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**GENETIC MECHANISMS OF MIR AGAINST A  
NECROTROPHIC FUNGUS AND CROSSTALK WITH AN  
ABIOTIC STRESS**

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## **ABSTRACT**

## 1. Abstract

Nowadays, social warning for the natural environment is increasing and the use of agrochemicals in crop management is being reduced due to pollution and health issues and they are becoming less accepted by the sociality. For this reason, many researches are addressed to look for more environmental friendly alternatives. One interesting alternative that is being investigating lately is the use of beneficial microorganisms, like mycorrhiza fungi, in crop management and crop protection. Mycorrhiza are soil fungi that form symbiotic associations with plants roots worldwide, providing the plant a better nutrition as well as a more efficient response against different challenges. In this research study, we analysed the impact on tomato plants of an arbuscular mycorrhizal fungus *Rizophagus irregularis* from a genetic point of view, against two different stresses. On the one hand, an abiotic stress that consisted on the subjection of the plants to a transient N starvation. On the other hand, plants faced a biotic stress, an infection by the necrotrophic fungus *Botrytis cinerea*. The combination of both stresses have also been studied. To do that we carried out an analysis of some genes expression in the plant leaves involved in plant defense and in nitrate uptake and signalling.

## **INTRODUCTION**



## 2. Introduction

### 2.1. Plant defense mechanisms and plant-pathogen interaction

In nature, plants are constantly exposed to changing conditions and they have to face several abiotic stresses such as drought, soil or water salinity or winter cold and biotic stresses such as viruses, pathogenic bacteria or fungi and pests.

To defend itself the plant has defensive barriers that can be constitutive or inducible. Constitutive barriers are passive defences, present before the attack, and they are physical and chemical barriers. Physical barriers include the cuticle, cellular walls, the trichomes and the stomata. Chemical barriers consist on antimicrobial compounds called anticipins such as saponines or piretrines (Osbourn, 1996; Ingle et al. 2006) and other secondary metabolites such as volatiles that can attract natural enemies of pests. On the other hand, if these defences are not enough plants have mechanisms to detect pathogens and induce defensive responses after a pathogen attack that produce changes in their metabolism.

Plants are commonly exposed pathogen challenges but the disease is rarely developed thanks to the plant defense mechanisms coordinated by the plant immune system. With this system the plant is able to recognise alien microorganisms by recognizing microbe associated molecules such as flagellin, lipopolysaccharides or peptidoglycans, which are termed microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by specific receptors that are located in the cell membranes called pattern recognition receptors (PRRs). This recognition produces an appropriate induction of defences in the host plant and leads to PAMP-triggered immunity (PTI). In reaction to this host defense, microbes have evolved effector proteins that are secreted into the host and suppress PTI, allowing successful pathogen colonization and disease development, thus causing effector-triggered susceptibility. In some cases, plants have proteins that are able to recognize pathogen effectors and lead to the activation of immune responses that are quicker and more effective than those in PTI called effector-triggered immunity (ETI). This system of plant-pathogen interaction has been described as the “zigzag” model (Figure 1) (Jones and Dangl, 2006). This fight between plant and pathogen continues until the plant is not able to recognise the pathogen effectors and it colonises the host or when the plant is able to defend itself against all the pathogen effectors.

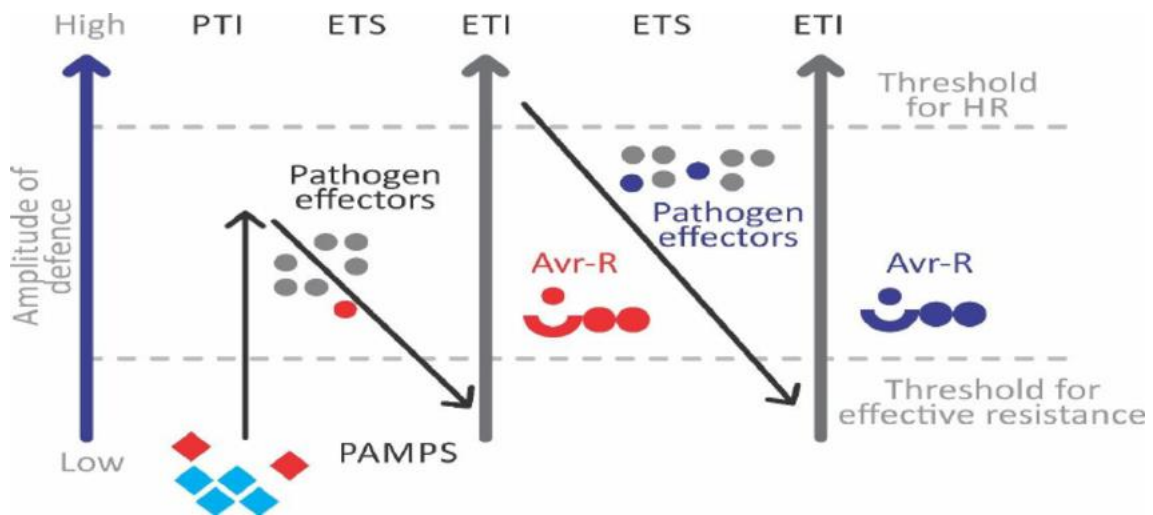


Figure 1. Zig-zag model. [www.nature.com](http://www.nature.com)

## 2.2. Arbuscular mycorrhizal fungi (AMF)

To defend themselves against challenging agents, plants have evolved a wide range of strategies. One strategy is to form associations with beneficial soil microorganisms, like arbuscular-mycorrhizal fungi (AMF). Mycorrhizal associations are mutualistic and reciprocally beneficial symbiotic relationships between plant root and some specific soil-borne fungi (Song et al 2015). It is probably the oldest and most widespread plant symbiosis on the Earth dating its emergence to 450 million years ago. Almost all plant species can form mycorrhizas and it can occur in almost all kind of ecosystems. It is estimated that over 80% of land plants form arbuscular mycorrhizas (AM) with fungi belonging to the phylum Glomeromycota (Brundrett, 2002). It is considered that mycorrhizal associations facilitated the colonization of land (Redecker et al., 2000).

Arbuscular mycorrhizal fungi are obligate biotrophs that require the host plant to complete their cycle. Plant allocates photosynthates to the fungus while the AMF improve plant acquisition of water and mineral nutrients (Rivero et al., 2015). The fungus colonizes the root cortex and forms intracellular structures called arbuscules where the exchange of nutrients between the partners takes place (Jung et al., 2012). The extracellular hyphal network spreads widely into the surrounding soil improving the supply of inorganic nutrients, specially phosphate and nitrate (Smith et al., 2011).

Plant roots exude a diverse array of biological compounds, for the interaction between plants and AMF, Strigolactones have been identified as AMF-recruiting signals. These hormones stimulate hyphal branching in AMF helping the fungus to localise host roots and facilitate infection (Cameron et al. 2013). In order to colonise the roots, the fungus has to overcome plant defences. The plant is able to recognise the AMF by recognition of its MAMPs and this recognition can trigger initially, an immune response. The initial stages of root colonization by AMF are

accompanied by transient induction of selected plant defences, followed by localised suppression at later stages of the interaction (Kapulnik et al. 1996).

Besides the improvement of plant nutrition, the AMF also provides other beneficial effects. The fungus increases the absorption surface in the soil helping the plant to acquire essential micronutrients such as copper, zinc, manganese and cobalt. It improves the soil structure by making the soil particles more stable and it favors plant diversity. AMF colonised plants have more competitive ability in the medium (León, 2013).

This relation implies important changes in plant primary and secondary metabolism and have a deep impact on plant physiology, altering the plant ability to cope with biotic and abiotic stresses. The symbiosis acts commonly improving host tolerance to unfavourable environmental conditions and resistance to pests and pathogens (Gianinazzi et al., 2010; Jung et al., 2012; Ruiz Lozano et al., 2012; Selosse et al., 2014). It confers the plant an enhanced defensive capacity against pathogens named "mycorrhiza induced resistance" (MIR).

Previous studies have proven that MIR enhance plant resistance against a wide range of attackers including biotrophic and necrotrophic pathogens, nematodes and herbivorous arthropods. For example, mycorrhizal colonization improved tomato resistance to an array of diseases caused by *Erwinia carotovora* (García-Garrido and Ocampo, 1988), *Fusarium oxysporum* f. sp. *lycopersici* (Akköprü and Demir, 2005), *Phytophthora nicotianae* var. *parasitica* (Cordier et al., 1996), *P. parasitica* (Cordier et al., 1998), *Pseudomonas syringae* (García-Garrido and Ocampo, 1989) and to foliar disease of early blight (Fritz et al., 2006).

### **2.3. Induced resistance: Defense Priming**

Numerous studies have determined that colonization of plant roots with certain beneficial microbes, including AMF, causes the induction of a specific physiologic state in plants called "priming". Primed plants show faster and stronger activation of various cellular defense responses induced following the exposure to either pathogens, herbivore insects or abiotic stress (Kuc, 1987; Conrath et al., 2006; Pastor et al., 2010; Pastor et al., 2013; Rivero et al., 2015). "Priming" sets the plant in an "alert" state in which defences are not actively expressed but in which the response to an attack occurs more effectively compared to plants not previously exposed to the priming stimulus, efficiently increasing plant resistance (Jung et al., 2012). The primed state can also be induced by treatment of plants with various natural and synthetic compounds, such as  $\beta$ -aminobutyric acid (BABA), jasmonic acid (JA), and salicylic acid (SA) (Jakab et al., 2001; Worrall et al., 2012).

## 2.4. Genes involved in defense pathways

Plant defense responses are coordinated by small molecules that act as signal transducers and regulate the coordinated expression of genes that code for defense-related proteins and compounds (Ausubel, 2005; Jones and Dangl, 2006). Among these molecules, the phytohormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) play key roles (Pieterse et al., 2009). According to the challenger lifestyle one signaling pathway will prevail over the others.

According to their lifestyles the pathogens can be classified in: biotrophs, necrotrophs and hemibiotrophs. Biotrophs feed on plant alive tissues whereas necrotrophs feed on plant dead tissue that they have killed previously. On the other hand, hemibiotroph organisms can have both types of lifestyle changing on the different stages of their life cycle.

Generally, in plants, SA signalling pathway regulates responses such as programmed cell death, effective against biotrophic and hemibiotrophic organisms whereas the JA pathway activates resistance against necrotrophic organisms, herbivores and wounding (Thomma et al., 1998).

In contrast to below-ground interactions, AM effects on pests or pathogens attacking the aerial parts of the plant are less studied. Early studies described that biotrophic pathogens appear to spread better on mycorrhizal plants. As for the hemibiotrophs, the effect of the symbiosis varies from no effect to reduction of the disease. However, the proliferation of pathogens with a necrotrophic lifestyle is hampered and disease symptoms are weaker in mycorrhizal plants (Jung et al., 2012). Therefore, AM plants are more resistant to necrotrophs and chewing insects, attackers targeted by JA-dependent defense responses.

One of the objectives of this study is to observe the effects in the gene expression of the inoculation of the arbuscular mycorrhiza *Rhizophagus irregularis* in tomato plants against the fungus *Botrytis cinerea*. *B. cinerea* is a necrotrophic fungus that causes damage to wide range of plant species, including tomato (*Solanum lycopersicon*).

Since this pathogenic fungus shows a necrotrophic lifestyle we will analyse genes involved in the JA synthesis pathway such as LOXD, AOS1 and JAR1. LOXD and AOS1 participate in early stages of the JA synthesis pathway (figure 2). JAR1 encodes for an amino acid conjugate of the JA, jasmonate isoleucine, a bioactive form of this hormone. We will also analyse the expression of PINII which is a JA marker (De Domenico et al., 2012).

To improve our analysis, we will also use a transgenic tomato genotype that overexpresses the prosystemin gene due to in tomato plants, systemic induction of JA-dependent defense responses is mediated by the molecule systemin.

Systemin is a signal peptide, formed by 18 amino acids (aa), released from a larger precursor of 200 aa, called prosystemin (Mcgurl et al. 1992). Systemin was the first identified plant bioactive peptide, isolated from tomato as a potent inducer of protease inhibitors (PIs) (Pearce et al. 1991).

Systemin has a key role on response upon wounding and herbivory due to the PIs degrade essential amino acids in the herbivore midgut (Chen et al. 2005; Mcgurl et al. 1994). More recently it has been observed that systemin has a wider role including its involvement in tomato resistance against necrotrophic phytopathogens (Diaz et al, 2002; El Oirdi et al. 2011).

We will also analyse the activity of NCDE3, a gene involved in the synthesis of abscisic acid (ABA). ABA is a plant hormone involved in many plant functions. However, its possible influence in mycorrhizal induced resistance has not yet been studied.

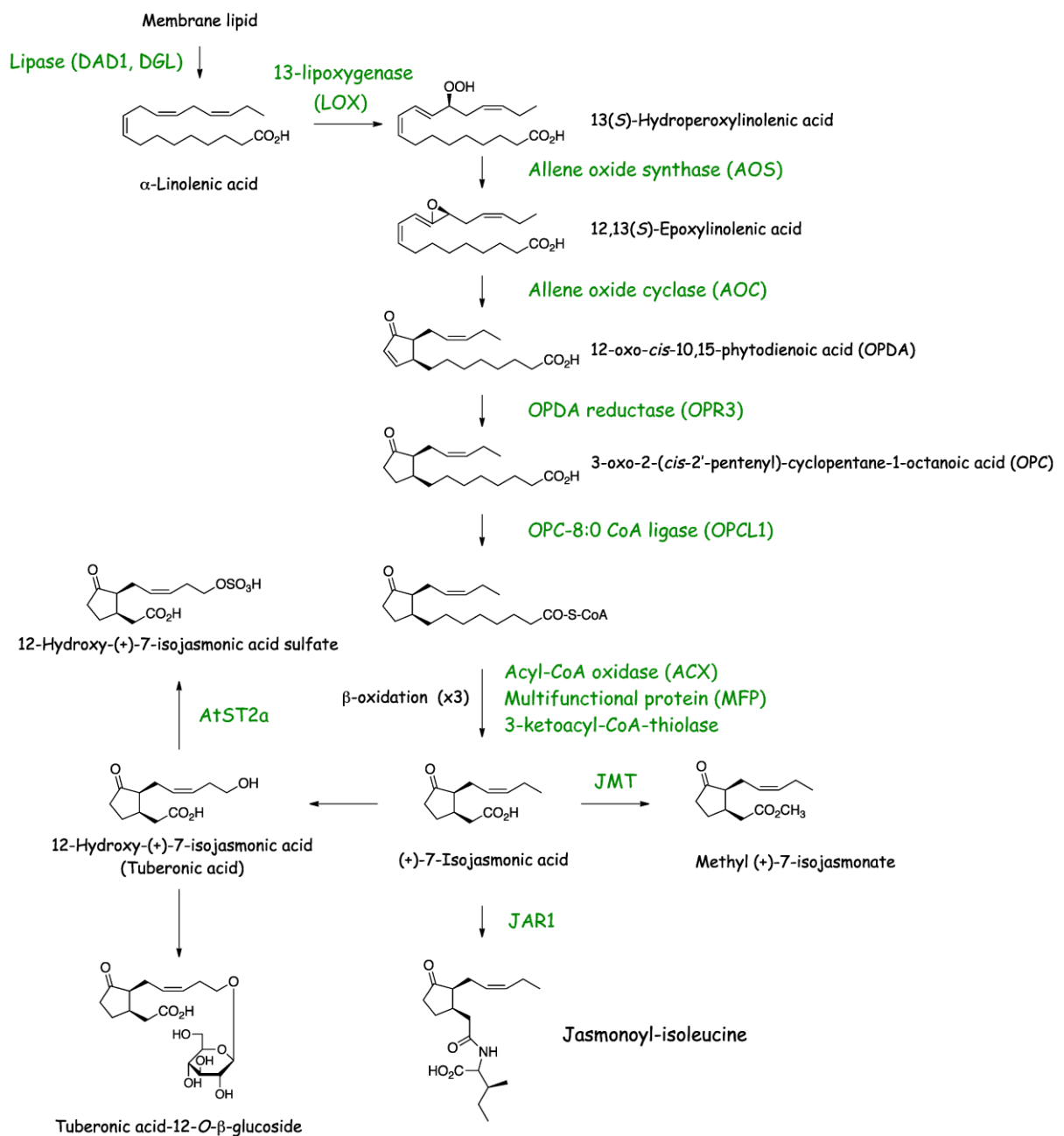


Figure 2. JA synthesis pathway. hormones.psc.riken.jp

## 2.5. Nitrate transceptors and immune defense responses

Nitrogen is a key element in plant growth and development. Nitrate ( $\text{NO}_3^-$ ) is the major nitrogen source that plants can find in the soil and it can act both as a nutrient and signal on plant metabolism and growth. Nitrate concentration can vary drastically in the soil and plants can react to the different concentrations with two different transport systems in order to uptake  $\text{NO}_3^-$ ; low affinity transport systems (LATS) and high affinity transport systems (HATS). LATS perform in the nitrate uptake when the concentration in the soil is high ( $>1\text{mM}$ ) whereas HATS take up nitrate when the concentration is low ( $<100\mu\text{M}$ ). (Glass et al. 1992; Crawford et al. 1998; Tsay et al. 2007).

There are two families of nitrate transporters that have been characterised in a variety of plants including tomato. Low affinity transporters are encoded by the NRT1 gene family while the NRT2 family of genes encodes high affinity transporters (Hildebrandt et al. 2002). In addition to nitrate transport function, nitrate transporters have been evidenced to be involved in nitrate sensing and act as so called transceptors (transporters and receptors) (Ho et al. 2009; Gojon et al. 2011). The term “transceptor” has been applied to membrane proteins that fulfill dual nutrient transport/signalling functions. For example, NRT1.1 and NRT2.1 can both perceive small amounts of nitrate and transmit signals to the plant in order to integrate growth with nutrient availability (Krouk et al. 2010).

Once in the root  $\text{NO}_3^-$  is either stored in vacuoles or assimilated to organic nitrogen and partitioned to plasmids (Orsel et al. 2002). Alternatively, nitrate is loaded into xylem vessels and transported to the aerial parts (Marschner et al. 1997). However molecular mechanisms of nitrate uptake and long distance transport are still poorly understood.

A previous study has also presented an additional role for NRT2.1 linked to plant resistance against the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*) (Camañes et al. 2012).

In tomato 5 genes induced by nitrate have been identified, LeNRT1.1, LeNRT1.2, LeNRT2.1, LeNRT2.2 and LeNRT2.3 (Ono et al. 2000; Hildebrandt et al. 2002).

It has been reported that among the nitrate transporters only le NRT2.3 had a higher expression in AMF colonized tomato roots than in non-colonized controls suggesting that AMF colonization affects nitrate uptake and allocation to the plant probably mediated by leNRT2.3 (Hildebrandt et al. 2002).

It has been determined that the protein sequence of LeNRT2.3 shows a 77% identity with AtNRT2.4 suggesting that this gene has the same function as a high affinity nitrate transporter. However, it is not clear, in a previous study it has been reported that LeNRT2.3 may encode a low-affinity transporter for nitrate as well as act as a long distance transporter (Fu et al. 2015).

Nitrogen metabolism has been found to be related with phytopathology. Nitrogen metabolism genes are strongly affected by pathogen infection, that might be a result of both defense activation and attempted pathogen manipulation of the host metabolism for nutritional purposes. Nitrogen supply affects plant- pathogen interaction, it is clear that N limitation has an impact on plant defence but we can find contradictory conclusions. It was found that N limitation reduced the resistance of *Arabidopsis* to *E. amilovora*, whereas N limitation reduced the susceptibility of *Arabidopsis* to *B.cinerea* (Fagard et al. 2014). There is a hypothesis of the impact of nutritional status on plant defense that predicts that under limiting conditions, available resources would be allocated to higher defense production (Massad et al., 2012).

In this study we will subject the plants to a modification the N supply in the substrate in order to analyse its effect in the genes expression and its influence in the mycorrhiza induced resistance (MIR). We will analyse some genes involved in the nitrate uptake and signalling: LeNRT2.1, LeNRT2.2 and LeNRT2.3.

## **MATERIALS AND METHODS**



### 3. Materials and methods

#### 3.1. Experimental design

For the experiment we used tomato plants (*Solanum lycopersicum*) from two genotypes, the variety Better Boy as a wildtype and an overexpressor of prosystemin 35S::PS. Plant treatments include mycorrhization, infection with the necrotrophic pathogen *Botrytis cinerea* and subjection to nitrogen starvation, all of them with their respective controls. Samplings were done 0h post-infection and 48h post-infection. The experiment treatments are the following:

Better Boy genotype: BB: control plants

BBAM: mycorrhized plants

BB-N: plans subjected to nitrogen starvation

BBAM-N: mycorrhized plants subjected to nitrogen starvation.

The same treatments were infected with *B. cinerea*, so we will use “inf” to design them: BB inf, BBAM inf, BB-N inf, BBAM-N inf.

The same treatments were applied to the 35S::PS genotype using “PS+”: PS+, PS+AM, PS+-N, PS+AM-N, PS+inf, PS+AM inf, PS+-N inf and PS+AM-N inf.

#### 3.2. Plant materials and growth conditions.

Tomato seeds were sterilised before sowing by keeping them 5 minutes shaking in 10% of commercial hydrochloric acid with tap water.

The seeds were sown in multiwell polystyrene trays 60cm<sup>3</sup> of capacity. Autoclaved vermiculite was used as germination substrate and then the seeds were watered with tap water. They were grown in a glass greenhouse at temperature between 18 and 26°C and a relative humidity between 60 and 90%. These conditions didn't change until the transplantation.

4 weeks after sowing, the seedlings had their first two true leaves and were transplanted into individual plant pots of 330cm<sup>3</sup> using autoclaved vermiculite as substrate. At this time half of the plants were mycorrhized. The plants were watered three times per week with tap water and fertilized once a week with Long Ashton nutrient solution (Hewitt, 1966) but modified with only 25% of the standard concentration of phosphorus (this improves the mycorrhization level).

Element	NO <sub>3</sub>	P	S	K	Mg	Ca	Na	B	Cu	Fe	Mn	Mo	Zn
µg/ml	170	41	48	156	36	160	31	0'5	0'06	5'6	0'6	0,05	0'07

Table 1. Long Ashton nutrient solution compounds



Image 1. Plant size at the transplantation time.

### 3.3. Micorrization

For the micorrization we used the inocula of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* (provided by the Experimental Station of Zaidín, Granada) that was propagated in a greenhouse by using clover plants (*Trifolium repens*) cultured in a substrate containing sepiolite and vermiculite in equal terms. A mixture of the substrate containing the inocula and autoclaved vermiculite was used for AM inoculation. The proportion was 10% of inocula substrate and the rest of vermiculite. The inoculation was carried out at the transplantation time. The plants were transplanted from the sowing trays to 330cm<sup>3</sup> pots containing the mixture.

The control pots were watered with a soil filtrate of the inocula substrate using a watman No 1 filter, to exclude possible effects of other soil microorganisms. The filtrate contained the natural soil populations without AMF inocula.

25 days after the inoculation the percentage of mycorrhization was evaluated in 5 plants randomly chosen. To evaluate the level of micorrization, the roots of the plants were washed with distilled water in order to eliminate the substrate and then they were cut in fragments about 2mm long. Then the roots were stained according to the Vierheilig et al. (1998) method. The roots were incubated in distilled water at 80°C during 13min with 10% of KOH for clarification. After washing them with distilled water several times we washed them once with 2% of acetic acid in distilled water. Then they were incubated in distilled water with 5% of ink (Parker ink) and 2% of acetic acid during 10min at 90°C. After that they were washed several times with distilled water in order to remove excess ink.

Once the roots were stained, they were placed in a petri dish and observed with a binocular magnifying glass. The percentage of micorrization was determined using the gridline intersection

method (Giovannetti and Mosse, 1980). It consists on randomly disperse the stained roots fragments in a petri dish with grid lines (each square has 1,2cm of side). Then we followed all horizontal and vertical lines and counted the intersections whit roots and mycorrhizas separately. The value 0 was given for roots and 1 for mycorrhizas. To calculate the percentage of colonised root length we divided the number of intersections with colonised roots by the total number of intersections.

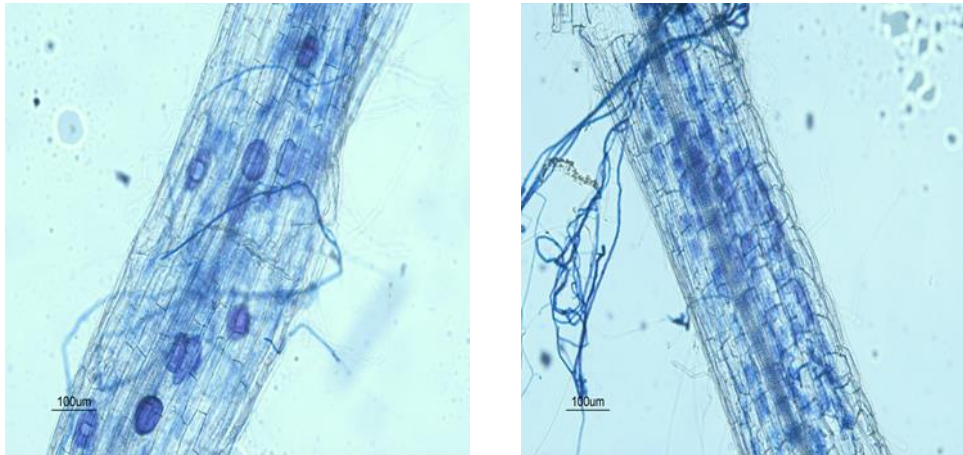


Image 2. Stained roots from mycorrhized plants. The fungal structures can be observed.

### 3.4. Nitrogen starvation

The next step consisted on subjecting some plants to a total nitrogen starvation during 48h. Their roots were washed with distilled water and then the plants were fertilized with the same nutrient solution than before (Long Ashton) but without nitrogen in hydroponic conditions. The hydroponic conditions consisted on placing the plants in little containers without substrate.



Image 3. Plants in hydroponic conditions

### 3.5. Pathogen inoculation

Half of the plants were infected with the necrotrophic fungus *Botrytis cinerea* CECT2100 (Spanish collection) after the 48h of nitrogen starvation. The pathogen was cultured for 15 days in a Petri dish with 19g/L PDA growth culture (Potato, Dextrose and Agar) at 24°C with a 12h photoperiod.

For the pathogen inoculation first it is needed to extract the spores and calculate the right concentration for the infection.

To extract the spores we took a sample of the fungus tissue and placed it inside a tube with about 20ml of magnesium sulfate ( $MgSO_4$  10mM) and we shook it so that the spores will be suspended in the liquid. In order to remove the mycelium and only keep the spores, we put a little piece of cotton inside a syringe and we made the liquid containing the spores pass through it collecting the liquid in a new tube. Then we centrifuged it at 4000rpm during 2 min. After that we removed the supernatant ( $MgSO_4$ ) with a pipette and kept the pellet (spores) in the ependorff tube. Then we added 500  $\mu$ l of sucrose and 500  $\mu$ l of  $KH_2PO_4$  in 50  $\mu$ l of Gambor B5 (germinating spores medium) to the tube containing the spores and we waited for 2h.

In order to count the spores we used an hemacytometer, an optical glass device containing a grid that is used for counting cells or other suspended particles. 10 $\mu$ l of the liquid containing the spores were taken with a pipette and poured in the hemacytometer near the cover (the liquid reaches and covers the grid by capillarity).

Then we observed the grid with a microscope and started to count spores. You have to count a significant number of squares. In this experiment we counted the spores that were in three lines of the grid. Only the spores that were inside the square were counted, and those that were touching the edges were discarded.

Calculation of the spore concentration:

<b>4</b>	<b>2</b>	<b>2</b>	<b>1</b>	<b>3</b>	<b>4</b>	<b>2</b>	<b>2</b>	<b>1</b>	<b>0</b>	<b>2</b>	<b>3</b>
<b>1</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>0</b>	<b>1</b>	<b>4</b>	<b>3</b>	<b>4</b>	<b>2</b>	<b>3</b>
<b>2</b>	<b>1</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>0</b>	<b>3</b>	<b>6</b>	<b>4</b>	<b>3</b>	<b>4</b>

Table 1. Representation of hemacytometer grid with the number of spores.

Total of spores: 81       $81/36 = 2,25$  spores/square

Each square has a volume of  $0,00025\text{mm}^3$

$2,25/0,00025 = 9000\text{sp}/\mu\text{l} = 9 \times 10^6$  spores/ml

We need  $10^5$  spores in 15ml

Using the formula:  $C_0 \times V_0 = C_f \times V_f$

The volume we need to take is:  $V_0 = \frac{15 \times 10^5}{9 \times 10^6} = 0,1667\text{ml}$

Once we had the spore concentration that we needed to make the infection we pulverized the third and fourth leaf of the plants with a spray. Then the plants were kept in transparent plastic boxes in order to keep 100% of relative humidity to make a favourable environment for *B. cinerea* infection. Plants were kept in the glass greenhouse under the same conditions mentioned above.

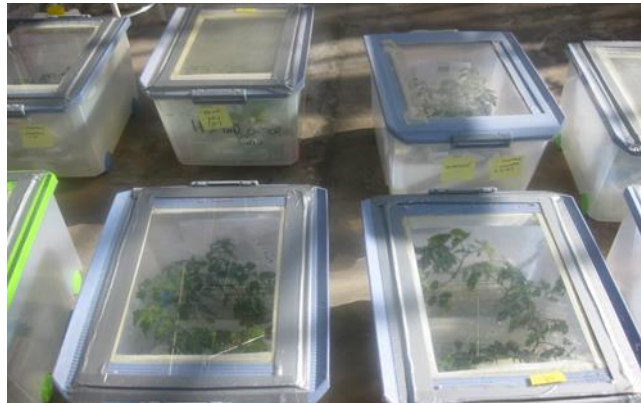


Image 4. Plants inside plastic boxes

### 3.6. Harvesting

Samples were taken at 0h post-infection (still non-infected plants) and 48h post-infection. We took plants of all treatments and we harvested the third and fourth leaf of each plant. Then they were stored in the fridge at  $-80^\circ\text{C}$ .

### 3.7. RNA extraction

To make the RNA extraction first we grinded the samples inside a mortar with liquid nitrogen ( $\text{N}_2$ ,  $-176^\circ\text{C}$ ) until they became powder and we place them in a 2ml Eppendorf tube.

A technique of dual extraction was carried out, which combines isolation of RNA and metabolites. The following technique is an adaptation of an already described protocol which allows the

combined isolation of metabolites, DNA, long RNAs, small RNAs and proteins from plants and microorganisms (Valledor et al., 2014).

First we added 800µl of buffer (MeOH:CHCl<sub>3</sub>:H<sub>2</sub>O, 2'5:1:0'5) to the Eppendorf tube containing the fresh tissue, vortex it and then centrifuge 20000g during 6min at 4°C. After that we removed the supernatant, placed it in new Eppendorf tubes and this samples were stored at -20°C for future metabolites analysis. The pellet was washed immediately with 1ml of Trizol (38ml of Aqua Phenol, 11'82g(X-1) of Guanidine Thiocyanate, 7'6g(X-3) of Ammonium Thiocyanate, 3'34ml(X-2) of Sodium acetate 3M, 5ml of Glycerol, up to 100ml of mQ water) and vortex for 30 seconds. The samples were kept 5min at room temperature and then centrifuged at 13000rpm, 4°C during 5min. After that the supernatant was transferred to a new 2ml Eppendorf tube, 220µl of CHCl<sub>3</sub> were added and then vortex for 15 second. The tubes were centrifuged at 13000rpm, 4°C and 5min and then the aqueous phase was transferred to a new 2ml Eppendorf tube. Then 350 µl of 2-propanol and 350 µl of 0,8M citrate/1,2mM NaCl were added and the tubes were kept at room temperature during 10min. They were centrifuged again at 13000rpm, 4°C during 15 min. After that we removed the liquid and rinsed twice with 0,5ml of 70%EtOH and centrifuge during 1min with the same conditions as before. Finally we air dried the pellet and dissolved it in 50µl nuclease-free water. The samples were kept at -20°C.

The RNA of two technical replicates of each treatment was isolated.

The quantity of isolated RNA was measured with the nanodrop.

### 3.8. Retrotranscription

A reverse transcription was carried out in order to turn RNA into complementary DNA (cDNA) due to RNA is very unstable and it is easier to work with cDNA which is more stable.

Before the retrotranscription we cleaned the RNA in order to remove any other molecule that could have been isolated with the RNA. To do so we used a commercial kit of Takara. The cleaning is based on the activity of a Dnasa that eliminates de DNA.

To make the cleaning we placed samples of the isolated RNA in 150µl Eppendorf tubes. The volume we needed to take depends on the concentration of RNA on each sample (it was measured with the nanodrop). We calculate the necessary volume that we had to take in order to get 150ng of RNA.

The volume of RNA plus mQ water should reach 4,9 µl, then we add 0,7µl of Dnase and 0,7 µl of the buffer that we found in the kit. Then the tubes were placed in a thermocycler at 37°C during 30min. After that we added 0,7 µl of Dnasa stop in order to stop the activity of the Dnasa due to

the next step is to transform RNA into cDNA. The tubes were placed again in the thermocycler at 65°C during 10min. Then we had the RNA cleaned.

Next step was to make the retrotranscription. For that, we used another commercial Takara kit called PrimeScript RT Reagent kit (Perfect Real Time).

The components of the kit were added to an Eppendorf tube: 2µl of PrimeScript Buffer, 0'5 µl of PrimeScript RT Enzyme, 0'5 µl of Oligo dT primer and 7 µl of our RNA. The tubes were place in the thermocycler at 37°C during 15min and then at 85°C during 5 seconds.

### **3.9. Quantitative RT-PCR**

The differential expression of selected genes was analysed by using real time-polymerase chain reaction (RT-PCR) using the cDNA samples that we obtained before from all the treatments. We used the Step One Real-Time PCR System (Applied Biosystems) thermocycler.

First all the cDNA samples were diluted to a concentration of 1/10. In order to make the calibration curves of the genes we made a pool (mixture of 1ml of each sample), and then we diluted the pool to the concentrations of 1/10, 1/100, 1/1000 and 1/10000.

To analyse the samples by PCR first we prepared a plaque that has 48 holes. On each hole we put a mix of 5µl of SYBR Green, 0'5 µl of each primer (forward and reverse), 3µl of mQ water and 1µl of our cDNA. 6 replicates of each treatment were analysed in order to obtain more accuracy in our data.

The program used for real-time PCR was 10 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 1o seconds at 55°C and 20 seconds at 72°C. The melting curve was at 60°C during 10 seconds and 95°C during 15 seconds.

The genes analysed were PINII, LOXD, JAR1, AOS1, PROSYS, NRT 2.1, NRT 2.2, NRT 2.3 and NCED3. The gene  $\alpha$  EF1 was used as the housekeeping (its expression does not change with the experimental conditions).

The sequence of the primers used are listed in Table 1.

Gene	Primer sequence (5' a 3')
LePIN2	F: 5'-CGT TCA CAA GGA AAA TCG TTA AT-3' R: 5'-CTT GGG TTC ATC ACT CTC TCC-3'
LeLOX-D	F: 5'-GAC TGG TCC AAG TTC ACG ATC C-3' R: 5'-ATG TGC TGC CAA TAT AAA TGG TTC C-3'
LeJAR1	F: 5'-CAT TGA AAC CAT CTC CTT GA-3' R: 5'-TAA ACT GCT TGC TGC TGT AAA-3'
LePROSYS	F: 5'-AAT TTG TCT CCC GTT AGA-3' R: 5'-AGC CAA AAG AAA GGA AGC AAT-3'
LeNCED	F: 5'-ACC CAC GAG TCC AGA TTT C-3' R: 5'-GGT TCA AAA AGA GGG TTA GC-3'
LeAOS2	F: 5'-AGA TTT TCT TCC CGA ATA TGC TGA A-3' R: 5'-ATA CTA CTG ATT CAT CAA CGG CAT -3'
LeNRT2.1	F: 5'-TTC CTG TTA CAT TTT GTC ATT TCCC C-3' R: 5'-CAG ATT CAA GAC TAT CCA TTC CTC A-3'
LeNRT2.2	F: 5'-TCA AGG GAA CGG AAG AAC ATT ATT A-3' R: 5'-GCT CAT TGA ACT AAA GAT TGA CGA T-3'
LeNRT2.3	F: 5'-AAT GCA TGG TGT TAC TGG TAG AGA-3' R: 5'-CTA ATA ATA GGG ACT AAA GGG GCT G-3'
Sl $\alpha$ EF1	F: 5'-GAT TGG TGG TAT TGG AAC TGT-3' R: 5'-AGC TCG TGG TGC ATC TC-3'

Table 2. Primer sequences

### 3.10. Data treatment

The RT-PCR analysis provides data of the Ct of each sample defined by the following exponential equation:

$$Ct = a \times \log_{10}[\text{concentration}] + b$$

With the calibration curves that we made we obtained equations of the line for each gene:

$$y = ax + b$$

So we can isolate the concentration of each gene:

$$[\text{concentration}] = 10 \times \left( \frac{Ct - b}{a} \right)$$

Then we related the concentration to the housekeeping:

$$\frac{[conc.]_{gene}}{[conc.]_{\alpha EF1}}$$

We calculated the mean and the standard deviation of the 6 replicates of each treatment.



The data were normalized to the control (BB). The control acquires the value of 1 and then all the data of the other treatments are divided by the control.

Finally, the data were plotted in a bar graphic, representing the means with their error bars dividing the standard deviation (SD) by the square root of the number of replicates (n):  $SD/\sqrt{n}$ .

For the data treatment the Excel program was used.

### **3.11. Statistical analysis**

In order to analyse significant differences between treatments an ANOVA analysis and an LSD test (95% of confidence interval) were made using the Statgraphics program.

## **OBJECTIVES**

#### 4. Objectives

As discussed in the introduction mycorrhizal fungi act as resistance inductors in plants enhancing their defense responses. One of the main goals of this study is to understand the molecular mechanisms governing the mycorrhiza induced resistance. To achieve this main goal, we proposed the following objectives:

- Study the influence of the mycorrhiza in plants that were not challenged with any stress, this is at timepoint 0 hours post infection.
- Study mycorrhizal impact in the defense-gene expression against an infection by the fungus *Botrytis cinerea* 48h after pathogen inoculation.
- Analyse the effect of mycorrhiza against a transient N depletion.

The second main objective is to analyse and understand how transient nitrogen depletions can affect mycorrhiza induced resistance (MIR). To achieve this main goal, we purpose the following objectives:

- Observe how a transient nitrogen depletion alone influence the expression of defense-related genes and genes encoding for nitrate transporters.
- Observe how a plant subjected to a transient nitrogen starvation respond against a pathogen infection.
- Analyse whether the nitrogen depletion has an influence MIR. Therefore, to study the molecular responses of the mycorrhiza following combined stresses such as a N transient depletion and an infection by a necrotrophic fungus.

The third main goal is to understand the role of the systemin in mycorrhiza induce resistance. In order to achieve this goal, the following objectives were purposed:

- Observe the prosystemin overexpression effects in the expression of defense genes.
- Observe the effect of mycorrhization in a prosystemin overexpression mutant.
- Analyse how a prosystemin overexpressor plants respond against an infection.
- Analyse the possible effect of prosystemin in MIR.
- Observe how a prosystemin overexpressor mutant respond against a transient nitrogen depletion.

## **RESULTS**

## 5. Results

### 5.1. Effect of mycorrhiza and N starvation in the absence of infection.

The following figures show the relative expression of different genes at the timepoint 0, the basal levels of the genes before the infection. The figures show the control plants(BB) compared with three treatments: BBAM, BB-N, BBAM-N.

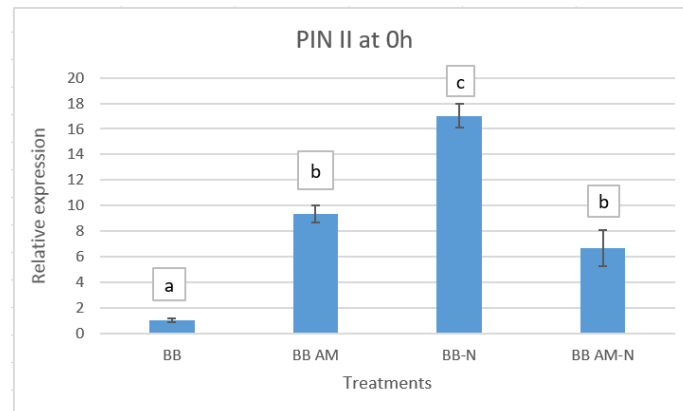


Figure 3. Relative expression of PIN II in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mycorrhizal plants show about 9-fold higher expression of *PINII* compared to the control. Nitrogen starvation (BB-N) increases the expression of *PINII* compared to mycorrhizal plants and 16-fold compared to the control. However, N starvation in mycorrhizal plants show the same expression levels as the mycorrhiza alone. This suggests that mycorrhiza is buffering the effect produced by the N depletion in the expression of *PINII* at timepoint 0.

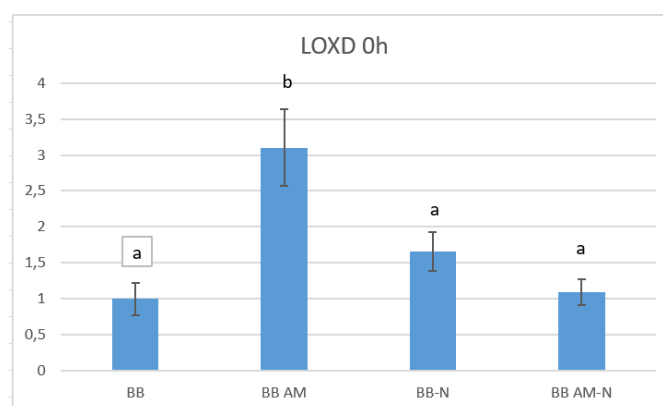


Figure 4. Relative expression of LOXD in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

The basal levels of *LOXD* were increased in plants colonized by AMF compared with the control (BB). Interestingly, a transient N starvation (BB-N, BBAM -N) restores basal levels of *LOXD* expression.

The N starvation did not significantly change the expression of *LOXD* compared to the control. This result suggest that a possible benefit of AM plants prior infection by triggering *LOXD* is antagonized by a transient N starvation.

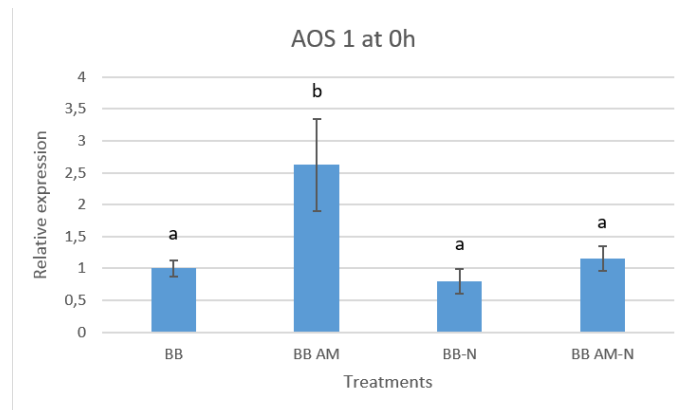


Figure 5. Relative expression of AOS1 in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

AMF colonized plants show an increased expression level of *AOS1* compared to the control, (about 2.5-fold compared to the control). However, plants subjected to a transient nitrogen starvation show similar levels of *AOS1* expression in both mycorrhized and non mycorrhized plants. This levels showed no differences with the control(BB) (figure 5). This gene expression profile is observed for both genes in the biosynthesis of oxylipins, *LOXD* and *AOS1*.

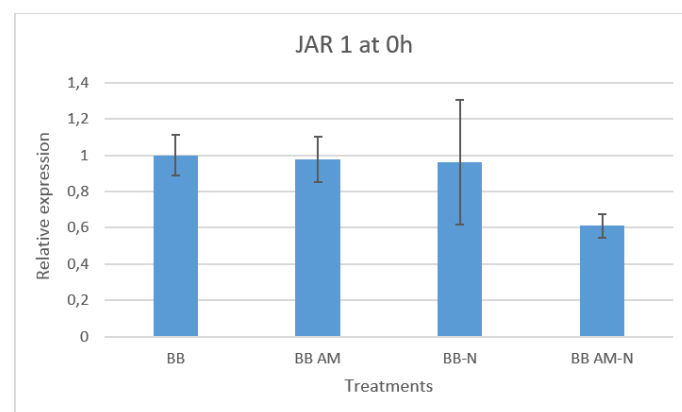


Figure 6. Relative expression of JAR1 in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Neither AMF colonization nor a N depletion alters the basal levels of *JAR1* gene expression, contrastingly with other oxylipin biosynthetic genes. Note that *JAR1* is involved in the synthesis of JA-Ile that is a branched pathway following JA synthesis.

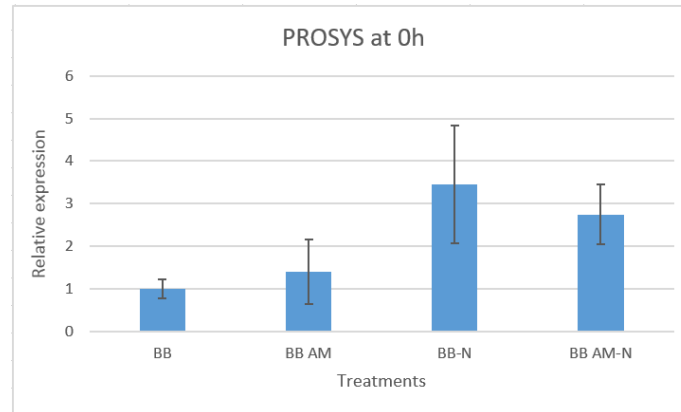


Figure 7. Relative expression of PROSYS in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

The *PROSYS* expression barely changed at basal levels, there are not significant differences between different treatments. Despite the N depletion shows a trend increasing Prosystemin gene expression, this is a slight non-significant change.

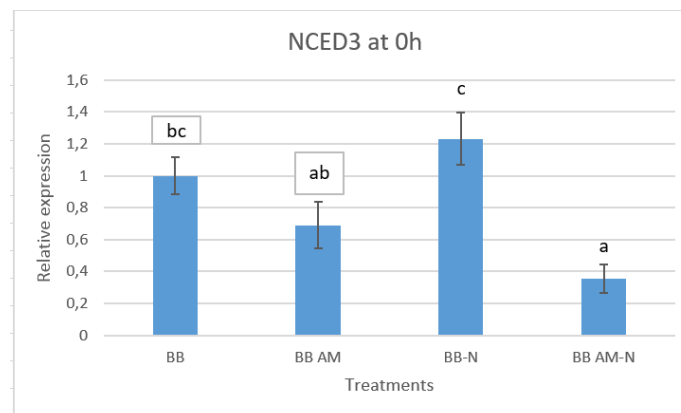


Figure 8. Relative expression of NCED3 in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

There *NCED3* accumulation was similar in both mycorrhizal and non-mycorrhizal plants (BB and BBAM) showing a slightly reduction of its expression in BBAM. Mycorrhizal plants showed lower expression of *NCED3* in plants subjected to N starvation, showing similar levels with the mycorrhiza treatment alone (Figure 8). It seems that AMF colonization has the same effect in both control and N deficient plants in the expression of *NCED3*, that clearly suggest an antagonistic function of mycorrhizal colonization and ABA biosynthesis in the absence of infection.

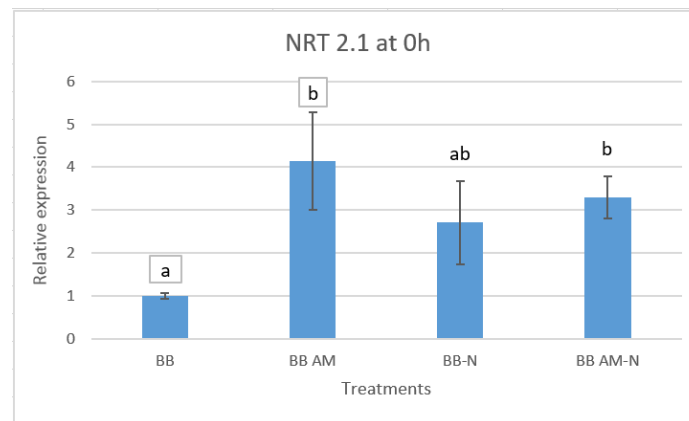


Figure 9. Relative expression of NRT2.1 in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Regarding to the expression of genes involved in the nitrate transport. There was an increase in the *NRT2.1* accumulation in response to mycorrhizal colonization in both BBAM and BBAM-N compared to the control(BB). BBAM has an *NRT2.1* expression about 4-fold the control and BBAM-N is about 3.5-fold the control (Figure 9). The N starvation seems to trigger a *NRT2.1* transcript accumulation (BBA-N and BBAM-N). This finding suggests that *NRT2.1* may play a similar role in tomato as in Arabidopsis since it is induced following a transient N starvation. In addition, mycorrhization has a positive effect in the induction of *NRT2.1* expression at an early timepoint (0h).

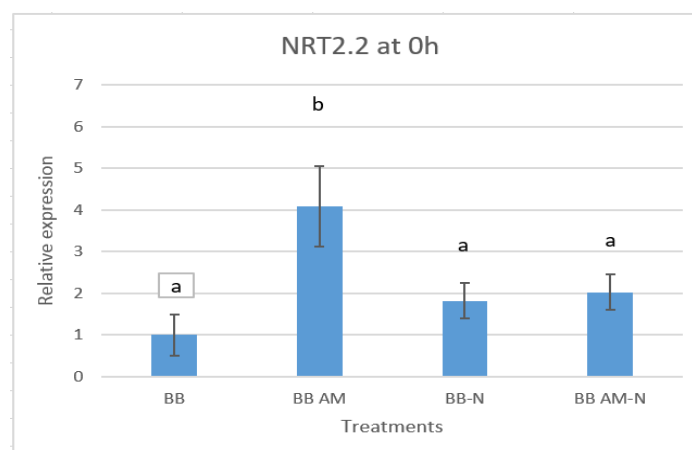


Figure 10. Relative expression of NRT2.2 in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

On the other hand, *NRT2.2* gene expression is triggered by in mycorrhizal plants showing an enhancement of four times compared to the control. Plants subjected to N starvation showed no significant differences with the control. Thus, *NRT2.2* may have a different roles compared to *NRT2.1* since N depletion cannot induce its expression, at least in leaves.



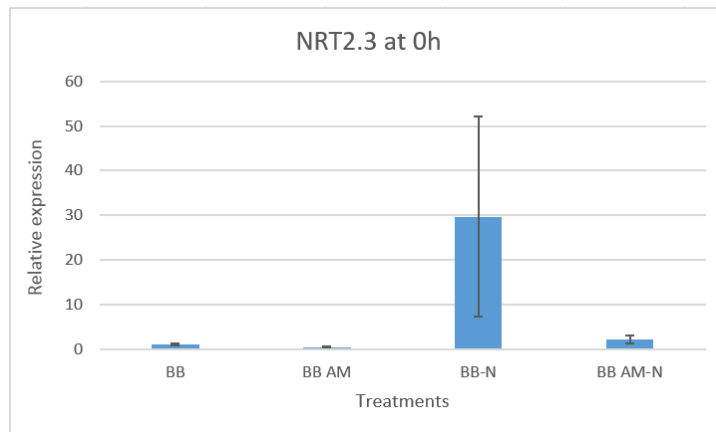


Figure 11. Relative expression of *NRT2.3* in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

*NRT2.3* expression level was extremely low in leaves. For this reason, in some treatments the data represented is based in a few number of replicates.

Significant differences in the expression of *NRT2.3* between different treatments at basal levels were not observed. However, it seems clear a trend in induction of *NRT2.3* expression in the absence of N. Notably, this gene is not triggered in AM plants therefore its role in mycorrhization may be different compared with the other two genes of the NRT family.

## 5.2. Effect of mycorrhiza and a transient N depletion following infection by *Botrytis cinerea*.

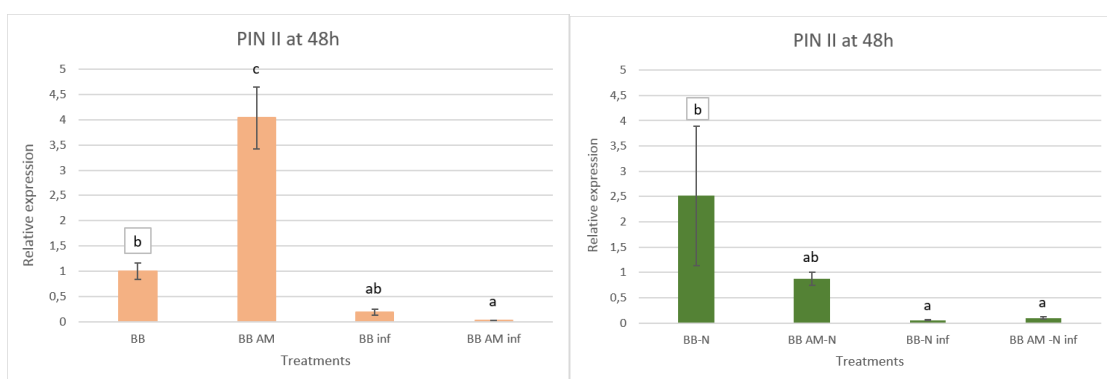


Figure 12. Relative expression of *PINII* at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mycorrhizal plants show a higher expression of *PINII* compared to control plants, as it happened at timepoint 0, and also compared with the other treatments. Surprisingly, the infection does not show significant differences with the controls (BB) in the expression of *PINII*. Mycorrhizal infected

plants do not show differences with control infected plants (BB inf), however the expression of *PIN1* is lower compared with the control (BB) (Figure 12).

Expression level of *PIN1* was reduced in mycorrhizal plants after N starvation compared with BBAM alone. The infection produced a reduction in the expression of *PIN1* in plants subjected to a N starvation (BB-N inf and BBAM-N inf) compared with non-infected plants with the same treatments (BB-N and BBAM-N) (figure 12) but there are no differences between the expression of *PIN1* in BB-N inf and BBAM-N inf.

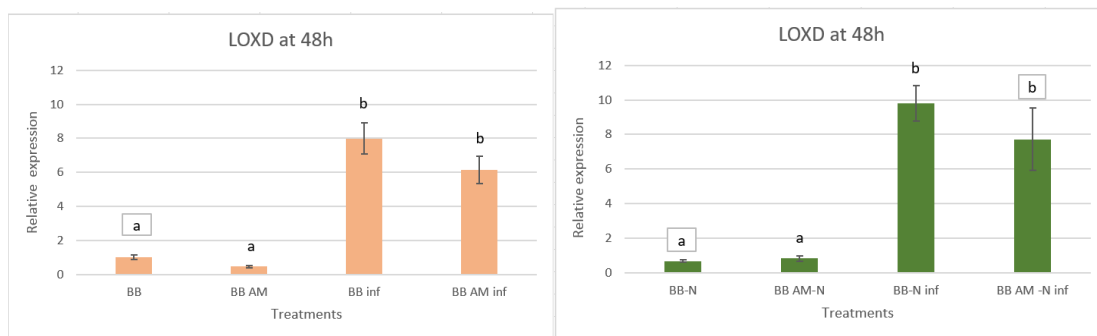


Figure 13. Relative expression of LOXD at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Control and mycorrhizal plants show no differences in the expression of *LOXD* (figure 13). After the infection the expression of *LOXD* was higher in both BB-N and BBAM-N than in non-infected plants but there are not significant differences between the two treatments.

The N starvation has a similar effect in *LOXD* expression that follows the same pattern as in plants normally fertilized (Figure 13). There are no differences between control and AM plants subjected to a N Starvation (BB-N and BBAM-N). The infection increases notably the expression of *LOXD* compared to non-infected plants with similar levels in mycorrhizal and non-mycorrhizal plants (BB-N inf and BBAM-N inf).

Thus the accumulation of *LOXD* response against pathogen infection is clear, however the AMF colonized plants did not differ regarding to the *LOXD* expression at 48h after infection.

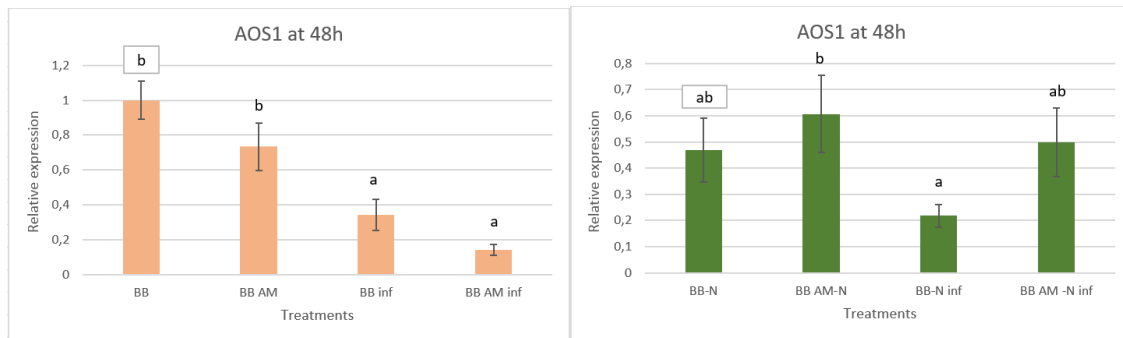


Figure 14. Relative expression of AOS1 at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

AOS1 accumulation was similar between mycorrhizal and non-mycorrhizal plants in both infected and non-infected treatments. Infected plants showed lower levels of AOS1 expression in both mycorrhizal and non-mycorrhizal plants compared to non-infected plants. In this case we cannot see any effect of the mycorrhization either in the presence or in the absence of infection.

After a transient N starvation the infection produced a decrease in the AOS1 accumulation compared to the other treatments and the control. This reduction was antagonized in mycorrhizal plants, although this expression levels did not differ statistically it is observed a clear trend (figure 14).

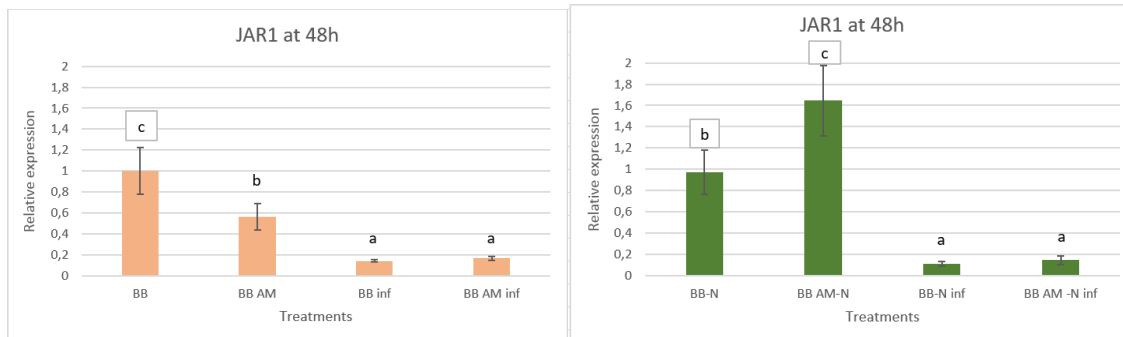


Figure 15. Relative expression of JAR1 at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mycorrhization induced a slight reduction of JAR1 expression in the nascence of infection, however infected plants showed a reduction in JAR1 gene expression. (Figure 16).

In plants subjected to N starvation, mycorrhiza showed a very similar profile of JAR1 that was higher in healthy plants. However, JAR 1 expression was significantly suppressed in infected plants compared with the healthy ones (BB-N and BBAM-N) in both mycorrhizal and non-mycorrhizal plants (BB-N inf and BBAM-N inf).

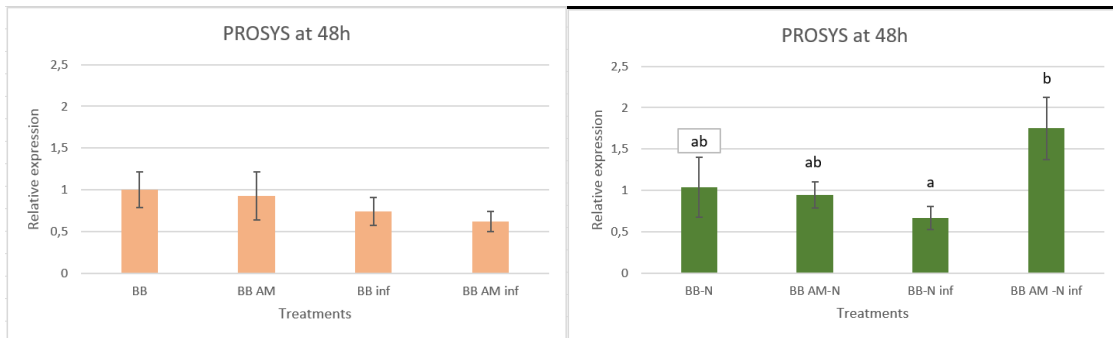


Figure 16. Relative expression of PROSYS at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

There were similar expression levels of *PROSYS* in all treatments with complete nutrition, without significant differences between them (Figure 16).

In response to N starvation mycorrhiza did not induce a higher accumulation of *PROSYS* compared with non-mycorrhizal plants (BB-N). However, mycorrhization produced a higher accumulation of *PROSYS* in infected plants (BBAM-N inf) compared to non-mycorrhizal infected plants (BBAM-N inf) (Figure 16).

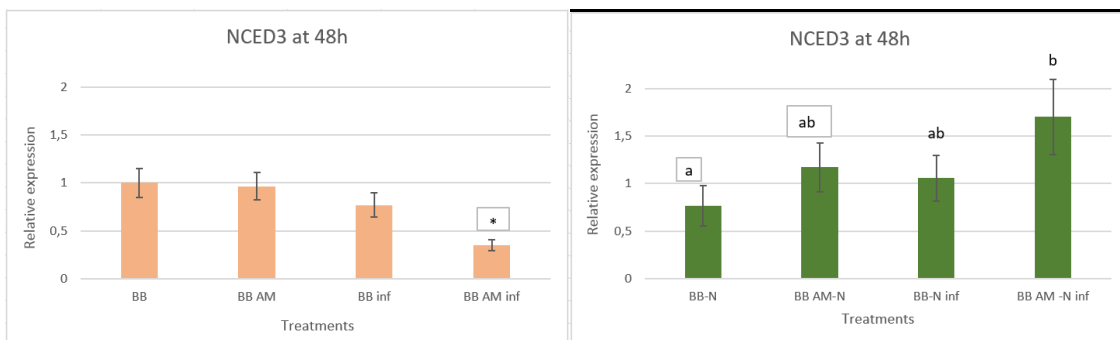


Figure 17. Relative expression of NCED3 at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Regarding to ABA biosynthesis genes, the expression of *NCED3* was not changed by any of the experimental conditions in normally fertilized tomatoes with the exception of BBAM infected plants that showed a reduction by 50% in *NCED3* gene expression.

After N starvation this situation was reverted since, only BBAM-N inf treated plants showed a higher expression of *NCED3* compared with the control. The rest of the treatments showed similar accumulation of this gene (Figure 17).

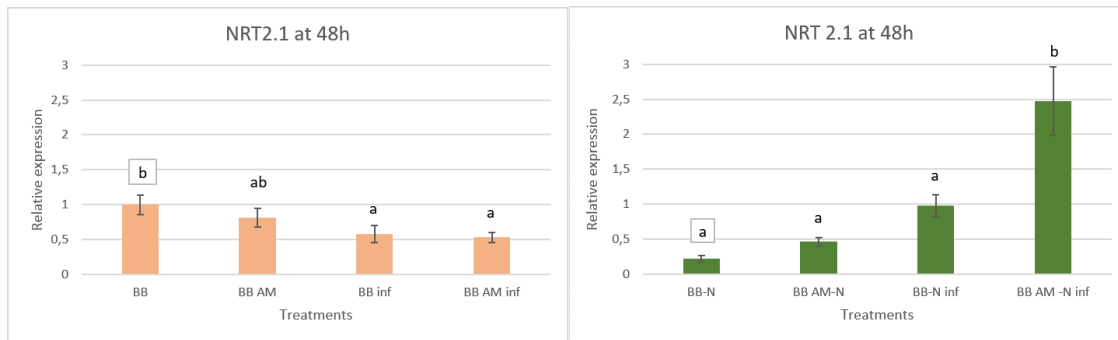


Figure 18. Relative expression of *NRT2.1* at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

At 48hpi mycorrhization did not change significantly the levels of *NRT2.1* compared to the control while the infection produced a small decrease in the expression of *NRT2.1* in both control (BB inf) and mycorrhizal plants (BBAM inf) compared to the non-infected control (BB).

In response to N starvation mycorrhizal infected plants showed significantly higher accumulation of *NRT2.1* compared to the other treatments (figure 18). This suggests that mycorrhiza produces a more pronounced response to the pathogen infection when subjected to a transient N starvation.

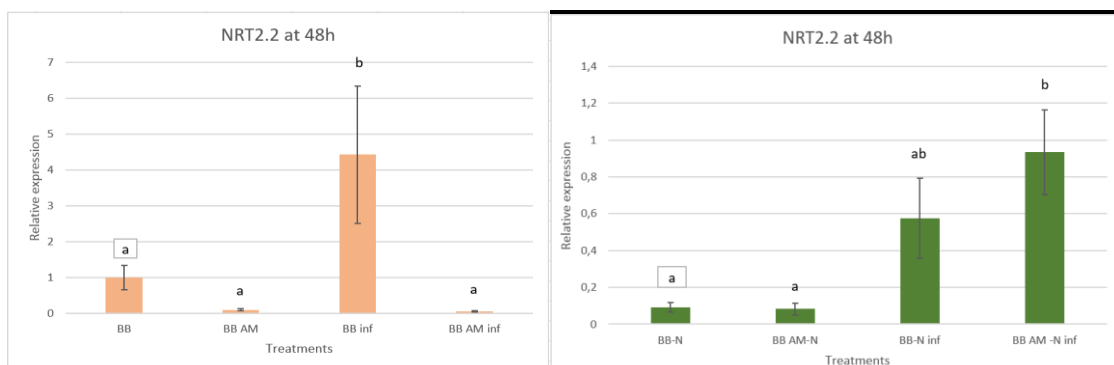


Figure 19. Relative expression of *NRT2.2* at 48h after infection. Different letters mean significant differences, ANOVA and LSD test (95%).

The infection alone produced a great increase in the expression of *NRT2.2*. The rest of the treatments showed similar *NRT2.2* accumulation with the control (Figure 19).

In plants subjected to N starvation the infection produced an increase in the *NRT2.2* expression compared with non-infected plants especially in mycorrhizal plants, as it happened with the *NRT2.1*. BBAM-N inf showed about 10-fold *NRT2.2* expression compared to the control (Figure 19).

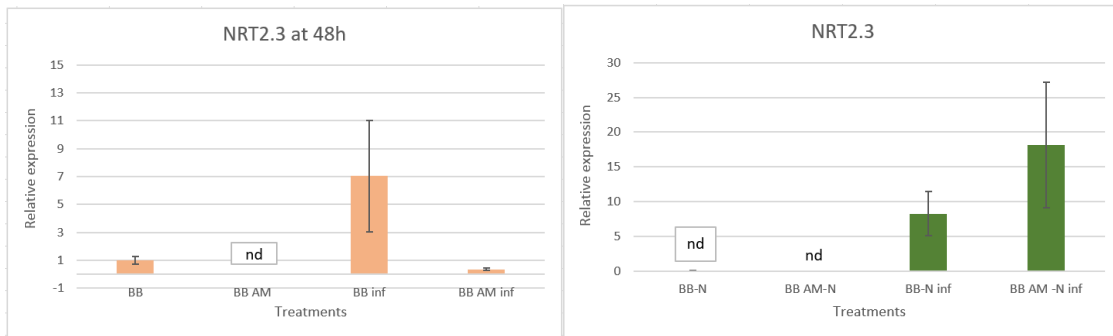


Figure 20. Relative expression of *NRT2.3* at 48h after infection. Nd: non detected levels. Different letters mean significant differences, ANOVA and LSD test (95%).

It seems that the infection alone increased the expression of *NRT2.3*, however, we cannot see significant differences between treatments (Figure 20).

In plants subjected a N starvation we obtained data only for the infected plants. Both mycorrhized and non-mycorrhized plants showed similar levels of *NRT2.3* accumulation. Mycorrhiza colonization slightly increased its accumulation (Figure 20).

### 5.3. Effects of prosystemin overexpression in MIR

#### 5.3.1. Influence of prosystemin overexpression in the absence of infection.

In order to determine the influence of the systemin in mycorrhization process and its interplay with *B. cinerea* infection and nutritional cues we performed experiments with an overexpressor of the *PROSYSTEMIN* gene that encodes the precursor protein that after cleavage rend the systemin peptide.

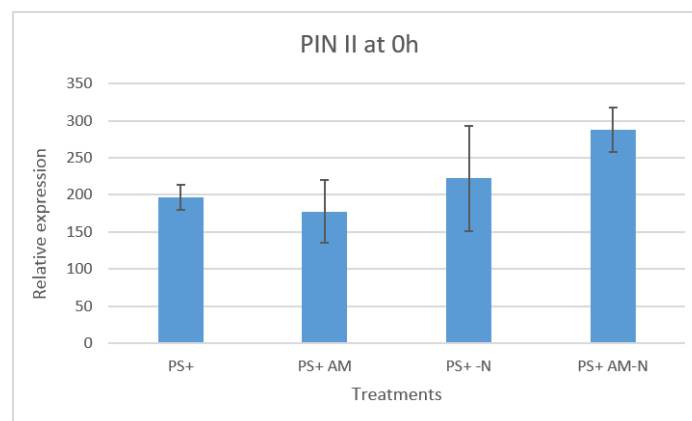


Figure 21. Relative expression of *PINII* prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Prosystemin overexpression has a big impact on *PINII* expression compared to the wildtype (Figure 3). The accumulation of *PINII* was about 200-fold higher than the control wildtype BB.

However, there are no significant differences between the different treatments showed in figure 21, overexpression of prosystemin did not produce a differential expression of *PIN11* neither in mycorrhized plants nor in plants subjected to N starvation.

*PIN 11* was notably expressed at basal levels but not as response of the different experimental conditions.

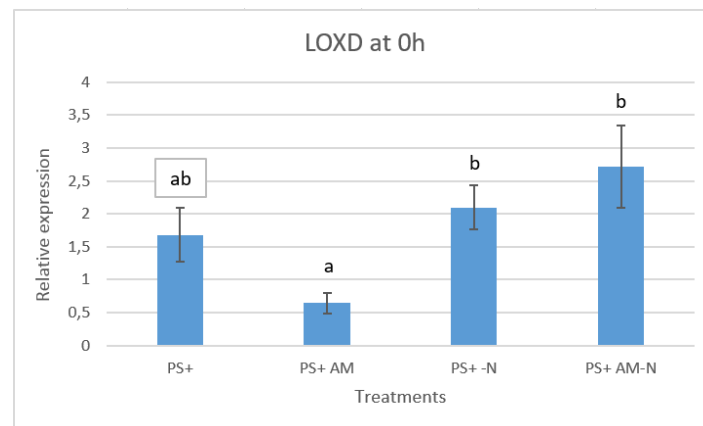


Figure 22. Relative expression of LOXD in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Levels of *LOXD* were similar in the mutant and the wildtype in the absence of infection or N starvation (Figure 4). However, the expression pattern in the different treatments changed.

Noteworthy, the expression of *LOXD* in PS+ is higher than in BB (Fig 7) plants although not as high as expected since there is around a 40% of increase.

Any of the treatments (AM, -N and AM-N) showed significant differences with the mutant control (PS+), however the expression of *LOXD* was lower in mycorrhizal plants than in the other treatments.

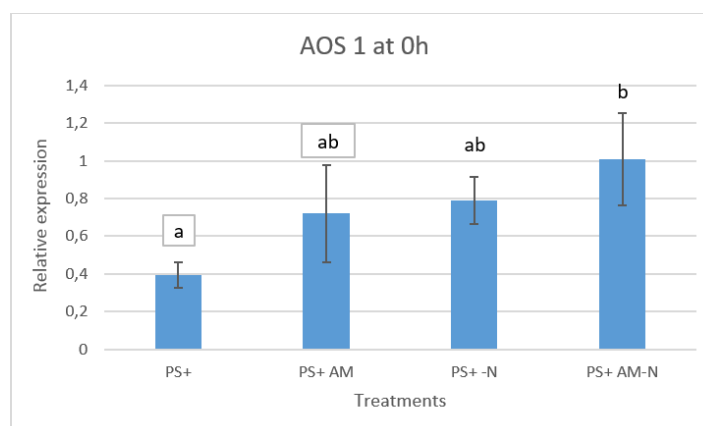


Figure 23. Relative expression of AOS1 in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

In general, *AOS1* accumulation was lower in the PS+ mutant than in the wildtype in the absence of infection.

Only mycorrhizal plants subjected to N starvation showed significantly higher accumulation of *AOS1* in the mutant at basal levels.

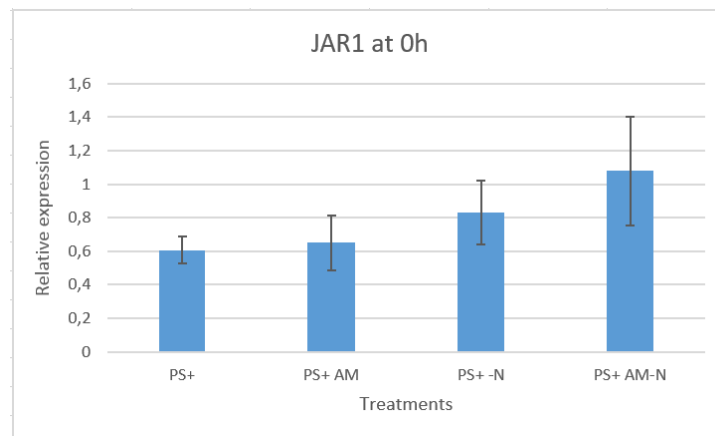


Figure 24. Relative expression of *JAR1* in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Levels of *JAR1* expression were slightly lower in the PS+ mutant compared to the wildtype at basal levels. The expression of *JAR1* between different treatments was similar without significant differences, as it happened in the wildtype (Figure 6). This results suggest that *JAR1* activity is not affected by any of the treatments at basal levels.

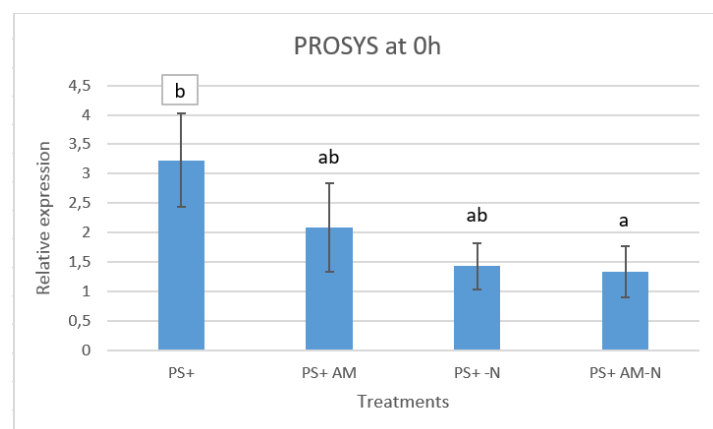


Figure 25. Relative expression of *PROSYSYS* in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

*PROSYSYS* levels were higher comparing the PS+ control to the wildtype control (BB).



Mycorrhizal plants showed similar levels of *PROSYS* compared to the control (PS+). At basal levels the N starvation produced a decrease in the *PROSYS* expression especially in mycorrhizal plants compared to the control (PS+) (Figure 25), suggesting that this peptide has not a significant role in response to N starvation.

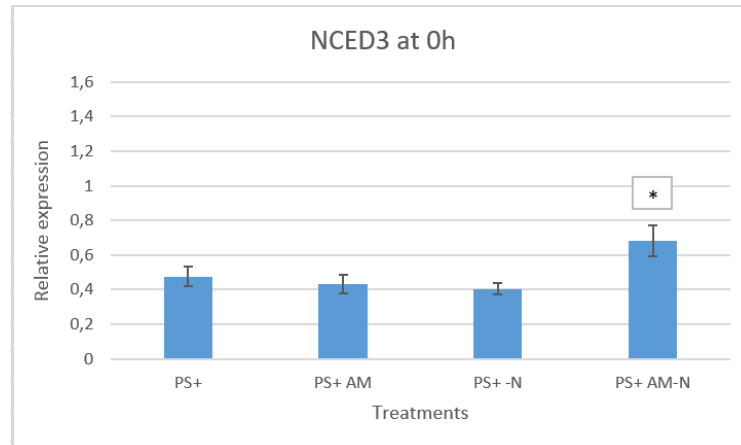


Figure 26. Relative expression of *NCED3* in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

The mutant showed higher accumulation of *NCED3* in mycorrhizal plants subjected to N starvation compared to the other treatments and the control that had similar levels between them (Figure 26). Interestingly, the observed gene expression is the opposite compared to wild type plants since the micorrhization in the absence of N suppressed *NECD3* (Fig 8) while in the Ps+ plants subjected to the same treatments it is observed an increase in *NECD3* expression (Fig 26)

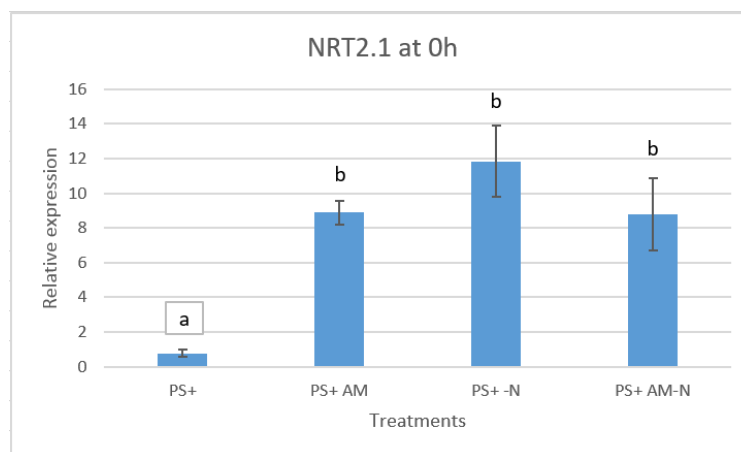


Figure 27. Relative expression of *NRT2.1* in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mycorrhizal plants showed higher levels on *NRT2.1* expression than the control of about 8-fold. In plants subjected to N starvation the expression of *NRT2.1* was increased in both treatments, PS+ -N and PS+ AM-N.

Here we can see the effect of the mycorrhiza alone, producing an increase of *NRT2.1* expression and also an increase in the accumulation of this nitrate transporter after a transient nitrogen starvation. This strengthens the fact that this gene is responsive to nitrogen starvation, additionally the overexpression of systemin seems not to have a direct effect on *NRT2.1* expression.

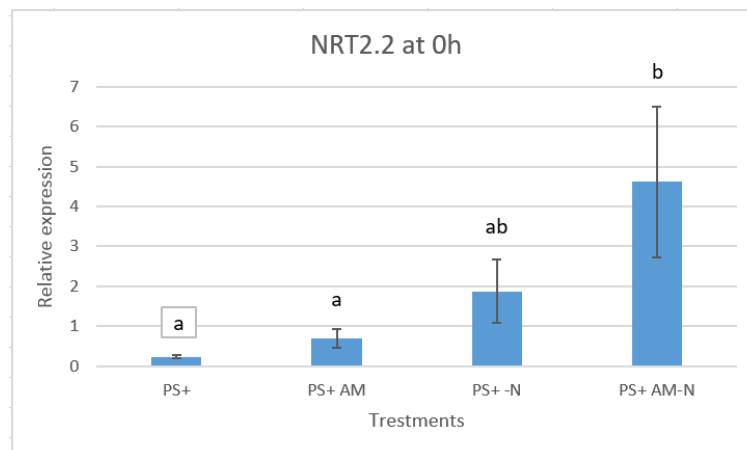


Figure 28. Relative expression of *NRT2.2* in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mycorrization alone slightly increased *NRT2.2* expression compared to the control at basal levels but there were not significant differences. N starvation produced higher accumulation of *NRT2.2* especially in mycorrhizal plants.

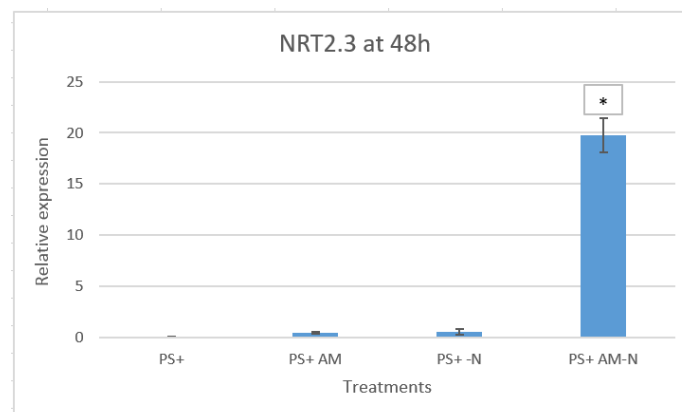


Figure 29. Relative expression of *NRT2.3* in prosystemin overexpressor plants in the absence of infection. Different letters mean significant differences, ANOVA and LSD test (95%).

*NRT2.3* accumulation was significantly increased in mycorrhizal PS+ plants after N starvation compared with the rest of the treatments at timepoint 0.

This suggest that mycorrhiza is inducing a notably accumulation of *NRT2.3* when the plant has been subjected to a transient N starvation.

### 5.3.2. Effect of prosystemin overexpression in MIR after infection and crosstalk with a transient N depletion.

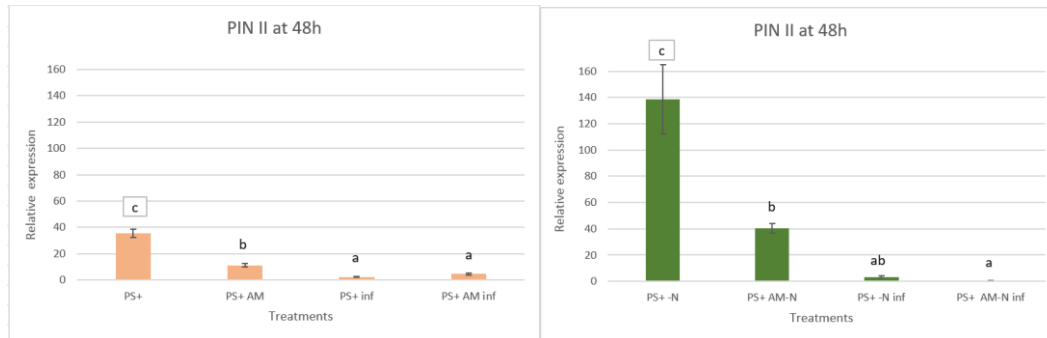


Figure 30. Relative expression of PINII in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

*PINII* accumulation was lower in mycorrhizal plants (PS+ AM) than in the control (PS+). Mycorrhizal and non-mycorrhizal infected plants showed a similar accumulation but lower levels of *PINII* expression compared to non-infected plants (figure 30). Therefore, expression of *PINII* in PS+ plants was not induced neither by infection nor by mycorrhiza, suggesting that *PINII* has not a defensive role against this pathogen. However, it seems that mycorrhization is buffering the response produced by the N starvation in PS+ -N lowering the accumulation of *PINII* to levels similar to the control (PS+) in PS+AM-N.

In response to nitrogen starvation the same pattern was followed. *PINII* expression was more induced in non-infected control plants subjected to N starvation in both control and mycorrhizal plants compared to the same treatments with the complete nutrition (Figure 30). After N starvation infected plants showed lower levels in the expression of *PINII* compared to non-infected plants.

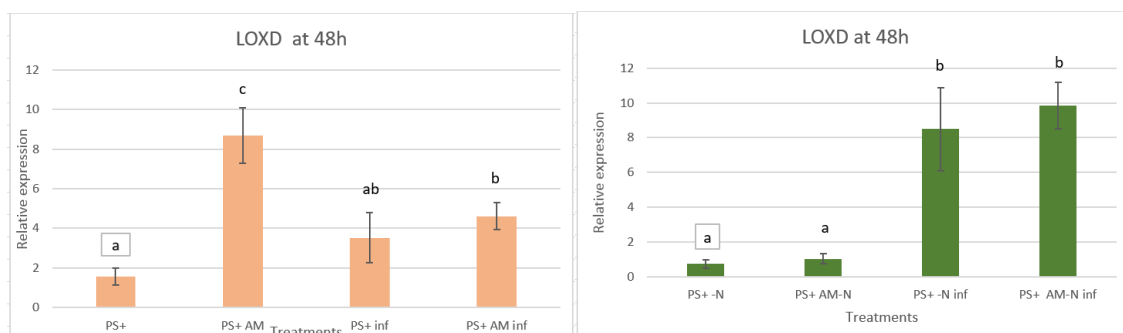


Figure 31. Relative expression of LOXD in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

*LOXD* expression was increased in mycorrhizal plants about 8-fold compared to the control. After the infection, *LOXD* expression was also higher than in the control especially in

mycorrhizal plants (Figure 31). Here we can observe the effect of the mycorrhiza in the expression of *LOXD*. Mycorrhiza induced accumulation of *LOXD* in both healthy and infected plants.

After N starvation the infection notably increased the expression of *LOXD* in both mycorrhizal and non-mycorrhizal plants compared with the non-infected plants (figure 31). The mycorrhiza slightly increased the accumulation of *LOXD* in infected and non-infected plants but significant differences are not shown. This results suggest the important role of *LOXD* against the infection of the pathogen.

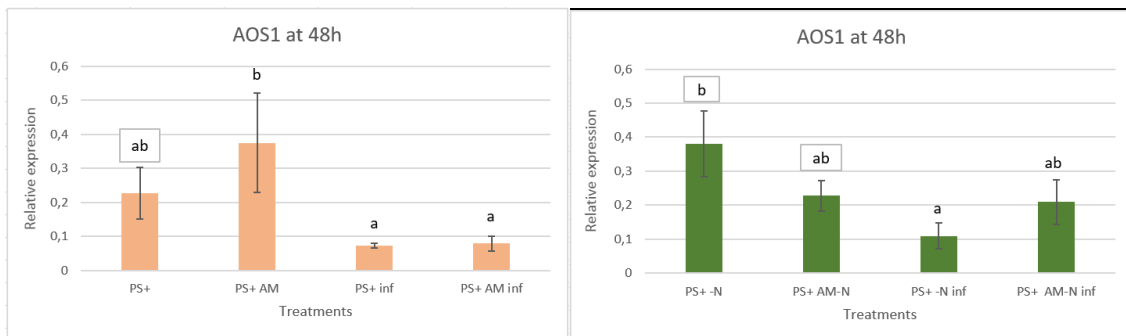


Figure 32. Relative expression of *AOS1* in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mychorrization did not lead to significantly higher levels of *AOS1* expression in healthy plants. However, the infection reduced the expression of *AOS1* in both PS+inf and PS+AM inf compared with the mycorrhiza alone.

In response to N starvation *AOS1* accumulation was similar in all the treatments except for non-mycorrhizal plants after infection that showed lower levels of *AOS1* (Figure 32).

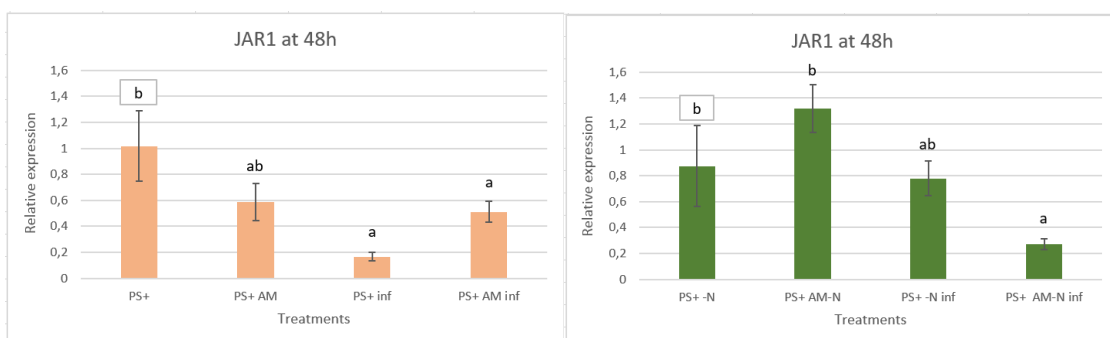


Figure 33. Relative expression of *JAR1* in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Infection reduced the *JAR1* expression compared to the control PS+ plants. Mycorrhization seems not to have an influence in the *JAR1* gene expression (Figure 33).

In plants subjected to N starvation slightly induced *JAR1* accumulation in healthy plants but there are not significant differences. The infection lowered the levels of *JAR1* in both mycorrhizal and non-mycorrhizal plants (Figure 33).

The expression of *JAR1* in PS+ mutant at 48h after pathogen inoculation follows the same pattern as in the wildtype at the same timepoint.

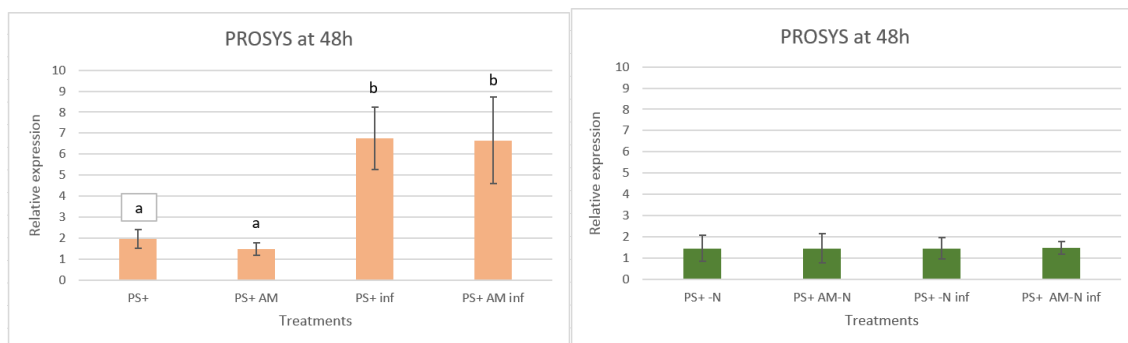


Figure 34. Relative expression of PROSYS in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

*PROSYS* was higher accumulated in infected plants than in non-infected plants in both mycorrhizal and non-mycorrhizal plants. The two infected treatments (PS+ inf and PS+AM inf) showed 3 times more accumulation of *PROSYS* than the control(PS+) and the sole mycorrhization (PS+AM) (Figure 34) suggesting the role of prosystemin in defense against the pathogen infection.

After N starvation *PROSYS* levels remain at very similar levels in all treatments without significant differences among them, lower than the levels in plants with the complete nutrition (Figure 34).

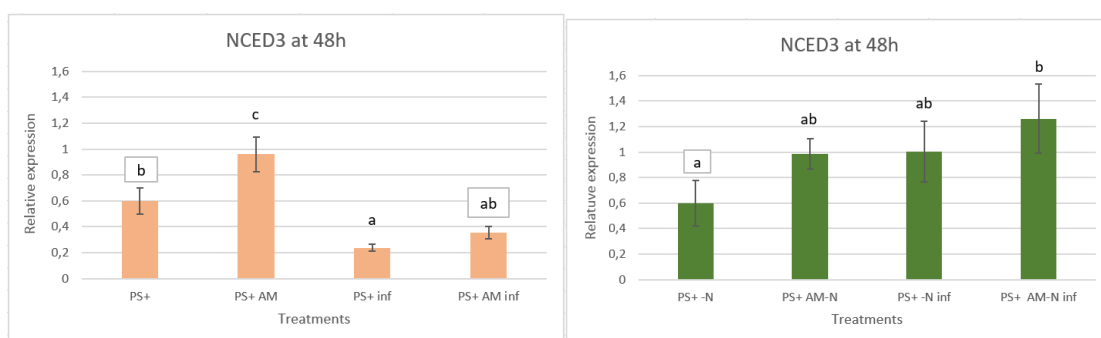


Figure 35. Relative expression of NCED3 in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Mycorrhization alone produced an increase in *NCED3* expression compared to the control in PS+ mutant at 48h in non-infected plants. In response to pathogen inoculation levels of *NCED3* were lowered in both PS+ inf and PS+AM inf especially in PS+ inf (figure 35).

After a transient N starvation *NCED3* expression did not have a changing effect compared to the control neither in healthy nor in infected plants. However, we can see a significantly higher induction of *NCED3* in PS+AM-N inf plants compared to the control (PS+-N) (Figure 35).

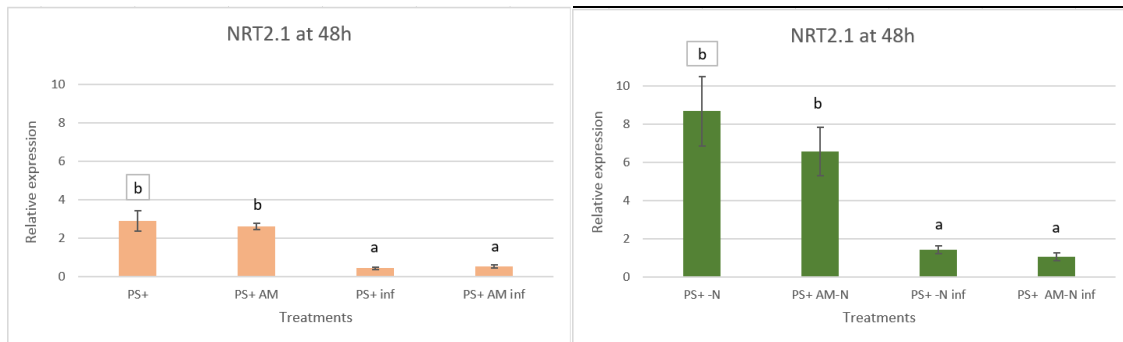


Figure 36. Relative expression of NRT2.1 in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Infection produced a reduction in the level of *NRT2.1* expression in both mycorrhizal and non-mycorrhizal plants. In this case the mycorrhiza is not having any effect in the accumulation of NRT2.1 in plants with complete nutrition, the levels are similar to the controls: PS+AM has the same levels as PS+ and PS+AM inf has the same levels as PS+inf (Figure 36).

The same pattern can be observed in plants subjected to a transient N starvation but with higher levels of NRT2.1 accumulation compared with plants with complete nutrition (Figure 36). Noteworthy, N starvation activates NRT2.1 expression as it happened in BB what suggests that Prosystemin may have not an influence in the regulation of this transporter. We can also see that the mycorrhiza slightly reduced the accumulation of NRT2.1.

This results suggest that the overexpression of prosystemin is not producing a mycorrhizal effect on the expression of *NRT2.1*.

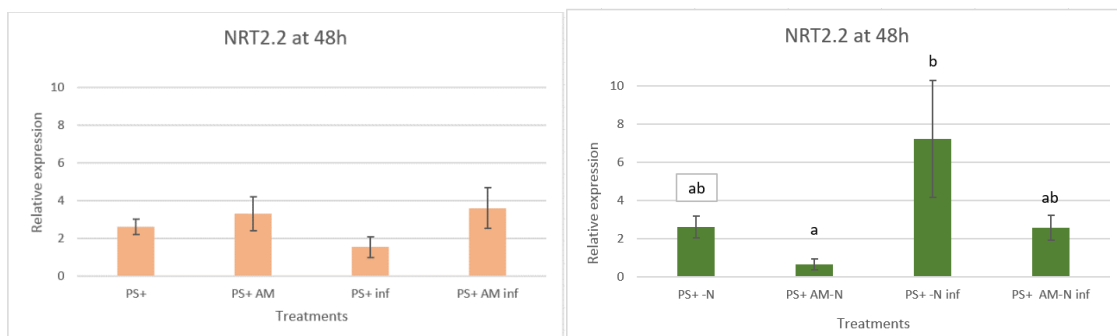


Figure 37. Relative expression of NRT2.2 in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

Expression of *NRT2.2* did not change in the different treatments of plants with the complete nutrition neither in healthy plants nor in infected plants (Figure 37).

In plants subjected to a N starvation we can see that the mycorrhiza slightly lowers the expression of *NRT2.2* in both healthy and infected plants. The same happened with the *NRT2.1* expression but in the *NRT2.2* expression this effect is more pronounced.

This suggest that the mycorrhiza is buffering the effect produced in response to a transient N depletion.

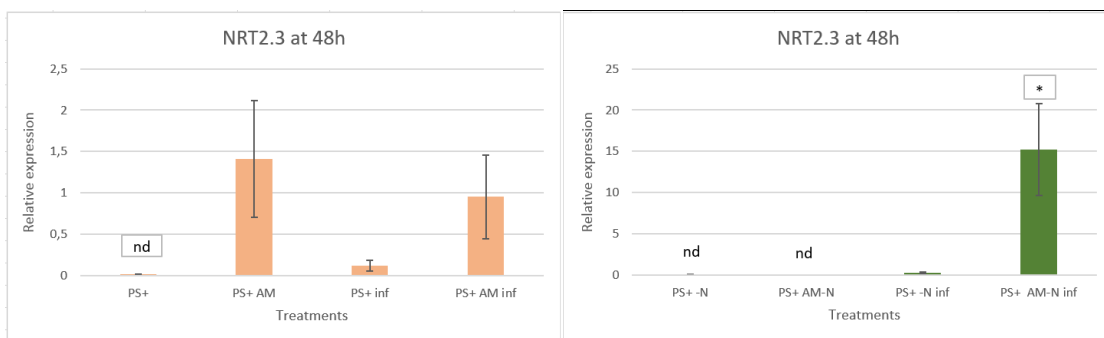


Figure 38. Relative expression of NRT2.3 in prosystemin overexpressor plants at 48h post infection. Different letters mean significant differences, ANOVA and LSD test (95%).

There were not significant differences in the expression of *NRT2.3* in plants with the complete nutrition at 48h as it happened with *NRT2.2*.

After N starvation we only obtained data of *NRT2.3* expression in the infected plants. The same as happened in the wildtype. Mycorrhizal plants showed significantly higher accumulation of *NRT2.3* in response to N starvation (Figure38).

This results make us think that the plant does not need to induce the nitrate transporter when it has a correct nutrition. In contrast these gene is induced when there is a N depletion, and the mycorrhiza makes this induction more pronounced.

## **DISCUSSION**



## 6. Discussion

A phenotype analysis was carried out by other members of the lab, which showed that mycorrhizal plants were more resistant to *B.cinerea* than non-mycorrhizal plants. Following a transient nitrogen starvation, the plants were more susceptible but the plants colonized by *R. irregularis* were still less affected by the pathogenic fungus than the controls (non-mycorrhizal plants), suggesting that MIR was still working although its efficiency was reduced. In this study we are going to discuss the molecular mechanisms that regulate the effects observed in the phenotype analysis in the plant aerial parts.

JA signalling pathway is known to play an important role in defense against necrotrophic pathogens and in the mediation of AMF-primed defense in tomato plants (Song et al 2015). In the present study we analyse the expression three important genes of the JA biosynthesis pathway, *LOXD*, *AOS* and *JAR1*.

Mycorrhiza itself induced the accumulation of two of these genes before the infection, showing higher expression than the control. It induced accumulation of *LOXD* and *AOS1* at timepoint 0 in wildtype genotype, thus providing the plant a state where the plant will be more able to mount an efficient defensive response after pathogen attack.

*LOXD* showed a clear induction after the necrotrophic pathogen infection. This gene was accumulated following the pathogen inoculation compared with non-infected plants. This occurred in both genotypes, *BetterBoy* and the *35S::PS* mutant, at 48h after pathogen inoculation and it was also observed in plants subjected to a transient N starvation. Mycorrhiza colonization resulted in many cases at a positive inductor of *LOXD* in plants infected with the pathogen, increasing its accumulation compared to non-mycorrhizal.

In contrast with the analysed *LOXD* expression, we could not find the same induction effect of the infection and the mycorrhiza colonization in the expression of *AOS1* and *JAR1*. *AOS1* induction promoted by the mycorrhiza was observed only at timepoint 0 suggesting that *AOS1* may function in plant defense being accumulated before the infection in order to prepare the plant to a possible future attack thus priming the plant. *JAR1* may have not been induced due to this gene participates in late steps of the JA biosynthesis pathway encoding for a JA bioactive conjugate, jasmonate isoleucine (JA-Ile) (De Domenico et al., 2012).

The gene Proteinase inhibitor 2 (*PIN II*) plays a role in defense against plant tissue damage produced by herbivory insects as well as is a JA marker due to its synthesis is regulated by this phytohormone (Howe, 2004). In our results we observed that *PINII* expression is not induced by the infection. The prosystemin overexpression mutant the expression of *PINII* dramatically increased in the absence of infection compared to the wildtype. This is explained because the systemin is involved in the systemic induction of JA (Coppola et al., 2014), so a prosystemin

overexpression may lead to an overaccumulation of systemin that triggers an accumulation of JA. Therefore, accumulation of *PINII* can be induced.

Systemin has been reported to be involved in tomato resistance against necrotrophic pathogens (El Oirdi et al. 2011). To determine its possible role in defense against *B. cinerea* we have analysed the expression of the *PROSYS* gene in tomato *BetterBoy* and prosystemin overexpressor mutant genotypes. We did not observe an effect of the infection in the induction of *PROSYS* in the wildtype. However, infection increased *PROSYS* expression in PS+ plants at 48h after pathogen inoculation, suggesting that systemin is participating in the defense against the necrotrophic pathogen in the background PS+. In our study, an influence in the mycorrhization in the accumulation the *PROSYS* gene was not observed.

Abcisic acid (ABA) is a plant phytohormone that plays a role in response to different stresses including defense-related responses (Pieterse et al., 2009). To analyse the possible influence of ABA in MIR we analysed the expression of *NCED3*, a gene that participate in the ABA synthesis pathway. Some studies have reported that ABA is induced in plant roots after mycorrhiza colonization and may play a role in MIR signalling (Cameron, 2013). In our study the *NCED3* expression followed alternate patterns at different timepoints and treatments under the same environmental conditions. It seemed that the mycorrhization produced a more pronounced response of *NCED3* accumulation to both infection or N starvation, either alone or in combination by altering the gene expression levels. Previous studies have reported that promotion of ABA depend on the stage of the plant-pathogen interaction and its potential role in systemic MIR signal may be transient (Cameron et al., 2013).

As commented above, AMF I colonization has been reported to help the plant to cope with biotic and abiotic stresses (Jung et al., 2012). Mycorrhiza can produce a buffering effect in response to some stresses. In response to the stress the plant tends to highly induce the expression of specific genes. Mycorrhizal colonization of these plants may act differently in response to the same stress by maintaining the gene accumulation to the same levels as the control (without stress stimulus). In our study we have seen this buffering effect in response to a transient nitrogen depletion. It happened with *PINII* at timepoint 0 in wildtype plants and at 48h in prosystemin overexpressor plants. This effect was also observed in the expression of *NRT2.2* in PS+ plants at 48h timepoint in response to transient N depletion.

In contrast, in some cases was the N starvation which was hampering the positive effect produced by the mycorrhization. We could observe this effect clearly in the expression of *LOXD* and *AOS1* in the absence of infection (0h) in the wildtype genotype.

As we know from previous studies mycorrhizal associations improve the plant supply of inorganic nutrients specially phosphate and nitrate (Smith et al., 2011). After analysing the expression of *NRT2.1* and *NRT2.2*, we observed that mycorrhizal colonization itself induced an enhanced accumulation of these genes at basal levels in both the wildtype and the PS+ mutant, thus contributing to increase the transporter activity in the nitrate uptake.

We could see that that plants subjected to a transient N depletion also showed a higher accumulation of *NRT2.1* in PS+ plants at timepoint 0 and of both *NRT2.1* and *NRT2.2* at timepoint 48h. This result is correlated with the fact that *NRT2* gene family are high affinity transporters that are activated when the concentration of nitrate in the soil is low (Gojon et al. 2011). Noteworthy, these genes may also be acting as a trancceptor by signalling to the plant the low levels of nitrogen in the soil. On the other hand, *NRT2.1* was not induced in plants normally fertilized when inoculated with the pathogen. When the concentration of nitrogen in the soil is sufficient the plant does not need to activate a high affinity transporter and instead it may be allocating its energy in the activation of defense mechanism in order to defense itself against the pathogen.

Some authors have determined that the *NRT2.3* gene is mostly induced in rizhodermal and pericycle cells in roots (Hildelbrandt et al 2002; Fu et al., 2015). *NRT2.3* gene expression levels were difficult to detect in our study due its low expression level in the shoots, reasonable in some experimental conditions the expression levels were below the detection limits even when we tried to optimize the qPCR using a range of annealing temperatures. However, we could observe some cases where the *NRT2.3* was significantly induced. Infected plants seemed to induce activity *NRT2.3* specially when plants were subjected to N starvation in wildtype plants at 48h timepoint. In PS+ mutants, mycorrhization had a significant influence by increasing the *NRT2.3* expression in plants subjected to N starvation at 0h and in infected plants subjected to N starvation at 48h. This suggest that the *NRT2.3* expression is highly activated in response to N starvation as well as in response to pathogen infection and that the mycorrhiza makes those responses more pronounced.

To completely understand the mechanisms that produced what we found in the phenotype analysis further research is needed, we should analyse other defense-mechanisms that plants use in order to respond to the pathogen challenging such as metabolites, which were not analysed in the current study.

## **CONCLUSION**

## 7. Conclusion

Arbuscular mycorrhizal fungi are natural worldwide plant-microorganism associations that are being studied as a potential alternative to agrochemicals in crop management and crop production. AMF may contribute to a more sustainable agriculture.

This research study has improved our understanding of the molecular mechanisms underlying the mycorrhiza induced resistance and its crosstalk with an abiotic stress in tomato leaves. Genes involved in the oxylipin pathway appeared to play an important role in MIR against the necrotrophic fungus *B. cinerea*. Mycorrhization produced in some cases an induction of these genes providing the plant a primed state. A transient N depletion lead to a less efficient MIR and an induction of nitrate transceptors but in many cases we could observe that mycorrhiza had a buffering effect over this abiotic stress. The phytohormone abscisic acid (ABA) seems not to play a role in MIR.

However, further research is needed. To complete this study an interesting purpose would be to carry out a metabolome analyses in order to amplify our knowledge in plant defense mechanisms induced by arbuscular mycorrhizal fungi.

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