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THÈSE DE DOCTORAT

**Symbiose fixatrice d'azote versus nutrition
minérale azotée : conséquence sur l'interaction
entre *Medicago truncatula* et le puceron du pois
*Acyrtosiphon pisum***

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Symbiose fixatrice d'azote versus nutrition minérale azotée : conséquence sur l'interaction entre *Medicago truncatula* et le puceron du pois *Acyrtosiphon pisum*

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RESUMÉ

Les symbiotes jouent un rôle crucial dans le phénotype de leur hôte et dans son adaptation à l'environnement. Cependant, jusqu'à récemment, les interactions plantes-insectes étaient étudiées sans tenir compte de la présence de bactéries symbiotiques chez les partenaires impliqués. De nouvelles découvertes ont démontré que les communautés racinaires et aériennes des plantes sont liées. Dans ce contexte, mon doctorat s'interroge sur la façon dont les interactions entre les espèces végétales et les insectes sont modulées par leurs symbiotes respectifs.

Dans un premier temps, j'ai analysé le rôle de la symbiose fixatrice d'azote (NFS) chez la légumineuse *Medicago truncatula* (A17) dans l'interaction avec des lignées de pucerons du pois *Acyrtosiphon pisum* portant différents endosymbionts facultatifs (FS). Pour ce faire, j'ai comparé la croissance de plantes de *M. truncatula* inoculées avec la bactérie nodulante *Sinorhizobium meliloti* (NFS) ou arrosées avec une solution de nitrate (non inoculées ; NI) infestées par des lignées de pucerons du pois provenant d'un même clone génétique (YR2) soit sans FS ou avec *Hamiltonella defensa*, *Serratia symbiotica* ou *Regiella insecticola*. La croissance des plantes NSF et NI est réduite par l'attaque des pucerons, tandis que la croissance des pucerons (mais pas leur survie) a été fortement réduite sur les plants NFS. En présence de pucerons la capacité de fixation d'azote des plantes NFS est réduite suite à l'induction d'une sénescence précoce des nodules. Enfin, chez les plantes NFS, toutes les lignées de pucerons ont déclenché l'expression du gène *Pathogenesis-Related-1* (*PR1*), un marqueur de la voie salicylique (SA), et du gène *Proteinase inhibitor* (*PI*), un marqueur de la voie jasmonique (JA), tandis que chez les plantes NI, seule l'expression de *PR1* a été déclenchée. Ainsi, le statut symbiotique de la plante influence clairement les interactions plante-puceron et la réponse de la plante à l'infestation, alors que le statut symbiotique du puceron ne fait que moduler l'amplitude de cette réponse.

Il a été démontré que le génotype de la plante et du puceron sont tous deux importants dans le résultat de leur interaction, j'ai donc étudié plus en détail comment la NFS affecte l'interaction entre différents génotypes de plantes et de pucerons. Pour cela, j'ai utilisé trois génotypes différents d'*A. pisum* dépourvus de FS (LL01, YR2, T3-8V1) et deux génotypes de *M. truncatula* (A17 et R108) en présence ou en absence *S. meliloti*. La performance de chaque génotype de puceron sur les deux génotypes de plantes et l'effet des différents génotypes de pucerons sur la croissance des plantes et la capacité de fixation de l'azote des plantes de SNF ont été mesurés. Nous avons également estimé la réponse de défense médiée par le génotype de *M. truncatula* déclenchée par les différents génotypes de pucerons en utilisant différents gènes marqueurs des voies de défense JA et SA. J'ai constaté que les génotypes plantes-insectes ainsi que la présence de *S. meliloti* affectent de manière significative les interactions plantes-aphides.

Ainsi, les interactions génétiques interspécifiques entre la plante hôte et les pucerons ainsi que leur statut symbiotique peuvent influencer la dynamique de la population et la structure de la communauté. Ces résultats montrent que l'interaction plante-insecte est fortement influencée par la génétique des espèces et par leur statut symbiotique, ajoutant un nouveau niveau de complexité qui reste à explorer.

Mots clefs

Puceron du pois (*Acyrtosiphon pisum*), *Medicago truncatula*, symbiose facultative, symbiose, rhizobium, fixation de l'azote, défense des plantes.

SUMMARY

Symbionts play a crucial role in shaping their host phenotype and driving its adaptation to the environment. However, until recently plant-insect interactions were studied disregarding the symbiotic bacterial presence in the involved partners. New findings have now demonstrated that above- and belowground plant communities are linked through biotic interactions. In this context, my PhD questions how the interaction between plant-insect species are modulated by their respective symbionts.

In the first part of my work I have analysed the effect of the nitrogen fixing symbiosis (NFS) in the leguminous *Medicago truncatula* (A17) in interaction with pea aphid *Acyrtosiphon pisum* lines bearing different facultative endosymbionts (FS). For this, first I have compared the growth of *M. truncatula* plants either inoculated with the nodules inducing bacteria *Sinorhizobium meliloti*

(NFS) or supplemented with nitrate (non-inoculated; NI), infested with pea aphid lines derived from the same genetic clone (YR2) and bearing either no FS or *Hamiltonella defensa*, *Serratia symbiotica* or *Regiella insecticola*. As expected, growth of both NFS and NI plants was reduced by the aphid attack, while aphid growth (but not survival) was strongly reduced on NFS compared to NI plants. Interestingly, most aphid lines decreased the plant nitrogen fixation capacity of NFS plants by inducing an early nodule senescence. Finally, in NFS plants all aphid lines triggered the expression of *Pathogenesis Related Protein 1* (*PR1*), a marker of the salicylic (SA) pathway, and of *Proteinase Inhibitor* (*PI*), a marker of the jasmonic (JA) pathway, while in NI plants only *PR1* expression was triggered. Thus, the plant symbiotic status influences clearly the plant-aphid interactions and the plant response while the aphid symbiotic status only modulates the response amplitude.

Since both plant and aphid genotypes are important in the outcome of their interaction, I further studied how plant symbiosis affect the plant-insect genotype x genotype interaction. For this, I used three different pea aphid genotypes devoid of FS (LL01, YR2, and T3-8V1) and two *M. truncatula* genotype (A17 and R108) combinations in the presence or absence of rhizobacteria. The performance of each aphid genotype on both plant genotypes and the effect of different aphid genotypes on the plant growth and nitrogen fixation capacity of NFS plants were measured. We also estimated *M. truncatula* genotype-mediated defence response triggered by the different aphid genotypes using multiple gene markers of the JA and SA defence-pathways. I found that the plant-insect genotypes as well as the rhizobacteria presence significantly affect plant-aphid interactions.

These results show that the outcome of the plant-insect interaction is strongly impacted by the genotype of the species and by their symbiotic status, rising a new level of complexity that remains to be explored.

Key words

Pea aphid (*Acyrtosiphon pisum*), *Medicago truncatula*, Facultative symbiont, symbiosis, rhizobium, nitrogen fixation, plant defence.

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LIST OF ABBREVIATIONS

In alphabetical order:

| | |
|-------------|---------------------------------------|
| AOS | Allene Oxide Synthase |
| Ap | <i>Acyrtosiphon pisum</i> |
| Avr | Avirulence |
| ET | Ethylene |
| ETI | Effector Triggered Immunity |
| FS | Facultative Symbiont |
| ISR | Induced Systemic Resistance |
| JA | Jasmonic Acid |
| LOX | Lipoxygenase |
| Mt | <i>Medicago truncatula</i> |
| NFS | Nitrogen Fixing Symbiosis |
| NI | Non-inoculated |
| PAMP | Pathogen Associated Molecular Pattern |
| PI | Proteinase inhibitor |
| PR | Pathogenesis Related |
| ROS | Reactive Oxygen Species |
| PTI | PAMP Triggered Immunity |
| SA | Salicylic Acid |
| SAR | Systemic Acquired Resistance |

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 Interactions and Evolutionary Processes, the Holobiont Theory

Most of the organisms living in the same environment directly or indirectly interact with each other. Each species thus evolves in a wide network of interactions with a great diversity of life forms. In nature, these interactions can be classified according to their durability and their cost / benefit: beneficial for one species and costly for the other (i.e. predation, parasitism), beneficial for both (mutualism), costly for both (competition) or beneficial for one and neutral for the other (commensalism and phoresy). Competition and predation are not considered durable interactions, unlike parasitism and mutualism, and have less impact on the evolution of the organisms involved. These beneficial, neutral, or unfavourable relationships influence the growth, survival and reproduction of individuals of a given species and can exert significant selective pressures on the population.

Among the interactions between living organisms, those involving microbes and their hosts play a pivotal role. In most cases, the functions of microbial partners influence the growth, development and health of the host. In exchange, the host provides a favourable environment for the growth and multiplication of microbes. The current host-microorganisms interactions result from ancient co-evolutionary processes of adaptation and specialization which have largely modulated the evolution of living organisms. These host-microorganism interactions have given rise to the use of the term "Holobiont" to describe an individual host not as a single entity but as a more complex entity made up of the host and the hosted microbial communities in all types of ecosystems (Figure1) (Simon et al., 2019). The spectrum of interactions in holobionts therefore ranges from pathogenesis/parasitism to mutualism and from facultative to obligatory symbioses. Among the mutualistic symbioses, microorganisms can live inside the host (endosymbionts) or even inside specific host cells (endocytobionts), often set up by the host. Endocytobiont-host interactions provide examples of extreme metabolic and genetic coadaptation, specifically in cases of old symbioses. In most of the cases, such symbionts are vertically transmitted from the mother to the offspring, which leads to a lower virulence of microbes. Insect symbiosis are a model for this symbiotic interactions, while ectosymbionts and intestinal microbiomes such as observed in humans are seemingly looser symbioses that have a more complex mode of

transmission (Browne et al., 2017; Ferretti et al., 2018). Microorganisms have important roles in the biology of multicellular organisms including digestive process or proper host development. From a genomic aspect, the hologenome concept, suggests that the physiology of any macroscopic organism derives from the integrated activities of its own genome and all the genomes of its microbiome (Figure1). The hologenome theory of evolution (HTE), (Zilber-Rosenberg and Rosenberg, 2008; Brucker and Bordenstein, 2012, 2013) states that a host is inseparable from its associated microbiome (collectively, a holobiont), and they constitute together a "unit of selection" in evolution. Although this theory is still debated wondering about the definitions of holobiont and hologenome (Theis et al., 2016). The concepts hologenome and holobiont are now routinely used in the literature and applied for a wide variety of organisms.

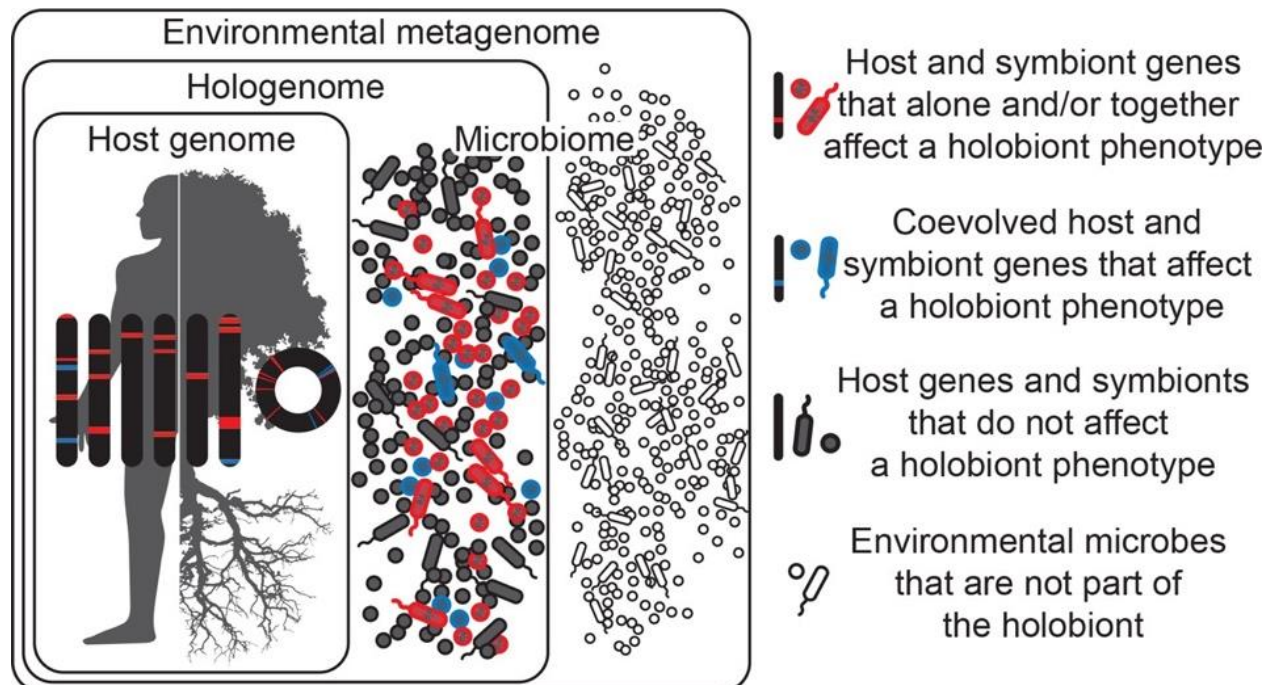


Figure 1 Holobionts and hologenomes. An holobiont is an entity formed by a host and its symbiotic microbes. This includes the microbes that affect the holobiont's phenotype and co-evolved (blue) or not (red) with the host, and the ones that do not affect the holobiont's phenotype (grey). Microbes may be transmitted vertically or horizontally and acquired from the environment. Holobiont phenotypes can change in time and space according to the microbes present in the holobiont. Microbes in the environment are not part of the holobiont (white). Hologenomes refer to the genomes of the host and its microbes at any given time point. Adapted from (Theis et al., 2016).

In addition, the hologenome could change under different environmental stresses either due to changes in the host of the symbiont genome, or the interaction between both. This indicates that the genetic richness of various microbial symbionts may play an important role in the adaptation and evolution of higher organisms.

1.2 Microbes, the New Players in the Plant-Insect Interactions

Plants and insects have co-existed and interacted for over 400 million years (Sugio et al., 2015). These interactions shaped the first terrestrial ecosystems, leading to the development of complex relationships ranging from antagonism to mutualism (Gatehouse, 2002). Insects can be either beneficial to plants, as pollinators or predators of harmful insects, or directly detrimental as herbivorous or indirectly as phytopathogenic vectors. Selection has thus acted on plants to reduce insect attacks through various physical and chemical defence mechanisms. In response, insects have developed a range of offensive strategies to manipulate the defence response of plants to successfully evolve on plants and adapt to different conditions. In the last decades, microorganisms were shown to play a significant role in the modulation of plant-insect interactions and they are therefore currently widely studied (Dean et al., 2009; Pineda et al., 2010; Sanchez-Arcos et al., 2016). Plant and insect symbionts can indeed shape their host phenotype and drive its adaptation to the environment (Kanvil et al., 2014b; Simon and Peccoud, 2018). Among these interactions, microbe-mediated plant-phytophagous insect interactions are described as “tri-trophic” or three-way interactions” (Biere and Bennett, 2013; Biere and Tack, 2013(a)). This three-way interaction includes positive or negative, direct or indirect, and trophic or non-trophic relationships between partners (see an example in Figure 2). Plant-associated microbes have been shown to affect insect performance in terms of attractivity, feeding efficiency, metabolism and ability to manipulate the host plant physiology (Dean et al., 2009; Pineda et al., 2010; Brunner et al., 2015; Sugio et al., 2015; Giron et al., 2017). In parallel, insect endosymbionts directly affect insect hosts reproduction, immunity and plant use (Sugio et al., 2015; Giron et al., 2017; Simon and Peccoud, 2018). Furthermore, they can also indirectly interfere with plant signal transduction pathways by either repressing or counteracting defence-related responses or altering plant metabolism (Clark et al., 2010; Su et al., 2013; Guo et al.,

2017). These multitrophic interactions play a relevant role in shaping the structure and diversity of living communities both in natural and agricultural ecosystems.

Although a solid fundamental understanding of direct plant-microbe and insect-microbe interactions has been acquired over the years, the effect of each organism symbiosis on the global interaction remains to be elucidated. Symbionts are enclosed in hosts, which are included in communities, which belong to an ecosystem. The goal is to discover how individual partners interact, connect, and communicate with each other and their environment. Exploring such relations both at an ecological and molecular level will help us better understand how symbiosis shapes the evolution of partner interaction.

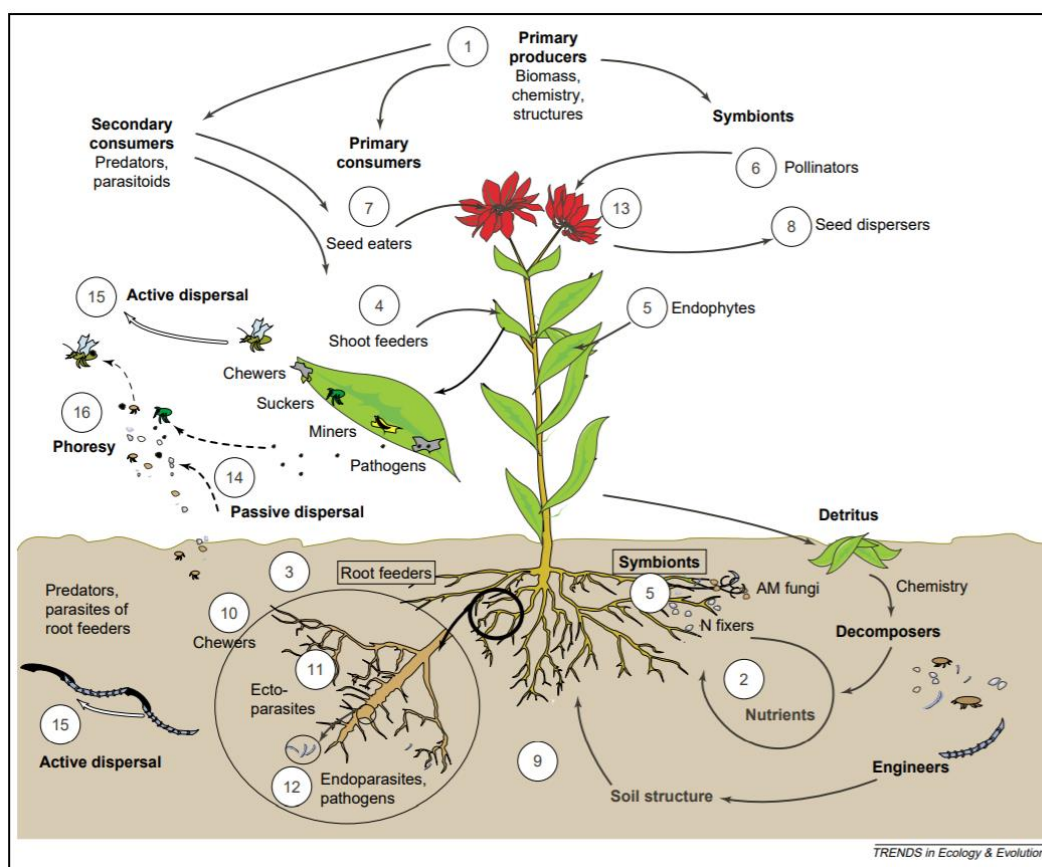


Figure 2 Interrelationship between the belowground and aboveground biodiversity. Aboveground plant biomass and chemical and structural composition (1) drive the abundance and diversity of aboveground levels. These aboveground characteristics depend upon the activity of soil decomposers and symbionts (5), which make nutrients available (2), and on aboveground and belowground herbivores and pathogens (3,4), which reduce plant growth. Heterotrophic organisms that interact with plants affect plant metabolism (3-5). In the longer term, pollinators (6) as well as seed eaters (7) and dispersers (8) affect the persistence of the plant species. Soil organisms interacting with a single plant root system are subsets of

the total species present in the direct surrounding soil (9). Although active roots have high turnover rates and are distributed throughout the soil, root herbivores and pathogens (3) can account for this ‘unstable food’ source by being relatively mobile (10, 11), similar to many aboveground chewing insects and free-living suckers, by adapting a specialized endoparasitic plant association (12) or by having an aboveground life phase enabling targeted active dispersal (15). Large aboveground and belowground organisms might disperse actively in a directional way (15), by flying, walking, crawling or borrowing, whereas smaller organisms (or small structures of larger organisms, such as seeds) disperse more randomly (14) by air, water or via phoresy (16). Abbreviations: AM fungi, arbuscular mycorrhizal fungi; N-fixers, nitrogen-fixing microorganisms. Adapted from (De Deyn and Van Der Putten, 2005).

My PhD work focused on the involvement of bacterial partners in the interaction between leguminous plants and pea aphids. To this purpose, I used the legume model *Medicago truncatula* in interaction with the pea aphid *Acyrtosiphon pisum*, and its bacterial partners. The rest of the introduction focus on description of these two model organisms and their involvement with bacterial partner.

1.3 Plant Symbiosis

Given that plants live fixed in the environment, they face multiple challenges to acquire resources, grow and defend themselves. However, they establish symbiotic relationship to fulfil these different functions. In fact, plants show a compatible phylogeny with symbiotic ectomycorrhizal fungi, suggesting that they have co-evolved with microorganisms since their first appearance on land, more than 500 million years ago (Cairney, 2000; Martin et al., 2017).

In the environment, microbes colonize plants and form with the complex interactions. These interactions normally occur in the rhizosphere, endosphere and phyllosphere (Figure 3), with soil being essential as the major source of microbial diversity (Gopal and Gupta, 2016; Compant et al., 2019). The selection of microbes from the soil pool inside host plants is driven by complex interactions between host plants and microorganisms. The plant genotype, its root exudates, soil types and properties, and environmental factors influence plant microbiome (Figure 3). These interactions range from mutualism to parasitism, and their outcome is important for the plant performance.

Plants form beneficial symbioses with a wide variety of microorganisms, among which the root associated arbuscular mycorrhizal fungi, endophytes, ectomycorrhizal fungi, rhizobacteria and actinobacterial symbioses are some of the most studied and understood. These microorganisms

provide several services and benefits to host plants. In exchange, they receive the carbon and reduced metabolites necessary for their development and multiplication.

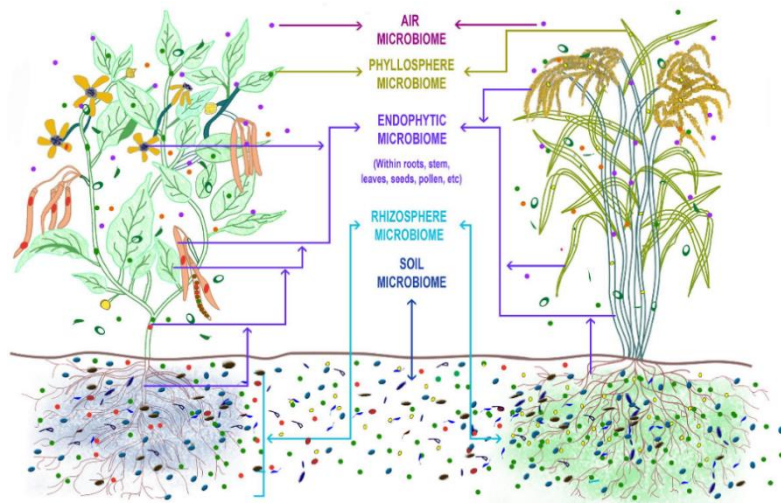


Figure 3 The plant microbiome.

The plant microbiome refers to the genomes of all the microbial communities present in the surface extend and internal tissues of the plant. Microbial communities mainly reside in the rhizosphere, endosphere and phyllosphere of the plant. From (Gopal and Gupta, 2016).

It has been extensively shown that plant-growth-promoting rhizobacteria (PGPR) and Arbuscular Mycorrhizae (AM) can improve crop productivity. PGPR and AM are naturally occurring soil microorganisms that aggressively colonize plant roots and benefit plants. For example, AM can help plants to capture phosphorus, nitrogen (N) and other nutrients from the soil (Jansa et al., 2019). AM also improve the tolerance of plants to stress (Begum et al., 2019). PGPR, such as free-living diazotrophs, provide N to a wide range of plants (Backer et al., 2018). In addition, PGPR can help plants to overcome abiotic stress. For instance, *Pseudomonas putida* and *P. fluorescens* respectively provide drought stress tolerance in chickpea (Gontia-Mishra et al., 2016) and rice plants (Etesami et al., 2007), while *Bacillus amyloliquefaciens* provides salt stress tolerance to maize (Chen et al., 2016). PGPR and AM also improve plant biotic stress tolerance by triggering Induced Systemic Resistance (ISR) in plants, which activates pathogenesis-related genes mediated by phytohormone signalling pathways and defence regulatory proteins, priming plants against potential pathogens and insects attacks (Pieterse et al., 2014). For example, *B. amyloliquefaciens*, now a commercial PGPR used for the management of a wide range of foliar diseases on agricultural crops, protects against the pathogenic fungus *Rhizoctonia solani* (Solanki et al., 2015). PGPRs also act against insects although with different outcomes depending on the species: *P. fluorescens*-induced ISR had an adverse impact on growth and development of the

generalist herbivore *Spodoptera exigua*, but not on the specialist *Pieris rapae* (Van Oosten et al., 2008). Conversely, it has a positive effect on the performance of the generalist aphid *Myzus persicae*, but no effect on the crucifer specialist aphid *Brevicoryne brassicae* (Pineda et al., 2012). Overall, PGPR constitutes to the plant defence mechanism against pathogens and insect enemies, thus playing an important role in plant evolution and adaptation to different stresses.

1.3.1 Plant Bacteria Symbiosis: The Leguminous Plants

Leguminous plants belong to *Fabaceae* family, the third largest family of flowering plants, which comprises around 800 genera and 20,000 species (Stagnari et al., 2017). The leguminous family consists of woody tree species in the tropical rainforests of Latin America and Africa, but also more temperate herbaceous plants, some of which are of major agronomic importance, such as peas, beans, chickpeas, lentils, lupin beans, soy or alfalfa (Cronk, 2006). In terms of agronomic importance, the leguminous family is the second most cultivated family after cereal crops worldwide. In 2014, legumes were spread over 190 million hectares, almost 13% of the world's arable land (Stagnari et al., 2017). They are grown as fodder plants for animal, but also as a protein crop for animal and human nutrition. Legume seeds, also known as legumes with protein seeds, account for 33% of the nitrogen requirements for human nutrition (mainly soybean and peanut). In addition to their crucial role in food security, legume plants are also used for industrial purposes, including manufacture of biodegradable ink and plastic and more recently, the production of biodiesel (Graham and Vance, 2003).

In addition to their importance for food and forage, legumes can provide economically sustainable benefits for agriculture and overcome the global climate crisis. Legumes can establish a facultative symbiotic relationship with nitrogen-fixing bacteria that belong to various groups of α - and β -proteobacteria and are collectively called rhizobia (Chen et al., 2003). Rhizobia transform atmospheric nitrogen (N_2) directly into assimilable ammonia (NH_4^+), making leguminous plants important players in the biogeochemical nitrogen cycle. Legume-rhizobia symbiosis is approximately 60-million-year-old and was likely a response to a changing climate characterised by increasing atmospheric CO_2 concentrations. The ability of legumes to fix nitrogen allowed them to benefit from higher CO_2 concentrations by increasing their N nutrition (Sprent, 2007).

The biological nitrogen fixation provides gradual enrichment of soil organic nitrogen from generation to generation (Spiertz et al., 2010), increasing soil fertility and colonization by other plant species. As a result, a legume-based crop rotation strategy could enrich the soil with nitrogen, leading to a decrease in the external inputs of mineral nitrogen fertilizers in agriculture and consequently a reduction in water and air pollution (Stagnari et al., 2017). Given the growing demand for protein-rich food and increased economic and environmental pressure, inclusive legume production systems can play crucial roles in providing multiple services in accordance with the principles of sustainability.

1.3.2 The Nitrogen Fixing Symbiosis

Plant and symbiotic bacteria live independently in nutrient-rich soils. When nitrogen levels in the soil become limiting for plant growth, a selective molecular exchange takes place between the plant and the rhizobacteria. Plant roots exude signals that initiate the legume-rhizobia symbiosis process (Hurst et al., 2011; Oldroyd, 2013). The successful implementation of a functional rhizobia symbiosis requires the completion of three sequential steps: recognition of each of the partners by setting up a molecular dialogue; the infection of plant tissues with the bacterium *Sinorhizobium meliloti* and the establishment of a primordium nodule. The *de novo* organogenesis of the nodules will then create the environment necessary for the efficient implementation of the nitrogen fixation process.

Among the different root exudates secreted by plants, *M. truncatula* releases a cocktail of flavonoid compounds into the rhizosphere (Broughton et al., 2003; Oldroyd et al., 2011; Oldroyd, 2013) (Figure 4). In response, soil bacteria such as *Rhizobium meliloti* produce and release lipo-chito-oligosaccharides called Nod factors (NFs). These bacterial NFs are specifically recognized by the plant through NF receptors present in the root epidermis and the cortex. The perception of NFs factors triggers calcium signalling pathways leading to calcium oscillation first in epidermal cells and then in cortical cells. NFs recognition induces the bending of the root hairs and the formation of an infection pocket in which the bacteria divide and induce the formation of infection thread, a tubular structure allowing the entry of bacteria into the plant. Subsequently, the infection thread invades plant cells, allowing the invasion of rhizobia into the

root tissue. In parallel, the perception of NFs triggers cell differentiation and division in the cortex, resulting in the initiation of the nodule meristem (Oldroyd, 2013).

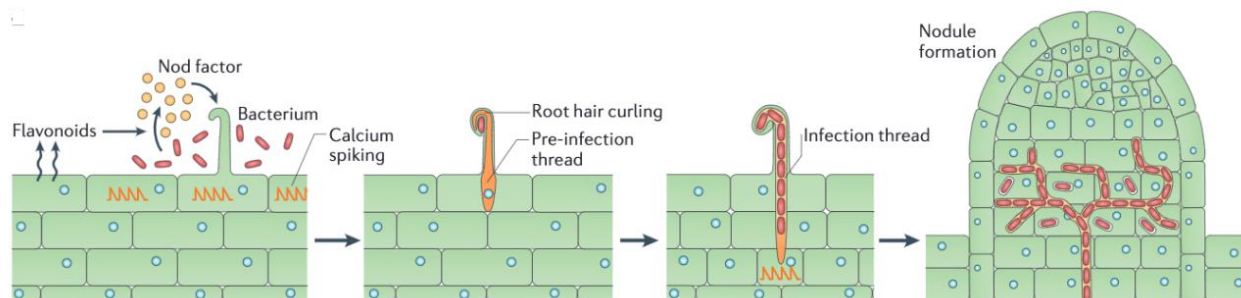


Figure 4 Schematic representation of the molecular dialogue between two symbiotic partners. Plant roots secrete flavonoids, which act as a signal to rhizobia to produce nodulation factors (Nod factors). Nod factors lead to the activation of the plant symbiosis signalling pathway. Rhizobia enter the plant root via root hair cells that trap the bacteria in a root hair curl. Infection threads allow invasion of rhizobia into the root tissue. Rhizobia invasion leads to the formation of a nodule meristem in the root cortex below the site of bacterial infection. The infection threads grow and ramify in the nodule tissue. (From Oldroyd, 2013)

The NFs induce the endoreduplication of plant DNA and cell differentiation to allow the entry of the bacteria. Rhizobia enter cortical cells by endocytosis, then differentiate into nitrogen-fixing bacteroides, thus creating a new plant organelle called the symbiosome. The symbiosome is surrounded by a peri-bacteroid membrane originating from the plant plasma membrane involved in regulating nutrient exchange (Figure 5). Finally, the bacteroides undergo a process of differentiation that involves the induction of nitrogen-fixing genes and the extinction of the ammonium assimilation genes, thus transforming the symbiosome into an ammonium exporting organelle.

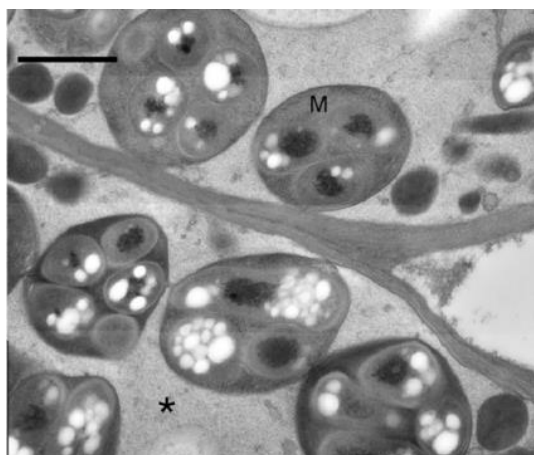


Figure 5 Electronic microscopic image of the cytoplasm and the symbiosome matrix from mature nodules in soybean root. Observation of typical symbiosomes with a matrix (M) containing bacteroids embedded into an intact host plant cell cytoplasm (asterisk). (From Radwan et al., 2012).

1.3.3 The Nodules Structure

Legume species form either a determinate or indeterminate type of root nodule depending on the activity of the apical meristem, the host plant and the bacterial interaction (Oldroyd, 2013). For instance, tropical legumes such as soybeans (*Glycine max*) or beans (*Vicia faba*) form the determinate type of nodules in which the nodule meristem is transient and each symbiosome contains multiple bacteroid which are, morphologically similar to free-living bacteria. Alfalfa (*Medicago sativa*) or pea (*Pisum sativum*) form the indeterminate type of nodules (Hirsch, 1992) in which the module meristem is persistent and each symbiosome contains only one bacteroid. These bacteroid are greatly enlarged due to DNA endoreduplication and the loss of the ability to return to a free-living bacterial state. This terminal differentiation is triggered by Nodule Cysteine Rich (NCR) peptides produced by the host plant (Mergaert et al., 2017; Pan et al., 2018). *M. truncatula* plants, which retain the apical meristem of the nodule, have indeterminate root nodules. These plants maintain nodule growth through a process of continuous cell division, thus generating elongated nodules (Figure 6).

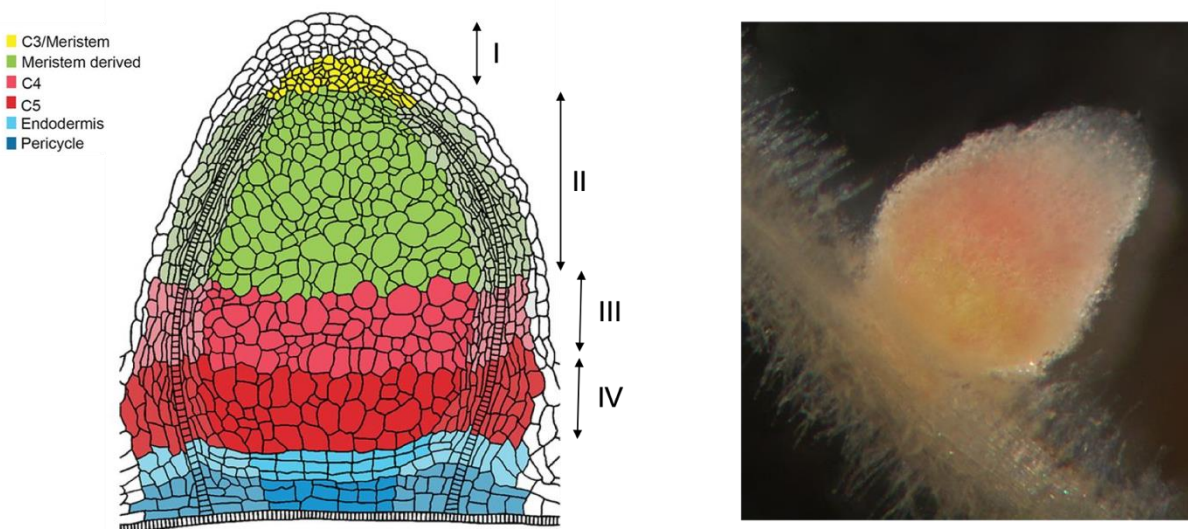


Figure 6 Structure of indeterminate nodules. Left, schematic drawing of the nitrogen fixing nodules showing the different tissue compartments. I: meristem in division (C3), II: prefixation zone (infection), III: nitrogen fixation zone (C4) and IV: senescent zone (C5) (from Xiao et al., 2014). Right, photograph of a *M. truncatula* roots showing a pinkish active nodule (commons.wikimedia.org).

1.3.4 Nodule Nitrogen Fixation Process

In legume-rhizobia symbiosis, atmospheric nitrogen (N_2) is reduced to ammonia by the bacterial nitrogenase, an enzyme made up of components encoded by the bacteria. It is a 250 kDa heterotetrameric protein with two α subunits and two β subunits (Hoffman et al., 2014) that uses metal ion as cofactors, including iron and an unusual molybdenum ion. The nitrogen-fixing reaction is believed to be carried out by the complex of iron, sulfur and molybdenum ions. A chain of cofactors, including ferredoxin or flavodoxin, supply electrons to this MoFe-cluster. The Nitrogenase hydrolyses 16 ATP molecules for one transformed N_2 molecule (Hoffman et al., 2014; Siegbahn, 2019). Bacteria obtain this energy by using plant carbohydrates. The ammonium produced by the nitrogenase enzyme is delivered in the cytosol of plant cells in the form of NH_4^+ (Ammonium)/ NH_3 (Ammonia). There are two pathways involved in the transport of ammonium across the membranes of the symbiosome: via an NH_3 channel and via a cation channel which transports K^+ , Na^+ and NH_4^+ . In plant cells, ammonium is integrated into amino acids mainly by the Glutamate synthase and aspartate aminotransferase (GS-GOGAT) pathways (Patriarca and Iaccarino, 2002; van Heeswijk et al., 2013).

The Nitrogenase is highly sensitive to oxygen and reactive oxygen species. The nitrogen fixing zone is thus under microaerophilic conditions. To allow the production of a large amount of ATP in an environment with a low free oxygen content, the plant strongly expresses an oxygen carrier and hemoprotein, the leghemoglobin (Lb), in the cells that contain the bacteroids. This protein allows the respiration of the bacteroid and is required for a proper nitrogen fixation (Ott et al., 2009). Environmental factors affect the levels of Lb in root nodules. For instance, Lb levels and nitrogen fixation activity in the nodules of pigeon pea and broad bean quickly decreases due to drought stress (Nandwal et al., 1991).

1.3.5 Nodule Growth and Activity are Regulated by A Feedback Mechanism

Plant leaves provide reduced carbon to root nodules by downward translocation in the phloem sap. In exchange, the root and nodules export nitrogen via the xylem flow. (Parsons et al., 1993), proposed that the growth of nodules and nitrogen fixation activity are strictly regulated by the presence of nitrogen-containing compounds in the phloem sap. They suggested that the presence of an elevated concentration of nitrogen sources (amino acids and amides) in the phloem sap could decrease the activity and growth of nodules (Layzell et al., 1979; Parsons et al., 1993). In

contrast, a lower amount of these compounds could accelerate the activity of the nodules. In addition, multiple studies have shown that high levels of nitrogen deposits in the soil lead to a breakdown of the plant-rhizobia symbiosis. For instance, long-term nitrogen additions led to the evolution of less effective bacterial rhizobia in the *Trifolium-Rhizobium* symbiosis (Weese et al., 2015). Nitrogen fixation activity in nodules is also strongly affected by abiotic stresses such as high salt, drought, extreme temperature, flooding and aluminium toxicity, which can impair nodules initiation, growth, development and function (Valentine et al., 2018).

1.3.6 Nodule Senescence

Nodular senescence occurs in the basal zone of the nodule (Figure 6) and it is visible by colour shift in N₂-fixing zone from red/pink associated with the functional leghemoglobin protein, to green, associated with the degradation of its heme-group (Van De Velde et al., 2006). In indeterminate nodules, senescence extends in a proximal-distal direction and spreads to the distal part of the nodule, resulting in a conically shaped senescence zone. Senescence is also observed in nodules of the determinate type but begins in the centre of the nodule. For determinate nodules, degradation of infected cells leads to the release of bacteroids, which return to the rhizosphere as free living bacteria. In indeterminate nodules, the senescence zone provides nutrients to the free-living bacteria present in the nodule. Nodule senescence is correlated with the induction of multiple proteases. Indeed, it leads to the activation of proteolytic activities in many plant models, including *Glycine max* and *M. truncatula* (Pladys and Vance, 1993). More specifically, the induction of cysteine proteases has been demonstrated in senescent nodules of soybeans (Alesandrini et al., 2003), astragalus (Alesandrini et al., 2003), peas (Kardailsky & Brewin, 1996) alfalfa (Fedorova et al., 2002). In addition, the reduction of the expression of the cysteine protease AsNodF32 expression induced via RNA-interference delays the establishment of the senescence process leading to increased nitrogen fixation in mature nodules (Li et al., 2007). The transcriptomic analysis of (Van De Velde et al., 2006) also identified two proteases whose expression was induced early during the senescence process, a papain-like protease (MtCP6) and a legume protease (Vacuolar Processing Enzyme, MtVPE) (Pierre et al., 2013). These proteases are involved in the early senescence response mediated by stress, for instance dark-induced stress (Guerra et al., 2010). These observations are in line with the increased

activity of cysteine proteases observed during nodule senescence in peas (Pladys and Vance, 1993; Groten et al., 2006).

1.4 Plant Disease and Immune Response

A disease is broadly defined as any physiological abnormality or disruption in the normal health of a plant. Disease can be caused by (biotic) agents such as, insect, fungi and bacteria, among others, or non-living environmental (abiotic) factors such as drought, nutrient deficiency, lack of oxygen, ultraviolet radiation or pollution.

Plant aggressors deploy two prominent strategies to attack plants depending on their behavior and feeding strategies. The first group includes necrotrophic pathogens and chewing-biting herbivores that kill host cells and then metabolize their contents. A prime and important example of a broad-spectrum necrotrophic pathogen is *Sclerotinia sclerotiorum*, a fungus that infects the leaves, flowers, fruits and stems of many host plants (Smolińska and Kowalska, 2018). Among the chewing-biting herbivores, many Coleopteran and Lepidopteran species are serious pests in many parts of the world. The second group includes biotrophs and piercing-sucking insects which thrive on living plant cells to promote their own growth and reproduction. Examples of such species include the powdery mildew fungi (Ascomycota phylum) (Hacquard et al., 2013) and aphids such as *Acyrtosiphon pisum*, the pea aphid (Dedryver et al., 2010).

Plant and pests/pathogens interactions are often specialized, thus making most of the induced diseases largely specific to a few plants or a small taxon. To protect themselves, plants have evolved both constitutive (i.e. innate) and inducible defences. The first ones are divided in physical defences as the cell wall, bark or waxy cuticles, and chemical ones such as the production of compounds, repellent or toxic, associated with secondary metabolism (Wittstock and Gershenzon, 2002; War et al., 2012). The inducible defences, triggered by the detection of an injury or pathogen, include the activation of specific pathways that can lead to production of specific chemicals and enzymes that degrade the pathogen or induce cell suicide (necrosis, apoptosis) to limit the spread of the disease to adjacent tissues (War et al., 2012).

1.5 Plants Defence Responses

An invading pathogen can induce several coordinated plant defence mechanisms in order to limit the growth of the pathogen and/or destroy it. Defence strategies include strengthening the cell wall, producing phytoalexin, or accumulating antimicrobial proteins (Fürstenberg-Hägg et al., 2013; Ramirez et al., 2018). The spatial and temporal regulation of these responses is the most determining factor for the outcome of the interaction. An incompatible reaction, which is the pathogen's failure to cause disease, is associated with the death of a small number of cells at the site of infection, known as the hypersensitive response (HR) (Balint-Kurti, 2019). The initiation of resistance mechanisms in plants requires the perception of a type of signal molecules called elicitors, either synthesized by the invading pathogen or released from the cell walls of plants. The production of these elicitors, based on the activation of existing components rather than on the biosynthetic machinery of the cells, is rapid, transient and occurs mainly on the surface of plant cells. An example of such reactions is the release of reactive oxygen species (ROS) in a process called oxidative burst, protect against invading pathogens and act as a signal to activate other plant defence reactions, including HR of infected cells (Bolwell and Wojtaszek, 1997; O'Brien et al., 2012). Changes in extracellular pH and a membrane potential, ion fluxes, changes in protein phosphorylation patterns and oxidative immobilization of plant cell proteins are also examples of early plant defence responses.

1.5.1 Molecular Mechanisms in Plant Pathogen Interactions

Molecular and genetic analyses have shown that plant-inducible resistance genes act as an immune system that gives plants the ability to recognize and respond to a particular pathogen. Pathogen recognition and defence response occur at two different levels (Balmer et al., 2013). First, pathogens are recognized by chemical patterns on their surface, called Pathogen Associated Molecular Patterns (PAMPs), Microbe-Associated Molecular Patterns (MAMPs), Danger-Associated Molecular Pattern (DAMP) and Herbivory Associated Molecular Pattern (HAMP) (Acevedo et al., 2015; Erb and Reymond, 2019) (Figure 7). These are complex chemical compounds, such as fungal chitin (Lu et al., 2016), that are not found in plants and whose structure does not change over time. These PAMPs are recognized by transmembrane pattern

recognition receptors (PRRs) (Boller and Felix, 2009), which triggers the activation of a specific cell signalling pathway, the PAMP-triggered immunity (PTI) (Schwessinger and Zipfel, 2008). Second, while pathogens have developed countermeasures to suppress or compromise PAMP-triggered immunity, plants have evolved specific resistance genes (R-genes) encoding for receptors that detect a pathogen effector and induce an effector-triggered immunity (ETI) defence reaction (Figure 7) (Göhre and Robatzek, 2008). ETI mostly results in a hypersensitive reaction involving the production of (ROS) that leads to death of the infected tissue and its adjacent cells within a short time (Torres et al., 2006).

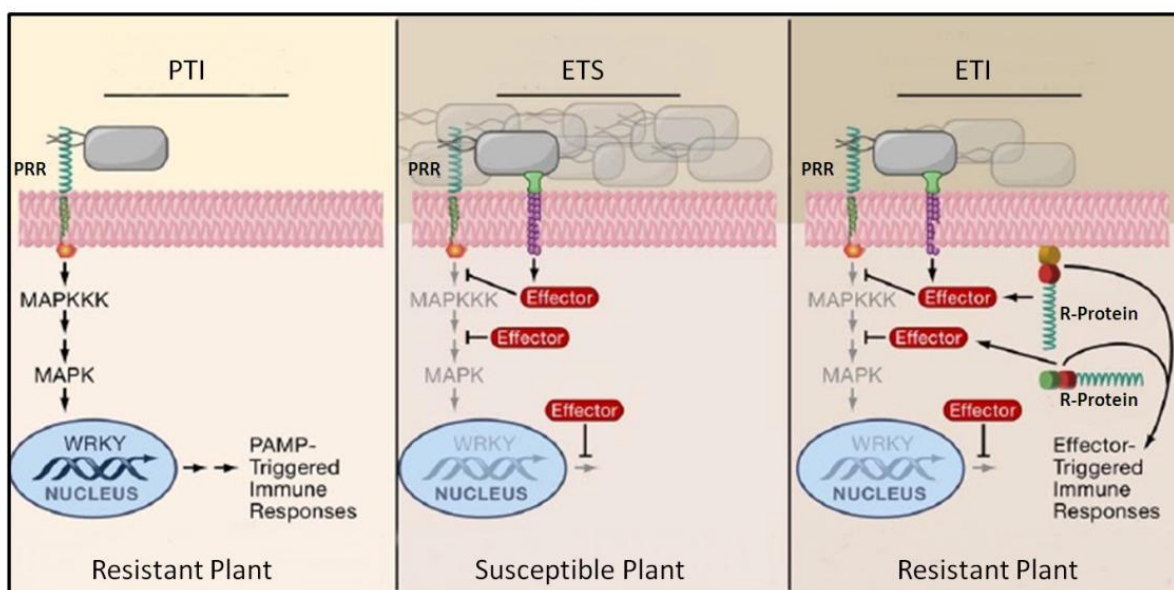


Figure 7 Different layers of plant-pathogen interaction. Plants detecting PAMPs induce defence mechanisms against the pathogen. Pathogens that comprise effectors can overcome the PTI and render the plant susceptible. To defend invading pathogens, plants have evolved R-gene encoded receptors which detect pathogen effectors and again induce a defence reaction, the so called effector-triggered immunity (ETI) (Chisholm et al., 2006).

Recognition of a specific effector protein by a R-receptor falls in the context of a gene-for-gene model hypothesis (for reviews Flor, 1971). The gene-for-gene hypothesis considers that for each specific gene controlling avirulence (*Avr*) in the pathogen, there is a corresponding recognition gene controlling resistance in the plant host. The interaction between the R-gene and the *Avr* gene product can either be direct or indirect. In a direct interaction, the effector is directly detected by the R-gene product in a “receptor-ligand” model, triggering a defence response. Indirect interactions require a host target protein (the guardee), which is the link between the

effector and the R-gene product. The effector targets the guard cell and manipulates it to make the plant susceptible. This alteration of the guard cell is then detected by the R-gene product, which consequently initiates the resistance response. This was postulated as the “Guard hypothesis” by (Van Der Biezen and Jones, 1998).

In general, unlike the PRR functions, which are widely conserved across families, both the ETI receptors and the pathogen effectors are extremely diverse from one species to another. PTI is generally effective against non-adapted pathogens in a phenomenon called “non-host response” whereas ETI is active against adapted pathogens. Local induction of both PTI and ETI often triggers broad-spectrum immunity against subsequent pathogen attacks in distal tissues, a phenomenon called acquired systemic resistance (SAR) (Fu and Dong, 2013).

Last, evidence now supports the hypothesis that certain components of plant defence are relatively non-specific, providing resistance to multiple diseases (MDR). MDR might be controlled by clusters of R genes that evolved under selection by pathogen-specific genes and/or individual genes dispersed in the plant genome (Wiesner-Hanks and Nelson, 2016).

1.5.2 Systemic Acquired Resistance and Induced Systemic Resistance

Plants are capable of generating two types of long-distance response that are effective against microbial pathogens and insect herbivores. The long-term, broad-spectrum induced resistance against pathogens is called Systemic Acquired Resistance (SAR) (Fu and Dong, 2013; Kachroo and Robin, 2013; Shavit et al., 2013) and Induced Systemic Resistance (ISR) (Figure 8). These responses are triggered by pathogens or specific strain of non-pathogenic plant growth-promoting rhizobacteria (PGPR) or fungi (Van Loon and Van Strien, 1999; Romera et al., 2019). Although SAR and ISR are phenotypically similar, they are regulated by different signalling pathways. SAR is primarily controlled by endogenous accumulation of salicylic acid (SA), and characterized by the activation of Pathogenesis-Related (PR) genes and proteins with antimicrobial activity (Fu and Dong, 2013; Kachroo and Robin, 2013). In contrast, ISR is not linked with an increase in the expression of defence-related genes. It is the establishment of a primed state of defence, which implies a faster induction of the defence-related response upon a pathogen or insect attack (Pieterse et al., 2014; Romera et al., 2019). Jasmonic acid (JA) is a crucial regulator of ISR. It has also been suggested that the PGPR-mediated ISR response benefits plants in their battle against insect herbivores (Pineda et al., 2010).

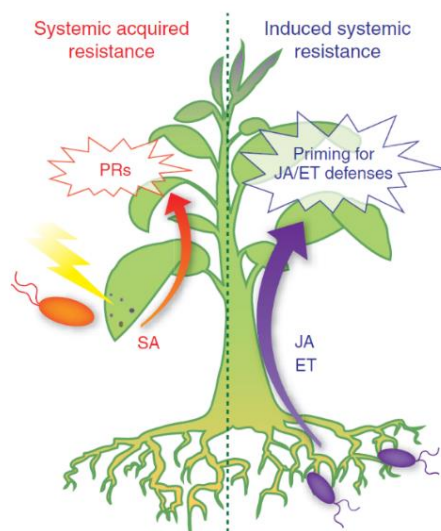


Figure 8 Schematic representation of systemically induced immune responses. SAR is typically induced in healthy tissues of locally infected plants. Signals at the site of infection travel to distal tissues via the phloem to activate plant defence responses. ISR is instead triggered when beneficial microorganisms colonize the plant roots. ISR primes plants for a faster expression of defence upon pathogen attack. (from Matyssek et al., 2013).

1.5.3 Hormones Involved in Plant Defence

Once the prime line of defence is activated, plant cells recognize danger signals and activate the downstream defence response. These responses trigger an influx of Ca^{2+} into the cytosol, leading to the production of ROS due to the activation of the MAPK kinase signalling cascade (Erb and Reymond, 2019). In the general model of MAPK signalling, membrane-bound Ras proteins facilitate the conversion of GTP to GDP, phosphorylating MAPKKK (Raf) proteins, which then phosphorylate MAPKK (MEK) proteins, leading to phosphorylation of MAPK (ERK) proteins. These signalling pathways activates diverse families of transcription factors, this triggering the hormonal-dependent defence response. Hormones operating downstream of pathogen detection provide another level of regulation and include many forms: salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), nitric oxide (NO), cytokinin (CK), gibberellin (GA), auxin, and brassinosteroids (BR). Among those, Salicylic Acid (SA), Jasmonic Acid (JA), and Ethylene (ET) have emerged as essential plant defence hormones. Finally, the hormone immune signal triggers systemic transcriptional reprogramming in plant tissues, resulting in broad SAR. The production of these hormones varies greatly depending on the amount, composition and timing of pathogen or insect species involved (De Vos et al., 2006, 2007; Erb and Reymond, 2019).

1.5.3.1 Salicylic Pathway (SA)

Biotrophic pathogens or sap-sucking insects mainly mobilize the SA pathway and cause *de novo* synthesis of PR proteins, many of which directly affect the growth of pathogens and the proliferation of diseases. SA is a small phenolic compound mainly synthesized via the isochorismate pathway in chloroplasts via the isochorismate synthase 1 (ICS1). The produced SA induces changes in cell transcription by acting on several transcriptional factors and cofactors such as the central signalling regulator and possible receptor of SA, Nonexpressor of pathogenesis related-1 (NPR1), NPR1-associated TGA transcription factors, the NPR1 paralogous SA receptors, NPR3 and NPR4, and WRKY transcription factors (Dong, 2004; Mukhtar et al., 2009; Saleh et al., 2015). The current model is that NPR1 homologs, NPR3 and NPR4, perceive SA thereby regulating the accumulation of the NPR1 protein (Pajerowska-Mukhtar et al., 2013). NPR1 controls the expression of more than 95% of the SA-responsive genes through interactions with specific transcription factors. Interestingly, it has been shown that one-third of the Arabidopsis genome is differentially expressed in response to SA, with 45 distinct clusters showing temporal changes in gene expression (Hickman et al., 2019). Since NPRs are conserved proteins in the plant kingdom, the expression of their genes is frequently used as robust marker to determine and characterize the SA-response during the acquired SAR (Wang et al., 2006; van Verk et al., 2011).

1.5.3.2 Jasmonic Pathway (JA)

Unlike biotrophic or necrotrophic pathogens, leaf-chewers insects mainly induce the JA pathway. JA is produced through the octadecanoid pathway from linolenic acid released from chloroplast membranes (Howe and Schilmiller, 2002; Wasternack, 2007; Howe and Jander, 2008). Allene Oxide Synthase (AOS) and Lipoxygenase (LOX) are key enzymes in the JA biosynthesis pathway (Pieterse et al., 2009, 2012). Then, JA conjugated to isoleucine (Ile) under the action of an amido synthetase of the GH3 family, leading to the bioactive form of JA (JA-Ile). JA and its precursors and derivatives known as jasmonates (JAs), are important molecules in the regulation of many physiological processes in plant growth and development. JAs can induce stomatal opening, inhibit the Rubisco biosynthesis and affect the uptake of nitrogen and phosphorus and the transport of nutritional compounds such as glucose. Under normal condition, the level of JA is low and the activation of the expression of JA-responsive genes is constrained

by repressor proteins of the JASMONATE ZIM domain (JAZ) family, which bind to transcription factors regulated by JA. The increase in the JA level induces a burst of transcriptional activity, activating dozens of transcription factors and increasing the expression of hundreds of genes, generating over time various expression patterns which target specific biological processes (Liu et al., 2016; Hickman et al., 2017; Wang et al., 2017).

1.5.3.3 Ethylene Pathway (ET)

Similar to the JA pathways, the ethylene pathway (ET) is activated rapidly and transiently after leaf injury. Ethylene is a very simple gas molecule, used by plants to modulate various developmental programs and coordinated responses to a multitude of external stress factors such as seed germination and dispersal, cell elongation, fertilization, fruit ripening or defence against pathogens (for reviews Guo and Ecker, 2004; Etheridge et al., 2005).

The key enzymes in ethylene biosynthesis, ACC synthase and ACC oxidase, mediate the interaction between external signals and the setup of complex responses. The ethylene signal transduction pathway is a linear pathway from perception to transcriptional regulation: Ethylene is perceived by a family of endoplasmic receptors showing similarity to histidine kinases. The ethylene receptors act on the Constitutive Triple Response 1 (CTR1) which in turn activates a MAPK cascade. CTR1 is normally thought to be inactivated, suppressing downstream signalling. Its activation triggers the cleavage of a membrane-bound protein, Ethylene Insensitive 2 (EIN2) protein. Its C-terminus will be translated to the nucleus to indirectly stabilize the transcription factors EIN3 and the EIN3-like (EIL) proteins and regulate the expression of a variety of genes involved in metabolism, defence and transport. Both transcriptional and post-transcriptional mechanisms regulate the expression of components in the ethylene signal transduction pathway. When ET is produced in combination with the JA, it antagonizes SA-mediated signalling to prioritize JA-ET-dependent defence signalling against pathogens (Pieterse et al., 2012).

1.5.3.4 Crosstalk Between SA and JA

As shown in Figure 9, the SA, JA and ET signalling pathways mutually affect each other, mainly through a negative crosstalk (Ellis et al., 2002; Kunkel and Brooks, 2002). Still, this crosstalk is interdependent and acts in a complex network. In nature, plants can be simultaneously attacked by various pathogens with different strategies and lifestyles. Therefore, the SA/JA crosstalk

allows fine tuning of transcriptional programs, determining the resistance to invaders and trade-offs with plant development.

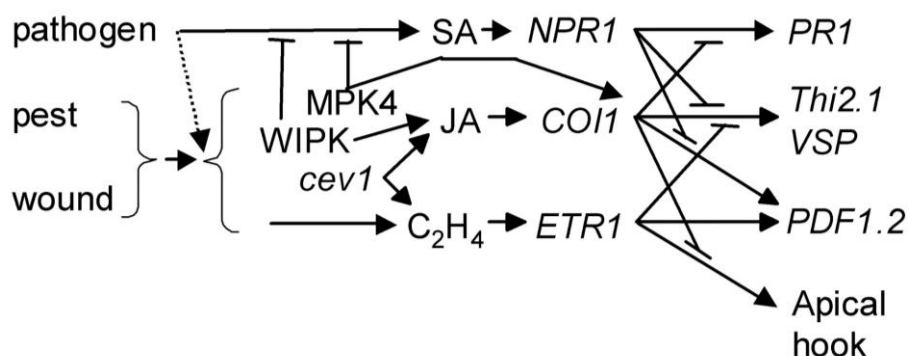


Figure 9 Model showing signalling pathways induced in plants by pathogen infection or wounding, such as caused by foraging insects. In all cases, a salicylic acid-independent pathway is triggered involving both JA and ET. Infection with a pathogen predominantly results in SA-dependent SAR leading to the accumulation of SA-inducible PRs. In addition, JA- and ethylene- responses are triggered. Depending on the invading pathogen, the production of defensive compounds could vary between SA and JA/ethylene pathways. Mitogen-activated protein kinases named MPK4 and WIPK are transcribed minutes after wounding (from Ellis et al., 2002).

(Doares et al., 1995) demonstrated that SA could inhibit the accumulation of proteinase inhibitors in response to JA treatment or wounding. This was one of the first reports of the antagonistic crosstalk between SA and JA signalling pathways. More recent studies demonstrated that SA treatment or activation of SA by a pathogen lowered resistance to pathogens or insects vulnerable to JA-mediated defence. For instance, in different models, induction of the SA pathway by *Pseudomonas syringae* suppressed the fungus JA signalling (Spoel et al., 2007). Whiteflies attack induced SA-dependant gene expression and suppressed the expression of JA-responsive genes (Zhang et al., 2013). Similarly, prior inoculation with the SA-inducing biotrophic pathogen *Hyaloperonospora arabidopsidis* suppressed JA-mediated defences activated by caterpillars feeding (Koornneef et al., 2008). Recent work indicates that suppression of the JA response by SA is predominantly regulated at the gene transcription level (Van der Does et al., 2013). SA may keep JA-responsive transcription factors away from their target genes by sequestering them in the cytosol or in complexes with repressor proteins in the nucleus. SA also affects JA-induced transcription by inducing degradation of transcription factors playing an activating role in JA signalling (Caarls et al., 2015).

1.5.3.5 Plant Defence Response Against Herbivores Insects

Plants defences affect herbivores preference for plant feeding or survival on the host plant, and indirectly attract natural enemies of insect pests. The JA pathway activation induces secondary metabolites (e.g. glucosinolates or alkaloids) and defence proteins – (polyphenol oxidase (PPO), Lipoxygenase (LOX), or peptidase inhibitors) that have anti-appetizing, anti-digestive, or toxic effect on insects. In addition, it induces the production of volatile compounds which can either attract predators and parasitoids of the herbivore insect (Frago et al., 2017), or induce defensive reactions in other parts of the plant (Das and Roychoudhury, 2014; Okada et al., 2015) and even in other surrounding plants (Moreira et al., 2016).

1.6 Insects Symbiosis

Insects represent the largest group of metazoans on the planet, and it is now clear that the evolution of herbivory has played a fundamental role in the diversification of the species of this group (Mitter et al., 1988; Mayhew, 2007; Wiens et al., 2015). During their evolution, herbivorous insects have established intimate relationships with their host plants, which represent both a habitat, a source of food and a place for reproduction. To interact successfully with their host plants, insects need to adapt to their phenology, nutrient composition and physiochemical defences (Gatehouse, 2002; Giron et al., 2018). To date, one million species of insects have been identified and half of them feed on plants (Wu and Baldwin, 2010; Wiens et al., 2015) showed a higher diversification rate in herbivorous insects, thus explaining the richness of the species of this clade. These phytophagous insects feed either by chewing plant tissues, in case of caterpillars or sucking the sap using specialized mouthparts, in case of aphids. In addition to these strong interactions with plants, insects have developed intimate relationship with the bacterial/microbial community they host. These microbial communities harboured by insects contribute to the diversification and specialization of the host (Hansen and Moran, 2014; Sugio et al., 2015). In addition, these microbial interactions also provide fertile ground for horizontal gene transfer (HGT), which is often responsible for the acquisition of new functions in insects, allowing them to exploit new ecological niches (Acuña and Wade, 2013; Wybouw et al., 2016). There is also evidence that the microbial community of insects may also interfere with the plant's defence mechanisms (Chaudhary et al., 2014; Shikano et al., 2017). For instance, the triggering

of the SA mediated pathway by the microbial community could affect the most effective JA-dependent defence response against the herbivores, which could positively benefit the insect herbivore (Diezel et al., 2009; Chung et al., 2013a) or *Hamiltonella defensa* infected whiteflies suppressed JA-mediated defence on tomato plants (Shikano et al., 2017). In contrast, the insect microbial community could also trigger plant defence response that are unfavourable to insects (Chaudhary et al., 2014). Given this evidence, it is suggestive that microbial partners play an important role in insect survival and fitness.

1.6.1 The Aphids

Aphids (Hemiptera, Aphidoidea, Aphididae) are sap-sucking, i.e. phloemophagous insects, from the order of Hemiptera, which includes more than 100,000 described species. They form the largest group of heterometabolous insects, of which 90% of the species are herbivores (IAGC 2010). Out of the 5000 known aphid species, 450 are widespread on crop plants, and 250 are considered as agricultural pests (Coeur d'Acier et al., 2010). Aphids are small in size and present characteristic features such as a pair of long antennae and a mouthpart stylet that can pierce the plant and suck the phloem sap. They are therefore specialized phloem feeders, leading to substantial yield losses in many agricultural crops. Aphids feed on the plant elaborate sap thanks to specialized mouthparts, especially the stylet, which can pierce the plant leaves and stem cuticle and navigate between the plant cells to reach the phloem tubes (Figure 10). Aphids are vectors of phytopathogenic viruses and have a high reproductive potential since they are mainly parthenogenetic, which makes them serious agricultural pests. The most economically-destructive aphids on crop plants include the Russian wheat aphid (*Diuraphis noxia*), potato aphid (*Macrosiphum euphorbiae*), green peach aphid (*Myzus persicae*), cotton melon aphid (*Aphis gossypii*) and aphids of grain and pasture legumes such as soybean aphid (*Aphis glycine*), cowpea aphid (*Aphis craccivora*), spotted alfalfa aphid (*Therioaphis trifolii*), bluegreen aphid (*Acyrtosiphon kondoi*) and pea aphid (*Acyrtosiphon pisum*).

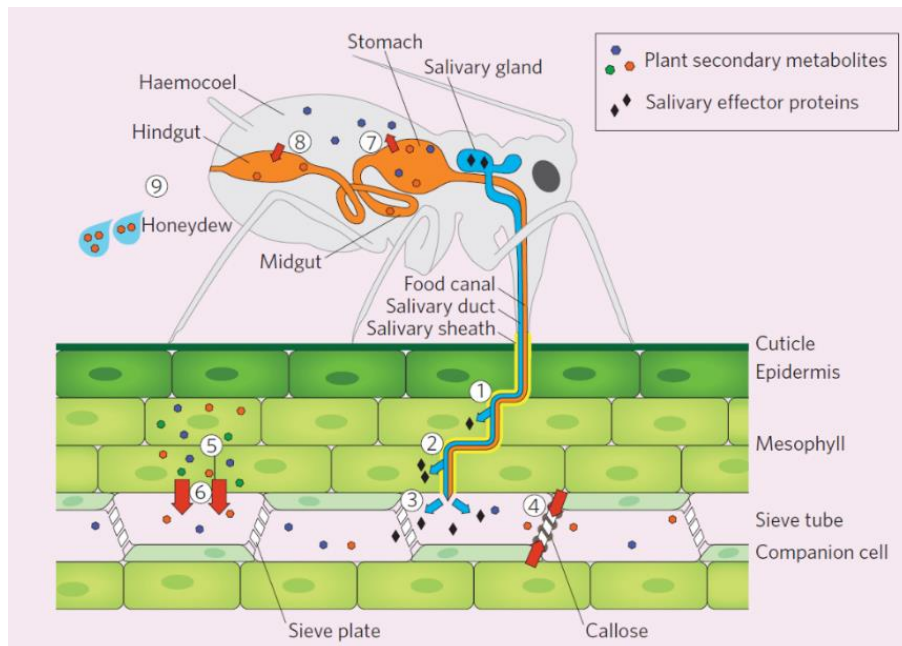


Figure 10 Schematic representation of aphid feeding and plant responses. Aphids insert their stylet between individual plant cells and secrete gelling saliva that encases the stylet in a salivary sheath (1). Aphids inject mesophyll cells with watery saliva containing effector proteins and suck back some liquid to assess plant quality (2). After reaching the phloem, aphids alternate between sap ingestion and saliva secretion into the phloem (3) to prevent callose deposition at sieve plates (4). Plant cells synthesize defensive secondary metabolites into the phloem (5-6). These secondary metabolites are then ingested by aphids and may reach their haemocoel (7), where they may accumulate or be excreted back into the hindgut (8) and eliminated via aphid's honeydew (9) (From Züst and Agrawal, 2016).

A sequence of behavioural steps takes place before an aphid decides to exploit a host plant (for review (Powell et al., 2006)). At long distances, aphids can orient themselves according to visual (plant reflectance spectrum) and olfactory cues by perceiving the volatile compounds emitted by plants (Guerrieri and Digilio, 2008; Schröder et al., 2014; Bruce, 2015). Once aphids reach the surface of the plant, they pierce the cuticle with their stylet which “navigates” towards sieve elements of the phloem (Figure 10). During this process, the aphid stylet quickly punctures and tastes the epidermal cells along the pathway in a probing behaviour that will decide of the compatibility the interaction (Pompon and Pelletier, 2012).

As mentioned above, the establishment of the aphid-plant interaction requires a phase of detection of the host plant followed by a phase of perception and acceptance of the plant. The pea aphid acceptance on legumes is a consequence of their adaptation to *Fabaceae*. The rapid expansion of the family of genes encoding chemosensory receptors and their diversification

under a positive selection mechanism highlights their important role in pea aphid adaptation to plants (Smadja et al., 2009). In addition, aphids inject salivary effector proteins into plants to facilitate phloem feeding that can also elicit defence reactions of the host plant (Hogehout and Bos, 2011). Since host plants differ in their defence reactions, salivary effectors have probably evolved according to the different pressures exerted by the hosts (Boulain et al., 2019).

1.6.2 Pea Aphid Model, *Acyrtosiphon pisum*

Commonly known as the pea aphid, *Acyrtosiphon pisum* belongs to the family of Aphididae, composed of more than 4000 representatives. About 90 species of this genus are distributed throughout the world, from Western Europe to East Asia, with some native species from North America. The pea aphid *Acyrtosiphon pisum* is present on all the continents (except in regions with extreme temperatures¹). *A. pisum* is about 4 mm long of green or pink colour and runs its entire life cycle on a single host plant (Figure 11). From spring to autumn, there are about twenty generations of meiotic parthenogenesis, the viviparous females giving birth to dozens of nymphs. This phase of asexual reproduction ensures a high growth rate of the aphid populations. Different environmental conditions, different plant conditions, seasons, and particularly days length can trigger changes from asexual to sexual reproduction (Mackay et al., 1983). Eggs are known to represent a form of resistance to cold conditions in winter (Figure 11).

A. pisum parthenogenetic reproduction ensures that nymphs are genetically identical to the mother. However, some aphid individuals remain polyphenic, meaning that individuals with the same genotype can develop several alternative phenotypes due to changing environmental conditions. The stress response and population densities can trigger the production of highly reproductive wingless (apterous) and winged (alate) aphids in the same clone (Braendle et al., 2006). Winged aphids are induced by abiotic or biotic parameters and they can passively disperse and colonize new plants.

¹ <https://www.cabi.org/isc/datasheet/3147#toDistributionMaps>

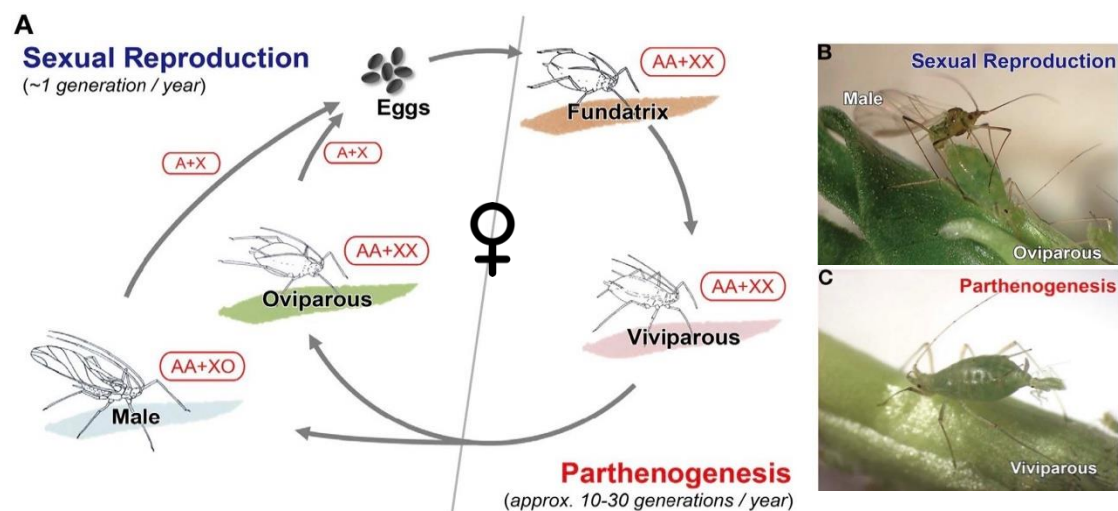


Figure 11 Typical annual life cycle of *A. pisum* aphids. **A.** Aphids reproduce by thelytokous parthenogenesis in spring and summer. Males and oviparous females produce fertilized eggs in late fall for overwintering. Each egg turns into a wingless female called fundatrix which reproduces asexually (parthenogenesis) and gives birth to other parthenogenetic viviparous females. The number of parthenogenetic generations depends on environmental factors. Parthenogenesis in aphids occurs without meiosis and genetic recombination. Accordingly, the offspring of a single fundatrix is genotypically identical but may express alternative phenotypes. **B.** Male and oviparous female mating. **C.** Viviparous female giving birth. (From Ogawa and Miura, 2014).

The pea aphid *A. pisum* is one of the best studied aphid species and is now considered as a biological model organism notably for genomic approaches (Brisson and Stern, 2006; Tagu et al., 2008, 2010, 2014). Its genome was the first Hemiptera genome to be entirely sequenced (IAGC, 2010).

1.6.3 Primary or Obligatory Symbiont *Buchnera aphidicola*

Plant phloem is an unbalanced source of nutrients, very rich in sugar but with low amounts of vitamins and essential amino acids such as methionine and tryptophan (Sandström and Moran, 1999; Douglas et al., 2006b). For example, essential amino acids represent only 8.2% of the total amino acids in the phloem of the fava beans (Douglas et al., 2006b). Compensation for this unbalanced diet is ensured by the obligatory symbiosis established with the bacteria *Buchnera aphidicola* (Baumann et al., 1997; Sapp, 2002). This is one of the oldest symbiosis, established 160-180 million years ago, which is likely at the origin of the diversification of aphid phloemophagous insects. *B. aphidicola* is a γ -proteobacterium confined to specialized aphid cells, known as primary bacteriocytes (Figure 12). These symbionts are vertically transmitted

from the mother to the offspring and account for a large part of the aphid microbiome. Numerous studies have shown that aphids can synthesize the nine essential amino acids in association with this endosymbiotic bacteria (Douglas et al., 2006b; Douglas, 2014; Boulain et al., 2018). In the course of evolution, the genome of *Buchnera* has been drastically reduced (about 500 kb), notably due to the loss of genes involved in its pathogenicity, redundant genes and regulatory genes (Gil et al., 2002; Van Ham et al., 2003). In contrast, genes involved in the biosynthesis of essential amino acids have been conserved, unlike those that were part of non-essential amino acid synthesis pathways.

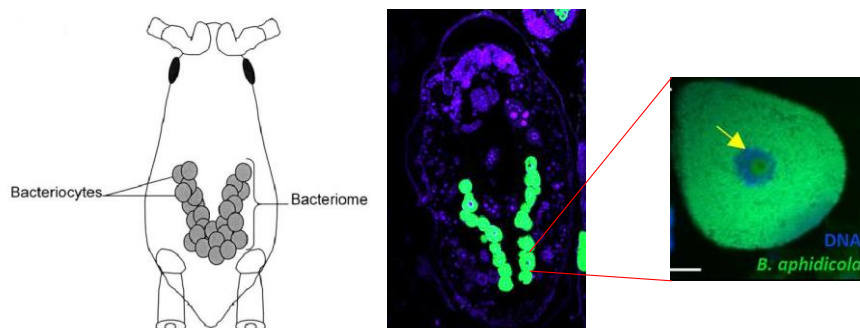


Figure 12 The bacteriome and bacteriocytes. Bacteriocytes localize to the aphid abdomen and surround the gut. The ensemble of bacteriocytes constitutes the aphid bacteriome (left). Staining of *B. aphidicola* (green) reveals each bacteriocyte of the bacteriome (centre). Right, Magnification of one bacteriocyte filled with green *B. aphidicola* cells (yellow arrow pointing the nucleus).

1.6.4 Facultative Symbionts

In addition to a primary symbiont, *A. pisum* individuals can host one or two (rarely more) other facultative symbionts, also called secondary symbionts. To date, nine have been described in aphids, including *Serratia symbiotica*, *Regiella insecticola*, *Hamiltonella defensa*, *Spiroplasma sp.*, *Rickettsia sp.*, *Fukatsuia symbiotica* (previously PAXS), *Rickettsiella sp* and *Wolbachia* (Oliver et al., 2010; Guo et al., 2017; Guyomar et al., 2018). These secondary symbionts can be found alone in most of the cases or with another symbiont in aphid individuals. Some associations are often observed between *Serratia* and *Rickettsiella*. Single symbiont infection has been proven to be more stable than multiple infection (Frantz et al., 2009; Guay et al., 2010; Tsuchida et al., 2011). Some of these facultative symbionts can be located in specialized cells bordering the bacteriocytes (sheath cells), in secondary bacteriocytes, in the aphid hemocytes or

freely circulating in the hemolymph (Figure 13) (Moran et al., 2005; Oliver et al., 2010; Schmitz et al., 2012). These symbionts are also maternally inherited but may also be transmitted sporadically horizontally (Oliver et al., 2010). Most of these facultative symbionts are pleomorphic (i.e. change in their morphology depending on the conditions) under different symbiotic conditions.

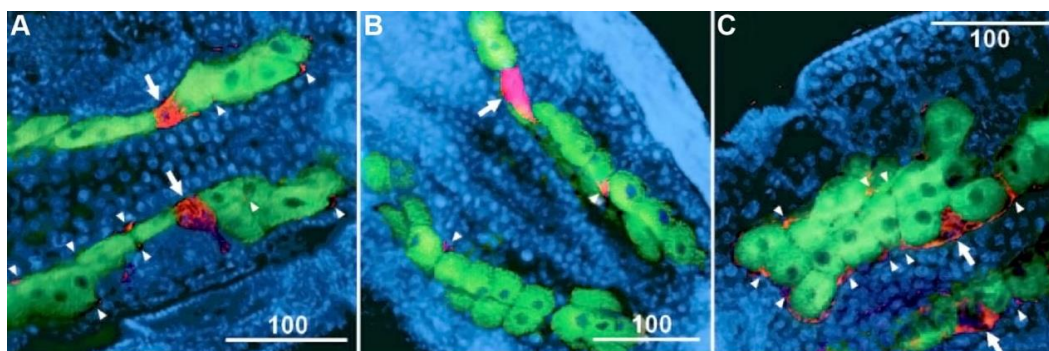


Figure 13 Localization of *A. pisum* secondary symbionts in its host tissues. *In situ* hybridization of *B. aphidicola* (green, A-C) and *S. symbiotica* (A), *H. defensa* (B) and *R. insecticola* (C) (red) in aphid embryos. The nucleus is stained in blue. Arrows indicate secondary symbionts in bacteriocytes; arrowheads indicate secondary symbionts in sheath cells, which localize to the bacteriome periphery and associate with the primary bacteriocytes. Scale bar, 100 μ m (adapted from Moran et al., 2005).

The presence of specific symbiont mainly depends on the aphid biotype (for pea aphid, the legume plant on which the biotype is specialized), the host plant and the presence of natural enemies. For instance, *S. symbiotica* and *Rickettsia sp.* are common in aphids' biotypes infecting peas or beans; *R. insecticola* is specially found in the clover biotype; *H. defensa* in the alfalfa and *Spiroplasma sp.* in the clover and alfalfa biotypes (Frantz et al., 2009).

Thanks to their extended phenotypes, facultative symbionts can greatly influence the ecology and the physiology of their hosts, and in several ways (Oliver et al., 2010). They can notably help aphids to deal with various different stresses (Table 1) (Oliver et al., 2010). When temperature increases, aphid performance (survival, development time and fecundity) is improved in the presence of *S. symbiotica*. (Russell and Moran, 2006). *H. defensa* protects aphids against parasitoid wasps thanks to the presence of a bacteriophage (APSE), some strains producing toxins that kill the wasp embryo (Oliver et al., 2003; Oliver and Higashi, 2019).

Table 1. Main phenotypic effects of pea aphid facultative symbionts.

| Symbionts | Phenotypic effects |
|------------------------------------|---|
| <i>Hamiltonella defensa</i> | Protection against parasitoids [Oliver et al., 2003] Alteration of defensive behavior [Dion et al., 2011b] |
| <i>Regiella insecticola</i> | Protection against fungal pathogens [Łukasik et al., 2013] Adaptation to host plant [Tsuchida et al., 2004] |
| <i>Fukatsuia symbiotica</i> (PAXS) | Protection against parasitoids [Guay et al., 2009] Heat resistance [Guay et al., 2009] |
| <i>Serratia symbiotica</i> | Heat resistance [Russell & Moran ,2005] Protection against parasitoids [Oliver et al., 2003] |
| <i>Rickettsia</i> sp. | Protection against fungal pathogens [Łukasik et al., 2013] Heat resistance [Montllor et al., 2002] |
| <i>Rickettsiella viridis</i> | Color change [Tsuchida et al., 2010] Protection against fungal pathogens [Łukasik et al., 2013] Protection against parasitoids [Leclair et al., 2017] |
| <i>Spiroplasma</i> sp. | Protection against fungal pathogens [Łukasik et al., 2013] Reproductive manipulation [Simon et al., 2011] |

The facultative symbiont *R. insecticola* induces aphid resistance to entomopathogenic fungus (Ferrari, 2004; Scarborough et al., 2005; Łukasik et al., 2013a, 2013b). Facultative symbionts can also influence the interaction between aphid and their predators. The symbiont *Rickettsiella* can change aphid colour from pink to green, which protects aphids from predators on its host plants (Tsuchida et al., 2011). Finally, several studies pointed the role of facultative symbionts in the adaptation to the plant. A better performance on the pea and clover plants was associated with the presence of *R. insecticola* (Tsuchida et al., 2004) but this phenotype seems to depend on the complex association with host aphid and plant genotypes (Ferrari et al., 2007; Wulff and White, 2015). The *Arsenophonus* symbiont presence improved the performance of the soybean aphid (*Aphis glycines*) on its host plant (Wulff et al., 2013; Wulff and White, 2015) and that of the spotted alfalfa aphids on their black locust trees hosts (Wagner et al., 2015).

Overall, these symbionts can help aphids improve their food performance, protect them against fungi and parasitoids, give them tolerance to high temperatures, and cause variations in their colour. Some of them also have an impact on the immune components (Schmitz et al., 2012; Laughton et al., 2016). Facultative symbionts thus influence the fitness of their host either positively or negatively. A better understanding of these traits could help us develop symbiont-based strategies to manage pests and diseases.

1.6.5 Pea Aphid: Host Plant Specialization

Pea aphids have a wide range of host plants within the *Fabaceae* family, with about 50 host plants referenced (Hopkins et al., 2017), and several hundreds of species as potential hosts. *A. pisum* populations are very dense on clover (*Trifolium pratense* purple clover and *T. repens* white clover), cultivated alfalfa (*Medicago sativa*), peas (*Pisum sativum*) and beans (*Vicia faba*). Within the same insect species, notably variations in plant use have been frequently documented. Ecologically and genetically distinct populations are referred to as “biotype”, “host race” or “ecotype” (Diehl and Bush, 1984). *A. pisum* is often considered as a single insect species but it rather consists of at least 15 biotypes with distinct genetic structuration associated to their preferred host plant (not the geographic location) and differential fitness on specific host plants (Peccoud et al., 2009, 2015). These aphid biotypes are specialized to one or a few host plants and form a sympatric population due to partial reproductive segregation. The current view is that the pea aphid has undergone rapid diversification about 10 000 years ago at the time of the development of agriculture, which led to the formation of the different biotypes through host plant specialization. Interestingly, all pea aphid biotypes characterized to date perform extremely well on the universal host *Vicia faba*.

It is interesting to note that the infection and distribution of facultative symbionts vary considerably with biotypes (Peccoud et al., 2015). For example, *H. defensa* is particularly associated with pea aphids feeding on Ononis, Genista, Lotus or *Medicago* in France. In turn, *R. insecticola* and *S. symbiotica* were more commonly associated with pea aphids collected on *Trifolium* and *Cytisus*, respectively. A field study on alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*) and hairy vetch (*Vicia villosa*) in North America reported that most aphids (mean = 74.2%) were infected with at least one facultative symbiont. *H. defensa* was found more often associated with aphids on alfalfa, *Regiella* with those on clover, while *Serratia* and *Regiella* were found at the same frequency on aphids on hairy vetch (Russell et al., 2013). Field studies also indicate the presence of multiple aphid-associated symbiont strains on a plant species, varying infection levels for seven species of common symbionts, and the frequent occurrence of coinfection by several species of symbionts. There are also geographical differences in the distribution of symbionts. However, how secondary symbionts influence the use of the host plant in pea aphid remains unclear. Phylogenetic analysis suggests that acquisition of symbionts often accompanies aphid host change. It is not known whether this is

related to host use or other ecological factors correlated with the transition to a new host. Indeed, Tsuchida et al., 2004 found that the removal of *Regiella* reduced the capacity of a pea aphid clone to feed on clover, while introduction of this same *Regiella* in a naive aphid host (*Megoura crassicauda*) improved its performance on the same plant (Tsuchida et al., 2004).

1.6.6 Specific Plant Defence Reaction Against Aphids

Several recent studies have examined the impact of aphid feeding on plant transcription profiles and identified the putative defensive responses that occur in susceptible and resistant hosts. They demonstrated a strong induction of SA, ET, and abscisic acid (ABA) pathways after aphid infestation (Moran and Thompson, 2001; De Vos et al., 2005; Guerrieri and Digilio, 2008; Kerchev et al., 2012; Jaouannet et al., 2014; Nalam et al., 2019), whereas JA signalling seemed to be repressed (De Vos et al., 2007; Kerchev et al., 2013) hypothesized that the aphids could manipulate the SA-pathways to suppress the JA-pathway, which could be more damaging to this insect. Although manipulation of the crosstalk JA-SA was observed in several insects (Diezel et al., 2009; Chung et al., 2013b), experimental evidence supporting this hypothesis still remains unclear for plant-aphid interaction (Kerchev et al., 2013). Moreover, despite changes in the expression of marker genes, suggesting an activation of different signalling pathways, there is no increase in phytohormones levels JA, SA, and ET when *A. thaliana* is exposed to *M. persicae* (De Vos et al., 2005). Non-adapted *A. pisum* clones to alfalfa induce significantly higher levels of SA and JA compared to adapted ones (Sanchez-Arcos et al., 2016).

Aphids cause very little physical damage to the host plant, compared to chewing insects, thus making difficult to understand the involvement of the defence associated with JA against aphids. It is also worth noting that the role of SA and JA in plant-aphid interactions may vary among plant species. In *Arabidopsis*, aphid bioassays on mutant lines with altered JA or SA signalling suggest that JA limits the growth of aphid populations, whereas SA induction has neutral or even positive effects on the aphid performance.

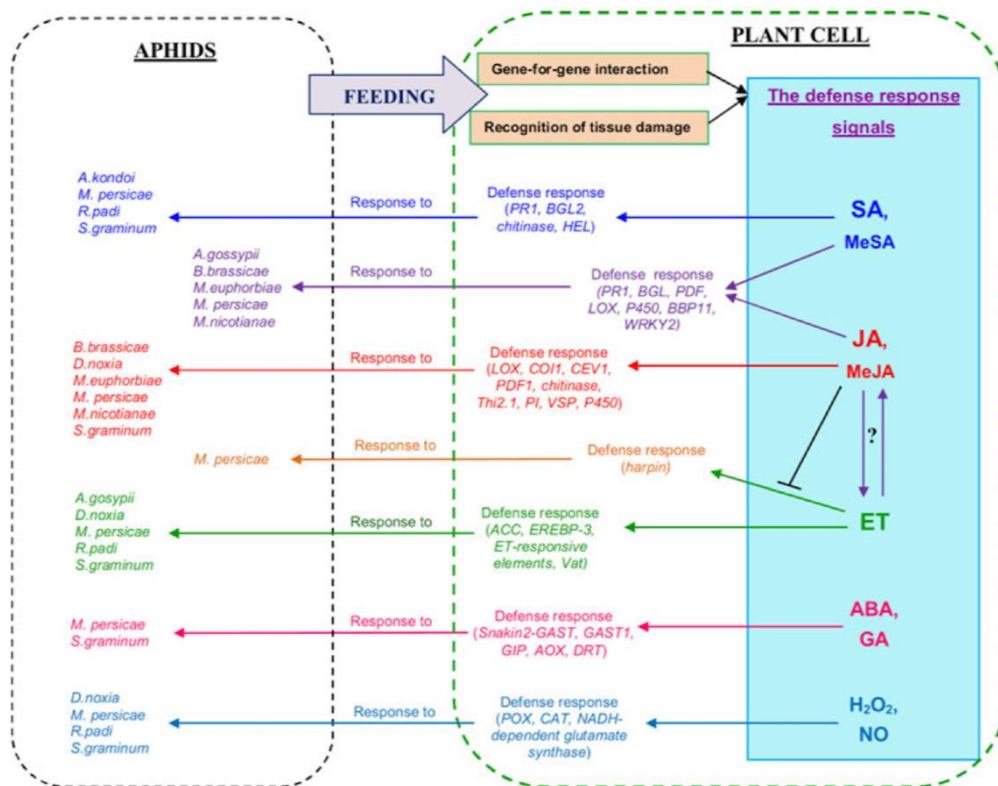


Figure 14 Multiple plant signalling pathways involved in defence response to aphid feeding. Chart displaying plant signalling pathways involving defence responses to aphid feeding. The arrows in the figure indicate activation pathways. Positive regulatory interaction between these signalling pathways are indicated by arrows, antagonistic interactions by blocked lines. (Morkunas et al., 2011).

Several aphid resistance genes coding for cytoplasmic NB-LRR receptors have been identified in plants (Dogimont et al., 2010). For instance, *Mi-1* genes provide tomato plants resistance against *M. euphorbiae* aphid (Pallipparambil et al., 2015), *Vat* genes confer resistance against certain genotypes of the cotton aphid (*Aphis gossypii*) (Boissot et al., 2016), and *RA* gene grant resistance against the lettuce root aphid (*Pemphigus bursarius*) (Wroblewski et al., 2007). However, the resistance gene response is highly variable among the pea aphid biotypes and *M. truncatula* genotypes (Stewart et al., 2009; Kanvil et al., 2014b).

Aphids can manipulate the host plant responses in a variety of ways (Figure 14). They can induce morphological changes (e.g. galls) at the plant level (Havelka, 2009), impacting nutrient allocation in their favour (Girousse et al., 2005), or suppress plant defences (Klingler et al., 2007). Manipulation of plant defence responses may be related to the injection of insect effectors as described in plant-pathogen interactions (Hogenhout and Bos, 2011). These insects have also

developed enzymatic activities allowing them to metabolize secondary metabolites that are constitutively produced by plants or induced during plant-herbivore interaction (Simon et al., 2011, 2015). These mechanisms of detoxification are closely related to the adaptation of herbivorous insects to their host plants and often associated with processes of antagonistic coevolution (Edger et al., 2015) or host change (Bass et al., 2014) favourable to the emergence of new species.

OBJECTIVES

2. OBJECTIVES

The legume family is the second most cultivated family and includes peas, soybeans and alfalfa among other important crop varieties. The symbiotic relationship between legumes and nitrogen-fixing bacteria is one of the best examples of biological mutualism in nature. Their capacity to fix atmospheric nitrogen in ammonia by the biological fixation of nitrogen makes legumes important economically and socially and is crucial for our study. Aphids are sap-sucking insects and one of the main insect pests and phytopathogenic virus carriers, notably on legumes. Unlike the majority of insects, aphids reproduce by parthenogenesis, which results in a rapid increase in the population. This results in high yield losses which represent a real threat to the cultivation of legumes. During evolution, aphids have developed an obligatory long-term relationship with the bacteria *Buchnera aphidicola* that provides essential amino acids they cannot find in obtaining plant phloem. In addition, aphids establish a symbiotic relationship with facultative symbionts that can confer specialization on the host plant, resistance to heat or to parasitoids or fungi. Thus, there are not sufficient countermeasures to control aphids either by chemical or agricultural measures.

In this study, we sought to understand the impact of nitrogen-fixing bacteria from legumes on the pea aphid and its endosymbionts and vice versa by comparing different parameters between plants in symbiosis and plants fed with nitrates. To date, and surprisingly, this has not been performed. We hypothesized that the presence of nitrogen-fixing bacteria could affect the fitness of aphids by modulating the defence response of the plant. Therefore, the resistance induced by nitrogen-fixing bacteria would potentially offer a promising strategy for controlling the growth of aphids. To this end, we have used *Medicago truncatula* as a legume plant model and *Acyrtosiphon pisum* as an aphid model with their respective symbiont(s). The objective was to integrate the knowledge of the two teams on *M. truncatula* and *A. pisum* and its symbionts to analyse the multitrophic interaction and possibly improve the resistance of crops to aphids and propose an approach to fight pests in the field.

3. CHAPTER 1

In this chapter, I have investigated how plant nitrogen-fixing symbiosis influence plant-aphid and aphid-endosymbiont interaction and *vice versa*. In particular, we examined how rhizobacteria influence the pea aphid (endosymbiont) performance and the induction of plant defence response. In addition, we investigated how aphid (endosymbionts) impact the plant fitness and plant nitrogen fixation level. The article is submitted to Proceeding B of the Royal society.

Preliminary Experiments

To our knowledge, no study had addressed yet the crosstalk between legume plants, plant nitrogen-fixing symbionts, aphids and their facultative endosymbionts. Given the absence of similar models in the literature, I spent the first 6 months of my PhD setting-up the appropriate experimental conditions to investigate such interactions.

Since the different lines of pea aphids were reared on the universal plant *Vicia faba*, I first used this plant to define the abiotic experimental conditions suitable for plants and aphids. In a second time, we produced two types of plants, with nitrogen-fixing symbiosis or fed with nitrates. Plants were sown and grown in a pot (one plant per pot) and then infested with 5 aphids that were removed 24 hours later. In both cases, I did not observe any significant difference in the performance of aphids (survival measured for 12 days and weight) and only a very small effect on the plant parameters analysed. I did not observe any significant differences in the aphid performance and very little effect on plant parameters analysed (plant biomass and nitrogen fixing activity) between the two plant conditions. I will not present these results into detail. One likely reason for that was the low number of infesting aphids used in relation to size of *Vicia faba*. Using more aphids would probably have been necessary to detect a possible effect on this legume.

This concern and the genetic and molecular knowledge available on *Medicago truncatula*, a model plant, then led us to change our biological model. This time, it was the small size of *M. truncatula* which required a period of adaptation to define the adequate number of aphids per plant, namely sufficient for statistical studies but not impacting too much the growth and survival of the plant.

Set up of the experiences with *Medicago*

First, I used 5 *Medicago truncatula* plants sown in the same pot by condition. Each pot was either inoculated with nitrogen-fixing bacteria (NFS), or supplemented with KNO₃ (non-inoculated, NI). In both cases, I infested each pot with 3 adult aphids in ventilated plastic boxes. I removed the adult aphids 24 hours after the infestation and recorded the number of aphid nymphs born. The aphid nymphs then developed into adult aphids over the next 12 days. I then recorded the number of adult aphids remaining on the plant 12 days after the infestation and

calculated the survival rate and the weight of the aphids. At the same time, I collected plant shoots and nodules to evaluate the aphids' impact on the plant biomass and the nitrogen fixing activity.

However, we noticed that the number of aphid nymphs produced per adult aphid 24 hours after the infestation was highly variable between biological replicas. We therefore decided to directly infest *Medicago truncatula* plants with synchronized L1 aphid nymphs. To this end, I first put adult aphids on *V. faba* plants. These aphids produced aphid nymphs over the next 24 hours, which developed into adult aphids within the next 12 days. I then collected these 12-day aphids and put them in a petri dish with *Vicia faba* leaves for 24 hours to produce synchronized aphid nymphs. The second experimental configuration I tested consisted of 6 *Medicago truncatula* plants per pot and an infestation condition of 10 aphid nymphs for 12 days. Using this experimental approach, we observed significant differences in the above-mentioned parameters of the aphid and the plant. We therefore established these conditions as optimal for studying crosstalk between plants, the symbionts fixing nitrogen in plants, aphids and endosymbionts of aphids. We have used this model in all the following experiments presented in the next two chapters of the results.

Introduction of the 1st publication

Plants are continuously exposed to above- and belowground herbivores and symbionts. Aboveground herbivores affect plant defence responses, thus altering plant-herbivore interactions. Plant symbionts, such as rhizobacteria, also have an impact on the plant defence response against herbivores and their interactions. To our knowledge, no study has addressed the crosstalk between plants, plant nitrogen-fixing symbionts, aphids and aphid endosymbionts to date. In the following research manuscript, we investigated how plant nitrogen-fixing symbionts influence plant-aphid and aphid-endosymbiont interaction and vice versa as well as the mechanisms underlying this crosstalk. To this purpose, we used *M. truncatula* A17 plant genotype as a model for legume-rhizobacteria symbiosis, and *A. pisum* aphids belong to YR2 genotype carrying with or without single facultative symbionts (5 different lines) as a model for aphid-bacteria symbiosis. Here, we demonstrated for the first time that both symbiotic plant and aphid partners influence plant-aphid interactions.

Publication 1: Nitrogen-fixation symbiosis affects legume-aphid interactions and plant defence.

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Nitrogen-fixation symbiosis affects legume-aphid interactions and plant defence.

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Running title: Legume symbiosis and aphid interactions

Key words: Pea aphid (*Acyrtosiphon pisum*), *Medicago truncatula*, symbiosis, rhizobium, nitrogen fixation, plant defence.

Number of Figures: 5

Abstract

Legumes can meet their nitrogen requirements through root nodule symbiosis, which could also trigger plants systemic resistance against pests. The pea aphid *Acyrtosiphon pisum*, a major pest of legumes, can harbour different facultative symbionts providing extended phenotypes. It is therefore worth determining if and how the symbionts in the host plant and in the aphid modulate their interaction. We used different *Acyrtosiphon pisum* lines without facultative symbiont or with a single one (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*) to infest *Medicago truncatula* plants inoculated with *Sinorhizobium meliloti* (Nitrogen-Fixing Symbiosis (NFS)) or supplemented with nitrate (non-inoculated (NI)). Growth of both NFS and NI plants was reduced by aphid attack, while aphid growth (but not survival) was reduced on NFS compared to NI. Infestation by most aphid lines decreased NFS plant nitrogen fixation capacity by inducing early nodule senescence. Finally, all aphid lines triggered the expression of *Pathogenesis Related Protein 1 (PRI)* and *Proteinase Inhibitor (PI)*, marker genes for salicylic and jasmonic pathways, respectively, in NFS plants, compared to only *PRI* in NI plants. Our results demonstrate that the plant symbiotic status influences plant–aphid interactions while the symbiotic status of the aphid can modulate the amplitude of the defence plant response.

Introduction

Symbiosis, the intimate relationship between two or more living organisms, is recognized as an evolutionary force shaping life on our planet [1]. Among the most studied examples are the extended phenotypes provided by bacterial symbiosis in plants and insects. Legumes (Fabaceae) are characterized by their ability to establish a symbiosis with nitrogen-fixing soil bacteria, Rhizobia [2,3], which can reduce atmospheric nitrogen (N_2) to ammonia that can be used by plants. This biological nitrogen-fixing symbiosis (NFS) occurs in new highly specialized plant organs called root nodules induced by the bacterium [3]. Symbiotic nitrogen fixation improves the productivity of leguminous crops and indirectly increases soil fertility, reduces greenhouse gas emissions and is beneficial for associated crops in intercropping [4,5]. The presence of rhizobacteria is also beneficial for the plant response to different pathogens and herbivores through several mechanisms such as nutrient competition and induced resistance [6]. Indeed, rhizobacteria trigger plant-Induced Systemic Resistance (ISR) that largely contributes to resistance against pests and pathogens [7]. ISR shows similarity to pathogen-induced Systemic Acquired Resistance (SAR), both of which render parts of uninfected plants more resistant to a broad spectrum of plant pathogens. Plant hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), are major players in the network of defence signalling pathways to resist pathogens and insects [8]. Several rhizobacteria trigger the salicylic acid (SA)-dependent SAR pathway by producing SA at the root surface whereas other rhizobacteria trigger a different signalling pathway independent of SA [9]. However, the effectiveness of the ISR-triggered plant defence depends on genetic and biotic/abiotic environmental factors. Overall, microbe-plant-insect interaction, referred as the “three-way interaction”, is a new and expanding research area [10]. Aphids are a serious pest of many crops, ornamental plants or forest trees. Of the 4000 species of aphids known worldwide, 450 thrive on crops and about 200 cause severe damage by feeding on plant phloem, reducing plant growth, and, more importantly, transmitting plant viruses [11,12]. Most aphids live in obligate symbiosis – since more than 150-200 My - with the gamma-proteobacterium *Buchnera aphidicola*, which provides essential amino acids for their metabolism, absent from the plant phloem [13,14]. *Buchnera* bacteria are housed in specialized cells, primary bacteriocytes, whose formation results from an evolutionary adaptation to symbiosis, and they are vertically transmitted to the next generation [15]. In addition to *Buchnera*, aphids can host one or more heritable facultative symbionts (FS), for instance among

the nine species described so far in the pea aphid [16,17]. These bacteria are not essential for the survival and reproduction of the host and may even be costly in terms of fitness [18,19], but they provide the host with extended phenotypes including resistance to parasitoid wasps, pathogenic fungi and heat [20]. The pea aphid (*Acyrtosiphon pisum*) is specialized on a limited range of host plants covering closely related species. A series of host-adapted biotypes specialized on these different host plants has been described with at least 15 different biotypes characterized so far and few hybrids between them in nature [21,22]. Interestingly, each of this biotype differ in its symbiotic complement [23,24], suggesting that facultative symbionts may also increase host performance on specific plants [17,20,25].

Since the economic impact of aphids is linked to successful colonization and establishment on host plants, understanding how these traits are influenced by their association with facultative symbionts could be essential for the management of these pests. Surprisingly, among the studies that provided these results almost none specified the symbiotic state of the legumes used, usually fava beans for the pea aphid, a universal plant for all biotypes. This while the symbiotic state of the plant could have an impact on the phenotypes observed in aphids.

Thus, on the one hand, the plant-associated symbionts affect the performance of insects in terms of feeding efficiency, metabolism, and ability to manipulate the physiology of the host plant [6] and, on the other hand, insect endosymbionts can directly affect the performance of insect hosts by affecting their reproduction and immunity depending on the nutritional status of the plant [26,27]. Furthermore, they may also indirectly interfere with plant signal transduction pathways by either repressing or neutralizing defence-related responses or by altering plant metabolism [28]. Although solid fundamental knowledge on plant-microbe and insect-microbe interactions has been acquired over the years, indirect relationships need to be further investigated. Exploring such relationships at the ecological and molecular level will help better understanding the role of three-way interactions in the evolution of plants, microbes and insects, and in the functioning of food webs and communities. Therefore, since specific aphid lines can be produced through symbiont elimination/injection techniques, we studied the potential influence of the facultative symbionts on legume-aphid interactions, considering plant in symbiosis with nitrogen-fixing bacteria (Nitrogen-Fixing Symbiosis, NFS) or supplemented with nitrate (non-inoculated, NI). We used *Medicago truncatula*, a legume-rhizobia symbiotic model, and pea aphid *A. pisum* lines of the same genetic background (YR2 clone), deprived of facultative symbionts or hosting one of

their most common facultative symbionts in the field (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*) [20,29]. One objective was to test for an influence of the plant NFS on the development and growth of aphids depending on the facultative hosted symbiont as well as of each facultative symbiont on the NFS plant. Results show that biological nitrogen fixation reduces aphid fitness independently of the aphid lines compared to nitrate feeding (NI) conditions. The infection by the majority, but not all, of the aphid facultative symbionts decreases significantly plant nitrogen fixation efficiency by affecting the root nodules function estimated by chemical assay and by specific root nodules genes expression (*leghemoglobin* and *cysteine protease 6*). Finally, all aphid lines trigger expression of *pathogenesis related protein 1* (*PR1*), a well define plant gene marker for salicylic defence pathway [30,31], and of *proteinase inhibitor* (*PI*), a plant marker gene for jasmonate defence pathway [32], in NFS plants while only *PR1* expression is triggered in NI plants. Overall, our results demonstrate that the outcome of plant–aphid interactions is influenced by the plant symbiotic status and modulated by the aphid hosted symbiont.

Materials and Methods

Plant Material and Growth Conditions

We used *Medicago truncatula* Jemalong A17 since this line is mainly susceptible to the pea aphid [33]. The seeds were scarified for 3 min with 10% (v/v) commercial bleach and washed five times with sterile water [34,35]. Seeds were placed on 0.4% agar plates in the dark for 2 days at 4°C and then for 2 days at 20°C. After germination, 6 seedlings were transferred to a round pot (diameter x height: 7.5 x 7.5 cm) containing a mixture of vermiculite and sand (2:1) and all the pots were moved to a growth chamber at 23°C (16h light) and 20°C (8h dark), 60 ± 5 % relative humidity and watered with nitrogen-free Fahraeus medium [36]. One week after the transfer, the pots were separated in two groups: one group was inoculated with the *Sinorhizobium meliloti* 2011 strain (NFS plants) [37], and the other was supplemented with 10 mL of water containing 5 mM potassium nitrate (KNO₃) (NI plants) [38]. For inoculation, *S. meliloti* 2011, a streptomycin- resistant strain, was cultured on Luria-Bertani medium supplemented with 2.5mM CaCl₂ and MgSO₄ and streptomycin at 200 µg/mL for 3 days at 30°C. Subsequently, the bacteria were grown in LBMC liquid medium for 24hrs, the bacterial cells

were pelleted at 5000g, washed twice with sterile distilled water and resuspended in sterile distilled water to a final optical density of 0.05 (OD 600 nm) [36]. Each NFS plant was supplemented with 10 mL of *S. meliloti* suspension.

Aphids Rearing and Infestation

Five *Acyrtosiphon pisum* aphid lines with the same YR2 genetic background were used in the experiments [29]. YR2 is a clone collected in 2002 in York (UK) on red clover (*Trifolium pratense*), a legume capable of establishing symbiosis with rhizobia [39]. The YR2 lines used differ only in their facultative secondary symbiont composition: the natural clone YR2-*Ri*(n) hosts a *Regiella insecticola* strain (Ri^{YR2}); the YR2-Amp line derives from YR2-*Ri*(n) by ampicillin treatment and is thus devoid of secondary symbionts [29]. YR2-Amp was used to produce the lines YR2-*Ri*(a) ((a) for artificial), YR2-*Hd* and YR2-*Ss* by injection of respectively, *R. insecticola* from the T3-8V1 clone (Ri^{T3-8V1} strain), *Hamiltonella defensa* from the L1-22 clone and *Serratia symbiotica* from the P136 clone [29,40]. The YR2-*Ss* created line was co-infected with *Rickettsiella viridis* due to its presence in the P136 donor clone. All these pea aphid lines were stable and reared in aerated cages on 4-week-old *Vicia faba* plants at 20°C with a 16/8h light/dark photoperiod. The symbiotype was controlled by PCR at different times during the experiment as previously described [41].

Experimental Design and Analysis of the Biological Material

Plants infestation by aphids was done one-week post-inoculation with *S. meliloti* or nitrate supplementation since the dry weight of the two plant types was almost identical at this time. Six NFS and six NI pots of six plants were then randomly distributed into six groups (see figure 1). Five groups were infested, each with one of the five different YR2 aphid lines (-Amp, -*Ri*(n), -*Ri*(a), -*Hd* and -*Ss*; ten L1 nymphs per pot), and one group, left non-infested, served as control. To obtain synchronized L1 aphids for infestation, five apterous adult females of each line were placed on separated fava bean plants 24h before infestation and allowed to give birth to young nymphs. L1 nymphs were then collected and transferred to plants. After aphid infestation, the pots were isolated individually in an aerated plastic box and maintained at 20°C under a 16:8h light/dark photoperiod, with a 70% relative humidity. The aphids were kept on the *M. truncatula* plants for 12 days after the beginning of the aphid infestation (before aphids reached their adult reproductive phase).

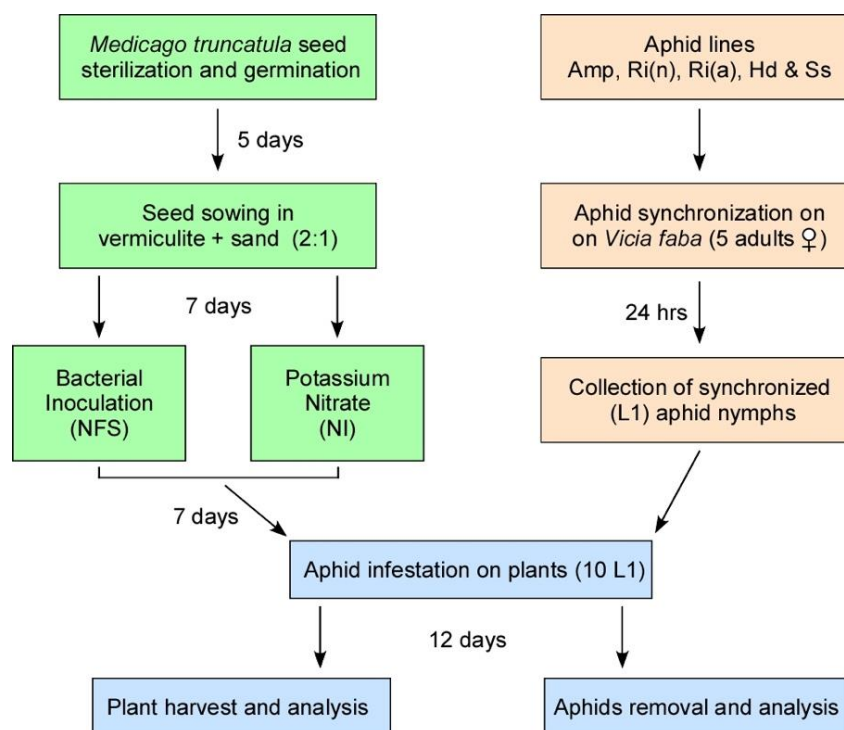


Figure 1. Schematic representation of the experimental design

Analyses of Aphid Fitness and Aphid Effect on Plants

Aphid survival was evaluated daily and the average weight of aphids was estimated on day 12 by weighting the surviving aphids. For this experiment, eighteen biological repeats were used. The effect of the different aphid lines on NFS and NI plants was estimated by measuring the weight of the plant shoots after removal of the aphids (day 12). For dry weight, the plant shoot was placed in a drying oven at 80°C for 3 days and weighed on a precision balance (OHAUS Corp, PA214; accuracy $\pm 0.1\text{mg}$). The dry weight of the shoots of the six individual plants from the three pots (18 plants in total) of each condition was measured on three separated experiments.

Nitrogen Fixation Assay

The nitrogen fixation assay was performed on the roots of the NFS plants immediately after removal of the aphids. The nodulated roots were incubated at 28°C for 1hr in rubber-capped glass bottles containing acetylene (29). Nitrogen fixing ability of the nodules was estimated

indirectly by the reduction of acetylene to ethylene by the nitrogenase (acetylene reduction assay: ARA). Gas conversion was measured by gas chromatography (6890N GC network system, Agilent). After ARA measurement, the nodules were separated from the roots, counted and weighted. The ARA values were expressed in nmol of ethylene \times hr⁻¹ \times mg of nodule⁻¹ [ARA/(hr \times mg nod)] and in nmol of ethylene \times hr⁻¹ \times plant⁻¹ [ARA/plant]. The experiment was performed for the three separate biological experiments, for which six individual plants from each pot were divided into two samples.

Gene Expression Analysis by Quantitative RT-PCR

The six plant shoots and nodules of *M. truncatula* plants from different conditions were collected immediately after the aphid removal, pooled and frozen in liquid nitrogen. For RNA extraction, the plant material was grinded in liquid nitrogen using a mortar to obtain a fine powder. Total RNAs were then isolated using RNazol® RT (Sigma), quantified (NanoDrop 2000 spectrophotometer), and the purity was assessed by Bio-analyser chips (Agilent) and electrophoresis on 1.5% agarose gel. DNA digestion (RQ1 RNase-free DNase) and reverse transcription (GoScript™ Reverse Transcription) were performed as described by the manufacturer (Promega). The quantitative PCR was performed using the qPCR Master Mix plus CXR (qPCR kit; Promega). Each reaction was carried out with 5 μ l of cDNA template diluted 40-fold and each set of specific primers (see Table S2). For defence related genes, we used the *Medicago* gene *Medtr2g435490*, annotated as *PR1* in database (NCBI protein ref XP_013463163.1) and the gene *Medtr4g032865*, proteinase inhibitor PSI-1.2, a potato type II proteinase inhibitor family protein, thereafter named proteinase inhibitor *PI* (NCBI protein ref KEH29269.1). For the root/nodule function: we used *Medtr5g066070*, the leghemoglobin 1 gene *MtLb1* (NCBI protein ref XP_003615280.1) [42] and *Medtr4g079800* encoding the senescence-specific cysteine protease SAG39 (also named *Cp6*; NCBI protein ref XP_003607574.1) [43]. Real-time RT-qPCR was performed as follows: 95°C for 3 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec (AriaMx Real-time PCR machine, Agilent). The primers efficiency was evaluated on a slope of a standard curve generated using a serial dilution of the samples. Cycle threshold values (Ct) were normalized to the average Ct of two housekeeping genes commonly used as a constitutive control in *Medicago*, the gene *Medtr2g436620* coding for *MtC27*, and the gene *Medtr4g109650.1* also named *a38* [37]. The expression of these two genes

was not affected by the treatments in our experiments. The original Ct values were obtained from the machine software (Ariamix software; Agilent) and further calculations were done using the RqPCRBase package [44] using RStudio Version 1.1.453 (<https://www.rstudio.com>). The results of the qPCR analysis were generated from four independent biological repeats with three technical repetitions per experiment.

Statistical Analysis

All experimental data are expressed as mean \pm standard error (SE). To test whether the survival and weight of aphid lines were affected by the treatment of plants (NFS and NI conditions), these data were analysed using a two-way ANOVA. A Two-way analysis of variance was performed on the influence of two independent variables (plant condition (NFS or NI); different aphid lines (Amp, -*Ri*(n), -*Ri*(a), -*Hd* and -*Ss*) on the weight and survival of aphids at 12 dpi. To test for differences between the aphid lines, the Sidak's multiple comparison test was performed. Data generated on the dry weight of the plant, the nitrogen fixation assay per plant or per mg of nodule, the number of root nodules per plant and the weight of the nodules per plant were analysed using a one-way ANOVA. The results from these experiments on NFS and/or NI plants were compared independently based on the treatment using the one-way ANOVA. Then, Tukey multiple comparison tests were performed in independent treatments to identify possible statistical differences between the aphid lines. All experimental data, except expression analysis results, were analysed using Prism v7 (GraphPad software, USA) and results are available in Supp file 1.

Results

Pea Aphid Lines Performance on Nitrogen-fixing symbiosis and Non-Inoculated Plants

To ascertain that the *A. pisum* YR2 lines were able to develop on *M. truncatula* NFS (Nitrogen Fixing Symbiosis) and NI (Non-Inoculated) plants (figure 1), the number of surviving adult aphids and their weight twelve days post-infestation were used as parameters to evaluate the aphid fitness (figure 2). Aphid survival was very high (about 90%) on both types of plants, with no significant difference between the lines and the conditions (figure 2A). In contrast, the

average weight of surviving aphids on NI plants was significantly higher (at least 40%) compared to those maintained on NFS plants, regardless of the line (figure 2B). Thus, the treatment of plants had no significant effect on the survival of aphids while the NFS treatment affected the growth of aphids, suggesting a lower acceptance by aphids (lower feeding rate) or a lower nutritional quality of the NFS plant sieve.

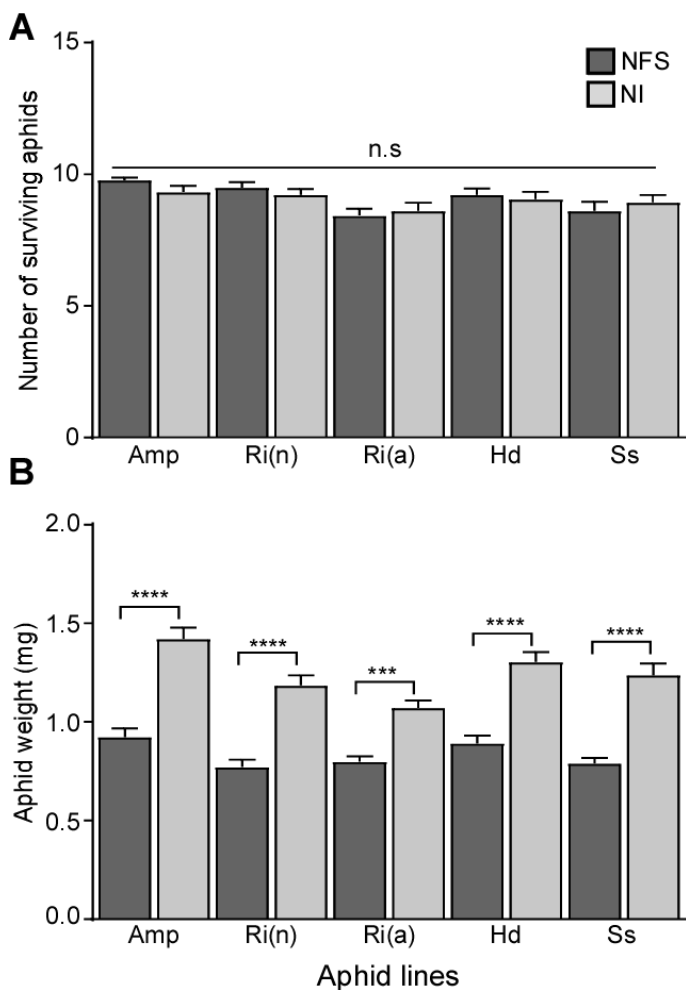


Figure 2. Performance of pea aphid lines on NFS and NI *M. truncatula* plants. **A)** Mean number of adult aphids surviving after 12 days on NFS (dark grey) and NI plants (light grey); (Mean \pm S.E.; n= 18). **B)** Mean individual weight of surviving adult aphids from the different lines on NFS and NI plants; (Mean \pm SE, n=18); statistically significant differences: p \geq 0.05 not significant; **, P \leq 0.01; *** p \leq 0.001; ****p \leq 0.0001.

Effects of Aphid Infestation on the Biomass of *M. truncatula* Plant Shoots

At the beginning of the experiment, the dry weight of the plant shoots was equivalent for the NFS control plants and the control plants fed with nitrates (figure S1) while by the end of the experiment it was about two time lower for NFS plants than for NI (figure 3) [38]. For NFS plants, infestation with YR2-Amp, -Ri(a) and -Ss aphid lines significantly reduced the dry weight

(about 25%) compared to control plants (figure 3A), unlike YR2-*Ri*(n) and -*Hd* for which the average reduction of 10% was not significant. For NI plants, infestation with YR2-Amp, -*Ri*(n), -*Ri*(a) and -*Hd* lines significantly reduced the dry weight (about 25%) compared to control plants, unlike infestation by YR2-*Ss* (non-significant 15% reduction) (figure 3B). Overall, the aphid infestation reduced the plant development regardless of their mode of nitrogen nutrition, but the amplitude of the effect was modulated according to the hosted facultative symbionts.

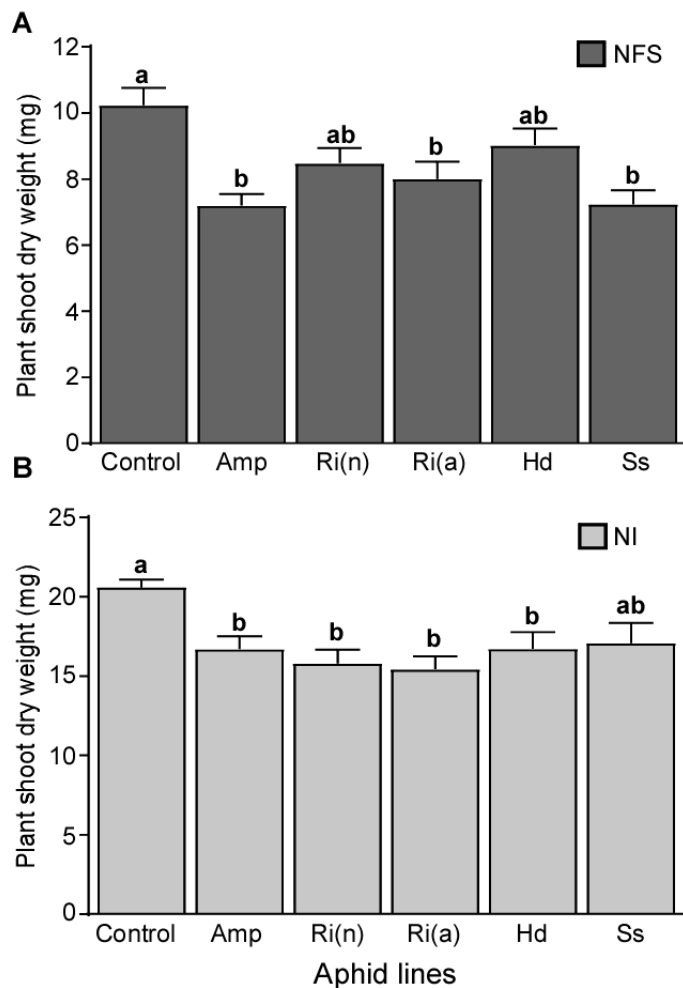


Figure 3. Effect of pea aphid lines on the dry weight of *M. truncatula* plant shoots. Dry weight of *M. truncatula* NFS (**A**) and NI (**B**) plant shoots after 12 days of infestation with the different pea aphid compared to their respective non-infested control plants (control); (Mean \pm S.E., n=3). Statistical differences between the means are indicated by different letters ($p \leq 0.05$).

Effect Of Aphid Lines on the Nitrogen Fixation of Inoculated Plants

The effect of the different pea aphid lines on the biological nitrogen fixation was evaluated only on NFS plants (capable of fixing atmospheric N_2 by forming root nodules) (figure 4). First, we counted and weighed the nodules as they are macroscopic markers for the establishment of NFS in plants [45]. The number of root nodules per plant was significantly lower in the presence of

aphids compared with non-infested control plants, except for the line YR2-*Hd* (figure 4A). The highest effect was observed for the YR2-*Ss* and -*Amp* lines with a reduction of 50% in the number of nodules. In agreement with this, the total weight of nodules per plant decreased after aphid infestation, but significantly only for the YR2-*Ss* and -*Amp* lines (figure 4B).

We then measured the nitrogen-fixing activity of the root nodules using the acetylene reduction assay (ARA) (figure 4C and 4D, respectively). When the ARA was expressed per mg of nodule, the plants infested with the lines YR2-*Amp*, -*Hd* and -*Ss* showed a significant reduction in the nitrogen fixation activity while this reduction in ARA was not significant for the YR2-*Ri*(n) and YR2-*Ri*(a) lines despite a 20% decrease compared to the control. The ARA per plant gave the same result, except for the effect of YR2-*Ri*(a) which was no more significant. Nitrogen fixation was therefore affected by the aphid infestation. To further investigate this effect on the biological function of nodules, we estimated the expression rate of two specific genes of nodules by qRT-PCR: i) the leghemoglobin gene *Mt1b1*, whose expression is correlated with optimal nitrogen fixation since it participates in the protection of the nitrogenase from oxygen denaturation and provides oxygen for bacterial respiration [46], ii) the *CP6 cysteine protease* (*Cp6*), a gene expressed during both developmental and stress-induced nodule senescence [43]. The expression of *Mt1b1* in NFS plants decreased following infestation by aphid lines except with YR2-*amp* (figure 4E) for which the large variation between replicates likely explains the lack of significance. In contrast, we observed a 5-to-23-fold increase in the expression of *Cp6* in NFS plants infested with the different aphid lines (figure 4F), except for the line YR2-*Ri*(n) for which the increase was not significant. Overall, these results suggest an early induction of nodule senescence in NFS plants after the infestation of aphids and therefore a decrease in metabolic efficiency.

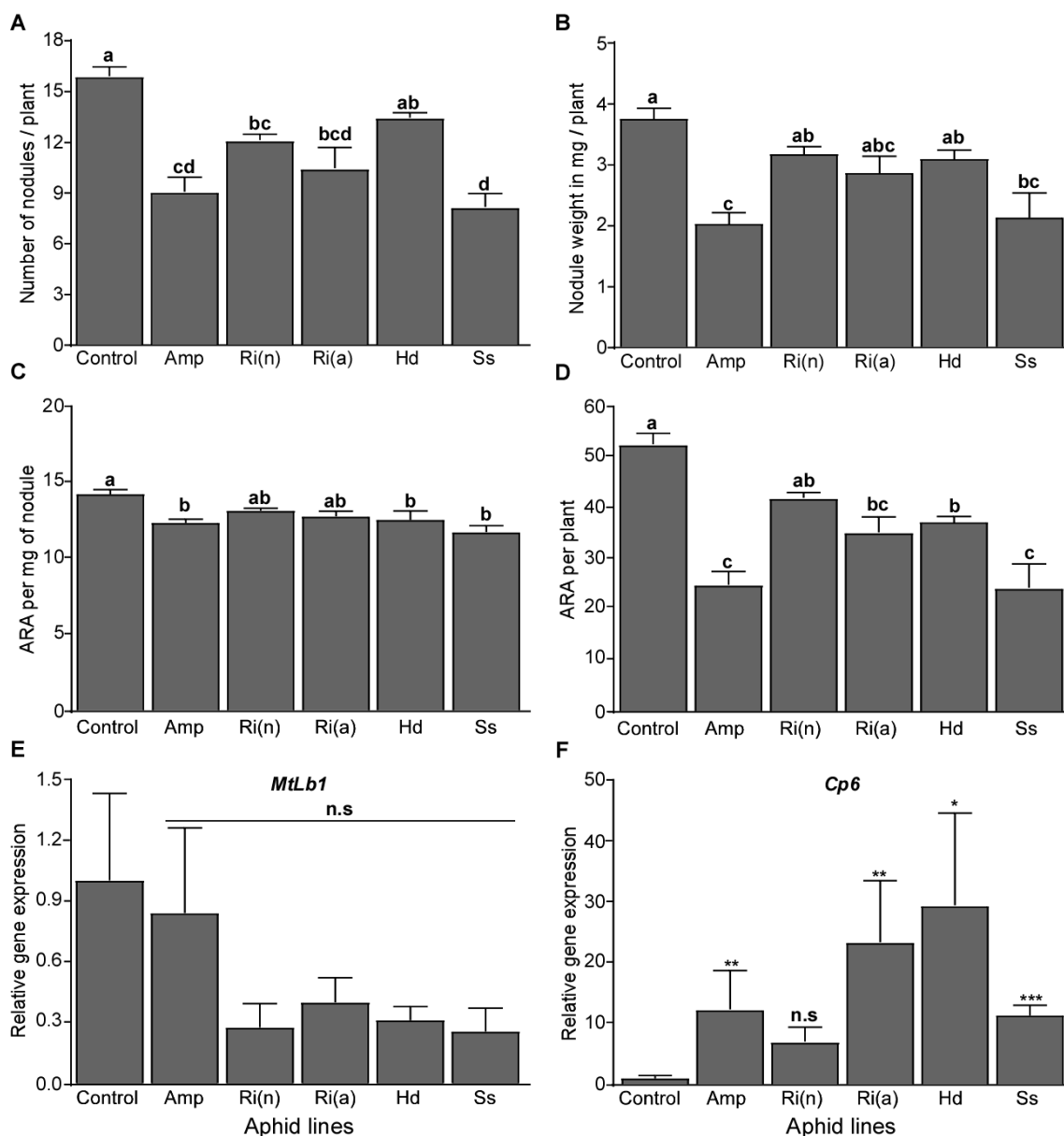


Figure 4. Effects of pea aphid infestation on biological nitrogen fixation. **A, B:** Number (**A**) and Weight (**B**) of root nodules from NFS plants not infested (control) or infested with the different YR2 pea aphid lines for 12 days. **C, D:** Mean acetylene reduction activity expressed per milligram of nodule (nmol ethylene \times $\text{hr}^{-1} \times \text{mg nodule}^{-1}$) (**C**) and per plant (nmol ethylene \times $\text{hr}^{-1} \times \text{plant}^{-1}$) (**D**) after infestation with pea aphids compared to non-infested plants (control). **E, F:** Relative expression of *MtLb1* (**E**) and *MtCp6* (**F**) estimated by qPCR in the nodules of plants infested by pea aphid lines compared to those of non-infested plants (control). In panel (**A-D**), (mean \pm S.E., $n=3$); statistical differences among means are indicated by different letters ($p \leq 0.05$). In panel (**E-F**), (mean \pm SE, $n=4$); Statistical differences: n.s., not significant ($p \geq 0.05$); *, $p \leq 0.05$; **, $P \leq 0.01$; *** $p \leq 0.001$.

Analysis of Expression of JA and SA Plant Defence Pathways

To prevent damage from insect pests, plants have developed an array of defensive strategies, including the production of various biochemical compounds that can affect insect growth and development [47]. Studies of plant defence signalling in plant-aphid models revealed that aphid feeding induces expression of gene markers of the salicylic acid (SA) pathway, such as the *Pathogenesis Related 1 protein (PRI)* gene [48–50]. It has also been speculated that activation of the SA-signalling pathways counteracts the activation of defence responses related to jasmonic acid (JA). Here, we have estimated by qRT-PCR the expression of the *PRI*, [49–51], and of *PI*, a marker of the jasmonic acid (JA) pathway [32], at the end of the aphid infestation period, under the two plant nutritional conditions. Expression of *PRI* and *PI* was similar on the shoots of non-infested NFS and NI control plants (Supplemental figure 2), suggesting that the basal defences of the plant were not affected by their mode of nutrition. After aphid infestation, a significant increase in the expression of *PRI* was induced regardless of the nutrition mode except for the line YR2-*Hd* on NI plants (figure 5A), suggesting an activation of the SA pathway. The induction of *PRI* ranged from 9-fold in NFS plants infested by the YR2-Amp line to 50-fold in non-inoculated plants infested with the YR2-*Ri(a)* line. The most striking result, however, was the contrasting level of expression of *PI* after infestation: NFS plants showed a significant 5-to-8-fold *PI* induction, suggesting activation of the JA pathway (figure 5B), while no induction was observed in NI plants (figure 5B). Therefore, under our conditions, the plant defence mechanisms induced by the aphid infestation seemed to be regulated differentially depending on the presence of the plant symbiont and to a lesser extent on the aphid FS.

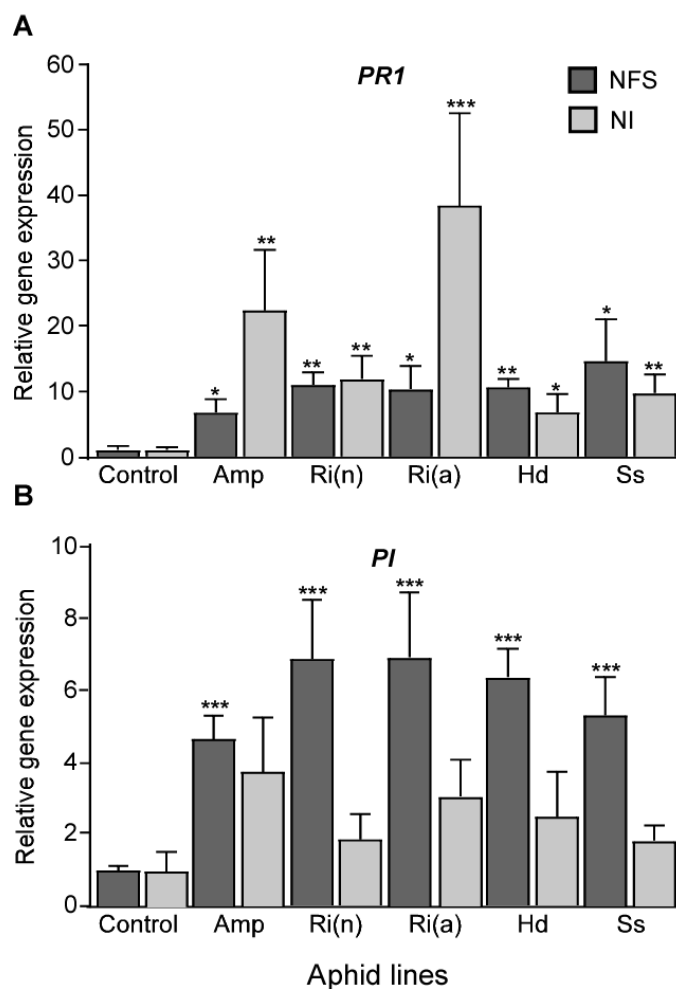


Figure 5. Level of expression of defence related genes. A, B: Expression of *PR1* (A) and *PI* (B) estimated by qPCR in shoots of NFS and NI plants 12 days after infestation by the different YR2 aphid lines. Non-infested plants serve as control. (Mean \pm SE, n=4); Statistical differences (t-test): **, $P \leq 0.01$; *** $p \leq 0.001$; ****, $P \leq 0.0001$.

Discussion

The purpose of this study was to analyse the possible effect of the nitrogen source of legumes (fertilizer versus symbiosis) on the growth of pea aphids, and that of aphid infestation on plant growth, biological fixation of nitrogen and defence pathways. Moreover, we wanted to test whether and how the presence of facultative aphid symbionts could differentially affect these plant parameters.

Aphid Growth is Mainly Affected by the Mode of Nitrogen Nutrition of the Plant

Facultative aphid symbionts (FS) have important known effects on the phenotype of their host. In addition, strong associations exist for instance in *A. pisum* between the biotypes adapted to a given leguminous plant and the FS symbiotype [20,52]. The role played by FS in adapting to the plant is still little known, but their removal can affect the fitness of aphids on a given plant [25]. Conversely, as an example, the transfer of *R. insecticola* to the aphid *Megoura crassicauda* allowed this species to develop on clover, a plant on which it could not normally feed [53]. The pea aphid biotype used here, YR2, was originally caught on clover [29] and naturally hosts the facultative symbiont *R. insecticola* (*Ri*^{YR2} strain). This biotype is undoubtedly not the best adapted to *Medicago*, but different symbiotic lines from this clone, containing none or a single FS were available to test the FS role on the plant-aphid interaction. Under our experimental conditions, we did not observe any significant aphid mortality, indicating that all the aphid lines were able to feed, grow and develop on the plants provided, as previously reported [54]. Although we did not quantify the progeny produced, we also observed that adult females from all lines raised under both plant nutrition conditions were able to reproduce when kept on the plant after the end of the experiment (PG personal observation). In the field, pea aphids infected with *Hd* and *Ri* are commonly found on *Medicago* plants, unlike those infected by *Ss* [55]. However, although variations exist, we did not notice any significant difference in the individual growth of aphids from the different lines, the main clear effect being due to the nutritional status of the plant. The aphid growth was indeed much lower (about 40-50% less) on rhizobium-inoculated plants which show a weaker development at the end point of the experiments (see further).

Aphid Infestation Affect Plants According to the Facultative Symbiont Hosted and The Nitrogen Nutrition Mode Of The Plants

While at the beginning of the experiment, the NFS and NI plants had the same dry weight, the NI plants grew twice as fast as the NFS ones during the experimental time [53]. Under both nutritional states, aphid station reduced the dry weight of the shoots but the amplitude of the decline was dependent on the FS hosted. *Hd* and *Ri* have retained pathogenic features, but not *Ss*, and *Ri*^{YR2} present in YR2-*Ri*(n) and *Ri*^{T3-8V1} present in YR2-*Ri*(a) are two different bacterial strains [16]. It is therefore not surprising that these bacteria can more or less affect their aphid host and the host plant. Indeed, aphids inject saliva containing proteins and metabolites to facilitate feeding and modulate plant physiology [50]. Some of these salivary components modulate the plant defences [56], among which symbiont derived proteins, such as the bacterial chaperone GroEL, have been described as elicitors [57]. As the aphids feed on the plant, competition for metabolites can also be altered by the presence of FS. To date, the study of the effect of facultative symbionts on the metabolic needs of aphids and the salivary components [58], as well as the role of components transmitted by the FS is still in its infancy and it is thus difficult to draw a clear conclusion. However, the significant differences observed deserve attention and open the way for a more in-depth study of the underlying mechanisms.

Aphid Infestation Affects the Biological Nitrogen fixation

If aphid infestation impacts the growth of both NI and NFS plants, it has led in NFS plants to three major congruent effects related to nitrogen fixation: the reduction in the number of root nodules, the decrease in the efficiency of nitrogen fixation per nodule (ARA) and the decrease of the expression of the *leghemoglobin* gene (*Mtlb1*), an indicator of optimal nitrogen fixation. They result in a less efficient nitrogen fixation in plants, which may also partly explain the lower growth of infested inoculated plants. The reduced number of nodules suggests that the process of rhizobium infection and/or the nodule meristem formation are affected. The upregulated expression of the *Cp6* gene, a nodule senescence marker, suggests an earlier induction of senescence in infested plants [59,60], which could at least partly explain the dysfunction of the nodule with a reduced nitrogen fixing activity.

Studies considering plant-pathogens interaction as a factor limiting the establishment of a nitrogen fixing symbiosis are still scarce [61]. Co-inoculation of *Rhizoctonia solani* or *Sclerotinia sclerotiorum* with rhizobia reduces the number of nodules and dry matter of the roots [62]. *M. truncatula* infection with the leaf pathogen *Pseudomonas syringae* also leads to a decrease in the number of nodules [63]. Surprisingly, the infestation of *Alnus viridus* by the genus-specific aphid *Prociphilus tessallatus* Fitch, which failed to establish feeding colonies, increased nitrogen-fixing activity and plant growth [64]. The question of how the aphids impact the root nodules (and therefore the plant symbiosis) is important and should be addressed in the future. One hypothesis could be the occurrence of a trade-off in the plant, the infestation by aphids leading to the interruption of the costly formation of nodules to compensate for the uptake of metabolites of the sieve. This in turn decreases the availability of nitrogen-containing metabolites, such as amino acids, for the aphids and can decrease their appetite for the plant. Another way in which aphid infestation may affect the function of nodules is through the activation of plant defence pathways (see below).

Aphid Infestation Affects Differentially the Defences of the NFS and NI plants

Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the three major signalling networks involved in the defence responses induced by aphids [65,66]. Pea aphids elicits these plant defence pathways through cell damage and reactive oxygen species production during penetration of the stylet and through recognition of their saliva components by the plant [65]. The crosstalk between SA and JA pathways has a crucial role in initiating a defence reaction against pathogens [67,68]. In general, JA production activates the defences against necrotrophic microbes and chewing herbivores [69,70] while SA production activates defences against biotrophic pathogens by stimulating the transcription of defence response genes, such as pathogenesis-related genes [68]. Aphid feeding therefore increases the transcription of mRNA of several PR genes and other genes associated with the salicylic acid (SA)-dependent response, leading to increased in some enzymatic activity including those of peroxidases and chitinases [50]. In *Arabidopsis thaliana*, aphids feeding on leaves induced a 10-fold increase in the transcription of *PR-1* [48] and the infestation of the potato (*Solanum tuberosum* L.) by *M. persicae* induced the production of transcripts encoding *PR1*, which gradually increased over the feeding-time of aphids [71]. Previous studies showed that the feeding of pea aphids on *M.*

truncatula led to a 2-3-fold increase of the expression of *PR5* during the first 3 days post infestation [33]. Here we observed a strong up-regulation of *PR1* expression by the pea aphid infestation in almost all our conditions, suggesting that the SA-dependent plant defences are activated whatever the plant nitrogen nutrition. Activation of the SA pathway is also suggested to be a general mechanism of antibiosis or aphid repellence [72,73], but the level of activation being the same under the two nutritional conditions of plants, it cannot explain the observed difference in the aphid growth.

Unlike the SA pathway, the JA pathway was activated differently by aphid infestation between NFS and NI plants: the proteinase inhibitor marker gene (*PI*) was significantly upregulated in NFS plants but not in non-inoculated ones. This last observation agrees with the results of Gao et al., (2008) [33] who observed no increase in *PI* expression in non-inoculated *M. truncatula* during pea aphid infestation. Several thousand genes are regulated up or down in root tissues during the establishment of the *Sinorhizobium* symbiosis, including some genes from the JA pathway [74]. Moreover, some strains of *S. meliloti* induced similar defence responses in *M. truncatula* as the pathogenic *P. syringae* strain DC3000, although this may be a transient activation [63]. The JA pathway thus could be sensitized in the presence of *S. meliloti*, the feeding of aphids being then enough to trigger it to a significant level. JA has many roles in plants on top of its defence activation such as promoting growth and development, including the formation of leaves and roots, as well as in the control of reproduction and senescence [75]. Whether it acts on the nodules also has to be clarified.

One interesting point is that the JA and SA signalling pathways have been shown to interact antagonistically in dicotyledonous plants [76]. In the NFS plants, both the SA and JA pathways seemed to be activated. Although this may have occurred at different time points during the 12 days of infestation, their dual effect was still visible at the end of the infestation period. It will be interesting to further understand whether and how the presence of *S. meliloti* modifies the JA/SA interplay in case of stress.

Conclusion

Sowing and inoculating plant seeds with rhizobia is a method introduced into agricultural systems to improve plants growth and help them adapt to poor nitrogen conditions, improve soil fertility and limit the use of chemical fertilizers. Beneficial soil microbes can also help the plant defend itself against various pathogens and insect herbivores by the mechanism of Induced Systemic Resistance (ISR) [77]. Rhizobacteria mediated ISR resembles that of systemic resistance induced by pathogens in that both types of induced resistance make uninfected part of plant more resistant to a wide range of pathogens. Our results on the legume/rhizobium/aphid tripartite interaction system reveals an interplay between rhizobia and aphid infestation through the modulation of plant growth, symbiosis and defence responses. The presence of rhizobium does not seem to protect *Medicago* from attack and feeding by aphids. However, aphid infestation has a major impact on root nodules and nitrogen fixation of the plant. This effect could be counterproductive in the perspective of using legumes for an improved soil management system, with a reduced level of nitrate. Indeed, the reduction in the number of plant nodules and N_2 fixation will directly affect plant yields and soil quality. Our results also open new research perspectives at the physiological and molecular levels in this tripartite model, although their generalization has yet to be tested. Here, we have used a single *M. truncatula* genotype, one aphid genotype and one bacterial genotype, but both the plant and aphid genotypes are known to be potentially important in the outcome of the interaction [78]. Despite its predictable complexity, a more in-depth analysis of the effect of the genetic diversity of the different partners on the plant symbiosis will therefore be a major challenge to improve our understanding of multitrophic interactions in natural and agricultural environments. In a context of more widespread use of legumes, this study shows that plants in symbiosis and without symbiosis may interact with pests in a very different way.

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Author contributions

Supervision and funding acquisition: M.P., P.F., J.-L.G.; Experimental design and Conceptualization: M.P., P.F., J.L.G. and G.P.; Data acquisition: G.P.; Statistical analysis: G.P. and P.F.; Supply of aphid biological resources: J-C.S.; Original draft preparation: G.P.; Writing-Review and Editing: G.P., J.-C.S., J.-L.G., M.P. and P.F.

Competing financial interests: The authors declare no competing financial interests.

Conflict of interest: The authors declare no conflict of interest.

Supplementary Figures

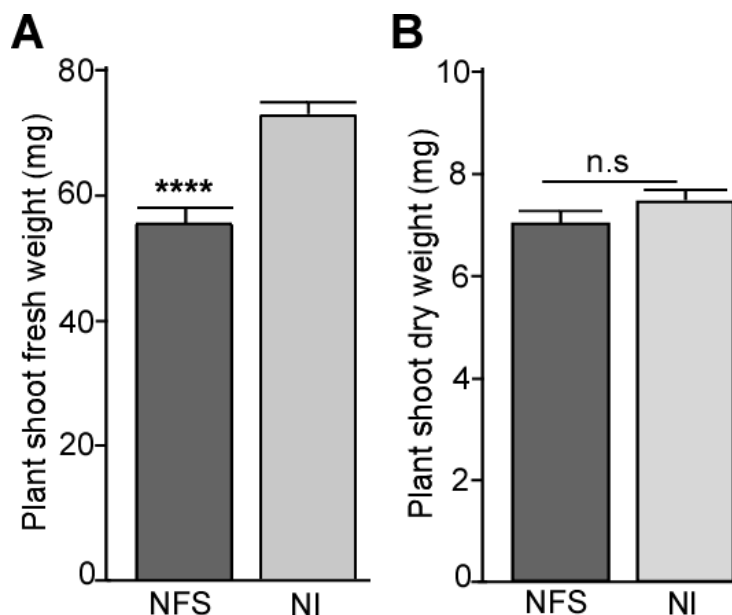


Figure S1. Comparison of the dry and fresh weight of NFS and NI plants before aphid infestation. A) Fresh weight and B) Dry weight of 2-week-old NFS and NI plants just before aphid infestation. Mean \pm SE n=4. t-test: n.s., non-significant $p > 0.05$; **** $P < 0.0001$

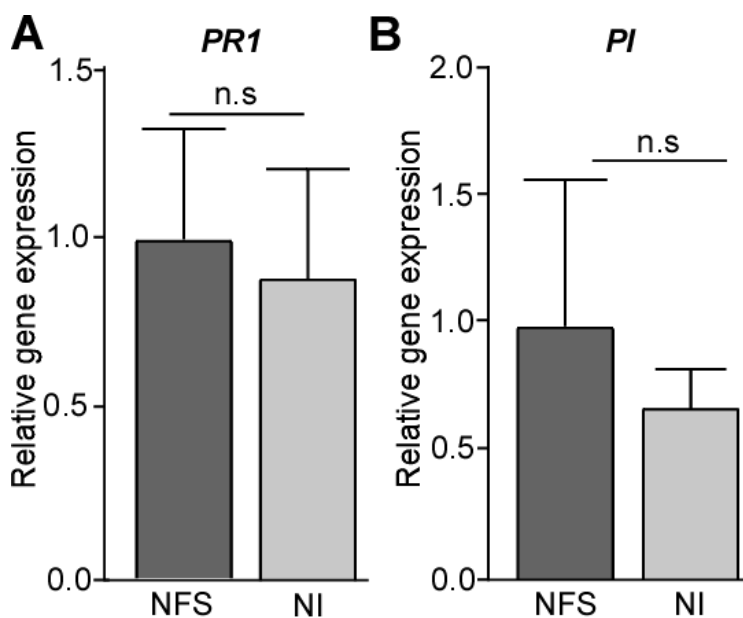


Figure S2. Relative gene expression of A) PR1 and B) PI genes in NFS or NI control plants. PR 1 and PI gene expression were set to 1 for NFS plant shoots and rescaled accordingly for NI plant shoots. Mean \pm SE n=4. t-test:n.s., Non-significant.

Supplementary File 1: Primer sequences and statistical data

Table S1. Primer sequences for RT-qPCR analysis.

| Description | Name | Forward primer | Reverse primer | Genomic ID | References |
|----------------------------|--------------|---------------------------|-----------------------|---------------|------------------------------|
| Housekeeping gene | <i>Mtc27</i> | TGAGGGAGCAACCAAATACC | GCGAAAACCAAGCTACCATC | Medtr2g436620 | Del Guidice et al., 2011[37] |
| Housekeeping gene | <i>a38</i> | TCGTGGTGGTGGTTATCAAA | TTCAGACCTTCCCATTGACA | Medtr4g109650 | Del Guidice et al., 2011[37] |
| Pathogen related protein-1 | <i>PR-1</i> | TTCGGGTTGGATGTGCTAAG | GGTTGAAGCTCAATGGCACT | Medtr2g435490 | This work |
| Protease inhibitor | <i>PI</i> | TGTGGTGCAATTCTTTCAGG | ATTTTGGGGTGAGGTGTTGA | Medtr4g032865 | This work |
| Leghemoglobin-1 | <i>MtLb1</i> | ATAGCTCATATGAGGCATTCAA | GAGTTGAGGACTATCTGTACT | Medtr5g066070 | Li X et al., 2018 [42] |
| Cysteine proteinase | <i>Cp6</i> | CCTGCTGCTACTATTGCTGGATATG | CACTCGCATCAATGGCTACGG | Medtr4g079800 | Pierre et al., 2014 [43] |

Tables S2 to S10: Statistical results for each experiment

Table S2. Number of aphid survival on NFS and NI. Results of Two-way ANOVA analysis showing the absence of significant effect of the plant condition (NFS and NI) on the aphid survival $F(1, 170) = 0.2217$, $P = 0.6384$; In contrast, significant differences were observed among the different aphid lines $F(4, 170) = 5.338$, $P = 0.0004$.

| Multiple comparison | Significance | p value |
|--------------------------|--------------|---------|
| NFS Ri(n) vs. NFS Hd | ns | 0.9992 |
| NFS Ri(n) vs. NFS Ss | ns | 0.3572 |
| NFS Ri(n) vs. NFS Amp | ns | 0.9992 |
| NFS Ri(n) vs. NFS. Ri(a) | ns | 0.1399 |
| NFS Ri(n) vs. NI Ri(n) | ns | 0.9992 |
| NFS Ri(n) vs. NI Hd | ns | 0.9744 |
| NFS Ri(n) vs. NI Ss | ns | 0.8999 |
| NFS Ri(n) vs. NI Amp | ns | >0.9999 |
| NFS Ri(n) vs. NI Ri(a) | ns | 0.3572 |
| NFS Hd vs. NFS Ss | ns | 0.8351 |
| NFS Hd vs. NFS Amp | ns | 0.8999 |
| NFS Hd vs. NFS Ri(a) | ns | 0.5444 |
| NFS Hd vs. NI Ri(n) | ns | >0.9999 |
| NFS. Hd vs. NI Hd | ns | >0.9999 |
| NFS Hd vs. NI Ss | ns | 0.9992 |
| NFS. Hd vs. NI Amp | ns | >0.9999 |
| NFS. Hd vs. NI Ri(a) | ns | 0.8351 |
| NFS. Ss vs. NFS Amp | ns | 0.0685 |
| NFS Ss vs. NFS Ri(a) | ns | >0.9999 |
| NFS Ss vs. NI Ri(n) | ns | 0.8351 |
| NFS. Ss vs. NI Hd | ns | 0.9744 |
| NFS. Ss vs. NI Ss | ns | 0.9967 |
| NFS. Ss vs. NI Amp | ns | 0.6571 |
| NFS Ss vs. NI Ri(a) | ns | >0.9999 |
| NFS Amp vs. NFS Ri(a) | * | 0.0175 |
| NFS Amp vs. NI Ri(n) | ns | 0.8999 |
| NFS Amp vs. NI Hd | ns | 0.6571 |
| NFS Amp vs. NI Ss | ns | 0.4527 |
| NFS Amp vs. NI Amp | ns | 0.9744 |
| NFS Amp vs. NI Ri(a) | ns | 0.0685 |
| NFS Ri(a) vs. NI Ri(n) | ns | 0.5444 |
| NFS Ri(a) vs. NI Hd | ns | 0.8276 |
| NFS Ri(a) vs. NI Ss | ns | 0.942 |

| | | |
|------------------------|----|---------|
| NFS Ri(a) vs. NI Amp | ns | 0.3482 |
| NFS Ri(a) vs. NI Ri(a) | ns | >0.9999 |
| NI Ri(n) vs. NI Hd | ns | >0.9999 |
| NI Ri(n) vs. NI Ss | ns | 0.9992 |
| NI Ri(n) vs. NI Amp | ns | >0.9999 |
| NI Ri(n) vs. NI Ri(a) | ns | 0.8351 |
| NI Hd vs. NI Ss | ns | >0.9999 |
| NI Hd vs. NI Amp | ns | 0.9992 |
| NI Hd vs. NI Ri(a) | ns | 0.9744 |
| NI Ss vs. NI Amp | ns | 0.9898 |
| NI Ss vs. NI Ri(a) | ns | 0.9967 |
| NI Amp vs. NI Ri(a) | ns | 0.6571 |

Table S3. Weight of aphids on NFS and NI plants. Results of Two-way ANOVA analysis showing a significant effect of the plant condition (NFS and NI) on the aphid weight $F(1, 170) = 209.2$, $P < 0.0001$ and of the aphid line $F(4, 170) = 9.073$, $P < 0.0001$. There was no significant interaction between “aphid line” and the weight of aphids $F(4, 170) = 1.763$, $P = 0.1386$.

| Multiple comparison | Significance | p value |
|----------------------------|---------------------|----------------|
| NFS Ri(n) vs. NFS Hd | ns | 0.5642 |
| NFS Ri(n) vs. NFS Ss | ns | >0.9999 |
| NFS Ri(n) vs. NFS Amp | ns | 0.226 |
| NFS Ri(n) vs. NFS. Ri(a) | ns | >0.9999 |
| NFS Ri(n) vs. NI Ri(n) | **** | <0.0001 |
| NFS Ri(n) vs. NI Hd | **** | <0.0001 |
| NFS Ri(n) vs. NI Ss | **** | <0.0001 |
| NFS Ri(n) vs. NI Amp | **** | <0.0001 |
| NFS Ri(n) vs. NI Ri(a) | **** | <0.0001 |
| NFS Hd vs. NFS Ss | ns | 0.7689 |
| NFS Hd vs. NFS Amp | ns | >0.9999 |
| NFS Hd vs. NFS Ri(a) | ns | 0.8633 |
| NFS Hd vs. NI Ri(n) | **** | <0.0001 |
| NFS. Hd vs. NI Hd | **** | <0.0001 |
| NFS Hd vs. NI Ss | **** | <0.0001 |
| NFS. Hd vs. NI Amp | **** | <0.0001 |
| NFS. Hd vs. NI Ri(a) | ns | 0.0743 |
| NFS. Ss vs. NFS Amp | ns | 0.3996 |
| NFS Ss vs. NFS Ri(a) | ns | >0.9999 |
| NFS Ss vs. NI Ri(n) | **** | <0.0001 |
| NFS. Ss vs. NI Hd | **** | <0.0001 |
| NFS. Ss vs. NI Ss | **** | <0.0001 |

| | | |
|------------------------|------|---------|
| NFS. Ss vs. NI Amp | **** | <0.0001 |
| NFS Ss vs. NI Ri(a) | *** | 0.0002 |
| NFS Amp vs. NFS Ri(a) | ns | 0.521 |
| NFS Amp vs. NI Ri(n) | *** | 0.0006 |
| NFS Amp vs. NI Hd | **** | <0.0001 |
| NFS Amp vs. NI Ss | **** | <0.0001 |
| NFS Amp vs. NI Amp | **** | <0.0001 |
| NFS Amp vs. NI Ri(a) | ns | 0.2675 |
| NFS Ri(a) vs. NI Ri(n) | **** | <0.0001 |
| NFS Ri(a) vs. NI Hd | **** | <0.0001 |
| NFS Ri(a) vs. NI Ss | **** | <0.0001 |
| NFS Ri(a) vs. NI Amp | **** | <0.0001 |
| NFS Ri(a) vs. NI Ri(a) | *** | 0.0003 |
| NI Ri(n) vs. NI Hd | ns | 0.5809 |
| NI Ri(n) vs. NI Ss | ns | 0.997 |
| NI Ri(n) vs. NI Amp | ** | 0.0037 |
| NI Ri(n) vs. NI Ri(a) | ns | 0.6331 |
| NI Hd vs. NI Ss | ns | 0.9778 |
| NI Hd vs. NI Amp | ns | 0.617 |
| NI Hd vs. NI Ri(a) | ** | 0.0041 |
| NI Ss vs. NI Amp | ns | 0.0626 |
| NI Ss vs. NI Ri(a) | ns | 0.1371 |
| NI Amp vs. NI Ri(a) | **** | <0.0001 |

Table S4. Dry weight of NFS plants. Results of the Tukey multiple-comparison test analysing the effect of the different pea aphid lines (YR2 genotype) on the **dry weight of NFS plants**; One-way ANOVA of the whole experiment, $F(5, 102) = 5.989$, $P < 0.0001$.

| Multiple comparison | Significance | p value |
|---------------------|--------------|---------|
| Control vs. Amp | *** | 0.0002 |
| Control vs. Ri(n) | ns | 0.1022 |
| Control vs. Ri(a) | * | 0.0152 |
| Control vs. Hd | ns | 0.4733 |
| Control vs. Ss | *** | 0.0003 |
| Amp vs. Ri(n) | ns | 0.4011 |
| Amp vs. Ri(a) | ns | 0.8334 |
| Amp vs. Hd | ns | 0.0772 |
| Amp vs. Ss | ns | >0.9999 |
| Ri(n) vs. Ri(a) | ns | 0.9808 |
| Ri(n) vs. Hd | ns | 0.9627 |
| Ri(n) vs. Ss | ns | 0.4315 |
| Ri(a) vs. Hd | ns | 0.6463 |
| Ri(a) vs. Ss | ns | 0.8567 |
| Hd vs. Ss | ns | 0.0872 |

Table S5. Dry weight of NI plants. Results of the Tukey multiple-comparison test analysing the effect of the different pea aphid lines (YR2 genotype) on the dry weight of NI plants; One-way ANOVA of the whole experience, $F(5, 102) = 4.035$, $P = 0.0022$.

| Multiple comparison | Significance | p value |
|---------------------|--------------|---------|
| Control vs. Amp | * | 0.039 |
| Control vs. Ri(n) | ** | 0.0046 |
| Control vs. Ri(a) | ** | 0.0018 |
| Control vs. Hd | * | 0.0409 |
| Control vs. Ss | ns | 0.0847 |
| Amp vs. Ri(n) | ns | 0.9812 |
| Amp vs. Ri(a) | ns | 0.9227 |
| Amp vs. Hd | ns | >0.9999 |
| Amp vs. Ss | ns | 0.9997 |
| Ri(n) vs. Ri(a) | ns | 0.9998 |
| Ri(n) vs. Hd | ns | 0.9791 |
| Ri(n) vs. Ss | ns | 0.9159 |
| Ri(a) vs. Hd | ns | 0.9173 |
| Ri(a) vs. Ss | ns | 0.7942 |
| Hd vs. Ss | ns | 0.9997 |

Table S6. Number of nodules / NFS plants. Results of the Tukey multiple-comparison test analysing the effect of the different pea aphid lines (YR2 genotype) on the number of nodules per plant for NFS plants; One-way ANOVA of whole experiment, $F(5, 30) = 14.54$, $P < 0.0001$.

| Multiple comparison | Significance | p value |
|---------------------|--------------|---------|
| Control vs. Amp | **** | <0.0001 |
| Control vs. Ri(n) | * | 0.0156 |
| Control vs. Ri(a) | *** | 0.0002 |
| Control vs. Hd | ns | 0.2316 |
| Control vs. Ss | **** | <0.0001 |
| Amp vs. Ri(n) | ns | 0.0755 |
| Amp vs. Ri(a) | ns | 0.7837 |
| Amp vs. Hd | ** | 0.0036 |
| Amp vs. Ss | ns | 0.9593 |
| Ri(n) vs. Ri(a) | ns | 0.6313 |
| Ri(n) vs. Hd | ns | 0.8107 |
| Ri(n) vs. Ss | * | 0.0105 |
| Ri(a) vs. Hd | ns | 0.0844 |
| Ri(a) vs. Ss | ns | 0.3003 |
| Hd vs. Ss | *** | 0.0004 |

Table S7. Nodule weight / plant. Results of the Tukey multiple-comparison test analysing the effect of the different pea aphid lines (YR2 genotype) on the weight of nodules in NFS plants (mg / plant); One-way ANOVA of whole experiment, $F(5, 30) = 7.195$, $P < 0.0002$.

| Multiple comparison | Significance | p value |
|---------------------|--------------|---------|
| Control vs. Amp | *** | 0.0003 |
| Control vs. Ri(n) | ns | 0.552 |
| Control vs. Ri(a) | ns | 0.1356 |
| Control vs. Hd | ns | 0.419 |
| Control vs. Ss | *** | 0.0008 |
| Amp vs. Ri(n) | * | 0.0284 |
| Amp vs. Ri(a) | ns | 0.1895 |
| Amp vs. Hd | * | 0.0474 |
| Amp vs. Ss | ns | 0.9997 |
| Ri(n) vs. Ri(a) | ns | 0.9464 |
| Ri(n) vs. Hd | ns | >0.9999 |
| Ri(n) vs. Ss | ns | 0.0563 |
| Ri(a) vs. Hd | ns | 0.9841 |
| Ri(a) vs. Ss | ns | 0.314 |
| Hd vs. Ss | ns | 0.0909 |

Table S8. ARA per mg of nodules. Results of the Tukey multiple-comparison test analysing the effect of the different pea aphid lines (YR2 genotype) on ARA per mg of nodules in NFS plants; One-way ANOVA of whole experiment, $F(5, 30) = 5.285$, $P < 0.0013$.

| Multiple comparison | Significance | p value |
|---------------------|--------------|---------|
| Control vs. Amp | * | 0.0118 |
| Control vs. Ri(n) | ns | 0.3023 |
| Control vs. Ri(a) | ns | 0.0811 |
| Control vs. Hd | * | 0.0289 |
| Control vs. Ss | *** | 0.0005 |
| Amp vs. Ri(n) | ns | 0.6566 |
| Amp vs. Ri(a) | ns | 0.9617 |
| Amp vs. Hd | ns | 0.9991 |
| Amp vs. Ss | ns | 0.8543 |
| Ri(n) vs. Ri(a) | ns | 0.9808 |
| Ri(n) vs. Hd | ns | 0.8551 |
| Ri(n) vs. Ss | ns | 0.1113 |
| Ri(a) vs. Hd | ns | 0.9973 |
| Hd vs. Ss | ns | 0.6556 |

Table S9. ARA per plant. Results of the Tukey multiple-comparison test analysing the effect of the different pea aphid lines (YR2 genotype) on ARA per plant; One-way ANOVA of whole experiment, $F(5, 30) = 15.15$, $P < 0.0001$.

| Multiple comparison | Significance | p value |
|---------------------|--------------|---------|
| Control vs. Amp | **** | <0.0001 |
| Control vs. Ri(n) | ns | 0.1004 |
| Control vs. Ri(a) | ** | 0.0014 |
| Control vs. Hd | ** | 0.0062 |
| Control vs. Ss | **** | <0.0001 |
| Amp vs. Ri(n) | ** | 0.0017 |
| Amp vs. Ri(a) | ns | 0.1154 |
| Amp vs. Hd | * | 0.0333 |
| Amp vs. Ss | ns | >0.9999 |
| Ri(n) vs. Ri(a) | ns | 0.5216 |
| Ri(n) vs. Hd | ns | 0.8474 |
| Ri(n) vs. Ss | ** | 0.0011 |
| Ri(a) vs. Hd | ns | 0.9927 |
| Hd vs. Ss | * | 0.0225 |

Table S10. Analysis of gene expression data using the t-test.

| | p value | p value | | p value | p value | p value | p value |
|------------------|---------------|------------|--------------|---------------|----------------|--------------|---------------|
| | <i>MtLb-1</i> | <i>Cp6</i> | | <i>PRI NI</i> | <i>PRI NFS</i> | <i>PI NI</i> | <i>PI NFS</i> |
| Amp NFS | 0.780022 | 0.00961 | Amp | 0.005208 | 0.015178 | 0.108835 | 0.000478 |
| Ri(n) NFS | 0.141657 | 0.147555 | Ri(n) | 0.002926 | 0.00413 | 0.457504 | 0.00073 |
| Ri(a) NFS | 0.251172 | 0.00127 | Ri(a) | 0.000892 | 0.011606 | 0.132567 | 0.001503 |
| Hd NFS | 0.134981 | 0.024828 | Hd | 0.099349 | 0.00384 | 0.525784 | 9.92E-05 |
| Ss NFS | 0.174654 | 0.000791 | Ss | 0.004047 | 0.01034 | 0.243729 | 0.000885 |

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Additional Information: 12 dpi Different Aphid Lines Infested and Non-infested NI and NFS plants

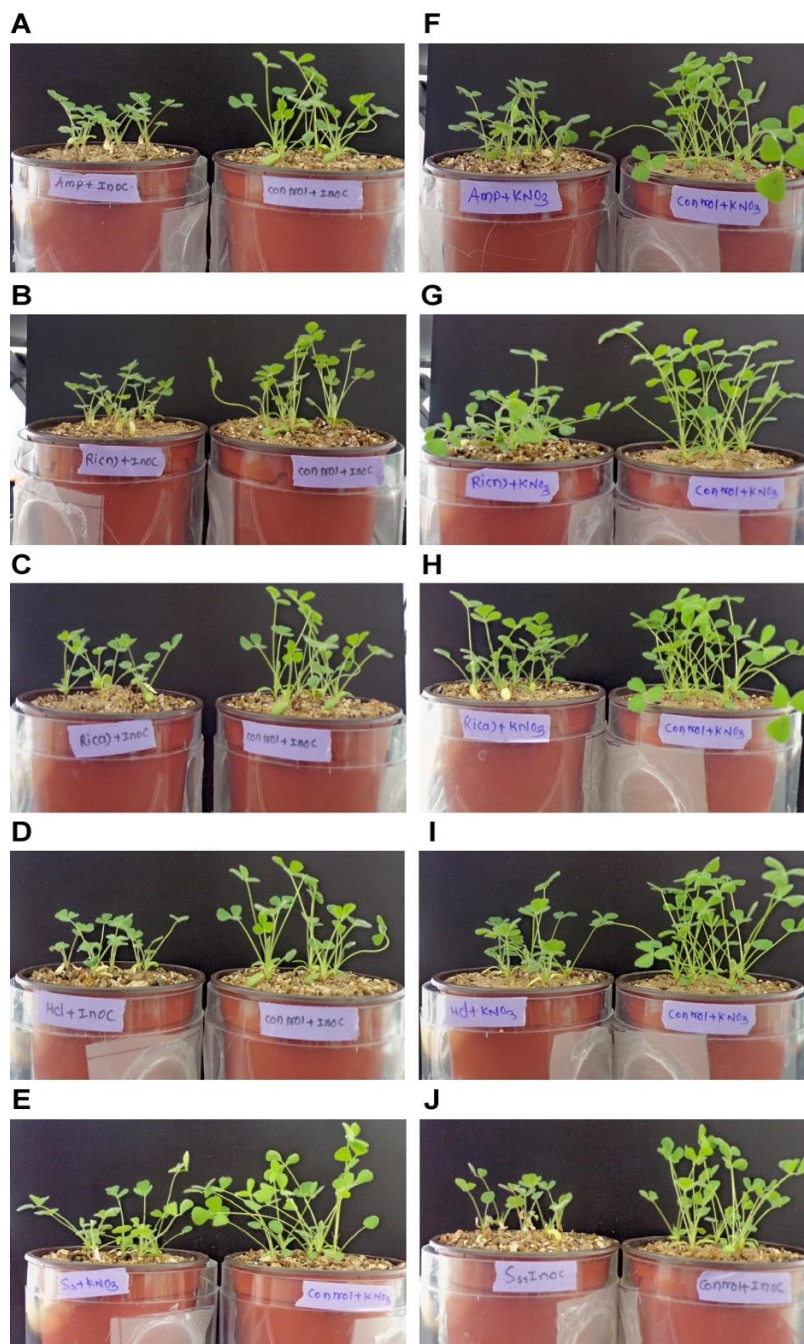


Figure A1 : Photos of *M. truncatula* A17 NI and NFS plants infested with different aphid lines and control plants at 12 dpi (A, B, C, D, E) Amp, Ri(n), Ri(a), Hd and Ss infested and control NFS plants respectively. (F, G, H, I, J) Amp, Ri(n), Ri(a), Hd and Ss infested and control NI plants respectively.

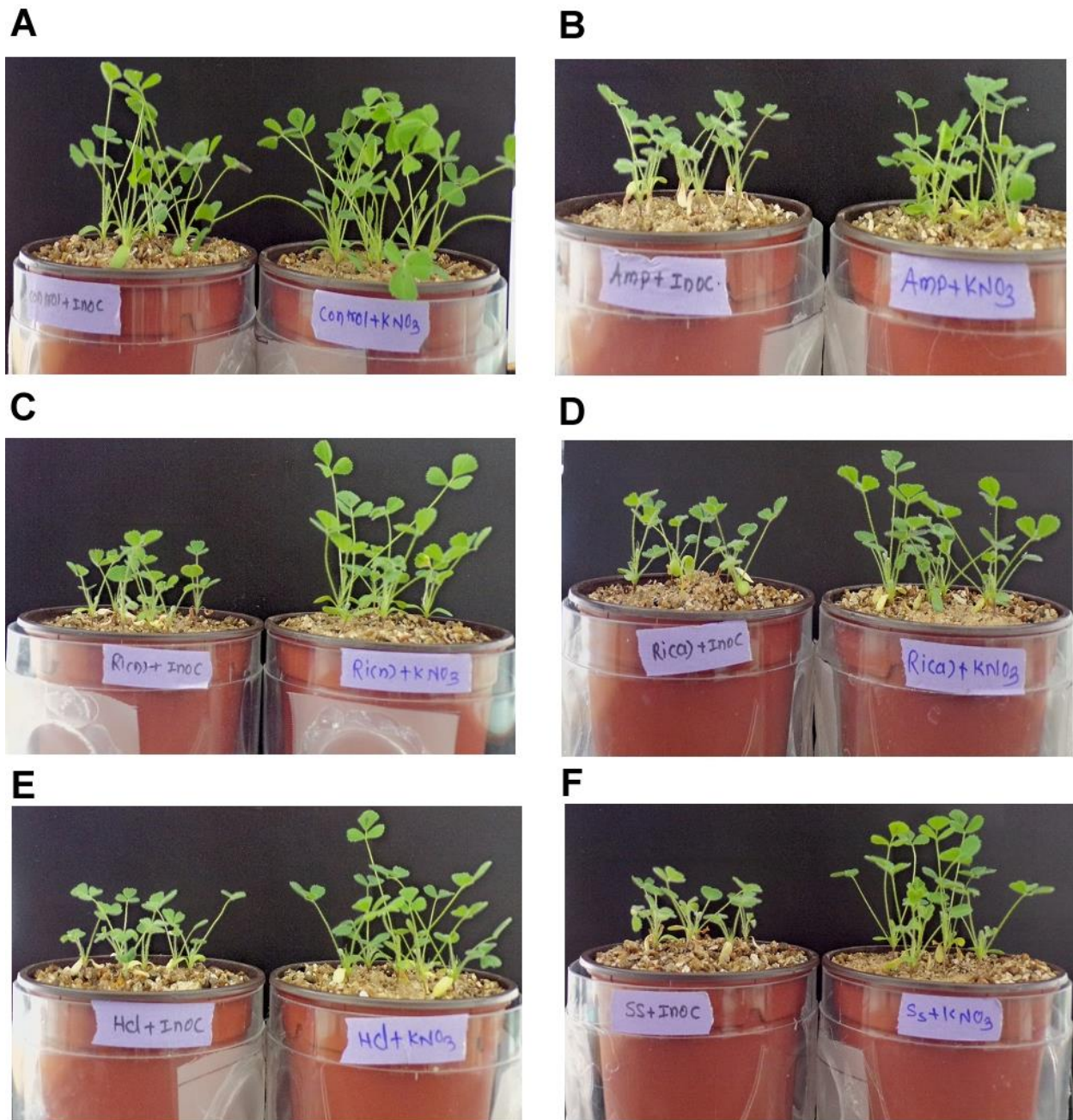


Figure A2: Comparison between NI and NFS *M. truncatula* A17 plants at 12 dpi. (A) Control, (B) Amp (C) Ri(n), (D) Ri(a), (E) Hd, and (F) Ss

4. CHAPTER 2

This chapter present analyses which were performed to test the genericity of the results of the previous article on plant-aphid-symbiont interactions by considering the genetic diversity in *M. truncatula* and *A. pisum*. Indeed, both the plant genotype and the aphid genotype and their interaction could influence the traits in the interactions. We therefore tested the existence of such effects in the presence or absence of rhizobacteria, aiming to analyse the importance of both plant and aphid genotypes in the interactions between plants and aphids. As in the first chapter, we analyse the performance of plant and aphid during the interaction and we looked to the induction of plant defence by aphid infestation using different *M. truncatula* cultivars and different aphid biotypes. To analyse potential different induction of the defence mechanisms, a time course analysis was performed.

Introduction for 2nd Publication

Legume plants directly or indirectly interact with a plethora of above- and belowground species. It is well-accepted that plant belowground interactions regulate plant aboveground interactions and vice versa. Several studies incorporating plant genetic variation suggest that plant-mediated indirect interactions between above- and belowground species may play an essential role in modulating the ecology and evolution of species within a community. In the following research manuscript, we investigated whether rhizobacteria influence genotype-mediated plant-aphid interactions. To this purpose, we used three different *A. pisum* aphid genotype (LL01, YR2, and T3-8V1) and two *M. truncatula* ecotypes (A17 and R108) and one rhizobacterial species (*S. meliloti 2011*). Given that plant defence response varies by aphid-plant genotype and time of infestation, we examined defence related gene expression at different aphid feeding time. We finally measured how aphid genotypes impact plant fitness and nitrogen fixation levels. Here, we show plant genotype strongly influence aphid-genotype mediated interaction. In addition, rhizobacteria differentially affect aphid fitness and plant defence responses depending on the plant genotype, thus adding another level of complexity to genotype-mediated plant-aphid interactions. While some more experiments need to be performed, we plan to submit these data for publication before the end of 2020.

Publication 2: Plant-aphid genotypic diversity and rhizobacteria community influence each other in three-way interactions

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(in progress)

Plant-aphid genotypic diversity and rhizobacterial symbiosis influence other in three-way interactions

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Running title: Aphid-plant genotypic and rhizobacterial mediated interaction

Key words: Pea aphid (*Acyrtosiphon pisum*), *Medicago truncatula*, Genotype, symbiosis, rhizobium, nitrogen fixation, plant defence.

Number of Figures: 10

Abstract

The genetic diversity in both plants and aphids influence their interaction and the physiological responses of both partners. Pea aphid (*Acyrtosiphon pisum*) feed on a restricted range of legume plants, including *Medicago truncatula*, and genetic variations of this host plant species influence aphid preference and performance. In this study, we investigated how the presence of the rhizobia symbionts affects the aphid genotype x plant genotype interactions, with a particular focus on the induction of the defence pathways. To this purpose, we used three different pea aphid genotypes without facultative symbionts (LL01, YR2, T3-8V1) feeding on two *M. truncatula* genotypes (JA17 and R108) watered with nitrate (NI) or infected with the nitrogen fixing bacteria (NFS) *Sinorhizobium meliloti*. Here we show that the expression of plant defence-related gene expression varies according to different *M. truncatula* and aphid genotype combination as well as in the presence or absence of rhizobacteria. We also demonstrate that the duration of aphid feeding and the compatibility of plant-aphid interactions modulated defence related gene expression. The low levels of SA-and-JA related gene expression on native LL01 genotype correlated with a significantly better performance on A17 plants. Similarly, in T3-8V1 aphid line modifies plant defence response and their fitness depending on the plant genotype. Our results indicate that rhizobacterial inoculation on *M. truncatula* plants led to changes in aphid fitness and defence response in relation to plant genotype. Altogether, this study adds a level of complexity in aphid-plant genotype-genotype interaction mediated by rhizobacteria.

Introduction

Aphids represent a major group of agricultural pests that limit the productivity of many crops by causing serious damage to plants due to both direct feeding and indirect disease vectoring. Aphid species differ in their host range, some are restricted to a plant family while others are polyphagous and feed on different plant species (Simon et al., 2015). For instance, the green peach aphid *Myzus persicae* attacks more than 400 plant species while *Brevicoryne brassicae* and *Acyrtosiphon pisum* are respectively specialized to *Brassicaceae* and *Fabaceae* families, respectively (Le Guigo et al., 2012; Silva et al., 2012). Aphid specialization depends on their intimate relationship with host plants during their life cycle: this involves adaptations to cope with the plant phenology, nutrient composition and chemical/physical defences. However, the aphid performance depends also on the host-plant genotype or ecotype (Ferrari et al., 2008; Zytynska and Preziosi, 2013) and the aphid genotype or biotype (Loxdale and Balog, 2018). Many studies have questioned this plant genotype x insect genotype interaction, but most of them have neglected the role of the beneficial symbiosis in these interacting organisms. It is only during the last decade that the importance of plant–microbe, plant–arthropod and arthropod–microbe has been shown to play an essential role in the selection, adaptation and evolution of plant–arthropod interactions, thus giving rise to the concept of three-way interactions (Tétard-Jones et al., 2012; Biere and Bennett, 2013). Such three-way interactions between plants, arthropods and microbes may have important consequences for the performance of other species in their local community.

The role played by rhizobacteria on the interaction of plants with arthropods has been much less studied. (Tétard-Jones et al., 2007) showed that interactions between barley genotypes and aphid clones are affected by the presence or absence of rhizobacteria and evidenced the importance of the genotype [aphid]x[barley]x[rhizobacteria] on aphid fitness during the multitrophic interactions. Recently, (Pineda et al., 2010; Grunseich et al., 2020) suggested that plant growth-promoting rhizobacteria generally have negative effects on herbivore performance or abundance, most likely through Induced Systemic Resistance (ISR) in host plants. In addition, presence of rhizobacteria has positive effects on sap-sucking insects likely due to an increase in phloem nutrient levels. Rhizobacteria negatively affect herbivory natural enemies in the field, either by changing plant attractiveness or by decreasing the nutritive quality of their host (Pangesti et al., 2015; Peterson et al., 2016).

The Leguminous family is the second most important crop family in the world and *Medicago truncatula* (barrel medic) is a model plant to explore leguminous plant and aphid interactions. *M. truncatula*, as other legumes, establish nitrogen fixing symbiotic (NFS) relationships with rhizobia (*Sinorhizobium meliloti*). This association provides a nitrogen source for the plant when nitrogen is in limited supply (Mitra and Long, 2004). Moreover, NFS also modulates plant defence responses against different herbivores (Dean et al., 2014; Franco et al., 2017). Multiple studies have shown that *M. truncatula* genotype plays a critical role in determining the interaction outcome with aphids that range from a fully compatible to a non-compatible plant-aphid interaction (Ferrari et al., 2008; Kanvil et al., 2014; Stewart et al., 2016). For instances, the *M. truncatula* Jester ecotype provides resistance against the blue-green aphid *Acyrtosiphon kondoi* and the pea aphid *A. pisum* Harris (Gao et al., 2008; Kamphuis et al., 2019). In contrast, the closely related ecotype A17 provides no significant resistance against aphids (Klingler et al., 2005, 2007; Stewart et al., 2009). The variation in virulence among aphid genotypes on this different *M. truncatula* ecotypes led to the description of a molecular mechanism involving a ‘gene-for-gene’ recognition between resistance (R) genes in plants associated with avirulence (Avr) genes in aphids (Stewart et al., 2009; Kanvil et al., 2014). Similarly, variation in the induction of phytohormone-dependent plant response to aphid feeding depends on the plant and aphid genotypes. The blue green aphid induces the expression of genes associated with the salicylic acid-response (SA) pathway in both *M. truncatula* resistant and susceptible lines early after its attack, although with different induction kinetics (Gao et al., 2008). In contrast, genes associated with the Jasmonic acid-response (JA) pathway were exclusively or predominantly induced in the resistant line (Gao et al., 2007, 2008). Pea aphid infestation also increases the SA-pathway genes in both susceptible and resistant *M. truncatula* plant genotypes and did not induce changes in the JA pathway genes in susceptible plants (Gao et al., 2007, 2008).

In a previous study we showed that there is an interplay between the presence of nitrogen fixing rhizobia and pea aphid infestation through the modulation of the plant growth, the nitrogen fixing symbiosis and the plant defence responses (Pandharikar et al., 2020 submitted). However, in these experiments we only used one aphid genotype and one plant genotype. Since both plant and aphid genotypes may be important in the outcome of the interaction (Stewart et al., 2016), here we used three different *A. pisum* genotypes and two *M. truncatula* genotypes to investigate

how plant and aphid genotypes modulate the effect of the nitrogen fixing symbiosis on the plant-aphid interaction. Among *M. truncatula* ecotypes, genome has been fully sequenced, while the R108 ecotype is used for genetic transformation (Salzer et al., 2004; Wang et al., 2014). It has been demonstrated that A17 differs from R108 in their phenotype and developmental response and display different physiological responses to biotic (Gaige et al., 2012) and abiotic stress (Li et al., 2014; Wang et al., 2014). In addition, both ecotypes can easily establish in both nature and laboratory a symbiotic relationship with the nitrogen-fixing rhizobium bacteria *S. meliloti* (Mitra and Long, 2004). For the pea aphid genotypes, we used three pea-aphid lines (YR2-amp, T3-8V1-amp and LL01) that were originally collected in France and England and represent two European biotypes (Simon et al., 2011): YR2 and T3-8V1 are clover biotypes and LL01 is an alfalfa biotype.

In the current study, we used the rhizobacteria- *M. truncatula* -*A. pisum* model to examine the effect produced by NFS on the G x G plant - aphid interaction, including the time course induction of salicylic acid defence pathway and jasmonic acid biosynthesis and defence pathways. We observed that the rhizobacteria presence significantly affects plant-aphid interactions and that this effects depends on the plant and aphid genotypes. These results show that the outcome of the plant-insect interaction is strongly impacted by the symbiotic status of the plant and the genotype of both interacting species. This adds another levels of complexity in the understanding of the ecological relationships between species in nature.

Materials and Methods

Plant Material and Growth Conditions

Two *Medicago truncatula* ecotypes Jemalong A17 and R108 were used. Seeds of both ecotypes were scarified for 10 min in H₂SO₄ 96%, rinsed five times with sterile water, sterilized for 3 min with 10% (v/v) commercial bleach, and washed five times with sterile water (Barker et al., 2006; Nelson et al., 2015). Seeds were placed on 0.4% agar plates in the dark for 2 days at 4°C and then for 2 days at 20°C. After germination, 6 plantlets were transferred to a round pot (diameter x height: 7.5 x 7.5 cm) containing a mixture of vermiculite and sand (2:1) and all pots were moved to a growth chamber at 23°C (16h light) and 20°C (8h dark), relative humidity 60 ± 5 % and watered with nitrogen-free plant-pod medium (Oger et al., 2012). One week after

transferring the six plantlets, *M. truncatula* A17 and R108 pots were separated into two groups: one group was inoculated with the *Sinorhizobium meliloti* 2011 strain (NFS plants) (del Giudice et al., 2011), and a second group was supplemented 5 mM potassium nitrate (KNO₃) in water (NI plants) (Moreau et al., 2008). For inoculation, the *S. meliloti* 2011 strain was cultured on Luria-Bertani medium supplemented with 2.5 mM CaCl₂ and MgSO₄ (LBMC) and 200 µg/mL streptomycin for at for 3 days at 30°C. Subsequently, bacteria were grown in LBMC liquid medium for 24h, bacterial cells were pelleted at 5000g, washed twice with sterile distilled water, resuspended in sterile distilled water to a final optical density of 0.05 (OD 600) (Oger et al., 2012). Each NFS plant was supplemented with 10 mL of *S. meliloti* suspension.

Biological Materials (Aphid Lines)

The three pea-aphid (*A. pisum*) lines used (YR2, T3-8V1 and LL01) were originally collected from field sites in France and England and represent two European biotypes. Two are consider as clover biotypes (YR2 and T3-8V1) and one (LL01) as an alfalfa biotype (aphid Table 1). The YR2 (original name YR2-amp) and T3-8V1 (original name T3-8V1-amp) lines were freed from secondary symbiont after ampicillin treatment of the original field clones (Simon et al., 2011; Schmitz et al., 2012). All aphid lines are stable (more than 15 years old) and were maintained in cages on the universal plant host fava bean, at 20°C, under a 16:8h light/dark cycle. The absence of known symbiont was tested by PCR as described during the time of the experiments.

Aphid Rearing and Infestation

One-week post-inoculation with *S. meliloti* (necessary time to the formation of nodules) or nitrate supplementation, plants were infested with different aphid lines. Inoculated (NFS) and non-inoculated (NI) pots with six plants were then randomly distributed in different groups (see Figure. 1 from publication 1). To synchronize the age of the aphids for the experiments, 20 apterous female adults (LL01, YR2-Amp and T3-8V1-Amp) were placed on separated *Vicia faba* plants and allowed to reproduce. After 24 h, 10 nymphs (L1) of each aphid line were collected and used for infestation of one pot of each group of *M. truncatula* plants. The development of the different pea aphid lines was measured 1, 4, 6, 8 and 12 days after aphid infestation (dpi). Survival and weight of aphids (average weight of all alive aphids on a plant)

were measured as fitness parameters. For each time point different sets of plant and aphids were used on six separated replicates. For each conditions and time point non-infested NFS and NI plant pots were maintained. These plants served as control. All pots were individually isolated in an ventilated plastic box and maintained at 20°C under 16:8h light/dark photoperiod with a 70% relative humidity.

Aphid Lines Effect on Plants

To estimate the effect of the different aphid lines on A17 and R108 inoculated and non-inoculated plants, the dry weight of the plant shoot was measured at 4, 8 and 12 dpi. The plant shoot was placed in a drying oven at 80°C for 3 days and weighed on a precision balance (OHAUS Corp, PA214; accuracy \pm 0.1 mg). The shoot dry weight of the six individual plants from three pots (in total 18 plants) per condition was measured on three separated experiments.

Nitrogen Fixation Assay

To study the effect of aphid infestation on biological nitrogen fixation, acetylene reduction activity (ARA) was quantified in control and aphid-infested plants. Only NFS plants were analyzed given their ability to fix atmospheric N₂ via the formation of root nodules. Nodulated roots were incubated at 28°C for 1 hr, in a rubber-capped glass bottles containing acetylene (Johanna Döbereiner, 1966; El Msehli et al., 2011). Nitrogen fixation capability of nodules was estimated indirectly based their ability to reduce acetylene to ethylene via their nitrogenase activity (ARA). Gas conversion was measured by gas chromatography (6890N GC network system, Agilent). To study the time effect of the aphid infestation, ARA was quantified at 4, 8 and 12 dpi After ARA measurement, nodules were separated from the roots, counted and weighted. The values from ARA are expressed in nmol of ethylene produced per hour and mg nodule (ARA/(hr x mg nod)) and the total plant roots production, ARA/plant (nmol of ethylene/(hr x no. of root nodules per plant). The experiment was performed for three independent biological repeats.

Gene Expression Analysis by Quantitative RT-qPCR

For *M. truncatula* A17 and R108 NFS and NI plant shoot harvesting, the aphid lines were removed from the plants using a paintbrush. Control plants were brushed in the same way as aphid-infested plants. Six plant shoot per condition and time points were collected immediately after aphids removal, pooled and frozen in liquid nitrogen. For RNA extraction, plant material was grinded in liquid nitrogen using a mortar to obtain a fine powder. Total RNAs were then isolated using RNazol® RT (Sigma), quantified (NanoDrop 2000 spectrophotometer), and analyzed Bio-analyzer chips (Agilent) and 1.5% agarose gel electrophoresis to assess the purity. DNA digestion (RQ1 RNase-free DNase) and reverse transcription (GoScript™ Reverse Transcription) were performed as described by the manufacturer (Promega). The quantitative PCR was performed using the qPCR Master Mix plus CXR (qPCR kit; Promega). Each reaction was carried out with 5 µl of cDNA template diluted 40-fold and each set of specific primers. As SA defence related genes, we used the *Medicago* gene *Medtr2g435490*, annotated as *PR1* in database (NCBI protein ref XP_013463163.1), *Medtr1g080800* annotated as Pathogenesis related protein-4 (*PR4*) in database (NCBI protein ref XM_013613520.2), and *Medtr1g062630* annotated as Thaumatin-like protein (*PR5*) (XM_013612651.2). As JA-related genes, we used linoleate 9S-lipoxygenase (*LOX5*) (XM_003627148.3), Allene Oxide Synthase (*AOS1*) (XM_013610584.2) and the gene *Medtr4g032865* a proteinase inhibitor PSI-1.2 named thereafter *PI* (XP_013455238.1), a potato type II proteinase inhibitor family protein. Real-time qPCR was performed, as follows: 95°C for 3 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec (AriaMx Real-time PCR machine, Agilent). The primers efficiency was evaluated on the slope of a standard curve generated using a serial dilution of the samples. Cycle threshold values (Ct) were normalized to the average Ct of two housekeeping genes (del Giudice et al., 2011), the gene *Medtr2g436620* coding for MtC27, which is commonly used as a constitutive control in roots and nodules for expression studies in *Medicago* and the gene *Medtr4g109650.1* (also name a38). The expression of these two genes was not affected by the treatments in our analyses. The original Ct values were obtained from the machine software (Ariamix software; Agilent) and further calculations were done using the RqPCRBASE package using RStudio Version 1.1.453 (<https://www.rstudio.com>). In order to measure change in plant basal defence response over the time, the expression level of non-infested control plant was quantified in each condition (NI and NFS). For each condition and time points the expression

levels of aphid infested plants were compared with non-infested control plants. The results of the qPCR analysis were generated from three independent biological repeats with three technical repetitions per experiment.

Statistical Analysis

All experimental data are expressed as mean \pm standard mean error (SE). To study whether the survival and weight of aphid lines (LL01, YR2 and T38-V1) were affected by the plant genotype (A17 and R108), plant treatment (NFS and NI conditions), these data were analysed using a two-way ANOVA with time points and aphid lines as categorical explanatory variables. To test the differences among time points and aphid lines, the Sidak's multiple comparisons test was performed. Data generated from the dry weight of the plant, the nitrogen fixation assay per plant or per nodule, the number of root nodules and the weight of the nodules were analysed using the repeated measure ANOVA test. The results obtained from these experiments on NFS and NI plants were compared independently on the basis of the treatment using the repeated measure ANOVA test. Then, Tukeys multiple comparison tests in independent treatments were performed to identify a possible significant statistical difference between the aphid lines. All experimental data, except expression analysis results (analyzed with RqPCRBASE package), were analysed using Prism v7 (GraphPad software, USA) and results are available in supplementary file.

Results

Impact of Nitrogen Fixing Bacteria and Plant Genotype on Pea Aphid Genotype Fitness

To determine whether the different aphid genotypes develop differently on *M. truncatula* A17 and R108 cultivars inoculated (nitrogen fixing symbiosis, NFS) or non-inoculated (NI plants), the number of surviving aphids was counted and their growth rate measured at 1, 4, 6, 8 and 12 days post infestation (dpi). The aphid survival was approximately 90% high with no significant difference between aphid lines, plant genotypes and plant conditions (Figure 1).

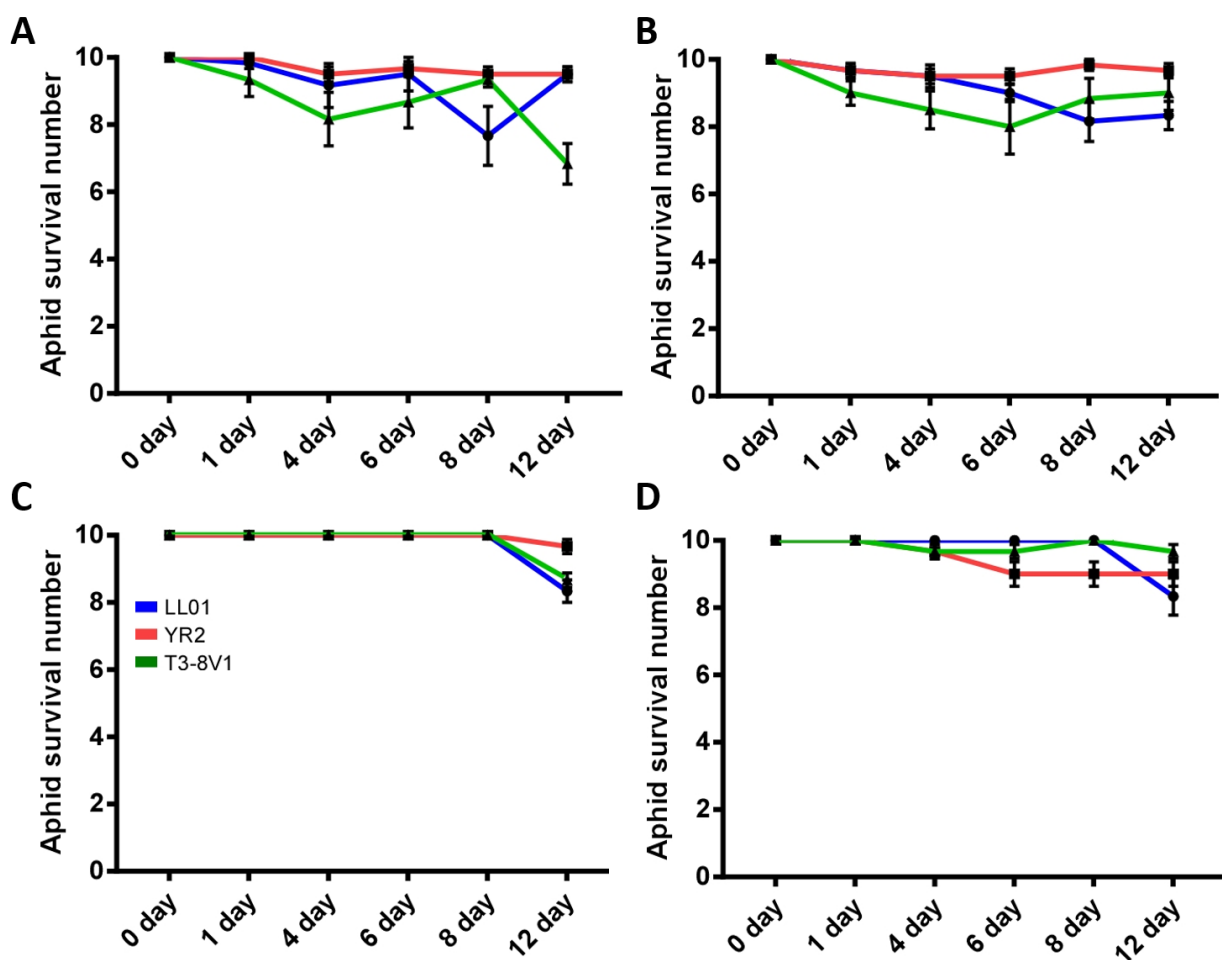


Figure 1: Survival of pea aphid lines on NI and NFS *M. truncatula* A17 and R108 plants. A,B) Mean number of adult aphids surviving over 12 days on *M. truncatula* A17 NI and NFS plants; (Mean \pm S.E.; n= 6). **C,D)** Mean number of adult aphids surviving over 12 days on *M. truncatula* R108 NI and NFS plants; (Mean \pm S.E.; n= 6); No stctically significant differences were observed

The growth rate of the three aphid genotypes was measured from L1/L2 (day 1) to adult L12 stage (day 12) after plant infestation. LL01 aphids grew significantly better on A17 plants (NFS

or NI) than YR2 and T3-8V1 (Figure 2 A,B) with a final LL01 adult weight almost 1.5 to 2 fold higher than the other lines. On R108 NI and NFS plants, T3-8V1 line appears to gain weight more rapidly than the other lines, but aphids stopped growing after 6 dpi on NI and 8 dpi on NFS (Figure 2 C, D). LL01 aphids grew also more rapidly than YR2 during the first days but they showed also a slowdown thereafter while YR2 continued to grow until the adult stage. At the 12 dpi, the weight of the three aphid genotypes was similar on NFS plants while it differed on NI plants. See all statistics in Tables S2 to S5. Thus, the aphid growth rate depends upon the plant genotype x aphid genotype interaction. Moreover, the aphid growth is not always modulated by the symbiotic state of the plant depending on the aphid and plant genotypes.

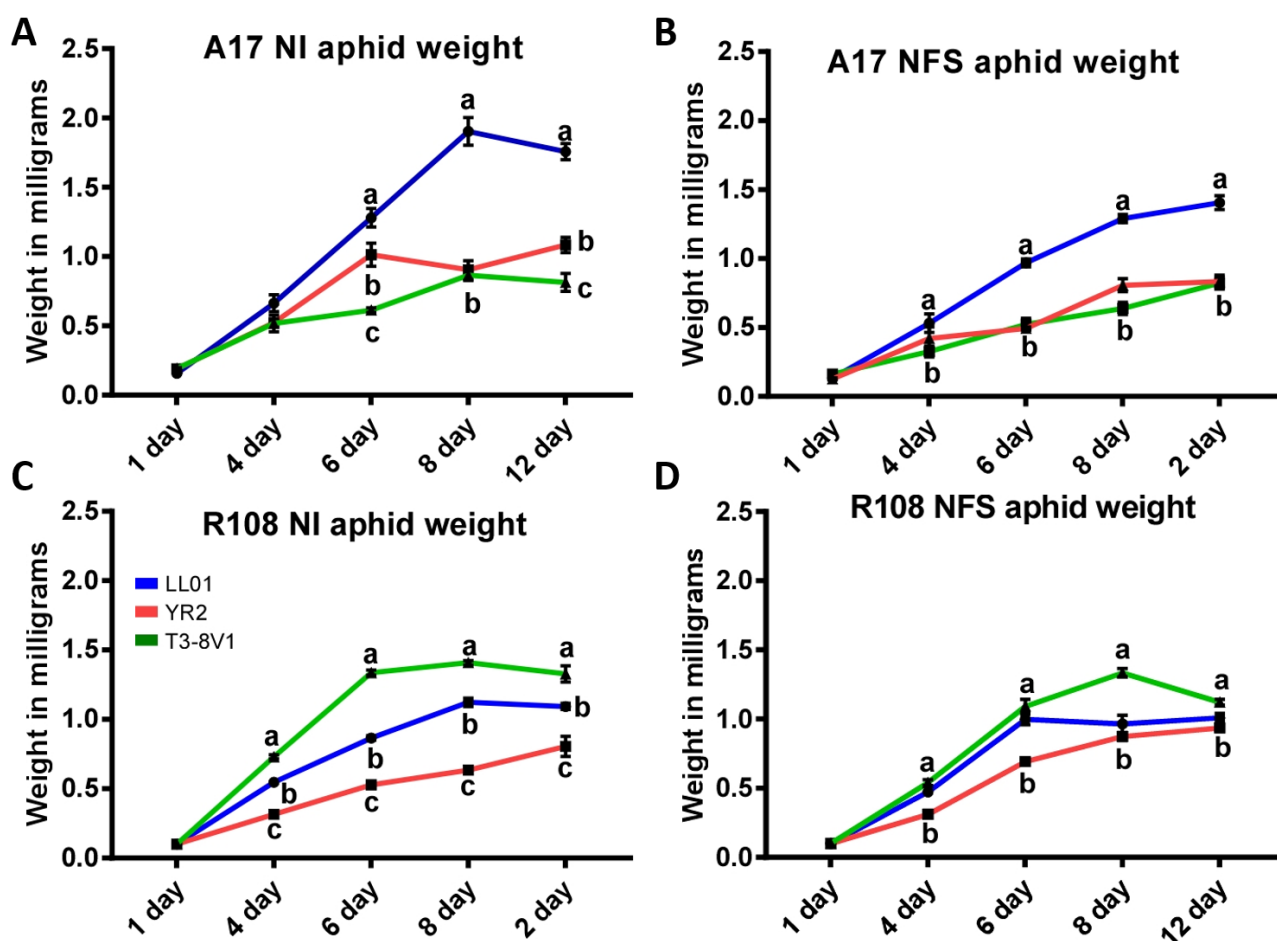


Figure 2: Weight of pea aphid lines on *M. truncatula* NI and NFS A17 and R108 plants. Mean individual weight of surviving adult aphids from the different lines at each time point over the experiment on *M. truncatula* plants; (Mean \pm SE, n=6). **A,B)** A17 genotype A) NI plants B) NFS plants. **C,D)** R108 genotype C) NI plants D) NFS plants. Statistical differences between the means are indicated by different letters ($p \leq 0.05$).

Effects of Aphid Infestation on the Biomass of *M. truncatula* Plant Shoots

At the beginning of the experiment the control plant shoot dry weight was similar between A17 and R108 NFS and NI plants (supplementary Figure 1), while at day 12, it was about 40% higher for NI compared to NFS plants. We assessed the effect of aphid infestation on plant development by weighting the dried plant shoot at 4, 8 and 12 dpi. The infestation with all aphid lines significantly reduced the dry weight of NFS and NI plants A17 and R108 compared to control non-infested plants, with few differences between them (Figure 3). LL01 aphids seem to have lower impact on the growth of A17 compared to the other lines. See all statistics in tables S6 to S9. The growth of aphid infested and non-infested A17 and R108 NFS and NI plants can be seen in Figure S2 to S5. These data indicates that while the development of aphids is affected by the plant/aphid genotypes, the plant development is similar for the two cultivars A17 and R108.

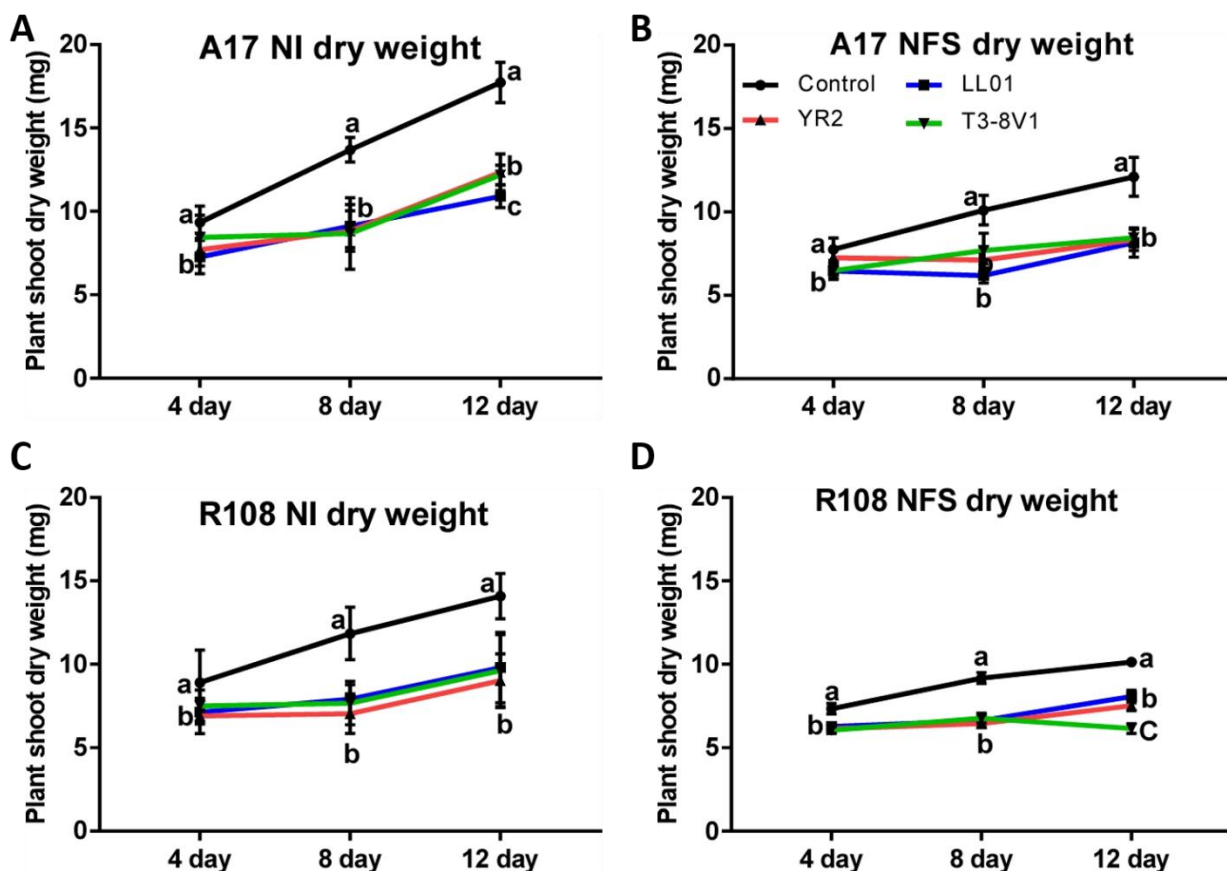


Figure 3: Effect of pea aphid lines on the dry weight of *M. truncatula* A17 and R108 NI and NFS plant shoots. Dry weight of *M. truncatula* A17 NI (A) and NFS (B) and *M. truncatula* R108 NI (C) and NFS (D) plant shoots at 4, 8 and 12 days of infestation with the different pea aphid lines compared to their respective non-infested control plants (control); (Mean \pm S.E., n=3). Statistical differences between the means are indicated by different letters ($p \leq 0.05$).

Effect of Aphid Lines on the Nitrogen Fixation of Inoculated Plants

The effect of the pea aphid lines on NFS plants nitrogen fixation capacity was estimated at 4, 8 and 12 dpi by measuring the number of nodules and their nitrogenase activity using the acetylene reduction assay (ARA). For both plant genotypes, the number of root nodules was about 2-fold reduced in infested plants at 8 dpi and this difference was maintained at 12 dpi, with A17 genotype developing more nodules than R108 plants (Figure 4). Accordingly, ARA per plant or per mg of nodule was significantly reduced in aphid infested plants at 8 dpi compared to the non-infested control plants, a reduction kept at 12 dpi. Interestingly, both R108 and A17 showed a similar ARA per plant level at 12 dpi despite a difference in their nodule number, usually correlated to a higher activity per nodule in the cultivar R108 (Figure 4). There was no or little difference between the aphid different aphid lines. Overall, these results suggest an reduction in NFS in plants after the infestation of aphids, however intensity appears to be modulated by plant-aphid genotype and time of infestation.

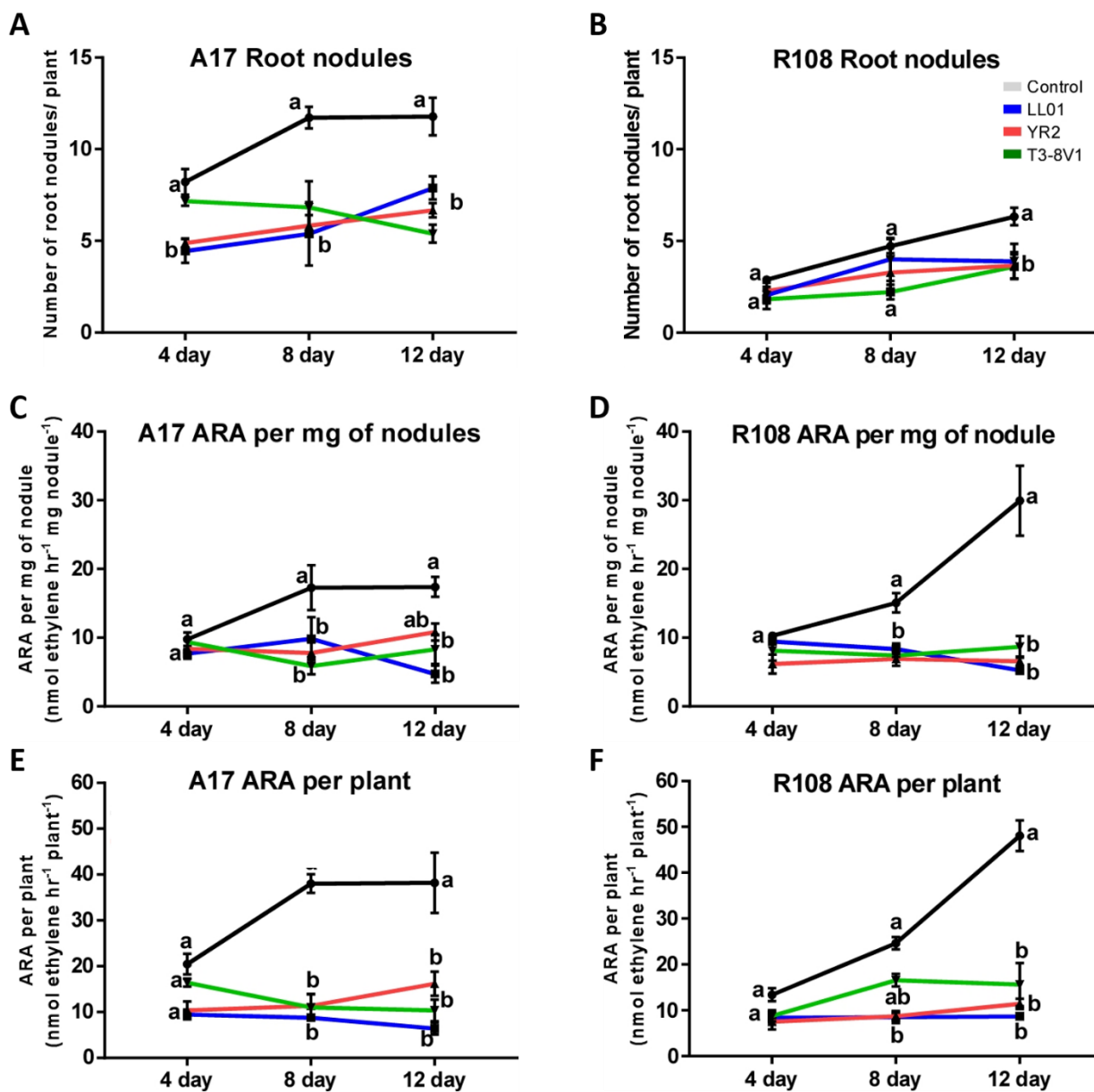


Figure 4: Effects of pea aphid infestation on biological nitrogen fixation of *M. truncatula* A17 and R108 plants. A, B: A17 (A) and R108 (B) Number of root nodules in NFS plants not infested (control) or infested with the different pea aphid lines for 4, 8 and 12 days. C, D: Mean acetylene reduction activity expressed per milligram of nodule ($\text{nmol ethylene} \times \text{hr}^{-1} \times \text{mg nodule}^{-1}$) of *M. truncatula* A17 plants (C) and R108 plants (D) after infestation with pea aphid lines compared to non-infested plants (control). E, F: Mean acetylene reduction activity expressed per plant ($\text{nmol ethylene} \times \text{hr}^{-1} \times \text{plant}^{-1}$) of *M. truncatula* A17 (E) *M. truncatula* R108 after infestation with pea aphid lines compared to non-infested plants (control); (Mean \pm S.E., $n=3$). Statistical differences between the means are indicated by different letters ($p \leq 0.05$).

Expression Analysis of Genes from the SA and JA Defence Pathway

Numerous studies have demonstrated that the SA pathway is involved in the plant defence response against aphids while the role played by the JA pathway in this process is still debated (Morkunas et al., 2011; Okada et al., 2015). Here we analysed the effect of the nitrogen fixing symbiosis on the plant defence response elicited by aphid infestation. To perform this analysis, we measured the expression level of different genes induced by the SA-mediated defence pathway (Pathogenesis-related protein genes *PR1*, *PR4* and *PR5*) and the JA-mediated defence pathway (the *Proteinase Inhibitors (PI)*, the Lipoxygenase (*LOX5*) and the Allene Oxide Synthase1 (*AOS1*) by qRT-PCR in plant shoots of non-infested control and aphid-infested NFS and NI A17 and R108 plants during a time course analysis. Pathogen-related genes were used to indicate a downstream activation of SA-mediated response after aphid infestation: basic *PR1* proteins marker for SAR response (Fu and Dong, 2013; Kamphuis et al., 2013), *PR4* (Chitinase class I and II) (De Zutter et al., 2017) and *PR5* (thaumatin-like protein) (Kamphuis et al., 2013). For JA-mediated response, we used lipoxygenase (*LOX5*) (Song et al., 2016) and allene oxide synthase (*AOS.1*) (Eyres et al., 2016) as marker genes for the JA biosynthesis pathway and *PI* (Protease Inhibitors) which is strongly induced by JA-induction.

Analysis of the Expression of Pathogenesis-Related Protein Gene *PR1*

In NI A17 plants, the infestation with YR2 and T3-8V1 led to a significant and strong increase in *PR1* expression at all the time points. The highest expression level was observed at 4 dpi (43 and 54-fold change, YR2 and T3-8V1 respectively) (Figure 5A). In contrast, presence of LL01 induced a 10-fold significant change in *PR.1* expression only at 12 dpi. In infested A17 NFS plants, *PR1* expression in plant shoots increased 7-8-fold compared to control plant shoots and this expression remained very stable over the time with few variations between aphid lines (Figure 5B). On R108 NI plants, LL01, YR2 and T3-8V1 infestation increased the *PR1* expression (4-to-26-fold change) with some variation dependent on the time point and the aphid line (Figure 5C, Table S18). The highest induced expression occurred at 8 dpi by the YR2 line (26-fold change).

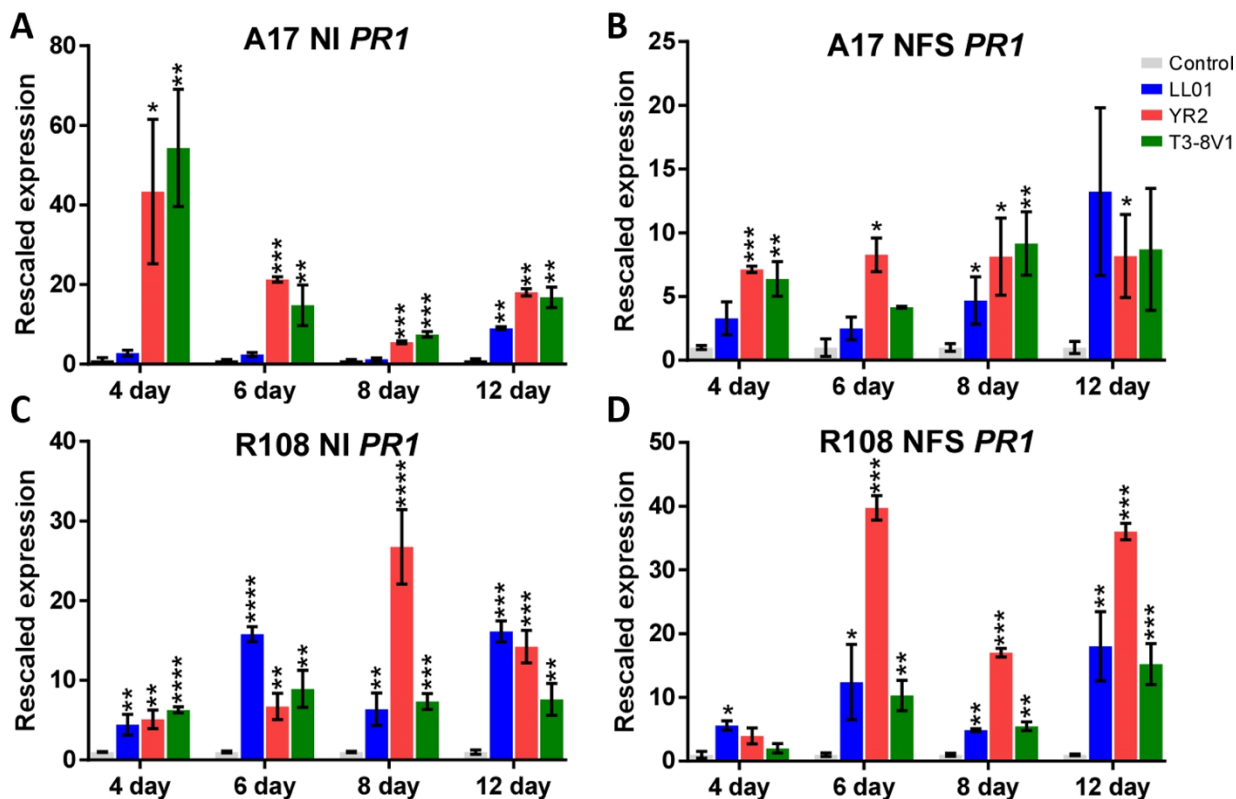


Figure 5: *PR1* expression levels estimated by qPCR at each time point of the experiment in shoots of NI and NFS *M. truncatula* A17 and R108 plants after infestation by the different aphid lines; (Mean \pm SE, n=3). A,B) *M. truncatula* A17 on (A) NI plants (B) NFS plants and C,D) *M. truncatula* R108 (C) NI plants D) NFS plants. Non-infested plants served as controls. Statistical differences (t-test): $p > 0.05$; * $P \leq 0.005$; ** $P \leq 0.01$; *** $p \leq 0.001$; ****, $P \leq 0.0001$.

Similarly, on R108 NFS plants, aphid infestation significantly increased *PR1* expression over time (5-to-39-fold change), being the strongest increase one induced by the YR2 line at 6 dpi (Figure 5D). Taken together, these results showed that *PR1* expression is modulated by both plant and aphid genotypes. Moreover, the symbiotic root colonization affected more the *PR1* expression profiles in A17 plants more than in R108 plants.

Analysis of the Expression of Pathogenesis-Related Protein Gene *PR4*

On A17 NI plants, YR2 and T3-8V1 infestation led to a significant increase in *PR4* expression levels, except for 6 dpi, being maximal (16-fold) at 8 dpi for T3-8V1 (Figure 6A). In contrast, LL01 infestation did not significantly increase *PR4* expression level over the time course of the experiment (Figure 6A).

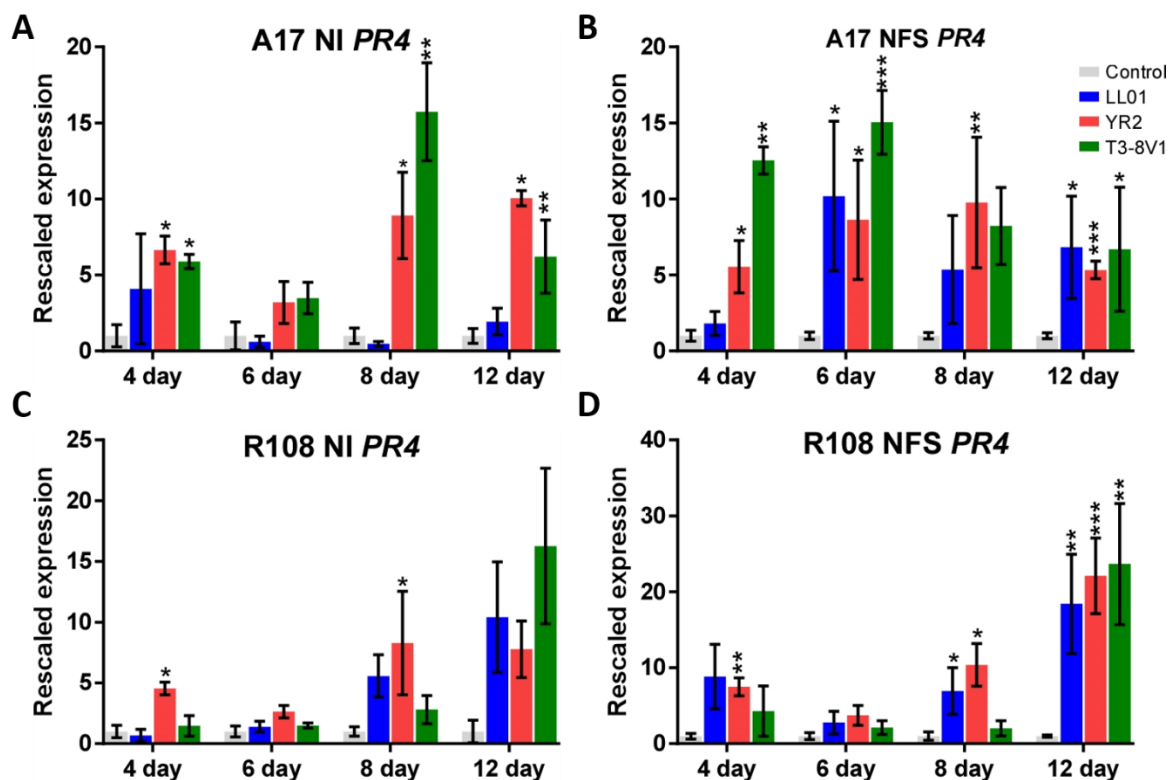


Figure 6: Expression levels of *PR4* estimated by qPCR in shoots of NI and NFS *M. truncatula* A17 and R108 plants at each time point of the experiment after infestation by the different aphid lines; (Mean \pm SE, n=3). **A,B) *M. truncatula* A17 (**A**) NI plants (**B**) NFS plants and **C,D)** *M. truncatula* R108 (**C**) NI plants (**D**) NFS plants. Non-infested plants served as control; Statistical differences (t-test): $p > 0.05$; * $P \leq 0.005$; ** $P \leq 0.01$; *** $p \leq 0.001$; ****, $P \leq 0.0001$.**

On A17 NFS plants, the expression level of *PR4* was significantly increased by YR2 and T3-8V1 infestation during the time course analysis. LL01 infestation significantly increased the *PR4* expression level only from at 6 dpi onwards and remained stable 12 dpi (Figure 6B). On R108 NI plants, slight induction of *PR4* with all lines, with only significant increase with YR2 line at 4 and 8 dpi (Figure 6C). On R108 NFS plants, the general pattern of expression was similar to this observed in NI plants, however aphid infested plants displayed a significant stronger increase in *PR4* expression at 12 dpi (up to 15-25-fold from the control) for the three aphid lines (Figure 6D). Overall, effect on *PR4* expression levels appeared to be higher on A17 and R108 aphid infested NFS plants compared to NI plants, suggesting an effect of the nitrogen fixing symbiosis on the expression of *PR4*.

Analysis of the Expression of Pathogenesis-Related Protein Gene *PR5*

PR5 expression levels showed a very different pattern over time among aphid-infested A17 and R108 NI and NFS plants (Figure 7). While LL01 line infestation had no effect on *PR5* expression on A17 NI plants (Figure 7A), it did have a strong but transient effect at day 6 dpi on A17 NFS plants. YR2 and T3-8V1 infestation of A17 NI plants induced a biphasic pattern of *PR5* expression with a strong induction at day 4, followed by a decrease at day 6 and an increase again at day 8 that was stronger for T3-8V1, and final decrease at 12 dpi. In A17 NFS plants, YR2, T3-8V1 and LL01 infestation had a similar induction, being the high induction at 6 dpi (Figure 7B, Tables S17 and S17).

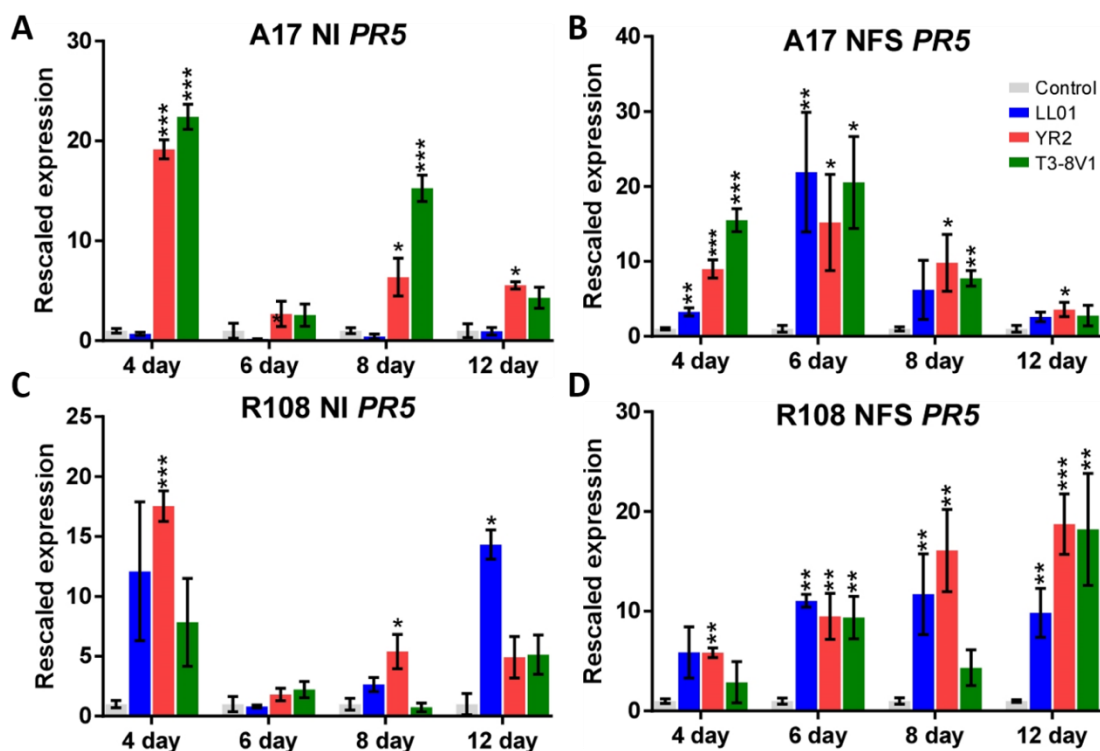


Figure 7: Expression levels of *PR5* estimated by qPCR in shoots of NI and NFS *M. truncatula* A17 and R108 plants at different time points of the experiments after infestation by the different aphid lines; (Mean \pm SE, n=3). A,B) *M. truncatula* A17 (A) NI plants (B) NFS plants; C,D) *M. truncatula* R108 (C) NI plants (D) NFS plants of NFS and NI plants. Non-infested plants served as control; Statistical differences (t-test): $p > 0.05$; * $P \leq 0.005$; ** $P \leq 0.01$; * $p \leq 0.001$; ****, $P \leq 0.0001$.**

In R108 NI plant shoots, YR2 at 4 and 8 dpi and LL01 at 12 dpi produced a significant induction of the *PR5* expression. In NFS plants, the *PR5* expression levels increased from 9 to

18-folds between 4 and 12 dpi in plant shoots infested by YR2. LL01 infestation significantly increased the level of *PR5* transcripts between 4 and 6 dpi, and the level remained significantly higher at 8 and 12 dpi (Figure 7C). In contrast, T3-8V1 line infestation did not significantly induce *PR5* expression at 4 and 8 dpi but a significant 9-18 fold increase was observed at 6 and 12 dpi (Figure 7D). See statistics in tables S18 and S19. In conclusion, the variation in *PR5* expression appears complex but is depends on plant nitrogen feeding conditions and plant and aphid genotype interactions.

Gene Expression Analysis of the JA Defence Pathway:

Lipoxygenase *LOX5*. *LOX5* expression levels remained unchanged upon infestation with any of the aphid lines in both inoculated and non-inoculated A17 and R108 plants (Figure 8). This result showed that this gene is not activated by the infestation regardless genotype of the plants and the aphids or the nitrogen feeding conditions.

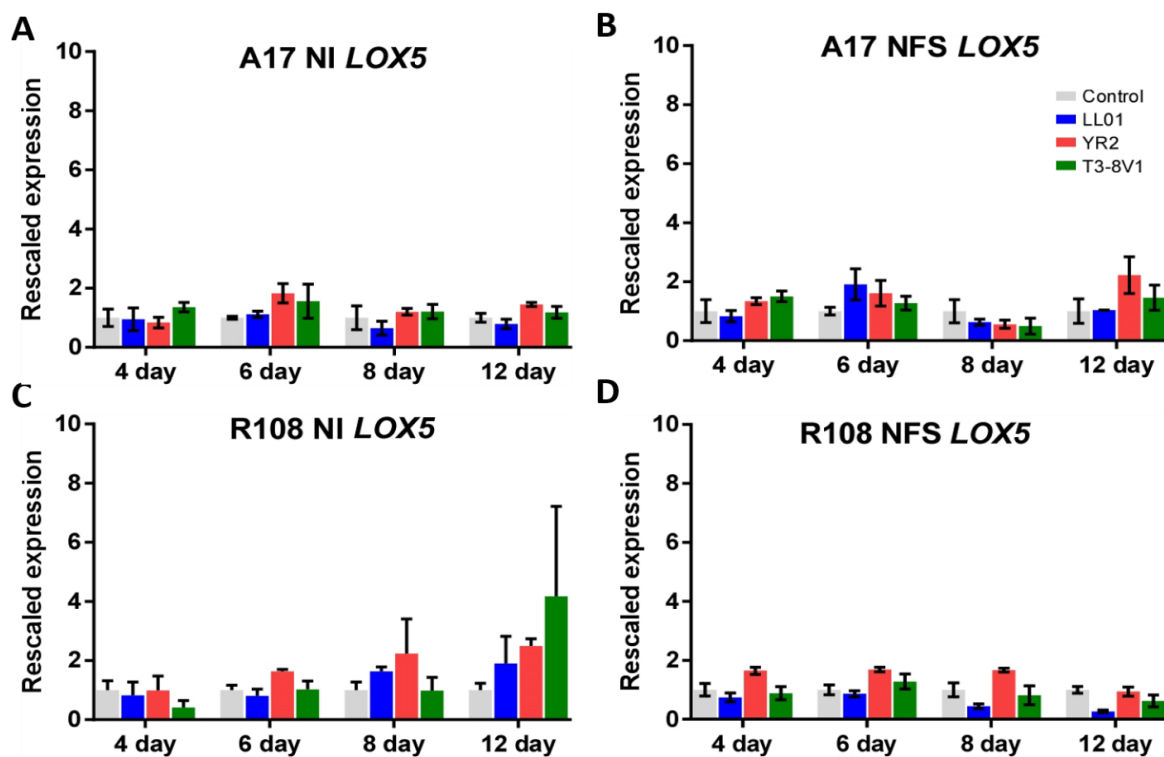


Figure 8: Expression levels of *Lox5* estimated by qPCR in shoots of NFS and NI plants of *M. truncatula* A17 and R108 plants after infestation by the different pea aphid lines. A,B) *M. truncatula* A17 on NI (A) NFS (B) and C,D) *M. truncatula* R108 NI (C) and NFS (D). Non-infested plants served as control. (Mean \pm SE, n=3); No statistical differences observed.

Allene Oxide Synthase1 AOS1. In A17 NI plants, T3-8V1 infestation had a significant effect on *AOS1* expression from day 4 to 8 dpi and YR2 infestation induced a significant increase at 6 and 8 dpi (Figure 9A). LL01 induced significant increase in *AOS1* level only at 8 dpi. In A17 NFS plants, only LL01 increased the expression at 6 and 12 dpi (Figure 9B). In R108 NI plants, YR2 infestation had no significant effect on *AOS1* expression. In contrast, T3-8V1 and LL01 infestation significantly induced *AOS1* expression at 12 dpi with a 30-fold and 10-fold increase, respectively, compared to control (Figure 9C). There was no significant differences after infestation by aphid lines in NFS R108 plants, meaning that no significant modification of *AOS1* expression occurred during the time course analysis in R108, except at 12 dpi for NI plants. See statistics in tables S22 and S23.

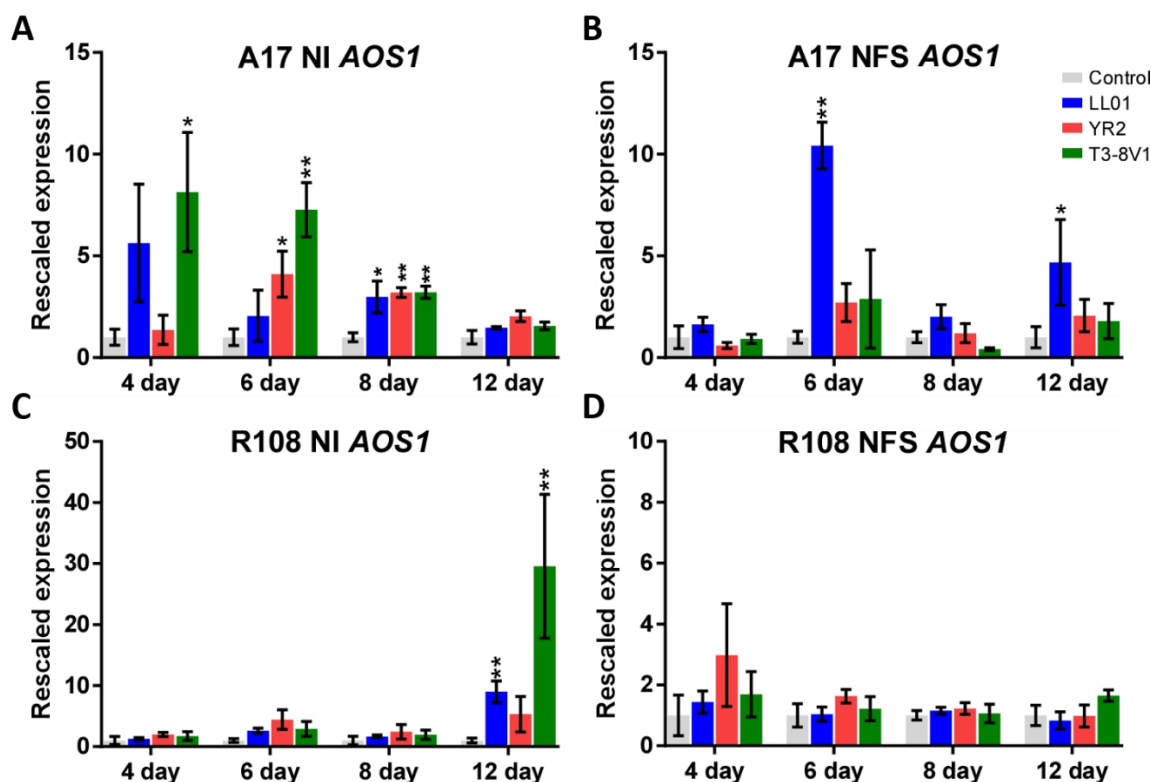


Figure 9: Expression levels of *AOS1* estimated by qPCR in shoots of NFS and NI *M. truncatula* A17 and R108 plants after infestation by the different aphid lines. A,B) *M. truncatula* A17 NI (A) NFS (B) and *M. truncatula* R108 NI (C) and NFS (D). Non-infested plants served as control. (Mean \pm SE, n=3); Statistical differences (t-test): $p > 0.05$; * $P \leq 0.005$; ** $P \leq 0.01$; * $p \leq 0.001$; ****, $P \leq 0.0001$.**

Taken together, these results showed that upon aphid infestation, *AOS1* expression is depends on plant genotype (i.e. A17 and R108 NI plants), aphid genotype (i.e. LL01 versus YR2 and T3-8V1 during infestation of A17 NFS plants) and nitrogen feeding plant conditions (R108 NI and

NFS plants).

Proteinase Inhibitors (PI). In A17 NI plants, the expression levels of *PI* were significantly increased 5-to-6-fold upon YR2 and T3-8V1 infestation. In contrast, LL01 infestation did not modified *PI* expression (Figure 10A). However, in A17 NFS plants, all aphid lines induced a significant 10-fold increase in *PI* expression at 12 dpi in addition to an increase at 4 dpi (YR2-amp) and 8 dpi (TR-8V1-amp) (Figure 10B Table S21). In aphid-infested in R108 NI plants, *PI* expression was not significantly modified (Figure 10C). However, on R108 NFS plants, YR2 aphid lines induced *PI* expression from 6 to 12 dpi and LL01 and T3-8V1 induced *PI* expression at 6 and 8 dpi (Figure 10D). See statistics in tables S23. These results showed that, as for *AOS1* expression, *PI* expression depends on the plant genotype (i.e. A17 NI versus R108 NI plants), on aphid line (i.e. YR2, T3-8V1 and LL01 on A17 NI plant) and on nitrogen feeding plant conditions (i.e. both R108 NI versus R108 NFS plants and A17 NI versus A17 NFS plants).

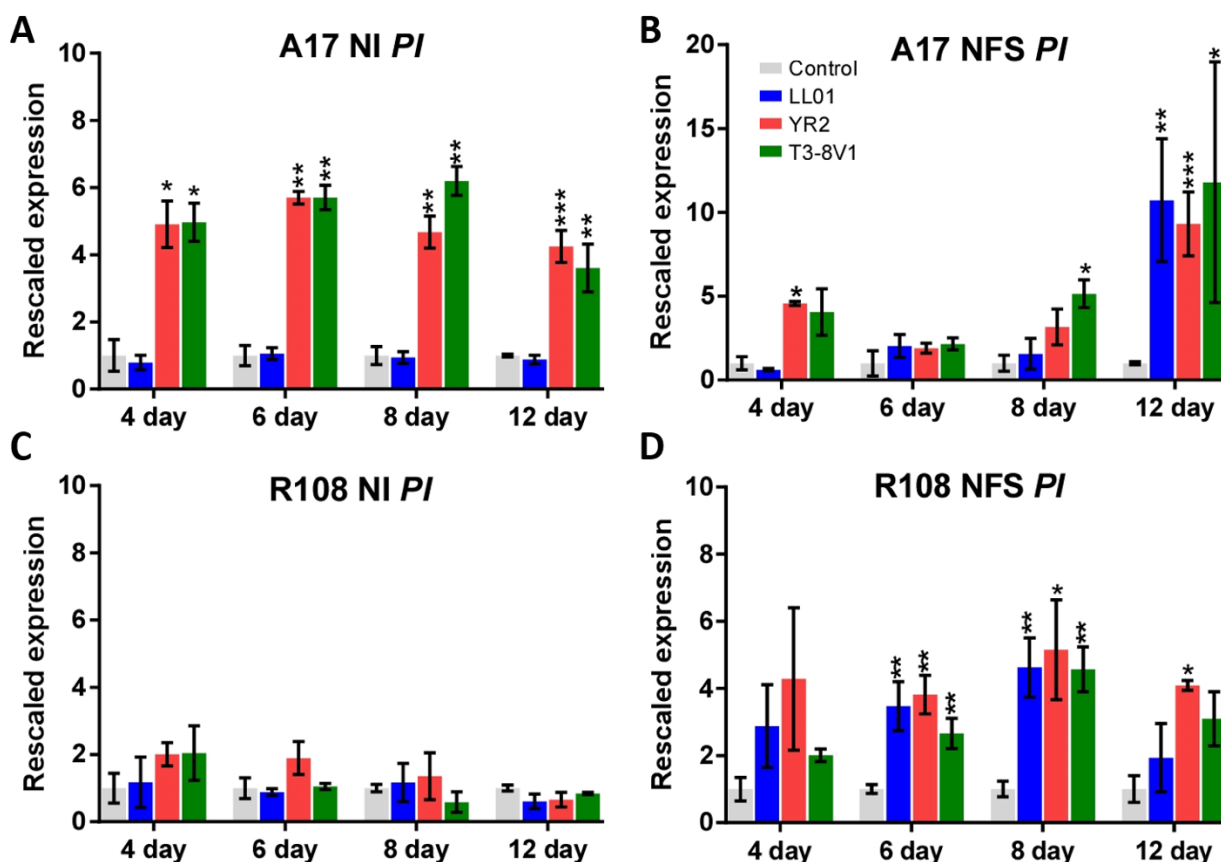


Figure 10: Expression levels of *PI* estimated by qPCR in shoots of NFS and NI plants of *M. truncatula* A17 and R108 plants. A,B) *M. truncatula* A17 on NI (A) NFS (B) and *M. truncatula* R108 NI (C) and NFS (D). Non-infested plants served as control. (Mean \pm SE, n=3); Statistical differences (t-test): p > 0.05; * P \leq 0.005; ** P \leq 0.01; * p \leq 0.001; ****, P \leq 0.0001.**

Discussion

The microorganisms associated with plant roots have important impacts on plant growth and physiology, including enhancing absorption or availability of nutrients, and affecting the production of compounds involved in defence against insect herbivores (Pineda et al., 2010). In a previous work we have shown that the presence of rhizobacterium on *M. truncatula* (*Mt*) modulates its interaction with the pea aphid *A. pisum* (*Ap*), and the induction of a JA defence related gene (Pandharikar et al., 2020). Conversely the presence of aphid on symbiotic plants affected the nodulation and thus the nitrogen assimilation process. The objective of the present work was to determine whether this effect was due to a specific aphid genotype x plant genotype combination. To ascertain and generalise these results, this study had to be extended to the interaction of host plant genotype, aphid genotype and the mode of nutrition of the plant (NI or NFS) i.e. the GxGxE interaction.

We used here two different *Mt* ecotypes in combination with three different *Ap* genotypes. Aphid species are known to feed on restricted range of host plant (Peccoud et al., 2009; 2015), and many aphid species exhibit host plant preferences among these groups. In the pea aphids, genetically distinguished host races or biotypes demonstrate strong preference for their host plant species from which they are collected (Ferrari et al., 2006; Zytynska et al., 2014). The three *Ap* lines LL01, YR2, T3-8V1 are long-established parthenogenetic clones that have different genotypes and are considered as potentially different biotypes (Simon et al., 2011; Boulain et al., 2018), LL01 being caught on alfalfa and the two other lines on clover (Simon et al., 2011). The lines used here are naturally or artificially free of facultative symbionts that could have interfere with the plant interaction (Kanvil et al., 2014; Pandharikar et al., 2020). For *Mt* we choose the two different ecotypes, Jemalong A17 and R108 that have been widely used in physiological and molecular studies. They differ in their phenotypes (Schnurr et al., 2007; Bolingue et al., 2010) and in their responses to abiotic and biotic stresses (Salzer et al., 2004; De Lorenzo et al., 2007; Gaige et al., 2012), and are able to form efficient symbiosis with *S. meliloti* although in a plant ecotype-specific manner at the molecular level (Salzer et al., 2004). Both were susceptible host for pea aphids (Gao et al., 2007; Kanvil et al., 2014). Using combinations of these aphids and plants genotypes we investigated how the plant nitrogen fixing symbiosis (NFS) affect the plant-aphid interaction, particularly the plant molecular defences pathways elicited by aphid predation using several SA and JA gene markers.

Aphid Fitness

Aphid fitness was estimated by their survival and their growth, and both the *Mt* and *Ap* genotypes have a significant effect on these aphid parameters. Under our conditions all aphids were able to survive and develop on the two *Mt* plants with both nutritional sources. LL01 aphids performed much better on A17 genotype than YR2 and T3-8V1 ones, and they reach a heavier weight in absence of NFS. Conversely, T3-8V1 aphids grew more rapidly on R108 than the two other aphid lines albeit their final weight was not extremely different, and NFS had almost no effect on the final weight. These results are in agreement with those of (Kanvil et al., 2014) indicating high virulence of LL01 aphid on different *M. truncatula* genotype, including A17 and R108, while survival of 6 other aphid lines was more affected on A17 than R108. In nature, incompatible plant-aphid interaction typically results in a lower growth of aphids and may lead to an early death (Giordanengo et al., 2010; Kanvil et al., 2014). Variation in aphid performance on different host species and between genotypes of the same species is related to specific ‘gene-for-gene’ recognition mechanisms associated with resistance (R) genes in plants and avirulence (Avr) genes in aphids (Kaloshian, 2004). Screening of *Mt* has identified ecotypes with resistance to aphid species (Crawford et al., 1989), and three independent resistance loci, TTR, AKR and APR, that confer resistance to spotted alfalfa aphid (*Therioaphis trifolii*), blue green aphid (*Acyrtosiphon kondoi*) and pea aphid, respectively (Gao et al., 2008). *Mt* A17 is reported to bear an aphid resistance locus (R gene) named the Aphid-induced lesions (AIL) locus responsible for a hypersensitive response (HR), that induced a rapid plant cell death (Klingler et al., 2009; Lei and Zhu-Salzman, 2015). An HR-like response may contribute to aphid resistance by decreasing the access to the sap; however, we did not observe significant aphid death response suggesting if HR occurs it had only an impact effect on the aphid growth.

In our experiment, the genotypic interactions (*Mt* x *Ap*) was the most important factor for the aphid growth, the plant symbiotic status introducing only some modulation in the growth rate but did not change the overall final results. Rhizobacteria presence is known to induce change in phloem-sap quality (Goundoudaki et al., 2004; Wurst et al., 2004) and can improve or decrease aphid fitness on plant-hosts. For instance, in a field study on soybean, presence of natural but not inoculated rhizobia or nitrogen fertilizer appears to decrease soybean aphids (*Aphis glycines*) density (Dean et al., 2009). However, barley supplementation with rhizobacteria was shown to

influence aphid fitness either positively (increased population size) or negatively (decreased population size) depending on plant genotype (Tétard-Jones et al., 2007, 2012; Zytynska et al., 2010). It should be noted that the most studies used adult aphids that are moved from one host plant (generally the universal host *Vicia fabae*) and followed their fitness (survival, reproduction) or their effect on the plant. Here we moved L1 nymph to the host plant to follow their development expecting that the transition from one host plant to another will be less drastic than moving adults. The different adult weight reached by the aphid lines in the different conditions may affect their reproductive output and thus the population dynamic, but this was not tested here.

Aphid Infestation Effect on Plants

In addition to monitoring aphid performance, different indicators of host responses to infestation were quantified: the plant growth, the nitrogen fixation for symbiotic plants, and SA and JA defence pathway genes expression.

Plant growth. While at the beginning of the experiment the shoots dry mass of NI and NFS plants was similar at the end of the 12 day of experiment, as expected, it was higher for the nitrate supplemented plants (Goh et al., 2013, 2016; Pandharikar et al., 2020). This lower biomass (or slower development) of NFS plants certainly results from the nitrogen metabolic lag with the NI plants, indeed these plants have immediately access to the nitrogen resource from the media while NFS plant need first to allow the rhizobium nodulation before to reach an optimum supply of nitrogen. The pea aphid infestation decreases the dry weight of both NI and NFS plant shoots in almost the same proportion, regardless of the aphid and plant genotypes, certainly through the withdrawal of a similar quantity of nutriment. This suggests that pea aphids seem to use NFS and NI *Mt* plants very similarly even if they may have a different sap quality. Infestation of soybean by the soybean aphid *Aphis glycines* also significantly reduced plant biomass, pod density, and total N concentration in aerial plant tissues of rhizobium inoculated and non-inoculated soybean plants (Brunner et al., 2015).

Nodules and nitrogen fixation. In agreement with our previous study, the number of root nodules per plant was decreased in aphid infested plants. While *Mt* A17 had a higher number of nodules per plant than R108, both ecotypes showed about 50% less nodules at the end of infestation. No important effect of the aphid genotype on this decrease was observed. This

nodules number reduction is translated in a decrease of nitrogen fixation. Thus, NFS plants face a double challenge, first aphid withdraw sap and nutrient and second they lose the potential to acquire nitrogen by losing or not forming nodules. Reduction in shoot weight can also lead to a reduction in carbon acquisition, (Stark and Kytöviita, 2005), which in turn can negatively affect symbiotic relationship with rhizobia as indicated by multiple studies on legumes (Sirur and Barlow, 1984). In pea and soybean, aphid infestation also impacted number, weight and nitrogen fixation activity of root nodules (Sirur and Barlow, 1984; Riedell et al., 2009). However, these decreases appeared to be dependent upon the aphid genotype and the time after infestation. Here we used only one strain of rhizobacterium (*S. meliloti* 2011) but different strains may result in different interaction with different plant genotypes with the benefits of symbiosis depending on the partners genotypes (Spoerke et al., 1996; Bronstein et al., 2006; Gubry-Rangin et al., 2010). It will be interesting in the future to test whether plant infected with different rhizobacterium strains react similarly to the presence of aphids.

Salicylic pathway. We observed a complex SA response on *M. truncatula* to each aphid lines which is plant and aphid genotypes-, plant symbiosis-, and time-dependent for the change in expression of the three PR genes tested. For example, LL01 had few effects on *PR1* expression in A17 NI and NFS, no effect on *PR4* on A17 NI but an increase at after 6 days in NFS plants and a strong transient effect on *PR5* in A17 NFS but not NI. A completely different time-response expression for these genes in the R108 ecotype was observed with the same LL01 line. In their study, Gao et al., 2007 reports that *PR5* and *PR10* were not induced on NI A17 by the bluegreen aphid, a similar situation observed here with LL01. However, the same bluegreen aphid induced a strong induction of PRs on the resistant jester ecotype two to three days after infestation, indicating also a plant genotype dependent response. On *M. sativa*, native and non-native aphid lines (even those that were barely able to survive or reproduce), triggered both a SA and JA-Ile response, with amplitude that were also aphid-genotype and time dependent (Sanchez-Arcos et al., 2016). SA pathway may be a general mechanism of antibiosis or aphid repellence but its induction is clearly dependent from the aphid-plant genotype. This pathway could be induced by the piercing and injection of aphid salivary effectors that contains many proteins. These salivary proteins may suppress plant-defence responses in native host plants (Mutti et al., 2008; Pitino and Hogenhout, 2013) or trigger defence reactions in non-host plants

(Gao et al., 2008; Hogenhout and Bos, 2011). Indeed, different pea aphid biotypes showed different saliva composition (Boulain et al., 2018), suggesting that the ability of host races to feed on their native host plants may lie in their ability to manipulate defence pathways either by avoiding recognition or suppressing them much more effectively on their native than on non-native host plants.

Jasmonic pathway. The JA pathway has been associated with defence against necrotrophic pathogens and chewing insects mechanical wounding which, unlike aphid feeding, involve maceration of plant tissue. Lipoxygenases (LOXs) are the initial enzymatic step of the jasmonate (JA) pathway. LOXs catalyse the formation of oxylipins that can further activate diverse pathways, including those associated with allene oxide synthase (AOS) (López et al., 2011; Eyres et al., 2016; Babenko et al., 2017). In previous studies the different MtLOX genes (*LOX1* to *LOX6*) were used to estimate the JA response induction against aphids and pathogens (Gao et al., 2008; Jayaraman et al., 2014; Song et al., 2016). All LOX genes except *LOX6* showed a strong Methyl-jasmonate induction in the susceptible A17 and the resistant jester *Mt* but these genes were upregulated only in resistant jester plants 12 to 24 h after bluegreen aphid infestation but not in A17 ones (Gao et al., 2007). Here we observed a similar absence of aphid induction for *LOX5* in the two susceptible *Mt* ecotypes used, that was not changed by the presence or absence of rhizobium (similar results were observed with *LOX1* and *LOX3*, not shown). In agreement, *AOS1* expression, although slightly induced, did not show important time changes whatever the conditions. Although *LOX5* and *AOS1* were not upregulated we observed a strong induction of *PI* by all aphid lines particularly at 12 days in A17 NFS plants. In A17 NI a basal increase was also induced in presence of YR2 and T3-8V1 and in R108 NFS plants with all aphid lines but not in R108 NI plants. We observed thus an aphid genotype-plant genotypes, plant nutritional condition and time dependent effect. Protease inhibitors block the insect gut protease activity essential for digesting proteins. As aphid feed exclusively on phloem sap poor in proteins, they may thus not be highly dependent on gut proteolytic digestion. However, increase in the level of proteinase inhibitors in pea plants or in artificial diet cause toxicity to aphids affecting their growth and reproduction (Rahbé et al., 2003b, 2003a). *PI* is downstream in the JA pathway, its induction without increase in other JA dependent genes suggests that activation of some defences can also occur also in a JA independent manner. Several lines of

evidence indicate that there is a crosstalk between response pathways, even antagonistic between SA and JA. Depending on the type of invader encountered, the plant appears to be capable of switching on the appropriate pathway or combination of pathways. It is possible also that plants will adjust these defences upon the time and the strength of the aggression.

Effect of rhizobium. From our results it is difficult to draw a clear picture of an effect of NFS on the SA and JA induced response by aphids. Multiple studies indicated that plant growth promoting bacteria such as *Sinorhizobium* promote a priming activation of the plant defence response named Induced Systemic Resistance (ISR) that resembles the pathogen induced defence response (Reviewed by Van Loon, 2007). ISR-primed plants induce faster and stronger expression of cellular defence responses upon pathogen or insect attack, resulting in an enhanced level of resistance to the aggressors (Pozo and Azcón-Aguilar, 2007; Van der Ent et al., 2009; Pineda et al., 2013; Sugio et al., 2015). Rhizobacteria trigger the salicylic acid (SA)-dependent pathway by producing SA at the root surface or trigger different signalling pathway dependent on jasmonic acid (JA) and ethylene. In our case the effect of the rhizobacteria was underlying this of aphids, and we remark that presence of NFS modulates the *PRs* and the *PI* responses, but this was also plant-aphid genotype dependent. *PR1* expression is strongly reduced in A17 by rhizobium presence after infestation with YR2 and T3-8V1 in agreement with our previous report (Pandharikar et al., 2020) but in contrast *PR4* response was increased. This is true also for *PI* that is more induced in A17 NFS plant at 12 days but not in R108.

Conclusion

In this work we have uncovered wide variation in performance of three different aphid lines across two *M. truncatula* host genotypes. Aphid fitness and plant-mediated defence responses were highly dependent on genotype of both aphid and host plants. In addition, we clearly confirmed that the rhizobacterial community is strongly impacted by aphid and this is independent from the plant and aphid genotype combination. From an agronomical point of view, this last result, if further confirm in different legume plant species, may have a strong impact on the use of these plants has a green fertilizer or for the expected yield of commercial rhizobacteria infected seeds.

Supplementary Figures

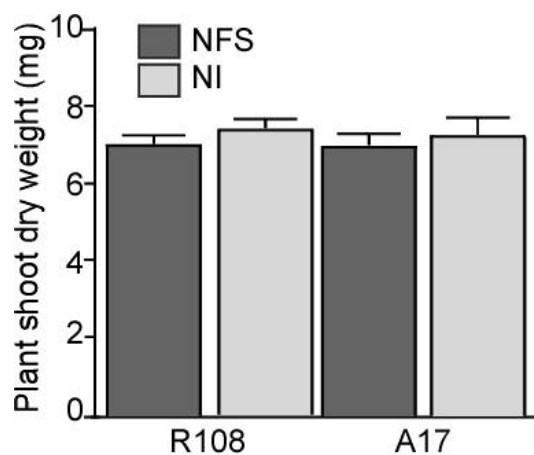


Figure S1. Comparison of 2-week-old A17 and R108 NFS and NI plants just before aphid infestation. (A) Dry weight of 2-week-old NFS and NI plants just before aphid infestation. No ststistic differences observed

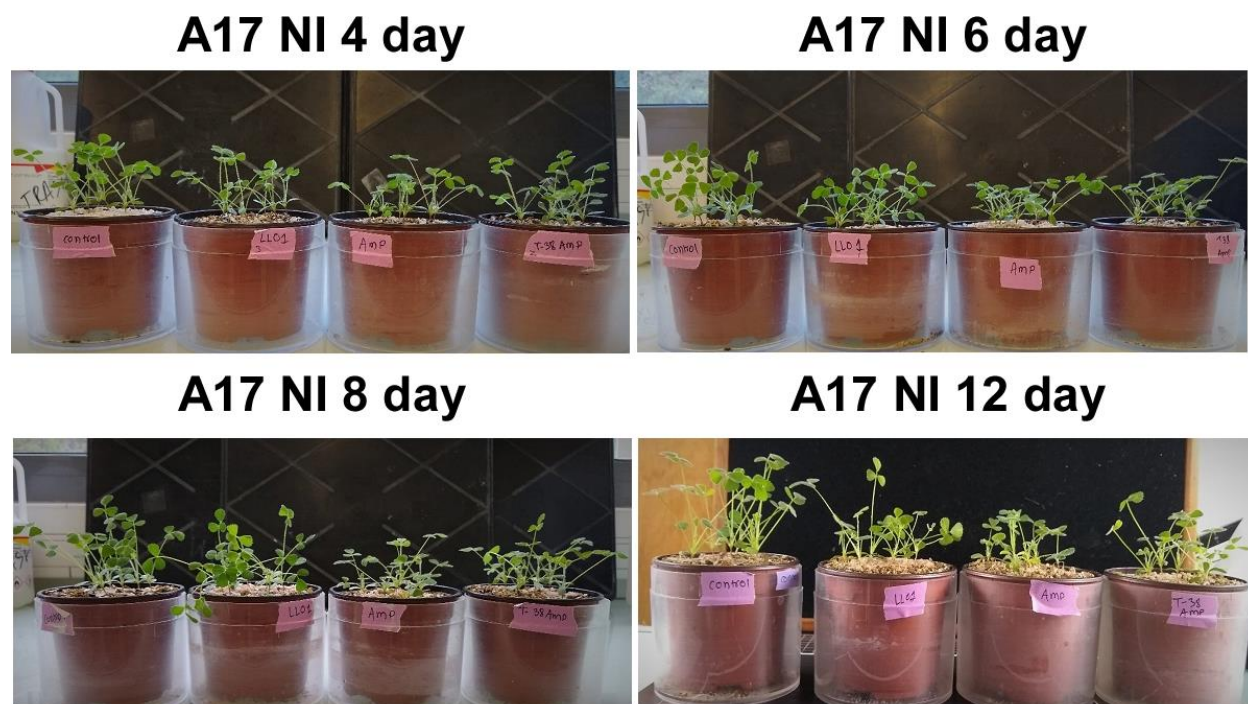


Figure S2: Photos of *M. truncatula* A17 NI plants aphid infested and control plants at different time points (A) 4 day (B) 6 day (C) 8 day (D) 12 day

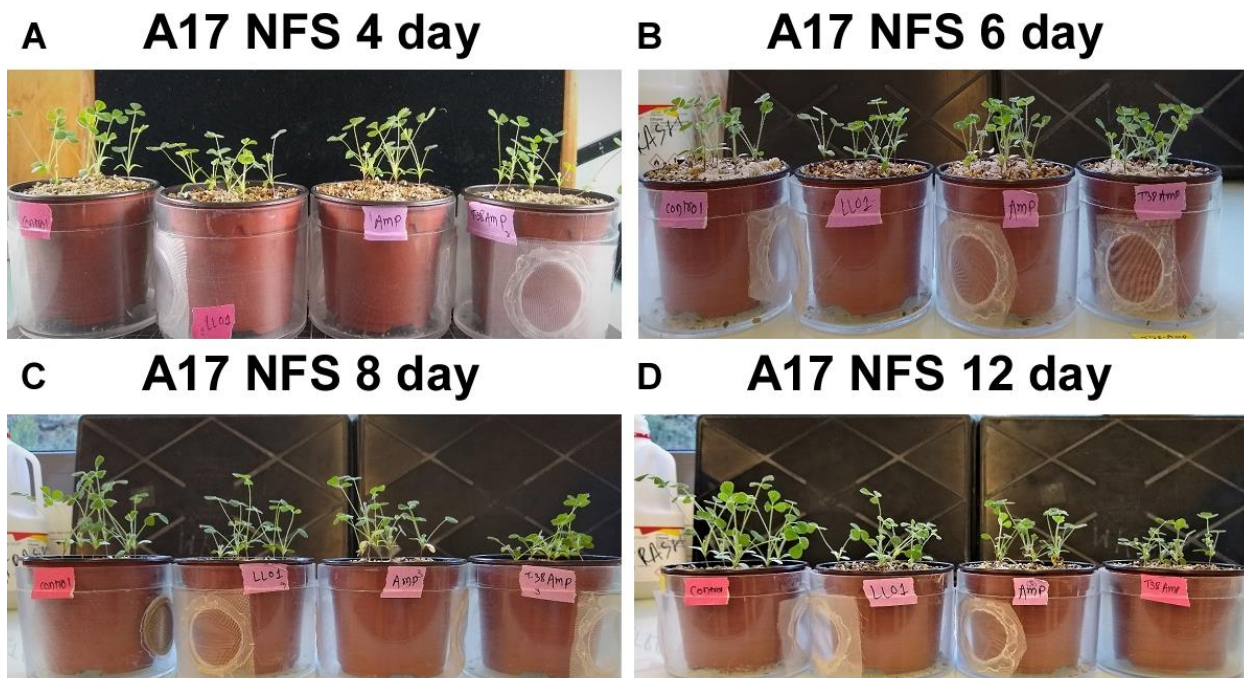


Figure S3: Photos of *M. truncatula* A17 NFS plants aphid infested and control plants at different time points (A) 4 day (B) 6 day (C) 8 day (D) 12 day

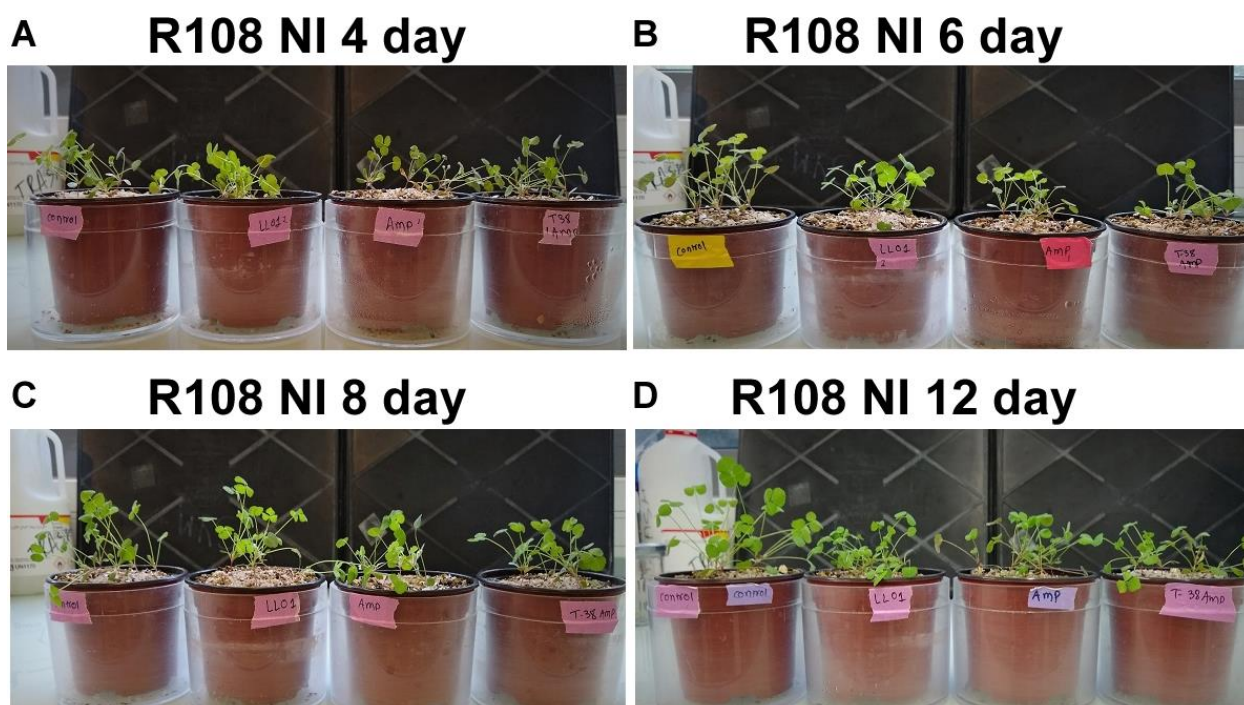


Figure S4: Photos of *M. truncatula* R108 NI plants aphid infested and control plants at different time points (A) 4 day (B) 6 day (C) 8 day (D) 12 day

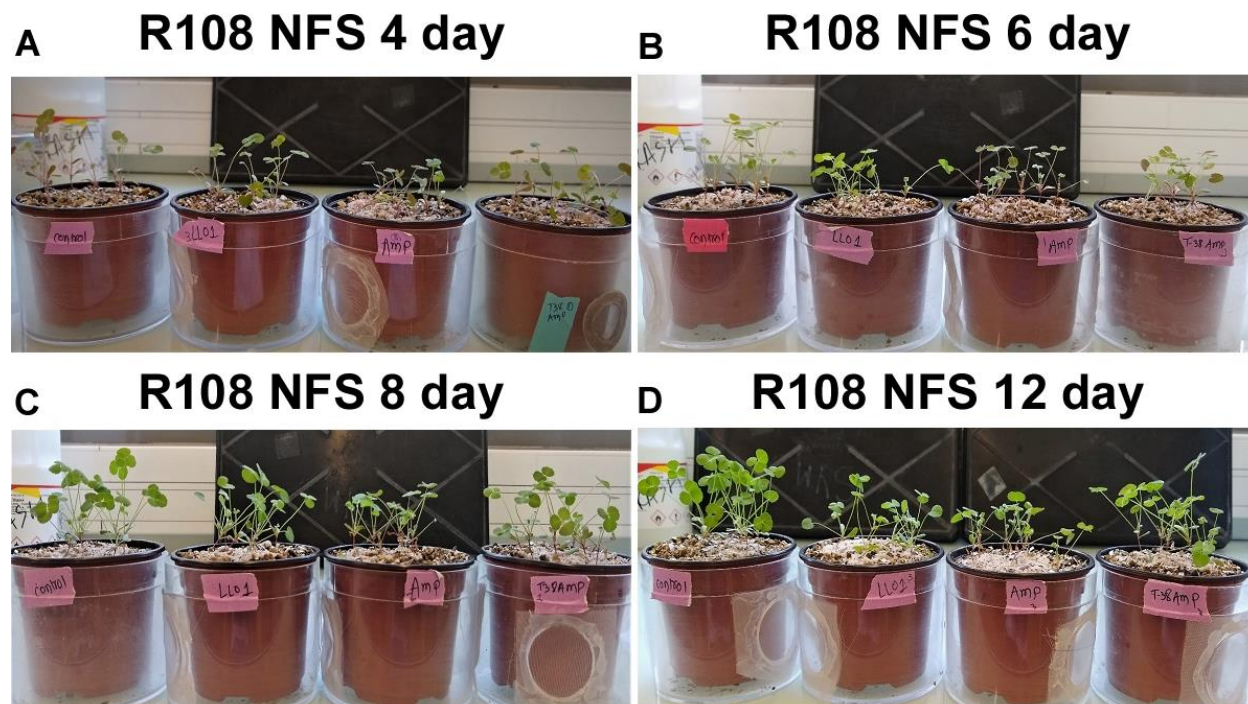


Figure S5: Photos of *M. truncatula* R108 NFS plants aphid infested and control plants at different time points (A) 4 day (B) 6 day (C) 8 day (D) 12 day

Supplementary Tables

Table 1 Origin of the aphid lines

| Line | Color | Plant of collection | Location | Collection date | Secondary symbiont | References |
|-------------|-------|---------------------|------------------|-----------------|--------------------|-----------------------------|
| LL01 | Green | Alfalfa | Lusignan, France | January 1987 | none | Jean-Christophe simon.,2011 |
| YR2-Amp* | Pink | Clover | York, UK | December 2002 | none | Jean-Christophe simon.,2011 |
| T3-8V1-Amp* | Green | Clover | Domagne, France | June 2003 | none | Jean-Christophe simon.,2011 |

* Artificial lines, treated with ampicillin in 2010 and secondary symbionts were removed

Table S2: Results of multi-comparisons test analysing **aphid weight** over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) on **A17 NI** plants; the Two-way ANOVA of whole experiment were, $F(2, 15) = 130.1$, $P < 0.0001$

| Multiple comparison | | P value |
|---------------------|------|---------|
| 1 day | | |
| LL01 vs. YR2 | ns | 0.9001 |
| LL01 vs. T3-8V1 | ns | 0.89 |
| YR2 vs. T3-8V1 | ns | 0.9997 |
| 4 day | | |
| LL01 vs. YR2 | ns | 0.1748 |
| LL01 vs. T3-8V1 | ns | 0.152 |
| YR2 vs. T3-8V1 | ns | 0.9971 |
| 6 day | | |
| LL01 vs. YR2 | ** | 0.0027 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| 8 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | ns | 0.8681 |
| 12 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | ** | 0.0024 |

Table S3: Results of multi-comparisons test analysing **aphid weight** over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) on **A17 NFS** plants; the Two-way ANOVA of whole experiment were, $F(2, 15) = 80.05$, $P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 1 day | | |
| LL01 vs. YR2 | ns | 0.8814 |
| LL01 vs. T3-8V1 | ns | 0.9963 |
| YR2 vs. T3-8V1 | ns | 0.8412 |
| | | |
| 4 day | | |
| LL01 vs. YR2 | ** | 0.0035 |
| LL01 vs. T3-8V1 | ns | 0.1738 |
| YR2 vs. T3-8V1 | ns | 0.2719 |
| | | |
| 6 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | ns | 0.8685 |
| | | |
| 8 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | * | 0.0202 |
| | | |
| 12 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | ns | 0.982 |

Table S4: Results of multi-comparisons test analysing **aphid weight** over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) on **R108 NI** plants; the Two-way ANOVA of whole experiment were, $F(2, 15) = 324.4$, $P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 1 day | | |
| LL01 vs. YR2 | ns | >0.9999 |
| LL01 vs. T3-8V1 | ns | >0.9999 |
| YR2 vs. T3-8V1 | ns | >0.9999 |
| | | |
| 4 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | *** | 0.0001 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| | | |
| 6 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| | | |
| 8 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| | | |
| 12 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | **** | <0.0001 |

Table S5: Results of multi-comparisons test analysing **aphid weight** over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) on **R108 NFS** plants; the Two-way ANOVA of whole experiment were, $F(2, 15) = 104.4$, $P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 1 day | | |
| LL01 vs. YR2 | ns | >0.9999 |
| LL01 vs. T3-8V1 | ns | >0.9999 |
| YR2 vs. T3-8V1 | ns | >0.9999 |
| 4 day | | |
| LL01 vs. YR2 | *** | 0.0007 |
| LL01 vs. T3-8V1 | ns | 0.2504 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| 6 day | | |
| LL01 vs. YR2 | **** | <0.0001 |
| LL01 vs. T3-8V1 | ns | 0.0654 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| 8 day | | |
| LL01 vs. YR2 | ns | 0.0807 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | **** | <0.0001 |
| 12 day | | |
| LL01 vs. YR2 | ns | 0.1878 |
| LL01 vs. T3-8V1 | * | 0.0241 |
| YR2 vs. T3-8V1 | **** | <0.0001 |

Table S6: Results of multi-comparisons test analysing **A17 NI** plant shoot dry weight over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) infested and non-infested control plant shoots; the Two way ANOVA of whole experiment were, $F(3, 68) = 111.6, P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | *** | 0.0002 |
| Control vs. T3-8V1 | ns | 0.0968 |
| LL01 vs. YR2 | ns | 0.6748 |
| LL01 vs. T3-8V1 | * | 0.0151 |
| YR2 vs. T3-8V1 | ns | 0.2342 |
| 8 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.8807 |
| LL01 vs. T3-8V1 | ns | 0.666 |
| YR2 vs. T3-8V1 | ns | 0.9785 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ** | 0.0016 |
| LL01 vs. T3-8V1 | ** | 0.0062 |
| YR2 vs. T3-8V1 | ns | 0.9806 |

Table S7: Results of multi-comparisons test analysing **A17 NFS** plant shoot dry weight over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) infested and non-infested control plant shoots; the Two way ANOVA of whole experiment were, $F(3, 68) = 164.5$, $P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | ns | 0.1802 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ** | 0.0079 |
| LL01 vs. T3-8V1 | ns | 0.9995 |
| YR2 vs. T3-8V1 | * | 0.0113 |
| 8 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ** | 0.0011 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | ns | 0.1092 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.8892 |
| LL01 vs. T3-8V1 | ns | 0.6274 |
| YR2 vs. T3-8V1 | ns | 0.9633 |

Table S8: Results of multi-comparisons test analysing **R108 NI** plant shoot dry weight over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) infested and non-infested control plant shoots; the Two way ANOVA of whole experiment were, $F(3, 68) = 99.86$, $P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | ** | 0.0026 |
| Control vs. YR2 | *** | 0.0004 |
| Control vs. T3-8V1 | * | 0.0272 |
| LL01 vs. YR2 | ns | 0.9625 |
| LL01 vs. T3-8V1 | ns | 0.8794 |
| YR2 vs. T3-8V1 | ns | 0.6099 |
| 8 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.3084 |
| LL01 vs. T3-8V1 | ns | 0.9695 |
| YR2 vs. T3-8V1 | ns | 0.5741 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.394 |
| LL01 vs. T3-8V1 | ns | 0.9824 |
| YR2 vs. T3-8V1 | ns | 0.6242 |

Table S9: Results of multi-comparisons test analysing **R108 NFS** plant shoot dry weight over different time points for different aphid lines (LL01, YR2 and T3-8V1 genotypes) infested and non-infested control plant shoots; the Two way ANOVA of whole experiment were, $F(3, 68) = 60.42$, $P < 0.0001$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | * | 0.0274 |
| Control vs. YR2 | ** | 0.0096 |
| Control vs. T3-8V1 | ** | 0.005 |
| LL01 vs. YR2 | ns | 0.985 |
| LL01 vs. T3-8V1 | ns | 0.9446 |
| YR2 vs. T3-8V1 | ns | 0.9969 |
| 8 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.9658 |
| LL01 vs. T3-8V1 | ns | 0.9832 |
| YR2 vs. T3-8V1 | ns | 0.8377 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.4696 |
| LL01 vs. T3-8V1 | **** | <0.0001 |
| YR2 vs. T3-8V1 | ** | 0.0021 |

Table S10: Results of multi-comparisons test analysing **nodule numbers** over different time points for different aphid infested lines (LL01, YR2 and T3-8V1 genotypes) and non-infested control **A17 NFS** plant nodules; the Two way ANOVA of whole experiment were, $F(3, 8) = 17.54$, $P = 0.0007$

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | * | 0.0201 |
| Control vs. YR2 | * | 0.0457 |
| Control vs. T3-8V1 | ns | 0.8125 |
| LL01 vs. YR2 | ns | 0.9823 |
| LL01 vs. T3-8V1 | ns | 0.1296 |
| YR2 vs. T3-8V1 | ns | 0.2481 |
| 8 day | | |
| Control vs. LL01 | *** | 0.0001 |
| Control vs. YR2 | *** | 0.0003 |
| Control vs. T3-8V1 | ** | 0.0021 |
| LL01 vs. YR2 | ns | 0.9819 |
| LL01 vs. T3-8V1 | ns | 0.6232 |
| YR2 vs. T3-8V1 | ns | 0.8334 |
| 12 day | | |
| Control vs. LL01 | * | 0.0159 |
| Control vs. YR2 | ** | 0.0013 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.7365 |
| LL01 vs. T3-8V1 | ns | 0.1824 |
| YR2 vs. T3-8V1 | ns | 0.709 |

Table S11: Results of multi-comparisons test analysing **Mean acetylene reduction activity expressed per milligram of nodule** (nmol ethylene x hr⁻¹ x mg nodule⁻¹) over different time points for different aphid infested lines (LL01, YR2 and T3-8V1 genotypes) and non-infested **A17 NFS** control plant nodules; the Two way ANOVA of whole experiment were, F (3, 8) = 12.23, P = 0.0023

| Multiple comparison | | P value |
|----------------------------|-----|----------------|
| 4 day | | |
| Control vs. LL01 | ns | 0.8357 |
| Control vs. YR2 | ns | 0.9423 |
| Control vs. T3-8V1 | ns | 0.9985 |
| LL01 vs. YR2 | ns | 0.9924 |
| LL01 vs. T3-8V1 | ns | 0.9048 |
| YR2 vs. T3-8V1 | ns | 0.9778 |
| 8 day | | |
| Control vs. LL01 | * | 0.0308 |
| Control vs. YR2 | ** | 0.0045 |
| Control vs. T3-8V1 | *** | 0.0007 |
| LL01 vs. YR2 | ns | 0.8427 |
| LL01 vs. T3-8V1 | ns | 0.4037 |
| YR2 vs. T3-8V1 | ns | 0.8694 |
| 12 day | | |
| Control vs. LL01 | *** | 0.0002 |
| Control vs. YR2 | ns | 0.0661 |
| Control vs. T3-8V1 | ** | 0.0068 |
| LL01 vs. YR2 | ns | 0.0944 |
| LL01 vs. T3-8V1 | ns | 0.486 |
| YR2 vs. T3-8V1 | ns | 0.7471 |

Table S12: Results of multi-comparisons test analysing **Mean acetylene reduction activity expressed per plant** (nmol ethylene x hr⁻¹x per plant⁻¹) over different time points for different aphid infested lines (LL01, YR2 and T3-8V1 genotypes) and non-infested **A17 NFS** control plant nodules; the Two way ANOVA of whole experiment were, F (3, 8) = 38.27, P < 0.0001

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | * | 0.0393 |
| Control vs. YR2 | ns | 0.0647 |
| Control vs. T3-8V1 | ns | 0.7181 |
| LL01 vs. YR2 | ns | 0.9954 |
| LL01 vs. T3-8V1 | ns | 0.2921 |
| YR2 vs. T3-8V1 | ns | 0.4088 |
| 8 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.9041 |
| LL01 vs. T3-8V1 | ns | 0.9349 |
| YR2 vs. T3-8V1 | ns | 0.9997 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.0756 |
| LL01 vs. T3-8V1 | ns | 0.7287 |
| YR2 vs. T3-8V1 | ns | 0.4408 |

Table S13: Results of multi-comparisons test analysing **nodule numbers** over different time points for different aphid infested lines (LL01, YR2 and T3-8V1 genotypes) and non-infested control **R108 NFS** plant nodules; the Two way ANOVA of whole experiment were, $F(3, 8) = 5.901$, $P = 0.0200$

| Multiple comparison | | P value |
|----------------------------|----|----------------|
| 4 day | | |
| Control vs. LL01 | ns | 0.6749 |
| Control vs. YR2 | ns | 0.9136 |
| Control vs. T3-8V1 | ns | 0.8093 |
| LL01 vs. YR2 | ns | 0.9633 |
| LL01 vs. T3-8V1 | ns | 0.9951 |
| YR2 vs. T3-8V1 | ns | 0.9951 |
| 8 day | | |
| Control vs. LL01 | ns | 0.0602 |
| Control vs. YR2 | ns | 0.4303 |
| Control vs. T3-8V1 | ns | 0.8671 |
| LL01 vs. YR2 | ns | 0.6749 |
| LL01 vs. T3-8V1 | ns | 0.2544 |
| YR2 vs. T3-8V1 | ns | 0.8671 |
| 12 day | | |
| Control vs. LL01 | * | 0.0358 |
| Control vs. YR2 | * | 0.0405 |
| Control vs. T3-8V1 | ns | 0.0678 |
| LL01 vs. YR2 | ns | >0.9999 |
| LL01 vs. T3-8V1 | ns | 0.9904 |
| YR2 vs. T3-8V1 | ns | 0.9949 |

Table S14: Results of multi-comparisons test analysing **Mean acetylene reduction activity expressed per milligram of nodule** (nmol ethylene x hr⁻¹ x mg nodule⁻¹) over different time points for different aphid infested lines (LL01, YR2 and T3-8V1 genotypes) and non-infested **R108 NFS** control plant nodules; the Two way ANOVA of whole experiment were, F (3, 8) = 17.88, P = 0.0007

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | ns | 0.9867 |
| Control vs. YR2 | ns | 0.3677 |
| Control vs. T3-8V1 | ns | 0.8159 |
| LL01 vs. YR2 | ns | 0.559 |
| LL01 vs. T3-8V1 | ns | 0.9475 |
| YR2 vs. T3-8V1 | ns | 0.8637 |
| 8 day | | |
| Control vs. LL01 | ns | 0.055 |
| Control vs. YR2 | * | 0.0156 |
| Control vs. T3-8V1 | * | 0.0243 |
| LL01 vs. YR2 | ns | 0.9412 |
| LL01 vs. T3-8V1 | ns | 0.9819 |
| YR2 vs. T3-8V1 | ns | 0.9974 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.9506 |
| LL01 vs. T3-8V1 | ns | 0.5242 |
| YR2 vs. T3-8V1 | ns | 0.8312 |

Table S15: Results of multi-comparisons test analysing **Mean acetylene reduction activity expressed per plant** (nmol ethylene x hr⁻¹x per plant⁻¹) over different time points for different aphid infested lines (LL01, YR2 and T3-8V1 genotypes) and non-infested **R108 NFS** control plant nodules; the Two way ANOVA of whole experiment were, F (3, 8) = 55.97, P < 0.0001

| Multiple comparison | | P value |
|----------------------------|------|----------------|
| 4 day | | |
| Control vs. LL01 | ns | 0.3286 |
| Control vs. YR2 | ns | 0.2054 |
| Control vs. T3-8V1 | ns | 0.3991 |
| LL01 vs. YR2 | ns | 0.9908 |
| LL01 vs. T3-8V1 | ns | 0.999 |
| YR2 vs. T3-8V1 | ns | 0.9723 |
| 8 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | * | 0.0467 |
| LL01 vs. YR2 | ns | >0.9999 |
| LL01 vs. T3-8V1 | * | 0.046 |
| YR2 vs. T3-8V1 | ns | 0.0527 |
| 12 day | | |
| Control vs. LL01 | **** | <0.0001 |
| Control vs. YR2 | **** | <0.0001 |
| Control vs. T3-8V1 | **** | <0.0001 |
| LL01 vs. YR2 | ns | 0.7806 |
| LL01 vs. T3-8V1 | ns | 0.1026 |
| YR2 vs. T3-8V1 | ns | 0.4751 |

Table S16: Analysis of gene expression data using the t-test on **A17 NI** plants for **PR1**, **PR4** and **PR5** genes at different time points.

| Sample | A17 NI-PR1 | A17 NI-PR4 | A17 NI-PR5 |
|----------------|-------------|-------------|-------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.132503792 | 0.579071927 | 0.355124399 |
| YR2 4 days | 0.016326307 | 0.032699554 | 0.000323067 |
| T38Amp 4 days | 0.005097046 | 0.036426125 | 0.00026894 |

| Sample | A17 NI-PR1 | A17 NI-PR4 | A17 NI-PR5 |
|----------------|-------------|-------------|-------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.03830958 | 0.733624078 | 0.328609297 |
| YR2 6 days | 0.000119969 | 0.219635948 | 0.502885825 |
| T3-8V1 6 days | 0.002794645 | 0.113879011 | 0.242457872 |

| Sample | A17 NI-PR1 | A17 NI-PR4 | A17 NI-PR5 |
|----------------|-------------|-------------|-------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.793347322 | 0.395898221 | 0.147221252 |
| YR2 8 days | 0.000753933 | 0.017620251 | 0.011229938 |
| T3-8V1 8 days | 0.000516234 | 0.006595561 | 0.000789455 |

| Sample | A17 NI-PR1 | A17 NI-PR4 | A17 NI-PR5 |
|-----------------|-------------|-------------|-------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.008185927 | 0.326455017 | 0.728941653 |
| YR2 12 days | 0.00328447 | 0.005472456 | 0.032888582 |
| T3-8V1 12 days | 0.004449117 | 0.030136816 | 0.060619536 |

Table S17: Analysis of gene expression data using the t-test on **A17 NFS** plants for **PR1**, **PR4** and **PR5** genes at different time points.

| Sample | A17 NFS-PR1 | A17 NFS-PR4 | A17 NFS-PR5 |
|----------------|-------------|-------------|-------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.080016507 | 0.440774623 | 0.007159304 |
| YR2 4 days | 0.000275455 | 0.048263448 | 0.0005306 |
| T38Amp 4 days | 0.002962449 | 0.006846375 | 0.000130413 |

| Sample | A17 NFS-PR1 | A17 NFS-PR4 | A17 NFS-PR5 |
|----------------|-------------|-------------|-------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.19922972 | 0.023445254 | 0.010354629 |
| YR2 6 days | 0.034573657 | 0.030216879 | 0.030618405 |
| T3-8V1 6 days | 0.07307697 | 0.000989445 | 0.004439103 |

| Sample | A17 NFS-PR1 | A17 NFS-PR4 | A17 NFS-PR5 |
|----------------|-------------|-------------|-------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.040462573 | 0.196270141 | 0.094787516 |
| YR2 8 days | 0.032455782 | 0.086619241 | 0.035318908 |
| T3-8V1 8 days | 0.007393772 | 0.004745764 | 0.004102575 |

| Sample | A17 NFS-PR1 | A17 NFS-PR4 | A17 NFS-PR5 |
|-----------------|-------------|-------------|-------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.056830999 | 0.011504215 | 0.081438223 |
| YR2 12 days | 0.037945276 | 0.000886019 | 0.034592489 |
| T3-8V1 12 days | 0.079203064 | 0.028618601 | 0.267051053 |

Table S18: Analysis of gene expression data using the t-test on **R108 NI** plants for **PR1**, **PR4** and **PR5** genes at different time points.

| Sample | R108 <i>NI-PR1</i> | R108 <i>NI-PR4</i> | R108 <i>NI-PR5</i> |
|----------------|--------------------|--------------------|--------------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.007287006 | 0.489170129 | 0.164876244 |
| YR2 4 days | 0.002036829 | 0.032055192 | 0.000487391 |
| T38Amp 4 days | 2.70E-05 | 0.816155436 | 0.160170344 |

| Sample | R108 <i>NI-PR1</i> | R108 <i>NI-PR4</i> | R108 <i>NI-PR5</i> |
|----------------|--------------------|--------------------|--------------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 2.84E-05 | 0.529994667 | 0.826911723 |
| YR2 6 days | 0.002084924 | 0.12016152 | 0.283258322 |
| T3-8V1 6 days | 0.001614322 | 0.331940263 | 0.190034079 |

| Sample | R108 <i>NI-PR1</i> | R108 <i>NI-PR4</i> | R108 <i>NI-PR5</i> |
|----------------|--------------------|--------------------|--------------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.004413883 | 0.056949692 | 0.107841433 |
| YR2 8 days | 6.49E-05 | 0.048739166 | 0.037026162 |
| T3-8V1 8 days | 0.000223887 | 0.359335738 | 0.655401066 |

| Sample | R108 <i>NI-PR1</i> | R108 <i>NI-PR4</i> | R108 <i>NI-PR5</i> |
|-----------------|--------------------|--------------------|--------------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.00046349 | 0.086365061 | 0.027689784 |
| YR2 12 days | 0.000790602 | 0.062447205 | 0.097060148 |
| T3-8V1 12 days | 0.005529184 | 0.052312594 | 0.080021919 |

Table S19: Analysis of gene expression data using the t-test on **R108 NFS** plants for **PR1**, **PR4** and **PR5** genes at different time points.

| Sample | R108 NFS-PR1 | R108 NFS-PR4 | R108 NFS-PR5 |
|----------------|--------------|--------------|--------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.023526394 | 0.159041096 | 0.118733996 |
| YR2 4 days | 0.063898617 | 0.004920742 | 0.0016633 |
| T38Amp 4 days | 0.263381095 | 0.371589148 | 0.514089208 |

| Sample | R108 NFS-PR1 | R108 NFS-PR4 | R108 NFS-PR5 |
|----------------|--------------|--------------|--------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.010570458 | 0.319731471 | 0.002238602 |
| YR2 6 days | 0.000218884 | 0.128391144 | 0.007459459 |
| T3-8V1 6 days | 0.00318511 | 0.291160757 | 0.005802738 |

| Sample | R108 NFS-PR1 | R108 NFS-PR4 | R108 NFS-PR5 |
|----------------|--------------|--------------|--------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.002043783 | 0.04388512 | 0.00503282 |
| YR2 8 days | 0.000221654 | 0.014877406 | 0.002896797 |
| T3-8V1 8 days | 0.002447157 | 0.368392777 | 0.059419035 |

| Sample | R108 NFS-PR1 | R108 NFS-PR4 | R108 NFS-PR5 |
|-----------------|--------------|--------------|--------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.00146274 | 0.002482519 | 0.001455198 |
| YR2 12 days | 9.18E-06 | 0.000347605 | 0.000180683 |
| T3-8V1 12 days | 0.000492169 | 0.003461447 | 0.002834293 |

Table S20: Analysis of gene expression data using the t-test on **A17 NI** plants for **PI**, **LOX5** and **AOS1** genes at different time points.

| Sample | A17 NI-PI | A17 NI-LOX5 | A17 NI-AOS1 |
|----------------|-----------|-------------|-------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.857362 | 0.818479294 | 0.055192988 |
| YR2 4 days | 0.019817 | 0.75941151 | 0.926184555 |
| T38Amp 4 days | 0.017901 | 0.311038031 | 0.012749707 |

| Sample | A17 NI-PI | A17 NI-LOX5 | A17 NI-AOS1 |
|----------------|-----------|-------------|-------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.724592 | 0.409146447 | 0.932333641 |
| YR2 6 days | 0.008484 | 0.050048432 | 0.045949063 |
| T3-8V1 6 days | 0.008799 | 0.632814934 | 0.007132655 |

| Sample | A17 NI-PI | A17 NI-LOX5 | A17 NI-AOS1 |
|----------------|-----------|-------------|-------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.982157 | 0.515809286 | 0.038707254 |
| YR2 8 days | 0.0078 | 0.46787629 | 0.00581988 |
| T3-8V1 8 days | 0.003892 | 0.552076368 | 0.006635965 |

| Sample | A17 NI-PI | A17 NI-LOX5 | A17 NI-AOS1 |
|-----------------|-----------|-------------|-------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.419237 | 0.354997405 | 0.191365196 |
| YR2 12 days | 0.000305 | 0.059050728 | 0.072653478 |
| T3-8V1 12 days | 0.002779 | 0.515058822 | 0.177120056 |

Table S21: Analysis of gene expression data using the t-test on **A17 NFS** plants for *PI*, *LOX5* and *AOS1* genes at different time points.

| Sample | A17 NFS-PI | A17 NFS-LOX5 | A17 NFS-AOS1 |
|----------------|-------------|--------------|--------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.538071378 | 0.827180227 | 0.259074669 |
| YR2 4 days | 0.019332209 | 0.317613662 | 0.632267308 |
| T38Amp 4 days | 0.061169216 | 0.229816147 | 0.835199522 |

| Sample | A17 NFS-PI | A17 NFS-LOX5 | A17 NFS-AOS1 |
|----------------|-------------|--------------|--------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.222701244 | 0.200015901 | 0.002301391 |
| YR2 6 days | 0.198874046 | 0.297170717 | 0.198080427 |
| T3-8V1 6 days | 0.167258166 | 0.392922548 | 0.787069022 |

| Sample | A17 NFS-PI | A17 NFS-LOX5 | A17 NFS-AOS1 |
|----------------|-------------|--------------|--------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.64801985 | 0.438955518 | 0.170197467 |
| YR2 8 days | 0.136505303 | 0.320217247 | 0.809106266 |
| T3-8V1 8 days | 0.027485004 | 0.29111585 | 0.091982731 |

| Sample | A17 NFS-PI | A17 NFS-LOX5 | A17 NFS-AOS1 |
|-----------------|-------------|--------------|--------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.009437261 | 0.751229972 | 0.036826274 |
| YR2 12 days | 0.000781226 | 0.108835454 | 0.185157197 |
| T3-8V1 12 days | 0.028379572 | 0.377323748 | 0.469824207 |

Table S22: Analysis of gene expression data using the t-test on **R108 NI plants** for *PI*, *LOX5* and *AOS1* genes at different time points.

| Sample | R108 <i>NI-PI</i> | R108 <i>NI-LOX5</i> | R108 <i>NI-AOS1</i> |
|----------------|-------------------|---------------------|---------------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.911078197 | 0.458853206 | 0.41011869 |
| YR2 4 days | 0.140727722 | 0.828223493 | 0.176949255 |
| T38Amp 4 days | 0.264506549 | 0.228553561 | 0.47391528 |

| Sample | R108 <i>NI-PI</i> | R108 <i>NI-LOX5</i> | R108 <i>NI-AOS1</i> |
|----------------|-------------------|---------------------|---------------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.999488922 | 0.476944716 | 0.076207309 |
| YR2 6 days | 0.203753828 | 0.039904502 | 0.058559104 |
| T3-8V1 6 days | 0.692729313 | 0.921334436 | 0.18761009 |

| Sample | R108 <i>NI-PI</i> | R108 <i>NI-LOX5</i> | R108 <i>NI-AOS1</i> |
|----------------|-------------------|---------------------|---------------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.79528312 | 0.156451764 | 0.231498139 |
| YR2 8 days | 0.974046945 | 0.727900166 | 0.469987393 |
| T3-8V1 8 days | 0.239087052 | 0.689084988 | 0.327238107 |

| Sample | R108 <i>NI-PI</i> | R108 <i>NI-LOX5</i> | R108 <i>NI-AOS1</i> |
|-----------------|-------------------|---------------------|---------------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.157008474 | 0.9715774 | 0.007268605 |
| YR2 12 days | 0.182709867 | 0.029182958 | 0.090251111 |
| T3-8V1 12 days | 0.156940046 | 0.583295987 | 0.005787938 |

Table S23: Analysis of gene expression data using the t-test on **R108 NFS** plants for *PI*, *LOX5* and *AOS1* genes at different time points.

| Sample | R108 NFS- <i>PI</i> | R108 NFS- <i>LOX5</i> | R108 NFS- <i>AOS1</i> |
|----------------|---------------------|-----------------------|-----------------------|
| Control 4 days | 1 | 1 | 1 |
| LL01 4 days | 0.337286652 | 0.435938646 | 0.338702562 |
| YR2 4 days | 0.109265449 | 0.100274265 | 0.276562649 |
| T38Amp 4 days | 0.111853147 | 0.727445994 | 0.410804208 |

| Sample | R108 NFS- <i>PI</i> | R108 NFS- <i>LOX5</i> | R108 NFS- <i>AOS1</i> |
|----------------|---------------------|-----------------------|-----------------------|
| Control 6 days | 1 | 1 | 1 |
| LL01 6 days | 0.006190011 | 0.576450826 | 0.776181824 |
| YR2 6 days | 0.002877779 | 0.040111344 | 0.210897962 |
| T3-8V1 6 days | 0.009397873 | 0.4566853 | 0.754456763 |

| Sample | R108 NFS- <i>PI</i> | R108 NFS- <i>LOX5</i> | R108 NFS- <i>AOS1</i> |
|----------------|---------------------|-----------------------|-----------------------|
| Control 8 days | 1 | 1 | 1 |
| LL01 8 days | 0.008780027 | 0.047048361 | 0.437909696 |
| YR2 8 days | 0.022418715 | 0.066355715 | 0.42986007 |
| T3-8V1 8 days | 0.004949651 | 0.542158321 | 0.948215226 |

| Sample | R108 NFS- <i>PI</i> | R108 NFS- <i>LOX5</i> | R108 NFS- <i>AOS1</i> |
|-----------------|---------------------|-----------------------|-----------------------|
| Control 12 days | 1 | 1 | 1 |
| LL01 12 days | 0.437721765 | 0.004808609 | 0.845002464 |
| YR2 12 days | 0.013944304 | 0.724234534 | 0.960198358 |
| T3-8V1 12 days | 0.057444436 | 0.188668607 | 0.238965973 |

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GENERAL DISCUSSION AND CONCLUSION

5. GENERAL DISCUSSION AND CONCLUSION

Since the last decades, more and more studies have demonstrated that interactions between aboveground and belowground subsystems play a crucial role in regulating community structure and ecosystem functioning (for reviews, De Deyn and Van Der Putten, 2005; Rasmann and Turlings, 2008; Hol et al., 2010). It is now clear that microbes associated with plants and insects can profoundly influence plant-insect interactions. Recent studies have also demonstrated that co-evolving a microbe with host-protective properties within hosts drives the evolution of reduced aggressor virulence as a by-product of adaptation to the defensive microbe (Ford et al., 2016; King and Bonsall, 2017; Nelson and May, 2017). Defensive microbes thus might play a central role in host-aggressor interactions, by replacing or boosting host-based defences, engaging in within-host competition with aggressor or providing the host with protective or toxic molecules, and ultimately driving this tripartite co-evolutionary dynamics (Moran et al., 2008; Bruce, 2015; Vorburger and Perlman, 2018). Making practical use of such above-belowground interactions offers important opportunities for enhancing the sustainability of agriculture, as it could favour crop growth, nutrient supply, and defence against biotic and abiotic stresses.

Ecologically important symbiotic associations are mainly studied separately, and some systems have been well explored, such as the plant-rhizobacteria and the aphid-endosymbiont associations. However, such two-way interaction studies cannot always predict the response of the different organisms in the community. Indeed, many laboratories now developed tritrophic (Shikano et al., 2017; Turlings and Erb, 2018) and even tetratrophic models (Zytynska et al., 2010; Poelman et al., 2012; Nicholls et al., 2017) to explore how indirect ecological effects influence species dynamics and abundance.

My doctoral work has explored in this framework of aboveground-belowground interaction the potential bottom-up and top-down effects mediated by the host plant between the plant, the aphid and its symbionts. I used two well-known models, *A. pisum* and *M. truncatula*, whose genomes and different characterized genotypes are available (IAGC, 2010; Young et al., 2011; Pecrix et al., 2018) as well as those of the different facultative symbionts of pea aphid (Moran et al., 2008) and of the nitrogen-fixing bacteria *Sinorhizobium meliloti* (Galibert et al., 2001). This work also certainly includes one of the first explorations of the (G x G x E) interaction between the plant

genotype, the aphid genotype and the nitrogen-fixing symbiont as an environmental variable. In this discussion section, I will focus on the most obvious and general results of my thesis, in particular the effect of the nitrogen-fixing symbiont on the plant defence responses induced by aphids and the negative effect of the aphid presence on the plant nodulation.

6.1 Effect of Plant on Aphid's fitness

While under our experimental conditions, the aphid survival was not affected by the nutritional status of the plants, I observed a clear effect of the nitrogen source on the aphid growth, with a higher growth rate and final weight on nitrate-supplemented plants. The immediate availability of nitrates in NI plants certainly allows better plant growth and indirectly aphids, indicating a significantly better quality of the sap. Conversely, the installation of rhizobacteria can be costly for the plant (at least during its establishment) and their number / activity may not be sufficient during the experimental period to provide sufficient nitrogen to allow both plant and aphid growth. *M. truncatula* has a generation time of approximately 10 weeks from seed to seed in growth chambers. In the *M. truncatula/S. meliloti* symbiosis, the plant simultaneously undergoes a series of developmental changes, creating root nodules and allowing bacterial entry and differentiation. While the plant response to bacterial nod factors (NFs) occurs within minutes and genes activation follows within hours and days (Pucciariello et al., 2009; Maunoury et al., 2010; Moreau et al., 2011 ; Boscari et al., 2013; Lang and Long, 2015; Jardinaud et al., 2016), the first root cells divisions occur between 16 and 18 hours after inoculation and the first nodules form after 4-5 days (Pucciariello et al., 2009; Sugawara and Sadowsky, 2014). Their number increases with the growth of the plant to reach a plateau after several weeks, certainly due to a dynamic between creation and senescence of nodules.

We initially hypothesized that the presence of aphid facultative symbionts would either be costly or beneficial for aphids as they participate in the use of plants. The secondary symbionts may play a role in the adaptation of their aphid host to different plant species since they are non-randomly distributed between the biotypes associated with specific host plants (Tsuchida et al., 2002; Leonardo and Muiru, 2003; Simon et al., 2003; Ferrari, 2004). For example, the pea aphid hosting *R. insecticola* improves its performance on clover (*Trifolium*) (Tsuchida et al., 2004) and

H. defensa on alfalfa plants (*Medicago*) (McLean et al., 2011). Douglas et al. (2006a) studied the performance of eight pea aphid clones with different symbionts (including *H. defensa*, *R. insecticola* and *S. symbiotica*) on chemically defined diets with a modified sucrose and amino acid content and they concluded that the impact of secondary symbionts on aphid traits is very unlikely to have a purely nutritional basis. The variation in growth was observed only in the presence of certain secondary symbionts in NI plants and may be due to the cost of the multiplication of these symbionts during the development of aphids (Doremus and Oliver, 2017) in competition with that of *Buchnera* (Heddi et al., 2005; Simonet et al., 2016) and aphids for nutrients. *Buchnera* density was positively correlated with aphid dietary nitrogen levels, whereas that of the facultative symbiont *Serratia symbiotica* increased in aphids reared on a low-nitrogen diet, indicating possible regulatory mechanisms or nutritional needs distinct between obligatory and facultative symbionts in the same insect host (Wilkinson et al., 2007). Deciphering the effect of plant symbiosis on the multiplication and function of the different symbionts through metabolomics studies could be an interesting future development of this work.

The chemical composition of plants (phytometabolome) is dynamic and modified by environmental factors. Plant-friendly microbes influence their nutritional and phytochemical composition, which can positively or negatively modulate the insect fitness. Schweiger et al. (2014) performed a multi-species metabolomics studies on five plant species of different phylogenetic relatedness, including *M. truncatula*. The study revealed that, the leaves of the five plant species share a large part of their metabolome, a high degree of species-specificity in response to the same general arbuscular (AM) mycorrhizal fungus *Rhizophagus irregularis*. Phosphorus increased significantly in all species, but carbon and nitrogen were less affected. A considerable fraction of the leaf metabolomes has been modulated by AM colonization of the roots, covering different parts of the network of the plant metabolic pathways in a highly species-specific mode, except for the reduction in organic acids of the citric acid cycle that was a common response to all dicots. *M. truncatula* was the most sensitive to AM with a modulation of 14.7% of its metabolic features. Fungal mycorrhization of the pea (*Pisum sativum*) resulted in a delayed development, associated with higher seed biomass accumulation and a prolonged period of vegetation (Shtark et al., 2019). Metabolomics studies on roots and leaves of *Arabidopsis* treated with root-colonizing *Pseudomonas fluorescens* (van de Mortel et al., 2012), showed also

a change in metabolites and the glucosinolates, involved in plant defense against microbial pathogens (Clay et al., 2009) and generalist herbivores (i.e. leaf chewers and phloem feeders; Müller et al., 2014; Bakhtiari & Rasmann, 2020), were among the most differentially accumulating metabolites. In *Medicago sativa* metabolic profiling revealed that plants with Rhizobia accumulated more antioxidants (SOD, POD, GSH), osmolytes (sugar, glycols, proline), organic acids (succinic acid, fumaric acid, and alpha-ketoglutaric acid), and metabolites that are involved in nitrogen fixation (Song et al., 2017).

The examples above have shown the absence of apparent rules for determining *a priori* the change induced by the rhizobium symbiosis on the plant species and the effect of the elements of the sap on the interaction with the insects and on their fitness. It is of note that most of the studies were performed on mature plants which have already established a strong interaction with their symbionts. Interestingly, the change in sap composition induced by rhizobacteria affects the aphid diet, the honeydew collected from aphid colonies feeding on nodulating plants containing more total sugars but the same amino acids-N content as that from colonies feeding on non-nodulating plants (Whitaker et al., 2014). It is also well demonstrated that the performance of aphid clones/lines is related to the host plant species from which they were collected originally. Plant species generally differ distinctly in their sap composition (Sanchez-Arcos et al., 2019) but most of the present amino acids are in similar compositions in different plant species. Febvay et al., (1988) and Sandström and Pettersson (1994) analysed sugars and amino acids in the phloem sap of four *Medicago sativa* clones with different resistance to pea aphid. They found a negative correlation between the reproduction rate of the aphid and the sugar / amino acid ratio; however, the amino acid balance contributed to but did not explain the resistance of some cultivars. Thus, the total concentration of amino acids in phloem sap cannot probably fully explain the differences in the performance of pea aphid clones and lines. However, a recent study suggests that the amount of two amino acids, L-phenylalanine and L-tyrosine, in leaf cells explains a large proportion of the variation in differential acceptability of plants by *A. pisum* clones but that metabolic-linked compounds may also directly influence the insect behaviour (Hopkins et al., 2017).

M. truncatula is genetically resistant to different aphid species including the blue green aphid (BGA; *A. kondoi*), pea aphid and spotted alfalfa aphid (SAA; *Therioaphis trifolii*) (Klingler et

al., 2007; Guo et al., 2009). The resistance of the cultivar Jester to these three aphid species has been characterized in detail and involves antibiotic and antixenosis; in all three cases it is based on the phloem. Separate genes were identified for resistance to SAA (TTR) and BGA (AKR), both mapped on chromosome 3 but acting independently to reduce survival and growth of their target aphid species (Klingler et al., 2007). The position of the AP resistance gene, APR (resistance to *A. pisum*) on the genetic map shows that it is close to the AKR locus. It is therefore not clear whether there is one gene for AP resistance or several genes forming a R-genes cluster (Kanvil et al., 2014b; Kamphuis et al., 2016). Kanvil et al. (2014b) tested eight clonal genotypes of pea aphid collected from various legume hosts, on a species-wide panel of genotypes of *M. truncatula*. The interaction outcomes were highly dependent on specific combinations of aphid and host genotypes. Closely related pairs of Australian genotypes of *M. truncatula* differing in their resistance to aphids revealed no increased resistance to European pea aphid clones, and a single plant line was fully resistant to all clones. In the MtA17 ecotype, two independent loci, RAP1 and RAP2, are involved in the resistance to specific clones of pea aphids such as PS01, but have no effect against LL01 (Stewart et al., 2009; Kanvil et al., 2014a). We also observed that the LL01 clone adapted to alfalfa performed better on MtA17 than the two other aphid clones, but this difference in fitness was not retrieved on the MtR108 genotype, suggesting that none of the plant ecotypes was clearly resistant to the aphid clone used or that an aphid clone was avirulent.

In the phloem, many secondary plant metabolites are also recognized as aphid resistance factors such as phenolic compounds, hydroxamic acids, indole alkaloids, glucosinolates and cyanogenic glycosides. Among them, the saponin glycosides and their derivatives were found in *M. truncatula*, both in aerial and root tissues, and significant differences in the saponin content were observed among ecotypes (Lei et al., 2019). The differential accumulation of saponins in aerial and root tissues suggests that they play different roles in the plant fitness. In alfalfa, the number of infesting aphids was found to be negatively correlated to the content of saponins in the leaves (Goławska et al., 2008). The different amounts of saponins or their derivatives found in the different cultivars of *M. truncatula* may explain some of the differences I observed in the growth of aphid lines / clones. Alternatively, aphid lines / clones may also differentially eliminate, store or metabolize these compounds, perhaps using facultative symbionts.

6.2 Effect of Aphid on Plant

Aphids can damage plants by removing enough sap to cause wilting and death, resulting in significant yield losses in many crops. In my experiments, the feeding of pea aphids induced a reduction of 20 to 25% in the dry weight of the plant shoots whatever the genotypes of the plant and the aphid or the plant nutritional conditions. The decrease in the weight of the plant is generally proportional to the number of aphids; here, ten L1 nymphs for six plants were used in all conditions to give the plant the best chance of survival and I have rarely observed a plant death. Aphid survival was also very high (> 90%), and when a death occurred, it was generally towards the end of the 12-day period, suggesting only a marginal effect. As stated above, the phloem sap quality can differ between plants and affect the performance of aphids. In turn, aphid infestation can alter the chemical composition and nutritional value of phloem sap in a plant-dependent manner. In alfalfa, pea aphid feeding modifies the distribution of carbon and nitrogen in the stem and inhibits its longitudinal and radial expansion (Girousse et al., 2005). Girousse et al. (2005) noted similarities between the effects of pea aphids on alfalfa and the phenomenon of thigmotropism, the reduction in stem elongation in response to mechanical stimulation. Recently, Sanchez-Arcos et al. (2019) showed that the metabolic profiles of *M. sativa* and *T. pratense* plants infested with native aphid clones differ from those after infestation by non-native aphids or non-infested at all. Among the differential compounds identified were flavonoids, saponins, non-proteinogenic amino acids and peptides. As members of these classes of compounds are known for their activity against insects and particularly aphids, they may in turn be responsible for the differential performance of clones on native host plants vs. non-native ones.

The feeding of aphids induces various defence signalling mechanisms in plants (Morkunas et al., 2011; Jaouannet et al., 2014; Sugio et al., 2015) and mutualistic root associations are known to alter interactions with insects aboveground. Sap-feeding insects such as aphids and whiteflies induce defensive signalling mediated by SA, but JA also seems to participate in the case of aphids (Thompson and Goggin, 2006; De Vos et al., 2007). However, other defence pathways are also involved in the plant defence against aphids: indeed, transcripts associated with ethylene, abscisic acid (ABA) and gibberellic acid change after aphid infestation (Thompson and Goggin, 2006). My work confirmed that the SA pathway is activated during aphid feeding,

although the PR genes analysed showed a time-dependent and aphid and plant genotypes-dependent response. In addition, no clear effect was observed between plant SA and JA induced defences and the survival or fitness of aphids, suggesting that the SA and JA signalling pathways are weak or irrelevant for the induced defence against different aphid genotypes. In the early soybean-aphid interaction, SA and JA signalling seem to occur simultaneously, but expression of SA and ABA-linked marker genes increased over a 24-hour period (Selig et al., 2016) with a significant induction of PR1 and PR2 genes but no induction of the JA pathway. Although my data agree with this, since I observed no significant induction of lipoxygenase 5 (LOX5) or the genes of the allene oxide Synthase-1 (AOS1) coding for key enzymes in JA production, whose expression normally increases by positive JA feedback (Wasternack and Song, 2017), a sticking of the results was obtained for the proteinase inhibitor, a JA marker gene. This gene was clearly induced in a time, plant genotype, and nutritional conditions-dependent manner. This discrepancy may be due to a timing effect, the activation of other JA pathway genes being transient and occurring rapidly after aphid infestation or result from a bypass of the conventional JA pathway to induce PI under these specific conditions. One possibility would be a crosstalk between SA and the JA pathway since, in certain cases, SA affects JA-induced transcription by inducing the degradation of transcription factors having an activating role in JA signalling (Caarls et al., 2015). Although not time-activated, we observed that a basal increase in expression of some of the SA and JA pathway genes occurred in an aphid-plant genotypes manner. This may be a kind of priming effect or pre-reactive state of the plant, the plant sensing the aggression, but the alarm level having not reached a triggering threshold. Alternatively, the crosstalk between the SA and JA system (Caarls et al., 2015; Liu et al., 2016) maintains a *status quo* between the pathways activation, blocking a stronger plant defence reaction that could damage self-tissues. In some case this *status quo* is broken (maybe due to an accumulation of aphid effectors or a change in plant sensing mechanisms) and the plant reacts. In *Arabidopsis* interacting with *Myzus persicae*, the presence of the rhizobacterium *Pseudomonas fluorescens* primes the plant for enhanced expression of *LOX2*, a gene involved in (JA) pathway, and suppress the expression of *ABAI*, a gene involved in the abscissic acid (ABA) signalling pathway (Pineda et al., 2012). In contrast, almost no effect of the plant-microbe interaction with *Brevicoryne brassicae* was found.

In my work, a constant effect of aphid infestation has been on the formation and function of nodules. The results showed that, 3 weeks after inoculation of the rhizobacteria, the total number, the weight of the nodules, the nitrogen fixation were reduced after infestation of aphids compared to control plants. Further investigation of the level of gene expression of molecular markers of nodule function showed that i) there was no significant effect of aphid infestation on the level of *MtLb1* encoding for leghemoglobin which supplies oxygen to nitrogen-fixing bacteria and protects their nitrogenase enzyme from being inactivated (Appleby, 1984; Ott et al., 2009), and ii) the Cysteine protease-6 (*Cp6*) a marker for early nodule senescence (Guerra et al., 2010; Pierre et al., 2014) was upregulated after aphid infestation.

Nodule senescence typically occurs in 5 to 11-week-old nodules with a slow decrease in nitrogen fixing activity during this period (Puppo et al., 2005). In contrast, environmental stress conditions such as drought, saline stress, defoliation, continuous darkness and cold stress accelerate senescence and considerably decrease nitrogen fixation in the days following stress (Dupont et al., 2012). During the senescence of the nodules, both bacteroids and the host plant cells undergo sequential degradation. First, the bacteroids are degraded, then the host plant cells start to decay, leading to the complete degradation of the proximal part of the nodule (Van De Velde et al., 2006). One of the characteristics of this early senescence process is an increase in proteolytic activity, in particular of cysteine protease activities (Guerra et al., 2010; Pierre et al., 2014).

The kinetic study in the second paper suggests that the number of nodules remains almost constant under stress from aphids during the period observed, suggesting either that their formation is inhibited, or that formation and senescence occur at the same time. In addition, the nitrogen fixing function of these nodules is greatly altered after 8 days. Expression of the leghemoglobin gene appeared to decrease, but not significantly, and since leghemoglobin is synthesized in the bacteroid host cell (Verma and Bal, 1976), we can suggest that these cells are still functional. At the onset of induced senescence, expression of *MtCP6* was detected in cells which showed bacteroid degradation and were interposed between healthy binding cells (Guerra et al., 2010). It can therefore be suggested that the aphid presence has induced bacteroid degradation without affecting too much the host cells. Testing more genes on nodules obtained from a kinetic experiment and integrating some microscopy experiments to access the structure of nodules may help analyse this question.

6.3 How to explain this aphid effect?

Vasse et al. (1993) reported that a plant defence response could be involved in the formation of aborted infection threads during normal infection of *M. sativa* by *S. meliloti*. The plant reacts to the infection by eliciting a hypersensitive-like response. These authors suggest that this response is part of the mechanism by which the plant autoregulates nodulation. HR is a rapid localized cell death that occurs at the point of entry of pathogens induced by fungi, oomycetes, bacteria and viruses, but HR can also be induced by other organisms, such as insects (Rossi et al., 1998), which form long-term intimate interactions with their host plant. Interestingly, HR is a defence against aphids of the resistant genotype Jemalong, which developed necrotic lesions following infestation. Lesions were induced by stylet punctures and were similar to HR induced by the bacterial pathogen (Stewart et al., 2009).

During the consecutive stages of nodule differentiation of *S. meliloti*, a reprogramming of the transcription of the *M. truncatula* roots occurs. Among the differential expressed genes, some have suggested the activation of the jasmonate pathway in nodules, raising questions about the role of jasmonate during nodule development (Moreau et al., 2011). Jasmonate has also been shown to inhibit the signalling pathway for nodule formation in the *M. truncatula* roots (Sun et al., 2006) and proposed on the basis of indirect evidence (induction of lipoxygenase genes) to be involved in nodule senescence (Van De Velde et al., 2006).

Exogenous application of SA is reported to reduce nodulation in plants with undetermined nodule formation such as Medicago (Van Wees and Glazebrook, 2003). Stacey et al. (2006) induced transgenic expression of a bacterial salicylate hydroxylase (NahG) to decrease SA levels in *M. truncatula* plants, which led to enhanced nodulation and infection suggesting an inhibitory role for SA in nodulation. The regulation of ethylene production is also an important component in the symbiotic regulation and development of nodules (Guinel, 2015; Larrainzar et al., 2015). It has been involved in most of the stages leading to a mature nodule, and also in nodules senescence (Guinel, 2015). In the mutant sickle of plants, defective in the perception of ethylene, nodulation is not inhibited by ethylene, leading to an hypernodulation (Penmetsa and Cook, 1997). Ethylene is the most important hormone involved in plant resistance to pathogens and pests. The expression of genes involved in the production and signalling of ethylene is

upregulated in response to aphid infestation (Moran et al., 2002). Guo and Ecker (2004) found that the ethylene-insensitive mutant *sickle* has reduced resistance to aphids relative to the wild-type.

A subtle balance in the defence pathway is therefore necessary for the establishment of nodules and an imbalance in these pathways will lead to modification of the nodule formation and functioning. Aphids may play on this balance to drive nodulation toward the slope of senescence. Another possibility is that plants attacked by aphids impair the formation of nodules, thus restricting the supply of nitrogen necessary for the aphid development even if it costs fitness to the plant.

At last, it has been shown that the efficiency of nitrogen fixation is regulated by nodule-specific cysteine-rich (NCR) peptides in *M. truncatula* (Haag et al., 2011; Wang et al., 2018). Bacteroid differentiation is mediated in the host compartments by a large family of several hundred legume NCR peptides, which are transported in these compartments. A subset of them has antimicrobial activity *in vitro* and *in planta* and a BacA (a membrane protein) mutant of *Sinorhizobium* is sensitive to NCR peptides and unable to establish symbiosis (Haag et al., 2011). Interestingly, seven Bacteriocyte-specific Cysteine-Rich (BCR) peptides have been described in pea aphid (Shigenobu and Stern, 2013; Uchi et al., 2019); these cysteine-rich peptides are exclusively expressed in bacteriocytes hosting *B. aphidicola*. BCR and NCR peptides have structural similarities and BCR peptides have antimicrobial activity against *E. coli*. In addition, the sbmA mutant of *E. coli*, a bacA homolog of *S. meliloti*, was more sensitive to BCR peptides than the wild type. Although this may be a convergent evolution allowing the host to keep the symbiotic bacteria under control, it is tempting to speculate that pea aphid peptides could also participate in the control of rhizobacteria if they are injected into plants as active effectors. A recent study also indicated that a large family of cysteine-rich proteins was upregulated in the salivary glands (Boulain et al., 2019).

PERSPECTIVES

6. PERSPECTIVES

Little is still known about the interaction between plant-insect-bacterial partners and how these tritrophic or tetratrophic status modulate the general interaction between plants and insects (Frago et al., 2012; Sugio et al., 2015; Giron et al., 2017; Shikano et al., 2017). My work, although exploratory, has shown that based on well-known models, progress can be done even in these complex situations.

A central result of my thesis is that the presence of aphids impairs the correct development of nodulation of *S. meliloti* and blocks the nitrogen fixing activity of nodules in *M. truncatula*. This has occurred regardless of aphid clones, the presence of aphid facultative symbionts and plant genotypes, although all of these parameters may have modulatory effects. In the previous section, I discussed some of the possible reasons at the molecular level, but more information is still needed to assess the generality of this effect. More combinations of *M. truncatula* and pea aphid genotypes should be tested, and aphid inhibition of nodule formation and activity should be extended to other legume symbiosis models such as *Medicago sativa*, pea or clover. In my preliminary work, I tested the *Vicia faba* plant, but the data were not conclusive possibly due to an insufficient number of aphids used for the infestation. This plant is the universal host of all pea aphid biotypes and deserves more attention as it may respond differently compared to other plants. It will be interesting also to test if different species of aphids have the same effect on these plants and if the effect is reversible once the aphids are eliminated.

A second point to elucidate is the effect of the number of aphids. It is known that different plant responses to aphids occur several aphid-dependent modes. Here, we have used a fixed number of aphids; it will be interesting to identify the smallest number of aphids producing an effect and if the effect is aphid number-dependent. We also used young plants in contact with developing aphids. Testing the effect on nodule activity and senescence with older plants having reached maximum nodulation, in contact with young or adult aphids, would help to understand the genericity of the effect we observed.

In the current work we also used only one bacterial strain (*S. meliloti* 2011strain). It would be worth analysing the aphids effect on other available bacterial strains with different efficiencies in nodule formation and nitrogen fixation, depending on the accession of *M. truncatula* (Terpolilli et al., 2008; Kazmierczak et al., 2017).

Future work will also be needed to study the link between defence signalling, plant growth and the accumulation of defence metabolites in this interaction. To do this, a combined approach of transcriptomics and metabolomics will provide a holistic view of the modifications of plant metabolism during the interaction. Several recent studies have focused on the metabolomics approach to characterize the metabolic changes occurring in the plant-aphid interaction. However, to date, there is little information on the metabolomics changes in plants induced by nitrogen-fixing bacteria and linked to resistance to insect pests (Walker et al., 2011; Weston et al., 2012). In this context, metabolomics studies can provide detailed information on how nitrogen-fixing bacteria can modulate plant metabolites that affect the fitness of aphids. The time course analysis of the changes in plant metabolism will also be very important to analyse the plant / aphid interaction, since the race between the establishment of plant defences and the multiplication of aphids may be crucial for the outcome of the interaction. In our study, we focus on a few marker genes for SA- and JA-mediated defence responses. However, ethylene and abscisic acid are also important regulators in the plant-aphid interaction (Jaouannet et al., 2014). Given the importance of the various defence signalling pathways, transcriptomic analysis will be required to decipher the involvement of the diverse plant hormones in the plant response to aphids.

In our study, the indirect effect mediated by rhizobia seems to affect the fitness of aphids on NFS plants. Although the efficiency of nitrogen fixation in the plant-rhizobia symbiotic association is suboptimal after aphid infestation, the symbiosis process persists. This phenomenon is common in many legume-rhizobium symbioses in which the nodules are unable to supply the plant with sufficient nitrogen (Schumpp and Deakin, 2010). This maintenance of symbiosis may be associated with a protection of the plant against pathogens in addition to the partial nutritional advantage. Legumes constantly face multiple pathogenic challenges in their natural environment, which could provide an evolutionary advantage to the persistence of suboptimal nitrogen-fixing bacteria in nodules (Schumpp and Deakin, 2010). This hypothesis is essential to understand the possible role of microbes in the adaptation of legumes to the multiple challenges encountered. To identify the different roles of rhizobia in the nitrogen supply of plants and the induction of plant defence, we could construct rhizobia mutant strains defective at different stages of the symbiosis process and analyse the plant development and induced systemic resistance against aphids under different growth conditions. This approach will determine whether a process of functional

symbiosis is required for the initiation of a rhizobacterial-mediated defence response. In addition, a transcriptomic analysis of very few rhizobacterial strains has been conducted to unravel the plant molecular changes associated with induced systemic resistance (Cartieaux et al., 2003, 2008; Verhagen et al., 2004; Wang et al., 2005; Weston et al., 2012). This approach will help us to identify differentially regulated genes in the plant-aphid-bacteria interaction and could allow better understanding of the roles of the bacterial symbiont in the plant-aphid interaction.

Much more could be done such as monitoring the feeding behaviour of pea aphids on the plant under different nutritional conditions or including an additional level of complexity by adding natural enemies of the aphid to the system to check if the presence of rhizobacteria could attract natural enemies of aphids to protect plants.

Agronomical Interest

Climate change and food security are two of the greatest challenges that humanity will face in the near future. The population of Earth is expected to reach 9.8 billion in 2050 and 11.2 billion in 2100² increasing the demand for healthier food even if food is used more efficiently to avoid waste. Agriculture is already one of the main contributors to global warming; it is therefore also necessary to reduce its footprints. In the introduction to the recent issue on Legumes (Plant Cell Environ. 2019), Foyer et al., wrote: “The success of future agriculture depends on achieving an appropriate balance between environmental sustainability and yield-associated economic constraints. One economically efficient solution to protect soil quality in a sustainable manner is not only to return to legume-based conservation agriculture but also to simultaneously develop improved legume varieties and more effective rhizobial strains, which can be introduced across different cropping systems”.

Indeed, numerous studies and reviews favour the development of legumes as an alternative source of protein and as "green manure" thanks to the enrichment of the soil in nitrogen (thanks to their nitrogen fixing symbiont) thus reducing the use of nitrogen fertilizers. In addition, more and more rhizobacteria are used as plant growth promoters and to strengthen their defence system. However, our data have shown that nitrogen fixation in legumes can be altered when plants are attacked at an early stage by aphids. This can have a direct impact in the field by

² <https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html>

reducing plant growth and yield and indirect by limiting the nitrogen supplied to the soil by the crop.

Continuing this research is therefore essential to understand this aphid-rhizobacteria interaction and to develop better management of legume crops in the future.

7. GENERAL REFERENCES

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Les symbiotes jouent un rôle crucial dans le phénotype de leur hôte et dans son adaptation à l'environnement. Cependant, jusqu'à récemment, les interactions plantes-insectes étaient étudiées sans tenir compte de la présence de bactéries symbiotiques chez les partenaires impliqués. De nouvelles découvertes ont démontré que les communautés racinaires et aériennes des plantes sont liées. Dans ce contexte, mon doctorat s'interroge sur la façon dont les interactions entre les espèces végétales et les insectes sont modulées par leurs symbiotes respectifs.

Dans un premier temps, j'ai analysé le rôle de la symbiose fixatrice d'azote (NFS) chez la légumineuse *Medicago truncatula* (JA17) dans l'interaction avec des lignées de pucerons du pois *Acyrtosiphon pisum* portant différents endosymbionts facultatifs (FS). Pour ce faire, j'ai comparé la croissance de plantes de *M. truncatula* inoculées avec la bactérie nodulante *Sinorhizobium meliloti* (NFS) ou arrosées avec une solution de nitrate (non inoculées ; NI) infestées par des lignées de pucerons du pois provenant d'un même clone génétique (YR2) soit sans FS ou avec *Hamiltonella defensa*, *Serratia symbiotica* ou *Regiella insecticola*. La croissance des plantes NSF et NI est réduite par l'attaque des pucerons, tandis que la croissance des pucerons (mais pas leur survie) a été fortement réduite sur les plants NFS. En présence de pucerons la capacité de fixation d'azote des plantes NFS est réduite suite à l'induction d'une sénescence précoce des nodules. Enfin, chez les plantes NFS, toutes les lignées de pucerons ont déclenché l'expression du gène *Pathogenesis-Related-1* (*PR1*), un marqueur de la voie salicylique (SA), et du gène *Proteinase inhibitor* (*PI*), un marqueur de la voie jasmonique (JA), tandis que chez les plantes NI, seule l'expression de *PR1* a été déclenchée. Ainsi, le statut symbiotique de la plante influence clairement les interactions plante-puceron et la réponse de la plante à l'infestation, alors que le statut symbiotique du puceron ne fait que moduler l'amplitude de cette réponse.

Il a été démontré que le génotype de la plante et du puceron sont tous deux importants dans le résultat de leur interaction, j'ai donc étudié plus en détail comment la NFS affecte l'interaction entre différents génotypes de plantes et de pucerons. Pour cela, j'ai utilisé trois génotypes différents d'*A. pisum* dépourvus de FS (LL01, YR2, T3-8V1) et deux génotypes de *M. truncatula* (JA17 et R108) en présence ou en absence *S. meliloti*. La performance de chaque génotype de puceron sur les deux génotypes de plantes et l'effet des différents génotypes de pucerons sur la croissance des plantes et la capacité de fixation de l'azote des plantes de SNF ont été mesurés. Nous avons également estimé la réponse de défense médiée par le génotype de *M. truncatula* déclenchée par les différents génotypes de pucerons en utilisant différents gènes marqueurs des voies de défense JA et SA. J'ai constaté que les génotypes plantes-insectes ainsi que la présence de *S. meliloti* affectent de manière significative les interactions plantes-aphides.

Ainsi, les interactions génétiques interspécifiques entre la plante hôte et les pucerons ainsi que leur statut symbiotique peuvent influencer la dynamique de la population et la structure de la communauté. Ces résultats montrent que l'interaction plante-insecte est fortement influencée par la génétique des espèces et par leur statut symbiotique, ajoutant un nouveau niveau de complexité qui reste à explorer.

Puceron du pois (*Acyrtosiphon pisum*), *Medicago truncatula*, symbiose facultative, symbiose, rhizobium, fixation de l'azote, défense des plantes.