UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE,
BIOSCIENZE E TECNOLOGIE CHIRURGICHE

Curriculum Biologia Cellulare e Molecolare

XXX CICLO

# *Omyc analyzes reveal how plants respond to the symbiosis with the arbuscolar mycorrhizal fungi and symbiotic bacteria*

# *Analisi "omiche" mostrano come le piante rispondono alla simbiosi con i funghi micorrizici arbuscolari e con i batteri simbiotici*

Docente guida: Prof.ssa **Candida Vannini**

Tutor: Dott.ssa **Milena Marsoni**

Tesi di dottorato di:

**Francesca Ortolani**

Matr. 609184

Dip. Biotecnologie e Scienze della Vita – Università degli Studi dell'Insubria

Anno accademico 2016-2017

# Summary

Currently, there is an increasing body of evidence that the plant health depends on their tight associations with specialised soil microorganisms that have great effects on plant growth, protection and productivity. Among them, arbuscular mycorrhizal (AM) fungi (subphylum Glomeromycota, Schüßler et al., 2001) can establish a mutualistic association with most terrestrial plant species, including important agricultural and horticultural crops (Bonfante and Genre 2015). The AM symbiosis develops in roots, where the fungus colonizes the cortex supplying mineral nutrients to the plants in exchange of photosynthetic carbon compounds (Smith and Rweead, 2008). The exchanges between fungus and plant occur through specialized, branched and intracellular fungal structures called arbuscules (Bonfante and Anca 2009). Besides an improved mineral nutrition, plants colonized by AMF also receive other benefits, including an increased biomass, yield, and higher tolerance to biotic (pathogens) and abiotic (drought, salinity, heavy metals) stresses, leading to improved plant fitness (Gernns et al. 2001; Van der Heijden and Sanders 2002; Hildebrandt et al. 2007; Pozo and Azcón- Aguilar 2007; Aroca et al. 2008; Pozo et al., 2010; Lanfranco and Young 2012). Many plant roots also harbour a variety of soil beneficial bacteria that can benefit the plant by serving as plant-growth promoters. These bacteria, known as PGPR (plant-growth promoting rhizobacteria) can work as active rhizosphere components (Caballero-Mellado et al., 2007) or endophytic plant colonizers (Paungfoo-Lonhienne et al., 2014). The mechanisms that promote plant growth and development include: solubilization of minerals, nitrogen fixation, production of siderophores, plant growth regulators and organic acids, as well as protection by enzymes like chitinase, ACC-deaminase and glucanase (Berg, 2009; Glick et al., 2007; Hayat et al., 2010). These microorganisms can enhance biomass

production and tolerance of the plants to several soil conditions as salinity, drought and heavy metals (Baharlouei et al., 2011). However, knowledge on the interaction of PGPR with many plants of agronomic interest is still scarce (Castanheira et al., 2015). Among plants colonized by AMF and PGPR is the bread wheat (*Triticum aestivum*), one of the most important food supply and the third-most widely grown crop worldwide, after rice and corn. Despite its economic and social importance, the effects of wheat symbiosis with the above microbes, considered individually or together, have never been investigated, in contrast to many other crop plants. Thanks to their benefits, AMF and PGPR can be used as natural biofertilizers and bioprotectors in integrated strategies for a sustainable and healthy wheat production. Furthermore, some AMF may contain endobacteria in their cytoplasm. These bacteria have a reduced genome, which lacks some crucial metabolic pathways and reveals dependence on the host for nutrients and energy (Ghignone et al. 2012). In exchange, they give many benefits to fungal host, sustaining its presymbiotic growth (Lumini et al., 2007; Salvioli et al., 2015), increasing the success of AMF sporulation and mechanisms for ROS-detoxification, and eliciting AMF innate immune responses (Salvioli et al., 2015). The beneficial effects of mycorrhizae in the rhizosphere are the result of synergistic interactions among all rhizosphere microbes, which are crucial for plant growth (Linderman, 1992). Thus, the relationship between AMF, their associated bacteria and plants provides a very interesting example of a meta-organism (Bosch and McFall-Ngai, 2011) and may be of great importance for sustainable agriculture.

In view of the above observations, during this thesis work three aims were pursued.

The first was the characterization of the molecular responses of wheat roots and leaves in presence of AMF alone and with a leaf pathogen to test whether AM

fungi can be used as biofertilizer and bioprotector for enhancing plant growth and yield. We investigated the main pathways involved in enhancing plant biomass and mineral nutrition, and in promoting the bioprotective effect against a leaf pathogen. To address these issues, we combined phenotypic, metabolomic and molecular approaches, as detailed in Chapter 1.

The second aim was to analyze how proteome of wheat, in both roots and leaves, changes in response to colonisation by AMF and PGPR, considering single or double inocula. The purpose was to use these rhizosphere microbes in integrated strategies for a sustainable agriculture to improve plant health and yield. We wanted to achieve information about proteins that play pivotal roles in the molecular interactions of wheat with AMF and PGPR. In particular, this study has been set to obtain proteomic data providing a comprehensive picture of the intricate and yet mostly unknown cross-talk between wheat and AMF/PGPR. Methods and results of this study are reported in Chapter 2.

The third aim was to understand the effects of AMF endobacteria on both fungal and plant fitness. Thus, we analysed the proteomic profile of AMF spores with and without endobacteria, and after application of the synthetic strigolactone GR24 that, similarly to strigolactones produced by plant roots, is perceived by AM fungi, stimulating their energy metabolism and growth. The goal was to better explain with proteomics some morphological traits of the spore without bacteria. Moreover, we wanted to provide new insights into the molecular mechanisms mediating endosymbiosis and into how bacteria provide direct and/or indirect ecological benefits, not only for their fungal host, but also for the plant. A full account of this investigation is given in Chapter 3.

The research activities carried out during this thesis can be divided in the following three distinct, but interconnected parts.

In **Chapter 1**, we focused on the role of an AM fungus (*Funneliformis mosseae*) in the mineral nutrition of wheat, and on its potential protective effect against a leaf pathogen (*Xanthomonas translucens)*. To address these issues, phenotypical, metabolomic and molecular approaches have been combined. Several studies have shown that both model and agricultural plants colonized by AMF often display an increased mineral nutrition and biomass, and higher tolerance to biotic and abiotic stresses. Despite wheat being one of the major global crops, its response to AM symbiosis has been poorly investigated so far. In this study, morphological observations indicated that AM wheat plants displayed a growth effect, in terms of biomass and grain yield, as well as a reduction of the lesions produced by the pathogen. To elucidate the molecular mechanisms underlying the mycorrhizal phenotype, we investigated the local and systemic changes of transcripts and proteins in roots and leaves during the bipartite (wheat-AM fungus) and tripartite (wheat-AM fungus-pathogen) interaction. Transcriptomic and proteomic profiling identified the main pathways (nutrient transport, primary metabolism, defence mechanisms, hormone regulation) involved in enhancing plant biomass, mineral nutrient content and in promoting the bio-protective effect against the leaf pathogen. Interestingly, the pathways were differentially regulated depending on the plant organ/microbe relationship. Mineral and amino-acid contents in roots, leaves and seeds, and protein oxidation profiles in leaves supported the omics data, providing new insightin the mechanisms exerted by AM symbiosis to confer stronger productivity and higher resistance to *X. translucens* in wheat.

In **Chapter 2,** we studied the mechanisms behind PGPR (*Burkholderia graminis*) - wheat interactions and the synergic interaction between *B. graminis* and *F. mosseae* on plant, through a proteomic analysis of wheat roots and leaves. Thus, we investigated the proteome alterations triggered in wheat by the dual inoculation PGPR + AMF compared to the sum of the effects elicited by single

inocula. The main pathways identified concerned regulation of metabolic process, phytohormones, mineral transport and stress responses. In plants inoculated with *B. graminis*, the regulation of proteins involved in auxin pathways and the increase of N uptake efficiency may explain the observed root growth increase upon PGPR inoculation. Moreover, bacteria promoted the increase of several proteins involved in abiotic stress, in particular salt stress, and may contribute to improvement of the plant performance under stress conditions. Dual inoculation further led to the activation of many growth and defense-related proteins in roots and at systemic level. This result indicates that the dual inoculation in wheat enhances the biofertilizer and bioprotective effects of PGPR *B. graminis* and AMF *F. mossae* when co-inoculated. Phenotypic results also revealed that dual inoculation stimulates the growth of both roots and leaves of wheat plants with respect to the control.

Finally, in **Chapter 3,** we shed light on the interaction between AMF and their endosymbiont bacteria. RNA-seq analysis of the AMF *Gigaspora margarita* in the presence and absence of its endobacterium *Candidatus* Glomeribacter gigasporarum indicated that endobacteria are able to enhance fungal bioenergetic capacity. iTRAQ quantitative proteomics was used to identify differentially expressed proteins in *G. margarita* germinating spores with its endobacteria (B+), without endobacteria in the cured line (B-) and after application of the synthetic strigolactone GR24. Proteomic, transcriptomic and biochemical data identified several fungal and bacterial proteins involved in interspecies interactions. The greatest effects were on fungal primary metabolism and respiration, which was 50% higher in B+ than in B-. Quantification of carbonylated proteins indicated that the B- line had higher oxidative stress levels, which were also observed in two host plants. This study shows that endobacteria generate a complex interdomain network that affects AMF and fungal–plant interactions.

# Riassunto

Attualmente, c'è un crescente numero di prove secondo cui la salute delle piante dipende dalla loro stretta associazione con microrganismi specializzati del suolo che hanno grandi effetti sulla crescita, protezione e produttività delle piante. Tra questi, i funghi micorrizici arbuscolari (AMF) (subphylum Glomeromycota, Schüßler et al., 2001) hanno la capacità di stabilire un'associazione mutualistica con la maggior parte delle specie vegetali terrestri, tra cui importanti colture agricole e orticole (Bonfante e Genre 2015). La simbiosi micorrizica si instaura a livello delle radici, dove il fungo colonizza la corteccia e fornisce sostanze minerali alla pianta in cambio di composti carboniosi fotosintetici (Smith e Rweead, 2008). Gli scambi tra fungo e pianta avvengono attraverso particolari strutture fungine, intracellulari e ramificate, denominate arbuscoli (Bonfante e Anca, 2009). Oltre ad avere un migliore apporto di nutrienti minerali, le piante colonizzate dai funghi AMF ricevono anche altri vantaggi, tra cui un aumento della biomassa e della resa, e una maggiore tolleranza agli stress biotici (patogeni) e abiotici (siccità, salinità e metalli pesanti nel terreno). Tutto ciò porta ad un miglioramento del benessere delle piante (Gernns et al. 2001; Van der Heijden and Sanders 2002; Hildebrandt et al. 2007; Pozo and Azcón-Aguilar 2007; Aroca et al. 2008; Pozo et al., 2010; Lanfranco and Young 2012). Le radici di molte piante ospitano anche una grande varietà di batteri benefici del suolo che possono fornire diversi vantaggi alla pianta, agendo da promotori della crescita. Questi batteri, noti come PGPR (plant- growth promoting rhizobacteria) possono essere componenti attivi della rizosfera (Caballero-Mellado et al., 2007) o colonizzatori endofitici (Paungfoo-Lonhienne et al., 2014). I meccanismi che promuovono la crescita e lo sviluppo delle piante includono: solubilizzazione dei minerali, fissazione dell'azoto, produzione di siderofori, di regolatori di crescita delle piante e acidi organici, nonché protezione da enzimi come la chitina, l'ACC-

deaminasi e la glucanasi (Berg, 2009, Glick et al. 2007; Hayat et al., 2010). Questi microrganismi possono migliorare la produzione di biomassa e la tolleranza delle piante a diverse condizioni del suolo come salinità, siccità e metalli pesanti (Baharlouei et al., 2011). Tuttavia, le conoscenze sull'interazione dei PGPR con molte piante di interesse agronomico sono ancora scarse (Castanheira et al., 2015). Tra le piante colonizzate da AMF e PGPR c'è il frumento tenero (*Triticum aestivum*), una delle più importanti fonti di cibo e la terza coltura più diffusa al mondo, dopo riso e mais. Nonostante l'importanza economica e sociale del frumento, gli effetti della sua simbiosi con questi microrganismi, considerati singolarmente o insieme, non sono mai stati studiati, a differenza di molte altre piante. Grazie ai loro vantaggi, AMF e PGPR possono essere utilizzati come biofertilizzanti e bioprotettori naturali in strategie agricole integrate per una produzione di frumento sostenibile e sana. Alcuni AMF possono contenere endobatteri nel loro citoplasma. Questi batteri hanno un genoma ridotto, che manca di alcune vie metaboliche fondamentali e rivela la dipendenza dall'ospite per quanto riguarda l'apporto di nutrienti ed energia (Ghignone et al., 2012). In cambio, gli endobatteri forniscono molti vantaggi all'ospite fungino, sostenendo la sua crescita presimbiotica (Lumini et al., 2007, Salvioli et al., 2015), aumentandone il successo di sporulazione ed i meccanismi per la disintossicazione dalle ROS e suscitando risposte immunitarie innate (Salvioli et al., 2015). Gli effetti benefici delle micorrize possono quindi essere il risultato di interazioni sinergiche tra diversi microbi della rizosfera, che risultano fondamentali per la crescita vegetale (Linderman, 1992). Pertanto, la relazione tra AMF, i batteri a loro associati e le piante fornisce un esempio molto interessante di meta-organismo (Bosch e McFall-Ngai, 2011) e possono essere di grande importanza per l'agricoltura sostenibile.

Il primo obiettivo della tesi è stato quello di caratterizzare le risposte molecolari delle radici e delle foglie di frumento in presenza di AMF da solo e con un patogeno fogliare, per verificare se i funghi AM possono essere utilizzati come biofertilizzanti e bioprotettori per aumentare la crescita e la resa delle piante. Abbiamo indagato i principali processi coinvolti nell'aumento della biomassa vegetale, nella nutrizione minerale e nella stimolazione di effetti bioprotettivi nei confronti di un patogeno fogliare. Per affrontare questi argomenti abbiamo combinato approcci fenotipici, metabolomici e molecolari, come descritto nel Capitolo 1.

Il secondo obiettivo è stato quello di analizzare il proteoma del frumento, sia in radici che in foglie, in seguito alla colonizzazione da parte di AMF e PGPR, considerando l'inoculo singolo o doppio. Lo scopo era quello di utilizzarli nell'agricoltura sostenibile per migliorare la salute delle piante e la loro produttività. Abbiamo cercato informazioni sulle proteine che svolgono ruoli chiave nelle interazioni molecolari del frumento con AMF e PGPR. In particolare, questo studio prevedeva di ottenere dati proteomici per fornire un'immagine completa dell'intricato intreccio tra frumento e AMF/PGPR, non ancora descritto in letteratura in modo chiaro. Metodi e risultati di questo studio sono riportati nel Capitolo 2.

Il terzo obiettivo è stato capire l'effetto degli endobatteri sulla fisiologia dei funghi e delle piante. Pertanto, abbiamo analizzato il profilo proteomico delle spore AMF con e senza endobatterio e dopo l'applicazione di GR24, uno strigolattone sintetico che, similmente agli strigolattoni prodotti dalle radici delle piante, è percepito dai funghi AM e ne stimola il metabolismo energetico e la crescita. L'obiettivo era spiegare con la proteomica alcuni tratti morfologici della spora fungina senza batteri. Inoltre, abbiamo voluto fornire nuovi approfondimenti sui meccanismi molecolari che mediano l'endosimbiosi e su come i batteri forniscono

vantaggi ecologici diretti e/o indiretti, non solo per il loro ospite fungino ma anche per la pianta. Un resoconto completo di questa indagine è riportato nel Capitolo 3.

Nel **Capitolo 1** ci siamo concentrati sul ruolo del fungo AM *Funneliformis mosseae* nell'apporto nutrizionale del frumento e sul suo potenziale effetto protettivo contro un patogeno delle foglie (*Xanthomonas translucens*). Per affrontare questi argomenti, sono stati combinati approcci fenotipici, metabolomici e molecolari. Diversi studi hanno dimostrato che le coltivazioni agricole colonizzate da AMF mostrano spesso un aumentato apporto minerale e biomassa e una maggiore tolleranza agli stress biotiche e abiotiche. Nonostante il frumento sia uno delle principali coltivazioni a diffusione globale, la sua risposta alla simbiosi con funghi AM è stata ben poco indagata finora. In questo studio, osservazioni morfologiche hanno evidenziato che le piante micorrizate mostrano un aumento della crescita, in termini di biomassa e resa della granella, nonché una riduzione delle lesioni prodotte dal patogeno. Per chiarire i meccanismi molecolari sottostanti al fenotipo micorrizico, abbiamo studiato i cambiamenti di trascritti e proteine in radici e foglie, durante l'interazione doppia (frumento - fungo AM) e tripartita (frumento - fungo AM - patogeno). I profili di trascrittomica e proteomica hanno individuato i principali processi (trasporto dei nutrienti, metabolismo primario, meccanismi di difesa, regolazione ormonale) coinvolti nel miglioramento della biomassa vegetale, nell'assunzione delle sostanze nutritive minerali e nella promozione dell'effetto bioprotettivo contro un patogeno fogliare. È interessante notare che i processi sono regolati in modo differenziato a seconda delle relazioni tra organo vegetale e microorganismo. Il contenuto di minerali e aminoacidi nelle radici, nelle foglie e nei semi e i profili di ossidazione delle proteine nelle foglie hanno supportato i dati omici, fornendo una nuova visione dei meccanismi esercitati dalla simbiosi micorrizica per

conferire una maggiore produttività e una maggiore resistenza a *X. translucens* nel frumento.

Nel **Capitolo 2** abbiamo indagato i meccanismi che sottostanno alle interazioni tra PGPR (*Burkholderia graminis*) e frumento e l'interazione sinergica tra *B. graminis* e *F. mosseae* nei confronti della pianta, attraverso un'analisi proteomica delle radici e delle foglie di frumento. Pertanto, abbiamo studiato le alterazioni proteomiche attivate nel frumento dalla doppia inoculazione PGPR + AMF rispetto alla somma degli effetti prodotti dai singoli inoculi. I principali processi identificati riguardavano la regolazione del processo metabolico, i fitoormoni, il trasporto minerale e le risposte allo stress. Nelle piante inoculate con *Burkholderia*, la regolazione delle proteine coinvolte nel metabolismo dell'auxina e l'aumento dell'efficienza di assorbimento dell'N possono spiegare l'incremento della crescita radicale osservato. Inoltre, i batteri promuovono l'aumento di diverse proteine coinvolte nello stress abiotico, in particolare lo stress al sale, e possono contribuire a migliorare le prestazioni delle piante in condizioni di stress. L'inoculazione doppia porta ulteriormente all'attivazione di molte proteine legate alla crescita e alla difesa nelle radici e a livello sistemico. Questo risultato indica che l'inoculazione doppia nel frumento aumenta l'effetto biofertilizzante e protettivo del PGPR *B. graminis* e dell'AMF *F. mossae* quando sono co-inoculati. Anche i risultati fenotipici hanno rivelato che l'inoculazione doppia stimola la crescita di radici e foglie di piante di frumento rispetto al controllo.

Nel **Capitolo 3** abbiamo voluto approfondire gli effetti della presenza di un endobatterio sulla fisiologia dei funghi AMF. L'analisi RNA-seq dell'AMF *Gigaspora margarita* in presenza ed assenza del suo endobatterio *Candidatus* Glomeribacter gigasporarum ha indicato che l'endobatterio è in grado di aumentare la capacità bioenergetica fungina. La proteomica quantitativa fatta con iTRAQ (isobaric tags for relative and absolute quantification) è stata utilizzata per

identificare le proteine espresse differenzialmente nelle spore germinanti di *G. margarita* con gli endobatteri (B +), senza endobatteri nella linea trattata (B-) e dopo l'applicazione dello strigolattone sintetico GR24. I dati proteomici, trascrizionali e biochimici hanno identificato diverse proteine fungine e batteriche coinvolte nelle interazioni interspecifiche. I maggiori effetti si sono osservati sul metabolismo primario del fungo e la respirazione, che è stata superiore del 50% in B + rispetto a B-. La quantificazione delle proteine carbonilate ha indicato che le spore B- presentavano livelli di stress ossidativi più elevati. Questo studio dimostra che gli endobatteri generano un complesso intreccio tra diversi domini che riguarda gli AMF e le interazioni tra le piante e i funghi.

Summary……2

Riassunto……7

General Introduction……..14

*1. AGRICULTURE*……14

*2. ARBUSCULAR MYCORRHIZAL FUNGI*……15

*3. PLANT - AMF INTERACTIONS*……17

*4. AMF - PLANT GROWTH PROMOTING RHIZOBACTERIA INTERACTIONS*……20

*5. AMF - ENDOBACTERIA INTERACTIONS*……22

Aim of the thesis……23

References……26

Chapter 1……37

Chapter 2……101

Chapter 3……138

# General Introduction

## *1. AGRICULTURE*

Between 1960 and the first decade of 2000, agricultural production more than tripled due to Green Revolution technologies and a significant expansion in land exploitation, intensive use of water and chemical inputs in crop production (Pingali, 2012). The negative side effects include land degradation, salinisation of cultivated areas, over-extraction of groundwater, build-up of pest resistance and limitation of biodiversity, emission of greenhouse gases, and soil and water pollution (Horlings et Marsden, 2011). A major concern is whether agriculture will be capable of meeting the needs of a global population that is projected to reach more than 9 billion by mid- century, while incorporating a more sustainable use of the world's natural resources. Under these conditions, improving crop productivity calls for innovative solutions.

In this context, of paramount interest is the complex network of interactions among plants and beneficial soil microorganisms that produce positive outcomes in plant production. Many rhizosphere microorganisms are plant growth promoters and/or biocontrol agents and interact with plants, increasing the uptake of water and nutrients from the soil, stimulating phytohormones metabolism, establishing competition with soil-borne pathogens, activating plant defence mechanisms and inducing a systemic response to pathogen attack (Andrews et al., 2010). The plant microbiota can be exploited as a natural bio-fertiliser and bio-protector in agronomic environments, to enhance crop yield and quality in environmentally sustainable agricultural practices. Among the beneficial organisms, arbuscular mycorrhizal fungi are an important group of soil microbes

able to influence plant metabolism (Rillig, 2004; Smith and Read, 2008; van der Heijden et al., 2008).

## *2. ARBUSCULAR MYCORRHIZAL FUNGI*

Arbuscular mycorrhizal fungi (AMF) are members of the subphylum *Glomeromycota* (Schüßler et al., 2001) and occur in soils of nearly all terrestrial ecosystems. AMF and their host plants form a mutualistic association, called mycorrhiza, in which both organisms have an advantage. It is probably the oldest and most widespread plant symbiosis in nature; indeed, phylogenetic evidence and fossil records date its existence back more than 450 million years (Smith and Read, 2008). Fungi colonise the roots of about 80% of all plant species, including important agricultural crops (Harrison, 2005). It has recently been suggested that, in natural environments, a non-mycorrhizal condition should be viewed as abnormal, although there is a marked diversity among AM fungal communities belowground, depending on plant species, soil type and season, or a combination of these factors (Smith and Smith, 2012).

AM fungi colonize the root cortex by developing intercellular hyphae and extensive intracellular branched hyphae (arbuscules), where the exchange of nutrients between the partners takes place. The fungus also forms massive networks of subterranean extra- radical hyphae, which form the mycelium and can explore extensive soil volumes inaccessible to plant roots. Moreover, being much thinner than roots, hyphae are able to penetrate smaller pores (Allen, 2011).

In the symbiosis, the plant provides the fungus with organic carbon derived from photosynthesis. AMF are obligate biotrophic symbionts relying on their host plant to proliferate and survive. In exchange, AM fungi improve the supply of water and most mineral elements, especially phosphorus (P) and nitrogen (N), from the

soil and transfer these to the root cells of the plant (Bonfante and Genre, 2010). Furthermore, they significantly facilitate the uptake of nutrients present in forms unavailable to plants, such as N present in organic compounds. Plants also receive other benefits from the AMF interaction, including increased tolerance to drought, heavy metal toxicity, pollutants, salinity and other abiotic stresses (Miransari, 2010, Smith et al., 2010, Meier et al., 2015).

More recently, protective effects of AM fungi against pathogens, pests and parasitic plants have been described for many plant species, including agriculturally important crop varieties (Jung et al., 2012). Reports of enhanced resistance of mycorrhizal plants to a wide range of belowground attackers have long been known. For example, it is reported that AM symbiosis alleviates damage caused by soil-borne pathogens such as the fungi *Rhizoctonia*, *Fusarium*, and *Verticillium spp*., oomycetes, including *Phytophthora*, *Pythium*, and *Alphanomyces spp*. (Whipps, 2004), and parasitic nematodes such as *Pratylenchus* and *Meloidogyne spp*. (de la Peña et al., 2006; Li et al., 2006; Vos et al., 2011). By contrast, induced resistance against shoot pathogens has been reported only in the last decade and the outcome of the interaction is variable, relying on the lifestyle and challenge strategy of the attacker (Pozo and Azcón-Aguilar, 2007; Koricheva et al., 2009; Campos-Soriano et al., 2012). An example of resistance to shoot pathogens is the enhanced resistance to *Xanthomonas campestris* induced in *Medicago truncatula* (Pozo and Azcón-Aguilar, 2007).

Several studies propose that mycorrhizal colonisation leads to a preconditioning of the plant, allowing a more effective activation of defence mechanisms in response to subsequent pathogen attack (Jung et al., 2012). This phenomenon is known as *priming* (Beckers and Conrath, 2007; Pozo and Azcón-Aguilar, 2007; Mauch-Mani et al., 2017), and because of the priming condition, the plant is

placed in a state of “alert” in which the response to an attack is faster and/or stronger (Jung et al., 2012).

Priming is not localised to the colonised root area, but occurs in the whole root system and even in the aerial parts of the host (Benhamou et al., 1994; Cordier et al., 1998; Li et al., 2006; Pozo et al., 2002; Yao et al., 2003; Mauch-Mani et al., 2017). Many efforts are aimed at unravelling plant-AMF interactions at the molecular level, but results are not yet conclusive, and especially for priming mechanisms, they are conflicting (Pozo and Azcón-Aguilar, 2007).

## *3. PLANT - AMF INTERACTIONS*

The establishment of a beneficial association requires mutual recognition and substantial coordination of plant and AMF responses (Zamioudis and Pieterse, 2012); this is the reason why rhizosphere microbiomes typically differ between plant species (Bisseling et al., 2009).

When a seed starts to germinate, large amounts of biological compounds, such as sugars, amino acids, vitamins and organic acids, are excreted into the surrounding environment, attracting a large population of microorganisms. As the seedling grows, roots and microorganisms communicate and interact with each other through the release of diffusible factors, such as strigolactones (SLs), Myc factors, auxin-like and other molecules, that are perceived by both partners (Igiehon and Babalola, 2017). For example, SLs are a group of carotenoid-derived signalling molecules, exuded by the plant roots, that promote AM hyphal branching and mycorrhiza establishment (Bouwmeester et al., 2007). Once the symbiosis is formed, SL production in the host plant decreases (Lòpez-Ràez et al., 2011).

As soon as direct contact between the symbionts takes place, AMF attach to the plant roots by forming the hyphopodium, thereby enabling colonisation of the root

(Cavagnaro et al., 2001; Dickson, 2004; Dickson et al., 2007). Inside root cells, the arbuscular structures of the fungus are completely enveloped by the plant plasma membrane, the so-called perisymbiotic or periarbuscular membrane, and thus remain within the apoplast of the plant cell, without direct contact with the symplast (Smith and Read, 2008). Although the periarbuscular membrane is a continuation of the plant plasma membrane, it is highly differentiated and equipped with symbiosis-specific transporters (Pumplin and Harrison, 2009). These transporters are specific for inorganic phosphate, ammonium and sulphur, and evidence for their role in the transfer of mineral nutrients between fungus and host has been obtained in studies on several plant species (Casieri et al., 2013). For example, three phosphate transporter genes in tomato (*LePT3*, *LePT4* and *LePT5*) are up-regulated in AM-colonised roots (Nagy et al., 2005). Since these specific transporters are expressed only during mycorrhization, they may be used as clear markers of that process.

The plant-fungal interface is separated by the periarbuscular space, an apoplastic region framed by the periarbuscular membrane of the plant and a specialised membrane of the fungus. This organisation of two facing plasma membranes is important with respect to the symbiotic transfer of nutrients to both partners (Smith and Smith, 2011).

Many findings suggest that beneficial fungi, sharing some similarities with biotrophic pathogens, are initially recognised as potential invaders and the plant immune response is triggered by perception of microbial molecules, known as microbe-associated molecular patterns (MAMPs), present both in pathogenic and beneficial microbes. In particular, when the specific receptors (pattern-recognition receptors; PRRs) in the host recognise MAMPs, a signalling cascade regulated by salicylic acid (SA) is initiated, resulting in MAMP-triggered immunity (MTI) (Zamioudis and Pieterse, 2011).

During the later stages of the interaction, the symbionts are able to cope with plant defence responses by repressing SA-regulated defences and activating a symbiotic program regulated by jasmonates (JAs); this allows successful colonisation of host roots (Pozo and Azcón-Aguilar, 2007). The mycorrhiza establishment has a profound impact on plant physiology, with important changes in plant primary and secondary metabolism (Hause and Fester, 2005). Moreover, in order to prevent excessive colonisation and carbon drainage, maintaining the interaction at mutualistic levels (Breuillin et al., 2010), the plant has to regulate fungal proliferation within the roots. This phenomenon is known as auto-regulation of the symbiosis (Vierheiling et al., 2008).

AMF colonisation also activates the plant's immune responses, through accumulation of defence-related regulatory molecules, such as transcription factors and MAP kinases (Beckers et al., 2009), which, in turn, play a key role in the regulation of JA responses. Indeed, many studies confirm the importance of the JA signalling pathway for mycorrhiza-induced resistance (MIR), leading to the activation of defence gene expression in local and systemic tissues (Jung et al., 2012). In mycorrhizal roots, there is evidence for the accumulation of other important defensive plant compounds, such as reactive oxygen species, specific isoforms of enzymes such as chitinases and glucanases, and activation of phenylpropanoid metabolism. This modulation of host defence, known as priming, may result in a preconditioning of the plant, a kind of sensitisation of the tissue, which allows a more effective activation of host responses to pathogen attack (Mauch-Mani et al., 2017) and influences plant interactions with other organisms. Primed responses are not restricted to AMF-colonised areas, but occur in the whole root system and even in the shoots. For example, mycorrhized tomato plants are systemically protected against *Phytophthora parasitica* infection, forming papilla-like structures around sites of pathogen infection, preventing the pathogen from spreading further (Pozo and Azcón-Aguilar, 2007). Priming

condition can be maintained throughout the plant's life cycle and can even be transmitted to subsequent generations (Mauch-Mani et al., 2017).

Several studies propose that the mechanisms involved in the bioprotective role exerted by AM fungi are not exclusively dependent on improved mineral nutrition (Liu et al., 2007), but result from the combination of multiple mechanisms that may operate simultaneously (Jung et al., 2012). Indeed, the AM association brings about competition for colonisation sites and photosynthates, changes in the root system and in rhizosphere microbial populations, alterations in the metabolic profile of the root and activation of plant defence mechanisms (Vos et al., 2013).

## *4. AMF - PLANT GROWTH PROMOTING RHIZOBACTERIA INTERACTIONS*

Many rhizosphere bacteria create dynamic and complex interactions with plant roots and other soil microorganisms. Both *in vitro* and field experiments indicate that some rhizobacteria have plant growth promoting effects on inoculated plants, with yield increases of 5% to 100% depending on the study conditions, plant species, bacterial strain and soil environmental conditions (Massena Reis et al., 2011). These effects are due to rhizobacteria ability to provide plants with utilisable nitrogen through fixation of atmospheric N and to make free phosphate available from insoluble P sources (Marella, 2014). These plant growth promoting rhizobacteria (PGPR) increase plant energy metabolism, production of growth regulators (as auxin, cytokinin and gibberellin) and promote amino acids and nucleotides synthesis. These processes, in addition to the increase of cell division and extensin synthesis, could explain root growth stimulation. They also enhance plant tolerance of abiotic stress, improving tolerance to heavy metal toxicity,

salinity and drought, and decreasing unfavourable effects of ethylene synthesis through their ACC deaminase activity (Hayat et al., 2010).

In addition, PGPR indirectly stimulate plant growth by competing with pathogens for space at the root surface, and by producing phytohormones, siderophores and exopolysaccharides, which could induce improved resistance to biotic stress (Grobelak et al., 2015). Siderophores in particular are molecules that chelate iron with great affinity, making it available to the plant root and unavailable to pathogens (Grobelak et al., 2015). PGPR also produce metabolites with antibiotic and/or antifungal properties, and protective effects have been reported against various phytopathogens including fungi (e.g., *Fusarium* head blight) and bacteria (e.g., *Xanthomonas translucens*) (Huang and Wong, 1998; Glick, 2005).

The presence of mycorrhiza influences plant-microbe interactions, as a result of a combination of diverse mechanisms. AM fungi compete for colonisation sites and photosynthates with other soil microbes and may lead to a shift in the microbial community of the rhizosphere, favouring certain beneficial microorganisms with the capacity to antagonise root pathogens (Badri and Vivanco, 2009). Moreover, they enhance the bacterial activity responsible for N fixing and P solubilisation (Linderman, 1992). On the other side, PGPR can enhance the rate of root exudation, stimulating hyphal growth and facilitating root penetration by the AM fungus (Jeffries et al., 2003). This facilitates the colonisation of plant roots by AMF (Hildebrandt et al., 2002; Jaderlund et al., 2008) and enhances growth of the fungal mycelium (Marulanda et al., 2006).

Synergistic interaction between these microbes not only enhances benefits to each other, but also promotes plant growth and health (Artursson et al., 2006; Yusran et al., 2009) by regulating nutritional and hormonal balance, improving water uptake, solubilising nutrients, producing plant growth regulators and inducing resistance against plant pathogens and abiotic stresses (Nadeem et al., 2013).

Moreover, co-inoculation with both PGPR and AMF increases plant antioxidant activities, an important means of protecting plant cells from the deleterious effects of reactive oxygen species (Ordookhani et al., 2010). For example, co-inoculation with *Pseudomonas fluorescens* and *Glomus mosseae* BEG12 increased growth of tomato plants (Egberongbe et al., 2010). However, the effects of these interactions depend upon the nature of the microbial strains involved, as well as plant species (Lau and Lennon, 2011).

## *5. AMF - ENDOBACTERIA INTERACTIONS*

Some AMF may host in their cytoplasm or vacuoles several gram-negative and rod- shaped bacteria, with an approximate size of 0.8–1.2 × 1.5–2.0 μm, as obligate symbionts. The first observations of these microorganisms date back to the 1970s, when many reports noted the presence of bacterium-like objects inside endomycorrhizal fungi (MacDonald and Chandler, 1981). Symbiotic relationships between fungi and endobacteria appear to be stable and have been evolutionarily maintained for 400 million years (Mondo et al., 2012). These bacteria are vertically transmitted through fungal generations and have a reduced genome, lacking crucial metabolic pathways and revealing dependence on the host for nutrients and energy (Ghignone et al., 2012). Indeed, the bacteria cannot be cultured outside of their host and this is the main obstacle to their study (Bonfante and Anca, 2009).

The endobacteria are important for optimal development of the fungal host: they sustain presymbiotic fungal growth, such as hyphal elongation and branching (Lumini et al., 2007; Salvioli et al., 2015). Furthermore, they increase the success of fungal sporulation, enhance mechanisms for detoxification of reactive oxygen species and enhance ATP production, thereby raising fungal bioenergetic

capacity. Then the bacteria provide AMF with additional tools with which to tackle environmental stresses, probably eliciting AMF innate immune responses (Salvioli et al., 2015).

AMF also benefit from the production of bacterial metabolites that are used directly by the fungus. These include organic acids, volatile compounds (ethylene) and non-volatile compounds (Horii and Ishii, 2006). From this point of view, mycorrhizal symbiosis is an interdomain network, in which organisms of three different kingdoms associate to produce a beneficial interaction. Endobacteria demonstrate the tripartite nature of mycorrhizas, and by providing benefits for fungal fitness (Bonfante and Anca, 2009), can exert an indirect positive influence on the plant host (Cruz and Ishii, 2011).

# Aim of the thesis

The overarching aim of this thesis was to obtain a better understanding of plant molecular responsiveness to AMF.

In particular, the following working hypotheses were addressed:

1. AMF root colonisation induces systemic changes in plants

2. AMF are able to induce bioprotective effects against a leaf pathogen

3. the synergy between AMF and PGPR enhances plant growth and its ability to deal with stress

4. endobacteria associated with AMF have an active role as a third component of the symbiosis.

In Chapter 1, the main goal was to improve knowledge of the molecular interactions between wheat (*Triticum aestivum* cv. Chinese Spring) and AMF

(*Funneliformis mosseae*) and to define plant responsiveness to the AM symbiosis in both roots and leaves (hypothesis 1). The main pathways involved in enhancing mineral nutrient uptake and plant biomass were examined, since AMF improve plant fitness. This involved exploring the plant growth effect exerted by AMF under both greenhouse and controlled conditions, combining phenotypical and molecular approaches. Using the same conditions and approaches, the ability of AMF to induce a bioprotective effect against pathogens was studied. This was achieved by evaluating the impact of the AM symbiosis on infection by *Xanthomonas translucens*, which is a specific pathogen of wheat leaves (hypothesis 2). A comprehensive functional overview of both local and systemic changes in roots and leaves during the mycorrhizal combination (plant and AMF), as well as during the tripartite interaction (plant, AMF and pathogen), was provided by integrating transcriptomic (RNA-seq) and proteomic (LC-MS/MS) analysis.

The other purpose of this thesis (Chapter 2) was to better clarify the synergy between AMF (*F. mosseae*) and PGPR (*Burkholderia graminis*) and their role in producing positive effects on wheat (*T. aestivum* cv. Chinese Spring) growth and protection (hypothesis 3). Proteomic analysis (LC-MS/MS) was carried out to probe wheat molecular responses and proteome changes induced by the double inoculation, both at local and systemic levels. In addition, a comparison was made of the alterations triggered by the dual inoculation and the sum of the effects elicited by single inoculation. Wheat plants, grown in glasshouse conditions, were single- or double-inoculated with AMF or/and PGPR, whereas control plants were not inoculated. Physiological and proteomic responses were then compared after 50 days later. Investigations focused on pathways involved in biomass production and synthesis of phytohormones and enzymes that modulate plant regulatory mechanisms.

Since AMF can harbour endobacteria in their cytoplasm, the aim of Chapter 3 was to supply new insights into the molecular mechanisms mediating endosymbiosis and the ecological benefits provided by the endobacterium to its fungal host and also to the plant. The host plant *Lotus japonicus*, the AMF *Gigaspora margarita* and its endobacterium *Candidatus* Glomeribacter gigasporarum, were used because they are considered as model organisms for studies of this type of tripartite interaction and some experimental evidence has already been obtained using this system. Both RNA-seq analysis and a gel-free proteomic approach (iTRAQ) were used to identify the transcriptional and proteomic profile of AMF spores, with (control sample) or without (cured line) endobacteria, and after application of the synthetic strigolactone GR24 (hypothesis 4).

In Chapters 1 and 2, the same experimental system based on *T. aestivum* as plant model and *F. mosseae* as AM fungus was used, because, even though wheat is one of the most widely grown crops worldwide, its response to AM symbiosis has been poorly investigated. *F. mosseae* was chosen since it showed a better frequency and intensity of mycorrhization in wheat root than another AMF, such as *Rhizophagus intraradices*.

In Chapter 3, *L. japonicus* was used as the host plant because of its small genome size, short life cycle of about 2 to 3 months and perennial nature, making this plant favorable to the study. This part of the work was focused on benefits of the tripartite association, regardless of host plant type. *G. margarita* was chosen as the AMF since it is considered a model organism for the study of the interactions between AMF and endobacteria.

## References

**Andrews M., Hodge S., Raven J.A. (2010**) Positive plant microbial interactions *Annals of Applied Biology* ISSN 0003-4746.

**Allen, M. F. (2011).** Linking water and nutrients through the vadose zone: a fungal interface between the soil and plant systems. *Journal of Arid Land* 3, 155–163. doi: 10.3724/SP.J.1227.2011.00155.

**Aroca R, Del Mar Alguacil M, Vernieri P, Ruiz-Lozano JM. (2008).** Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA- deficient mutant (sitiens). *Microb Ecol* 56(4): 704-719.

**Artursson V., Finlay R.D., Jansson J.K. (2006)** Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environ Microbiol*; 8: 1–10.

**Badri D. V. and Vivanco J.M. (2009)** Regulation and function of root exudates. *Plant, Cell and Environment* 32, 666–681.

**Baharlouei J., Khavazi K., Pazira E., Solhi M., (2011).** Evaluation of inoculation of plant growth- promoting rhizobacteria on cadmium uptake by Canola and Barley. International Conference on Environmental Science and Technology IPCBEE, vol. 6. IACSIT Press, Singapore.

**Beckers G. J. and Conrath U. (2007).** Priming for stress resistance: from the lab to the field. *Curr. Opin. Plant Biol*. 10:425–431.

**Beckers G. J. M., Jaskiewicz M., Liu Y., Underwood W. R., He S. Y., Zhang S., and Conrath U. (2009).** Mitogen-activated protein kinases 3 and 6 are

required for full priming of stress responses in Arabidopsis thaliana. *Plant Cell* 21:425–431.

**Benhamou N., Fortin J. A., Hamel C., Starnaud M., and Shatilla A. (1994).** Resistance responses of mycorrhizal Ri TDNA-transformed carrot roots to infection by Fusarium oxysporum f. sp. chrysanthemi. *Phytopathology* 84:958–968.

**Berg G. (2009)** Plant microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol*; 84:11–8.

**Bisseling T., Dangl J. L. and Schultze-Lefert P. (2009).** Next-generation communication. *Science* 324:691.

**Bonfante P. and Anca I. A., (2009)** Plants, Mycorrhizal Fungi, and Bacteria: A Network of Interactions. *The Annual Review of Microbiology*. 63: 363-83.

**Bonfante P. and Genre A. (2010)** Mechanisms underlying benefi cial plant – fungus interactions in mycorrhizal symbiosis. *Nature Communications* DOI: 10.1038/ncomms 1046.

**Bonfante P., Genre A. (2015).** Arbuscular mycorrhizal dialogues: do you speak 'plantish' or 'fungish'? *Trends Plant Sci* 20(3): 150-154.

**Bosch T. C. and McFall-Ngai M. J. (2011).** Meta-organisms as the new frontier. *Zoology* 114: 185-190.

**Bouwmeester H. J., Roux C., Lopez-Raez J. A., and Bécard G. (2007).** Rhizosphere communication of plants, parasitic plants and AM fungi. *Trends Plant Sci*. 12:224–230.

**Breuillin F., Schramm J., Hajirezaei M., Ahkami A., Favre P., Druege U., Hause B., Bucher M., Kretzschmar T., Bossolini E., Kuhlemeier C., Martinoia E., Franken P., Scholz U., and Reinhardt D. (2010).** Phosphate systemically inhibits development of arbuscular mycorrhiza in Petunia hybrida and represses genes involved in mycorrhizal functioning. *Plant J.* 64:1002–1017.

**Caballero-Mellado J., Onofre-Lemus J., Estrada-de los Santos P. and Martınez-Aguillar L. (2007)** The tomato rhizosphere, an environment rich in nitrogen-fixing Burkholderia species with capabilities of interest for agriculture and bioremediation. *Appl Environ Microbiol* 73, 5308–5319.

**Campos-Soriano L., García-Martínez J., and Segundo B. S. (2012).** The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defense-related genes in rice leaves and confers resistance to pathogen infection. *Mol. Plant Pathol*. doi:. doi:10.1111/j.1364- 3703.2011.00773.x.

**Casieri L., Ait Lahmidi N., Doidy J., Veneault-Fourrey C., Migeon A., Bonneau L., et al. (2013).** Biotrophic transportome in mutualistic plant-fungal interaction. *Mycorrhiza* 23, 597-625. 10.1007/s00572-013-0496-9.

**Castanheira N., Dourado A., Kruz S., Alves P.I., Delgado-Rodríguez A.I., Pais I., Semedo J., Scotti- Campos P., Sánchez C., Borges N., Carvalho G., Barreto Crespo M.T., Fareleira P. (2016)** Plant growth- promoting Burkholderia species isolated from annual ryegrass in Portuguese soils. *J Appl Microbiol.* 120(3):724-39. doi: 10.1111/jam.13025.

**Cavagnaro T.R., Gao L.L., Smith F.A., Smith S.E. (2001).** Morphology of arbuscular mycorrhizas is influenced by fungal identity. *New Phytologist* 151: 469-475.

**Conrath U., Beckers G. J. M., Flors V., García-Agustín P., Jakab G., Mauch F., Newman M. A., Pieterse C. M. J., Poinssot B., Pozo M. J., Pugin A., Schaffrath U., Ton J., Wendehenne D., Zimmerli L., and Mauch-Mani B. (2006).** Priming: Getting ready for battle. Mol. *Plant-Microbe Interact*. 19:1062–1071.

**Cordier C., Pozo M. J., Barea J. M., Gianinazzi S., and Gianinazzi-Pearson V. (1998).** Cell defense responses associated with localized and systemic resistance to Phytophthora parasitica induced in tomato by an arbuscular mycorrhizal fungus. *Mol. Plant-Microbe Interact.* 11:1017–1028.

**Cruz A., and Ishii T. (2011)** Arbuscular mycorrhizal fungal spores host bacteria that affect nutrient biodynamics and biocontrol of soil-borne plant pathogens. *Biol Open.* 1(1):52-7. doi: 10.1242/bio.2011014. Epub 2011 Nov 1.

**De la Peña E., Echeverría S. R., Van Der Putten W. H., Freitas H., and Moens M. (2006).** Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass Ammophila arenaria. *New Phytol*. 169:829–840.

**Dickson S. (2004).** The Arum-Paris continuum of mycorrhizal symbioses. *New Phytologist* 163: 187- 200.

**Dickson S., Smith F., Smith S. (2007).** Structural differences in arbuscular mycorrhizal symbioses: more than 100 years after Gallaud, where next? *Mycorrhiza* 17: 375-393.

**Egberongbe H.O., Akintokun A.K., Babalola O.O., Bankole M.O. (2010)** The effect of Glomus mosseae and Trichoderma harzianum on proximate analysis of soybean (Glycine max (L.) Merrill.) seed grown in sterilized and unsterilised soil. *J Agric Ext Rural Dev*; 2:54–8.

**Gernns H., von Alten H., Poehling H.M. (2001).** Arbuscular mycorrhiza increased the activity of a biotrophic leaf pathogen - is a compensation possible? *Mycorrhiza* 11(5): 237-243.

**Ghignone S., Salvioli A., Anca I., Lumini E., Ortu G., Petiti L., Cruveiller S., Bianciotto V., Piffanelli P., Lanfranco L. et al. (2012).** The genome of the obligate endobacterium of an AM fungus reveals an interphylum network of nutritional interactions. *ISME Journal* 6: 136–145.

**Glick B.R., (2005)** Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase.

*FEMS Microbiol*. Lett., 251, pp. 1-7.

**Glick BR, Cheng Z, Czarny J, Cheng Z, Duan J. (2007)** Promotion of plant growth by ACC deaminase-producing soil bacteria. *Eur J Plant Pathol*; 119:329–39.

**Grobelak A., Napora A., Kacprzak M., (2015)** Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecological Engineering* 84, 22-28.

**Harrison M. J. (2005)** Signaling in the arbuscular mycorrhizal symbiosis. *Annual Review of Microbiology* 59.19-42.

**Hause B. and Fester T. (2005).** Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta* 221:184–196.

**Hayat R., Ali S., Amara U., Khalid R., Ahmed I. (2010)** Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann Microbiol*; 60:579–98.

**Hildebrandt U., Janetta K., Bothe H. (2002)** Towards growth of arbuscular mycorrhizal fungi independent of a plant host. *Appl Environ Microbiol* 68:1919–24.

**Hildebrandt U., Regvar M., Bothe H. (2007).** Arbuscular mycorrhiza and heavy metal tolerance. *Phytochemistry* 68(1): 139-146.

**Horii S., Ishii T. (2006)** Identification and function of *Gigaspora margarita* growth-promoting microorganisms. *Symbiosis (Rehovot)* Vol.41 No.3 pp.135-141 ref.18.

**Horlings L.G. and Marsden T.K. (2011)** Towards the real green revolution? Exploring the conceptual dimensions of a new ecological modernisation of agriculture that could 'feed the world'. *Global Environmental Change* 21 (2011) 441–452.

**Igiehon N.O. et Babalola O.O. (2017).** Biofertilizers and sustainable agriculture: exploring arbuscular mycorrhizal fungi. *Appl Microbiol Biotechnol*. 101(12):4871-4881.

**Huang Y. and Wong P.T.W (1998)** Effect of Burkholderia (Pseudomonas) cepacia and soil type on the control of crown rot in wheat. *Plant and Soil* 203: 103–108.

**Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea J. (2003)** The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol Fertil Soils* 37:1– 16.

**Jaderlund L, Arthurson V, Granhall U, Jansson JK. (2008)** Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria: as revealed by different combinations. *FEMS Microbiol Lett* 287:174–80.

**Jung S.C., Martinez-Medina A., Lopez-Raez J.A., Pozo M.J. (2012)** Mycorrhiza-Induced Resistance and Priming of Plant Defenses. *J Chem Ecol* (2012) 38:651–664.

**Koricheva J., Gange A. C., and Jones T. (2009).** Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. *Ecology* 90:2088–2097.

**Lau J.A. and Lennon J.T. (2011)** Evolutionary ecology of plant–microbe interactions: soil microbial structure alters selection on plant traits. *New Phytol*; 192:215–24.

**Li H.-Y., Yang G.-D., Shu H.-R., Yang Y.-T., Ye B.-X., Nishida I., and Zheng C.-C. (2006)**. Colonization by the arbuscular mycorrhizal fungus Glomus versiforme induces a defense response against the root-knot nematode Meloidogyne incognita in the grapevine (Vitis amurensis Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3. *Plant Cell Physiol* 47:154–163.

**Linderman R.G. (1992).** Vesicular-arbuscular mycorrhizae and soil microbial interactions. In Mycorrhizae In Sustainable Agriculture, Vol. 54 (ed. G. J. Bethlenfalvay and R. G. Linderman), pp. 45-70. Madison: ASA.

**Liu J., Maldonado-Mendoza I., Lopez-Meyer M., Cheung F., Town C. D., and Harrison M. J. (2007)**. Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. *Plant J.* 50:529–544.

**López-Ráez J. A., Charnikhova T., Fernández I., Bouwmeester H., and Pozo M. J. (2011).** Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato. *J. Plant Physiol.* 168:294–297.

**Lumini E., Bianciotto V., Jargeat P., Novero M., Salvioli A., Faccio A., Becard G., Bonfante P. (2007).** Presymbiotic growth and sporal morphology are affected in the arbuscular mycorrhizal fungus Gigaspora margarita cured of its endobacteria. *Cellular Microbiology* 9(7), 1716–1729.

**MacDonald R.M, Chandler M.R. (1981).** Bacterium-like organelles in the vesicular-arbuscular, mycorrhizal fungus Glomus catedonius. *New Phytol*. 89:241–46.

**Marella S., (2014)** Bacterial Endophytes in Sustainable Crop Production: Applications, Recent Developments and Challenges Ahead. *International Journal of Life Sciences Researc* Vol. 2, Issue 2, pp: 46-56.

**Marulanda A., Barea J.M., Azcon R. (2006)** An indigenous drought tolerant strain of Glomus intraradices associated with a native bacterium improves water transport and root development in Retama sphaerocarpa. *Microb Ecol* 52:670–8.

**Massena Reis V., dos Santos Teixeira K., Pedraza R. (2011)** Bacteria in Agrobiology: Plant Growth Responses. *Springer-Verlag*, Berlin.

**Mauch-Mani B., Baccelli I., Luna E., and Flors V. (2017)** Defense Priming: An Adaptive Part of Induced Resistance *Annu. Rev. Plant Biol*. 2017. 68:485–512.

**Meier S., Cornejo P., Cartes P., Borie F., Medina J., Azcón R. (2015).** Interactive effect between Cu- adapted arbuscular mycorrhizal fungi and biotreated agrowaste residue to improve the nutritional status of Oenothera picensis growing in Cu-polluted soils. *J. Plant Nutr*. Soil Sci. 178, 126–135. 10.1002/jpln.201400092.

**Miransari M. (2010).** Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. *Plant Biol*. 12:563–569.

**Mondo S.J, Toomer K.H, Morton J.B, Lekberg Y, Pawlowska T.E. (2012).** Evolutionary stability in a 400-million-year-old heritable facultative mutualism. *Evolution* 66: 2564–2574.

**Nadeem S.M, Ahmad M., Zahir Z.A., Javaid A., Ashraf M. (2013)** The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnology Advances* 32 (2014) 429–448.

**Nagy R., Karandashov V., Chague V., Kalinkevich K., Tamasloukht M., Xu G., et al. . (2005).** The characterization of novel mycorrhiza-specific phosphate transporters from Lycopersicon esculentum and Solanum tuberosum uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. *Plant Journal*. 42, 236–250. 10.1111/j.1365-313X.2005.02364.x

**Ordookhani K., Khavazi K., Moezzi A., Rejali F. (2010)** Influence of PGPR and AMF on antioxidant activity, lycopene and potassium contents in tomato. *Afr J Agric Res*; 5:1108–16.

**Paungfoo-Lonhienne C., Lonhienne T.G.A., Yeoh Y.K., Webb R.I., Lakshmanan P., Chan C.X., Lim P.-E., Ragan M.A. et al. (2014)** A new species of Burkholderia isolated from sugarcane roots promotes plant growth. *Microb Biotechnol* 7, 142–154.

**Pingali P.L. (2012)** Green Revolution: Impacts, limits, and the path ahead. *Proc Natl Acad Sci U S A*. 109 (31): 12302–12308.

**Pozo M. J., Cordier C., Dumas-Gaudot E., Gianinazzi S., Barea J. M., and Azcón-Aguilar C. (2002).** Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to Phytophthora infection in tomato plants. *J. Exp. Bot*. 53:525–534.

**Pozo M.J. and Azcón-Aguilar C. (2007).** Unraveling mycorrhiza induced resistance. Curr. Opin.

*Plant Biol*. 10:393–398.

**Pozo M. J., López-Ráez J. A., Azcón-Aguilar C. (2010).** Impact of arbuscular mycorrhizal symbiosis on plant response to biotic stress: the role of plant defence mechanisms. In: Koltai H. KY ed. *Arbuscular Mycorrhizas: Physiology and Function*. Amsterdam: Springer, 193–207.

**Pumplin N., Harrison M.J. (2009).** Live-cell imaging reveals periarbuscular membrane domains and organelle location in Medicago truncatula roots during arbuscular mycorrhizal symbiosis. *Plant Physiol.* 151(2):809-19. doi: 10.1104/pp.109.141879.

**Rillig, M.C., (2004).** Arbuscular mycorrhizae, glomalin, and soil aggregation. *Canadian Journal of Soil Science* 84, 355-363.

**Salvioli A., Ghignone S., Novero M., Navazio L., Venice F., Bagnaresi P. and Bonfante P. (2015)** Symbiosis with an endobacterium increases the fitness of a mycorrhizal fungus, raising its bioenergetic potential. *The ISME Journal* **10,** 130–144; doi:10.1038/ismej.2015.91;

**Schüßler, A., Schwarzott, D., Walker, C., (2001).** A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research* 105, 1413-1421.

**Smith S.E. and Read D.J., (2008).** Mycorrhizal Symbiosis. *Academic Press*, San Diego and London.

**Smith S., Facelli E., Pope S., and Andrew Smith F. (2010).** Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. *Plant Soil* 326:3–20.

**Smith S.E. and Smith F.A., (2011)** Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales. *Annu. Rev. Plant Biol.* 2011. 62:227–50.

**Smith S.E. and Smith F.A., (2012)** Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Journal Mycologia* volume 104, 2012.

**Van der Heijden M.G.A., Bardgett R.D., van Straalen N.M., (2008).** The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11, 296-310.

**Vierheilig H., Steinkellner S., Khaosaad T., and Garciagarrido J. M. (2008).** The biocontrol effect of mycorrhization on soilborne fungal pathogens and the autoregulation of the AM symbiosis: One mechanism, two effects? pp. 307–320, in *A. Varma (ed.), mycorrhiza*. Springer, Berlin, Heidelberg.

**Vos C., Claerhout S., Awire R., Panis B., De Waele D., and Elsen A. (2011).** Arbuscular mycorrhizal fungi reduce root-knot nematode penetration through altered root exudation of their host. *Plant Soil* 354:335–345.

**Vos I.A., Pieterse C.M.J., van Wees S.C.M. (2013).** Costs and benefits of hormone-regulated plant defences. *Plant Pathology* 62 (Suppl. 1), 43–55.

**Whipps, J. M. (2004).** Prospects and limitations for mycorrhizas in biocontrol of root pathogens. *Can. J. Bot*. 82:1198–1227.

**Yao M. K., Désilets H., Charles M. T., Boulanger R., and Tweddell R. J. (2003).** Effect of mycorrhization on the accumulation of rishitin and solavetivone in potato plantlets challenged with Rhizoctonia solani. *Mycorrhiza* 13:333–336.

**Yusran Y., Volker R., Torsten M. (2009)** Effects of plant prowth-promoting rhizobacteria and Rhizobium on mycorrhizal development and drowth of Paraserianthes falcataria (L.) nielsen seedlings in two types of soils with contrasting levels of pH. *Proceedings of the international plant nutrition colloquium XVI.* UC Davis: Department of Plant Sciences;

**Zamioudis C. and Pieterse C. M. J. (2012).** Modulation of host immunity by beneficial microbes.*Mol. Plant-Microbe Interact*. 25:139–150.

# Chapter 1

## Omics approaches applied to wheat reveal how mycorrhizal symbiosis confers enhanced productivity and higher resistance to the leaf pathogen *Xanthomonas translucens*

Fiorilli V.*, Vannini C.**, Ortolani F.**, Garcia Seco D.***, Novero M.*, Moulin L.

***, Terzi V.****, Morcia C. ****, Bagnaresi P. ****, Bracale M.**, Bonfante P.* Affiliations:

*Dipartimento di Scienze della vita e Biologia dei Sistemi, Viale P.A. Mattioli 25, 10125, Torino, Italy.

**Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, via J.H. Dunant 3, 21100 Varese, Italy.

***IRD, Cirad, Univ. Montpellier, Interactions Plantes Microorganismes Environnement (IPME), 34394 Montpellier France.

****CREA-GB, Research Centre for Genomics and Bioinformatics, Via San Protaso 302, 29017-Fiorenzuola d'Arda, Italy.

### Abstract

Besides improved mineral nutrition, plants colonised by arbuscular mycorrhizal (AM) fungi often display increased biomass and higher tolerance to biotic and abiotic stresses. Several studies have focused on transcriptomic and proteomic changes in both model and agricultural plants. By contrast, notwithstanding the global importance of wheat (*Triticum aestivum* L.) as an agricultural crop, its response to AM symbiosis has been poorly investigated. Here, we focused on the role of an AM fungus on mineral nutrition of wheat, and on its potential protective effect against *Xanthomonas translucens*. To address these issues, phenotypical, metabolomic and molecular approaches have been combined. Morphological observations indicated that AM wheat plants displayed a growth effect, in terms of biomass and grain yield, as well as a reduction in lesion production following pathogen infection. To elucidate the molecular mechanisms underlying the mycorrhizal phenotype, we investigated local and systemic changes of transcripts and proteins in roots and leaves during the bipartite (plant-AM fungus) and tripartite (wheat-AM fungus-pathogen) interaction. Transcriptomic and proteomic profiling identified the main pathways (nutrient transport, primary metabolism, defence mechanisms, hormone regulation) involved in enhancing plant biomass and mineral nutrition and in promoting the bioprotective effect against the leaf pathogen. Interestingly, the pathways were differentially regulated depending on the organ/microbe relationship. Mineral and amino acid contents in roots, leaves and seeds, and protein oxidation profiles in leaves, supported the *omics* data, providing new insight into the mechanisms exerted by AM symbiosis to confer higher productivity and enhanced resistance to *X. translucens* in wheat.

## Introduction

In natural environments, plants interact simultaneously with a broad spectrum of both pathogenic and beneficial microorganisms that might influence plant

performance and survival. Among soil microbes, arbuscular mycorrhizal (AM) fungi (subphylum Glomeromycotina; Spatafora et al., 2016) establish a symbiosis with most plants living in wild and agroecosystems (Bonfante and Genre, 2015). AM fungi colonized the root cortex, supplying mineral nutrients to plants in exchange for carbon compounds, thanks to the development of highly branched intracellular structures called arbuscules (Gujahr and Parniske, 2012). Besides improved mineral nutrition, plants colonised by AM fungi often display increased biomass and yield grain and a higher tolerance to biotic and abiotic stresses leading to a general improvement in plant fitness (Gernns et al., 2001; Van der Heijden and Sanders, 2002; Hildebrandt et al., 2007; Pozo and Azcón-Aguilar, 2007; Aroca et al., 2008; Pozo et al., 2010; Cameron et al., 2014). Considering the range of benefits provided by the fungal partner, the management of AM fungi in crop production is a cornerstone for future low-input and sustainable agriculture.

Many studies have focused on local and systemic transcriptomic and proteomic changes in rice (Campos-Soriano et al., 2012; Gutjhar et al., 2015; Fiorilli et al., 2015), maize (Willmann et al., 2013), *Medicago truncatula* (Liu et al., 2007; Adolfsson et al., 2017) and tomato plants (Benabdellah et al., 2000; Fiorilli et al., 2009; Salvioli et al., 2012; Zouari et al., 2014; Cervantes-Gàmez et al., 2016). By contrast, even though wheat (*Triticum aestivum* L.) is a major global crop, cultivated on more than 200 million hectares with more than 600 million tons of annual production (Baloglu et al., 2014b; Inal et al., 2014; Okay et al., 2014), its response to AM symbiosis has been poorly investigated. The main reason for this shortcoming is that wheat has a hexaploid genome of 17 Gb in size, more than 80% of which is composed of repetitive transposable elements (Bennetzen et al., 2005). It is considered one of the most challenging genomes, since it has the genetic structure of three independent genomes in one species (*AABBDD* genome) (IWGSC, 2014). A meta-analysis highlights the beneficial effects of

mycorrhizal inoculation on wheat dry weight and phosphorus (P), nitrogen (N) and zinc (Zn) uptake (Pellegrino et al., 2015). However, the molecular determinants underlying the AM-related growth promotion and enhanced nutrient status in wheat are still poorly understood.

AM symbiosis is acknowledged to reduce damage caused by soil-borne pathogens including fungi, oomycetes (Whipps, 2004) and parasitic nematodes (de la Peña et al., 2006; Li et al., 2006; Vos et al., 2013). Similarly, in wheat, where pathogen attacks cause about 10–16% yield losses (Oerke, 2006), some of these stress events are alleviated by AM fungi (Al-Karaki et al., 2004; Zhou et al., 2015). The mechanisms involved in the bio-protective effect of AM fungi are not fully explained: they are not exclusively dependent on the improved mineral nutrition, but seem to be related to activation of plant defence mechanisms (Jung et al., 2012). In general, establishment of an AM symbiosis and production of AM signals activate defence-responsive genes in both shoot and root (Liu et al., 2007; Campos-Soriano et al., 2011; Hayek et al., 2014; Giovannetti et al., 2015). These processes are reminiscent of defence priming, i.e., the induction of effective defence in plant tissues by various natural and artificial compounds (Conrath et al., 2015). As a consequence, mycorrhizal plants are expected to be better protected against pathogen challenge than non-mycorrhizal plants: this phenomenon has been named *mycorrhiza-induced resistance* (MIR) (Pozo and Azcón- Aguilar, 2007; Cameron et al., 2012). MIR is dependent on the particular pathogen- mycorrhizal plant interaction and the plant organ under examination, i.e., root or shoot (e.g., Gernns et al., 2001; Whipps, 2004; Miozzi et al., 2011; Lingua et al., 2002; Fritz et al., 2006; Pozo et al., 2010; Fiorilli et al., 2011; Song et al., 2015a; Liu et al., 2007).

The contrasting results obtained in such studies suggest that induction of resistance against pathogens depends on multiple mechanisms that may operate

simultaneously (Pozo and Azcòn-Aguillar, 2007; Fiorilli et al., 2011; Miozzi et al., 2011; Song et al., 2015a). The potential protective effect of mycorrhizal symbiosis in wheat has never been investigated.

The main goals of this work were, first, to define the responsiveness of *T. aestivum* cv. Chinese Spring to AM symbiosis, and second, to elucidate the molecular mechanisms underlying the mycorrhizal phenotype. We looked for the main pathways involved in enhancing plant biomass and mineral nutrition, and in promoting the bio-protective effect against a leaf pathogen. To address these issues, we combined phenotypic, metabolomic and molecular approaches. We explored the plant growth effect exerted by AM fungi in both greenhouse and controlled environment conditions. In greenhouse experiments we misured some agronomic traits, such as tillering capacity, vegetative biomass and yield, as well as qualitative traits, such as kernel weight and size. Plants grown in controlled conditions were used to confirm the morphometric data recorded in the greenhouse and for omic analisys. Further we evaluated the impact of AM symbiosis against *Xanthomonas translucens*, which is a specific pathogen of wheat leaves. By integrating whole-transcriptome sequencing (RNA-seq) with shotgun nanoflow scale liquid chromatography-tandem mass spectrometry (LC-MS/MS), we provide a comprehensive functional overview of both local and systemic transcriptomic and proteomic changes in roots and leaves during the mycorrhizal combination (plant and AM fungus), as well as during the tripartite interaction (plant, AM fungus and pathogen). We demonstrate that AM symbiosis has strong positive effects on wheat development, and also confers resistance to a wheat pathogen.

## Material and Method

*Greenhouse trial*

Two pot experiments were conducted during the 2015-2016 seasons in the greenhouse of Genomics Research Centre (Fiorenzuola, PC, Italy) to evaluate the effect of *Funneliformis mosseae* (BEG.12; MycAgro, France) on some wheat agronomic traits. The experiments had two treatments, mycorrhizal and non-mycorrhizal plants and were carried out in 15x15x20 cm square pots, filled with a 50:50 mixture of sterile sand and field soil. This soil, from the Fiorenzuola's experimental farm located in Northern Italy (44°55'19.4"N 9°54'32.3"E), was sieved (2 mm) and dry treated at 100°C x 1 hour x3 times, during three consecutive days (Brito et al., 2009). The pasteurized soil resulted alkaline (pH 8.21), with the following characteristics: total carbonate 12.66%; inorganic carbon 15.19 g/kg; total carbon 28.1 g/kg; organic carbon 12.9 g/kg; organic matter 2.23%; total N 0.11%; C:N ratio 11.8; P2O5 35.4 mg/kg; cation exchange capacity 7.17 cmol(+)/kg. Before potting, all pots were washed with 0.1% HCl and deionized water and filled with 3.5 kg of soil. *F. mosseae* (formerly *Glomus mosseae* BEG.12) obtained from MycAgro Lab. (Technopole Agro-Environment, Bretenière, France) was inoculated 2-3 cm below the planting holes in thirty replicated pots. Other thirty pots were not inoculated. Seeds of bread wheat (*Triticum aestivum* Desf.; cv Chinese Spring) were surface-sterilized and germinated on wet filter paper in Petri dishes for 5 days in the dark, at 20°C. Five seedlings were transplanted into each pot and after 3 days the pots were thinned to three plants each. Plants were cultivated in greenhouse with natural light and temperature from January to July as randomised design. The plants were irrigated with tap water to keep substrate at 80% field capacity without any fertilization until full ripening. At the end of tillering stage, the number of tillers/plant was recorded. At Z92 cereal growth staging scale (Zadoks et al., 1974) all plants were harvested. Aboveground plant dry biomass and spike weights were recorded for each pot. The spikes were threshed and 1000-kernel weights were determined.

Grains were scanned using a flatbed scanner (Epson Expression 10000XL) and the images were processed with the GrainScan software (Whan et al., 2014) to determine grain area, length and width. The roots were recovered, thoroughly washed and used for DNA extraction. The presence of *F. mosseae* DNA was evaluated with quantitative real time-PCR analysis, using the *F. mosseae* BEG12 primers according to Alkan et al (2006).

*Plant growth condition, inoculation with AM fungus and the pathogenic bacterial strain*

For standard laboratory conditions, *Triticum aestivum* cv. Chinese Spring seeds were surface sterilized and then germinated in distilled water. The seedlings were then transferred to pots with sterile quartz sand. Inoculation of *Funelliformis mosseae* (BEG.12; MycAgro, France) were performed by mixing the inoculum with sterile quartz sand (30% v/v). Mycorrhizal and non-inoculated (control) plants were maintained under glasshouse conditions under cycles of 12 h of light at 21°C and 50% relative humidity (RH) and 12 h of dark at 21°C and 50% RH, watered twice a week with water, and once with a modified Long-Ashton solution containing a low phosphorous concentration (32 μM $Na_2HPO_4$*$12H_2O$) (Hewitt, 1966). After 49 days post AM fungal inoculation (dpi), a set of mycorrhizal and control plants were inoculated with *X. translucens type* strain CFBP 2054 (syn. ATCC 19319, DSM 18974, ICMP 5752, LMG 876, NCPPB 973; GeneBank acc. no. CAPJ01000000) as described by Garcia-Seco et al., 2017. Controls were inoculated following the same procedure but with water. For the transcriptomic and proteomic (*omics*) experiment the infiltrated zone was collected 1-day post-inoculation (dpi) by pathogen when symptoms were not visible. While for phenotypic observation infected plants were left to grow and symptoms (water-soaked lesions) were scored 14 dpi. For the “omics” experiments, plants were

harvested 50 dpi. Portions of the root system from each mycorrhizal plant were selected under stereomicroscope on the basis of the presence of external mycelium. These root portions were mixed and pooled together and then divided into two samples, one to assess the level of mycorrhizal formation (done over 80 cm of root per sample), and the other for RNA extraction. The mycorrhizal roots were stained with cotton blue and the level of mycorrhizal colonization was assessed according to Trouvelot et al. (1986). Root and shoot fresh biomass of control (C), mycorrhizal (M) plants were measured and evaluated with the analysis of variance. Parallel independent experiments were conducted to estimate the yield, in the same conditions of previous trials. Spikes weight were measured separately in the mature plants at the end of their natural cycle. Two plants were pooled per replicate, with three biological replicates per treatment. Once pulverized in liquid nitrogen, each sample was split into two aliquots, one for protein extraction and one for RNA extraction.

*Nucleic acid extraction, cDNA synthesis and Real-time quantitative RT-PCR*

Total genomic DNA was extracted from *F. mosseae* sporocarps as described in Fiorilli et al., 2009, and from *T. aestivum* shoots using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Plant and fungal genomic DNAs were used to test each primer pair designed for real-time PCR to exclude cross-hybridization.

Total RNA was extracted from *T. aestivum* shoots and roots of M, C plants, as well as control and mycorrhizal plants infected by the bacterial pathogen *X. translucens* (CX and MX) as described by Garcia-Seco et al., 2017. After DNase treatment and confirmation of RNA integrity using Nanodrop™, Experion™

Automated Electrophoresis System, and gel electrophoresis, the total RNA was used for mRNA preparation, fragmentation and cDNA synthesis.

Quantitative Real-time was used to measure the expression of nine genes shown to be differentially regulated by RNA-Seq. Single-strand cDNA was obtained as described in Vallino et al. (2014). Three biological replicates were conducted for each condition. Quantitative real time PCR experiments and data analysis were carried out as described in Vallino et al. (2014). The primer names and corresponding sequences are listed in Table S1.

*RNA-seq transcriptome sequencing*

The preparation of mRNA libraries was done by TrueSeq Stranded mRNA Sample (Illumina) Preparation. The library quality validation (fragment concentration and length) was performed by Fragment Analyzer using the standard and high sensitivity DNA kits (Agilent Technologies, Santa Clara, CA) and by qPCR (LightCycler 480; Roche Diagnostics, Meylan, France). RNA-seq library preparation and sequencing was carried out by MGX-Montpellier GenomiX platform using a HiSeq 2500 instrument (Illumina Inc., San Diego, CA). Six libraries were sequenced per lane (around 30 million reads per replicate), with 100 nucleotides sequence length per read.

*Bioinformatic analysis of transcriptome data*

Mapping and alignment of sequence reads was done with Burrows-Wheeler transform (BWA-MEM algorithm) using the BWA-MEM algorithm (Li, 2013). Reads were subsequently aligned to *Triticum aestivum* coding sequences (CDS) (IWGSC1.0 + popseq.29) and non-coding RNA (ncRNA). Subsequently, sequence counts were collected by Samtools idxstats filtering of bam alignment

files for reads with MAPQ ≥ 3. Differentially expressed genes (DEG) were called via DESeq2 1.10.0 Bioconductor package using local fit and betaPrior parameter set to False. Independent filtering was enabled (Love et al., 2014). A false discovery rate threshold of 0.05 was set for DEG calling. Sample clustering and principal component analyses were performed upon variance stabilization transformation of expression data. Transcripts were considered differentially expressed when the adjusted *p* values are below 0.05 and the logarithmic fold change over 0.5 or when the logarithmic fold change was over 2. The transcriptomic data related to roots and leaves of mycorrhizal plant and roots and leaves of mycorrhizal plants infected by X. *translucens* have been deposited in the European Bioinformatics Institute (EMBL-EBI) with the dataset identifier E-MTAB- 5898, while the transcriptomic data from control plants and control plants infected by *X. translucens* with the dataset identifier E-MTAB-5891 (Garcia-Seco et al., 2017).Unless otherwise stated, further graphical outputs were generated with custom R and Python scripts.

*Protein extraction and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis*

Total proteins were extracted from *T. aestivum* shoots and roots of M, C, CX and MX plants as described by Garcia-Seco et al., 2017. MS analysis was performed on a QExactive mass spectrometer coupled to a nano EasyLC 1000 (Thermo Fisher Scientific Inc., Waltham, MA). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. For each sample, 4 µL of peptides were loaded on a self- made column (75 µm × 150 mm) packed with reverse-phase C18 material (ReproSil-Pur 120 C18-AQ, 1.9 µm; Dr. Maisch GmbH, Ammerbuch, Germany) and eluted at a flow rate of 300 nL/min by a gradient from 2 to 35% B in 80 min, 47% B in 4 min and

98% B in 4 min. Samples were acquired in a randomized order. The mass spectrometer was operated in data-dependent mode (DDA), acquiring a full-scan MS spectra (300−1700 m/z) at a resolution of 70,000 at 200 m/z after accumulation to a target value of 3,000,000, followed by HCD (higher-energy collision dissociation) fragmentation on the twelve most intense signals per cycle. HCD spectra were acquired at a resolution of 35,000 using a normalized collision energy of 25 and a maximum injection time of 120 ms. The automatic gain control (AGC) was set to 50,000 ions. Charge state screening was enabled and singly and unassigned charge states were rejected. Only precursors with an intensity above 8300 were selected for MS/MS (2% underfill ratio). Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1010 and 445.1200.

*Proteomic data processing and bioinformatic analysis*

Mass spectrometer raw files were analyzed by MaxQuant (version 1.5.3.28 and 1.5.3.30) with the match between runs (matching time window of 2 min) and label free quantification (LFQ) options selected. Tandem MS spectra were searched against UniProt *T. aestivum* (Version 2015-10, 100.800 entries), Uniprot *Rhizophagus irregularis* (Version 2015-10, 29.847) and Uniprot *Xanthomonas translucens* (Version 2015-10, 14.378 entries). For the annotation of the unknown proteins a blast search was made against the Uniprot database viridiplantae (Version 2015-10, 3398870 entries), taking the first hit with a valid annotation. Trypsin/P was chosen as the protease, cysteine carbamidomethylation was set as fixed modification, and oxidation of methionine and acetylation of the N-terminal as variable modifications. Peptide tolerance was set to 4.5 ppm, while MS/MS tolerance was set to 0.5 Da. Peptide- spectrum matches (PSMs) and proteins were

validated with 1% FDR. Only PSMs with a minimum length of 7 amino acids were kept.

The raw data was first processed by Perseus (MaxQuant package) and the incorrected protein identifications (contaminants, decoy and "only identified by site" entries) were removed from the main data frame. LFQ intensities were Log2 transformed. Only protein groups detected in almost two of three biological replicate samples sharing the same treatment and tissue were considered unambiguously identified and were used for assessment of significant change. Missing value were estimated using the *R* package 'imputeLCMD' https://cran.r project.org/web/packages/imputeLCMD/). The experimental quality was checked using the multi-scatter plots tool for the analysis of Pearson correlation between the samples. To determine the differentially expressed proteins (DEPs) among considered conditions, we performed a multiple sample test using Anova with a permutation-based false discovery rate (FDR) cut-off of 0.01.

For the GO analysis of DEPs we used agriGO v2.0 (Tian et al., 2017) and for the cluster analysis, we used the "Profile plot" tool of Perseus program.

*Aminoacid analysis*

HPLC-grade water, HPLC-grade methanol (MeOH), formic acid, aspartic acid (Asp), asparagine (Asn), glutamine (Gln), glutamic acid (Glu), serine (Ser), threonine (Thr), cystine (Cys), alanine (Ala), proline (Pro), valine (Val), methionine (Met), tyrosine (Tyr), leucine (Leu), phenylalanine (Phe), tryptophan (Trp), lysine (Lys), histidine (His) arginine (Arg) ornithine (Orn), pipecolic acid (Pip), citrulline (Cit), GABA and two deuterated internal standards (L-Phenyl-d5-alanine and L-*alanine* $^{15}$N) were purchased from Sigma–Aldrich Co. (Dorset, UK). Stock solutions for all compounds were prepared in distilled water (10 mM).

A working mixture (1mM of each aminoacid, AA) was prepared and used for the calibration (range 2-15 μM).

For the AA extraction, leaf and root samples were lyophilized and 0.1 g of each sample was re-suspended in 10 mL of 0.1% (v/v) formic acid in water/methanol (50:50). 10 μl of 10 mM deuterated internal standards were added. The mixture was then vortexed for 4 h in the dark, sonicated for 15', centrifuged and the supernatant was collected.

The HPLC analysis was performed in a Finningan Surveyor MS plus HPLC system (Thermo Electron Corporation, CA, USA). Separation was achieved using C18 column (Phenomenex, synergi 4u fusion-RP 80a 150 x 2.00 mm). The mobile phase was composed of (A) water with 0.1% (v/v) formic acid and (B) methanol/water (50:50) 0.1% (v/v) formic acid with flow rate 150 μL/min; gradient 0–3.0 min/2% (v/v) B, 3–16 min/2–50% (v/v) B. For the mass spectrometry quantification, a Finningan LXQ linear ion trap mass spectrometer, equipped with an ESI ion source (Thermo Electron Corporation, CA, USA), was used. The analyses were done in positive (spray voltage 4,5 kV, capillary temperature 270˚C) and in the multiple reaction monitoring (MRM) mode. The optimization of collision energy for each substance, the tuning parameters and the choice of fragments to confirm the identity of target compounds were done in continuous flow mode, by using standard solution at concentration of 5μM (Table S2). The linearity of the method was considered adequate when square correlation coefficient ($R^2$) was higher than 0.98, based on peak area. The limits of detection (LOD) and quantification (LOQ) were fixed at 1 μM and at 2 μM, respectively.

*Minerals content analysis*

All frozen samples, were lyophilized, dried in an oven at 60°C for 8 h and then mineralized with 1 mL of HCl and 1 mL of hydrogen peroxide ($H_2O_2$). Samples were reconstituted in 1% $HNO_3$ in Milli-Q water. Blanks were made with the same solvents and chemicals employed in the treatment and digestion of the samples, or with just 1% $HNO_3$ in Milli-Q water. Calibration standard solutions were prepared from 1000 $mgLl^{-1}$ standard solutions of Mg, Fe, Cu, Zn and K (Baker Instra-Analyzed).

The determination of minerals was performed on a Thermo Fisher Solar M6 atomic absorption spectrometer. Ca, Mg, Fe, Zn, K were determined at ppm levels by flame atomic absorption spectrometry (FAAS) with deuterium lamp background correction; Cu was determined at ppb levels by graphite furnace atomic absorption spectrometry (GFAAS) and Zeeman background correction. All parameters such as the wavelength and the bandpass were set according to the recommendations of the instrument Cookbook.

The phosphorous content determination was performed as reported Chen and Toribara (1956).

*Detection of carbonylated proteins*

Carbonylated proteins were detected in all samples collected at 1 dpi and 14 dpi, as described previously (Garcia-Seco et al., 2017). Protein carbonylation values were expressed as carbonylation index (arbitrary units) after normalization for protein amounts. For each set (Leave and Roots) data (means ± SD, n = 4) were subjected to one-way analysis of variance (ANOVA).

**Results**

## Greenhouse experiments

Table 1 presents the results of a 2-year greenhouse trial to evaluate the impact of *Funneliformis mosseae* inoculation in Chinese Spring wheat. ANOVA showed that all measured agronomic traits, such as tillering capacity, vegetative biomass and yield, as well as qualitative traits, such as kernel weight and size, are significantly different between mycorrhizal (M) and non-mycorrhizal (C) plants. The presence of *F. mosseae* correlated with greater tillering capacity and plant biomass and also with increased yield, kernel weight and size (Fig. S1a). AM fungal inoculation also led to significant increases in concentrations of P, magnesium (Mg) and Zn in M seeds. In addition, total amino acid (AA) content increased (methionine, ornithine, tyrosine and tryptophan were more abundant), while lysine content decreased (Fig. S1b, c).

## Phenotypic assessment under controlled conditions

To confirm the morphometric data recorded in the greenhouse, C and M plants were grown in controlled conditions in a growth chamber, and the biomass of their epigeous and hypogeous parts was measured at 50 and 63 dpi. Growth of both tissues was increased significantly in M plants compared with C plants (Fig. 1a, b). To better investigate the effect of AM symbiosis on plant yield, spike weight was evaluated in M and C plants at the end of their natural life cycle. M plants displayed higher spike weight than C plants (Fig. 1c).

In the same experiment, 12 pots were devoted to investigating the impact of the AM fungus on leaf infection with *X. translucens*. These plants were identified as MX, while control plants infected with the pathogen were identified as CX. For all samples, mycorrhizal success was evaluated 50 days post inoculation (dpi) by calculating the total length of root colonisation (F%) and the total number of

arbuscules (A%) in M plants (F%: 53.5 ± 16.9; A%: 26.3 ± 9) and in MX plants (F%: 59.7 ± 12.6; A%: 34.5 ±

14.2). Similar colonisation values were detected 63 dpi in M (F%: 61.5 ± 16.3; A%: 38.5 ± 10.2) and MX plants (F%: 60.5 ± 19.2; A%: 40.5 ± 12.2), revealing that pathogen inoculation of the leaves did not inhibit root colonisation by the AM fungus, in the short term. At the same time (63 dpi, i.e., 14 days after inoculation with *X. translucens*), disease symptoms were evident. Lesion length was significantly reduced in MX plants compared with CX plants (Fig. 2a, b).

These experiments demonstrate that AM symbiosis exerts a positive effect on wheat growth and provides protection against *X. translucens*.

**A quantitative overview of transcript and proteomic data sets**

RNAs and proteins were isolated from leaves (L) and roots (R) of wheat plants, grown in the absence (LC and RC) or in the presence of the mycorrhizal fungus *F. mosseae* (LM and RM), and following infection with the bacterial pathogen *X. translucens* (LMX and RMX). For transcriptomic analysis, each treatment was sequenced in triplicates, with 37 million reads on average per replicate, and a minimum of 27 million and maximum of 41 million reads per replicate. Pearson correlation coefficients for biological replicate samples sharing the same treatment and tissue were always above 0.9 (Table S3), indicating a good level of reproducibility among replicates.

Also, for proteomic investigation, each treatment was analysed in triplicate leading to 2750 proteins identified on average per replicate, with a minimum of 2659 and a maximum of 2800 proteins per replicate. Pearson correlation coefficients for biological replicate samples sharing the same treatment and tissue

ranged from 0.95 to 0.99 (Table S4), indicating that the mass spectrometric analysis had robust reproducibility. All genes with a false discovery rate (FDR) below 0.05 and $log_2$FC over 0.5 and proteins with FDR below 0.01 and $log_2$FC over 0.3 were considered differentially expressed (differentially expressed genes, DEGs; and differentially expressed proteins, DEPs).

The overall changes in gene expression detected in the different comparisons are represented in a Venn diagram (Fig. 3). As expected, the AM fungus had a greater impact on the root system than bacterial infection (RM vs. RC: 5155 DEGs and RCX vs. RC: 150 DEGs, respectively). About the wheat AM marker genes identified in the RM vs. RC comparison, 377 out of 532 DEGs were shared by the RM vs. RC and RMX vs. RCX contrasts, whereas 30 DEGs, probably representing the specific AM core set, were differentially regulated during the RM vs. RC, RMX vs. RCX and RMX vs. RM contrasts (Fig. 3A). The presence of the AM fungus during pathogen infection (LMX vs. LCX) had a lower impact on the leaves (96 DEGs) than the single interactions without AM infection (LCX vs. LC: 8408 DEGs; LM vs. LC: 9097). By contrast, the presence of *Xanthomonas* on leaves of mycorrhizal plants (LMX vs. LM) led to a higher number of DEGs (13302) than other comparisons, and 43% (5777) of these DEGs were exclusively regulated in this contrast (Fig. 3B). These data suggest that the strong differential response detected during the tripartite interaction is due to a synergistic effect between the presence of the AM fungus in the roots (systemic effect) and the presence of the pathogen in the leaves (local effect). For a deeper analysis, we compared the expression of all identified proteins in the four samples (M, C, MX, X) by cluster analysis, on the basis of the modulation of their expression. Fig. 4 shows some of the protein profiles obtained.

**Differentially regulated genes and proteins in wheat leaves and roots following colonisation by *F. mosseae***

RNA-seq analysis revealed 3607 up-regulated genes and 1549 down-regulated genes in M roots (Fig. 5a; Table S5). An even higher gene modulation was observed in leaves: 6632 were up-regulated genes and 2464 were down-regulated (Fig. 5c; Table S6). RNA- seq analysis revealed 532 and 2220 transcripts that were exclusively expressed in roots (RM vs. RC) and leaves (LM vs. LC) of M plants, respectively.

Unlike wheat, rice has been much studied as a model plant for AM symbiosis (Güimil et al., 2005; Guthjar et al., 2015; Fiorilli et al., 2015). To determine whether a common core of genes responds to AM symbiosis, we compared the available AM-rice root RNA-seq data set (Fiorilli et al., 2015) with the wheat data in the present work. Using a Reciprocal Best Hits (RBH), we identified 114 of the 1088 up-regulated genes, which contained a corresponding sequence in mycorrhizal wheat roots (Table S7). Among them were several AM marker genes identified in other AM host plants: Glycerol-3- phosphate acyltransferase (*OsRAM2* homolog) (Gobbato et al., 2012), *Gibberellin response modulator protein* (*LjRAD1 homolog*) (Xue et al., 2015), LysM domain- containing protein (OsLysM homolog) (Fiorilli et al., 2015), *Ammonium transporter and Inorganic phosphate transporters* (see nutrient uptake paragraph) and ABC-2 type transporter (OsSTR1 homolog) (Gutjahr et al., 2012) (Table S7). These genomic communalities further support a key role for these genes in establishment of the AM symbiosis.

When RM samples were compared with RC samples, proteomic analysis revealed 586 up-regulated and 395 down-regulated proteins (Fig. 5b; Table S8), and a

comparison between LM and LC samples revealed 175 up-regulated and 226 down-regulated proteins (Fig. 5d; Table S9).

To gain an overview of the overlap between transcriptomic and proteomic data sets, differentially regulated genes and proteins detected using the two high-throughput techniques were compared. In roots and leaves of mycorrhizal plants, transcriptomic and proteomic data sets shared 192 (3.7% of DEGs and 19% of DEPs) and 82 (0.9% of DEGs and 20% of DEPs) elements, respectively (Fig. S2; Tables S10 and S11).

Taking advantage of bioinformatics tools such as agriGO v2.0 and over-represented Gene Ontology (GO) categories, we identified the pathways elicited by the AM fungus locally (root) and systemically (leaf) (Table S18). In the following paragraphs, we illustrate those pathways that might better explain the effects on growth and bio- protection detected in mycorrhizal plants.

***Nutrient uptake***

Nutrient uptake is a crucial trait of the AM symbiosis; however, in wheat, AM-induced nutrient transporter genes are still poorly characterised. To help fill this knowledge gap, the transcription profiles of some phosphate transporters (PTs), ammonium transporter 3 member 1 (*TaAMT3.1*), high-affinity sulfate transporter 2 (*TaSulfTr2*), potassium channel (*TaAKT1*) and oligopeptide transport (*TaOPT*) were investigated by both RNA-seq and qRT-PCR analyses. We monitored transcript levels of the PT genes previously described in wheat as highly induced in M roots (*TaPT10*, *TaPT11* and *TaPT12*) (Sisaphaithong et al., 2012; Duan et al., 2015), and clustered with the AM- induced PT genes (*OsPT11*; *MtPT4*; *LjPT4*; Paszkowski et al., 2002; Javot et al., 2007; Guether et al., 2009) (Fig. S3) as well as the putative inorganic phosphate transporter 1- 13 (*TaPT13*), which

shows high homology with the AM-induced *OsPT13* (Yang et al., 2012) (Fig. S3). These PT genes were strongly induced in RM and slightly induced in RC (Fig. S4). The transcripts of *TaAMT3.1*, which shows high similarity to the AM-induced *OsAMT3.1* from rice (Vallino et al., 2013; Fiorilli et al., 2015), were detected exclusively in M roots (Fig. S4). The same gene expression profile was observed for *TaAKT1* and *TaOPT*, whose transcripts were detected only in M roots. By contrast, although *TaSulfTr2* was strongly induced in M roots, it was also expressed in C roots (Fig. S4). A comparable expression profile was detected for *LjSultr1;2* which was induced in *L. japonicus* by both sulphur starvation and mycorrhizal formation (Giovannetti et al., 2014).

AM colonisation led to the differential regulation of several proteins involved in nutrient uptake. In agreement with the transcriptomic data, TaPT10 and TaAMT3.1 proteins were accumulated in RM. In addition, two proteins involved in N uptake were up-regulated in RM vs. RC: an AMT which shows high similarity to OsAMT3.2 and a nitrate transporter with similarity to the tomato AM-inducible LeNRT2;3 (Hildebrandt et al., 2002). Accordingly, two glutamine synthases, involved in N assimilation, were more strongly expressed in RM than in RC. An AA transporter with high homology to the mycorrhiza-inducible LjLHT1.2 from *L. japonicus* (Guether et al., 2011) was also up-regulated in RM. Several proteins involved in iron (Fe) uptake also accumulated in RM: one Fe-phytosiderophore transporter, some nicotianamine synthases (NAS) and two deoxymugineic acid synthases. Finally, a $H^+$- ATPase and a copper (Cu)-transporting ATPase were also induced in RM. $H^+$-ATPase shows high homology to OsHa1 and MtHA1, which energise nutrient uptake during mycorrhizal symbioses in rice and *Medicago truncatula* (Wang et al., 2014). The Cu-transporting ATPase is 90% similar to HMA4 of *Oryza sativa*, which is involved in Cu accumulation in root vacuoles (Xin-Yuan et al., 2016).

To validate the omics data, the mineral profiles of C and M plants were determined in both roots and leaves (Table 3). In agreement with the increased expression of phosphate and Cu transporters, P and Cu contents were significantly higher in RM. Mg, potassium (K) and Fe concentrations were lower in RM and higher in LM suggesting that AM promotes mineral translocation towards the shoot. These results may partially explain the increased expression of NAS in RM, since NAS expression is differentially regulated by Fe status (Bonneau et al., 2016). In conclusion, transcriptomic and proteomic data sets revealed a strong activation of nutrient transporter genes and proteins in M wheat plants, leading to an increased mineral content in both roots and leaves.

***Primary metabolism and AA content***

Both transcriptomic and proteomic data sets revealed differential expression of genes and proteins involved in primary metabolism in leaves and roots of M plants (Figs. S5 and S6). Several genes and proteins involved in carbohydrate metabolism (glycolysis, tricarboxylic acid [TCA], OPP pathways) were induced in RM, to provide energy and carbon skeleton during AM colonisation (Fig. S6). Due to the AM requirement for reduced plant C, metabolic pathways producing glucose (the sugar that is taken up by the AM fungus during its intraradical phase; Bago et al., 1999) were expected to be up- regulated. Enzymes involved in sucrose cleaving, such as sucrose synthases, invertases and fructokinases 1 and 2, were indeed up-regulated in wheat M roots. Recent work also demonstrated lipid movement from host plants to AM fungi, a process requiring strong activation of lipid metabolism in the arbuscule-containing cells of mycorrhizal roots in *Medicago*, lotus and carrot (Bravo et al., 2017; Luginbuehl et al., 2017; Jiang et al., 2017, Keymer et al., 2017). Fatty acid biosynthesis was also strongly up-regulated in wheat RM: we detected increased transcripts and protein levels of

key enzymes of this pathway, including 3-ketoacyl-CoA synthases, plastid Acetyl-CoA carboxylase and 3- oxoacyl-[acyl-carrier-protein] reductase 3. Several other genes and proteins involved in lipid metabolism, including *Triacylglycerol lipase 1 precursor*, *GDSL-like lipase/acylhydrolase*, plastid omega-3 fatty acid desaturase, Aestivum stearoyl-ACP desaturase and *lipid-transfer protein* (LTP35), were strongly induced in RM vs. RC. For example, in RM samples, *glycerol-3-phosphate dehydrogenase* (*G3PDH*) and *glycerol kinase* (*GK*), key enzymes involved in Glycerol-3-phosphate (G3P) biosynthesis, were up-regulated. At the proteomic level, the accumulation of Acyl-CoA dehydrogenase family member 10, and Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a, which are involved in fatty acid beta-oxidation, provided further support for the role of lipid metabolism in the AM symbiosis.

Focusing on the shoots of M plants, and on the list of common DEGs and DEPs, we identified the up-regulation of genes and proteins involved in photosynthesis and related processes. We identified genes and proteins related to Rubisco large subunit-binding protein, Photosystem II 10 kDa polypeptide, two Sucrose synthases 1 and one cell wall invertase, the last responsible for mediating sucrose cleavage yielding UDP-glucose and fructose. Proteomic results also showed the accumulation of ferrochelatase 2 (FC2) in LM, which produces heme for the photosynthetic machinery, and two Delta- aminolevulinic acid dehydratases involved in chlorophyll biosynthesis. In LM, we also detected the accumulation of Peptidyl-prolyl cis-trans isomerases (PPIases), similar to AtCYP38, and Protease Do-like 5, both involved in photosystem II repair (Sipio et al., 2008).

The enrichment analysis of DEPs detected in the comparisons RM vs. RC and LM vs. LC indicated that protein metabolism is one of the most up-regulated biological process in both roots and leaves of M plants (Table S18). In addition, transcriptomic and proteomic results revealed modulation of AA metabolism in

response to the AM fungus in both leaves and roots (Figs. S5 and S6). To confirm these data, free AA content was measured by LC-MS/MS analysis in both organs from C and M plants (Table 2). We observed in RM a significant increase in phenylalanine (Phe) and threonine, as expected by the down-regulation of lactoylglutathione lyase, which is involved in threonine degradation. In LM vs. LC, we found an increase in Phe and tyrosine and a decrease in threonine, valine, serine and tryptophan. The decreased tryptophan might be related to the formation of N-benzoylanthranilate (involved in phytoalexin biosynthesis), as indicated by the down-regulation of anthranilate phosphoribosyl transferase and the increase in anthranilate N-benzoyltransferase protein 1. Significant decreases in glutamine, lysine and arginine were also detected in both RM and LM samples.

Transcriptomic and proteomic data sets provide strong evidence that AM fungi enhance primary metabolism in wheat, leading to corresponding changes in sugar, lipid and AA pathways. These dynamic changes in roots are mirrored by increased photosynthetic activity in leaves.

*Phytohormones regulation*

Both transcriptional and proteomic analyses highlight the regulation of genes and proteins involved in phytohormones pathways. When M plants were compared with C plants, several genes involved in auxin and abscisic acid (ABA) metabolism and transport were affected (Table S12). Accumulation of transcripts for *Auxin-responsive GH3-like*, *Auxin efflux carrier component 1*, *Auxin transporter-like protein* and (*SAUR*)- *like* genes was observed in leaves of M plants. By contrast, the genes and proteins induced in M roots were mostly involved in auxin homeostasis; these included IAA-AA conjugate hydrolase and indole-3-acetic acid-amido synthetase. When RM was compared with RC, a

putative homolog of the strigolactones biosynthetic gene *More Axillary Branches 1* (*MAX1*) from *Arabidopsis* was strongly up- regulated in RM.

Since phytohormones can mediate plant immune responses and resistance induction (Pieterse et al., 2009; 2014), we monitored the expression profiles of genes involved in Ethylene (ET), Salicylic Acid (SA), Jasmonic Acid (JA) and ABA metabolism. As observed in the shoots of mycorrhizal tomato plants (Fiorilli et al., 2009; Cervantes- Gàmez et al., 2016), among the most up-regulated genes we detected in LM were several enzymes involved in ET biosynthesis: *ACC synthase 1* (*S-adenosyl-L- methionine methylthioadenosine-lyase 1-, 1-aminocyclopropane-1-carboxylate oxidase; ACC oxidase 1* and *ACC oxidase 3*). Accordingly, in the RM sample, GO analysis showed the enrichment of DEPs involved in methionine and S-adenosylmethionine metabolism, both precursors in ET biosynthesis. Several lipoxygenase (LOX) genes and proteins, and 12-oxophytodienoate reductase 1, were modulated in both organs of M plants. LOXs are crucial for lipid peroxidation processes and contribute to JA (through the 13-LOX pathway) and oxylipin biosynthesis, which play a pivotal role as signalling and protective molecules in plants responding to biotic stress (Blee, 2002; Prost et al., 2005). In addition, *allene oxide synthase* (*AOS4*) and two *AOS2* genes, which catalyse the first step in the biosynthesis of JA, were highly expressed only in leaves of M plants. Comparison of leaves and roots of M and C plants found that genes involved in SA biosynthesis or response were not differentially expressed. When M plants were compared with C plants, *9-cis-epoxycarotenoid dioxygenase* (*NCED*), involved in *de novo* ABA biosynthesis, was strongly up-regulated in both roots and leaves. Several genes involved in ABA catabolism (e.g., *ABA 8'-hydroxylase* and *ABA-responsive protein-related*) were also up-regulated in both organs.

Proteomic results indicated the involvement of gibberellin (GA) in mycorrhization, with the induction of the GA receptor GID1L2 in RM and LM, and Ent-kaurenoic acid oxidase, involved in GA biosynthesis, in RM.

Taken as a whole, the dynamics of wheat phytohormones levels following *F. mosseae* inoculation confirms the scenario already described for other AM-host plant interactions (Foo et al., 2013; Gutjahr, 2014; Pozo et al., 2015).

*Local and systemic induction of defence genes and proteins in mycorrhizal plants*

Although several studies have reported that defence reactions are activated in roots during the establishment of AM symbiosis, few have investigated such responses at the systemic level (Pozo and Azcón-Aguilar, 2007). Analysis of the over-represented GO categories, detected in the comparison RM vs. RC, highlighted the strong activation of genes involved in oxidation-reduction processes (Fig. S6). Also at the proteomic level, some ROS-scavenging enzymes, such as glutathione S-transferase (GST), ascorbate peroxidase, catalase, peroxiredoxin IIF, glutaredoxin and gamma- glutamyltranspeptidase 1, were differentially expressed in RM vs. RC. M roots showed a significant up-regulation of some proteins involved in plant defence, including three acidic endochitinases, elicitor responsive gene 3, hypersensitive-induced reaction protein 3 and the cysteine-rich receptor-like protein kinases, Crk 25 and Crk6. *crk25* mutants are less resistant to pathogen infection (Bourdais et al., 2015), while Crk6 is required for *X. oryzae*- and BTH-induced immune responses (Chern et al., 2016). Several Germin-like genes and proteins (GLPs) were up-regulated in RM. GLPs, which are involved in defence responses, such as the oxidative burst, are up-regulated in roots following mycorrhizal colonisation (Güimil et al., 2005; Liu et al., 2007; Fiorilli et al., 2015; Song et al., 2015).

In the comparison LM vs. LC, we identified 29 genes as exclusively expressed in the leaves of M plants and which are known to be involved in responses to biotic stress. These include a pathogenesis-related protein *PR-1*, three barley mildew resistance locus o (*MLO*) genes and putative homologs of Resistance to *Pseudomonas syringae* pv. maculicola 1 (*RPM1*) protein (Table S13). We also identified the induction of *Riboflavin synthase-like* (*RS*), an enzyme involved in the last steps of riboflavin biosynthesis. Riboflavin has been related to defence priming in *Arabidopsis* (Zhang et al., 2009; 2014; 2015), and it was also found to be over-accumulated in leaves of M tomato plants following *Botrytis cinerea* infection, suggesting its involvement in MIR (Sanchez-Bel et al., 2016). Systemic responses were confirmed by proteomic analysis of LM vs. LC, where an increase in some proteins involved in response to biotic stimuli, such as chitinase, PR protein, GLP and phenylalanine ammonia-lyase, was detected. Some enzymes involved in cell wall remodelling accumulated in the LM sample: they included the Beta-D-xylosidases 6 and 7, beta glucanase, beta-glucosidase 6, GDP-D-mannose 4,6-dehydratase and the non-specific lipid-transfer protein LPT142, the last of which might play a role in wax or cutin deposition and in intracellular signal transduction. Finally, we found an increase in two PPIases that play a role in plant responses to heat, salinity and oxidative stress. Recent studies demonstrate that PPIases participate in responses to biotic stress in several plants, including wheat (Yang et al., 2016).

When compared with their respective controls (LC and LCX), leaf protein profile 4A in both the LM and LMX samples exhibited induction of several proteins putatively involved in plant defence. Among them, we found W5ALA0, a protein highly homologous to ERDJ3B in *Arabidopsis*, involved in regulating protein folding in the endoplasmic reticulum lumen and which is necessary for pathogen-associated molecular pattern (PAMP)-triggered immunity (Nekrasov et al., 2009);

W5B9I7, a protein showing 80% identity with NAA10 in *Arabidopsis*, and which is involved in plant immunity by maintaining homeostasis of the immune receptors SNC1 and RPM1 (Xu et al., 2015); and the Cell Division Cycle 5-like protein (CDC5) that, in addition to its function during mitosis, is also a core component of protein complexes that positively regulate defence responses through splicing and small RNA processing (Halter et al., 2015).

In conclusion, mycorrhizal symbiosis leads to the activation of many defence-related genes not only in roots, but also in leaves of wheat. Taken as a whole, these genetic determinants, some never reported previously in an AM symbiosis, could predispose wheat to priming (Gianinazzi, 1991; Pozo et al., 2009).

*Transcriptomic and proteomic changes induced by a leaf pathogen in wheat leaves and roots in the presence of an AM fungus*

Our results (Fig. 2a, b) revealed that the AM fungus exerted a bio-protective effect against the wheat leaf pathogen *X. translucens*. Although wheat responses to this pathogen have already been investigated (Garcia-Seco et al., 2017), here we explored whether the pathogen triggered changes in transcript and proteomic profiles of leaves and roots in M plants, with special attention to genes related to pathogen resistance. For the "omics" experiment, we inoculated leaves of C and M plants with *X. translucens* (49 days following inoculation with the AM fungus) and collected the infiltrated zones and roots after 24 h. Global transcriptional and proteomic analyses were performed on both organs using RNA-seq and LC-MS/MS, respectively.

To gain an overview of the differentially expressed transcripts and proteins, we analysed the DEGs and DEPs obtained comparing leaves and roots of C and M plants following pathogen infection (LMX vs. LCX; RMX vs. RCX). In leaves,

we detected 28 up- and 69 down-regulated genes (Table S14), and 96 up- and 196 down-regulated proteins (Table S15) (Fig. 6 a-d). Omics data revealed an unexpected metabolic repression in both plant organs when the pathogen was inoculated on mycorrhizal plants. In this LMX vs. LCX comparison, no over-represented biological process GO terms were identified; however, in LM plants, the genes and proteins repressed during pathogen infection are mainly involved in AA metabolism (i.e., 3-deoxy-7- phosphoheptulonate synthase, Aspartate aminotransferase; asparagine synthetase, glutamine-dependent asparagine synthetase), and defence responses (i.e., PR proteins, GSTs, defensin genes). The core set of 29 genes expressed exclusively in LM plants and involved in responses to biotic stress (Table S13) was not differentially expressed in the other comparisons LMX vs. LM, LMX vs. LCX and LCX vs. LC. This expression profile probably reflects the general down-regulation of plant metabolism imposed by the pathogen rather than any active role in the MIR revealed by the reduced lesions (Fig. 2).

The most up-regulated gene in LMX vs. LCX was *premnaspirodiene oxygenase*, a cytochrome P450 enzyme, which functions in heme-iron binding and possesses oxidoreductase activity. The translated protein shared 80% identity with OsCYP71 from rice. *OsCYP71* is involved in early responses against *Xanthomonas oryzae pv. oryzae* (*Xoo*), and *OsCYP71*-overexpression lines confer resistance to *Xoo* (Niño et al., 2016). Interestingly, this transcript was strongly down-regulated in LCX vs. LC (Garcia-Seco et al., 2017). Another cytochrome P450 monooxygenase (CYP99A2), a precursor of the diterpenoid phytoalexins momilactones A and B, was also strongly induced in LMX vs. LCX. Momilactones have been well characterised, and antimicrobial activity against fungal pathogens has been reported (Tamogami and Kodama, 2000, Hasegawa et

al., 2010). In addition, OsCYP99A2 is involved in benzothiadiazole (BTH)-mediated priming in rice leaves (Akagi et al., 2014).

Nutrient availability has a strong impact on plant defence (Pasto et al., 2014 a,b), and nutrient uptake is a major factor in AM symbiosis (Fellbaum et al., 2012). In agreement with this statement, in the LMX vs. LCX comparison, we identified the up-regulation of nitrate/chlorate transporters (NTRs). Leaf protein profile 6A (Fig. 4) shows the exclusive induction in LMX of several proteins involved in plant defence responses, including two glucanases and cinnamoyl- CoA reductase 2, which were suggested to participate in the hypersensitive response to *X. campestris* (Lauvergeat et al., 2001). H3.3, a protein that regulates gene body DNA methylation, belongs to the same profile. H3.3 knockdown in *Arabidopsis* reduces transcription of genes responsive to environmental cues (Wollmann et al., 2017). In addition, some proteins involved in cell redox homeostasis, such as glutathione peroxidase and PDIL5, were up-regulated in LMX vs. LCX.

To evaluate the systemic impact of leaf pathogen infection on the root, we analysed the DEGs and DEPs obtained in the RMX vs. RM comparison. We detected 1174 up- and 1531 down-regulated genes (Table S16), and 223 up- and 288 down-regulated proteins (Table S17). As expected, all the putative homologs of the AM marker genes (i.e., PTs and AMT genes) identified in the RM vs. RC comparison were also detected in the RMX treatment; there was even a slight up-regulation of the genes in RMX vs. RM. This up- regulation could be related to the slight increase in fungal colonisation detected in RMX vs. RM plants. Analysis of over-represented GO categories among the DEGs detected in the comparison of RMX vs. RM highlighted a statistically significant enrichment of genes involved in responses to stress, including L-phenylalanine catabolic process, response to chitin, response to wounding, detection of biotic stimuli and defence response to fungi and bacteria (Fig. S7). The most up-regulated genes

were as follows: a predicted protein (Traes_2AL_3A3918F92.1) that shares 85% identity with a predicted antimicrobial peptides (AMPs) 1 protein from *Brachypodium distachyon*, which is involved in non-specific host defence (Nawrot et al., 2014), and three PR proteins. A huge perturbation of genes belonged to the oxidation-reduction process category (98 up- and 88 down-regulated), and a down-regulation of genes involved in the L- phenylalanine catabolic process was also observed.

Proteomic analysis revealed a statistically significant enrichment of DEPs involved in protein folding and ATP synthesis. In addition, GSTs, peroxidases, acidic endochitinases and heat shock proteins were increased (Table S16). To understand whether these responses were related to specific pathogen-induced oxidative damage, we compared the protein carbonylation profiles in leaves of M, C, CX and MX plants. Twenty-four hpi, both samples exposed to *X. translucens* (LCX and LMX) exhibited reduced protein carbonylation compared with non-infected plants (LC and LCX). This suggests that the antioxidant defence systems in LCX and LMX were able to cope with the oxidative stress induced by pathogen infection, thereby avoiding irreversible protein oxidation. However, LMX plants showed a stronger ROS- scavenging ability than LCX plants (Fig. 7A). Fourteen dpi, the level of oxidative damage in LCX and LMX plants was higher than in LM and LC plants. Again, the level of carbonylated proteins was consistently lower in LMX plants than LCX plants (Fig. 7B).

Overall, these results suggest that the AM fungus alleviates the symptoms caused by *X. translucens* through a broad down-regulation of transcript and protein levels, particularly evident in leaves. Here, a change in the level of protein oxidation, and a specific activation of genes involved in disease resistance and nutrient transport, suggests a specific MIR reaction.

Fourteen days after *X. translucens* inoculation, the content of most AAs was strongly reduced in LCX plants; the decrease was less pronounced in LMX plants (Table 2). These data validate the transcriptomic and proteomic results, showing that cellular AA metabolism was down-regulated in the LMX vs. LCX treatments.

## Discussion

Although wheat is one of the most important sources for food, animal feed and industrial raw materials, limited attention has been paid to its response to AM fungi. This is mainly due to the difficulty in overcoming the challenges imposed by the large wheat genome, comprising three complete genomes, multiple gene duplications and extensive regions of suppressed recombination (IWGSC, 2014). These genome features make traditional genetic tools, such as mutant availability and genetic transformation protocols, time-consuming and costly, making this host plant less suitable for the functional characterisation of the AM symbiosis.

### *AM symbiosis leads to short- and long-term benefits in wheat*

The high level of genetic diversity of wheat is mirrored by a diverse responsiveness to AM symbiosis when different genotypes are considered (Kapulnik and Kushnir et al., 1981; Hetrick et al., 1992; Zhu et al., 2001; Singh et al., 2012; Lehnert et al., 2017), and by contrasting results in terms of perceived benefits (i.e., biomass and nutrient acquisition) (Ryan et al., 2005; Al-Karaki et al., 2004; Pellegrino et al., 2013). Our greenhouse experiments revealed that all measured agronomic traits were higher in mycorrhizal wheat (cv. Chinese Spring) plants, including an improvement in nutrient and AA content of seeds. The higher levels of Mg, Zn and P that were detected in seeds suggest potential benefits to the offspring, such as faster growth, a better chance of surviving in mineral-

deficient soils and, especially for crops, improved grain yield and quality (Rengel and Graham et al., 1995; Ekiz et al., 2008; White and Venneklass, 2012; Gerendàs and Führs, 2013; Chen et al., 2016). Although increases in seed size, yield and germination rate have been observed in different plant species in symbiosis with AM fungi (Lewis and Koide, 1990; Koide and Lu, 1995; Heppell et al., 1998; Allison, 2002; Poulton et al., 2002; Berta et al., 2014; Bona et al., 2016), effects on wheat grain have received little attention. Our results demonstrate substantial changes in seed morphology and nutrient content, opening the way to further investigations of long-term benefits for offspring and of transgenerational mycorrhizal effects on progeny phenotype.

By determining genome-wide transcriptional changes during mycorrhizal establishment, DEGs involved in numerous processes in plants were identified. In both dicots and monocots, massive transcriptional changes occur during mycorrhizal establishment, and a core of AM symbiosis marker genes, considered the functional signature of the symbiosis, have been identified (i.e., Güimil et al., 2005; Fiorilli et al., 2009; Gomez et al., 2009; Handa et al., 2015; Fevre et al., 2014). Focusing on rice, for which data sets from whole-genome transcript profiling of mycorrhizal roots are available (Güimil et al., 2005; Guthjar et al., 2015; Fiorilli et al., 2015), we wondered whether the well- annotated rice genome could provide a template to decipher AM responses in wheat. Comparative analysis highlighted an extensive overlap in the transcriptional responses of rice and wheat mycorrhizal roots, allowing us to decipher several AM marker genes in wheat roots. Among them, several nutrient transporter genes, including PTs and AMTs, were identified, as confirmed by the increased content of several mineral nutrients in roots and leaves. Mg and Fe contents were lower in RM plants and higher in LM plants, suggesting a root to aboveground organ translocation. Considering that Fe and Mg are essential macronutrients involved in numerous

physiological processes, including photosynthetic $CO_2$ fixation, biomass production and grain yield (Ravet et al., 2012; Cakmak, 2013; Briat et al., 2015; Jezek et al., 2015), it is tempting to speculate that AM symbiosis might also improve wheat development via Fe and Mg acquisition. These data support the concept that positive mycorrhizal growth responses arise largely from the increased uptake of growth-limiting nutrients (Smith and Read, 2008). Our work also reports a strong impact exerted by the AM fungus on primary metabolism and phytohormones regulation of the host plant at both the local and systemic level, suggesting that other metabolic pathways are involved in these growth responses.

Meyer and colleagues (2007) proposed that the increased biomass in mycorrhizal *Arabidopsis* is orchestrated by a combination of metabolites that act synergistically. Among them, sugars such as sucrose, members of the TCA cycle and metabolites involved in membrane/phospholipid biosynthesis such as G3P are involved in the control of plant growth and development. Several wheat genes and proteins involved in these carbohydrate and lipid pathways were strongly induced in both RM and LM plants, suggesting that the increased biomass in AM-infected plants is related to the general mechanisms described for *A. thaliana*.

In conclusion, both greenhouse and growth chamber experiments demonstrated growth benefits to wheat following AM inoculation. The omics results confirm previous findings (e.g., improved expression of nutrient transporters), but also provide novel information: a complex pattern of local and systemic changes in gene expression with similarities and differences between roots and leaves, changes in nutrient and AA contents, and effects on seeds.

*At least two modalities guarantee the bio-protection effect exerted by AM symbiosis against a leaf pathogen*

The bio-protective role of AM symbiosis against crop pathogens has long been recognised on the basis of experimental data showing significant reductions in disease symptoms (Fritz et al., 2006; Fiorilli et al., 2011; Campos-Soriano et al., 2012; Maffei et al., 2014). The molecular basis of such protective responses includes elicitation of defence-responsive genes, accumulation of defensive compounds at both local and systemic levels (Spanu et al., 1989; Dumas-Gaudot et al., 2000; Pozo et al., 2002; Gange, 2006; Liu et al., 2007; Fiorilli et al., 2009; Campos-Soriano et al., 2012, Jung et al., 2012) and modulation of defence-related hormones (Pozo and Azcon-Aguilar, 2007; Van Wees et al., 2008; Van der Ent et al., 2009; Pozo et al., 2010; Jung et al., 2012; Song et al., 2015; Mora-Romero et al., 2015).

In leaves of mycorrhizal rice plants, a two-step mechanism has been proposed to control the enhanced resistance against aboveground pathogens: a preliminary induction of defence mechanisms in the absence of the pathogen, followed by a faster and stronger activation of *PR* genes upon pathogen infection (Campos-Soriano et al., 2012). Our experiments revealed that, in wheat, the AM-induced bio-protective effect requires the integration of multiple mechanisms. As with other host plants, wheat roots activated a set of ROS-scavenging enzymes, as well as acidic endochitinases and PR proteins. We detected 29 defence-responsive genes (e.g., *PR proteins*, *RPM1*, *MLO*) exclusively regulated in the leaves of mycorrhizal wheat, as well as genes and proteins (e.g., *RS*, GLP, PAL) involved in the categories "response to biotic stimuli" and "plant immunity". These data suggest that wheat activates a "*broad-spectrum defence*" response, where genes and proteins playing a regulatory role in the host immune system are activated. The differential regulation of genes involved in the biosynthesis of defence-related hormones (i.e., JA, ET and ABA) (Pieterse et al., 2009; 2014), as well as changes in AAs, whose role in orchestrating plant-microbe interactions (Moe,

2013) and the plant immune system (Zeier, 2013) is emerging, reveal that the "*broad-spectrum defence*" response is part of a larger reprogramming scenario induced by the presence of AM fungi in the roots. The main emerging question is to understand whether such a "broad-spectrum defence" response, which leads to a priming state, is sufficiently effective to provide protection against a leaf pathogen, i.e., to activate MIR (Pozo and Azcon-Aguilar, 2007; Cameron et al., 2014). Although some pathways systemically elicited by the AM fungus, and listed as a "*broad-spectrum defence*" response, were also maintained during interaction with the pathogen, our results reveal that, following pathogen infection, leaves of mycorrhizal plants strongly induce different defence genes and proteins that we identify as a "*pathogen-specific defence*" response. Even if there was a general repression of many metabolic pathways, to counter pathogen attack, LMX plants accumulated transcripts and proteins involved in redox homeostasis, phytoalexin production and the hypersensitive response, all of which represent a "*pathogen-specific defence*" response. An example of such a "*pathogen-specific defence*" response is the elevated expression of two cytochrome P450 monooxigenase genes (*premnaspirodiene oxygenase* and *CYP99A2*) in LMX plants that have been reported in other plants to play a key role in hampering bacterial infection and to be involved in the priming response (Niño et al., 2016; Tamogami and Kodama, 2000; Hasegawa et al., 2010; Akagi et al., 2014). Changes in the abundance of carbonylated proteins, considered to be markers of oxidative stress, indicate that, at 14 dpi, when lesions are clearly visible on leaves, the presence of the AM fungus in the roots lightens the symptoms caused by the pathogen. In summary, deep omics analysis supported by metabolic data showed that AM symbiosis confers on wheat higher productivity and resistance to *X. translucens* infection. The higher productivity is accompanied by local and systemic activation of pathways involved in nutrient uptake, primary metabolism and phytohormone regulation. These benefits seem

to have long-term effects, since they are also effective on seeds. In addition to this large-scale metabolic reprogramming, we also identified defence-related pathways that have been described previously in roots of many other AM plants, as well as a novel core set of genes exclusive to leaves of mycorrhizal plants. While this represents a "broad-spectrum defence", we also detected a second "*pathogen-specific defence*" response, which occurs following bacterial infection and promptly activates genes already identified as operational in resistance to *Xanthomonas*. Together with the regulation of phytohormones and AA biosynthesis, we suggest that this two-step response contributes to MIR.

In conclusion, we propose that the activation of a "broad-spectrum defence" response, occurring in roots and leaves of mycorrhizal plants, makes wheat ready to switch to a "pathogen-specific defence" response upon bacterial infection, leading eventually to effective protection against the invader.

## ACKNOWLEDGEMENTS

This work was supported by the MIC-CERES grant funded in the frame of the CERES initiative by Agropolis Cariplo Fondations. F.O. is a PhD student of the "Biotechnology, Biosciences and Surgical Technology" course at Università degli Studi dell'Insubria.

## AUTHOR CONTRIBUTIONS

V.F, D.G.S, LM design the experimental work. V.F, D.G.S, M.N, F.O. carried out the majority of the experiments. V.F. C.V., F.O., P.B. M.B. analysed the datasets and wrote the manuscript; V.T. and M.C. performed Greenhouse experiments;

P.Bagn. performed the bioinformatics data analysis. P.B. and M.B. coordinate the project. All authors read and approved the final manuscript.

## REFERENCES

**Adolfsson L, Nziengui H, Abreu IN, Šimura J, Beebo A, Herdean A, Aboalizadeh J, Široká J, Moritz T, Novák O, Ljung K, Schoefs B, Spetea C** (2017) Enhanced Secondary- and Hormone Metabolism in Leaves of Arbuscular Mycorrhizal Medicago truncatula. Plant Physiology **175:** 392-411

**Akagi A, Fukushima S, Okada K, Jiang CJ, Yoshida R, Nakayama A, Shimono M, Sugano S, Yamane H, Takatsuji H** (2014) WRKY45-dependent priming of diterpenoid phytoalexin biosynthesis in rice and the role of cytokinin in triggering the reaction. Plant Mol Biol **86:** 171-183

**Al-Karaki G, McMichael B, Zak J** (2004) Field response of wheat to arbuscular mycorrhizal fungi and drought stress. Mycorrhiza **14:** 263-269

**Allison VJ** (2002) Nutrients, arbuscular mycorrhizas and competition interact to influence seed production and germination success in Achillea millefolium. Functional Ecology **16:** 742-749

**Aroca R, Del Mar Alguacil M, Vernieri P, Ruiz-Lozano JM** (2008) Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA-deficient mutant (sitiens). Microb Ecol **56:** 704-719

**Bagnaresi P, Bonfante P** (2014) From root to fruit: RNA-Seq analysis shows that arbuscular mycorrhizal symbiosis may affect tomato fruit metabolism. BMC Genomics **15:** 221

**Bago B, Pfeffer PE, Douds DD, Brouillette J, Bécard G, Shachar-Hill Y** (1999) Carbon Metabolism in Spores of the Arbuscular Mycorrhizal Fungus

Glomus intraradices as Revealed by Nuclear Magnetic Resonance Spectroscopy. Plant Physiology **121:** 263-272

**Baloglu MC, Inal B, Kavas M, Unver T** (2014) Diverse expression pattern of wheat transcription factors against abiotic stresses in wheat species. Gene **550:** 117-122

**Benabdellah K, Azcón-Aguilar C, Ferrol N** (2000) Alterations in the plasma membrane polypeptide pattern of tomato roots (Lycopersicon esculentum) during the development of arbuscular mycorrhiza. Journal of Experimental Botany **51:** 747-754

**Bennetzen JL** (2005) Transposable elements, gene creation and genome rearrangement in flowering plants. Curr Opin Genet Dev **15:** 621-627

**Berta G, Copetta A, Gamalero E, Bona E, Cesaro P, Scarafoni A, D'Agostino G** (2014) Maize development and grain quality are differentially affected by mycorrhizal fungi and a growth-promoting pseudomonad in the field. Mycorrhiza **24:** 161-170

**Blée E** (2002) Impact of phyto-oxylipins in plant defense. Trends Plant Sci **7:** 315-322

**Bona E, Scarafoni A, Marsano F, Boatti L, Copetta A, Massa N, Gamalero E, D'Agostino G, Cesaro P, Cavaletto M, Berta G** (2016) Arbuscular mycorrhizal symbiosis affects the grain proteome of Zea mays: a field study. Scientific Reports **6:** 26439

**Bonfante P, Genre A** (2015) Arbuscular mycorrhizal dialogues: do you speak 'plantish' or 'fungish'? Trends Plant Sci **20:** 150-154

**Bonneau J, Baumann U, Beasley J, Li Y, Johnson AA** (2016) Identification and molecular characterization of the nicotianamine synthase gene family in bread wheat. Plant Biotechnol J **14:** 2228-2239

**Bourdais G, Burdiak P, Gauthier A, Nitsch L, Salojärvi J, Rayapuram C, Idänheimo N, Hunter K, Kimura S, Merilo E, Vaattovaara A, Oracz K, Kaufholdt D, Pallon A, Anggoro DT, Glów D, Lowe J, Zhou J, Mohammadi O, Puukko T, Albert A, Lang H, Ernst D, Kollist H, Brosché M, Durner J, Borst JW, Collinge DB, Karpiński S, Lyngkjær MF, Robatzek S, Wrzaczek M, Kangasjärvi J, Consortium C** (2015) Large-Scale Phenomics Identifies Primary and Fine-Tuning Roles for CRKs in Responses Related to Oxidative Stress. PLoS Genet **11:** e1005373

**Bravo A, Brands M, Wewer V, Dörmann P, Harrison MJ** (2017) Arbuscular mycorrhiza-specific enzymes FatM and RAM2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. New Phytol **214:** 1631-1645

**Briat J-F, Rouached H, Tissot N, Gaymard F, Dubos C** (2015) Integration of P, S, Fe, and Zn nutrition signals in Arabidopsis thaliana: potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). Frontiers in Plant Science **6:** 290

**Cakmak I** (2013) Magnesium in crop production, food quality and human health. Plant and Soil **368:** 1-4

**Cameron DD, Neal AL, van Wees SC, Ton J** (2013) Mycorrhiza-induced resistance: more than the sum of its parts? Trends Plant Sci **18:** 539-545

**Campos-Soriano L, García-Martínez J, San Segundo B** (2012) The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. Mol Plant Pathol **13:** 579-592

**Campos-Soriano L, Segundo BS** (2011) New insights into the signaling pathways controlling defense gene expression in rice roots during the arbuscular mycorrhizal symbiosis. Plant Signal Behav **6:** 553-557

**Cervantes-Gámez RG, Bueno-Ibarra MA, Cruz-Mendívil A, Calderón-Vázquez CL, Ramírez-Douriet CM, Maldonado-Mendoza IE, Villalobos-**

**López MÁ, Valdez-Ortíz Á, López-Meyer M** (2016) Arbuscular Mycorrhizal Symbiosis-Induced Expression Changes in Solanum lycopersicum Leaves Revealed by RNA-seq Analysis. Plant Molecular Biology Reporter **34:** 89-102

**Chen PS TT, Warner H** (1956) Microdetermination of Phosphorus. Analytical chemistry **28:** 1756–1758

**Chern M, Xu Q, Bart RS, Bai W, Ruan D, Sze-To WH, Canlas PE, Jain R, Chen X, Ronald PC** (2016) A Genetic Screen Identifies a Requirement for Cysteine-Rich–Receptor-Like Kinases in Rice NH1 (OsNPR1)-Mediated Immunity. PLoS Genetics **12:** e1006049

**Conrath U, Beckers GJM, Langenbach CJG, Jaskiewicz MR** (2015) Priming for Enhanced Defense. Annual Review of Phytopathology, Vol 53 **53:** 97-119

**de la Peña E, Echeverría SR, van der Putten WH, Freitas H, Moens M** (2006) Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass Ammophila arenaria. New Phytol **169:** 829-840

**Duan J, Tian H, Drijber RA, Gao Y** (2015) Systemic and local regulation of phosphate and nitrogen transporter genes by arbuscular mycorrhizal fungi in roots of winter wheat (Triticum aestivum L.). Plant Physiol Biochem **96:** 199-208

**Dumas-Gaudot E, Gollotte A, Cordier C, Gianinazzil S, Gianinazzi-Pearson V** (2000) Modulation of Host Defence Systems. *In* Arbuscular Mycorrhizas: Physiology and Function. Springer Netherlands, Dordrecht, pp 173-200

**Ekiz H, Bagci SA, Kiral AS, Eker S, Gultekin I, Alkan A, Cakmak I** (1998) Effects of zinc fertilization and irrigation on grain yield and zinc concentration of various cereals grown in zinc-deficient calcareous soils. Journal of Plant Nutrition **21:** 2245-2256

**Fellbaum CR, Gachomo EW, Beesetty Y, Choudhari S, Strahan GD, Pfeffer PE, Kiers ET, Bücking H** (2012) Carbon availability triggers fungal nitrogen

uptake and transport in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A **109:** 2666-2671

**Fiorilli V, Catoni M, Miozzi L, Novero M, Accotto GP, Lanfranco L** (2009) Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus. New Phytol **184:** 975-987

**Fiorilli V CM, Francia D, Cardinale F, Lanfranco L** (2011) The arbuscular mycorrhizal symbiosis reduces disease severity in tomato plants infected by *Botrytis cinerea*. *In*, Vol 93. Journal of Plant Pathology, pp 237-242

**Fiorilli V, Vallino M, Biselli C, Faccio A, Bagnaresi P, Bonfante P** (2015) Host and non-host roots in rice: cellular and molecular approaches reveal differential responses to arbuscular mycorrhizal fungi. Frontiers in Plant Science **6:** 636

**Foo E, Ross JJ, Jones WT, Reid JB** (2013) Plant hormones in arbuscular mycorrhizal symbioses: an emerging role for gibberellins. Annals of Botany **111:** 769-779

**Fritz M, Jakobsen I, Lyngkjær MF, Thordal-Christensen H, Pons-Kühnemann J** (2006) Arbuscular mycorrhiza reduces susceptibility of tomato to Alternaria solani. Mycorrhiza **16:** 413

**Gange AC** (2007) Insect-mycorrhizal interactions: Patterns, processes, and consequences. Cambridge University Press, London

**Garcia-Seco D, Chiapello M, Bracale M, Pesce C, Bagnaresi P, Dubois E, Moulin L, Vannini C, Koebnik R** (2017) Transcriptome and proteome analysis reveal new insight into proximal and distal responses of wheat to foliar infection by Xanthomonas translucens. Sci Rep **7:** 10157

**Gerendás J, Führs H** (2013) The significance of magnesium for crop quality. Plant and Soil **368:** 101-128

**Gernns H, von Alten H, Poehling HM** (2001) Arbuscular mycorrhiza increased the activity of a biotrophic leaf pathogen - is a compensation possible? Mycorrhiza **11:** 237-243

**Gianinazzi S** (1991) Vesicular–arbuscular (endo)mycorrhizas: cellular, biochemical and genetic aspects. Agriculture, Ecosystems and Environment **35:** 105–119.

**Giovannetti M, Mari A, Novero M, Bonfante P** (2015) Early Lotus japonicus root transcriptomic responses to symbiotic and pathogenic fungal exudates. Front Plant Sci **6:** 480

**Gobbato E, Marsh JF, Vernié T, Wang E, Maillet F, Kim J, Miller JB, Sun J, Bano SA, Ratet P, Mysore KS, Dénarié J, Schultze M, Oldroyd GE** (2012) A GRAS-type transcription factor with a specific function in mycorrhizal signaling. Curr Biol **22:** 2236-2241

**Gomez SK, Javot H, Deewatthanawong P, Torres-Jerez I, Tang Y, Blancaflor EB, Udvardi MK, Harrison MJ** (2009) Medicago truncatula and Glomus intraradices gene expression in cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. BMC Plant Biol **9:** 10

**Guether M, Neuhäuser B, Balestrini R, Dynowski M, Ludewig U, Bonfante P** (2009) A mycorrhizal-specific ammonium transporter from Lotus japonicus acquires nitrogen released by arbuscular mycorrhizal fungi. Plant Physiol **150:** 73-83

**Gutjahr C** (2014) Phytohormone signaling in arbuscular mycorhiza development. Curr Opin Plant Biol **20:** 26-34

**Gutjahr C, Parniske M** (2013) Cell and developmental biology of arbuscular mycorrhiza symbiosis. Annu Rev Cell Dev Biol **29:** 593-617

**Gutjahr C, Radovanovic D, Geoffroy J, Zhang Q, Siegler H, Chiapello M, Casieri L, An K, An G, Guiderdoni E, Kumar CS, Sundaresan V, Harrison MJ, Paszkowski U** (2012) The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. Plant J **69:** 906-920

**Gutjahr C, Sawers RJ, Marti G, Andrés-Hernández L, Yang SY, Casieri L, Angliker H, Oakeley EJ, Wolfender JL, Abreu-Goodger C, Paszkowski U** (2015) Transcriptome diversity among rice root types during asymbiosis and interaction with arbuscular mycorrhizal fungi. Proc Natl Acad Sci U S A **112:** 6754-6759

**Güimil S, Chang HS, Zhu T, Sesma A, Osbourn A, Roux C, Ioannidis V, Oakeley EJ, Docquier M, Descombes P, Briggs SP, Paszkowski U** (2005) Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization. Proc Natl Acad Sci U S A **102:** 8066-8070

**Güther M, Volpe V, Balestrini R, Requena N, Wipf D, Bonfante P** (2011) LjLHT1.2—a mycorrhiza-inducible plant amino acid transporter from Lotus japonicus,

**Halter T, Navarro L** (2015) Multilayer and interconnected post-transcriptional and co-transcriptional control of plant NLRs. Curr Opin Plant Biol **26:** 127-134

**Handa Y, Nishide H, Takeda N, Suzuki Y, Kawaguchi M, Saito K** (2015) RNA-seq Transcriptional Profiling of an Arbuscular Mycorrhiza Provides Insights into Regulated and Coordinated Gene Expression in Lotus japonicus and Rhizophagus irregularis. Plant Cell Physiol **56:** 1490-1511

**Hasegawa M, Mitsuhara I, Seo S, Imai T, Koga J, Okada K, Yamane H, Ohashi Y** (2010) Phytoalexin accumulation in the interaction between rice and the blast fungus. Mol Plant Microbe Interact **23:** 1000-1011

**Hayek S, Gianinazzi-Pearson V, Gianinazzi S, Franken P** (2014) Elucidating mechanisms of mycorrhiza-induced resistance against Thielaviopsis basicola via targeted transcript analysis of Petunia hybrida genes. Physiological and Molecular Plant Pathology **88:** 67-76

**Heppell KB, Shumway DL, Koide RT** (1998) The effect of Mycorrhizal infection of Abutilon theophrasti on competitiveness of offspring. Functional Ecology **12:** 171-175

**Hetrick BAD, Wilson GWT, Cox TS** (1992) Mycorrhizal dependence of modern wheat varieties, landraces, and ancestors. Canadian Journal of Botany **70:** 2032-2040

**Hildebrandt U, Regvar M, Bothe H** (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry **68:** 139-146

**Hildebrandt U, Schmelzer E, Bothe H** (2002) Expression of nitrate transporter genes in tomato colonized by an arbuscular mycorrhizal fungus. Physiologia Plantarum **115:** 125-136

**Huang XY, Deng F, Yamaji N, Pinson SR, Fujii-Kashino M, Danku J, Douglas A, Guerinot ML, Salt DE, Ma JF** (2016) A heavy metal P-type ATPase OsHMA4 prevents copper accumulation in rice grain. Nat Commun **7:** 12138

**Inal B, Türktaş M, Eren H, Ilhan E, Okay S, Atak M, Erayman M, Unver T** (2014) Genome-wide fungal stress responsive miRNA expression in wheat. Planta **240:** 1287-1298

**Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ** (2007) A Medicago truncatula phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proceedings of the National Academy of Sciences **104:** 1720-1725

**Jezek M, Geilfus CM, Bayer A, Muehling KH** (2015) Photosynthetic capacity, nutrient status, and growth of maize (Zea mays L.) upon MgSO4 leaf-application,

**Jiang Y, Wang W, Xie Q, Liu N, Liu L, Wang D, Zhang X, Yang C, Chen X, Tang D, Wang E** (2017) Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. Science **356:** 1172-1175

**Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ** (2012) Mycorrhiza-induced resistance and priming of plant defenses. J Chem Ecol **38:** 651-664

**Kapulnik Y, Kushnir U** (1991) Growth dependency of wild, primitive and modern cultivated wheat lines on vesicular-arbuscular mycorrhiza fungi. Euphytica **56:** 27-36

**Keymer A, Pimprikar P, Wewer V, Huber C, Brands M, Bucerius SL, Delaux P-M, Klingl V, von Röpenack-Lahaye E, Wang TL, Eisenreich W, Dörmann P, Parniske M, Gutjahr C** (2017) Lipid transfer from plants to arbuscular mycorrhiza fungi. eLife **6:** e29107

**Koide RT, Lu XH** (1995) On the cause of offspring superiority conferred by mycorrhizal infection of Abutilon theophrasti. New Phytologist **131:** 435-441

**Lauvergeat V, Lacomme C, Lacombe E, Lasserre E, Roby D, Grima-Pettenati J** (2001) Two cinnamoyl-CoA reductase (CCR) genes from Arabidopsis thaliana are differentially expressed during development and in response to infection with pathogenic bacteria. Phytochemistry **57:** 1187-1195

**Lehnert H, Serfling A, Enders M, Friedt W, Ordon F** (2017) Genetics of mycorrhizal symbiosis in winter wheat (Triticum aestivum). New Phytol **215:** 779-791

**Lewis JD, Koide RT** (1990) Phosphorus supply, mycorrhizal infection and plant offspring vigor. *Functional Ecology* **4:** 695-702

**Li Q, Xie QG, Smith-Becker J, Navarre DA, Kaloshian I** (2006) Mi-1-Mediated aphid resistance involves salicylic acid and mitogen-activated protein kinase signaling cascades. Mol Plant Microbe Interact **19:** 655-664

**Lingua G, D'Agostino G, Massa N, Antosiano M, Berta G** (2002) Mycorrhiza-induced differential response to a yellows disease in tomato. Mycorrhiza **12:** 191-198

**Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD, Harrison MJ** (2007) Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. Plant J **50:** 529-544

**Liu J, Wu L, Wei S, Xiao X, Su C, Jiang P, Song Z, Wang T, Yu Z** (2007) Effects of arbuscular mycorrhizal fungi on the growth, nutrient uptake and glycyrrhizin production of licorice (Glycyrrhiza uralensis Fisch). Plant Growth Regulation **52:** 29-39

**Luginbuehl LH, Menard GN, Kurup S, Van Erp H, Radhakrishnan GV, Breakspear A, Oldroyd GED, Eastmond PJ** (2017) Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. Science **356:** 1175-1178

**Maffei G, Miozzi L, Fiorilli V, Novero M, Lanfranco L, Accotto GP** (2014) The arbuscular mycorrhizal symbiosis attenuates symptom severity and reduces virus concentration in tomato infected by Tomato yellow leaf curl Sardinia virus (TYLCSV). Mycorrhiza **24:** 179-186

**Marjohn CN, Jae-Young S, Franz Marielle N, Me-Sun K, Yu Jin J, Kwon-Kyoo K, Illsup N, and Yong-Gu C** (2016) Overexpression of rice premnaspirodiene oxygenase reduces the infection rate of <italic>Xanthomonas oryzae</italic> pv. <italic>oryzae</italic>. J Plant Biotechnol **43:** 422-431

**Meyer RC, Steinfath M, Lisec J, Becher M, Witucka-Wall H, Törjék O, Fiehn O, Eckardt A, Willmitzer L, Selbig J, Altmann T** (2007) The metabolic signature related to high plant growth rate in Arabidopsis thaliana. Proc Natl Acad Sci U S A **104:** 4759-4764

**Miozzi L, Catoni M, Fiorilli V, Mullineaux PM, Accotto GP, Lanfranco L** (2011) Arbuscular mycorrhizal symbiosis limits foliar transcriptional responses to viral infection and favors long-term virus accumulation. Mol Plant Microbe Interact **24:** 1562-1572

**Moe LA** (2013) Amino acids in the rhizosphere: from plants to microbes. Am J Bot **100:** 1692-1705

**Mora-Romero GA, Gonzalez-Ortiz MA, Quiroz-Figueroa F, Calderon-Vazquez CL, Medina-Godoy S, Maldonado-Mendoza I, Arroyo-Becerra A,**

**Perez-Torres A, Alatorre-Cobos F, Sanchez F, Lopez-Meyer M** (2014) PvLOX2 silencing in common bean roots impairs arbuscular mycorrhiza-induced resistance without affecting symbiosis establishment. Functional Plant Biology **42:** 18-30

**Nawrot R, Barylski J, Nowicki G, Broniarczyk J, Buchwald W, Goździcka-Józefiak A** (2014) Plant antimicrobial peptides. Folia Microbiol (Praha) **59:** 181-196

**Nekrasov V, Li J, Batoux M, Roux M, Chu Z-H, Lacombe S, Rougon A, Bittel P, Kiss-Papp M, Chinchilla D, van Esse HP, Jorda L, Schwessinger B, Nicaise V, Thomma BPHJ, Molina A, Jones JDG, Zipfel C** (2009) Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. The EMBO Journal **28:** 3428-3438

**Oerke EC** (2006) Crop losses to pests. Journal of Agricultural Science **144:** 31-43

**Okay S, Derelli E, Unver T** (2014) Transcriptome-wide identification of bread wheat WRKY transcription factors in response to drought stress. Mol Genet Genomics **289:** 765-781

**Pastor V, Balmer A, Gamir J, Flors V, Mauch-Mani B** (2014) Preparing to fight back: generation and storage of priming compounds. Front Plant Sci **5:** 295

**Pastor V, Gamir J, Camañes G, Cerezo M, Sánchez-Bel P, Flors V** (2014) Disruption of the ammonium transporter AMT1.1 alters basal defenses generating resistance against Pseudomonas syringae and Plectosphaerella cucumerina. Frontiers in Plant Science **5:** 231

**Paszkowski U, Kroken S, Roux C, Briggs SP** (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A **99:** 13324-13329

**Pellegrino E, Bedini S** (2014) Enhancing ecosystem services in sustainable agriculture: Biofertilization and biofortification of chickpea (Cicer arietinum L.) by arbuscular mycorrhizal fungi. Soil Biology & Biochemistry **68:** 429-439

**Pellegrino E, Opik M, Bonari E, Ercoli L** (2015) Responses of wheat to arbuscular mycorrhizal fungi: A meta-analysis of field studies from 1975 to 2013,

**Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC** (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol **5:** 308-316

**Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA** (2014) Induced systemic resistance by beneficial microbes. Annu Rev Phytopathol **52:** 347-375

**Poulton JL, Bryla D, Koide RT, Stephenson AG** (2002) Mycorrhizal infection and high soil phosphorus improve vegetative growth and the female and male functions in tomato. New Phytologist **154:** 255-264

**Pozo M. J. JSC, López-Ráez J. A., Azcón-Aguilar C.** (2010) Impact of arbuscular mycorrhizal symbiosis on plant response to biotic stress: the role of plant defence mechanisms. *In* KY Koltai H., ed, Ar*buscular Mycorrhizas: Physiology and Function*. Springer, Amsterdam, pp 193–207

**Pozo MJ, Azcón-Aguilar C** (2007) Unraveling mycorrhiza-induced resistance. Curr Opin Plant Biol **10:** 393-398

**Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM, Azcón-Aguilar C** (2002) Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to Phytophthora infection in tomato plants. J Exp Bot **53:** 525-534

**Pozo MJ, López-Ráez JA, Azcón-Aguilar C, García-Garrido JM** (2015) Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. New Phytol **205:** 1431-1436

**Pozo MJ VA, Garcia–Andrade J, Garcia JM, Azcon-Aguilar C** (2009) Priming plant defence against pathogens by arbuscular mycorrhizal fungi. *In* BJ Azcon-Aguilar C, Gianinazzi S, Gianinazzi-Pearson V, ed, Mycorrhizas—functional processes and ecological impact. Springer, Heidleberg, pp 137–149

**Prost I, Dhondt S, Rothe G, Vicente J, Rodriguez MJ, Kift N, Carbonne F, Griffiths G, Esquerré-Tugayé MT, Rosahl S, Castresana C, Hamberg M, Fournier J** (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol **139:** 1902-1913

**Ravet K, Reyt G, Arnaud N, Krouk G, Djouani E-B, Boucherez J, Briat J-F, Gaymard F** (2012) Iron and ROS control of the DownSTream mRNA decay pathway is essential for plant fitness. The EMBO Journal **31:** 175-186

**Rengel Z, Graham RD** (1995) Importance of seed Zn content for wheat growth on Zn-deficient soil .2. grain-yield. *Plant and Soil* **173:** 267-274

**Ryan MH, Herwaarden AFv, Angus JF, Kirkegaard JA** (2005) Reduced growth of autumn-sown wheat in a low-P soil is associated with high colonisation by arbuscular mycorrhizal fungi. Plant and Soil **270:** 275-286

**Salvioli A, Zouari I, Chalot M, Bonfante P** (2012) The arbuscular mycorrhizal status has an impact on the transcriptome profile and amino acid composition of tomato fruit. BMC Plant Biology **12:** 44-44

**Singh AK, Hamel C, DePauw RM, Knox RE** (2012) Genetic variability in arbuscular mycorrhizal fungi compatibility supports the selection of durum wheat genotypes for enhancing soil ecological services and cropping systems in Canada. Canadian Journal of Microbiology **58:** 293-302

**Sirpiö S, Khrouchtchova A, Allahverdiyeva Y, Hansson M, Fristedt R, Vener AV, Scheller HV, Jensen PE, Haldrup A, Aro EM** (2008) AtCYP38 ensures early biogenesis, correct assembly and sustenance of photosystem II. Plant J **55:** 639-651

**Sisaphaithong T, Kondo D, Matsunaga H, Kobae Y, Hata S** (2012) Expression of plant genes for arbuscular mycorrhiza-inducible phosphate transporters and fungal vesicle formation in sorghum, barley, and wheat roots. Biosci Biotechnol Biochem **76:** 2364-2367

**Smith S.E. and Read D.J., (2008).** Mycorrhizal Symbiosis. *Academic Press*, San Diego and London.

**Song Y, Chen D, Lu K, Sun Z, Zeng R** (2015) Enhanced tomato disease resistance primed by arbuscular mycorrhizal fungus. Frontiers in Plant Science **6:** 786

**Song YY, Simard SW, Carroll A, Mohn WW, Zeng RS** (2015) Defoliation of interior Douglas-fir elicits carbon transfer and stress signalling to ponderosa pine neighbors through ectomycorrhizal networks. Sci Rep **5:** 8495

**Song YY, Simard SW, Carroll A, Mohn WW, Zeng RS** (2015) Defoliation of interior Douglas-fir elicits carbon transfer and stress signalling to ponderosa pine neighbors through ectomycorrhizal networks. Sci Rep **5:** 8495

**Spanu P, Boller T, Ludwig A, Wiemken A, Faccio A, Bonfante-Fasolo P** (1989) Chitinase in roots of mycorrhizal Allium porrum: regulation and localization. Planta **177:** 447-455

**Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, James TY, O'Donnell K, Roberson RW, Taylor TN, Uehling J, Vilgalys R, White MM, Stajich JE** (2016) A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia **108:** 1028-1046

**Tamogami S, Kodama O** (2000) Coronatine elicits phytoalexin production in rice leaves (Oryza sativa L.) in the same manner as jasmonic acid. Phytochemistry **54:** 689-694

**Tian T, Liu Y, Yan H, You Q, Yi X, Du Z, Xu W, Su Z** (2017) agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res

**Vallino M, Fiorilli V, Bonfante P** (2014) Rice flooding negatively impacts root branching and arbuscular mycorrhizal colonization, but not fungal viability. Plant Cell Environ **37:** 557-572

**Van der Ent S, Van Wees SC, Pieterse CM** (2009) Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. Phytochemistry **70:** 1581-1588

**van der Heijden MGA SI** (2002) Mycorrhizal Ecology,

**Van Wees SC, Van der Ent S, Pieterse CM** (2008) Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol **11:** 443-448

**Vos C, Schouteden N, van Tuinen D, Chatagnier O, Elsen A, De Waele D, Panis B, Gianinazzi-Pearson V** (2013) Mycorrhiza-induced resistance against the root-knot nematode Meloidogyne incognita involves priming of defense gene responses in tomato. Soil Biology & Biochemistry **60:** 45-54

**Wang E, Yu N, Bano SA, Liu C, Miller AJ, Cousins D, Zhang X, Ratet P, Tadege M, Mysore KS, Downie JA, Murray JD, Oldroyd GE, Schultze M** (2014) A H+-ATPase That Energizes Nutrient Uptake during Mycorrhizal Symbioses in Rice and Medicago truncatula. Plant Cell **26:** 1818-1830

**Whipps JM** (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Canadian Journal of Botany-Revue Canadienne De Botanique **82:** 1198-1227

**Whipps JM** (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Canadian Journal of Botany-Revue Canadienne De Botanique **82:** 1198-1227

**Willmann M, Gerlach N, Buer B, Polatajko A, Nagy R, Koebke E, Jansa J, Flisch R, Bucher M** (2013) Mycorrhizal phosphate uptake pathway in maize:

vital for growth and cob development on nutrient poor agricultural and greenhouse soils. Frontiers in Plant Science **4:** 533

**Wollmann H, Stroud H, Yelagandula R, Tarutani Y, Jiang D, Jing L, Jamge B, Takeuchi H, Holec S, Nie X, Kakutani T, Jacobsen SE, Berger F** (2017) The histone H3 variant H3.3 regulates gene body DNA methylation in Arabidopsis thaliana. Genome Biology **18:** 94

**Xu F, Huang Y, Li L, Gannon P, Linster E, Huber M, Kapos P, Bienvenut W, Polevoda B, Meinnel T, Hell R, Giglione C, Zhang Y, Wirtz M, Chen S, Li X** (2015) Two N-Terminal Acetyltransferases Antagonistically Regulate the Stability of a Nod-Like Receptor in Arabidopsis. The Plant Cell **27:** 1547-1562

**Xue L, Cui H, Buer B, Vijayakumar V, Delaux P-M, Junkermann S, Bucher M** (2015) Network of GRAS Transcription Factors Involved in the Control of Arbuscule Development in Lotus japonicus. Plant Physiology **167:** 854-871

**Yang S-Y, Grønlund M, Jakobsen I, Grotemeyer MS, Rentsch D, Miyao A, Hirochika H, Kumar CS, Sundaresan V, Salamin N, Catausan S, Mattes N, Heuer S, Paszkowski U** (2012) Nonredundant Regulation of Rice Arbuscular Mycorrhizal Symbiosis by Two Members of the PHOSPHATE TRANSPORTER1 Gene Family. The Plant Cell **24:** 4236-4251

**Yang Y, Yu Y, Bi C, Kang Z** (2016) Quantitative Proteomics Reveals the Defense Response of Wheat against Puccinia striiformis f. sp. tritici. Sci Rep **6:** 34261

**Zeier J** (2013) New insights into the regulation of plant immunity by amino acid metabolic pathways. Plant Cell Environ **36:** 2085-2103

**Zhang S, Yang X, Sun M, Sun F, Deng S, Dong H** (2009) Riboflavin-induced priming for pathogen defense in Arabidopsis thaliana. J Integr Plant Biol **51:** 167-174

**Zhang Y, Jin X, Ouyang Z, Li X, Liu B, Huang L, Hong Y, Zhang H, Song F, Li D** (2015) Vitamin B6 contributes to disease resistance against Pseudomonas syringae pv. tomato DC3000 and Botrytis cinerea in Arabidopsis thaliana. J Plant Physiol **175:** 21-25

**Zhang Y, Liu B, Li X, Ouyang Z, Huang L, Hong Y, Zhang H, Li D, Song F** (2014) The de novo biosynthesis of vitamin B6 is required for disease resistance against Botrytis cinerea in tomato. Mol Plant Microbe Interact **27:** 688-699

**Zhou X, Fornara D, Wasson EA, Wang DM, Ren GD, Christie P, Jia ZJ** (2015) Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland. Soil Biology & Biochemistry **91:** 76-83

**Zhu YG, Smith SE, Barritt AR, Smith FA** (2001) Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. Plant and Soil **237:** 249-255

| Agronomic traits | Treatments | |
|---|---|---|
| | Control | Myc |
| Number of tillers/plant | 9.95 ± 2.5 | 11.25 ± 1.86 |
| Vegetative tissue DW (g $pot^{-1}$) | 12.9 ± 5 | 17.6 ± 5.1 |
| Grain yield (g $pot^{-1}$) | 5.5 ± 2.4 | 8.4 ± 2.4 |
| Thousand kernel weight (g) | 30.15 ± 3.8 | 35.7 ± 2.4 |
| Kernel area ($mm^2$) | 15.06±1.01 | 16 ± 0.37 |
| Kernel major ellipse (mm) | 5.7 ± 0.2 | 5.8 ± 0.1 |
| Kernel minor ellipse (mm) | 2.7 ± 0.2 | 2.9 ± 0.1 |
| *F.mosseae* detection in root tissue by qRT-PCR | - | + |

**Table 1**. **The mean values of agronomic and qualitative traits are reported**. The mycorrhization of root tissues have been evaluated with qPCR assay and the results obtained are reported as absence (-) or presence (+) of *Funneliformis mosseae* DNA. ANOVA showed that all the measured agronomic traits, are significantly different between control and mycorrhizal (myc) plants.

| | 50 dpi | | 63 dpi | | |
|---|---|---|---|---|---|
| | **RM/RC** | **LM/LC** | **LM/LC** | **LX/LC** | **LMX/LX** |
| *Aspartic acid* | 1.34 | 0.96 | 0.81 | 0.78 | 0.89 |
| *Alanine* | nd | nd | 0.96 | **0.44*** | **2.57**** |
| *Arginine* | 2 | 0.88 | 1.37 | 0.74 | 0.77 |
| *Asparagine* | nd | **0.72**** | 0.90 | **0.29*** | **3.13*** |
| *Cystine* | 0.99 | 0.97 | **2.85*** | 1.37 | 1.66 |
| *Citrulline* | 0.93 | 1.16 | 0.86 | 0.75 | **0.46*** |
| *Ethanolamine* | 0.99 | 1.07 | **0.17*** | 0**.28*** | 1.47 |
| *Phenylalanine* | **1.19*** | **1.49*** | 1.11 | **0.32**** | **2.06*** |
| *GABA* | 0.72 | 1.23 | 1.30 | 0.95 | **1.52*** |
| *Glycine* | nd | nd | 0.74 | **0.32*** | **2.04*** |
| *Glutamine* | 0.68 | **0.52**** | **0.43*** | **0.19*** | **1.35*** |
| *Histidine* | 1.11 | 0.75 | 0.77 | **0.12**** | **1.80**** |
| *Leucine* | 1.62 | 0.7 | 1.11 | **0.21**** | **1.35**** |
| *Lysine* | **0.46*** | **0.42**** | **0.47*** | **0.31*** | 1.06 |
| *Methionine* | 1 | 1.05 | **0.52**** | **0.28**** | 1.61 |
| *Ornithine* | 1 | 0.96 | 0.74 | **0.16*** | **2.83*** |
| *Pea* | nd | nd | 1.14 | 0.94 | 1.27 |
| *Proline* | 1.74 | 0.76 | 0.76 | **0.52*** | 1.05 |

| | | | | | |
|---|---|---|---|---|---|
| *Serine* | 1.15 | **0.58**** | **0.17**** | **0.10**** | **2.25**** |
| *Tyrosine* | 1.32 | **1.47*** | 0.79 | 0.70 | **1.45*** |
| *Treonine* | **1.50*** | **0.74*** | **0.92**** | **0.65**** | 1.62 |
| *Tryptophan* | 1.27 | **0.46**** | 0.71 | **0.39*** | 1.36 |
| *Valine* | 1.11 | **0.67*** | 0.76 | **0.17**** | **2.31*** |

**Table 2: Changes in free amino acids content.** Values represent the amino acid content ratio between roots and leaves of control (C) and mycorrhizal (M) plants collected 50 days post AM fungus inoculation. LMX/LCX column represent the content ratio between leaves of control and mycorrhizal plants upon pathogen infection (CX and MX) at 14 days post *X. translucens* infection (corresponding to 63 days post AM fungus inoculation) (two-tailed *t* test; **P < 0.01, and *P < 0.05).

| | | Roots | Leaves |
|---|---|---|---|
| **Mineral** | **Unit** | RM / RC | LM / LC |
| **Ca** | µg/mg | 2.09 | **0.58*** |
| **K** | µg/mg | **0.87*** | **1.19*** |
| **Zn** | µg/mg | 0.78 | 1.19 |
| **Cu** | ng/mg | **2.58*** | **1.86*** |
| **Mg** | µg/mg | **0.49*** | **1.38*** |
| **Fe** | µg/mg | 0.60 | **1.64*** |
| **P** | µg/mg | **1.67*** | 0.97 |

**Table 3: Changes in mineral content in roots and leaves of control and mycorrhizal plants.** Values represent the mineral nutrient content ratio between roots and leaves of control (C) and mycorrhizal (M) plants collected 50 days post AM fungus inoculation (two-tailed *t* test; **P < 0.01, and *P < 0.05).

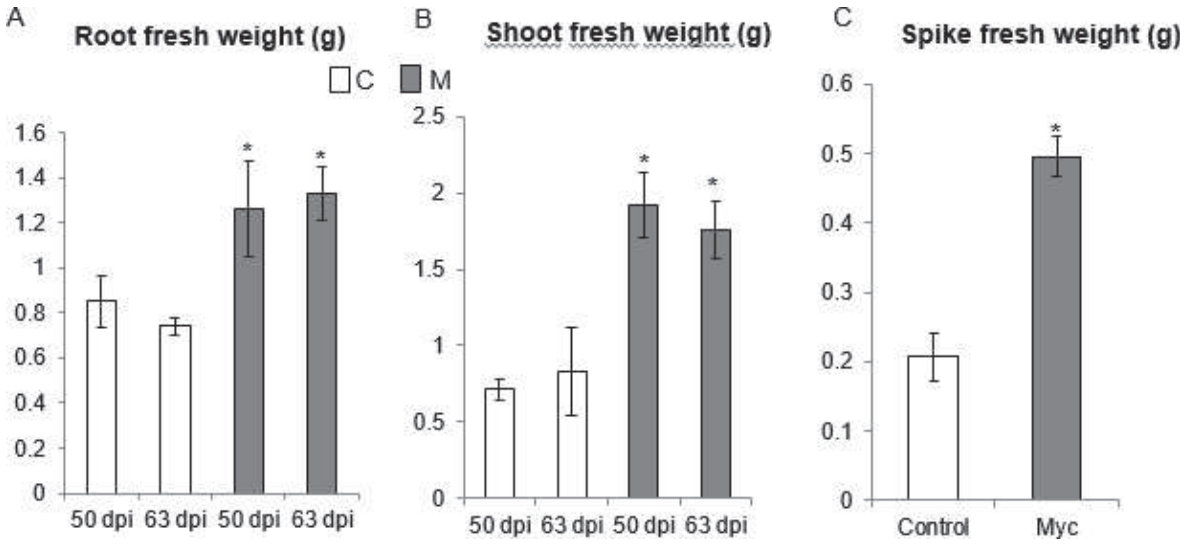



**Fig. 1: Effect of AM symbiosis on wheat biomass in different plant organs**

Fresh weight of roots (a) and leaves (b) of the control (C) and mycorrhizal (M) wheat plants harvested at 50 and 63 days post AM fungus inoculation. (C) Spike fresh weight of control and mycorrhizal plants evaluated at the end of wheat natural life cycle.

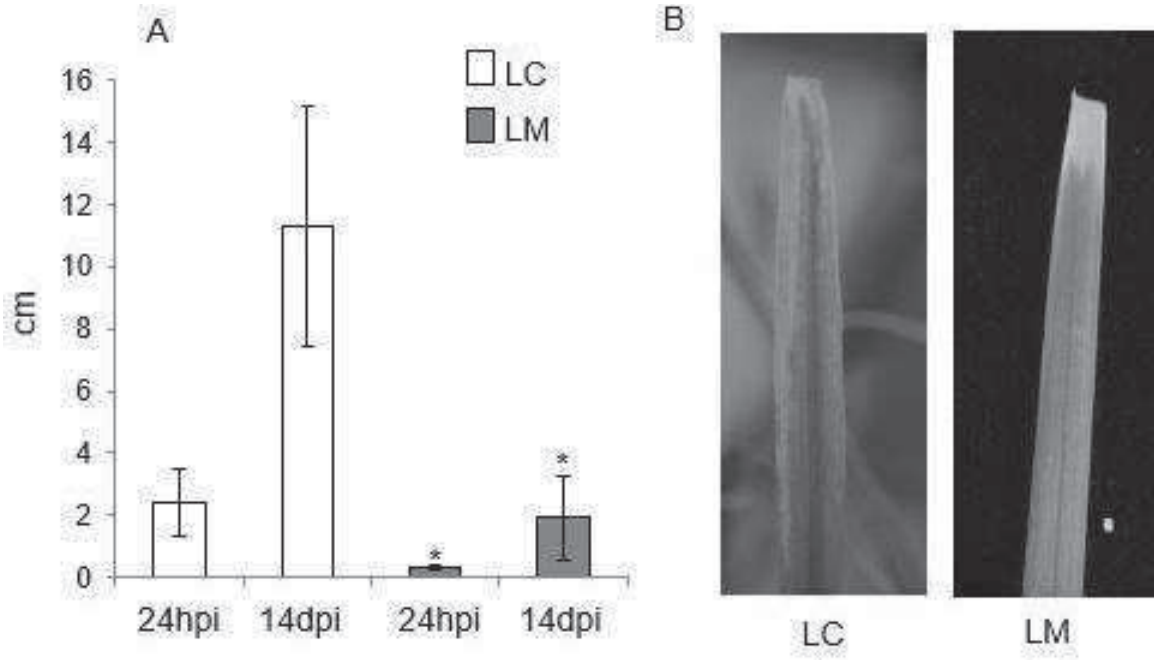



**Fig. 2: Phenotypic evaluation of disease symptoms caused by the bacterial pathogen *Xanthomonas translucens* in control (C) and mycorrhizal plants**

(A) Disease area (cm) was assessed on leaves from control (LC) and mycorrhizal (LM) plants 24 h post inoculation (hpi) and 14 days post inoculation. (B) The pictures show lesions provoked by *X. Translucens* on LC and LM 14 dpi.

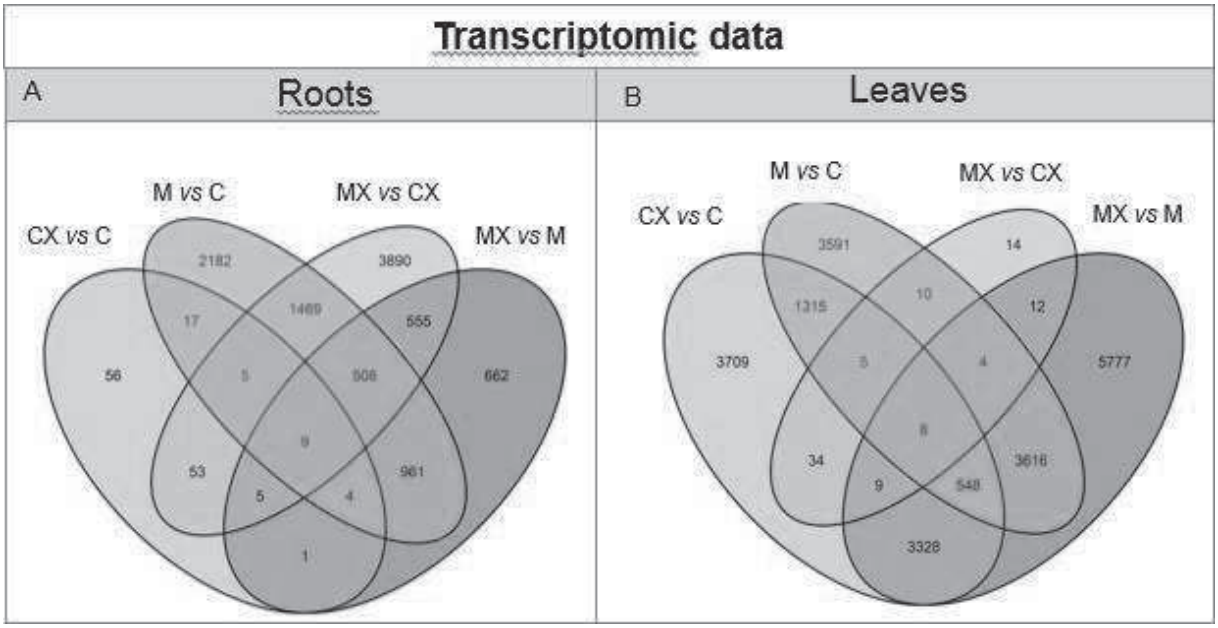



**Fig.3 Venn diagrams of DEGs modulated in the different comparisons in roots and leaves.** Venn diagrams illustrating the relationships between DEGs in the different contrasts among the same organ (A) roots and (B) leaves in the absence (C) or presence (M) of the AM fungus and following (CX; MX) or not (C; M) pathogen infection.

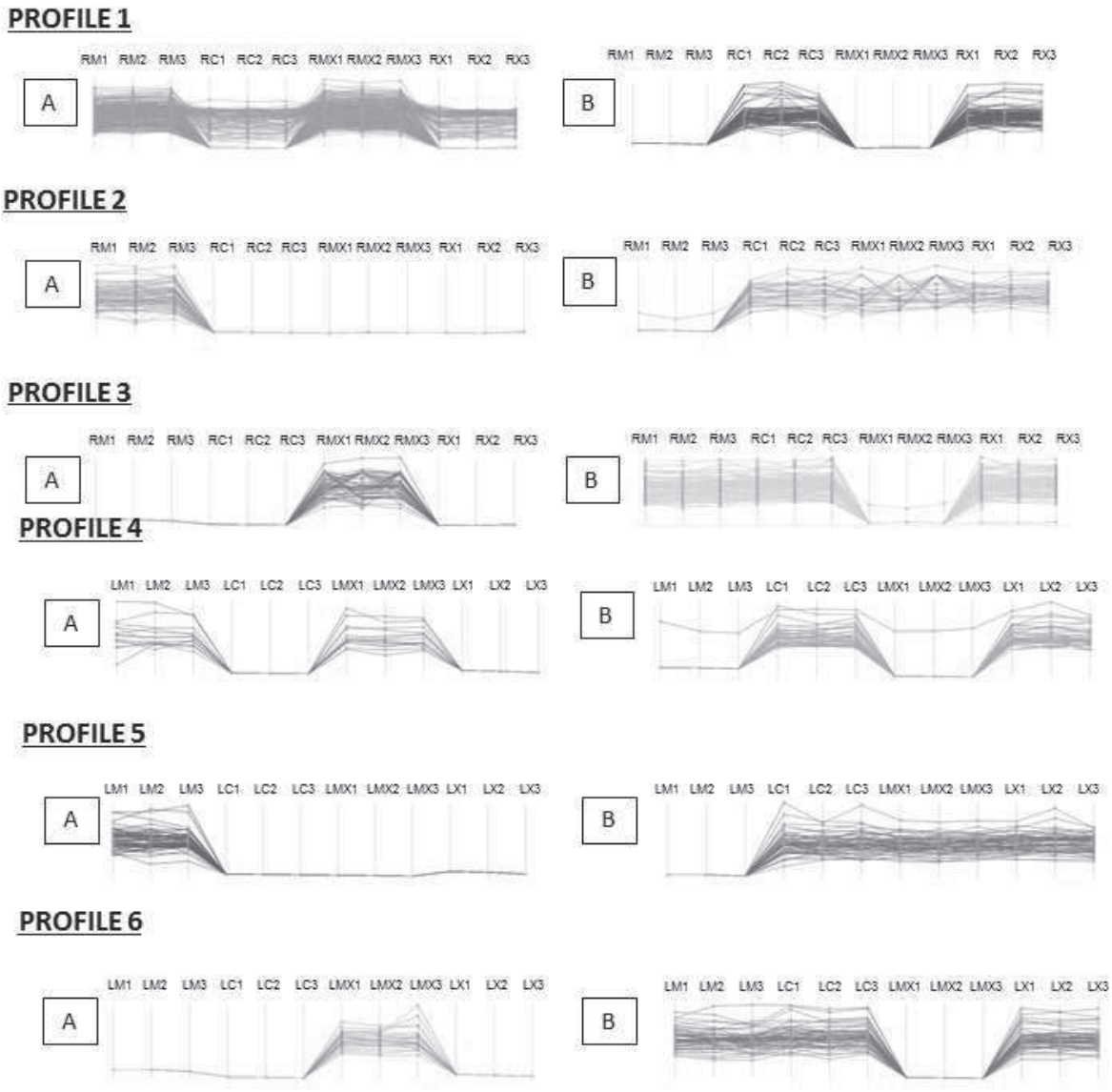



**Fig. 4: Protein profiles of roots and leaves.** For each sample three biological replicas were considered (indicated as 1, 2, 3). A and B indicate profiles containing respectively up or down regulated proteins in mycorrhizal samples (RM, RMX, LM, LMX).

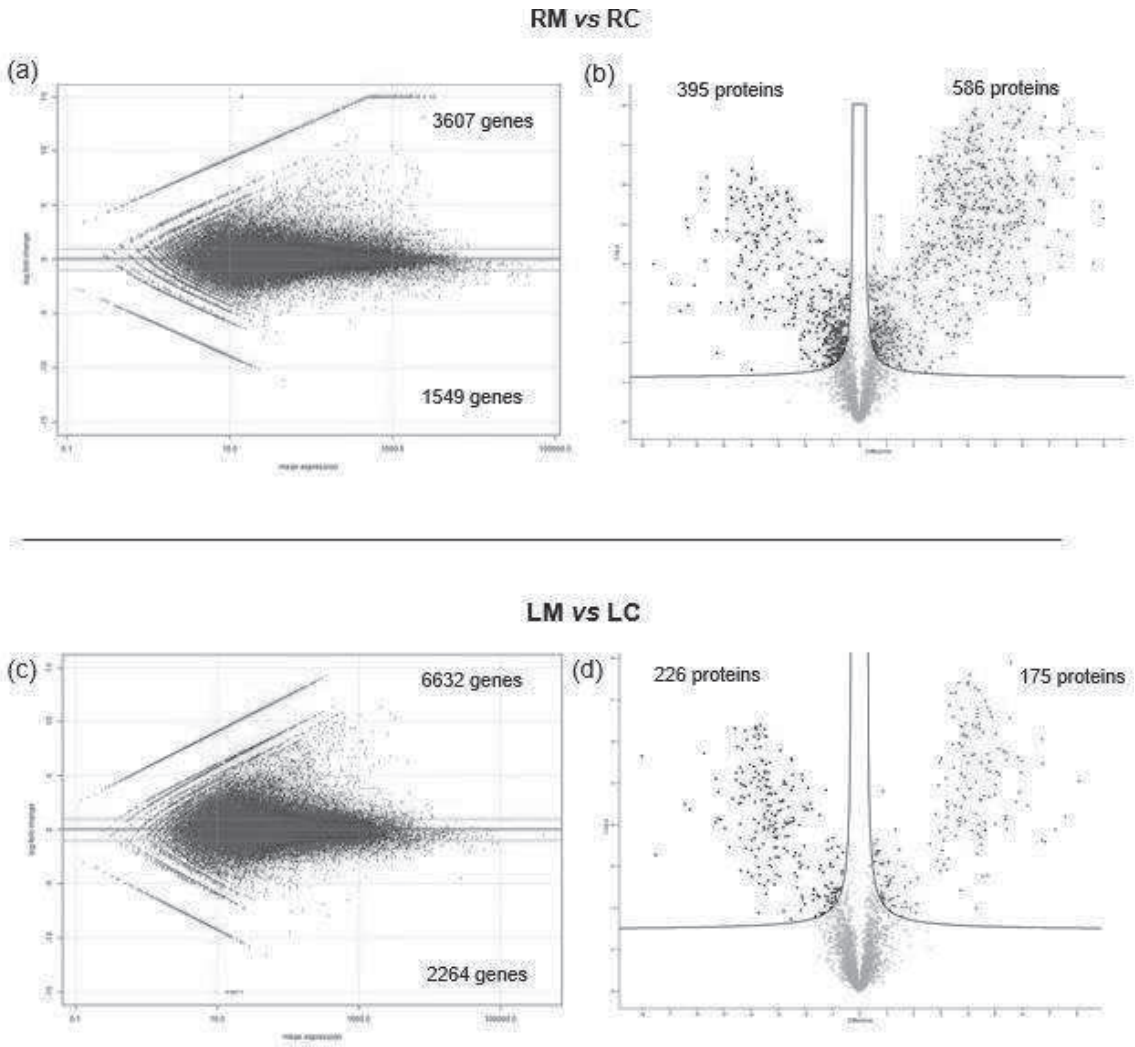



**Fig. 5: Global overview of the transcriptional and proteomic changes in the two organs (leaves -L and roots- R) in the absence (C) or presence (M) of the AM fungus.** Mean expression versus log2 fold change plots (MA-plots, left side; Volcano plot, right side) were computed for these comparisons: (A,B) RM vs RC, and (C,D) LM vs LC. Called DEGs (A,C) and DEPs (B,D) (FDR 0.05) are plotted in color. Volcano plot are with FDR=0.01 and s0=0.1. Blue spots indicate the down-regulated proteins while red ones indicate the up-regulated.

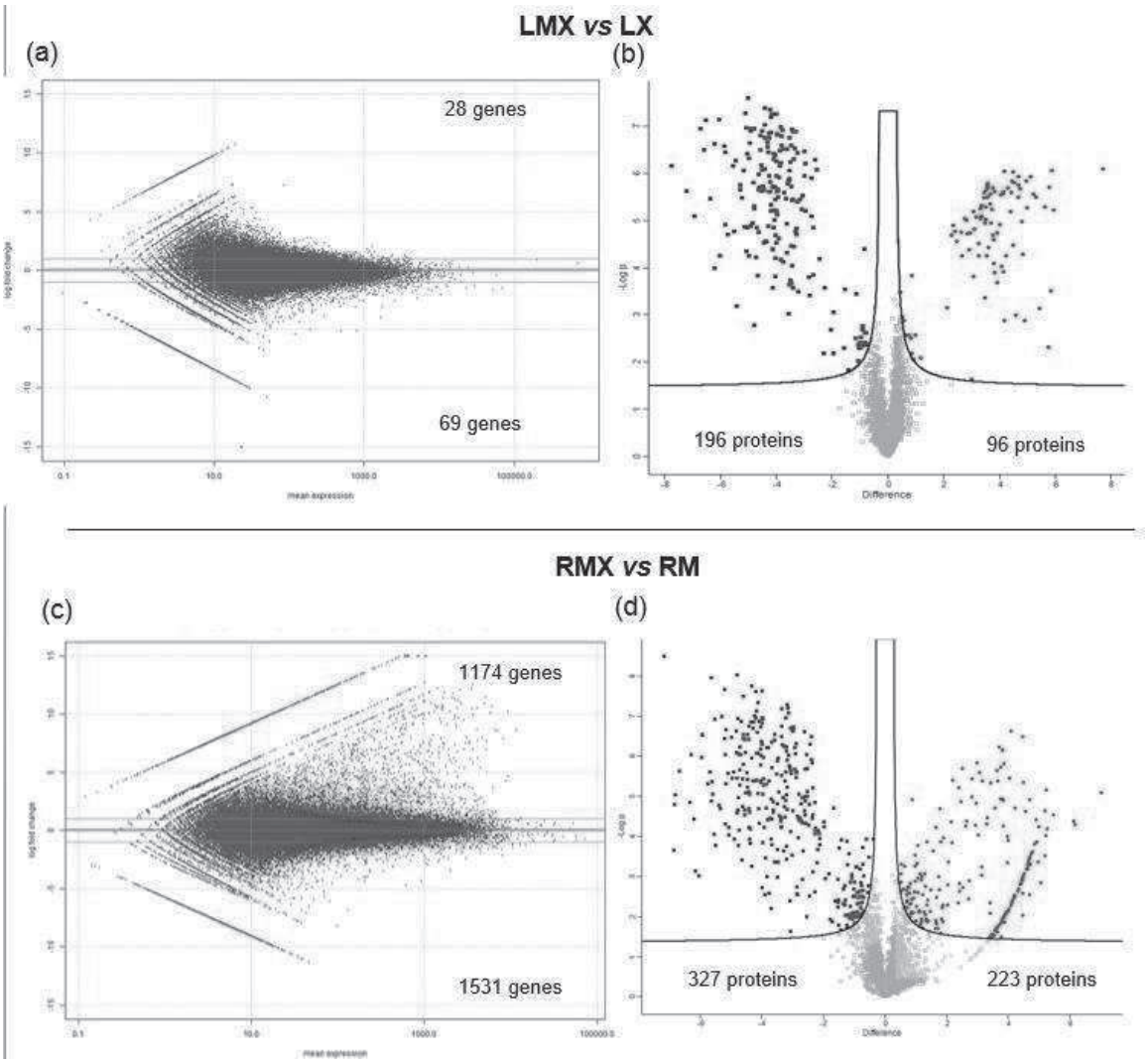



**Fig. 6: Global overview of the transcriptional and proteomic changes in the two organs (leaves -L and roots- R) following pathogen infection in non-mycorrhizal wheat plants (CX) and in mycorrhizal plants (MX).** Mean expression versus log2 fold change plots (MA-plots, left side; Volcano plot, right side) were computed for these comparisons: LMX vs. LCX (a,b), RMX vs. RM (c,d). Called DEGs (a,c) and DEPs (b,d) (FDR 0.05) are plotted in color. Volcano plot are with FDR=0.01 and s0=0.1. Blue spots indicate the down-regulated proteins while red ones indicate the up- regulated.

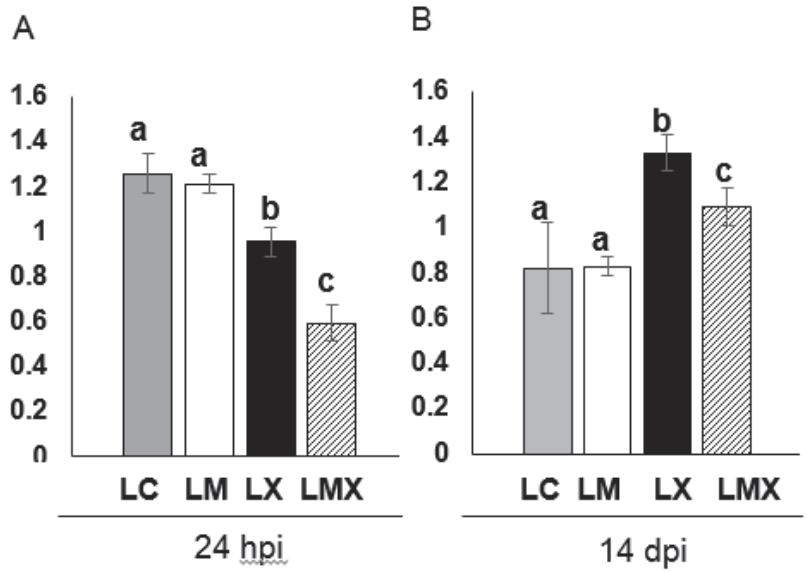



**Fig.7**: **Protein carbonylation profiles in leaves of control and mycorrhizal wheat plants after Xanthomonas infection at earlier (24 hpi) (A) and later (14 dpi) (B) time points**. Each bar represents the protein carbonylation index. Data (means ± SD, n = 3) were subjected to one-way analysis of variance (ANOVA). Bars not accompanied by the same letters are significantly different at the 5% level using Tukey's test.

## Supporting Information

Additional supporting information may be found at the link:

https://drive.google.com/drive/folders/0ByLbVnSXW-J3bEJPeTJxaldsR1E?usp=sharing

# Chapter 2

## Proteomic analysis of the molecular effects of single and co-inoculation of PGPR and AMF on wheat at the local and systemic level

Ortolani F., Vannini C., Marsoni M., Domingo G., Chiodaroli L., Bracale M.

(Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, via J.H. Dunant 3, 21100 Varese, Italy.)

### Abstract

PGPR are soil beneficial bacteria that can benefit the plant by serving as plant-growth promoters. Indeed, these microorganisms can enhance plant biomass production and tolerance to several soil conditions as salinity, drought and heavy metal toxicity thanks to solubilization of minerals, nitrogen fixation, and production of siderophores, plant growth regulators and organic acids. In this work, we studied the mechanisms behind PGPR - wheat interactions and the synergic interaction between PGPR and AMF on plant, analyzing wheat proteome in both roots and leaves. The aim was to provide a comprehensive picture of the intricate and yet mostly unknown cross-talk between wheat and AMF/PGPR, to exploit these interactions in integrated strategies for a sustainable agriculture. Proteomic analysis, focused on pathways involved in biomass production and synthesis of phytohormones and enzymes that modulate plant regulatory mechanisms, indicates that PGPR and even more the co-inoculation of PGPR and AMF in wheat have important biofertilizer and bioprotective effects. Phenotypic results also revealed that dual inoculation stimulates the growth of both roots and leaves of wheat plants with respect to the control.

## INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) play a central role in plant health. These organisms promote plant biomass production and fitness by enhancing the uptake of unavailable soil nutrients and water, increasing the biosynthesis of phytohormones and enzymes that modulate plant regulatory mechanisms and maintaining a proper antioxidant cellular environment (Liu et al., 2000; Bianciotto & Bonfante 2002; Singh et al. 2011; Gosling et al. 2006, Ordookhani et al., 2010, Marella, 2014). They also enhance the plant's adaptation to environmental stress and systemic resistance against phytopathogens (Mo et al., 2016, Schouteden et al., 2015, Medeitros et al., 2017; Cameron et al., 2013).

Moreover, PGPR can increase the rate of root exudation, which stimulates hyphal growth and facilitates root penetration by the fungus (Jeffries et al., 2003). Thus, bacteria facilitate the colonisation of plant roots by AMF (Hildebrandt et al., 2002; Jaderlund et al., 2008). On the other hand, AM fungi enhance the activity of nitrogen-fixing and phosphorus-solubilising bacteria (Linderman, 1992). For example, as a result of synergistic interactions, dual inoculation with *G. mosseae* and *Trichoderma spp*. increase the yield, seed quality and seed composition of soybean, and the growth of tomato plants is enhanced by co-inoculation with *P. fluorescens* and *G. mosseae* BEG12 (Egberongbe et al., 2010; Gamalero et al., 2004). Dual inoculation with PGPR (*Pseudomonas putida*, *B. polymyxa* and *Azotobacter chroococcum*) and AMF (*Glomus intraradices*) also increases root and shoot biomass in *Stevia rebaudiana* compared with single inoculation (Vadafar et al., 2014).

Several studies also show that the colonisation of both PGPR and AMF enhances the host's resistance to abiotic stress (Sharma & Nowak, 1998; Estrada-de los

Santos et al., 2001; Nowak & Shulaev, 2003; Compant et al., 2005; Sessitsch et al., 2005; Ait Barka et al., 2006) and leads to a preconditioning state (called priming) that allows the whole plant to respond more rapidly and strongly to pathogen attack compared with the control (Jung et al., 2012; Beckers and Conrath 2007; Conrath et al. 2006; Pozo and Azcòn-Aguilar 2007). For example, combined treatment with *Rhizophagus irregularis* and two strains of *Pseudomonas* enhances tomato resistance against the root-knot nematode *Meloidogyne incognita* to a greater degree than individual inoculation (Sharma and Sharma, 2017). The same results are obtained using *Glomus versiforme* or *Glomus mosseae* and *Bacillus polymyxa* (Liu et al., 2011).

Among PGPR, those belonging to genus *Burkholderia* are ubiquitous, rhizosphere colonisers that form a beneficial association with various crops such as maize, coffee (Estrada-de los Santos et al. 2001), tomato (Caballero-Mellado et al. 2007) and sugarcane (Perin et al. 2006; Castro-Gonzalez et al. 2011; Paungfoo-Lonhienne et al. 2014). These bacteria also protect plants against phytopathogenic microorganisms, especially fungi (Compant et al. 2008; Li et al. 2009; Tran Van et al. 2000), other bacteria, protozoa (Cain et al., 2000) and nematodes (Meyer et al., 2000). For example, *B. cepacia* significantly reduces symptoms of crown rot caused by *Fusarium graminearum* in wheat (Huang and Wong, 1998). The species *B. graminis* is a common endophytic rhizobacterium of corn, pasture grasses and wheat in Australia and France (Viallard et al., 1998).

Overall, the genus *Burkholderia* comprises nearly 90 species with remarkable functional diversity and distribution over a variety of habitats (Coenye and Vandamme 2003). Several *Burkholderia* species have been recognised as opportunistic human pathogens: this is the case for *B. cepacia* complex species (Mahenthiralingam et al. 2008). Other species are recognised as primary pathogens in humans and animals, as they are directly responsible for diseases

such as melioidosis and glanders (Coenye and Vandamme 2003). Some *Burkholderia* species infect plants and induce plant diseases, while others are endosymbionts of phytopathogenic fungi or plant-associated insects (Compant et al. 2008). Finally, several members of *Burkholderia* have beneficial interactions with plants, functioning as active rhizosphere components (Estrada-de los Santos et al. 2001; Caballero-Mellado et al. 2007), endophytic plant colonisers (Estrada et al. 2002; Caballero-Mellado et al. 2004; Gasser et al. 2011; Paungfoo-Lonhienne et al. 2014) or microsymbionts in legume root nodules (Chen et al. 2005).

The variety of benefits that PGPR exert on plants, together with their remarkable capacity to adapt to different environments, has stimulated growing interest in their use in agriculture (Castanheira et al., 2015). However, elucidating and quantifying the impact of PGPR on roots and the whole plant remain challenging (Vacheron et al., 2013) and limited information is currently available about the molecular responses of plants to inoculation with PGPR and AMF.

The aim of this study was to perform proteomic analysis to investigate the long-term effects of changes in wheat seedling growth induced by *B. graminis* at both local and systemic level. Moreover, we investigated the proteomic changes triggered in wheat by dual inoculation with *B. graminis* and the AMF *Funneliformis mosseae* compared with the sum of the effects elicited by single inoculation. The results of this study increase our understanding of the molecular mechanisms underlying the tripartite plant–PGPR–AMF interaction, which could facilitate the biotechnological exploitation of these soil microbiota.

## MATERIALS AND METHODS

*Bacterial strains and growth conditions*

The B. *graminis* C4D1M (eGFP tagged) strain was used in this study.

Bacteria were cultivated 24 hours at 28°C in YMA medium (yeast extract, 3 g; mannitol, 10 g; KH2PO4, 0.5 g; MgSO4, 0.2 g; NaCl, 0.1 g; agar, 18 g; distilled water,1 L; pH 7.0–7.2) containing the appropriate antibiotic. Bacteria were collected by centrifugation, washed twice and finally resuspended in water. The concentration of the inoculum was adjusted to $OD_{600}$ =1, and 1ml per plant were inoculated just after pre-germination.

*Plant growth condition, inoculation with AM fungus and bacterial strain*

*Triticum aestivum* cv. Chinese Spring seeds were surface sterilized, germinated in distilled water and then transferred to pots with sterile quartz sand. Seeds were single- or double-inoculated at the same time with *B. graminis* or *Funneliformis mosseae*. Inoculation of *F. mosseae* (BEG.12; MycAgro) were performed by mixing the inoculum with sterile quartz sand (30% v/v). Control plants were not inoculated. Plants were maintained under glasshouse conditions under cycles of 12 h of light at 21°C and 50% relative humidity (RH) and 12 h of dark at 21°C and 50% RH, watered twice a week with water, and once with a modified Long-Ashton solution containing a low phosphorous concentration (32 µM $Na_2HPO_4*12H_2O$) (Hewitt, 1966). Assessments of the *B. graminis* and dual colonization were evaluated 50 days post inoculation (dpi). The mycorrhizal success was evaluated as described in Fiorilli et al. (2018). Two plants were pooled per replicate, with three biological replicates per treatment.

*Proteomic analysis and data processing*

Total proteins were extracted from *T. aestivum* leaves (L) and roots (R) of Control plants (C) and plants inoculated with *Burholderia* (B), Mycorrhiza (M), and

*Burholderia* + Mycorrhiza (BM). Then, samples were digester and analysed by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) as described by Garcia-Seco et al., (2017).

Mass spectrometer raw files were analysed by MaxQuant (version 1.5.8.3) with the match between runs 0.7 and label free quantification (LFQ) options enabled. Tandem MS spectra were searched against UniProt *T. aestivum* (Version 2017-1, 150.716 entries), Uniprot *Rhizophagus irregularis* (Version 2015-10, 29.847) and Uniprot *Burholderia graminis* (Version 2015-10, 6.732). Trypsin/P was chosen as the protease, cysteine carbamidomethylation was set as fixed modification, and oxidation of methionine and acetylation of the N-terminal as variable modifications. Peptide tolerance was set to 4.5 ppm, while MS/MS tolerance was set to 0.5 Da. Peptide- spectrum matches (PSMs) and proteins were validated with 1% FDR. Only PSMs with a minimum length of 7 amino acids were kept. For the annotation of the unknown proteins a blast search was made against the Uniprot database viridiplantae (Version 2015-10, 3398870 entries), taking the first hit with a valid annotation.

The row data was processed by an in-house tool: i) the incorrected protein identifications (contaminants, decoy and "only identified by site" entries) were removed from the main data frame; ii) LFQ intensities were Log2 transformed; iii) we filtered out the ambiguously identification, only protein groups detected in almost two of three biological replicate samples sharing the same treatment and tissue were considered and were used for assessment of significant change; iv) Missing value were estimated.

To compare the differences (differentially expressed proteins, DEPs) among considered conditions, among analytical groups (C, M, B, BM) we performed an Anova based multiple sample coupled with Tukey test using the *R* package

LIMMA. Only proteins with FDR below 0.01 were considered differentially expressed (DEPs) within the various comparisons considered (B *vs* C, BM *vs* B, BM *vs* M).

The functional analysis was performed by AGRIGO v 2.0 (http://systemsbiology.cau.edu.cn/agriGOv2/index.php) using the single enrichment analysis (SEA) tool.

*Amino acid analysis*

HPLC-grade water, HPLC-grade methanol (MeOH), formic acid, aspartic acid (Asp), asparagine (Asn), glutamine (Gln), glutamic acid (Glu), serine (Ser), threonine (Thr), cystine (Cys), alanine (Ala), proline (Pro), valine (Val), methionine (Met), tyrosine (Tyr), leucine (Leu), phenylalanine (Phe), tryptophan (Trp), lysine (Lys), histidine (His) arginine (Arg) ornithine (Orn), pipecolic acid (Pip), citrulline (Cit), GABA and two deuterated internal standards (L-Phenyl-d5-alanine and L-alanine $^{15}$N) were purchased from Sigma–Aldrich Co. (Dorset, UK). Stock solutions for all compounds were prepared in distilled water (10 mM). A working mixture (1mM of each AA) was prepared and used for the calibration (range 2-15 µM). For the AA extraction, leaf and root samples were lyophilized and 0.1 g of each sample was re-suspended in 10 mL of 0.1% (v/v) formic acid in water/methanol (50:50). 10µl of 10 mM deuterated internal standards were added. The mixture was then vortexed for 4 h in the dark, sonicated for 15', centrifuged and the supernatant was collected.

The HPLC analysis was performed in a Finningan Surveyor MS plus HPLC system (Thermo Electron Corporation, CA, USA). Separation was achieved using C18 column (Phenomenex, Synergi 4u fusion-RP 80a 150 x 2.00 mm). The mobile phase was composed of (A) water with 0.1% (v/v) formic acid and (B)

methanol/water (50:50) 0.1% (v/v) formic acid with flow rate 150 µL/min; gradient 0–3.0 min/2% (v/v) B, 3–16 min/2–50% (v/v) B. For the mass spectrometry quantification, a Finningan LXQ linear ion trap mass spectrometer, equipped with an ESI ion source (Thermo Electron Corporation, CA, USA), was used. The analyses were done in positive (spray voltage 4,5 kV, capillary temperature 270˚C) and in the multiple reaction monitoring (MRM) mode. The optimization of collision energy for each substance, the tuning parameters and the choice of fragments to confirm the identity of target compounds were done in continuous flow mode, by using standard solution at concentration of 5µM (Table S0). The linearity of the method was considered adequate when square correlation coefficient ($R^2$) was higher than 0.98, based on peak area. The limits of detection (LOD) and quantification (LOQ) were fixed at 1 µM and at 2 µM, respectively.

*Detection of carbonylated proteins*

20 µg of total protein extract were derivatized with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl. The mixture was incubated for 30 min at and then neutralized by adding 1 volume of 2M Tris, 30% glycerol. After a 12% SDS-PAGE, proteins were transferred to polyvinylpyrrolidone (PVP) membrane (Serva Electrophoresis GmbH, Heidelberg, Germany). The oxidatively modified proteins were detected using anti- DNPH antibodies (anti-dinitrophenyl-group antibodies; Sigma, USA) and visualized by SuperSignal detection kit (Pierce Biotechnology, Rockford, IL, USA). Membrane images were acquired with Odyssey FC imaging systems (Li-COR, Biosciences, Lincoln, Nebraska, USA). Duplicate gel was stained by Colloidal Coomassie Brilliant Blue (CCBB) and acquired by GS-800 (Bio-Rad). Densitometric analysis of gel and membrane images were performed by ImageJ software (http://imagej.nih.gov/ij/).

Protein carbonylation index (arbitrary units) was measured as ratio between the optical density (OD) obtained from the whole lane of the immunoblot and the OD of Coomassie stain. For each set (Leave and Roots) data (means ± SD, n= 4) were subjected to one-way analysis of variance (ANOVA) with post-hoc Tukey's test.

## RESULTS AND DISCUSSION

*Phenotypic observations under controlled conditions*

Microscopic observations of roots at 20 dpi after inoculation with *B. graminis* harbouring green fluorescent protein (GFP) revealed the presence of green fluorescence both on the surface and in internal tissues of wheat roots, indicating that *B. graminis* invaded surface cells and endophytically colonised wheat roots (Fig. 1). At 20 dpi, a root endophytic population of *B. graminis* (~$5x10^2$ cfu $g^{-1}$ FW) was established in B and MB plants. No bacteria were detected in leaves (data not shown). The analysis of mycorrhizal colonisation level in M and MB plants (Fig. 2 A and B) showed no differences between them.

As shown in Fig. 3 a, b, at 50 and 72 dpi, the presence of *B. graminis* increased the root fresh weight of wheat plants by more than 30%, whereas it had no significant effect on shoot growth. By contrast, M plants showed a strong increase in both root and shoot fresh weight (Fig. 3 a, b). Double inoculation induced an increase in both root and shoot fresh weight that was significantly higher than that observed in C and B plants but not M plants. We evaluated the spike weight in all plants (data not showed) at the end of their natural cycle. Surprisingly, inoculation with *B. graminis* significantly increased total seed production, being the weight per spike in these plants twice that in C plants. In conclusion, although the PGPR *Burkholderia* can promote growth in several plants (Frommel et al., 1991; Nowak et al., 1995; Poupin et al., 2013), in our system, *B. graminis* promoted the growth

of *Triticum aestivum* roots but not the aerial parts of plants. Moreover, no additive effect was observed between *B. graminis* and *F. mosseae*.

*A quantitative overview of proteomics analysis*

We performed proteomic analysis of the leaves (L) and roots (R) of wheat plants grown in the absence (LC and RC) or presence of *B. graminis* (LB and RB), *F. mosseae* (LM and RM) and *B. graminis* plus *F. mosseae* (LBM and RBM). Each condition was analysed in triplicate, with an average of 925 proteins identified per replicate (minimum of 460 and maximum of 1293).

The Pearson correlation coefficients for biological replicate samples sharing the same treatment and tissue averaged 0.98±0.0068 in leaves and 0.98±0.0018 in roots, indicated that our mass spectrometric analysis had robust reproducibility (Fig. S2). Globally, 3066 and 3267 proteins were consistently identified and quantified in roots and leaves, respectively (supporting Fig. S3 and Table S1). Comparing the two datasets, there were 1394 common proteins, whereas 1672 were exclusively identified in roots and 1873 in leaves. In RM and RBM samples, approximately 6% of proteins were derived from the AMF (Table S11). No *B. graminis* proteins were detected.

ANOVA (FDR < 0.01) revealed an overall change in the levels of 2445 (80%) proteins in roots. Tukey test Post-Hoc comparisons between different conditions revealed 861 differentially expressed proteins (DEPs) in RB vs. RC (Table S6), 1077 DEPs in RBM vs. RM (Table S7) and 1291 DEPs in RBM vs. RB (Table S8). In leaves, we identified 1979 (60%) DEPs between samples. Statistical analysis revealed 651 DEPs in LB vs. LC (Table S2), 1173 in LMB vs. LM (Table S3) and 1135 in LMB vs. LB (Table S4). The presence of *B. graminis* on plants harbouring mycorrhizal fungi (RBM vs. RM and LBM vs. LM) led to a higher number of DEPs compared with control plants (RB vs. RC and LB vs. LC).

*Proteomic signatures of B. graminis in wheat roots*

Despite the consistent overlap in the proteins identified, only 64 proteins were differentially expressed in both tissues, while 797 and 587 proteins were expressed exclusively in roots (RB vs. RC) and leaves (LB vs. LC), respectively. In RB vs. RC (Table S6), 491 DEPs were upregulated and 370 were downregulated. Analysis of the over-represented gene ontology (GO) categories among DEPs highlighted the specific activation in RB samples of proteins involved in the generation of precursor metabolites and energy. In fact, some enzymes involved in the TCA cycle, such as two isocitrate dehydrogenase (NAD) subunits and malate dehydrogenase, were strongly upregulated in these samples. Moreover, the mitochondrial dicarboxylate/tricarboxylate transporter DTC was upregulated in the RB samples. This protein is important for plant metabolic functions requiring organic acid flux to or from the mitochondria, such as nitrogen assimilation, the export of reducing equivalents from the mitochondria and fatty acid elongation.

Accordingly, we found that one glutamine synthase (similar to AtGLN1;2,) with low affinity for ammonium was upregulated in RB, whereas two glutamine synthases with high affinity for ammonium were downregulated in these plants. *B. graminis* lacks nitrogenase activity, but it can scavenge trace amounts of ammonia and nitrogen oxides from the atmosphere (Hill and Postgate, 1969; Hurek et al., 1988; Castanheira et al., 2015). Moreover, *B. graminis* influences the nitrogen status of the host plant by increasing the expression of two nitrate transporters.

Proteomic analysis also revealed that amino acid (AA) metabolism was strongly modulated in wheat roots in response to *B. graminis* (Fig. S4, S5). To confirm this finding, we performed LC-MS/MS analysis to measure the free AA contents in

both organs from C and B plants (Table 1). In roots, the concentrations of almost all AAs were significantly higher in *B. graminis*-inoculated vs. control plants.

Several PGPR can solubilise phosphate, thus increasing P bioavailability in the soil. We found that a phosphate transporter homologous to OsPT8, a high-affinity transporter for external inorganic phosphate, was downregulated in response to *B. graminis*, whereas the phosphate import ATP-binding protein pstB1 was upregulated. Also, several ABC transporters, two sugar transporters and one peptide transporter were induced by the presence of the bacteria in the roots; this response could facilitate nutrient exchange from the plant host to the microbe (Badri et al., 2008, 2009; Loyola-Vargas et al., 2007; Sugiyama et al., 2007).

Many PGPR depend on plant root exudates for their survival (Glick et al., 1998). The accumulation of secondary compounds is also modified in several plants inoculated with PGPR (Vacheron et al. 2013). Accordingly, one of the most highly upregulated pathways in the RB samples was the terpenoid metabolism pathway (Table S6, Fig. S4.). Moreover, treatment with *B. graminis* increased the levels of proteins involved in lignin biosynthesis, such as caffeoyl CoA O-methyltransferase and cinnamyl-alcohol dehydrogenase 1. Two groups of proteins whose expression levels were altered by the presence of *B. graminis* are especially interesting, i.e., proteins related to hormone pathways and stress. Bacterial IAA production influences hormonal balance in the host plant, thus affecting its growth (Govindasamy et al., 2009). *B. graminis* can produce detectable amounts of IAA (Castanheira et al., 2015). We detected the accumulation of the IAA- amino acid hydrolase ILR1-like 3 protein and two auxin transporters (with high similarity to *Arabidopsis thaliana* ABCB4 and ABCG37) in roots in response to *B. graminis* treatment. These proteins are involved in auxin distribution and homeostasis in roots. The overexpression of 12-oxophytodienoate reductases 1 and allene oxide synthase in these roots suggests

that *B. graminis* stimulates the jasmonate signal transduction network. This hormone regulates a wide range of processes in plants, such as growth, development and responses to abiotic and biotic challenges. PGPR promote the establishment of mycorrhizal symbioses by increasing the rate of root exudation, thereby stimulating hyphal growth and facilitating root penetration by the fungus (Jeffries et al., 2003). We also found that a strigolactone esterase was upregulated in response to B. graminis treatment. This enzyme functions downstream of strigolactone synthesis as a component of hormone signalling and as an enzyme that participates in the conversion of strigolactone to the bioactive form.

The expression of several DEPs associated with biotic stress tolerance was altered in response to PGPR treatment, with some proteins downregulated (osmotin-like protein) and others upregulated (endochitinase, pathogenesis-related proteins 1, glucanases). These results are in accordance with the finding that PGPR modulate plant defence responses to enable efficient root colonisation and the establishment of rhizosphere microbial communities (Vacheron et al. 2016). Many *Burkholderia* species inhibit the growth of other bacteria, protozoa (Cain et al., 2000) and nematodes (Meyer et al., 2000). Their ability to suppress plant diseases was observed in many different crops, including corn, cotton, grapevine, pea, tomato and pepper (Compant et al., 2008).

PGPR improve plant resistance to abiotic stress, thereby enabling plants to survive under unfavourable environmental conditions (Belimov et al., 2001; Glick, 2010; Ma et al., 2011; Mayak et al., 2004b; Nadeem et al., 2007; Sandhya et al., 2009; Zahir et al., 2008). Accordingly, our proteomic data show that one of the over-represented GO categories among DEPs is related to the response to salt stress (proteins such as pyrroline-5-carboxylate reductase, ck29, pyridoxal kinase, histone deacetylase SAP18 and AGO1). In addition, proteins involved in drought resistance (for example, the dehydrin WZY1, two aquaporins) and temperature

stress (Cold shock protein-1, HSP20, HSP70-1) were upregulated. Finally, one of the most highly downregulated processes was the response to oxidative stress; in particular, several apoplastic peroxidases were differentially regulated by *B. graminis* inoculation (Fig. S4).

*Systemic protein regulation in B. graminis-inoculated plants*

Little is known about the effects of *Burkholderia* on the aerial parts of plants. In the LB vs. LC comparison (Table S2), 271 proteins were upregulated and 380 were downregulated. Roots inoculated with *B. graminis* exhibited a strong modulation in metabolism at the systemic level. In fact, the over-represented categories among upregulated DEPs included primary and protein metabolism. Whereas DEPs related to photosynthesis showed a contrasting pattern of expression, several proteins involved in sucrose and starch degradation, such as invertases, hexokinases, sucrose synthase and alpha amylase, were induced by *B. graminis* treatment. Soluble sugars such as fructose and glucose accumulated in the leaves of grapevine plantlets 4 weeks after root inoculation with *B. phytofirmans PsJN*. Perhaps the increased amounts of sucrose and starch degradation proteins detected in the present study increase hexose levels to fuel the observed plant growth promotion in roots and to facilitate cold acclimation in wheat, as demonstrated in grapevine (Fernandez et al., 2012). The activation of mitochondrial activity in the LB samples is supported by the observation that levels of proteins such as cytochrome C oxidase and ATP synthase increased in response to *B. graminis* inoculation. In addition, although some proteins involved in lipid transfer were downregulated in these plants, some enzymes involved in lipid synthesis (such as ketoacyl ACP synthase) were upregulated.

Contrary to what we observed in roots, AA analysis of the leaves of inoculated plants revealed an increase in (only) tryptophan levels and a significant decrease in the levels of glutamic acid, arginine and serine. The results of proteomic analysis at least partially explain these data. The downregulation of the TCA cycle (due to the observed decrease in malate dehydrogenase, aconitase and carbonic anhydrase levels) might explain the lower levels of AAs derived from intermediates in this cycle. Interestingly, we found that, in leaves, the levels of two proteins increased: a cytosolic glutamine synthetase highly similar to AtGln1;2 and a mitochondrial glutamate dehydrogenase 1 highly similar to GDH1; these proteins are both involved in ammonia assimilation under conditions of inorganic nitrogen excess (Lothier et al., 2011; Melo-Oliveira et al., 1996). In addition, we detected a strong increase in the levels of glutamate decarboxylase, which catalyses GABA biosynthesis. In plants, GABA is thought to be involved in many cellular processes in plants, such as the regulation of carbon and nitrogen metabolism/energy balance, osmoregulation and plant growth (Mei et al. 2016).

As observed in roots, lignin biosynthesis is also induced at the systemic level in response to *B. graminis* treatment based on the increased expression of caffeate O- methyltransferase, cinnamyl-alcohol dehydrogenase and phenylalanine ammonia-lyase (PAL). When present in plant tissues, bacteria induce defence mechanisms in the host plant (Rosenblueth and Martinez-Romero, 2006). Among these plant defence responses, the strengthening of cell walls and the establishment of surrounding material inside the xylem or cortex have been reported (Benhamou et al., 1998, 2000; James et al., 2002; Compant et al., 2005; Miché et al., 2006). Also, Frommel et al. (1991) detected an increase in the total plant lignin content in *Bp PsJN*- inoculated potato plantlets. Moreover, *Bp PsJN* inoculation increased the cell wall thickness in grapevine plantlets (Ait Barka et al., 2006), confirming our finding that *B. graminis* reinforces the host

cell wall. However, most DEPs belonging to biotic stress pathways (such as endochitinase, osmotin disease resistance-responsive family protein) and several HSPs were downregulated in response to *B. graminis* treatment.

Our proteomic analysis highlights the differential expression of proteins involved in phytohormones metabolism. In particular, proteins involved in jasmonate regulation (such as lipoxygenases) and \ acid (ABA) responses (such as the aldehyde oxidase 3 and the receptor PYR [pyrabactin resistance]) were upregulated in *B. graminis*-treated plants compared with the control. Crops overexpressing ABA exhibit increased drought tolerance (Gonzalez-Guzman et al., 2014). Interestingly, some proteins involved in abiotic stress tolerance, such as the chemocyanin-like protein TaCLP1, l-*myo*-inositol monophosphatase, aldose-1-epimerase and HSP101, were upregulated in the LB samples compared with the control. Finally, most DEPs with antioxidant activity were upregulated in LB, including ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), superoxide dismutase (SOD), glyoxalase I (Gly I) and glyoxalase II (Gly II).

*Proteomic changes induced by co-inoculation with B graminis and F. mosseae at the local and systemic levels*

Our phenotypic results (Fig.3 a, b) reveal that dual inoculation with *B. graminis* and *F. mosseae* stimulated the growth of both the roots and leaves of wheat plants compared with the control. The root growth of BM plants was comparable to that of B and M plants, while the shoot growth was comparable to that of M plants. These results suggest that the increased shoot growth of BM plants is due mainly to the presence of AMF in the dual inoculum.

To better understand the contribution of co-inoculation to the host plant, we compared the expression of all identified proteins in the four samples (C, M, MB, B) by profile analysis based on the modulation of their expression. Here, we paid particular attention to proteins upregulated in roots and leaves exclusively in response to the presence of *B. graminis* and *F. mosseae* in MB plants.

Profile 1A and 1B (Table S5) includes proteins that were upregulated and downregulated in RBM samples compared with all other treatment conditions, namely, proteins specifically induced in roots by the presence of the PGPR and the fungus.

Several enzymes related to glycolysis and the TCA cycle were upregulated in co-inoculated samples, including a fructose-bisphosphate aldolase, a glucose-6-phosphate isomerase and acetyl-coenzyme A synthetase. The A glucose-6-phosphate/phosphate translocator was also upregulated in this cluster. This enzyme is responsible for the transport of Glc6P into plastids of heterotrophic tissues where it can be used as a carbon source for starch biosynthesis, as a substrate for fatty acid biosynthesis or as a substrate for NADPH generation via the oxidative pentose phosphate pathway. Interestingly, some proteins related to transport, such as ammonium transporters, nitrate transporters, phosphate transporter and an Exporting 4 involved in the transport of macromolecules between the nucleus and the cytoplasm, were upregulated in duel-inoculated samples compared with the control. Several upregulated proteins in this profile are related to secondary metabolism. These proteins include a caffeic acid 3-O-methyltransferase involved in the phenylpropanoid and lignin biosynthesis pathways, a cinnamoyl-CoA reductase I, the first enzyme in the monolignol-specific branch of the lignin biosynthetic pathway and two norcoclaurine synthases, which catalyse the first committed step in benzylisoquinoline alkaloid biosynthesis.

Interestingly, dual inoculation upregulated a 5-pentadecatrienyl resorcinol O-methyltransferase involved in the biosynthetic pathway of the phytotoxin sorgoleone, a potent broad-spectrum allelochemical (active at micromolar concentrations) against many agronomical important monocot and dicot weed species. In addition, co- inoculation induced the upregulation of 2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase, an intermediate in the MEP pathway, which supplies isoprenoids to the plant. Several stress- and defence-related proteins were upregulated in RBM, including the following: four glutathione-S-transferases (GSTs); an annexin (an increase in annexin abundance was also found in salt-treated potato (Aghaei et al., 2008) and tomato (Manaa et al., 2011) plants vs. the control, indicating their role in abiotic stress signalling); an adenine nucleotide alpha hydrolases-like protein that responds to cold stress; a germin protein that plays a role in abiotic stress resistance in many plant species; a stress-upregulated Nod 19 that helps plants cope with oxidative stress generated under abiotic and biotic stress conditions; and two calnexins, which are integral membrane ER proteins that help monitor stress signalling and stress tolerance in plants. Other upregulated proteins include a β-1,3-glucanase that hydrolyses β-1,3- glucan (a major structural component of fungal cell walls) and promotes the release of cell wall-associated immune elicitors that stimulate plant defence reactions, and also WAX INDUCER1 (WIN1), which triggers wax production, enhances drought tolerance and modulates cuticular permeability when overexpressed in Arabidopsis (Kannangara et al., 2007).

It appears that dual inoculation did not alter the expression of proteins related to hormone metabolism, except for an ABA 8′-hydroxylase involved in the oxidative degradation of ABA that plays an important role in determining ABA levels to control postgermination growth. Proteomic profile 2A shows that 100 DEPs were upregulated in the LMB samples (Table S5). The most highly represented

functional category is related to protein synthesis and regulation, including several ribosomal proteins. In addition, one MPKK nearly identical to *Oryza sativa* MKK1 was upregulated in these samples. OsMKK1 functions in a signalling pathway that regulates salt stress tolerance in rice (Wang et al., 2014).

Dual inoculation also induced the accumulation of one Delta (24)-sterol reductase involved in brassinosteroids (BR) biosynthesis and two receptor-like cytoplasmic kinases highly similar to Arabidopsis BSK1 and BSK3, which regulate BR signalling. BSK1 also functions as a major regulator of plant immunity (Shi et al., 2013; Wang et al., 2017). Also, in leaves, dual inoculation induced the upregulation of seven proteins involved in cell transport, including an ammonium transporter highly similar to AMT1;1. This high-affinity ammonium transporter is likely involved in long-distance transport of ammonium to the shoots and the re-uptake of apoplastic ammonium derived from photorespiration in shoots. Moreover, duel inoculation upregulated the expression of OST3/6, a conserved subunit of the oligosaccharyl transferase complex (which is involved in innate immunity and abiotic stress tolerance) (Farid et al., 2013), and a mitochondrial ABC transporter that is highly similar to ATM3 (which is involved in heavy metal resistance and plays an important role in plant growth). In addition, two aquaporins were upregulated by dual inoculum. Finally, some other proteins related to biotic stress (such as the disease resistance protein RPM1, one chitinase and one trypsin inhibitor) were upregulated in duel-inoculated plants compared with the control.

Overall, these results indicate that dual inoculation has a synergistic effect on various wheat proteins. The proteins that are specifically induced in BM plants by the presence of the PGPR and fungus are mainly involved in primary and secondary metabolism, cellular transport and stress tolerance.

## CONCLUSION

Understanding the mechanisms behind PGPR–plant interactions is important for developing improved strategies for using these beneficial bacteria in agriculture. Our proteomic data indicate that the regulation of proteins involved in auxin pathways may explain, at least in part, the observed increase in root growth upon *B. graminis* inoculation. In addition, *B. graminis* promotes root growth by enhancing N uptake efficiency. Indeed, the constitutive expression of a high-affinity nitrate transporter in rice led to increased vegetative growth under low nitrogen conditions (Chen et al. 2016). Curiously, the aerial parts of wheat plants inoculated with this bacterial strain showed no increase in growth. A similar result was obtained by Castanheira et al. (2015). We speculate that such a response could be due to an increased metabolic demand for sugars and energy. The improved N uptake would be accompanied by a higher investment in C resources for the maintenance of the bacterial partner in the rhizosphere rather than increased plant growth. More investigations are needed to confirm this hypothesis.

In both leaves and roots, *B. graminis* promoted the increase in levels of several proteins involved in abiotic stress, particularly salt stress, which may help improve the plant's performance under stress conditions. We hypothesise that root colonisation by *B. graminis* generates abiotic stress priming, which improves the plant's ability to cope with adverse environmental conditions. Interestingly, dual inoculation also led to the activation of many growth- and defence-related proteins, not only in roots but also at the systemic level; some of these have not previously been reported. These results clearly indicate that dual inoculation with the PGPR *B. graminis* and the AMF *F. mosseae* in wheat enhances the biofertiliser and bioprotective effects of these organisms. Overall, these findings increase our understanding of the interactions between plants and beneficial

bacteria and provide novel information about the long-term effects of PGPR on plant development, opening new avenues for investigating these important biological associations.

## ACKNOWLEDGEMENTS

F.O. is a PhD student of the "Biotechnology, Biosciences and Surgical Technology" course at Università degli Studi dell'Insubria.

## REFERENCES

**Adolfsson L, Nziengui H, Abreu IN, Šimura J, Beebo A, Herdean A, Aboalizadeh J, Široká J, Moritz T, Novák O, Ljung K, Schoefs B, Spetea C** (2017) Enhanced Secondary- and Hormone Metabolism in Leaves of Arbuscular Mycorrhizal Medicago truncatula. *Plant Physiology*. DOI:10.1104/pp.16.01509

**Aghaei K, Ehsanpour AA, Komatsu S** (2008) Proteome analysis of potato under salt stress. *J Proteome Res* **7:** 4858-4868

**Ait Barka E, Nowak J, Clément C** (2006) Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, Burkholderia phytofirmans strain PsJN. *Appl Environ Microbiol* **72:** 7246-7252

**Artursson V, Finlay RD, Jansson JK** (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environ Microbiol* **8:** 1-10

**Badri DV, Loyola-Vargas VM, Broeckling CD, De-la-Peña C, Jasinski M, Santelia D, Martinoia E, Sumner LW, Banta LM, Stermitz F, Vivanco JM** (2008) Altered Profile of Secondary Metabolites in the Root Exudates of Arabidopsis ATP-Binding Cassette Transporter Mutants. *Plant Physiology* **146:** 762-771

**Badri DV, Loyola-Vargas VM, Du J, Stermitz FR, Broeckling CD, Iglesias-Andreu L, Vivanco JM** (2008) Transcriptome analysis of Arabidopsis roots treated with signaling compounds: a focus on signal transduction, metabolic regulation and secretion. *New Phytologist* **179:** 209-223

**Badri DV, Vivanco JM** (2009) Regulation and function of root exudates. *Plant Cell and Environment* **32:** 666-681

**Beckers GJ, Conrath U** (2007) Priming for stress resistance: from the lab to the field. *Curr Opin Plant Biol* **10:** 425-431

**Belimov AA, Safronova VI, Sergeyeva TA, Egorova TN, Matveyeva VA, Tsyganov VE, Borisov AY, Tikhonovich IA, Kluge C, Preisfeld A, Dietz KJ, Stepanok VV** (2001) Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. *Can J Microbiol* **47:** 642-652

**Benhamou N, Bélanger RR** (1998) Benzothiadiazole-Mediated Induced Resistance to Fusarium oxysporum f. sp. radicis-lycopersici in Tomato. *Plant Physiology* **118:** 1203-1212

**Benhamou N, Gagné S, Le Quéré D, Dehbi L** (2000) Bacterial-Mediated Induced Resistance in Cucumber: Beneficial Effect of the Endophytic Bacterium Serratia plymuthica on the Protection Against Infection by Pythium ultimum. *Phytopathology* **90:** 45-56

**Bianciotto V, Bonfante P** (2002) Arbuscular mycorrhizal fungi: a specialised niche for rhizospheric and endocellular bacteria. Antonie Van Leeuwenhoek **81:** 365-371

**Breen J, Bellgard M** (2010) Germin-like proteins (GLPs) in cereal genomes: gene clustering and dynamic roles in plant defence. *Funct Integr Genomics* **10:** 463-476

**Caballero-Mellado J, Martinez-Aguilar L, Paredes-Valdez G, Estrada-de los Santos P** (2004) Burkholderia unamae sp nov., an N-2-fixing rhizospheric and endophytic species. *International Journal of Systematic and Evolutionary Microbiology* **54:** 1165-1172

**Caballero-Mellado J, Onofre-Lemus J, Estrada-de los Santos P, Martínez-Aguilar L** (2007) The Tomato Rhizosphere, an Environment Rich in Nitrogen-Fixing Burkholderia Species with Capabilities of Interest for Agriculture and Bioremediation. *Applied and Environmental Microbiology* **73:** 5308-5319

**Cain CC, Henry AT, Waldo RH, Casida LJ, Falkinham JO** (2000) Identification and characteristics of a novel Burkholderia strain with broad-spectrum antimicrobial activity. *Applied and Environmental Microbiology* **66:** 4139-4141

**Cameron DD, Neal AL, van Wees SC, Ton J** (2013) Mycorrhiza-induced resistance: more than the sum of its parts? *Trends Plant Sci* **18:** 539-545

**Castanheira N, Dourado AC, Kruz S, Alves PI, Delgado-Rodríguez AI, Pais I, Semedo J, Scotti-Campos P, Sánchez C, Borges N, Carvalho G, Barreto Crespo MT, Fareleira P** (2016) Plant growth-promoting Burkholderia species isolated from annual ryegrass in Portuguese soils. *J Appl Microbiol* **120:** 724-739

**Castro-González R, Martínez-Aguilar L, Ramírez-Trujillo A, Estrada-de los Santos P, Caballero-Mellado J** (2011) High diversity of culturable Burkholderia species associated with sugarcane. *Plant and Soil*, Volume 345, Issue 1–2, pp 155–169

**Cervantes-Gámez RG, Bueno-Ibarra MA, Cruz-Mendívil A, Calderón-Vázquez CL, Ramírez-Douriet CM, Maldonado-Mendoza IE, Villalobos-López MÁ, Valdez-Ortíz Á, López-Meyer M** (2016) Arbuscular Mycorrhizal Symbiosis-Induced Expression Changes in Solanum lycopersicum Leaves Revealed by RNA-seq Analysis. *Plant Molecular Biology Reporter* **34:** 89-102

**Chen J, Zhang Y, Tan Y, Zhang M, Zhu L, Xu G, Fan X** (2016) Agronomic nitrogen-use efficiency of rice can be increased by driving OsNRT2.1 expression with the OsNAR2.1 promoter. *Plant Biotechnol J* **14:** 1705-1715

**Chen W-M, de Faria SM, Straliotto R, Pitard RM, Simões-Araùjo JL, Chou J-H, Chou Y-J, Barrios E, Prescott AR, Elliott GN, Sprent JI, Young JPW, James EK** (2005) Proof that Burkholderia Strains Form Effective Symbioses with Legumes: a Study of Novel Mimosa-Nodulating Strains from South America. *Applied and Environmental Microbiology* **71:** 7461-7471

**Coenye T, Vandamme P** (2003) Diversity and significance of Burkholderia species occupying diverse ecological niches. *Environmental Microbiology* **5:** 719-729

**Compant S, Nowak J, Coenye T, Clement C, Barka EA** (2008) Diversity and occurrence of Burkholderia spp. in the natural environment. *Fems Microbiology Reviews* **32:** 607-626

**Compant S, Reiter B, Sessitsch A, Nowak J, Clément C, Ait Barka E** (2005) Endophytic colonization of Vitis vinifera L. by plant growth-promoting bacterium Burkholderia sp. strain PsJN. *Appl Environ Microbiol* **71:** 1685-1693

**Conrath U** (2006) Systemic acquired resistance. *Plant Signal Behav* **1:** 179-184

**de Medeiros HA, de Araujo JV, de Freitas LG, Castillo P, Rubio MB, Hermosa R, Monte E** (2017) Tomato progeny inherit resistance to the nematode Meloidogyne javanica linked to plant growth induced by the biocontrol fungus Trichoderma atroviride. *Scientific Reports* 7:40216. doi: 10.1038/srep40216

**Egberongbe HO, Akintokun, A.K. „ Babalola OO, Bankole, M.O.** (2010) The effect of Glomus mosseae and Trichoderma harzianum on proximate analysis of soybean (Glycine max (L.) Merrill.) seed grown in sterilized and unsterilised soil. *In Journal of Agricultural Extension and Rural Development*, Vol 2, pp 54-58

**Estrada P, Mavingui P, Cournoyer B, Fontaine F, Balandreau J, Caballero-Mellado J** (2002) A N-2-fixing endophytic Burkholderia sp associated with maize plants cultivated in Mexico. *Canadian Journal of Microbiology* **48:** 285-294

**Estrada-De Los Santos P, Bustillos-Cristales R, Caballero-Mellado J** (2001) Burkholderia, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl Environ Microbiol* **67:** 2790-2798

**Farid A, Malinovsky FG, Veit C, Schoberer J, Zipfel C, Strasser R** (2013) Specialized Roles of the Conserved Subunit OST3/6 of the Oligosaccharyltransferase Complex in Innate Immunity and Tolerance to Abiotic Stresses. *Plant Physiology* **162:** 24-38

**Fiorilli V, Vallino M, Biselli C, Faccio A, Bagnaresi P, Bonfante P** (2015) Host and non-host roots in rice: cellular and molecular approaches reveal differential responses to arbuscular mycorrhizal fungi. *Frontiers in Plant Science* **6:** 636. doi: 10.3389/fpls.2015.00636.

**Frommel MI, Nowak J, Lazarovits G** (1991) Growth Enhancement and Developmental Modifications of in Vitro Grown Potato (Solanum tuberosum spp. tuberosum) as Affected by a Nonfluorescent Pseudomonas sp. *Plant Physiology* **96:** 928-936

**Gamalero E, Trotta A, Massa N, Copetta A, Martinotti MG, Berta G** (2004) Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. *Mycorrhiza* 14 (3): 185-192

**Gasser I, Cardinale M, Müller H, Heller S, Eberl L, Lindenkamp N, Kaddor C, Steinbüchel A, Berg G** (2011) Analysis of the endophytic lifestyle and plant growth promotion of Burkholderia terricola ZR2-12. *Plant and Soil*, 347:125-136 doi 10.1007/s11104-011-0833-8

**Glick BR** (2010) Using soil bacteria to facilitate phytoremediation. *Biotechnology Advances* **28:** 367-374

**Glick BR, Penrose DM, Li J** (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J Theor Biol* **190:** 63-68

**González-Guzmán M, Rodríguez L, Lorenzo-Orts L, Pons C, Sarrión-Perdigones A, Fernández MA, Peirats-Llobet M, Forment J, Moreno-Alvero M, Cutler SR, Albert A, Granell A, Rodríguez PL** (2014) Tomato PYR/PYL/RCAR abscisic acid receptors show high expression in root, differential sensitivity to the abscisic acid agonist quinabactin, and the capability to enhance plant drought resistance. *J Exp Bot* **65:** 4451-4464

**Gosling P, Hodge A, Goodlass G, Bending GD** (2006) Arbuscular mycorrhizal fungi and organic farming. *Agriculture Ecosystems & Environment,* Volume 113, Issues 1–4, Pages 17-35

**Govindasamy V, Senthilkumar M, Vellaichamy M, Annapurna K** (2009) Detection and Characterization of ACC Deaminase in Plant Growth Promoting Rhizobacteria. *Journal of Plant Biochemistry and Biotechnology* Volume 18, Issue 1, pp 71–76

**Gutjahr C, Sawers RJH, Marti G, Andrés-Hernández L, Yang S-Y, Casieri L, Angliker H, Oakeley EJ, Wolfender J-L, Abreu-Goodger C, Paszkowski U** (2015) Transcriptome diversity among rice root types during asymbiosis and interaction with arbuscular mycorrhizal fungi. *Proceedings of the National Academy of Sciences* **112:** 6754-6759

**Gutjahr C, Sawers RJH, Marti G, Andrés-Hernández L, Yang S-Y, Casieri L, Angliker H, Oakeley EJ, Wolfender J-L, Abreu-Goodger C, Paszkowski U** (2015) Transcriptome diversity among rice root types during asymbiosis and interaction with arbuscular mycorrhizal fungi. *Proceedings of the National Academy of Sciences* **112:** 6754-6759

**Hashem A, Abd_Allah EF, Alqarawi AA, Al-Huqail AA, Wirth S, Egamberdieva D** (2016) The Interaction between Arbuscular Mycorrhizal Fungi and Endophytic Bacteria Enhances Plant Growth of Acacia gerrardii under Salt Stress. *Frontiers in Microbiology* **7:** 1089

**Huang Y, Wong PTW** (1998) Effect of Burkholderia (Pseudomonas) cepacia and soil type on the control of crown rot in wheat. *Plant and Soil* **203:** 103-108

**James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PPM, Olivares FL, Ladha JK** (2002) Infection and Colonization of Rice Seedlings by the Plant Growth-Promoting Bacterium Herbaspirillum seropedicae *Z*67. *Molecular Plant-Microbe Interactions* **15:** 894-906

**Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea J** (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils*, Volume 37, Issue 1, pp 1–16

**Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ** (2012) Mycorrhiza-induced resistance and priming of plant defenses. *J Chem Ecol* **38:** 651-664

**Jäderlund L, Arthurson V, Granhall U, Jansson JK** (2008) Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria: as revealed by different combinations. *FEMS Microbiol Lett* **287:** 174-180

**Kannangara R., Branigan C., Liu Y., Penfield T., Rao V.,** and all (2007) The Transcription Factor WIN1/SHN1 Regulates Cutin Biosynthesis in Arabidopsis thaliana. *The Plant Cell*, Vol. 19: 1278–1294

**Lakshmanan V, Selvaraj G, Bais HP** (2014) Functional Soil Microbiome: Belowground Solutions to an Aboveground Problem. *Plant Physiology* **166:** 689

**Lareen A, Burton F, Schäfer P** (2016) Plant root-microbe communication in shaping root microbiomes. *Plant Mol Biol* **90:** 575-587

**Li J, Mahajan A, Tsai M-D** (2006) Ankyrin Repeat: A Unique Motif Mediating Protein−Protein Interactions. *Biochemistry* **45:** 15168-15178

**Linderman LG** (1992) Vesicular–arbuscular mycorrhizae and soil microbial interactions. *In Mycorrhizae in sustainable agriculture*, Vol 54. ASA Special Publication, Madison, Wisconsin, pp 45-70

**Liu A, Hamel C, Hamilton RI, Ma BL, Smith DL** (2000) Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (Zea mays L.) grown in soil at different P and micronutrient levels. *Mycorrhiza* **9:** 331-336

**Lothier J, Gaufichon L, Sormani R, Lemaître T, Azzopardi M, Morin H, Chardon F, Reisdorf-Cren M, Avice JC, Masclaux-Daubresse C** (2011) The cytosolic glutamine synthetase GLN1;2 plays a role in the control of plant growth and ammonium homeostasis in Arabidopsis rosettes when nitrate supply is not limiting. *J Exp Bot* **62:** 1375-1390

**Loyola-Vargas VM, Broeckling CD, Badri D, Vivanco JM** (2007) Effect of transporters on the secretion of phytochemicals by the roots of Arabidopsis thaliana. *Planta* **225:** 301-310

**Ma Y, Rajkumar M, Luo YM, Freitas H** (2011) Inoculation of endophytic bacteria on host and non-host plants-Effects on plant growth and Ni uptake. Journal of Hazardous Materials **195:** 230-237

**Mahenthiralingam E, Baldwin A, Dowson CG** (2008) Burkholderia cepacia complex bacteria: opportunistic pathogens with important natural biology. *Journal of Applied Microbiology* **104:** 1539-1551

**Manaa A, Ben Ahmed H, Valot B, Bouchet JP, Aschi-Smiti S, Causse M, Faurobert M** (2011) Salt and genotype impact on plant physiology and root proteome variations in tomato. *J Exp Bot* **62:** 2797-2813

**Mayak S, Tirosh T, Glick BR** (2004) Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiology and Biochemistry **42:** 565-572

**Mei X, Chen Y, Zhang L, Fu X, Wei Q, Grierson D, Zhou Y, Huang Y, Dong F, Yang Z** (2016) Dual mechanisms regulating glutamate decarboxylases and accumulation of gamma-aminobutyric acid in tea (Camellia sinensis) leaves exposed to multiple stresses. Sci Rep **6:** 23685

**Melo-Oliveira R, Oliveira IC, Coruzzi GM** (1996) Arabidopsis mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc Natl Acad Sci U S A **93:** 4718-4723

**Meyer SLF, Massoud SI, Chitwood DJ, Roberts DP** (2000) Evaluation of Trichoderma virens and Burkholderia cepacia for antagonistic activity against root-knot nematode, Meloidogyne incognita. Nematology **2:** 871-879

**Meyer SLF, Roberts DP, Chitwood DJ, Carta LK, Lumsden RD, Mao WL** (2001) Application of Burkholderia cepacia and Trichoderma virens, alone and in combinations, against Meloidogyne incognita on bell pepper. Nematropica **31:** 75-86

**Miché L, Battistoni F, Gemmer S, Belghazi M, Reinhold-Hurek B** (2006) Upregulation of jasmonate-inducible defense proteins and differential colonization of roots of Oryza sativa cultivars with the endophyte Azoarcus sp. Mol Plant Microbe Interact **19:** 502-511

**Nadeem SM, Ahmad M, Zahir ZA, Javaid A, Ashraf M** (2014) The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnol Adv **32:** 429-448

**Nadeem SM, Zahir ZA, Naveed M, Arshad M** (2007) Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Canadian Journal of Microbiology **53:** 1141-1149

**Nowak J, Shulaev V** (2003) Priming for transplant stress resistance in In vitro propagation. In Vitro Cellular & Developmental Biology - Plant **39:** 107-124

**Ordookhani K KK, Moezzi A and Rejali F** Influence of PGPR and AMF on antioxidant activity, lycopene and potassium contents in tomato. *In* African Journal of Agricultural Research, Vol 5, pp 1108-1116

**Paungfoo-Lonhienne C, Lonhienne TG, Yeoh YK, Webb RI, Lakshmanan P, Chan CX, Lim PE, Ragan MA, Schmidt S, Hugenholtz P** (2014) A new species of Burkholderia isolated from sugarcane roots promotes plant growth. Microb Biotechnol **7:** 142-154

**Paungfoo-Lonhienne C, Lonhienne TGA, Yeoh YK, Webb RI, Lakshmanan P, Chan CX, Lim P-E, Ragan MA, Schmidt S, Hugenholtz P** (2014) A new species of Burkholderia isolated from sugarcane roots promotes plant growth. Microbial Biotechnology **7:** 142-154

**Perin L, Martínez-Aguilar L, Castro-González R, Estrada-de los Santos P, Cabellos-Avelar T, Guedes HV, Reis VM, Caballero-Mellado J** (2006) Diazotrophic Burkholderia Species Associated with Field-Grown Maize and Sugarcane. Applied and Environmental Microbiology **72:** 3103-3110

**Poupin MJ, Timmermann T, Vega A, Zuniga A, Gonzalez B** (2013) Effects of the Plant Growth-Promoting Bacterium Burkholderia phytofirmans PsJN throughout the Life Cycle of Arabidopsis thaliana. Plos One **8**

**Pozo MJ, Azcón-Aguilar C** (2007) Unraveling mycorrhiza-induced resistance. Curr Opin Plant Biol **10:** 393-398

**Salvioli A, Zouari I, Chalot M, Bonfante P** (2012) The arbuscular mycorrhizal status has an impact on the transcriptome profile and amino acid composition of tomato fruit. BMC Plant Biology **12:** 44-44

**Sandhya V, Ali SZ, Venkateswarlu B, Reddy G, Grover M** (2010) Effect of osmotic stress on plant growth promoting Pseudomonas spp. Archives of Microbiology **192:** 867-876

**Schouteden N, De Waele D, Panis B, Vos CM** (2015) Arbuscular Mycorrhizal Fungi for the Biocontrol of Plant-Parasitic Nematodes: A Review of the Mechanisms Involved. Frontiers in Microbiology **6**

**Schwachtje J, Karojet S, Thormählen I, Bernholz C, Kunz S, Brouwer S, Schwochow M, Köhl K, van Dongen JT** (2011) A naturally associated rhizobacterium of Arabidopsis thaliana induces a starvation-like transcriptional response while promoting growth. PLoS One **6:** e29382

**Sessitsch A, Coenye T, Sturz AV, Vandamme P, Barka EA, Salles JF, Van Elsas JD, Faure D, Reiter B, Glick BR, Wang-Pruski G, Nowak J** (2005) Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *Int J Syst Evol Microbiol* **55:** 1187-1192

**Sharma DI, Sharma AK** (2017) Co-inoculation of tomato with an arbuscular mycorrhizal fungus improves plant immunity and reduces root-knot nematode infection. Rhizosphere Volume 4, Pages 25-28

**Sharma VK, Nowak J** (1998) Enhancement of verticillium wilt resistance in tomato transplants by in vitro co-culture of seedlings with a plant growth promoting rhizobacterium (Pseudomonas sp. strain PsJN). Canadian Journal of Microbiology **44:** 528-536

**Shi H, Shen Q, Qi Y, Yan H, Nie H, Chen Y, Zhao T, Katagiri F, Tang D** (2013) BR-SIGNALING KINASE1 Physically Associates with FLAGELLIN SENSING2 and Regulates Plant Innate Immunity in Arabidopsis. The Plant Cell **25:** 1143-1157

**Singh JS, Pandey VC, Singh DP** (2011) Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. Agriculture Ecosystems & Environment **140:** 339-353

**Singh VK, Singh AK, Kumar A** (2017) Disease management of tomato through PGPB: current trends and future perspective. 3 Biotech. 7(4):255. doi: 10.1007/s13205-017-0896-1.

**Sugiyama A, Shitan N, Yazaki K** (2007) Involvement of a Soybean ATP-Binding Cassette-Type Transporter in the Secretion of Genistein, a Signal Flavonoid in Legume-Rhizobium Symbiosis. Plant Physiology **144:** 2000-2008

**Swaroopa M** (2014) Bacterial Endophytes in Sustainable Crop Production: Applications, Recent Developments and Challenges Ahead. International Journal of Life Sciences Research **2:** 46-56

**Trân Van V, Berge O, Ngô Kê S, Balandreau J, Heulin T** (2000) Repeated beneficial effects of rice inoculation with a strain of Burkholderia vietnamiensison early and late yield components in low fertility sulphate acid soils of Vietnam. Plant and Soil **218:** 273-284

**Vacheron J, Desbrosses G, Bouffaud ML, Touraine B, Moënne-Loccoz Y, Muller D, Legendre L, Wisniewski-Dyé F, Prigent-Combaret C** (2013) Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci **4:** 356

**Vafadar F, Amooaghaie R, Otroshy M** (2014) Effects of plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungus on plant growth, stevioside, NPK, and chlorophyll content of Stevia rebaudiana. Journal of Plant Interactions **9:** 128-136

**Viallard V, Poirier I, Cournoyer B, Haurat J, Wiebkin S, Ophel-Keller K, Balandreau J** (1998) Burkholderia graminis sp. nov., a rhizospheric Burkholderia species, and reassessment of [Pseudomonas] phenazinium, [Pseudomonas] pyrrocinia and [Pseudomonas] glathei as Burkholderia. Int J Syst Bacteriol **48 Pt 2:** 549-563

**Wang F, Jing W, Zhang W** (2014) The mitogen-activated protein kinase cascade MKK1-MPK4 mediates salt signaling in rice. Plant Sci **227:** 181-189

**Wang ZY** (2012) Brassinosteroids modulate plant immunity at multiple levels. Proc Natl Acad Sci U S A **109:** 7-8

**Weston DJ, Pelletier DA, Morrell-Falvey JL, Tschaplinski TJ, Jawdy SS, Lu TY, Allen SM, Melton SJ, Martin MZ, Schadt CW, Karve AA, Chen JG, Yang X, Doktycz MJ, Tuskan GA** (2012) Pseudomonas fluorescens induces strain-dependent and strain-independent host plant responses in defense networks, primary metabolism, photosynthesis, and fitness. Mol Plant Microbe Interact **25:** 765-778

**Willmann M, Gerlach N, Buer B, Polatajko A, Nagy R, Koebke E, Jansa J, Flisch R, Bucher M** (2013) Mycorrhizal phosphate uptake pathway in maize: vital for growth and cob development on nutrient poor agricultural and greenhouse soils. Frontiers in Plant Science **4:** 533

**Yusran Y. RV, Mueller T.** (2009) Effects of Pseudomonas sp. "Proradix" and Bacillus amyloliquefaciens FZB42 on the Establishment of AMF Infection, Nutrient Acquisition and Growth of Tomato Affected by Fusarium oxysporum Schlecht f.sp. radicis-lycopersici. University of California

**Zahir ZA, Munir A, Asghar HN, Shaharoona B, Arshad M** (2008) Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (Pisum sativum) under drought conditions. J Microbiol Biotechnol **18:** 958-963

**Zouari I, Salvioli A, Chialva M, Novero M, Miozzi L, Tenore GC, Bagnaresi P, Bonfante P** (2014) From root to fruit: RNA-Seq analysis shows that arbuscular mycorrhizal symbiosis may affect tomato fruit metabolism. BMC Genomics **15:** 221

| **AA** | **RB/RC** | LB/LC |
|---|---|---|
| Aspartic acid | **2.20*** | 0.76 |
| Glutamic acid | **1.81*** | **0.52*** |
| Alanine | **6.51***** | 0.44 |
| Arginine | 1.08 | **0.69**** |
| Asparagine | **1.76***** | 0.86 |
| Cystine | 1.69 | 1.27 |
| Citrulline | 0.89 | 0.86 |
| Phenylalanine | **1.46***** | 1.04 |
| GABA | 1.38 | 1.24 |
| Glutamine | **3.62***** | 0.99 |
| Histidine | **1.97**** | 1.25 |
| Leucine | **3.03***** | 1.08 |
| Lysine | **3.63***** | 0.90 |
| Methionine | **1.75***** | 0.90 |
| Ornithine | **1.76***** | 0.86 |
| Proline | **1.96*** | 0.98 |
| Serine | **2.78**** | **0.51*** |
| Tyrosine | 1.39 | 0.96 |
| Threonine | **2.68***** | 0.80 |
| Tryptophan | 1.71 | **1.53*** |
| Valine | **1.95**** | 0.73 |

**Table 1.** Free AA contents in roots and leaves measured as B/C ratio. Two-tailed *t* test; **P < 0.01, and *P < 0.05

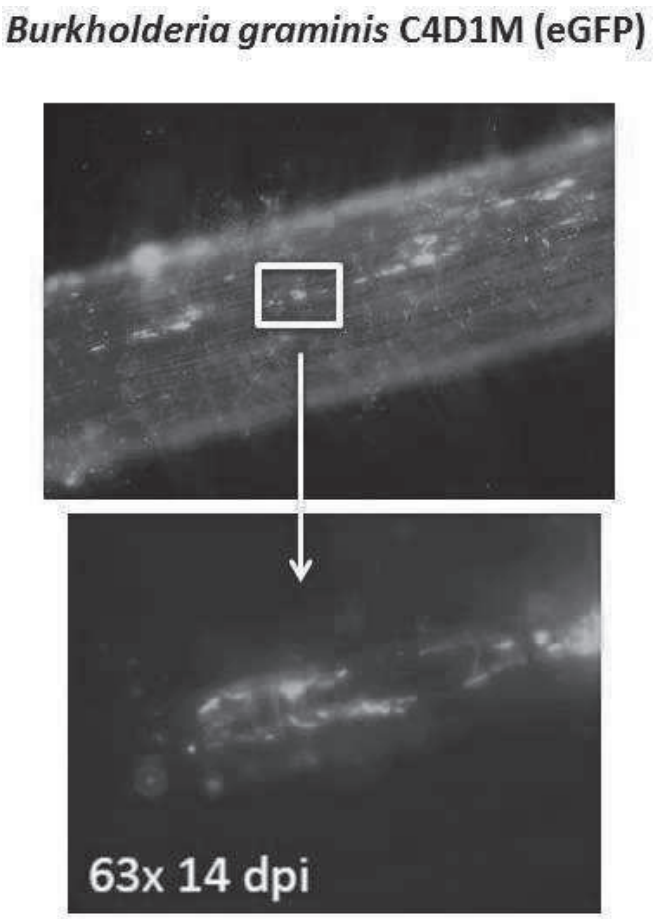
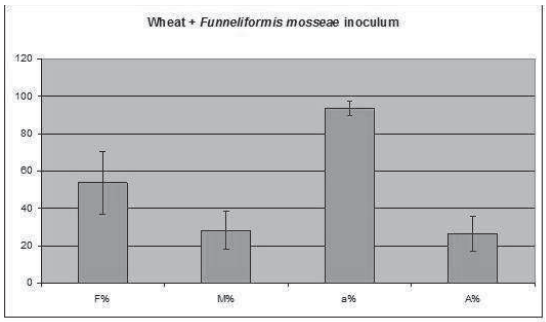


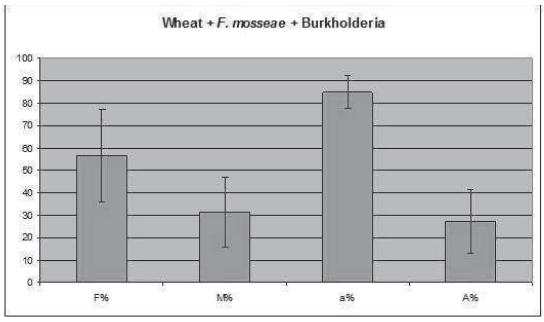

**Fig. 1:** Bacteria were tag with the green fluorescent protein eGFP and looked at roots 2 weeks post inoculation. *B. graminis* invades surface cells and can also colonize endophytically the wheat root.

a




| | F% | M% | a% | A% |
|---|---|---|---|---|
| Plant 1 | 33,75 | 17,55 | 94,17 | 16,53 |
| Plant 2 | 70,83 | 37,4 | 93,92 | 35,12 |
| Plant 3 | 70,24 | 40,25 | 93,18 | 37,51 |
| Plant 5 | 52,53 | 24,82 | 87,41 | 21,69 |
| Plant 6 | 40,35 | 21 | 98,5 | 20,68 |
| Mean | 53,54 | 28,204 | 93,436 | 26,306 |
| Standard Deviation | 16,91504 | 10,08129 | 3,962175 | 9,377443 |

b




| | F% | M% | a% | A% |
|---|---|---|---|---|
| Plant 1 | 56,58 | 33,83 | 95,14 | 31,85 |
| Plant 2 | 75,53 | 51,48 | 86,19 | 44,37 |
| Plant 3 | 45,95 | 18,89 | 77,64 | 14,67 |
| Plant 4 | 63,74 | 38,31 | 80,46 | 30,82 |
| Plant 5 | 22 | 8,35 | 77,8 | 6,5 |
| Plant 6 | 76,14 | 38,98 | 91,05 | 35,49 |
| Mean | 56,65667 | 31,64 | 84,71333 | 27,28333 |
| Standard Deviation | 20,50219 | 15,50337 | 7,30707 | 14,02647 |

**Fig. 2:** Detection of mycorrhizal colonization. To asses the rate of mycorrhization in (a) wheat + *Funneliformis mossae* (M plants) and (b) wheat + *Funneliformis mossae* + *Burkholderia* (MB plants), four parameters were considered: the percentage of segments showing internal colonization (F%); the average per cent colonization of root segments (M%); the average presence of arbuscules within the infected areas (a%); the presence of arbusculesin the whole root system (A%).

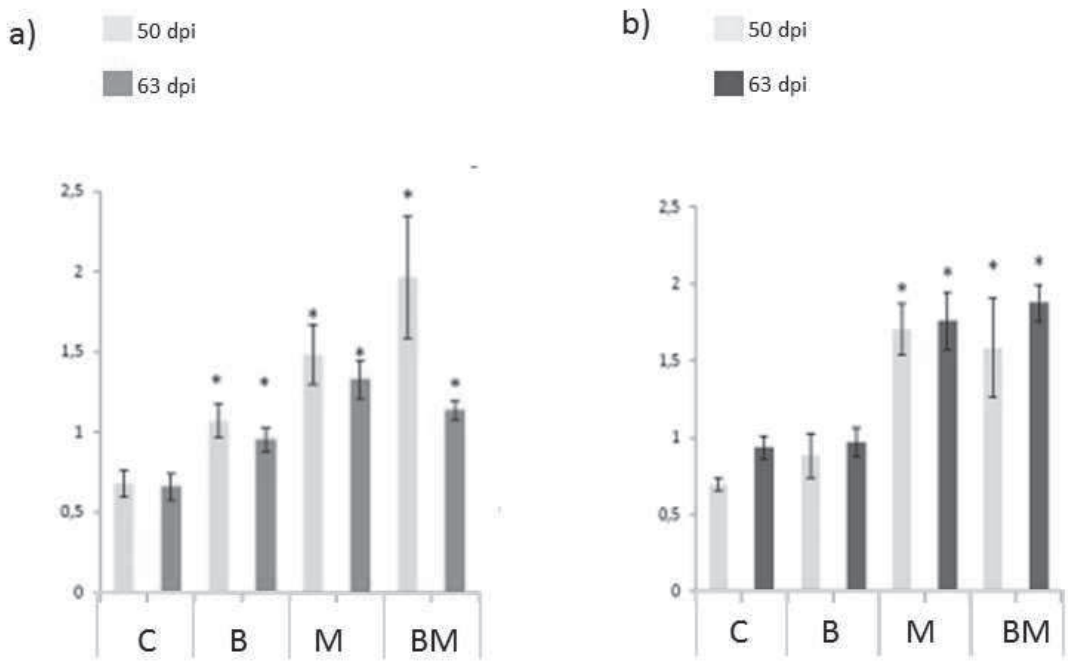



**Fig. 3**: Effect of *Burkholderia graminis* inoculation. Fresh weight of roots (a) and leaves (b) of the control (C), *B. graminis* (B), *Funneliformis mossae* (M), *Funneliformis mossae* + *B. graminis* (MB) inoculated plants harvested at 50 and 63 days post inoculation.

## Supporting Information

Additional Supporting Information may be found at the link:

https://drive.google.com/drive/folders/0ByLbVnSXW-J3WUREd0JKMWdVMW8?usp=sharing

# Chapter 3

**An interdomain network: the endobacterium of a mycorrhizal fungus promotes antioxidative responses in both fungal and plant hosts.**