF databases that would allow us to differentiate in future trials their possible origin. It would also be very interesting to observe the response of these AMF tissues to different stresses and compare them between distinct species in order to evaluate potential functional diversity.

- Phytohormones are key molecules regulating almost every developmental processes and defense signaling responses in plants (Pozo *et al.*, 2015). The present work points out, in agreement with previous reports, the crucial role of JA during MIR. However, nowadays there are increasing evidences of the role of other phytohormones in the coordination of defensive responses against biotic stress. Specifically, abscisic acid (ABA) has been described as coregulator of the MYC2 branch of the JA signalin pathway, regulating the defensive response against phytophagous insects (Kazan and Manners, 2013). In addition, it has been described that ABA plays a key role in primed

JA-regulated responses against herbivory (Vos *et al.*, 2015). In this sense, preliminary results in our group showed that in response to *S. exigua* herbivory, mycorrhizal plants beside showing a primed JA accumulation, also trigger stronger accumulation of ABA (Rivero *et al*., unpublished results). Thus, it would be interesting to evaluate the role of ABA in MIR through the use of transgenic lines or altered mutants in both synthesis and signaling of this phytohormone. In this way, we will increase our understanding about the regulation that different phytohormones exert in the protection of arbuscular mycorrhizas against biotic stresses.

- Among all the metabolites found to have a primed accumulation in mycorrhizal plants in response to herbivory In Chapter 3, only the direct effect of FP was tested. Therefore, it would be interesting to carry out tests where the effect of the other members of the PPCs, alkaloids and fatty acid derivatives observed. In addition, to fully explore the potential of direct or regulatory function of this compounds, besides their application in artificial diet, it would be interesting to proceed to its application on the plant, under *in vivo* conditions. These experiments could provide us with information beyond its possible individual direct effect, allowing to test potential synergisms with other compounds, and if they were mediating the synthesis of other metabolic derivatives that would harm the herbivore development.

- Plants are continuously exposed to multiple stresses from different nature. In this context, phytohormonal crosstalk during these multi-attacker interactions is essential to coordinate and prioritize plant defenses and stress responses (Vos *et al.*, 2015). In addition, plant phytohormone signaling and subsequent response to one stress can affect how plant responds to another (Rejeb *et al.*, 2014). Thus, it would be interesting to explore whether mycorrhizal modulation of defensive response to a specific stress could also confer protection against different disturbances. For example, the induction of SGA in mycorrhizal plants under salt stress may be trigger increased resistance against biotic attackers. In this way, we ideally could achieve enhanced protection against a specific stress through the application of a controled pulse of another stress. For example, this would be effective to counteract the suckers as aphids are not generally damaged when they feed on mycorrhizal plants because due to their feeding they hardly trigger defensive responses in the plant, passing unnoticed to the plant.

- Besides the induction of direct defenses, several studies have addressed the positive effect of AM-establishment on higher trophic levels, where the major recruitment of pests natural enemies (predators and parasitoids) have been reported (Guerrieri *et al*., 2004; Hoffman *et al*., 2011; Schausberger *et al*., 2012). Thus, it would be necessary to apply metabolomic approaches to study whether herbivore induced volatile compounds (HIPVs) emission is modulated as consequence of AM-establishment. If so, it would be interesting to deepen the identification of volatiles showing a primed profile, in addition to performing functional tests of the attraction of natural enemies through Y-tube choice trials.

CONCLUSIONS

Conclusions

1. A well-established arbuscular mycorrhizal symbiosis involves a strong metabolism rearrangement in the host roots. A common core of upregulated metabolic pathways by *F. mosseae* and *R. irregulare* was observed mainly in defensive routes: phenylpropanoids (lignins, lignans, and phenylpropanoid-polyamine conjugates), benzylisoquinoline alkaloids and oxylipins.

2. Besides common "mycorrhizal" changes, specific fingerprints were also observed for each AMF, which may underly the functional diversity reported for these fungi.

3. All the AMF tested, *F. mosseae*, *R. irregulare* and *C. etunicatum* increased tomato tolerance to overcome drought and salinity stresses. The degree of protection positively correlated with the severity of the stress.

4. The strongest protective effect was conferred by *C. etunicatum*, a strain isolated from an extremely dry and salty soil. This result highlights the potential of extreme environments as a source for beneficial microbes as bioprotectors.

5. AM-establishment enhanced the plant metabolic plasticity to cope with salinity stress. This effect was more pronounced in *C. etunicatum*, pointing out the importance of the reorganization of the metabolism to overcome stress.

6. The flavonoid catechin and B6 vitamers are involved in the AM-increased tolerance against salt stress.

7. Root colonization by *F. mosseae* resulted in an enhanced mortality of the generalist leaf-chewer insect *S. exigua* when feeding in the leaves of those plants.

8. AM establishment modulated metabolic profiles in response to herbivory in damaged leaflets. This modulation consisted in a primed accumulation of fatty acids derivatives, alkaloids and phenylpropanoid-polyamine conjugates.

9. The jasmonic acid signaling pathway is required for the primed accumulation of the phenylpropanoid-polyamine conjugates feruloyl putrescine in *F. mosseae* colonized plants.

10. Untargeted metabolomic is a useful and robust technique that allows an overview of general metabolic modulation in mycorrhizal plants, as well as the identification of key metabolic pathways and compounds relevant for the AM-enhanced plant stress tolerance.

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