University of Granada

Doctoral Programme in Fundamental and Systems Biology

Consejo Superior de Investigaciones Científicas (CSIC)

Estación Experimental del Zaidín (EEZ)
Department of Soil Microbiology and Symbiotic Systems
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Role of nitric oxide signalling in beneficial and pathogenic plant–fungal interactions

Leyre Pescador Azofra

DOCTORAL THESIS

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Role of nitric oxide signalling in beneficial and pathogenic plant–fungal interactions

Memory presented by Leyre Pescador Azofra, graduated in Biology and Science and Food Technology, to aspire to Doctor in Biology (with mention "International Doctor")

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V°B° Thesis supervisors

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La doctoranda / *The doctoral candidate* Leyre Pescador Azofra y las directoras de la Tesis / *and the Thesis supervisors:* María José Pozo Jiménez y/*and* María C. Romero Puertas

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Granada, 15 de junio de 2020

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ABSTRACT

Plants roots interact with a plethora of microorganisms. Whilst many of the interactions are detrimental, others benefit the plants by improving their growth and stress tolerance. Because of this, plants have evolved different mechanisms to differentiate among them and adjust their responses to promote the beneficial and restrict the detrimental ones. Signalling molecules as nitric oxide (NO) have a key role in the regulation of the interaction between plants and microorganisms.

NO is a gaseous lipophilic molecule that crosses cell membranes and plays multiple signalling roles in very short periods. It is involved in a wide range of processes along all developmental stages of the plant. Regarding plant–fungal interactions, increments in NO levels in the plant have been reported in response to both pathogenic and beneficial fungi. Nevertheless, little was known about NO regulation during the interaction with one of the most important soilborne fungal pathogens, *Fusarium oxysporum*. On the other hand, data regarding the role of NO in beneficial fungal interactions were also very scarce. Furthermore, there were no studies that compare interactions with both beneficial and pathogenic fungi to investigate the role of NO in the onset of both types of interplays.

The importance of beneficial fungi has raised as biostimulants and protectors of plants health as an alternative to traditional pest chemical control in crop management strategies. Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that are associated with the roots of most vascular plants. The fungus colonizes plants roots biotrophically, improving plant nutrition and their response to several biotic and abiotic stresses. Root colonization by AMF is a very fine–tuned process where the fungus is actively accommodated by the plant in the root cortex, and therefore this process relies on a finely regulated molecular dialogue. On the other hand, *Trichoderma* spp. are free–living soilborne fungi that colonize plants roots. They bring several benefits to the plants as priming of plant defenses, allowing a faster and more intense defense response to a subsequent pathogen attack leading to induced systemic resistance (ISR). Recently, it has also been

reported that *Trichoderma* volatile compounds can trigger ISR response without physical contact of the fungus with plant roots.

The present Doctoral Thesis aims to determine the regulatory role of NO in the establishment of beneficial and pathogenic interactions of plants with soil fungi and to decipher its possible implication in the ISR against pathogenic fungi triggered in plants by *Trichoderma* volatile compounds.

This PhD Thesis is focused on root interactions. In Chapters I and II we have compared the regulation of NO levels in tomato plants during its interaction with root colonizing beneficial fungi (*Rhizophagus irregularis* –obligate symbiont– and *Trichoderma harzianum* –free–living rhizospheric fungi–) and the root pathogen *Fusarium oxysporum*. This has been approached by performing time–course analyses of NO accumulation and the regulation of the tomato phytoglobins (PHYTOGB), as they are key pieces for controlling NO levels in plants.

In Chapter I we tried to decipher the role of NO in the early signalling that leads to the establishment of the arbuscular mycorrhizal symbiosis or the pathogenic interaction with *F. oxysporum* in tomato. Firstly, we showed that only tomato *PHYTOGB1* was responsive to NO. Then, we demonstrated that NO signatures differ between both interactions since the early steps of the interaction. We showed that NO levels are controlled by *PHYTOGB1* in the beneficial interaction at all analyzed time points. In contrast, during the interaction with the pathogen, the gene is downregulated driving to an uncontrolled increase in NO levels at later stages of the interaction. We also observed that the spatial distribution of the accumulated NO also depends on the type of interaction. While in response to the AMF the accumulation of NO was restricted to the outer cell layers and root hairs, the pathogen triggered NO accumulation in the entire root. Moreover, we found that both overexpressing or silencing *PHYTOGB1* gene in hairy roots lead to a deregulation of NO levels that resulted in altered mycorrhization and pathogen infection phenotypes. Hence, our results revealed that fine–tuned NO accumulation is required for the correct establishment of AM symbiosis and that *PHYTOGB1* is

induced during the early stages of the interaction to control NO levels to promote and control the symbiosis.

In Chapter II we tried to expand the study on NO signalling to other beneficial plant–fungal interactions, in this case with a well–characterized free–living biocontrol fungus. For this purpose, we followed a similar approach as in Chapter I, using the system tomato–*Trichoderma harzianum* T–78. We found that *PHYTOGB1* was the only *PHYTOGB* gene that was differentially regulated during the interaction. We discovered that the early interaction of *Trichoderma* and tomato plants triggered an early and transient burst of NO that also elicited the up–regulation of *PHYTOGB1*. In contrast with the results shown in Chapter I, *PHYTOGB1* was up–regulated along all the analyzed time. These results point out that different beneficial fungi trigger different NO signatures during the first steps of the interaction with plants. Besides that, we found that *PHYTOGB1* was upregulated while NO levels were maintained at a basal level in a well–established plant–*Trichoderma* symbiosis. We suggest that this regulation might favour the mutualistic symbiosis as the strategy followed by *Trichoderma* to colonize roots is mostly based on the early repression of plant immune responses to avoid the plant defenses.

Then, we reviewed and synthesized the existing literature regarding the NO signalling functions in plant–fungal interactions and discuss our results in this context in Chapter III. We tackled both above and belowground interactions concerning beneficial and pathogenic fungi. By compiling different published experimental data and discussing them with our results, we proposed different models regarding NO functions in the different interactions. Those models indicate that different NO regulation patterns point out different functions in the plant interaction with pathogenic or mutualistic fungi.

Finally, we further explored the role of NO in one of the most interesting benefits of plant interaction with beneficial fungi as the induction of systemic resistance (Chapter IV). NO has been proposed to be a key regulator in the ISR

response elicited by diverse beneficial microbes. Indeed, the root–specific gene *MYB72*, coding for a transcription factor that acts as a node of convergence in ISR elicited by several beneficial microbes is upregulated by NO. It has been recently demonstrated that *Trichoderma* volatile compounds can trigger ISR in plants, but the role of NO in this process remained unexplored. To address this question, we used the system *Trichoderma* volatile compounds–*Arabidopsis thaliana*–*Botrytis cinerea*. We found that plant perception of *T. harzianum* (T–78) or *T. asperellum* (T–34) volatiles triggered a burst of NO and also upregulated *MYB72*. We show that *MYB72* and NO signalling are mandatory for *Trichoderma* volatile compounds–mediated ISR against the shoot fungal pathogen *B. cinerea*. Additionally, we discovered the implication of NO signalling on *Trichoderma* volatile compounds–mediated priming of Arabidopsis plants for enhanced expression of defense genes, thus protecting plants against *B. cinerea* infection.

Altogether, in this PhD Thesis we have evidenced that NO harbours an important signalling role during plant–fungal interactions. We conclude that NO is a key signal in the establishment and fine–tuning of both mutualistic and pathogenic interactions, being its accumulation a common feature among them. However, the signature triggered differs quantitatively and in its spatio–temporal distribution in the different interactions. Indeed, this NO signature not only differs between a beneficial and a pathogenic fungus but also between different fungal mutualistic interactions. We further proved that these differences in NO signatures are concomitant with a differential transcriptional regulation of *PHYTOGB1*. This gene seems to exert a key role in controlling NO levels during the onset and in well–established beneficial interactions. Regarding pathogenic interplays, we have found that fungal pathogens might down–regulate *PHYTOGB1*, most likely to increase NO levels and promote favourable conditions for the invasion. Besides that, we further have confirmed that NO accumulation is triggered in Arabidopsis roots in response to *Trichoderma* volatile compounds. This accumulation is required for the upregulation of *MYB72*, that exerts a pivotal role in *Trichoderma*

volatile compounds–mediated ISR response. Thus, NO signalling acts upstream of *MYB72* and it is essential for triggering ISR.

RESUMEN

Las raíces de las plantas interaccionan con un sinfín de microorganismos. Mientras muchas de estas interacciones son perjudiciales para la planta, otras, por el contrario, le reportan numerosos beneficios que se pueden traducir en una mejora del crecimiento o de tolerancia a estreses. En consecuencia, las plantas han desarrollado diversos mecanismos para poder diferenciar entre microorganismos beneficiosos y perjudiciales y ajustar su respuesta ante ellos, favoreciendo las interacciones beneficiosas y restringiendo las perniciosas. Las moléculas señalizadoras como el óxido nítrico (NO) juegan un papel fundamental en la regulación de las interacciones entre plantas y microorganismos.

El NO es una molécula gaseosa de naturaleza lipofílica que es capaz de atravesar las membranas celulares, llevando así a cabo múltiples funciones señalizadoras en cortos periodos de tiempo. De esta manera, el NO está implicado en numerosos procesos durante todos los estadios de desarrollo de las plantas. En las interacciones de las plantas con hongos se ha comprobado que hay un incremento de NO en respuesta tanto a hongos beneficiosos como patogénicos. No obstante, poco se sabía acerca del NO en interacciones con uno de los hongos patógenos de suelo más importantes, como lo es *Fusarium oxysporum*. Por otro lado, los datos en relación al papel del NO en interacciones fúngicas beneficiosas eran muy escasos. Además, no había estudios comparativos sobre el papel del NO en el inicio de las interacciones fúngicas beneficiosas y patogénicas.

Por otro lado, el interés por los hongos beneficiosos ha ido creciendo en los últimos tiempos por su potencial como bioestimulantes y bioprotectores de plantas, ya que constituyen una alternativa sostenible al tradicional control químico de plagas. Entre estos organismos beneficiosos destacan los hongos micorrícico arbusculares (HMA), simbiontes obligados asociados con la mayoría de plantas vasculares. Estos hongos colonizan las raíces de forma biótrofa, mejorando la nutrición de la planta y su respuesta a diferentes estreses tanto bióticos como abióticos. El proceso de colonización de la raíz por los HMA requiere una alta regulación dado que el hongo es activamente acomodado en el córtex de la raíz por la planta, suponiendo este proceso un complejo diálogo molecular entre ambas

partes. Por otro lado, *Trichoderma* spp. son hongos de suelo de vida libre que colonizan las raíces confiriendo diversos beneficios a las plantas hospedadoras, entre ellos el *priming* de sus defensas. Favorecen que la planta desarrolle una respuesta más rápida e intensa tras el ataque por patógenos, dando lugar a la resistencia sistémica inducida (RSI). Recientemente ha sido demostrado que también los compuestos volátiles producidos por *Trichoderma* son capaces de producir esta RSI sin que exista un contacto físico entre el hongo y las raíces de la planta.

La presente Tesis Doctoral pretende determinar el papel regulador del NO en el establecimiento de las interacciones tanto beneficiosas como patogénicas de hongos del suelo con las raíces de las plantas. Asimismo, trata de elucidar la posible implicación del NO en la RSI producida por volátiles de *Trichoderma* frente a hongos patógenos.

Esta Tesis se centra en las interacciones de los hongos con las raíces de las plantas. En los Capítulos I y II hemos comparado la regulación de los niveles de NO en raíces de plantas de tomate durante las interacciones con los hongos beneficiosos como *Rhizophagus irregularis* (simbionte obligado) o *Trichoderma harzianum* (simbionte de vida libre); y el patógeno fúngico de raíz *Fusarium oxysporum*. Con este objetivo, hemos analizado la dinámica de acumulación de NO durante las primeras etapas de la interacción de las raíces de tomate con los hongos beneficosos o el patógeno. Para ello, se han llevado a cabo análisis de la acumulación de NO a lo largo del tiempo, así como de la regulación de los genes de fitoglobinas (*PHYTOGB*), al ser éstas piezas clave en la regulación de los niveles de NO en plantas.

En el Capítulo I hemos tratado de arrojar luz sobre el papel del NO en los primeros estadios de la interacción que dan lugar al establecimiento de la simbiosis micorrícica arbuscular o de la interacción patogénica con *F. oxysporum* en tomate. En primer lugar, comprobamos que solo el gen *PHYTOGB1* de tomate respondía al NO. Luego demostramos que los patrones de regulación de NO diferían entre ambas interacciones desde los primeros estadios. Demostramos también que los niveles de NO eran controlados por *PHYTOGB1* a todos los tiempos analizados en el caso de la

interacción beneficiosa. Por el contrario, durante la interacción con el patógeno, la expression del gen *PHYTOGB1* estaba inhibida, dando lugar a un incremento incontrolado de los niveles de NO en estadios más tardíos. Asimismo, observamos que la distribución espacial del NO también difería según el tipo de interacción. Mientras que en respuesta al HMA la acumulación de NO estaba restringida a las capas más externas de células de la raíz y a los pelos radicales, en la interacción con el patógeno se daba una acumulación de NO por toda la raíz. Por otro lado, también exploramos la función de *PHYTOGB1* en la regulación de las interacciones mediante una aproximación genética. Generamos plantas de tomate con raíces sobreexpresoras de *PHYTOGB1* o silenciadas para este gen. Encontramos que ambas líneas (sobreexpresora y silenciada) presentaban una desregulación de los niveles de NO que resultó en fenotipos tanto de micorrización como de patogénesis alterados. Por ello, nuestros resultados revelan que para el correcto establecimiento de la simbiosis micorrícico arbuscular es necesario un fino control de la acumulación de NO durante los primeros estadios de la interacción, y que *PHYTOGB1* es inducido durante estos inicios para controlar los niveles de NO con el fin de facilitar y controlar la simbiosis.

En el Capítulo II, tratamos de expandir el conocimiento de la señalización por NO a otras interacciones beneficiosas de las plantas con hongos del suelo. En este caso, con un hongo bien estudiado y conocido de vida libre, empleado también en biocontrol. Para ello, seguimos una metodología similar a la empleada en el Capítulo I, usando el sistema tomate–*Trichoderma harzianum* T–78. Encontramos que el gen *PHYTOGB1* era el único gen *PHYTOGB* diferencialmente regulado durante la interacción. A su vez, comprobamos que la interacción temprana de *Trichoderma* con las raíces de tomate producía un pico de NO temprano y transitorio, concordante con el incremento en la expresión de *PHYTOGB1*. Contrariamente a los resultados del Capítulo I, ahora *PHYTOGB1* se mantuvo inducido durante todos los tiempos analizados. Estos resultados ponen de manifiesto que diferentes hongos beneficiosos son capaces de producir diferentes patrones de regulación de NO durante los primeros estadios de la interacción con plantas. A su vez, encontramos que *PHYTOGB1* se mantenía inducido mientras que los niveles de NO se encontraban

en niveles basales en plantas con simbiosis bien establecida con *Trichoderma*. Sugerimos que esta regulación puede favorecer la simbiosis mutualista dado que la estrategia que sigue *Trichoderma* para colonizar las raíces se basa principalmente en la represión temprana de la respuesta inmune de la planta con el fin de evitar sus defensas.

Estos resultados permitieron completar la información disponible sobre las funciones del NO en interacciones planta–hongo. En el Capítulo III revisamos y sintetizamos la información existente en la literatura referente a las funciones de señalización del NO en las interacciones de las plantas con hongos. Para ello, abordamos tanto las interacciones de raíz como de parte aérea, considerando tanto a hongos beneficiosos como patogénicos. Recopilando los datos experimentales ya publicados y discutiéndolos con nuestros propios resultados, en este capítulo propusimos diferentes modelos para explicar las posibles funciones del NO en los distintos tipos de interacciones según el estilo de vida del hongo. Dichos modelos indican que diferentes patrones de regulación del NO muestran diferentes funciones en las interacciones con hongos beneficiosos o patogénicos.

Finalmente, decidimos investigar el papel del NO en uno de los beneficios más interesantes aportados a las plantas por la interacción de estas con hongos beneficiosos, como lo es la RSI (Capítulo IV). El NO ha sido propuesto como una molécula clave en la RSI producida por diversos microbios beneficiosos. Curiosamente, el gen específico de raíz *MYB72*, que codifica para un factor de transcripción que actúa como un nodo de convergencia en la RSI por diferentes microbios beneficiosos, es inducido por NO. A su vez, se ha demostrado recientemente que los compuestos volátiles producidos por *Trichoderma* son capaces de producir RSI en plantas. Sin embargo, el papel del NO en este proceso no se había explorado aún. Para arrojar luz sobre esta cuestión usamos el sistema: volátiles de *Trichoderma–Arabidopsis thaliana–Botrytis cinerea*. Descubrimos que la percepción de los volátiles de *T. harzianum* (T–78) o de *T. asperellum* (T–34) por la planta inducía la acumulación de NO y también inducía el gen *MYB72*. Demostramos que tanto *MYB72* como la señalización por NO son necesarios para el

establecimiento de la RSI mediada por volátiles de *Trichoderma* frente al patógeno foliar *B. cinerea*. Asimismo, también demostramos la implicación de la señalización por NO en el *priming* mediado por compuestos volátiles de *Trichoderma* para la expresión aumentada de genes de defensa, protegiendo de esta manera a las plantas contra la infección por *B. cinerea*.

En conjunto, en esta Tesis Doctoral evidenciamos que el NO ejerce un importante papel señalizador durante las interacciones planta–hongo. Concluímos que el NO es una molécula clave en el establecimiento y la regulación de interacciones mutualistas y patogénicas, siendo una característica de ambas. No obstante, el patrón de regulación de NO que produce cada interacción difiere tanto cuantitativamente como en su distribución espacio–temporal. De hecho, este patrón de regulación de NO no solo es diferente según la naturaleza beneficiosa o patogénica de la interacción, sino que además difiere entre las dos interaciones fúngicas de simbiosis mutualista estudiadas. También hemos comprobado que las diferencias en estos patrones de regulación de NO son concordantes con diferentes niveles transcripcionales de *PHYTOGB1*. Este gen parece que ejerce un rol clave en el control de los niveles de NO durante el inicio de las interacciones, así como en las interacciones beneficiosas bien establecidas. En cuanto a las interacciones patogénicas, hemos mostrado que los patógenos fúngicos podrían reprimir *PHYTOGB1*, probablemente con el fin de promover las condiciones favorables para su invasión. Además, también hemos confirmado que el NO se acumula en respuesta a los compuestos volátiles de *Trichoderma*. Esta acumulación es requerida para la inducción de *MYB72*, gen que ejerce un papel central en la RSI mediada por volátiles de *Trichoderma*. A su vez, hemos demostrado que el NO es esencial y actúa aguas arriba de *MYB72* en la RSI mediada por volátiles de *Trichoderma*.

INTRODUCTION

A. Nitric oxide

Nitric oxide is a free radical that transforms into more stable molecules by gaining or losing one electron. It has a short half–life and rapidly reacts with oxygen to form nitrogen dioxide, that converts into nitrite and nitrate (Umbreen *et al.*, 2018). Moreover, this gaseous free radical diffuses quickly through cell membranes due to its lipophilic nature and plays an important role in cell to cell signalling/communication (Beligni and Lamattina, 2001; Brouquisse, 2019).

Joseph Priestley described first NO as a colourless gas with an *in vivo* lifespan of 6 to 10 seconds (Priestley, 1772). In 1977, Murad showed that NO–releasing compounds such as nitroglycerin or sodium nitroprusside (SNP) were able to cause vasodilatation of smooth muscle and also to stimulate guanylate cyclase, therefore increasing cGMP levels in tissues (Katsuki *et al.*, 1977). Furchgott and Zawadzki (1980) discovered that endothelial cells produce the endothelium–derived relaxing factor (EDRF) which acts relaxing vascular smooth muscle. Parallel experiments by Moncada and colleagues hypothesized that EDRF might be a free radical (Moncada and Higgs, 2006). Some years later, Ignarro *et al*. (1988) and Furchgott (1988), based also on the studies of Moncada and collaborators, suggested that the EDRF was NO. Later on, NO was declared molecule of the year 1992 by Science magazine (Fig. 1) and in 1999 Furchgott, Ignarro and Murad were awarded by the Nobel Prize in Physiology and Medicine for their discoveries on the importance of NO, forgetting Moncada for the award.

Fig. 1. Cover of Science Magazine, year 1992.

In the second half of the 1980s, NO was reported to be produced from plant tissues, being detected as a side product from *in vivo* nitrate reductase activity assays (Dean and Harper 1986; Klepper, 1987). Since then, many studies have aimed to decipher the regulatory roles of NO in plants. Nevertheless, it was not till the end of the last century when the first reports on NO regulatory roles in plant–pathogen interactions were published (Delledonne *et al.* 1998; Durner *et al.*, 1998). From that point, NO has been thoroughly studied in plants, and it is now recognized as a signal molecule with a huge range of functions in plants. However, despite being intensively studied, neither NO production nor its signal transduction mechanisms are fully elucidated (Astier *et al.*, 2018).

A.1. NO production in plants

Due to the chemical nature of NO, in which the oxidation and reduced state of N may vary (nitrite, nitrate, amino groups, ammonium), plants can produce NO either through reductive or oxidative mechanisms.

A.1.1. Reductive routes of NO synthesis

The reductive pathway is the most studied way to produce NO via reduction from nitrite (NO_2^-) and several enzymes are reported to be implicated (Fig. 2). In higher plants, the **nitrate reductase (NR)–mediated pathway** and the **mitochondrial electron transport chain (mETC)–dependent pathway** are the most important sources of NO. **NR** is located in the cytosol and primarily catalyses the NADPH–dependent reduction of nitrate to nitrite, being this way key for nitrogen assimilation and metabolism in plants. Due to the nitrite toxicity for plants (Wang *et al.*, 2007), frequently, nitrite is then transported into the chloroplast and it is reduced to ammonium (NH_4^+) by the action of **Ferredoxin–Nitrite Reductases** (**NiR;** Joy and Hageman, 1966; Mikami and Ida, 1984). However, NR has also the ability to catalyze the conversion of nitrite to NO (Ni–NR activity). It is noteworthy that the efficiency of this reaction is low, and it only takes place in specific situations such as high nitrite concentration and low nitrate concentration or acidic or anoxic environments (Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000; Rockel *et al.*, 2002). Given that NR is the only source of nitrite in plants, nitrite–dependent NO production due to other sources is also attributed directly or indirectly to the nitrate reductase activity. Nevertheless, it has been recently described that in the unicellular algae *Chlamydomonas reinhardtii* NR can interact with the protein nitric oxide–forming nitrite reductase (NOFNiR) to form NO from nitrite under normoxic conditions (Chamizo–Ampudia *et al.*, 2017) and it is not inhibited by nitrate, in contrast to the Ni–NR activity. This mechanism has not been described yet in higher plants.

NO can also be generated by the **mETC pathway**, via nitrite reduction in the mitochondrial inner membrane. However, this pathway seems to be only active

under anaerobic/hypoxic conditions (Planchet *et al*., 2005; Gupta *et al.* 2005; Gupta and Igamberdiev, 2016), and this can serve as a way to preserve respiration using nitrite as an electron acceptor when oxygen is scarce (Gupta and Igamberdiev, 2011), as the reaction of nitrite reduction to NO is very sensitive to the inhibition by oxygen. Another way to produce NO via reduction of nitrite involves the peroxisomal enzyme **xanthine oxidoreductase** (**XOR**; Wang *et al.*, 2010). Nevertheless, this reaction only takes place under anaerobic conditions (Yu *et al.*, 2014). Rarely, NO is produced from nitrite **non–enzymatically** in specific conditions such as low pH or highly reducing environments, spontaneously in a light–mediated manner or in roots by the roots plasma membrane–bound nitrite:NO reductase (Cooney *et al.*, 1994; Durner *et al.*, 1998; Caro and Puntarulo, 1999; Stöhr *et al*., 2001; Bethke *et al*., 2004).

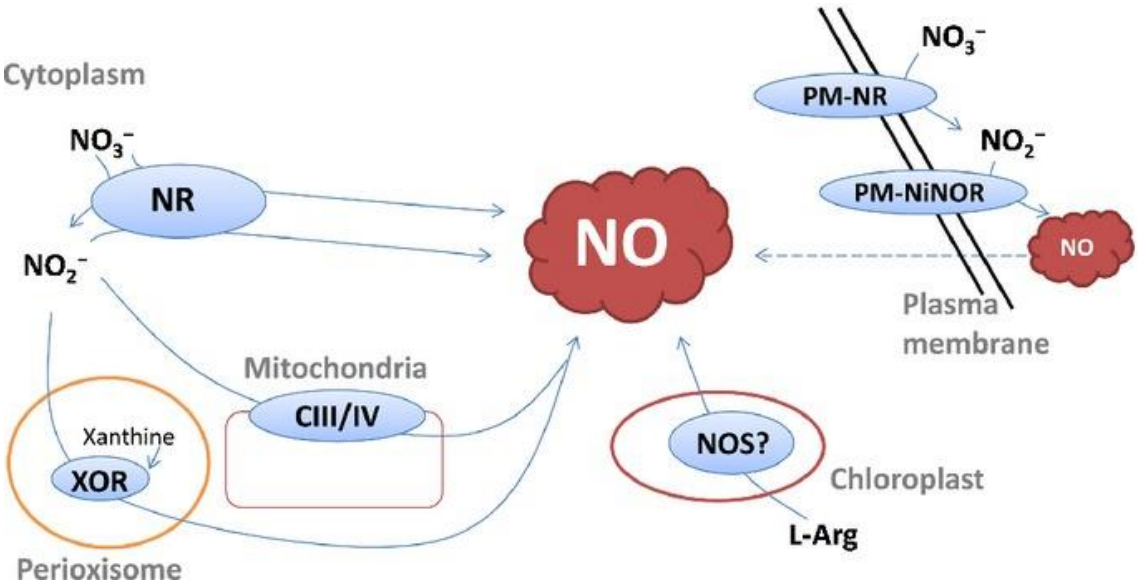


Fig. 2. Sources of NO in plants. Yu *et al.*, 2014. NR: nitrate reductase; NOS: NO synthase; PM: plasma membrane; NiNOR: nitrite–NO reductase; XOR: xanthine oxidoreductase.

A.1.2. Oxidative routes of NO synthesis

In mammals, NO production is catalysed by a family of enzymes so–called **Nitric Oxide Synthases (NOSs)**, which oxidize arginine to citrulline in a complex process (Alderton *et al*., 2001). Despite the efforts to find an enzyme structurally related to mammalian NO synthase (NOS) proteins, no gene analogous to mammalian NOS has been identified to date in the genomes of sequenced land plants (Jeandroz *et al.*, 2016). NOS enzymes found in the plant kingdom belong to algal species so far (Foresi *et al*. 2015; Jeandroz *et al*., 2016). Moreover, bioinformatics analyses in plant genomes and proteomes have not found highly conserved mammalian NOS motifs (Hancock and Neill, 2019) and the mammal NO–dependent signalling through cGMP has also been questioned in plants (Astier *et al*. 2019). Nevertheless, NOS–like activity has been extensively described in plants (Astier *et al.*, 2018) and the denomination "NOS–like" was adopted for this activity due to the similarities with the activity of mammalian NOS proteins, including the same substrate (NO and citruline are produced from L–Arginine) and the sensitivity to mammalian NOS inhibitors (Astier *et al*., 2018). **Hydroxylamine** has been proposed as another potential substrate for oxidative NO production in plants (Rümer *et al*. 2009) although the occurrence of this substrate naturally in plants is questionable and this substrate, in plants and cyanobacteria, appears to be reduced mainly by phytoglobins to ammonium under hypoxia (Sturms *et al*. 2011).

A.2. Regulation of NO levels

To be an effective signal molecule, NO levels must be tightly regulated. Cytosolic NO levels are determined by the balance of its synthesis and catabolism. Three main mechanisms control NO levels in plants: **reactive oxygen species (ROS)** that can react with NO; ***S*–nitrosoglutathione reductase (GSNOR)** which controls the GSNO content in the cell (Mur *et al.*, 2013a); and **phytoglobins** (previously known as non–symbiotic haemoglobins, Perazzolli *et al*., 2004; Qu *et al*., 2006; Nagata *et al*., 2008, 2009; Hill *et al*., 2016) that can oxidize NO to nitrate.

A.2.1. ROS

NO can react with oxygen, generating mainly nitrite and nitrate (Hancock, 2012). NO can be also scavenged by reacting with ROS, such as radical superoxide ($O_2^{\cdot-}$) generating peroxinitrite ($ONOO^-$), which is one of the most potent oxidant molecules in the cell; and with lipid peroxyl radicals ($LOO^{\cdot}$) through a still unknown mechanism, to produce nitro–fatty acids (NO_2–FAs; Rubbo, 2013). Recently, nitro–linolenic acid has been reported to be involved in plant development and plant response to different abiotic stresses (Mata–Pérez *et al.*, 2017).

A.2.2. GSNOR

Just–synthesized highly reactive NO can react with glutathione (glutamylcysteinylglycine, GSH), thus producing *S*–nitrosoglutathione (GSNO), considered as a reservoir of NO that provides NO signals for *S*–nitrosylation of proteins (Jahnová *et al.*, 2019; Yun *et al*., 2016). GSNO is metabolized to glutathione disulfide (GSSG) and ammonia (NH_3) by the cytosolic enzyme GSNOR (Frungillo *et al*., 2014; Yu *et al*., 2014).

A.2.3. Haemoglobins (Phytoglobins)

Haemoglobins (Hbs) are haem–proteins that typically comprise a haem prosthetic group and a polypeptide of 6 to 8 alpha–helix structures. The haem is an iron–protoporphyrin able to bind, among other ligands, diatomic gases such as O_2, CO and NO. While O_2 and CO_2 are bound exclusively when the haem iron is in ferrous form, NO is bound by ferrous iron with high affinity and by ferric iron with low affinity (Becana *et al.*, 2020). The homeostasis of NO can then be regulated through its oxidization to nitrate by some Hbs. Therefore, Hbs can regulate NO levels through either detoxification or delivery through *S*–nitrosylation reactions (Perazzolli *et al*., 2006).

Three main classes of Hbs have been identified in land plants: symbiotic–Hbs, the formerly known as non–symbiotic Hbs and truncated Hbs (Arredondo–Peter *et al.*, 1998; Garrocho–Villegas *et al.*, 2007). However, in 2016, during the XVIIth Conference on Oxygen–Binding and Sensing Proteins, another nomenclature

was proposed (Hill *et al.*, 2016) where symbiotic–Hbs have been renamed as symbiotic–phytoglobins and non–symbiotic Hbs are now just named as phytoglobins (Table 1).

Table 1. Phytoglobin classification and characteristics. Adapted from Hill *et al.*, 2016.

Nomenclature and characteristics accepted for Phytoglobins (Phytogb)			
FORMER PLANT GLOBIN NAME AND ABBREVIATION	**NEW NOMENCLATURE**	**PLANT ORIGIN**	**DISTINCTIVE CHARACTERISTICS**
Symbiotic haemoglobin (symHb)	SymPhytogb	Non–legume NO–fixing plants	Haeme–Fe mostly pentacoordinated Moderate to high affinity for O_2 Specifically localized in N_2–fixing nodules of actinorhizal plants or any other non–legume land plant
Leghaemoglobin (Lb)	Lb	N_2–fixing legumes	Haeme–Fe mostly pentacoordinated Moderate to high affinity for O_2 Specifically localized in legume N_2–fixing nodules
Nonsymbiotic haemoglobin (nsHb)	Phytogb0	Algae, bryophytes, gymnosperms	Moderate to high affinity for O_2 Found in any plant organs
Class/type1 nonsymbiotic haemoglobin (nsHb–1)	Phytogb1	Angiosperms	Haeme–Fe mostly hexacoordinated Extremely high affinity for O_2 Found in any plant organs
Class/type2 nonsymbiotic haemoglobin (nsHb–2)	Phytogb2	Angiosperms	Haaeme–Fe mostly pentacoordinated Moderate to high affinity for O_2 Found in any plant organs
Class/type3 nonsymbiotic haemoglobin (tr–Hb)	Phytogb3	Algae, land plants	Haeme–Fe penta– or hexacoordinate Moderate to high affinity for O_2 Found in any plant organ

Symbiotic phytoglobins (SymPhytogb), are phytoglobins found in plants that achieve nitrogen–fixing symbiosis but are not legumes, as *Parasponia* (Appleby *et al.*, 1983), *Casuarina* (Jacobsen–Lyon *et al.*, 1995) and *Chamaecrista* (Gopalasubramaniam *et al.*, 2008).

Leghaemoglobins (Lb) refer to those of the nitrogen–fixing legume species. This class of haemoglobins support the symbiosis with nitrogen–fixing bacteria via transporting free oxygen away from the oxygen–sensitive–nitrogenase enzyme (Gupta *et al*., 2011). They present a pentacoordinated structure (Gupta *et al*, 2011), the same as erythrocyte haemoglobin and other oxygen transporters. In fact, they also exhibit the characteristic red colour. This colour can be observed in nodules, where the concentration of this haemoglobin is high enough to appreciate the red colour at a macroscopic level. They have strong affinity for O_2 and NO (Hoy *et al*., 2008), but their pentacoordinate structure make possible O_2 to bind reversibly, allowing their storage and transportation functions.

Phytoglobins (Phytogb) encompass four classes of the formerly known non–symbiotic haemoglobins:

a) Phytogb0

Phytogb0 are found in algae, bryophytes, and gymnosperms, in any plant organ. Their structure can be penta– or hexa–coordinate and have moderate to high affinity for oxygen.

b) Phytogb1

Phytogb1 are found in angiosperms, in both monocotyledonous and dicotyledonous plants. They are mainly expressed in seeds, roots and stems (Arredondo–Peter *et al.*, 1998; Hill, 1998). Phytogb1 (as well as the other formerly known as ns–Hgs) is hexacoordinate (the iron coordinates with both the proximal and distal histidine, similarly to the haeme active site of cytochrome b5; Gupta *et al.*, 2011). This structure facilitates tight binding of

oxygen that can further accept an electron from iron and oxygenate NO resulting in the formation of nitrate (Perazzolli *et al*., 2004; Gupta *et al.*, 2011), functioning this way as NO scavenger. Indeed, Phytogb1 can scavenge NO using traces of oxygen (Perazzolli *et al*., 2004; Igamberdiev *et al.*, 2004, 2006), hence allowing Phytogb1 to scavenge NO at low oxygen content (Kolbert *et al.*, 2019). They were first described by Taylor and colleagues in 1994 and were shown to be up–regulated under hypoxia as well as in response to low ATP and nitrate (Nie *et al.*, 1997). Later, it was shown that NO is an inducer of Phytogb1 (Ohwaki *et al.*, 2005) and that NO transcriptionally regulates Phytogb1 (Bustos–Sanmamed *et al.*, 2011). The role of Phytogb1 in plant defense is based on the modulation of NO levels and the H_2O_2/NO ratio during the pathogenic interaction (Qu *et al.*, 2006). The regulation by NO of tomato Phytogb1 (Fig. 3) during beneficial and pathogenic fungal interactions has been analysed in this Doctoral Thesis and is described in Chapters I and II.

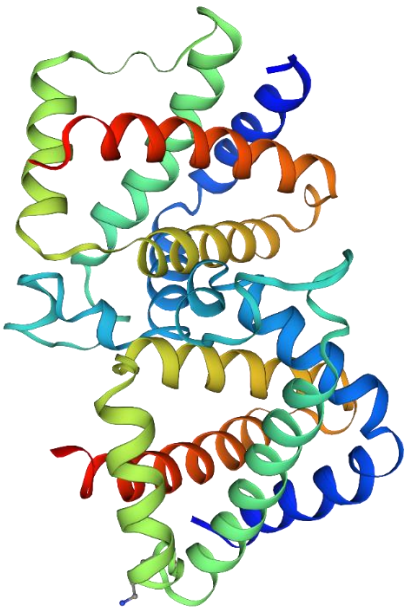


Fig. 3. Structural prediction of tomato Phytogb1. SWISS–MODEL automated protein modelling online software (swissmodel.expasy.org). NCBI protein reference: NP_001234498.1.

c) Phytogb2

Phytogb2 are found in angiosperms, predominantly present in dicotyledon plants and some monocotyledon ones (Smagghe *et al.*, 2009) as maize (Garrocho–Villegas *et al.*, 2007). They are mainly expressed in developing tissues and in vegetative and reproductive structures (Wang *et al.*, 2003; Ross *et al.*, 2004; Smagghe *et al.*, 2009). Phytogb2 are hexacoordinate, presenting lower affinity for oxygen than Phytogb1 (Gupta *et al.*, 2011) although their function is related to facilitate oxygen supply to developing tissues (Vigeolas *et al.*, 2011; Spyrakis *et al.*, 2011).

d) Phytogb3

Phytogb3, formerly known as truncated haemoglobins, is a group with very few similarities to Phytogb1 and Phytogb2, but share 40–45% sequence similarity with bacterial haemoglobins of the ''2–on–2'' structural motif. It seems to appear in plants due to a horizontal gene transfer from bacteria (Gupta *et al.*, 2011). Phytogb3 exhibits the two states of coordination (hexa– and penta–) but they have a low affinity for O_2 and NO (Watts *et al.*, 2001). Their functions remain mostly elusive, although there might be related to the regulation of oxygen delivery at high O_2 concentrations (Watts *et al.*, 2001).

A.3. Modes of action of NO

NO levels are known to influence multiple biological processes in plants and this can be achieved mainly through post–translational modifications (PTMs). NO–dependent post–translational modifications have important effects on the target proteins by regulating their activities, structure, subcellular location or interaction with biomolecules. NO–dependent PTMs results in the induction of different physiological responses and/or signalling processes as alteration of gene expression, metabolic changes and phytohormone signalling (Cui *et al.*, 2018; León and Costa–Broseta, 2019; Sánchez–Vicente *et al.*, 2019).

One of the first NO–dependent PTMs described was **metal–nytrosilation,** where NO binds the metal of a prosthetic group, such as iron in haem group from guanylate cyclase and haemoglobins (Horst and Marletta, 2018; Astier and Lindermayr, 2012). In plants, metal–nitrosylation data are scarce, and it has been described as a protective mechanism against oxidative stress related to high NO production (Astier and Lindermayr, 2012).

The most intensively studied signal transduction mechanism of NO is protein ***S*–nitrosylation/*S*–nitrosation.** This is the reversible process of adding NO to the sulfhydryl group of a cysteine residue to form an *S*–nitrosothiol (Hess *et al.*, 2005). Different studies have identified putative *S*–nitrosated proteins involved in primary metabolism and defense responses. In addition, recent studies pointed out that NO can directly regulate transcription by modifying several transcription factors (Romero–Puertas and Sandalio, 2016a; Lindermayr *et al.*, 2020). Interestingly, NO level is self–regulating and can regulate ROS through PTMs of producing enzymes and/or antioxidant system (Romero–Puertas and Sandalio, 2016b).

The other NO–dependent PTM is **tyrosine nitration**, which corresponds to the reaction of adding a nitro group ($–NO_2$) in the ortho position of the aromatic ring of a tyrosine residue of a protein (Stamler *et al.*, 2001; Vandelle and Delledonne, 2011).

It is noteworthy that several studies have postulate that NO regulates gene expression by changing **chromatin accessibility**. Therefore, the redox status of plant cells might have the potential to control chromatin modifications and epigenetic reprogramming of gene expression (Lindermayr *et al.*, 2020).

A.4. NO functions in plant biology

NO takes part in a plethora of plant processes, it is present in all plant organs at all developmental stages and it is also involved in the plant response to biotic and abiotic stresses.

This way, NO is entailed in the regulation of a wide range of plant developmental processes, for instance: seed dormancy emergence and seed

germination (Gibbs *et al.*, 2014; Albertos *et al.*, 2015; del Castello *et al.*, 2019); root development (Sanz *et al.*, 2015; Castillo *et al.*, 2018) as a promotor of adventitious roots, regulator of lateral root development and root hair formation and growth (del Castello *et al.*, 2019); flower development, flowering, pollen germination and breeding (Kwon *et al.*, 2012; Serrano *et al.*, 2015); fruit development (Manjunatha *et al.*, 2012; Du *et al.*, 2014) and fruit ripening (Corpas and Palma, 2018). NO has also been identified as a key piece involved in the plant response to several abiotic stress factors such as UV–light (Mackerness *et al.*, 2001), flooding and hypoxia (Dordas *et al.*, 2003; Perazzolli *et al*., 2004; Gupta and Igamberdiev, 2016), drought (García–Mata and Lamattina, 2001; Zhao *et al.*, 2001), heat and salt (Uchida *et al.*, 2005) or heavy metals (Kopyra *et al.*, 2003; Romero–Puertas *et al.*, 2018; Terrón–Camero *et al*., 2019). The first function assigned to NO in plants, however, was related to plant defense against biotic stresses.

A.4.1. NO in plant defense

During biotic stress, NO is produced in response to potential agresors as microbial pathogens and viruses, and it plays a role in a wide range of stress responses. These responses vary from the regulation of defense genes, to the production of hormones and the hypersensitive response (HR) development (Delledonne *et al.* 1998, Durner *et al.* 1998 Asai and Yoshioka 2009; Trapet *et al.*, 2015; Molina–Moya *et al*., 2019). Indeed, NO was shown to be rapidly generated in plants following a challenge with biotrophic and necrotrophic pathogens and is considered as a major defense activator (Mur *et al.*, 2013b).

Although plants face several biotic stresses, just a relatively small number of potential pathogens cause disease on plants. This is possible because only few phytopathogens can overcome the innate immunity of plants. Plant innate immunity is proposed to be a two–tiered immune system including pathogen–associated molecular pattern **(PAMP)–triggered immunity (PTI)** and **effector–triggered immunity (ETI)** (Fig. 4; Henry *et al*., 2012; Couto and Zipfel 2016).

The first layer of plant innate immunity relies on the basal resistance (Gómez–Gómez *et al.*, 1999; Zhang *et al.*, 2007), the primary immune response or

PTI (Fig. 4), that confers plants robust resistance to a broad spectrum of microbial pathogens, both adapted and non–adapted (Zipfel, 2008; Li *et al.*, 2016; Zhang *et al.*, 2010). The plant can recognize different molecular patterns associated to potential aggressors (Microbe–Associated Molecular Patterns –MAMPs– or Pathogen–Associated Molecular Patterns –PAMPs–) or cellular damage (Damage–Associated Molecular Patterns, DAMPs) via their Pattern Recognition Receptors proteins (PRR; Jones and Dangl, 2006), that induces both local and systemic immunity (Boutrot and Zipfel, 2017). Nowadays, it is well stated that NO produced in response to MAMPs, PAMPs or DAMPs exerts an important role in signalling pathways (Molina–Moya *et al*., 2019). Thus, NO leads to some nonspecific defense responses to a pathogen attack as the papillae formation to strengthen cell walls (Prats *et al.*, 2005); stomatal closure via NO mediation (Melotto *et al.*, 2006); or the accumulation of proteins related to phytoalexin synthesis (Noritake, 1996; Modolo *et al.*, 2002). Besides, several PAMPs can also induce a hypersentitive response (HR, Boutrot and Zipfel, 2017). HR is an early defense response that triggers programmed cell death, inducing tissue necrosis, and therefore restricting the growth of biotrophic pathogens.

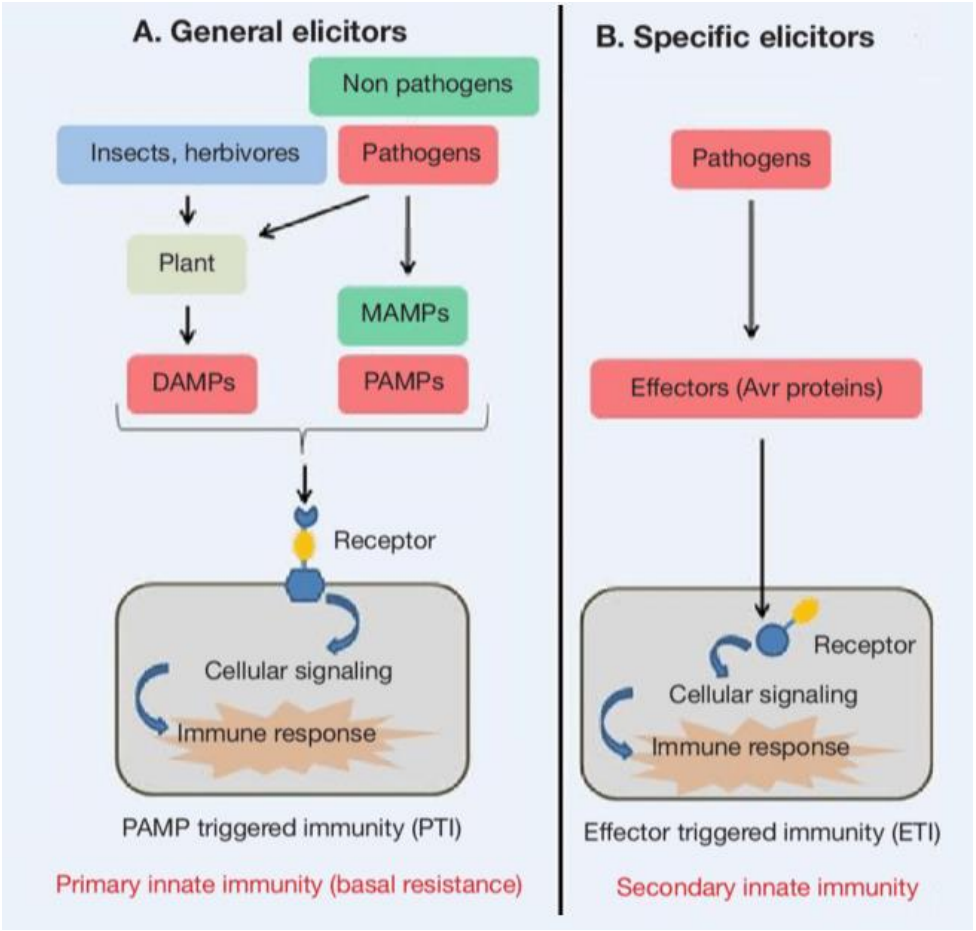


Fig. 4. **Plants innate immunity. Modified from Henry *et al.*, 2012. A**: General (or non–specific) elicitors include chemicals, MAMPs (Microbes–Associated Molecular Patterns); PAMPs (Pathogen–Associated Molecular Patterns) and DAMPS (Damage associated molecular patterns). **B**: Specific elicitors (or effectors) are produced by specialized pathogens and function only in plants carrying the corresponding disease resistance gene. Effectors typically lead to the secondary innate immunity after an intracellular receptor–mediated perception.

Race–specific host–adapted pathogens have evolved the mechanisms to escape from recognition or can produce effectors that suppress PTI as a result of coevolution with plants (Göhre and Robatzek, 2008; Deslandes and Rivas, 2012; Rafiqi *et al.*, 2012). In this case, plants have developed a second defense line, the **ETI** (Couto and Zipfel, 2016). As it is a coevolutionary competition, to fight against adapted pathogens, plants have evolved resistance (R) proteins inside plant cells (the receptors called NLR or NBS–LRR, for intracellular nucleotide–binding domain

leucine–rich repeat). These R proteins specifically recognize pathogen effectors and trigger a stronger immune response (Takken and Goverse, 2012). Thus, plants have evolved genotype–specific R genes. R proteins directly or indirectly recognize microbial effectors previously known as avirulence proteins (Avr). This implicates many signalling events at the cellular level such as ion fluxes (Ca^{2+}, K^{+}, NO_3^{-}, Cl^{-}) or production of ROS and NO. This, with other processes, finally results in the transcriptional reprogramming that leads to the activation of defense–related genes (Torres *et al.*, 2006; Besson–Bard *et al.*, 2008; Lindermayr *et al.*, 2010; Dubreuil–Maurizi *et al.*, 2011). ETI also typically involves the HR which leads to cell death (Romero–Puertas *et al.*, 2004; Stuible and Kombrink, 2004; Williams and Dickman, 2008; Coll *et al.*, 2011; Schlicht and Kombrink,2013). Cell death during HR seems to be dependent on the balanced production of NO and ROS (Delledonne *et al.* 2001), and it has been suggested that the kinetics of HR formation depends on the NO production rate (Mur *et al.*, 2005).

Therefore, NO is produced both during PTI and ETI, and the two of them provide resistance against a wide variety of potential pathogens. Only a few pathogens that have developed adaptations can successfully elude or suppress both defense layers and cause disease in plants.

A.4.1.1. Induced Resistance

It is noteworthy that the plant immune system not only acts to limit current pathogen invaders but can also lead the plant to an enhanced status of immunity called Induced Resistance (IR). Induced resistance relies, at least in part, on a potentiation of the plant ability to trigger defense responses upon attack, the so called "defense priming" (Conrath *et al*., 2016, Martínez–Medina *et al*., 2016, Mauch–Mani *et al*., 2017). Defense priming implies the preconditioning of plant tissues upon appropriate stimulation (priming stimulus), normally associated to transient and/or weak activation of defenses (Fig. 5). Despite of being transient or weak, some key regulatory elements may change in the plant (Mauch–Mani *et al*., 2017) so that the plant retains some stress memories, achieving an alert or "primed" state that results in a more efficient activation of defense responses upon a future

challenge (triggering stress) occurs. Indeed, in primed plants, a faster and stronger activation of defenses occurs upon attack by pathogens or herbivore insects, leading to enhanced resistance to the attacker encountered (Van Wees *et al.*, 2008). This effect is long lasting, and has been even shown to pass down generations by epigenetic modifications, conferring trans–generational immunity (Luna *et al.*, 2012).

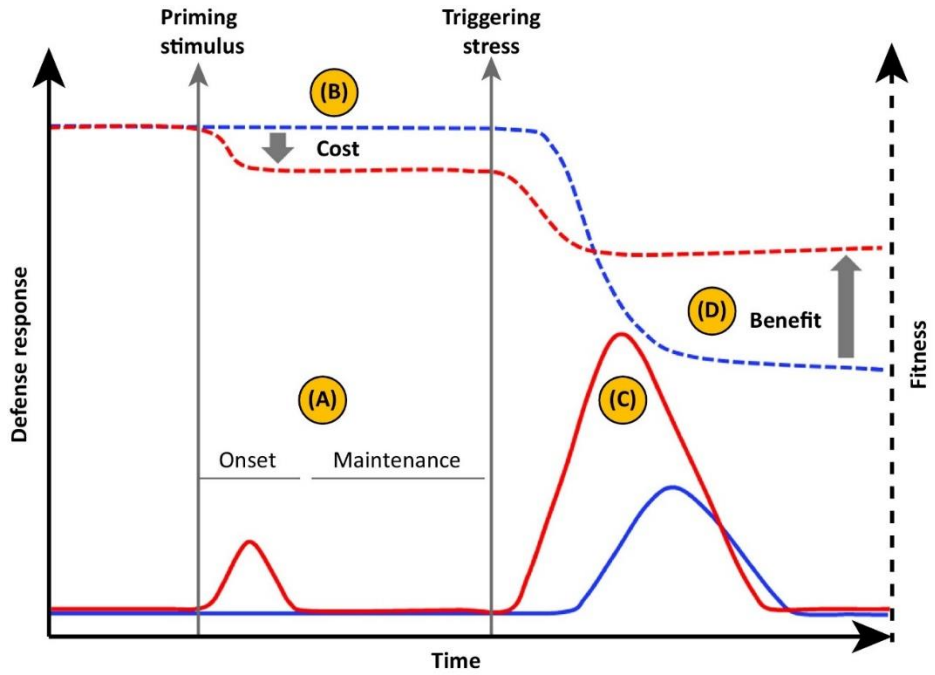


Fig. 5. Schematic representation of defense priming. Relation between defense responses and fitness in primed plants. Martínez–Medina *et al.*, 2016.
Solid lines: defense response; dashed lines: fitness. Red: primed plants; blue: unprimed plants. The following events define defense priming: **(A)** Stress memories: A priming stimuli leads to a transient and or weak activation of detenses, thus defenses before the triggering stress are low, but some molecular changes allow the plant to retain some stress memory that will allow the primed response upon challenge. **(B)** Low fitness costs: the maintenance of the primed state (before the triggering stress) has low fitness costs compared with direct activation of defenses. **(C)** A more robust defense response upon challenge: in response to the triggering stress, primed plants mobilize cellular defenses in a faster, earlier, stronger, and/or more sustained way than unprimed plants do. **(D)** Better performance: as a result, primed plants are expected to defend better against a given stressor than unprimed plants. Consequently, priming enhances plant fitness in hostile environments with minimal maintainance costs.

Plant induced resistance is usually classified in two main types depending on the triggering stimuli and the signalling pathways activated in the plant: Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). Remarkably, NO has been reported to have a regulatory role in both types of resistance.

a) Systemic Acquired Resistance (SAR)

Systemic acquired resistance (SAR, Sticher *et al.* 1997) is usually triggered by avirulent or attenuated pathogens, and is effective against a broad spectrum of pathogens. SAR is triggered upon local activation of a PTI or ETI response (Mishina *et al.*, 2007) produced by biotrophic or hemibiotrophic pathogens. This leads to enhanced resistance in tissues distal from the site of infection and involves one or more long–distance signals that propagate an enhanced defensive capacity in still undamaged plant parts (Dempsey *et al.*, 2012; Shah and Zeier, 2013). In SAR, salicylic acid (SA) accumulates both local and systemically, following pathogen infection. There is clear evidence that SA may work together with NO and ROS to establish SAR (Gao *et al.*, 2015; Mittler and Blumwald, 2015; Sami *et al.*, 2018). Indeed, SA, through the redox–regulated transcription cofactor NPR1 activates the expression of a large number of PR genes, involved in defense responses (Choudhary *et al.*, 2007; Romera *et al.*, 2019) (Fig. 6). Remarkably, NPR1 remains *S*–nitrosylated in its oligomeric form as an inactive form in the cytosol. When stimulating the defense responses, the oligomer form of NPR1 is dissociated into monomers due to changes in the redox state of the cell. This facilitates the migration of the protein into the nucleus to activate the transcription factor that positively regulates defense genes (Lindermayr *et al.*, 2010). In a second phase, NO exerts negative feedback by causing the *S*–nitrosylation of NPR1, turning the protein inactive again (Tada *et al.*, 2008; Yu *et al.*, 2014).

b) Induced Systemic Resistance (ISR)

ISR (van Loon *et al.* 1998) is also an enhanced defensive capacity of the whole plant against a broad range of pathogens and insect herbivores. ISR is

acquired upon local induction by MAMPs and other elicitors of beneficial microbes living in the rhizosphere, like bacteria and fungi (Pieterse *et al.*, 2014; Romera *et al.*, 2019). In contrast to SAR, ISR usually develops through a SA–independent pathway, where JA and ET play the main roles, and typically functions without major PR gene activation (Romera *et al.*, 2019; Fig. 6). Nevertheless, it has been demonstrated that NPR1 is also necessary for ISR, but the role of this protein may differ in both SAR and ISR (Pieterse *et al.*, 2014; Nie *et al.*, 2017). In fact, while NPR1 may function in the nucleus in SAR, in ISR, NPR1 seems to play a cytosolic function (Pieterse *et al.*, 2014), but the possible interaction of this protein with NO has not been described yet. Nevertheless, NO has been linked to ISR in Arabidopsis roots. In Arabidopsis, the root–specific transcription factor MYB72 (Van der Ent *et al.* 2008), that has been identified as a node of convergence in ISR elicited by diverse beneficial microbes (Pieterse *et al.*, 2014), is upregulated by NO (García *et al.*, 2010; Romera *et al.*, 2019).

It is noteworthy that signalling pathways involved in the induction of ISR can be different depending on the microbial and plant species involved in the interaction (Ryu *et al.*, 2003; Jankiewicz and Koltonowicz, 2012; Alizadeh *et al.*, 2013). Therefore, although JA/ET are the main participants in ISR, in some particular cases, SA accumulation might also be required (Ryu *et al.*, 2003; Alizadeh *et al.*, 2013). As an example of this, some latest studies have shown that the beneficial soil fungi *Trichoderma* (hereafter Trichoderma), can trigger in the plant both ISR and SAR responses, leading to a type of resistance called Trichoderma–ISR (TISR) (Martínez–Medina *et al.*, 2013). The overlap of both JA/ET and SA signalling pathways may induce defense responses against both necrotrophic and biotrophic pathogens, leading to a broad increase of disease resistance in plants (Martínez–Medina *et al.*, 2013; Pieterse *et al.*, 2014).

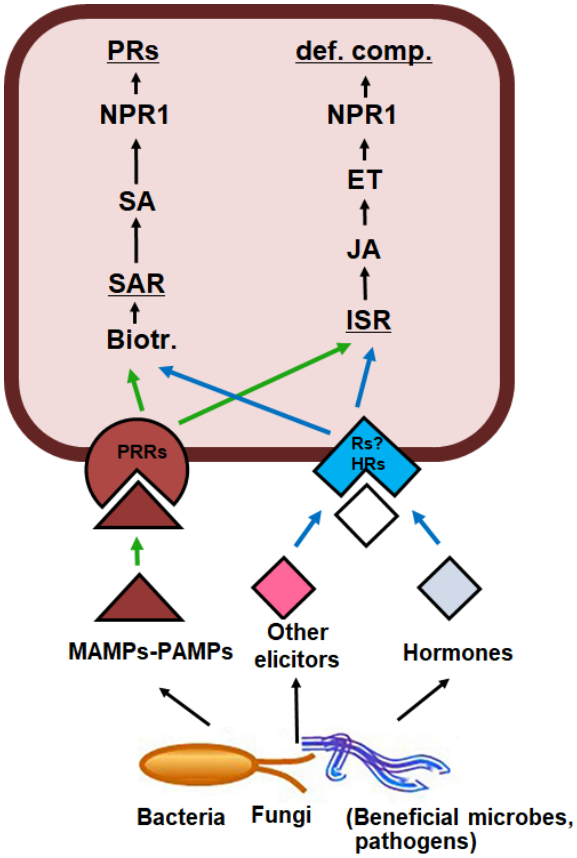


Fig. 6. Scheme of SAR and ISR. Modified from Romera *et al.*, 2019. Biotroph pathogens elicit SAR; beneficial microbes elicit ISR. Microbes can produce Microbe–Associated Molecular Patterns (MAMPs) or Pathogen–Associated Molecular Patterns (PAMPs), which are perceived by Pattern Recognition Receptors (PRRs), or other elicitors or hormones that are perceived by receptors (Rs) (some of them unknown). After the perception stage by receptors, signalling pathways are activated, leading to different responses. Def. comp. (defensive compounds), ET (Ethylene), HRs (Hormonal Receptors), ISR (Induced Systemic Resistance), JA (Jasmonic Acid), NPR1 (Nonexpressor of PR genes 1), PRs (Pathogenic–Related proteins), SA (Salicylic Acid), Rs (Receptors), SAR (Systemic Acquired Resistance).

B. Plant–fungal interactions

Numerous studies have demonstrated the importance of NO in the regulation of plant defense against fungal pathogens. As described in the previous section, NO triggers reprogramming of defense–related gene expression, the production of secondary metabolites with antimicrobial properties, and the HR (Mur *et al.*, 2017), although most studies have dealt with plant–bacteria interactions. Moreover, more recent studies have pointed to a role of NO during the establishment of plant–microbe beneficial interactions involving bacteria (Daminani *et al.*, 2016; Berger *et al.*, 2020), or fungi (Calcagno *et al.*, 2012; Espinosa *et al.*, 2014; Gupta *et al.*, 2014). Although these studies support a regulatory role of NO in the regulation of plant fungal interactions, the studies dealing with the precise function and molecular mechanisms mediating the role of NO in plant–fungal associations are still scarce.

This PhD Thesis aims to unravel the role of NO in the regulation of plant mutualistic or deleterious interactions with different fungi. We first explore the role of NO in the regulation of root interactions with beneficial and pathogenic soil fungi (Chapters I and II). Then, we compile and analyse the existing scientific literature regarding NO regulation in other plant–fungal interactions, and we discuss our results in this frame, aiming to identify potential general patterns of NO regulation/function according to the type of interaction. As a result, we generate different models partially filling some of the current knowledge gaps in the field (Chapter III). Finally, besides the role of NO in controlling the establishment of these plant–fungal symbioses, we further explore its potential role in mediating the beneficial impact of the interaction on plant health. For this purpose, we try to decipher the role of NO in ISR against foliar fungal pathogens induced by *Trichoderma*–derived volatile compounds (Chapter IV).

B.1. Beneficial soil fungi

The increasing trend to promote low–input and more sustainable agriculture has promoted the interest in beneficial soil microbes to reduce chemical inputs while promoting plant growth and efficient pest control. Many chemicals have been reported to have adverse effects on human health, the environment and living organisms and beneficial microbes are a promising alternative in sustainable agriculture. Beneficial microbes can have multiple applications as biostimulants and protectors of plant health, constituting an alternative for traditional pest control in crop management strategies.

Soil beneficial fungi have shown immense potential in improving plant growth and stress tolerance, leading to a variety of agricultural applications (Muller *et al.*, 2016; Hussain *et al.*, 2018). Indeed, due to their role in maintaining soil and plant quality and production, and due to its environmentally friendly characteristics, soil beneficial fungi have gained enormous attention as biofertilizers and bioprotectors (Mahanty *et al.*, 2017). Among them, special attention have received arbuscular mycorrhizal fungi (AMF) and Trichoderma species, with well–documented effects in improving plant nutrition and biocontrol of plant pathogens, respectively, and both widely distributed in nature.

B.1.1. Arbuscular mycorrhizal fungi

Most terrestrial plants are associated with mycorrhiza forming fungi (van der Heijden *et al.*, 2015, Vašutová *et al.*, 2019) to adequately grow and complete their life cycle in natural ecosystems (Smith and Read, 2008). The word "mycorrhiza", from the Greek *mykos* (fungus) and *rhiza* (root), describes the mutualistic association between certain soil fungi and plants. This term was introduced by the German botanist Albert Bernhard Frank in 1885 (Frank, 2005).

Arbuscular mycorrhizas (AM) are a type of endomycorrhizas that are the most widespread (Smith and Read, 2008) and with greater importance from a beneficial point of view for the plant (Parniske, 2008). The fungi implicated in this type of symbiotic association are obligate biotrophs that belong to the phylum

Glomeromycota (Wijayawardene *et al.* 2018). These fungi are associated with more than 70% of vascular plant species (Brundrett and Tedersoo, 2018). As obligate biotrophs, the fungus needs the host plant to complete its life cycle. This cycle begins in the rhizosphere, where a complex molecular dialogue takes place for the recognition of both partners, presenting a high degree of genetic and metabolic coordination. Plant roots release strigolactones (SLs) in the rhizosphere (Fig. 7). SLs are produced in low Pi conditions and can induce different fungal responses. Among those responses are spore germination, hyphal growth and hyphal branching, and the release of fungal molecules that trigger the symbiotic response in the plant (Waters *et al.*, 2017). On the other hand, the fungus produces other signalling molecules, the so–called MYC factors (lipochitooligosaccharides; –Maillet *et al.*, 2011– and short–chain chitin oligomers –Genre *et al.*, 2013–). These compounds allow the recognition of the fungus by the plant, which leads to the expression of host genes to establish the symbiosis. Plant receptors recognize MYC factors and trigger the "common symbiosis signalling pathway" that is shared with Rhizobium–legume symbiosis. This prepares the host root for the interaction with the fungus (Schmitz and Harrison, 2014) by stimulating the development of lateral roots that favour the contact between the two organisms.

When the physical contact is established, the fungal hypha develops the hyphopodium, a specialized hypha that attaches to the root epidermis before intracellular fungal penetration. This triggers the reorganization of plant cells cytoplasm, forming the prepenetration apparatus (PPA; Fig. 7). PPA is an ephemeral structure formed by an aggregation of cytosol, cytoskeleton and cell organelles. It allows the subsequent entrance of the hypha through the plant cell as it develops as a cytoplasmic bridge across the cell, surrounded by the endoplasmic reticulum, cytoskeleton and plasma membrane. This bridge leads the hypha across the invagination in the host cell (Genre and Bonfante, 2016; Pimprikar and Gutjahr, 2018).

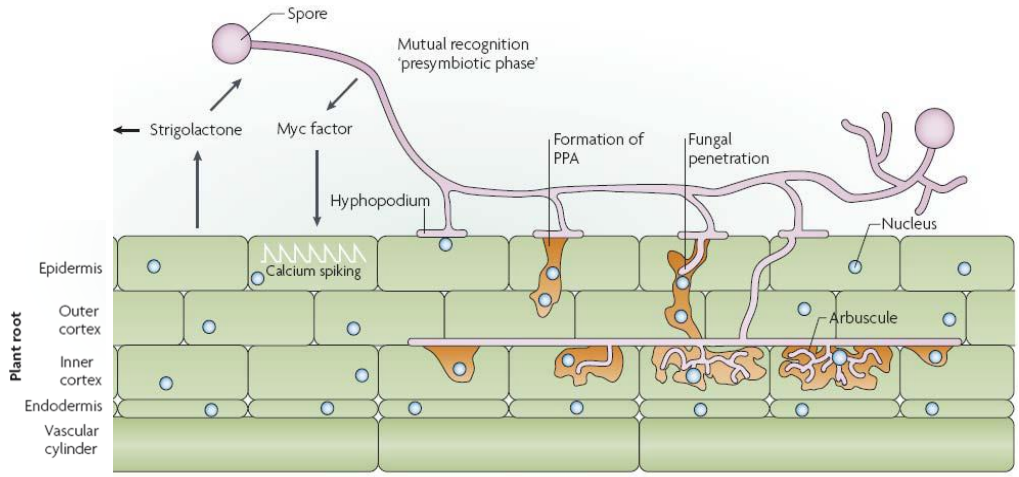


Fig. 7. Steps in arbuscular mycorrhiza development. Adapted from Parniske, 2008.

Once the fungus has reached the inner cortical cells, it develops a highly branched bush–like structure characteristic of this symbiosis, the arbuscles (Figs. 7, 8). Arbuscules occupy most of the cell volume, forming an extensive surface for water and nutrients exchange (Bonfante and Genre, 2010). Arbuscles are surrounded by a plant–derived peri–arbuscular membrane. This membrane prevents direct contact of the AMF with the plant cytoplasm (Parniske, 2008). The bidirectional nutrient exchange that benefits the plant and the AMF takes place in those arbuscles (Smith and Smith, 2011; Luginbuehl and Oldroyd, 2017). Arbuscles are not a constant structure but a dynamic one and their life spans about 2–8 days. Then, they collapse and the cell can recover the initial morphology and harbour a new arbuscle (Walter *et al.*, 2010; Kobae *et al.*, 2018).

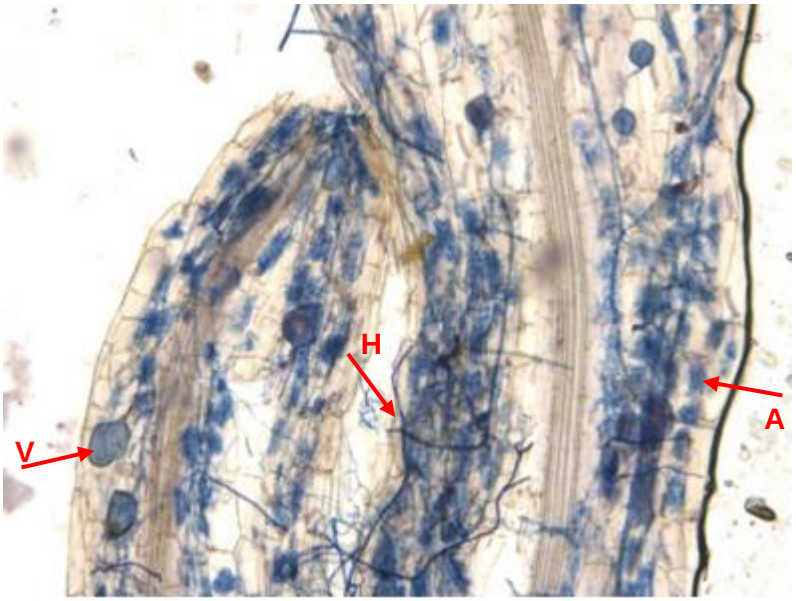


Fig. 8. Micrograph of a tomato mycorrhizal roots showing fungal structures –hyphae (H), arbuscules (A) and vesicles (V)– stained with trypan blue. Image by: Leyre Pescador.

Moreover, some AMF species also develop some structures inside the roots known as vesicles. Those are balloon–shaped structures that function as lipid storage for the fungus. In some AMF species, as in *Rhizophagus irregularis*, the vesicles can lead to the formation of spores inside the root (Smith and Read, 2008). Besides, some extraradical hyphae also turn into new spores, closing this way the cycle of the fungus (Fig. 7).

The fungus colonizes biotrophically the root cortex (Bonfante and Genre, 2010; Fig. 7) without causing damage or an apparent defense response. Then, when the first arbuscles are well–formed, a hyphal network is developed to act as a complementary root system. This hyphal network facilitates the water and mineral nutrients absorption by host plant (mainly Pi, but others such as nitrogen, copper and zinc –Smith and Smith, 2011; Ferrol *et al.*, 2016–). These extra–radical mycelia can explore a higher volume of soil, reaching much further than roots alone, thus functioning as a water and nutrient searcher for the plant. Accordingly, AM symbiosis has huge importance in plant nutrition in natural systems (Mauch–Mani *et al*., 2017; Kumar and Verma, 2018; Begum *et al*., 2019; Tian *et al*., 2020). In return,

the plant gives the fungus up to 20% of photosynthetically fixed carbon to complete its life cycle (Bago *et al.*, 2000). Moreover, it has been recently shown that the plant also provides lipids to the fungus (Keymer *et al.*, 2017; Luginbuehl *et al.*, 2017). Probably because of the costs of the symbiosis, plants try to control mycorrhizal colonization depending on nutrient availability and environmental conditions. Accordingly, AM colonization is a tightly regulated process where almost all known phytohormones seem to play a role in AM formation and/or functioning (Fig. 9; Pozo *et al.*, 2015). Salicylic acid (SA), ethylene (ET) and cytokinins control the early steps of the interaction, while gibberellins, abscisic acid (ABA), auxins and jasmonates (JA) regulate arbuscles formation and functioning (Pozo *et al.*, 2015). However, the potential regulatory role of NO in regulating AM symbiosis was unexplored.

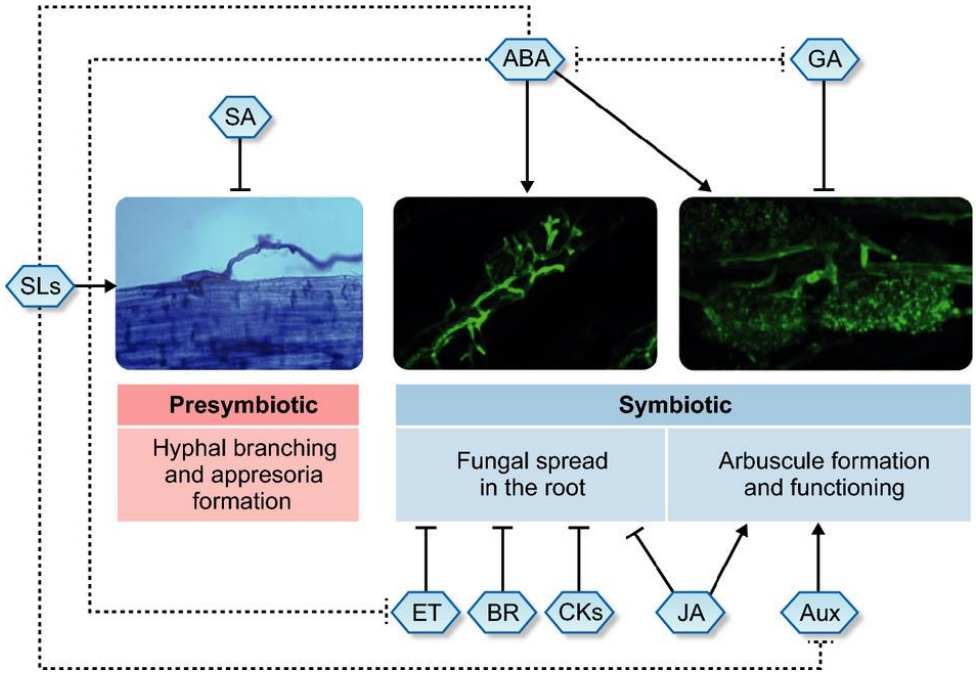


Fig. 9. Phytohormone regulation of arbuscular mycorrhiza (AM) formation and functioning. Pozo *et al.*, 2015. The scheme summarizes the role of plant hormones in regulating different stages of AM establishment and development. Positive and negative effects are illustrated by arrows and blunt-ended bars, respectively, and dashed lines indicate interactions suggested to play a role in AM regulation. Multiple functions can be envisaged for a particular hormonal group and, conversely, multiple hormones interact to fine-tune particular functions. ABA: abscisic acid; Aux: auxins; BR: brassinosteroids; CKs: cytokinins; ET: ethylene; GA: gibberellins; JA: jasmonates; SA: salicylic acid; SLs: strigolactones.

It has been proposed that the modulation of plant hormone levels during the symbiosis may have consequences also in the plants ability to cope with stressful situations. Indeed, it has been shown that the benefits from the symbiosis go beyond nutritional aspects. The symbiosis also improves the plant´s ability to cope with stress, and enhances tolerance to abiotic stresses such as drought and salinity (Sánchez–Romera *et al.*, 2016; Rivero *et al.*, 2018; Quiroga *et al.*, 2018); and resistance to pathogens and pests (Pozo *et al*., 2007; Jung *et al.,* 2012) have been demonstrated. This mycorrhiza–induced resistance is at least in part due to priming of plant defenses (Sanmartín *et al*., 2020). In agreement with all the potential benefits for the plant, many commercial inoculants use AMF (Chen *et al*., 2018; Szczałba *et al*., 2019) to improve crops.

B.1.2. *Trichoderma* spp.

The genus Trichoderma is formed by filamentous anamorphic fungi from Ascomycota division. Trichoderma (Fig. 10) are typical inhabitants of the soil that feed on organic matter primarily but also act as mycoparasites. Kubicek and collaborators (2011) found that mycoparasitism was the first mechanism of Trichoderma, that later turned to colonize the rhizosphere, being this favoured by the presence of other microorganisms and plant exudates in the soil. When interacting with plants, Trichoderma colonizes and penetrates the surface of plant roots, being the growth limited mostly to the apoplast, epidermis and first cortical cells layers (Mendoza–Mendoza *et al.*, 2018), thus not penetrating the vessels (Yedidia *et al.*, 1999; Chacón *et al.*, 2007; Samolski *et al.*, 2012; Carrero–Carrón *et al.*, 2018). Then, the fungus takes and advantage from roots exudates and the protection of the colonized niche (Woo and Lorito, 2007; Rubio *et al.*, 2012). Trichoderma is mostly recognized by its potential to control fungal pathogens through its capacity for mycoparasitism. In fact, Trichoderma has been used efficiently to control a large number of phytopathogens as *Rhizoctonia solani* (Harman *et al.*, 1981), *Botrytis cinerea* (Vos *et al.*, 2015) or *Fusarium oxysporum* (Martínez–Medina *et al.*, 2011).

Also, Trichoderma brings several benefits to the plants, as improving the nutritional status (Shoresh and Harman, 2008) or promoting the growth of lateral roots (Chang *et al.*, 1986; Yedidia *et al.*, 2001; Pelagio–Flores *et al.*, 2017) thus increasing plants productivity (Harman *et al.*, 2004). Indeed, Trichoderma is among the most used beneficial microorganisms in agriculture, being the most popular organism in commercial preparations (Szczałba *et al*., 2019).

Some Trichoderma strains have also been reported to increase the plant resistance to different types of pathogens when colonizing plant roots, and this resistance affects both below and aboveground tissues (Harman *et al.*, 2004). This enhanced resistance is related to the priming of plant defenses, allowing a faster and more intense defense response to a subsequent pathogen attack leading to ISR. This was shown for *B. cinerea* (Martínez–Medina *et al.*, 2013) and pathogenic nematodes as *Meloidogyne incognita* (Martínez**–**Medina *et al.*, 2017a). Recently, it has been shown that Trichoderma volatile compounds (VCs) are also able to prime plant defenses leading to ISR (Martínez–Medina *et al.*, 2017b).

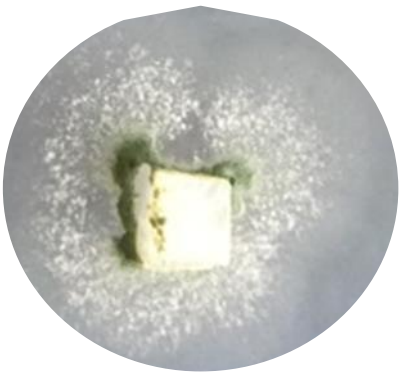


Fig. 10. Detail of *Trichoderma harzianum* (T–78) growing on Murashige and Skoog solid medium. Image by: Leyre Pescador.

B.2. Plant pathogenic fungi

Despite the many factors that can negatively impact crop productivity, losses attributed to pests are of utmost importance (Syed Ab Rahman *et al.*, 2018). Some studies estimated crop losses by pest and pathogens around 20–40% of total crop productivity (Oerke *et al.*, 2006). However, other authors criticized those data arguing that crop losses encompass many other factors as post–harvest quality losses and the accumulation of toxins (Savary *et al*., 2012). This way, estimating crop losses due to pests is a tough issue and no real data are available.

Some of the factors that favour the propagation of those diseases include the monocultures, the increase of international trade and the use of a limited number of cultivars (Syed Ab Rahman *et al.*, 2018).

In 2012, the Molecular Plant Pathology journal edited a number ranking the top ten fungal pathogens based on their scientific/economic importance. Among these common pathogens, *Botrytis cinerea* and *Fusarium oxysporum* were ranked in second and fifth position, respectively (Dean *et al.*, 2012).

B.2.1. *Fusarium oxysporum*

The *F. oxysporum* is a ubiquitous fungal species complex that includes plant, animal and human pathogens, and also a wide range of non–pathogens (Gordon, 2017). It belongs to Ascomycota division and has different hosts, being proposed as the first fungal multi–host pathogen (Ortoneda *et al*., 2004). Pathogenic strains of *F. oxysporum* are gathered into *formae speciales* (ff. spp.), according to the host species they infect (Armstrong and Armstrong, 1981; Di Pietro *et al*., 2003; van der Does *et al*., 2008; Dean *et al*., 2012). Each *forma specialis* (f. sp.) can be subdivided into races that are categorized by the virulence patterns on the resistant or susceptible varieties of the host species (Michielse *et al*., 2009).

F. oxysporum includes ubiquitous soil–borne plant pathogenic lineages of a wide range of plants that are the causal agents of vascular wilt, rots, and damping–off diseases (Bodah, 2017). Characteristic disease symptoms include vascular browning, leaf epinasty, stunting, progressive wilting, defoliation and plant death

(Agrios, 2005). *F. oxysporum* is a hemibiotrophic pathogen: it initially has a biotrophic relation with the plant and then subsequently cause cell death, switching to a necrotrophic lifestyle.

F. oxysporum reproduces mainly asexually, and there is no evidence of sexual reproduction under natural conditions (Gordon, 2017). *F. oxysporum* persist in the soil as resistant spores (chlamydospores) that can germinate when sensing plants exudates (Gordon, 2017). The spores germinate and extend their germ tubes until they contact plant roots. Then, the fungus can extensively colonize the root and forms the appressoria. The fungus penetrates plant cell walls via degradative enzymes (Michielse *et al*., 2009) and therefore it enters into epidermal cells and later into the root cortex. *F. oxysporum* can colonize plant roots without causing visible external damage, whereas producing necrosis at a microscopic level. The pathogen takes the nutrients from root cortex, and it can invasively grow and develop the disease. When it happens, *F. oxysporum* occludes the xylem provoking root rot, plant wilting and death. After this, the pathogen emerges from xylem to invade adjacent tissues (Gordon, 2017).

Cell colonization by *F. oxysporum* leads to plant defense responses, both local and systemically (Berrocal–Lobo and Molina, 2008). It is suggested that pathogen effectors suppress plant immune response during the endophytic colonization, whereas secondary metabolites with hormonal or toxic activity play a role during the necrotrophic phase (Di *et al*., 2016). *F. oxysporum* can manipulate plant defenses to enhance disease. Indeed, it can seize control of JA or ET signalling pathways (Chen *et al*., 2014). However, hormone manipulation depends on the fungal strain and the host plant involved (Di *et al*., 2016).

It is noteworthy that contrasting with the enormous host range species, individual isolates of *F. oxysporum* cause disease only on one or a few plant species (Armstrong and Armstrong, 1981; Gordon and Martyn, 1997). In this way, *F. oxysporum* f. sp. *lycopersici* (Fol), used in this Thesis, infects tomato causing tomato wilt (Fig. 11).

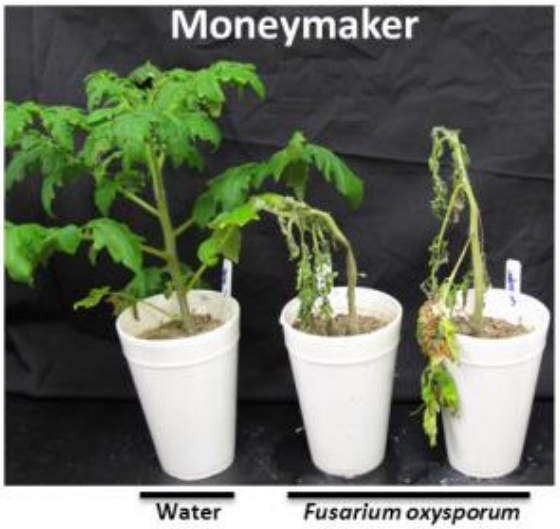


Fig. 11. **Fol symptoms on tomato plants cv. Moneymaker. Adapted from Ouyang *et al.*, 2014.**

B.2.2. Botrytis cinerea

B. cinerea is a filamentous fungus (Fig. 12) that is the causal agent of grey mould. This disease takes its name from the colour of the mycelia that the fungus develops within the host tissues. The genus *Botrytis* belongs to Ascomycota division. It is the asexual form (anamorph) that is more abundant in the field, while the sexual stage of the fungus (the teleomorph named *Botryiotinia fuckeliana*) is rarely observed in natural conditions. *Botrytis* is considered a necrotrophic model fungus (van Kan, 2006) due to its ability to infect more than 200 plant species, killing first the host cells and then colonizing the dead tissue.

During the asexual phase, the hyphae branch in the substrate (Elad *et al*., 2007) and the conidiophores are formed in the apical part of the aerial hyphae. With the appropriate conditions (high humidity and mild temperature) the conidia germinate generating new mycelia and initiating again the asexual cycle.

As a survival strategy, *B. cinerea* can form resistance structures called sclerotia. Sclerotia produce conidiophores and conidia, constituting, together with mycelia, the primary inoculum for crops (Williamson *et al*., 2007). For infecting plant tissues, *B. cinerea* produces numerous enzymes that degrade plant cell walls,

as well as toxins and other compounds such as oxalic acid. Oxalic acid may function as a pathogenesis cofactor, contributing to lowering the pH for optimal pathogenic enzyme functioning (Manteau *et al*., 2003). *Botrytis* is supposed to induce programmed cell death in the host, as a virulence strategy (Williamson *et al*., 2007). As a response for the infection, the plant activates a variety of defense reactions including antifungal metabolites and several PR proteins that are markers of SA, JA and ET signalling pathways (AbuQamar *et al*., 2017).

B. cinerea can infect all aerial parts of its host plants, causing huge damage both during plant growth (being more destructive on mature or senescent tissues of dicotyledonous hosts; Dean *et al.*, 2012) and in the post–harvest stage (during transport or cold storage; Fillinger and Elad, 2016). The cost of crop losses by *B. cinerea* is extremely difficult to calculate because of its broad host range (Dean *et al.*, 2012). Regarding its control in crops, fungicides remain the common method as no resistant plant varieties are known for this pathogen.

Fig. 12. *B. cinerea* hyphae and conidia. Colour modified for more contrast. Image by: Leyre Pescador.

INTEREST OF THE STUDY AND OBJECTIVES

Interest of the study

Plants constantly interact with a multitude of organisms, being many of them potentially deleterious for the plant. Despite the existence of many pests, crop diseases are the exception and not the rule. This is possible, as plants have evolved different mechanisms to detect potential aggressors and activate defense responses to fight them. Even so, when the protection mechanisms of the plant are altered or overpassed, the consequences might be devastating, with huge losses in both pre– and post–harvest production. Besides damaging interactions, there is also a myriad of beneficial organisms, including many soil microbes that establish mutualistic relations with the plant improving plant health. Thus, the plant needs to differentiate among beneficial and deleterious microorganisms, and to adjust their defense response accordingly to promote or contain the interaction. The study of the mechanisms that regulate plant interactions with beneficial and pathogenic microorganisms would reveal the molecular dialogue between the partners. Such dialogue is precisely regulated from the very early stages of the interaction to facilitate beneficial interactions and limit the detrimental ones. These studies are essential in plant biology, both from the basic knowledge point of view, to understand inter–kingdom communication, and from the practical point of view, to pave the way for biotechnological applications for crop protection, for instance, by using microbial inoculants or by the development of more resistant plant varieties.

Over the past twenty years, the reactive molecule nitric oxide has been shown as a signalling molecule involved in a plethora of physiological processes in plants. In particular, its role in the regulation of both plant–microbe pathogenic and beneficial interactions (mostly with bacteria) have been shown. However, despite the relevance of plant fungal interactions in plant health, little was known about the role of NO in regulating these interactions, particularly those occurring belowground. Beneficial fungi as the AMF and Trichoderma species have a great impact on plant health, and the contribution of NO to the fine regulation of their interaction with roots was largely unexplored. In addition, there were no studies regarding the possible function of NO in plant resistance to soilborne pathogens as

F. oxysporum that constitute a huge problem in agriculture. Besides the potential regulatory role of NO in the establishment of both beneficial and pathogenic interactions, little is known on its potential contribution to the induced systemic resistance triggered by beneficial soil fungi.

With this background, this Thesis aims to elucidate the role of NO in the establishment of diverse plant–fungal interactions of great relevance for the plant (the beneficial mycorrhizal and plant–Trichoderma symbioses, and the pathogenic interaction with *F. oxysporum*), and in the induced systemic resistance against pathogens triggered by volatile compounds from the beneficial fungi Trichoderma. For that, we used tomato (*Solanum lycopersicum*) and Arabidopsis (*Arabidopsis thaliana*) as model plants, *Rhizophagus irregularis* and *Trichoderma harzianum* as beneficial fungi and *Fusarium oxysporum* f. sp. *lycopersici* and *Botrytis cinerea* as pathogenic fungal species.

Objectives

The general aim of this PhD Thesis was to analyse the regulatory role of NO in the establishment of beneficial and pathogenic interactions with soil fungi, and to decipher its possible implication in Trichoderma–volatile compounds mediated ISR against pathogenic fungi.

To achieve this general objective, four specific objectives were defined and addressed, as follow:

1. **To discern the role of NO in the early signalling leading to the establishment of mycorrhizal or pathogenic interactions.** For this, we aimed to evaluate NO production dynamics in tomato during the endophytic colonization of the roots by *R. irregularis* or *F. oxysporum,* and to study the possible function of tomato phytoglobins in the regulation of NO levels during the process (Chapter I).

2. **To decipher the role of NO in the plant interaction with the free–living beneficial fungus *Trichoderma harzianum*.** For this, the NO production dynamic in the system tomato–*T. harzianum* and the possible function of the *PHYTOGB1* in the regulation of NO levels will be studied (Chapter II).

3. **To generate models of NO functions in plant–fungal interactions based in our results and the existing literature,** trying to identify common and differential patterns related to the pathogenic and mutualistic character of the associations, and their impacts on plant health (Chapter III).

4. **To explore the role of NO in the establishment of the ISR mediated by Trichoderma VCs**. For this purpose, the system Trichoderma VCs–Arabidopsis–*B. cinerea* will be used. We will study the production of NO in Arabidopsis roots by Trichoderma VCs, and by using a NO scavenger we will check the implication of NO in ISR (Chapter IV).

CHAPTER I

Nitric oxide and phytoglobin *PHYTOGB1* are regulatory elements in the *Solanum lycopersicum–Rhizophagus irregularis* mycorrhizal symbiosis

Adapted from:

Martínez–Medina A, **Pescador L**, Fernández I, Rodríguez–Serrano M, García JM, Romero–Puertas MC, Pozo MJ. 2019. Nitric oxide and phytoglobin *PHYTOGB1* are regulatory elements in the *Solanum lycopersicum–Rhizophagus irregularis* mycorrhizal symbiosis. New Phytologist 223, 1560–1574.

Abstract

The regulatory role of nitric oxide (NO) and phytoglobins in plant response to pathogenic and mutualistic microbes has been evidenced. However, little is known about their function in the arbuscular mycorrhizal (AM) symbiosis. We investigated whether NO and phytoglobin *PHYTOGB1* are regulatory components in the AM symbiosis.

Rhizophagus irregularis in vitro–grown cultures and tomato plants were used to monitor AM–associated NO–related root responses as compared to responses triggered by the pathogen *Fusarium oxysporum*. A genetic approach was conducted to understand the role of *PHYTOGB1* on NO signalling during both interactions.

After a common early peak in NO levels in response to both fungi, a specific NO accumulation pattern was triggered in tomato roots during the onset of the AM interaction. *PHYTOGB1* was upregulated by the AM interaction. By contrast, the pathogen triggered a continuous NO accumulation and a strong downregulation of *PHYTOGB1*. Manipulation of *PHYTOGB1* levels in overexpressing and silenced roots led to a deregulation of NO levels and altered mycorrhization and pathogen infection.

We demonstrate that the onset of the AM symbiosis is associated with a specific NO–related signature in the host root. We propose that NO regulation by *PHYTOGB1* is a regulatory component of the AM symbiosis.

Introduction

Plants encounter a myriad of microbes at the root–soil interface that can interact with roots with detrimental or beneficial outcomes for plant fitness. Prevalent beneficial associations between plants and microbes include the arbuscular mycorrhizal (AM) symbiosis. This symbiosis is estimated to be as old as land plants themselves, and plays a key role in terrestrial ecosystems regulating nutrient and carbon cycles, and influencing soil structure and ecosystem multifunctionality (Van der Heijden *et al*., 2015). In the AM symbiosis the AM fungus

inhabits the root cortical cells and provides the plant with an additional (fungal) pathway of mineral nutrient uptake from the soil (Smith *et al*., 2011). Besides its nutritional aspects, the symbiosis may enhance plant resistance and tolerance to multiple stresses (Jung *et al*., 2012; Barzana *et al*., 2014). In return, the plant supplies the fungus with carbon in the form of photosynthesis–derived sugars and lipids (Pfeffer *et al*., 1999; Jiang *et al*., 2017). Accordingly, plants have evolved sophisticated mechanisms to accommodate these beneficial symbionts (Bonfante and Genre, 2010). While promoting these and other beneficial relationships, plants must restrict the establishment of pathogenic associations. Achieving this balance requires the perception of potential invading microorganisms, followed by a rapid and tight regulation of immune responses to promote or contain the microbial colonization of root tissues (Zamioudis and Pieterse, 2012; Plett and Martin, 2017; Zipfel and Oldroyd, 2017). In the AM symbiosis, the plant actively accommodates the fungal partner, guiding it to the cortex where it forms the specialized, highly branched structures called arbuscules, where the exchange of nutrients takes place (Bonfante and Genre, 2010). The development of such intimate interaction relies on a continual signalling between the symbionts, and on the activation of an extensive genetic and developmental program in both partners (MacLean *et al*., 2017). Multiple signalling components operate in the establishment and the maintenance of the AM symbiosis including calcium spiking, reactive oxygen species and plant hormones (Pozo *et al*., 2015). The chemical communication between the host plant and the AM fungus is initiated in the rhizosphere, before the physical contact between the symbionts (Buee *et al*., 2000; Chabaud *et al*., 2011). The perception of fungal diffusible signals by the plant is translated in a transcriptional response that prepares the plant for the subsequent fungal colonization (Maillet *et al*., 2011; Genre *et al*., 2013). In this route, fungal signals are interpreted into a signalling pathway that regulates the activation of essential symbiotic genes required to promote the symbiosis (Chabaud *et al*., 2011; Genre *et al*., 2013). A second generation of signalling during the root colonization triggers a transcriptional reprograming in epidermal and cortical cells, with differential expression of many genes associated with transcriptional regulation, cell wall modification and defense responses. This

drives a strong cellular remodeling and the precise modulation of defense responses in the host root, which eventually leads to the establishment of the symbiosis (Liu *et al*., 2003; Siciliano *et al*., 2007; Genre *et al*., 2008; Gaude *et al*., 2012). For instance, it is proposed that the tight regulation of plant defense responses upon specific recognition of the fungal partner by the plant is essential for its active accommodation in the root tissues (García–Garrido and Ocampo, 2002; Siciliano *et al*., 2007). The degree of the symbiotic interaction is further regulated according to the plant needs and environmental conditions (Pozo *et al*., 2015). This regulation, which is partially controlled by the host plant, aims to maintain the mutualistic character of the symbiosis, avoiding excessive root colonization (Vierheilig, 2004). Despite a significant progress over the last years, understanding the signalling hardware governing the AM symbiosis is an ongoing challenge. This is due mostly to the complex genetic make–up of the AM fungus, its obligate biotrophic nature and the asynchronous character of the fungal colonization (Sedzielewska–Toro and Delaux, 2016).

The highly reactive signal molecule nitric oxide (NO) is a key component of the signalling pathways regulating plant immunity (Delledonne *et al*., 1998; Durner *et al*., 1998; Bellin *et al*., 2013). NO is produced rapidly in plant tissues during incompatible interactions with biotrophic pathogens as well as in compatible interactions with necrotrophic pathogens (van Baarlen *et al*., 2004; Romero–Puertas *et al*., 2004; Floryszak–Wieczorek *et al*., 2007). NO also can be produced by microbial pathogens to promote the infection of plant tissues (Arasimowicz–Jelonek and Floryszak–Wieczorek, 2016), and participates in the proper establishment of the mutualistic association between legumes and rhizobia (Hichri *et al*., 2015). In this symbiosis, NO is proposed to be involved in the activation of the developmental program required for nodule formation and development, and in the early repression of the plant defense reaction favoring symbiosis establishment (Ferrarini *et al*., 2008; Boscari *et al*., 2013). NO accumulation can be regulated by the activity of plant phytoglobins (previously known as non–symbiotic haemoglobins; Perazzolli *et al*., 2004; Qu *et al*., 2006; Nagata *et al*., 2008, 2009; Hill *et al*., 2016), that may function as NO dioxygenases that catalytically metabolize NO

to nitrate (Seregelyes *et al*., 2004; Hill, 2012). Indeed, NO triggers the expression of the phytoglobin gene *PHYTOGB1* in a number of plant species (Perazzolli *et al*., 2004; Bustos–Sanmamed *et al*., 2011; Bai *et al*., 2016); and the manipulation of the *PHYTOGB1* in transgenic lines evidenced its crucial role for NO bioactivity during plant–microbe interactions (Perazzolli *et al*., 2004; Shimoda *et al*., 2009; Mur *et al*., 2012; Bai *et al*., 2016; Fukudome *et al*., 2016).

Increasing evidence is showing that NO also is produced during other symbiotic interactions including mycorrhizal and lichen symbioses (Weissman *et al*., 2005; Calcagno *et al*., 2012; Espinosa *et al*., 2014). Moreover, genome–wide analysis of transcription patterns revealed *PHYTOGB1* as one of the mycorrhiza–early activated genes in the epidermal layer of *Medicago truncatula* roots interacting with different AM fungi (Siciliano *et al*., 2007; Hogekamp and Küster, 2013). Together these observations suggest a potential role of NO and phytoglobins in AM symbiosis establishment. However, the role(s) of NO in the AM symbiosis remains elusive so far, and its regulation during the establishment and functioning of the symbiosis is still puzzling.

In the present contribution, we hypothesized that NO is a signalling component of the regulatory pathway that is activated in the host root during the onset of the AM symbiosis.We also hypothesized that the AM symbiosis–related signalling is associated with a specific NO signature, different to that associated with immunity related signalling. We further explored the role of *PHYTOGB1* in the regulation of NO bioactivity in mutualistic and pathogenic plant–microbe interactions. Our results demonstrate that the AM onset is associated with a specific NO–related signature and a specific regulation pattern of the *PHYTOGB1* gene in the host root. By using transgenic hairy roots silenced and overexpressing the *PHYTOGB1* gene, we demonstrated the role of *PHYTOGB1* in the regulation of NO levels in tomato roots, and in the regulation of the AM establishment and pathogen infection.

Materials and methods

Plant and fungal material

Tomato (*Solanum lycopersicum* cv. Moneymaker) seeds were surface–sterilized in 4% sodium hypochlorite and germinated in sterile water at 25°C in darkness. After 1 week, seedlings were transferred to hydroponic conditions in 3–l tanks containing Long Ashton nutrient solution (Hewitt, 1966) with constant aeration. Plants were grown in the hydroponic tanks (six plants per tank) at 16 h: 8 h, light (24°C): dark (16°C) cycle at 70% relative humidity for two weeks before use. The AM fungus *Rhizophagus irregularis* (Schenck and Smith DAOM 197198) was grown in monoxenic cultures, using Ri T–DNA (*Agrobacterium rhizogenes*)–transformed carrot (*Daucus carota* clone DC2) according to St–Arnaud *et al*. (1996). Cultures were established according to Chabot *et al*. (1992) in 100 x 20 mm Petri plates, placed in 150 x 25 mm Petri plates (Fig. 1a, b) to allow separating the root compartment from the hyphal compartment. Petri plates were incubated in the dark at 24°C until the hyphal plate, which contained M medium without sucrose, was profusely colonized by the fungus (*c*. 12 weeks; Fig. 1c). The root plate was then removed, and plates were used for the experiments. *Fusarium oxysporum* f. sp. *lycopersici* was grown on potato dextrose agar (PDA) at 28°C in dark conditions for 5 days.

Early interaction experiment set–up

A small orifice (3–mm diameter) was made in the side and the lid of the Petri dishes containing the *R. irregularis* or the *F. oxysporum* cultures. Two–week–old tomato plants, grown in the hydroponic tanks were transferred to the Petri plates, one plant per plate, placing the roots on the surface of the culture and the stem in the hole, letting the shoot expand outside the plate, in open air conditions (Fig. 1d) as described by Voets *et al*. (2005). Petri plates were closed and covered to keep the root system in the dark, and plants were kept in a growth chamber at 16 h: 8 h, light

(24°C): dark (16°C) cycle at 70% relative humidity. At 4, 8, 24, 48, 72 and 96 h after setting up the experiment, plants were harvested and root material was collected.

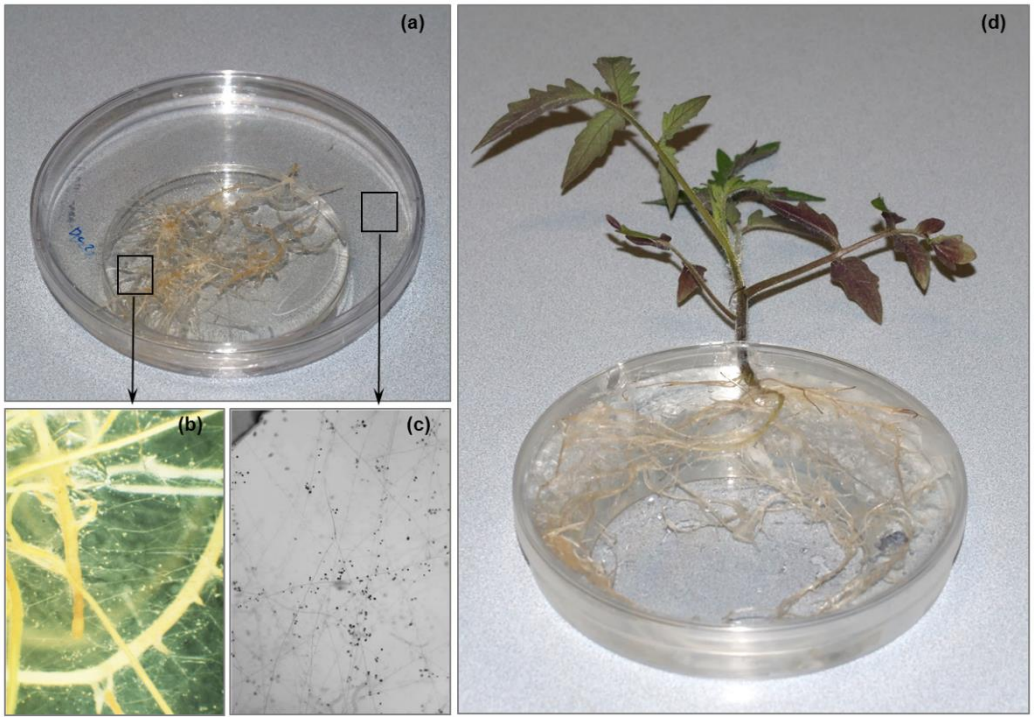


Fig. 1. Early interaction experiment set–up. (a) The arbuscular mycorrhizal fungus *Rhizophagus irregularis* was grown in monoaxenic cultures using Ri T–DNA–transformed carrots. The plate containing the root culture was placed in a bigger plate containing M medium without sucrose **(b).** When the M medium was profusely colonized by the fungus **(c)**, the plate containing the root cultures was removed, and tomato plants were transferred to the plates, with the roots placed on the surface of the colonized medium and the shoot extending beyond the plate **(d).**

Fungal elicitors

Exudates were obtained from *c*. 1 x 10^8 germinating spores of *R. irregularis* and *F. oxysporum*. Sterile spores were germinated in 30 ml sterile distilled water for 1 week at 24°C in dark (germination rate was *c*. 80%). The germinating spore suspensions were then collected and filtrated first through 0.45–µm and later through 0.22–µm Millipore filters. We denote the resulting filtrate as germinating spore exudates. Homogenates of *R. irregularis* and *F. oxysporum* cell wall were obtained from *R. irregularis* monoxenic cultures grown as described above, and *F.*

oxysporum grown in potato dextrose broth media on a shaker for 5 days in dark conditions. The mycelium of the *R. irregularis* monoxenic culture was carefully removed with 10mM sodium citrate to liquefy the culture media. The cell wall material from both fungi was prepared according to Ren and West (1992) and then ground to fine powder and lyophilized. Roots were treated for 3, 6 and 24 h with 3 ml germinating spore exudates from *R. irregularis* and *F. oxysporum*, or with 3 ml ground lyophilized cell walls resuspended in distilled sterile water at 0.1% (w/v).

Chemical treatments

The roots of 2–weeks–old tomato plants grown in hydroponic tanks were treated with the nitric oxide (NO)–releasing compounds sodium nitroprusside (SNP; 200 µM; Sigma–Aldrich, St Louis, MI, USA), *S*–nitrosoglutathione (GSNO; 350 µM; Calbiochem, San Diego, CA, USA) and DETA–NONOate (500 µM; Cayman Chemicals, Ann Arbor, MI, USA), for 1 and 3 h. In the case of SNP, a control treatment with 200 µM of sodium ferricyanide was run in parallel (Bethke *et al*., 2006).

NO detection and quantification

Quantitative NO determination was performed through spectrofluorometry as described previously (Nakatsubo *et al*., 1998; Besson–Bard *et al*., 2009). Briefly, 0.2 g of fresh root samples were ground in 0.8 ml extraction buffer (50 mM Tris–HCl, pH 7.8; 0.1 mM EDTA; 0.2% triton X–100; 10% glycerol; 2% PVPP) with a mortar. Homogenates were centrifuged at 11300 g for 30 min. Aliquots of supernatants were immediately diluted 50–fold in HEPES buffer (50 mM, pH 7.5). DAF–2 (Merck Biosciences) was added at 2 µM final concentration and the reaction mixtures were incubated at 37°C in the dark for 2 h. Fluorescence was measured in a RF–540 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 485 and 515 nm, respectively. NO detection by microscopy was performed as described in Sandalio *et al*. (2008): segments of plant roots were incubated for 1 h in darkness with 10 µM 4–amino–5–methylamino–20,70–difluorofluorescein diacetate (DAF–FM DA; Merck Biosciences, Darmstadt,

Germany), prepared in 10 mM Tris–HCl (pH 7.4). As a negative control, roots segments were similarly incubated with the NO scavenger 2–4–carboxyphenyl–4,4,5,5–tetramethylimidazoline–1–oxyl–3–oxide (cPTIO; Sigma) at a final concentration of 500 μM. The segments were washed three times for 15 min each in 10 mM Tris–HCl (pH 7.4) to remove dye excess. The fluorescence emitted by DAF–FM DA was detected by excitation at 495 nm and emission at 515 nm using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Real–time quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Extraction of total RNA from plant roots and synthesis of cDNA was performed according to Martínez–Medina *et al*. (2013). Real–time qRT–PCR reactions and relative quantification of specific mRNA levels were performed according to Martínez–Medina *et al*. (2013) and by using the gene–specific primers described in Table S1. The data were normalized using the housekeeping gene *SlEF* (X14449) encoding for the tomato translation elongation factor–1α, whose expression remained stable in the different lines and conditions. mRNA sequences of the tomato phytoglobin genes *PHYTOGB1* (AY026343), *PHYTOGB2* (AY026344) and *PHYTOGB3* (AW036344) were found in the online database NCBI. Gen structure information was obtained using the on–line database SOL Genomics Network (http://solgenomics.net/).

Generation of the *PHYTOGB1* RNAi and OE vectors and transformation by *Agrobacterium rhizogenes*

For the generation of the RNAi vector a PCR fragment of 201 bp including part of the 30–UTR and coding region of the *PHYTOGB1* gene was amplified using tomato cDNA as template and the primers RNAi–*PHYTOGB1* Fw: 5´–CACCGGTT AGTGCTATCAAGACTGAGATGAAG–3´ and RNAi–*PHYTOGB1* Rv: 5´–GCACACAAATTAGATTATAAAATTTTGCAACG–3´. PCR was performed using Taq polymerase Poof–reading (Roche) according to manufacturer's instructions. The

PCR product was purified by using the DNA clean and concentrator kit (Zymo Research, Irvine, CA, USA), and then cloned into pENTR–TOPO (Invitrogen, Carlsbad, CA, USA) according to manufacturer's indications. Subsequently a Gateway reaction was performed with destination expression vector pRedRoot (Limpens *et al*., 2004). The inserts were verified by restriction digests and sequencing. The pRedRoot vector without insert (empty vector) was used for controls. The vectors were introduced into *A. rhizogenes* strain MSU440 by electroporation. *A. rhizogenes* was grown for 2 days at 28°C under spectinomycin selection (50 μg ml^{-1}). The integrity of the constructs was checked by sequencing. Tomato seeds were surface–sterilized in 4% sodium hypochlorite and germinated for 5 days in darkness in sterile conditions. The germinated seeds were transferred to a half strength Murashige and Skoog (MS) vitamin agar–solidified medium (pH 5.8) and grown for 5 days at 21°C with a photoperiod of 16 h: 8 h, light: dark. Tomato seedlings were transformed with *A. rhizogenes* containing the appropriate constructs according to Chabaud *et al*. (2006) with some modifications. Briefly, the roots of the seedlings were cut out and the seedlings were co–cultivated with MSU440 for 6 day at 21°C with a photoperiod of 16 h: 8 h, light: dark, in half–strength MS vitamin agar solidified medium. Seedlings were then transferred to MS agar–solidified medium supplemented with 500 μg ml^{-1} cefotaxime and 50 μg ml^{-1} kanamycin for 3 days at 25°C with a photoperiod of 16 h: 8 h, light: dark, to select positive transgenic individuals. Seedlings were then transferred to MS agar–solidified medium supplemented with 300 μg ml^{-1} cefotaxime and 50 μg ml^{-1} kanamycin and roots were cut out and then grown for 21 days at 25°C with a photoperiod of 16 h: 8 h, light: dark. Emerging roots were periodically screened for DsRED1 fluorescence. Red–fluorescent roots were retained, whereas all nonfluorescent roots were removed by excision.

For overexpression, the *PHYTOGB1* full–length open reading frame was amplified from tomato cDNA by using the specific primers OE–*PHYTOGB1* Fw: 5′–CACCATGAGTAGCT TTAGTGAAGAACAAGAAGC–3′ and OE–*PHYTOGB1* Rv: 5′–CTTCATCTCAGTCTTGATAGCACTAACC–3′, and cloned into pENTR–TOPO (Invitrogen) as described for the generation of the RNAi vector. Subsequently a

Gateway reaction was performed with destination expression vector pAtUbq10_DsRed (Kryvoruchko *et al*., 2016). The empty vector was used for controls. The verified construct was then introduced into *A. rhizogenes* strain MSU440 by electroporation and transformants were selected by resistance to streptomycin and spectinomycin. Generation of composite *S. lycopersicum* plants was performed according to Ho–Plágaro *et al*. (2018).

Colonization bioassays

Transformed plants were transferred to 100–ml pots containing a sterile sand: vermiculite mixture (1: 1, v/v). Inoculation with *R. irregularis* was achieved according to Rivero *et al*. (2015). The *R. irregularis* inoculum consisted of *R. irregularis* kept in a soil–sand mixture containing extraradical mycelium and spores, and mycorrhizal root fragments of *Trifolium repens* (Rivero *et al*., 2015). Plants were placed in a completely randomized design in a growth chamber at 16 h: 8 h, light (24°C): dark (16°C) cycle at 70% relative humidity. Plants were watered three times a week with nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration. Six weeks after transplanting into pots, plants were harvested and root material was collected. Mycorrhizal structures were stained with trypan blue (Phillips and Hayman, 1970). Quantification of the different fungal structures within the roots was performed according to Trouvelot *et al*. (1986), using a Nikon Eclipse 50i microscope, Nikon, Tokyo, Japan. Molecular quantification of *R. irregularis* within the roots was performed by qRT–PCR using *Ri–EF1α* primers specific for the constitutively expressed elongation factor 1a from *R. irregularis* (Helgason *et al*., 2003). The functionality of the mycorrhizal symbiosis was checked by analyzing the expression of the tomato *LePT4*, encoding a phosphate transporter specific for the AM symbiosis which is expressed in arbusculated cells (Balestrini *et al*., 2007).

Results

NO levels oscillate in tomato roots differently during early steps of mycorrhizal and pathogenic interactions

Nitric oxide is involved in the plant responses to different microbes, including pathogens and rhizobial bacteria (Bellin *et al*., 2013; Hichri *et al*., 2015). To understand whether NO also is a signalling component of the AM symbiosis establishment, we first investigated NO levels in tomato roots during early stages of the AM interaction with *R. irregularis* by using a *R. irregularis in vitro*–grown cultures (Fig. 1) and the fluorescent indicator for the detection of NO DAF–2. To further investigate the *in vivo* spatiotemporal fluctuation of NO accumulation in roots, we used the cell–permeable NO–specific probe DAF–FM DA and confocal laser microscopy. Moreover, to discern whether the AM symbiosis signalling is associated to specific patterns of NO accumulation, we studied in parallel the NO accumulation pattern in tomato roots during early stages of the pathogenic interaction with *F. oxysporum*. We detected a transient burst of NO in tomato roots 4 h after the contact with the AM fungus (Fig. 2a, b). After this first NO peak, NO production oscillated in time, showing two more peaks at 48 and 96 h. NO levels in *R. irregularis*–roots at 8, 24 and 72 h was similar to that observed in control plants. *R. irregularis*–induced NO accumulation was observed mainly in the outer cell layers (epidermal and cortical cells) and in root hairs (Fig. 3a, b). Incubation of roots with the NO scavenger cPTIO extinguished the fluorescence induced by *R. irregularis*, confirming that NO production was being detected (Fig. S1).

The interaction of the roots with the pathogen *F. oxysporum* also induced a strong and transient NO burst at 4 h (Fig. 2a, b). After 8 h, NO levels in *F. oxysporum*–roots returned to basal levels. However, 24 h after the contact with the pathogen, NO accumulation increased over time. It was remarkable that by contrast with the AM interaction, the pathogen–triggered NO accumulation was evenly distributed over the root fragments analyzed (Fig. 3b). Together our findings demonstrate that NO is accumulated in tomato roots during the early steps of both the mutualistic and pathogenic interactions. However, NO accumulation triggered by the AM interaction

showed a specific spatiotemporal pattern, which differed significantly to that observed during interaction with the fungal pathogen.

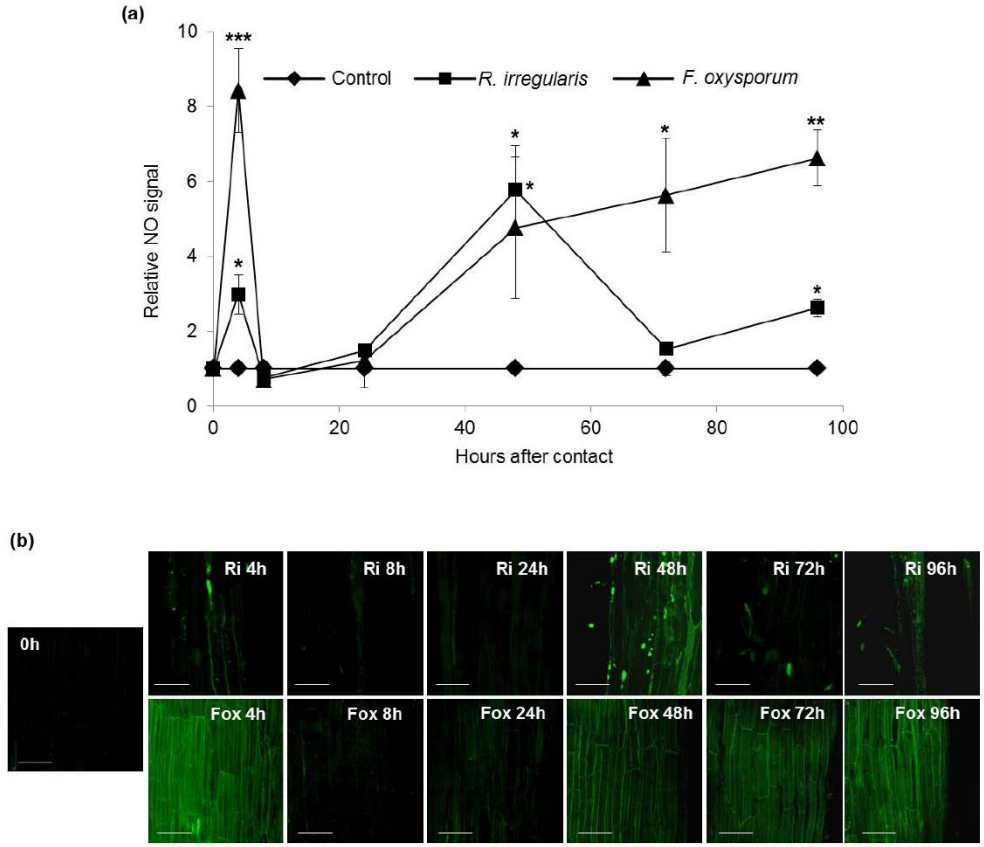


Fig. 2. Nitric oxide (NO) accumulation in tomato roots after contact with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* or the pathogenic fungus *Fusarium oxysporum*. (a) NO was detected by fluorimetry by using the specific NO detector DAF–2 at 4, 8, 24, 48, 72 and 96 h after the contact with the different fungi. *In vitro*–grown cultures of *R. irregularis* and *F. oxysporum* were used in the experiments. NO levels are reported as the fold control plants at ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ according to Dunnett test. **(b)** Imaging of NO production in tomato roots by confocal laser increase relative to that of the control plants at each time point ± SE ($n = 4$ biological replicates). Asterisks indicate significant differences compared to microscopy. Images are projections of several optical sections collected by confocal microscopy showing the NO–dependent DAF–FM DA fluorescence (green; excitation at 495 nm, emission at 515 nm) from plants at 0, 4, 8, 24, 48, 72 and 96 h after contact with *R. irregularis* (Ri) or *F. oxysporum* (Fox). Bars, 50 µm. One independent representative of four biological replicates is shown. These results are representative for one of three independent experiments.

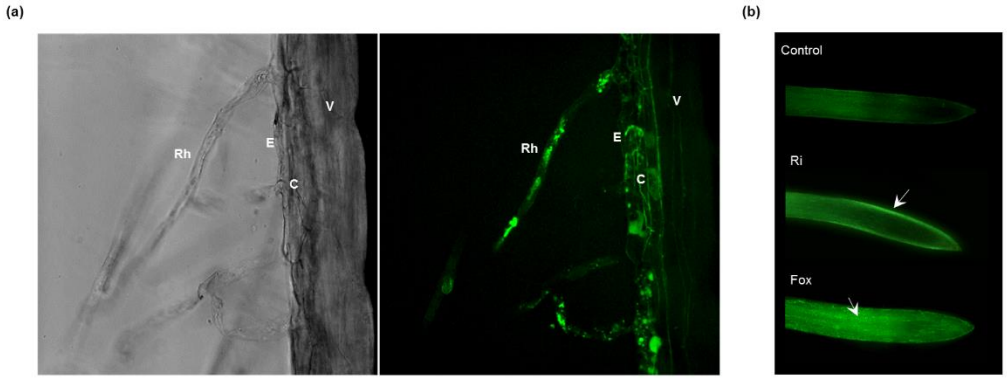


Fig. 3. Tissue–specific visualization of nitric oxide (NO) in tomato roots 48 h after contacting with *Rhizophagus irregularis* (a). Bright field (left panel) and fluorescence (right panel) images were taken by confocal microscopy. Green indicates NO–dependent DAF–FM DA fluorescence (excitation at 495 nm, emission at 515 nm). V, vascular bundle; E, epidermis; C, cortex; Rh, root hair. **(b)** NO also was visualized by fluorescence microscopy in roots of tomato plants mock inoculated (control) or 48 h after contacting with *R. irregularis* (Ri) or *Fusarium oxysporum* (Fox). Green indicates NO–dependent DAF–FM DA fluorescence (excitation at 495 nm, emission at 515 nm). The arrows point to NO–dependent DAF–FM DA signal confined mostly to the outer cell layers of *R. irregularis*–roots, and evenly distributed over the *F. oxysporum*–roots. Representative images are shown.

Exudates from *R. irregularis* germinating spores induce NO accumulation in tomato roots

During the presymbiotic stages of the AM symbiosis diffusible molecules released by the AM fungus, the so–called MYC factors, activate early symbiotic responses in the roots (Maillet *et al*., 2011). We reasoned that plant perception of MYC factors might trigger a NO–related response in the host root. To investigate this, we monitored NO accumulation in tomato roots after treatment with germinating spore exudates from *R. irregularis*. We found that the exudates triggered an early burst of NO, which occurred within the first 3 h post–treatment (Fig. 4a). The NO signal declined to basal levels after 6 h of treatment. A further burst of NO was observed 24 h after the application of the *R. irregularis* germinating spore exudates (Fig. 4a). It is remarkable that germinating spore exudates from the pathogenic fungus *F. oxysporum* did not significantly alter NO levels in the roots (Fig. 4a).

Fungal cell wall components also are known to elicit early plant defense responses, functioning as microbe–associated molecular patterns that can be recognized by the plant immune system (Zipfel and Robatzek, 2010). We analyzed whether cell wall components from *R. irregularis* or *F. oxysporum* elicit a NO–related response in the host roots. The application of a suspension of homogenized fungal cell walls from *R. irregularis* did not affect NO levels in tomato roots (Fig. 4b), whereas *F. oxysporum* cell walls induced a slight, although not significant, transient increase in NO accumulation after 6 h. These findings indicate that plant perception of bioactive molecules present in the AM fungal exudates triggers a NO–related signalling during the presymbiotic stage of the AM symbiosis.

The tomato *PHYTOGB1* gene is upregulated in tomato roots in response to NO

The tomato genome contains three genes encoding phytoglobins: one class 1 phytoglobin (*PHYTOGB1*), one class 2 phytoglobin (*PHYTOGB2*) and one truncated phytoglobin (*PHYTOGB3*) (Fig. S2). Previous studies provided compelling evidence that phytoglobin genes can be induced by NO, playing a major role in plant protection against nitrosative stress (Perazzolli *et al*., 2004). We investigated whether tomato phytoglobin genes also are regulated by NO. To this end, we analyzed the transcriptional regulation of the set of tomato phytoglobin genes in roots after the treatment with the NO donors SNP, DNN and GSNO. Incubation with the different NO donors triggered the upregulation of the *PHYTOGB1* gene at 1 and 3 h post–treatment (Fig. 5a). NO donors did not significantly induce the expression of the other two phytoglobin genes (Fig. 5b, c). These results demonstrate that the tomato phytoglobin gene *PHYTOGB1* is consistently upregulated by NO, and suggest a potential role for *PHYTOGB1* in NO metabolism in tomato roots.

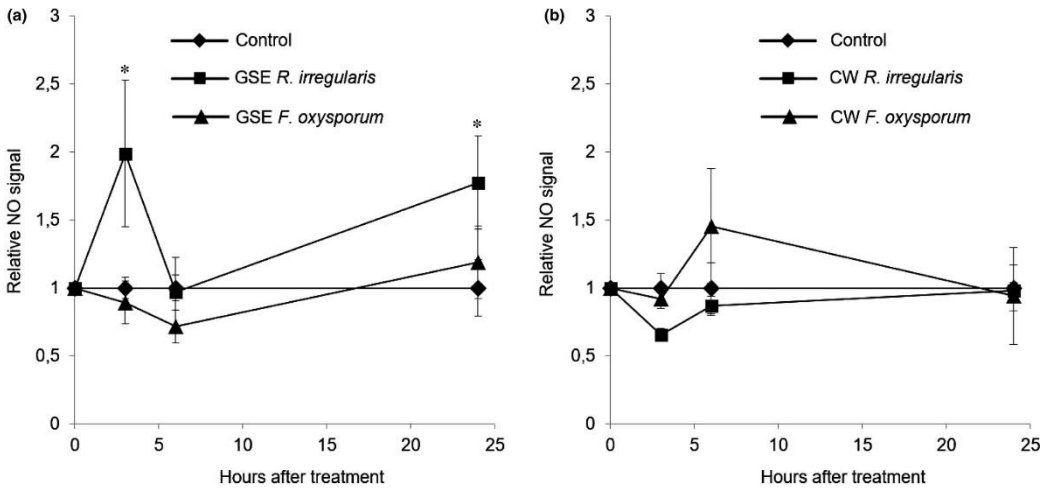


Fig. 4. Effect of exudates from germinating spores (GSE) and a suspension of cell walls (CW) from *Rhizophagus irregularis* and *Fusarium oxysporum* on endogenous nitric oxide (NO) root accumulation. NO was detected by fluorimetry by using the specific NO detector DAF–2 in tomato roots at 3, 6 and 24 h post–treatment with the GSE **(a)** or fungal CW **(b).** NO levels are showed as the fold increase relative to that of the control plants at each time point ± SE (n = 4 biological replicates). Asterisks indicate significant differences compared to control plants (Dunnett test, $P < 0.05$). These results are representative from one of two independent experiments.

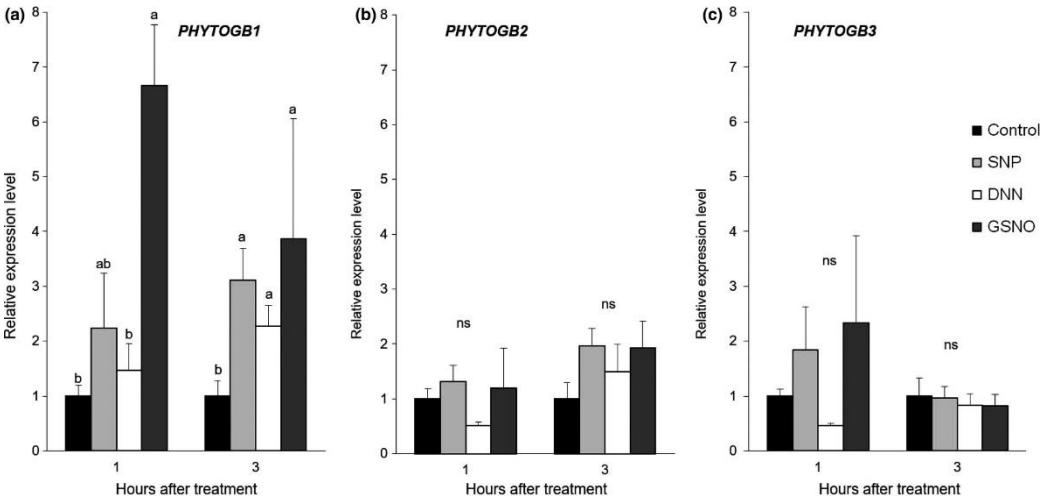


Fig. 5 Effect of nitric oxide (NO) on the regulation of tomato phytoglobin genes. Expression of **(a)** *PHYTOGB1*, **(b)** *PHYTOGB2* and **(c)** *PHYTOGB3* were analyzed in roots of tomato plants 1 and 3 h after treatment with the NO donors sodium nitroprusside (SNP), DETA–NONOate (DNN) and *S*–nitrosoglutathione (GSNO). Results were normalized by using the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the control plants not treated with the NO donors at each time point ± SE (n = 3 biological replicates). Data not sharing a letter in common at each time point differ significantly according to Tukey's honest significant difference test ($P < 0.05$). ns, not significant. These results are representative for one of two independent experiments.

The mycorrhizal and pathogenic interactions differentially regulate *PHYTOGB1* gene expression in tomato roots

Given the responsiveness of *PHYTOGB1* to NO and the impact of the AM and pathogenic interactions on NO accumulation, we reasoned reasoned that these interactions might elicit an early activation of *PHYTOGB1* in tomato roots. To investigate this, we analyzed the regulation of the tomato phytoglobin genes in roots during the early stages of the interaction with *R. irregularis* and with *F. oxysporum*. *PHYTOGB1* transcription was induced already 4 h after the contact with both fungi (Fig. 6a; Table S2). *R. irregularis*–triggered upregulation of *PHYTOGB1* was further observed at 8, 48 and 96 h after the interaction.

By contrast to the sustained induction of *PHYTOGB1* by the interaction with the AM fungus, the pathogen led to an initial increase of *PHYTOGB1* at 4 h after the interaction, but followed by a strong decrease later on (Fig. 6a). It is remarkable that *PHYTOGB1* upregulation induced by the pathogen at 4 h was *c*. 10 times higher than that seen in roots interacting with the AM fungus (Fig. 6a). The levels were still higher, although to a lesser extent, at 8 h. However, at later time points *PHYTOGB1* expression strongly decreased in the host roots, with transcripts barely detected by 72 or 96 h after contact (Fig. 6a). A similar inhibition of *PHYTOGB1* expression was found in tomato roots and shoots upon infection with the root and foliar pathogens *Phytophthora parasitica* and *Botrytis cinerea*, respectively (Fig. S3). By contrast to *PHYTOGB1*, *R. irregularis* did not upregulate the expression of *PHYTOGB2* and *PHYTOGB3* throughout the monitored timespan (Fig. 6b, c). Indeed, *R. irregularis* reduced the expression levels of *PHYTOGB2* in tomato roots (Fig. 6b).

We observed a similar reduction in *PHYTOGB2* in plants in contact with the pathogen, specifically from 48 h after the contact (Fig. 6b). These observations indicate that *PHYTOGB1* is specifically upregulated by the AM interaction, and may suggest a role for *PHYTOGB1* during the onset of the AM symbiosis. Remarkably, in agreement with the NO accumulation pattern described earlier (Fig. 4), only germinating spore exudates from the mycorrhizal fungus, and not from *F. oxysporum*, significantly induced the expression of the *PHYTOGB1* gene in tomato roots (Fig. S4).

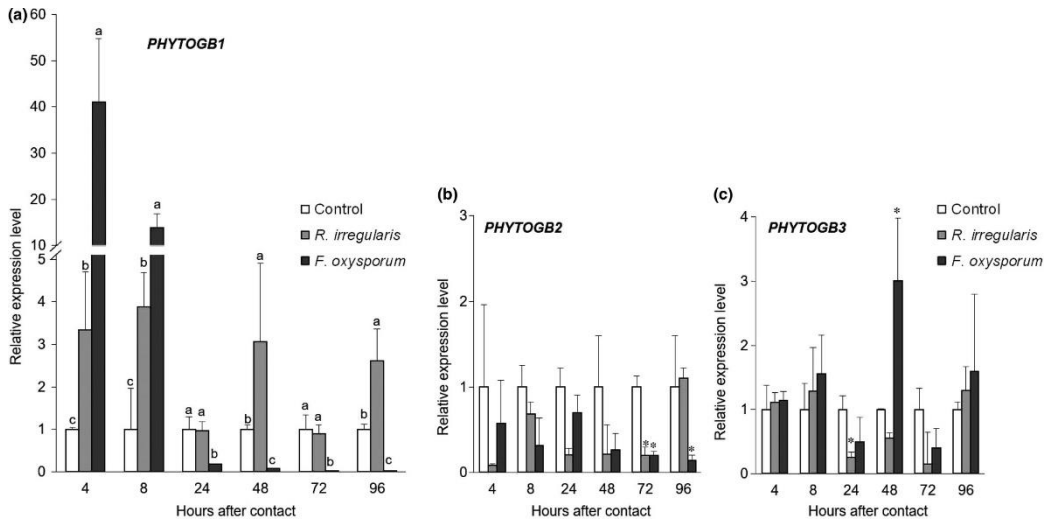


Fig. 6. Time course of expression of the tomato phytoglobin genes after contact with *Rhizophagus irregularis* or *Fusarium oxysporum*. The expression levels of **(a)** *PHYTOGB1*, **(b)** *PHYTOGB2* and **(c)** *PHYTOGB3* were analyzed in roots of tomato plants 4, 8, 24, 48, 72 and 96 h after contact with the arbuscular mycorrhizal fungus R. *irregularis* or the pathogen *F. oxysporum*. Results were normalized to *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the control plants at each time point ± SE (n = 4 biological replicates). In (a) data not sharing a letter in common at each time point differ significantly according to Tukey's honest significant difference test (P < 0.05). In (b) and (c) asterisks indicate significant differences in each time point compared to control plants (Dunnett test, P < 0.05). These results are representative for one of three independent experiments.

Altered *PHYTOGB1* levels in tomato roots leads to changes in NO and impacts mycorrhizal root colonization

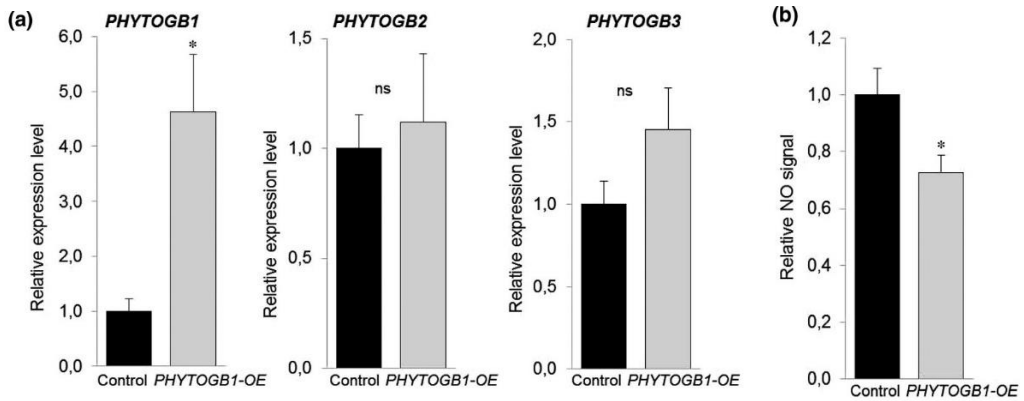
In order to confirm whether *PHYTOGB1* is involved in NO metabolism in tomato, we generated composite plants with *PHYTOGB1* overexpressing (*PHYTOGB1*–OE) and the corresponding empty vector control roots. qRT–PCR analysis confirmed that the lines carrying the overexpressing construct had significantly increased *PHYTOGB1* expression levels compared with control roots carrying the empty vector, whereas the expression of the other phytoglobin genes remained unaltered (Fig. 7a). NO levels in *PHYTOGB1*–OE were lower compared to control roots transformed with the empty vector (Fig. 7b). As shown in Fig. 7c, a higher frequency (F%) and intensity (M%) of mycorrhizal colonization was found in the root system of *PHYTOGB1*–OE lines compared to plants transformed with the empty vector. Moreover, the intensity of the colonization (m%) within the colonized

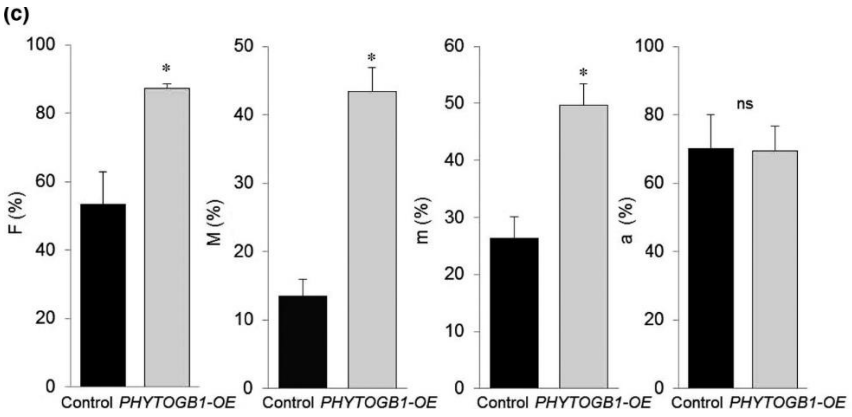
root fragments also was higher in *PHYTOGB1*–OE lines. It is remarkable that overexpressing *PHYTOGB1* did not affect the arbuscule abundance in the mycorrhizal parts (a%). The results from the histochemical analysis were further verified by molecular analysis. A higher accumulation of *Ri–EF1α* gene transcripts were detected in *PHYTOGB1*–OE roots compared to roots carrying the empty vector (Fig. 7d). Similarly, a stronger expression of *LePT4* , which encodes an AM–specific plant phosphate transporter, was found in the *PHYTOGB1*–OE roots (Fig. 7d). We further investigated whether silencing of *PHYTOGB1* also had phenotypic effects on NO accumulation and mycorrhizal colonization patterns. With this aim a hairpin construct was created that targeted 201 bp of the tomato *PHYTOGB1* sequence. Composite plants were generated with *PHYTOGB1* silencing (*PHYTOGB1*–RNAi) and its corresponding empty vector (control) roots. qRT–PCR analysis showed that the RNAi construction significantly decreased the *PHYTOGB1* expression compared with control roots, but it did not alter significantly the expression of any of the other phytoglobin genes (Fig. 8a). A strong increase in NO accumulation was observed in *PHYTOGB1*–RNAi lines compared to control roots transformed with the empty vector (Fig. 8b), further demonstrating the role of *PHYTOGB1* in regulating NO root metabolism. A higher frequency (F%) and intensity (M%) of mycorrhizal colonization was found in the *PHYTOGB1*–RNAi lines compared to plants transformed with the empty vector (Fig. 8c). Although not significant, *PHYTOGB1*–silenced lines also showed a slight increased in colonization intensity in the roots fragments (m%). As in the overexpressing lines, silencing *PHYTOGB1* did not affect arbuscule abundance in the mycorrhizal parts (a%). These results were further corroborated by the higher *Ri–EF1α* and *LePT4* transcript levels in *PHYTOGB1*–RNAi roots (Fig. 8d). Altogether our results demonstrate the importance of NO regulation by *PHYTOGB1* specifically during the early stages of mycorrhizal establishment, related to root colonization, but not in the development of the arbuscules.

We investigated whether this alteration could be related to changes in plant defenses associated to the altered NO levels. With this aim, we tested whether alteration of NO levels by exogenous application of a NO donor (GSNO) and the NOS–like inhibitor aminoguanidine, or by altered levels of the *PHYTOGB1* lead to altered

defense gene expression in tomato roots. The application of GSNO triggered a significant transient induction of several defense genes in tomato roots, whereas they were repressed by the application of aminoguanidine (Fig. S5). However, lower NO levels in the *PHYTOGB1*–OE lines were associated to higher basal levels of some defense–related genes, suggesting that NO can be a positive or negative regulator of defenses depending on its concentration and timing (Fig. S5). Remarkably, the increase of some defense genes triggered by the mycorrhizal colonization in control roots transformed with the empty vectors was not found in the *PHYTOGB1* overexpressing and silenced roots, supporting a release of the plant control over the fungus that may lead to higher mycorrhizal colonization (Fig. S5).

In analogy to the mycorrhizal interaction, deregulation of *PHYTOGB1* had an impact in the interaction with the root pathogen. We found an enhanced infection by *F. oxysporum* in the *PHYTOGB1*–RNAi roots displaying elevated NO levels (Fig. S6). By contrast, a lower incidence of *F. oxyporum* was found in *PHYTOGB1*–OE compared to plants transformed with the empty vector (Fig. S6).


(a)
PHYTOGB1
PHYTOGB2
PHYTOGB3
Relative expression level
6,0
5,0
4,0
3,0
2,0
1,0
0,0
1,5
1,0
0,5
0,0
2,0
1,5
1,0
0,5
0,0
*
ns
ns
Control
PHYTOGB1-OE
(b)
Relative NO signal
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
Control
PHYTOGB1-OE


(c)
F (%)
M (%)
m (%)
a (%)
100
80
60
40
20
0
50
40
30
20
10
0
60
50
40
30
20
10
0
*
*
*
ns
Control
PHYTOGB1-OE

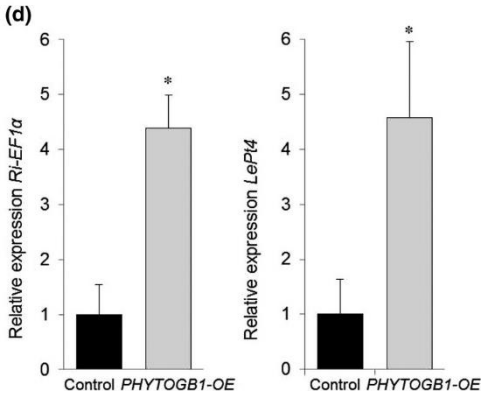

(d)
Relative expression Ri-EF1α
Relative expression LePt4
6
5
4
3
2
1
0
*
*
Control
PHYTOGB1-OE

Fig. 7. Impact of overexpressing tomato *PHYTOGB1* on the expression of tomato phytoglobin genes, nitric oxide (NO) root accumulation and mycorrhizal colonization. Tomato plants were transformed with empty ectors (control) or *PHYTOGB1* overexpressing constructs (*PHYTOGB1*–OE). **(a)** Expression level of *PHYTOGB1*, *PHYTOGB2* and *PHYTOGB3* was analyzed in empty vector controls and in *PHYTOGB1*–OE roots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the empty–vector control roots ± SE (n = 6 biological replicates). **(b)** NO accumulation was detected in empty vector controls and in *PHYTOGB1*–OE roots by fluorimetry using the specific NO detector DAF–2. NO levels are reported as the fold change relative to that of the empty–vector control roots ± SE (n = 6 biological replicates). **(c)** Arbuscular mycorrhiza fungal structures within the roots were analyzed in empty–vector controls and *PHYTOGB1*–OE roots 6 weeks after inoculation with *Rhizophagus irregularis* in pots. F%, frequency of colonization in the root system; M%, intensity of colonization in the root system; m%, intensity of colonization within the mycorrhizal fragments; a%, arbuscule abundance in mycorrhizal parts. **(d)** Relative expression of the *R. irregularis* constitutive gen *Ri–EF1α* and the mycorrhizal functionality marker gene *LePT4* in empty–vector controls and *PHYTOGB1*–OE roots 6 weeks after inoculation with *R. irregularis* in pots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the empty–vector control root ± SE (n = 6 biological replicates). The asterisks indicate a statistically significant difference in comparison to the empty–vector control root according to Student's t–test ($P < 0.05$); ns, not significant. These results are representative from one of two independent experiments.

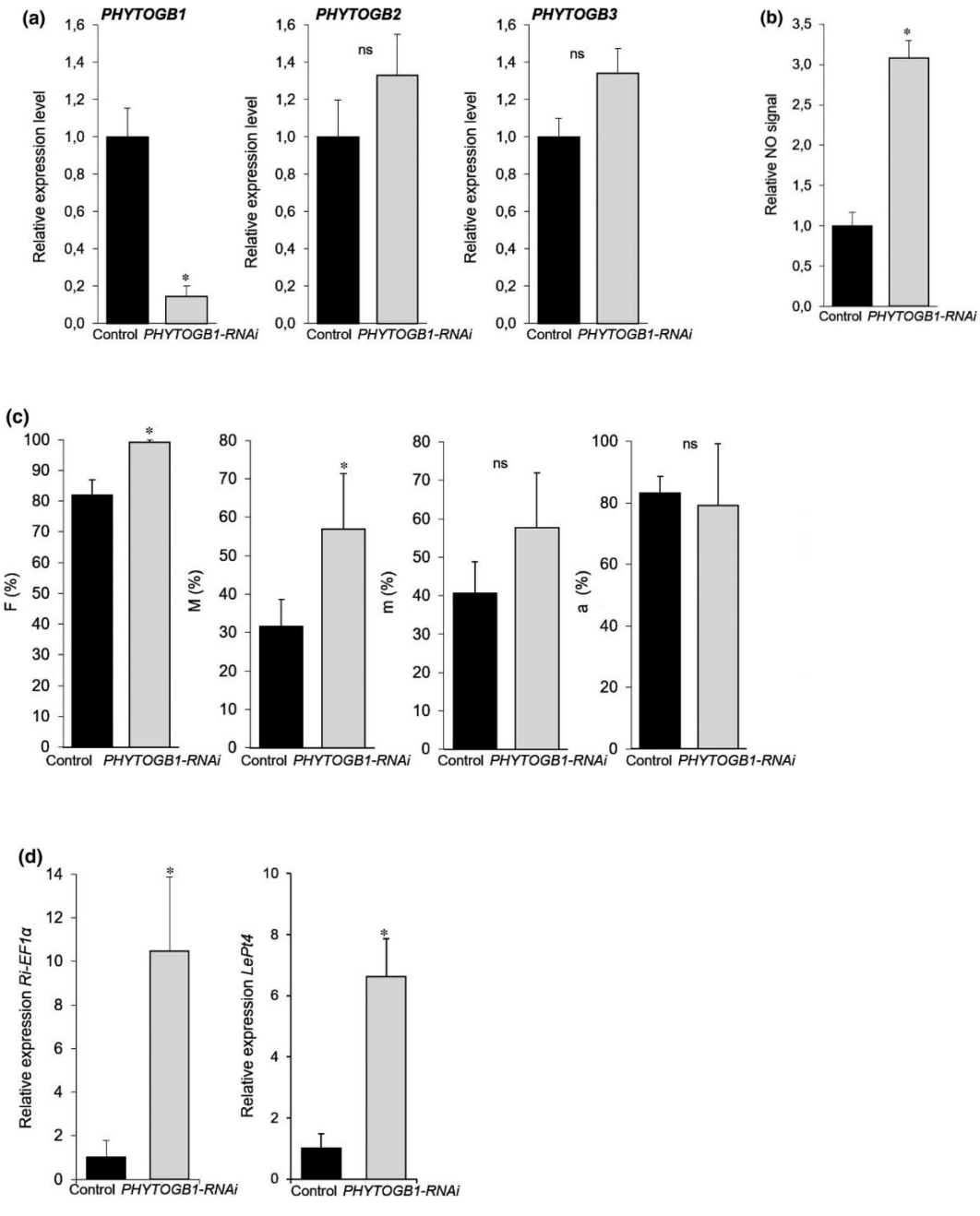

(a)
PHYTOGB1
PHYTOGB2
PHYTOGB3
Relative expression level
ns
*
Control
PHYTOGB1-RNAi
(b)
Relative NO signal
(c)
F (%)
M (%)
m (%)
a (%)
(d)
Relative expression Ri-EF1α
Relative expression LePt4

Fig. 8. Impact of silencing tomato *PHYTOGB1* on the expression of tomato phytoglobin genes, nitric oxide (NO) root accumulation and mycorrhizal colonization. Tomato plants were transformed with empty vectors (control) or *PHYTOGB1*–silenced constructs (*PHYTOGB1*–RNAi). **(a)** Expression level of *PHYTOGB1*, *PHYTOGB2* and *PHYTOGB3* was analyzed in empty–vector controls and in *PHYTOGB1*–RNAi roots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the empty–vector control roots ± SE (n = 6 biological replicates). **(b)** NO accumulation was detected in empty–vector controls and in *PHYTOGB1*–RNAi roots by fluorimetry using the specific NO detector DAF–2. NO levels are reported as the fold change relative to that of the empty–vector control roots ± SE (n = 6 biological replicates). **(c)** Arbuscular mycorrhiza fungal structures within the roots were analyzed in empty–vector controls and *PHYTOGB1*–RNAi roots 6 weeks after inoculation with *Rhizophagus irregularis* in pots. F%, frequency of colonization in the root system; M%, intensity of colonization in the root system; m%, intensity of colonization within the mycorrhizal fragments; a%, arbuscule abundance in mycorrhizal parts. **(d)** Relative expression of the *R. irregularis* constitutive gen *Ri–EF1α* and the mycorrhizal functionality marker gene *LePT4* in empty–vector controls and *PHYTOGB1*–RNAi roots 6 weeks after inoculation with *R. irregularis* in pots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the empty–vector control root ± SE (n = 6 biological replicates). The asterisks indicate a statistically significant difference in comparison to the empty–vector control root according to Student's t–test ($P < 0.05$). Ns, not significant. These results are representative for one of two independent experiments.

Discussion

Nitric oxide (NO) accumulation in plant cells is an early component of the signalling pathways activated in plants during immune responses to pathogens, and also during rhizobial symbiosis establishment (Besson–Bard *et al*., 2008; Hichri *et al*., 2015). In several plant–microbe interactions NO bioactivity is regulated partially by the activity of class 1 phytoglobins (Qu *et al*., 2006; Nagata *et al*., 2008; Hill, 2012; Mur *et al*., 2012). However, NO occurrence, function and regulation remain obscure in the case of the arbuscular mycorrhizal (AM) symbiosis. In the present study, we studied whether NO and its regulation by phytoglobins are regulatory components in the establishment and control of the AM symbiosis. We further addressed the specificity of the NO–related signature in the AM symbiosis by analyzing in parallel the NO accumulation pattern and NO–related responses triggered during the pathogenic interaction.

NO oscillations and *PHYTOGB1* regulation are components of the signalling pathway regulating the onset of the AM symbiosis

Our study revealed the accumulation of NO during early stages of the interaction between tomato roots and the AM fungus *Rhizophagus irregularis* (Fig. 2). We found that NO accumulation oscillates in response to the AM fungus from the earliest time point monitored until the end of the study. These results suggest a potential role(s) for NO from the early host recognition to the transduction pathway leading to the symbiosis establishment upon contact with the AM fungal hyphae. Similarly, Espinosa *et al*. (2014) showed an increase in NO levels in roots of olive seedlings 1 h after contacting with *R. irregularis*. Although the authors did not monitor the temporal modulation of the NO signalling, these previous observations reinforce the idea that the early AM interaction is associated with NO–related signalling in the host roots.

Imaging of NO production further revealed that the AM fungus–triggered NO accumulation is located mainly in the outer cell layers of the root and in root hairs (Fig. 3). These specific root zones have been associated previously with a fast

triggering of the calcium (Ca^{2+}) signalling in response to exudates from AM fungal germinating spores and AM fungal hyphopodia (Chabaud *et al*., 2011; Genre *et al*., 2013). This overlap between AM–triggered NO and Ca^{2+} signalling might suggest an interplay between both signalling components in the onset of the AM symbiosis. Indeed, NO has the capacity to act as a Ca^{2+} mobilizing intracellular messenger (Courtois *et al*., 2008), and Ca^{2+} has been suggested to be linked to downstream NO generation through the action of calmodulin–like proteins (Ma *et al*., 2008). Our results reveal that the AM interaction triggers an early NO–related response in root cell types that previously have been associated with early AM signalling. Indeed, NO accumulation in the roots occurred within the few first hours after contact with the AM fungus. This could imply that the early NO signalling is triggered by diffusible fungal factors which activate the AM symbiosis pathway, and/ or by fungal cell wall components that could act as general microbe–associated molecular patterns activating a rapid and unspecific defense reaction (Boller and Felix, 2009). To clarify this, we treated tomato roots with exudates from *R. irregularis* germinating spores and with *R. irregularis* cell wall extracts. We found that NO signalling was specifically triggered by components in the exudates from the germinating spores, but not by the cell wall extracts (Fig. 4). These results indicate that the early NO signalling observed is triggered specifically by bioactive molecules present in the AM fungal exudates. This is in agreement with previous observations by Calcagno *et al*. (2012) revealing a transient accumulation of NO in *Medicago truncatula* root cultures in response to exudates from the AM fungus *Gigaspora margarita*. Our results reinforce the idea that NO signalling is a component of the early plant response to diffusible factors in the exudates from AM fungal germinating spores.

It is noteworthy that we found a temporal overlap between the AM fungus–triggered NO accumulation and the regulation of the specific tomato phytoglobin gene *PHYTOGB1* (Fig. 2). *PHYTOGB1* has been shown to be NO–inducible in other plant species (Ohwaki *et al*., 2005; Bustos–Sanmamed *et al*., 2011; Bai *et al*., 2016), and here we confirm that *PHYTOGB1* was the only NO–inducible tomato phytoglobin gene (Fig. 5). This concomitant regulation of NO and *PHYTOGB1* suggests a role for *PHYTOGB1* in regulating NO bioactivity during the onset of the

AM symbiosis. Indeed, although the potential function(s) of *PHYTOGB1* remained largely unknown, previous studies showed an upregulation of this gene in the model plant *M. truncatula* in response to the early interaction with the AM fungi *G. margarita* and *R. irregularis* (Siciliano *et al*., 2007; Hogekamp and Küster, 2013). Altogether, our results point to a potential role of *PHYTOGB1* and NO signalling in the signalling pathway activated during the AM symbiosis establishment.

The AM symbiosis displays a specific signature of NO accumulation in the host roots

We next investigated whether the NO–related response triggered by the AM fungus results from the specific plant recognition of its fungal symbiont or instead, it is part of a general immune response. To this end, we compared NO oscillations elicited by the AM interaction with those triggered by the pathogen *F. oxysporum*. The NO signatures elicited by the two interactions were significantly different (Fig. 2). For instance, the early (4 h) plant response to the pathogen was associated with a stronger accumulation of NO compared to that triggered by the fungal symbiont. In analogy to our observations, previous studies demonstrated that early NO–related responses elicited by mutualistic and pathogenic bacteria differ significantly (Nagata *et al*., 2008; Espinosa *et al*., 2014). It is noteworthy that the stronger NO burst triggered in the pathogenic interaction was accompanied by a stronger upregulation of the NO–inducible *PHYTOGB1* (Fig. 6). At later stages, the pathogen induced a continuous increase in NO, which was spread through the complete root (Fig. 2). This contrasts with the more regular oscillations of NO levels observed in the AM interaction, which was restricted to the outer cell layers. In the mycorrhizal system, *PHYTOGB1* expression followed an oscillatory pattern similar to that of NO levels. However, it is intriguing that during the pathogenic interaction, the increased NO accumulation triggered at later stages was accompanied by a strong downregulation of *PHYTOGB1*, despite the NO–inducible character of this gene (Fig. 5). These results suggest the ability of *F. oxysporum* for actively repressing *PHYTOGB1* expression, most likely to promote high levels of NO and create

favorable conditions for the invasion (Arasimowicz–Jelonek and Floryszak–Wieczorek, 2016). In line with our observations, the symbiotic rhizobium *Mesorhizobium loti* and the pathogens *Ralstonia solanacearum* and *Pseudomonas syringe* triggered differential patterns of NO accumulation and regulation of the class 1 phytoglobin gene *LjHB1* in Lotus japonicus, being *LjHB1* transcriptional activation blocked by the pathogens (Nagata *et al*., 2008). Interestingly, here we confirmed a similar repression pattern for tomato *PHYTOGB1* during other pathogenic interactions with the root oomycete *Phytophthora parasitica* and with the shoot necrotrophic fungus *Botrytis cinerea* (Fig. S3). These results pinpoint *PHYTOGB1* as a key target for pathogenesis in different systems.

It also is interesting that by contrast to AM fungal diffusible signals, exudates from germinating spores of the pathogen did not trigger NO accumulation and *PHYTOGB1* upregulation in tomato roots (Figs 4, S4). This finding strongly suggests that the NO–related response triggered by the *R. irregularis* diffusible factors is not a general response to fungi, but most likely is specific to AM fungi. In general, our observations indicate that although NO production is a common component of plant responses to the AM symbiont and the pathogen *F. oxysporum*, there are clear differences between the NO signatures elicited by both interactions. Such differences probably reflect different biological functions of NO and a differential regulation by *PHYTOGB1* in both interactions.

PHYTOGB1 regulates NO levels in tomato and is involved in the regulation of mycorrhizal colonization

The role of class 1 phytoglobins as regulators of NO levels in plant–microbe interactions has been established in some legume plants and Arabidopsis (Shimoda *et al*., 2009; Bustos–Sanmamed *et al*., 2011; Fukudome *et al*., 2016). To investigate whether *PHYTOGB1* also is involved in NO regulation in tomato, and if it is a regulator of the AM symbiosis, we generated tomato *PHYTOGB1* overexpressing and silenced hairy roots. We found that, indeed, the accumulation of NO was strongly reduced in the overexpressing lines and enhanced in the silenced ones when

compared to their respective control roots (Figs 7, 8). These findings demonstrate that *PHYTOGB1* control endogenous NO levels in tomato roots, consistently with its previously reported ability to catalytically metabolize NO to nitrate in other systems (Seregelyes *et al*., 2004; Hill, 2012).

Our results further evidenced a stronger frequency and intensity of mycorrhizal colonization in the *PHYTOGB1* overexpressing roots compared to those carrying the empty vector (Fig. 7). Remarkably, overexpression of *PHYTOGB1* did not alter the abundance of arbuscules in the colonized areas, supporting a role of *PHYTOGB1* in the regulation of the early events of the interaction leading to root colonization and its extension, but not in arbuscule formation. These results are in line with previous studies showing an upregulation of *PHYTOGB1* specifically in cells harboring the first mycorrhizal infection sites in M. truncatula roots (Siciliano *et al*., 2007; Hogekamp and Küster, 2013). Intriguingly, a higher mycorrhizal colonization was also found in *PHYTOGB1*–silenced plants (Fig. 8). These findings support the hypothesis that precise fine–tuning of NO levels is required for the control of the AM symbiosis establishment and extension. Previous studies showed a similar contrasting role of NO in the control of nodulation in the rhizobial symbiosis: NO has been shown to promote nodule formation (Pii *et al*., 2007), and to be deleterious to nodule production (Shimoda *et al*., 2009). Our results evidenced that both, higher and lower NO accumulation in *PHYTOGB1*–silenced and overexpressing plants promoted mycorrhizal colonization. Taking into consideration the role of NO in the regulation of plant defenses (Fig. S5), we hypothesize that NO might be involved in the plant regulation of the degree of AM colonization by regulating plant defenses, however, the specific impact of NO on plan defenses during the mycorrhizal interaction remains unknown.

In analogy to the mycorrhizal interaction, deregulation of *PHYTOGB1* affected the interaction with the root pathogen (Fig. S6). An enhanced infection was found in the *PHYTOGB1*–silenced lines, whereas a lower incidence of the pathogen was observed in the overexpressing lines. These findings indicate that *PHYTOGB1* bioactivity is required for the plant to restrict the pathogen infection, and reinforce the idea that blocking the transcriptional activation of *PHYTOGB1* can be a pathogen

strategy to increase NO levels to favor infection (Nagata *et al*., 2008). Our results reveal a major role of tomato *PHYTOGB1* in regulating NO levels and root–fungi interactions, particularly in the establishment of the AM symbiosis. They also indicate that *PHYTOGB1* is involved in the control by the host plant of the extension of mycorrhizal colonization, most likely by regulating NO bioactivity in host roots.

Conclusion

We demonstrated that NO accumulation and *PHYTOGB1* transcriptional regulation are early components of the regulatory pathway that is activated in tomato roots during the onset of the AM symbiosis with *R. irregularis*. We further demonstrated that although NO–related signalling is a common regulatory component in mutualistic and pathogenic plant–microbe interactions, the NO–related signature and *PHYTOGB1* regulation shows different patterns in both interactions. We propose that fine–tuned NO accumulation is required for proper AM establishment, and that *PHYTOGB1* is triggered during the interaction to control NO levels in order to promote and control the AM symbiosis.

Supplementary data

Table S1. List of primers used in the analyses.

ID	Target gene	Sequence (5' → 3')
AY026343	*PHYTOGB1*	ATGCTGGTGAATGGGGTCTC TCCCTCACCACAACCTTTCC
AY026344	*PHYTOGB2*	GGACTCTGATGAACTTCCTGAGAATAAT TCGTTTCTGAAGATGGATGGAT
AW036344	*PHYTOGB3*	GTAAAGAACATGCCATTAGGAATC ATGGCGTCCAATTAATGGTGG
X14449	*SlEF* (Elongation factor 1α)[1]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC
AY885651	*LePT4* (Phosphate transporter)[2]	GAAGGGGAGCCATTTAATGTGG ATCGCGGCTTGTTTAGCATTTC
Q2V9G7	*Ri-EF1α* (Elongation factor 1α)[3]	TTGCTTTCGTCCCAATATCC AGTGGAAGACGAAGGGGTTT

[1]Rotenberg *et al*. (2006); [2]Balestrini *et al*. (2007); [3]Helgason *et al*. (2003).

Table S2. Basal expression levels of tomato phytoglobin genes in control (non inoculated plants) through the time course analysis. Expression level of *PHYTOGB1*, *PHYTOGB2* and *PHYTOGB3* was analyzed in roots of control plants at 4, 24, 48, 72 and 96 hours after transferring to the control plates. Results were normalized to the *SlEF* gene expression in the same samples. The relative expression levels are reported ± SE (*n* = 4 biological replicates).

Time after transferring the plants	*PHYTOGB1*	*PHYTOGB2*	*PHYTOGB3*
4 h	0,108 ± 0,012	0,101 ± 0.098	0,177 ± 0,053
8 h	0,208 ± 0,198	0,060 ± 0,017	0,227 ± 0,064
24 h	0,425 ± 0,115	0,206 ± 0,040	0,635 ± 0,067
48 h	0,130 ± 0,029	0,165 ± 0,098	0,273 ± 0,012
72 h	0,392 ± 0,076	0,110 ± 0,021	1,301 ± 0,402
96 h	0,453 ± 0,063	0,541 ± 0,304	0,811 ± 0,100

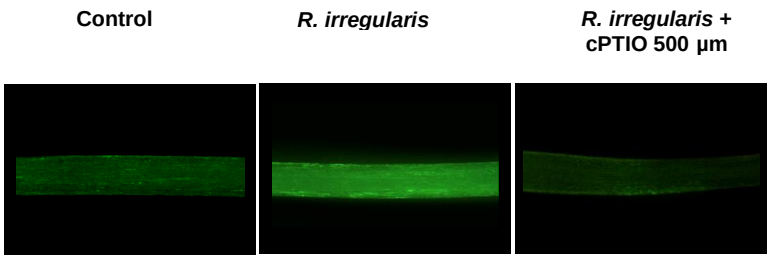


Fig. S1. Imaging nitric oxide (NO) production in tomato roots interacting with *Rhizophagus irregularis* and treated with cPTIO. NO–dependent DAF–FM DA fluorescence was visualized by fluorescence microscopy in control roots (left panel), in roots 48 h after contact with *R. irregularis* (middle panel) and in roots 48 h after contact with *R. irregularis* and treated with 500 µm cPTIO (right panel). The green fluorescence of the DAF–FM DA (excitation at 495 nm, emission at 515 nm) is observed.

(a)

Gene	Accession No.	Location	Length	E1	I1	E2	I2	E3	I3	E4	Protein
PHYTOGB1	AY026343	ch07:2965697-2967964	2268	375	485	117	863	115	107	206	153
PHYTOGB2	AY026344	ch03:41282493-41284402	1910	446	76	117	732	115	245	179	157
PHYTOGB3	AW036344	ch08:54369971-54375358	5388	199	1630	131	2958	145	93	232	170

(b)

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PHYTOGB1  MSSFSEEQEALV- VKSWGSMKKDAG----------EWGLKFFLKIFEIAP----SAKKMFS
PHYTOGB2  MG-FTDKQEALV-RDSWEFMKQDIP----------QLSLRFFSLILEIAP-----VAKNMFS
PHYTOGB3  MQSLQQKASEWSGVDPNDAFAIDETNLFEKLGLQAFINLSTNFYNRVYDDEEEWFRSIFS

PHYTOGB1  FLKDSNVPLDQNPKLKIHAKSVLVMT------CEAAVQLRKAGKVVVRDSTLKKIGATHFKYG
PHYTOGB2  FLKDSDELPENNPKLRAHAVKVFKMT------CESAIQLREKGEVVVGETTLKYLGSIHLQKR
PHYTOGB3  NSSKEDAIRNQYEFFVQRMGGPPLYSERKGHPALIGRHRPFPVTHKAADRWLQHMQQALD

PHYTOGB1  VVDEHFEVTKYALLETIKEASQEMWSVEMKNAWGEAYDQLVSAIKTEMK---------
PHYTOGB2  VADPHFEVVKEALLRTVKEATGNKWKDEMKEAWSEAYDQLASAIKAEMHAEAAA
PHYTOGB3  SVTDIDEDSKTKMMNFFRHTAFFLVAGDELKNQNQSVACKHAANKPAAE---------
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Fig. S2. Exon–intron compositions of *Solanum lycopersicum* phytoglobin genes, and alignment of *S. lycopersicum* phytoglobins. (a) Exon–intron compositions of *PHYTOGB1*, *PHYTOGB2* and *PHYTOGB3*. The gene structure appears to be the same for the three phytoglobins with four exons and three introns. Lengths of exons (E) and introns (I) are given in base pairs. Lengths of proteins are given in number of aminoacid residues. **(b)** Alignment of *S. lycopersicum* phytoglobins. The conserved aminoacids are highlighted.

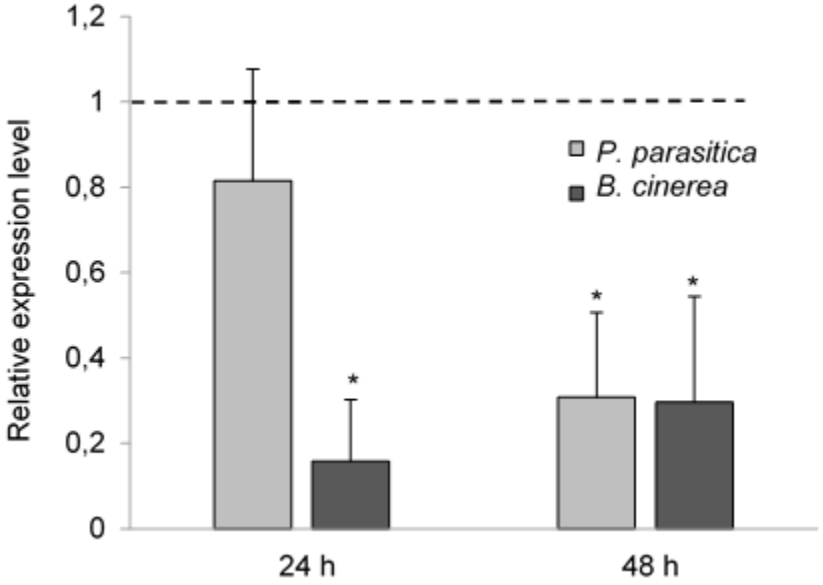


Fig. S3. ***Solanum lycopersicum PHYTOGB1*** **gene expression in tomato plants after contact with the pathogens** ***Phytophthora parasitica*** **and** ***Botrytis cinerea***. Expression level of *PHYTOGB1* was analyzed in roots of plants 24 and 48 hours after contact with *P. parasitica* and in mock treated roots. *PHYTOGB1* expression was also analyzed in shoot of tomato plants 24 and 48 hours after contact with *B. cinerea* and in and in mock treated shoots. Results were normalized to the *SlEF* gene expression in the same samples and expressed as a fold change with respect to their mock controls at each time point. Data are means ± SE (n = 4 biological replicates). The asterisk indicates a statistically significant difference in comparison mock controls (Student's t –test, $P < 0.05$).

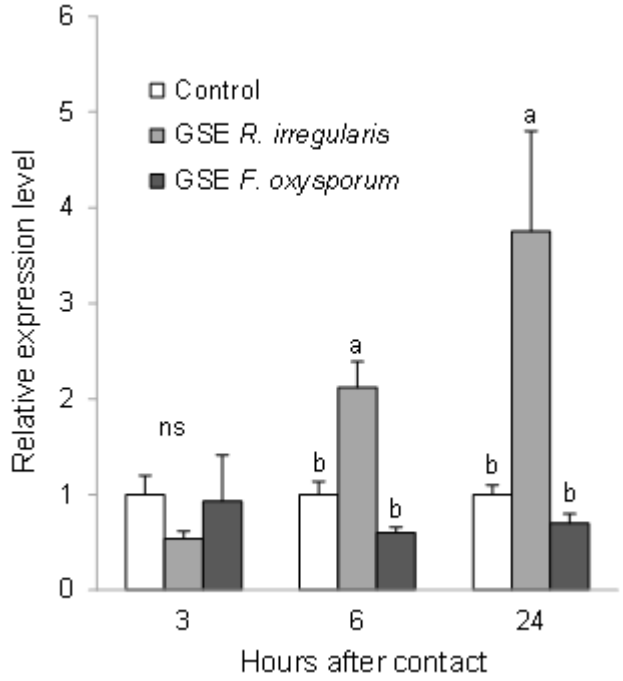
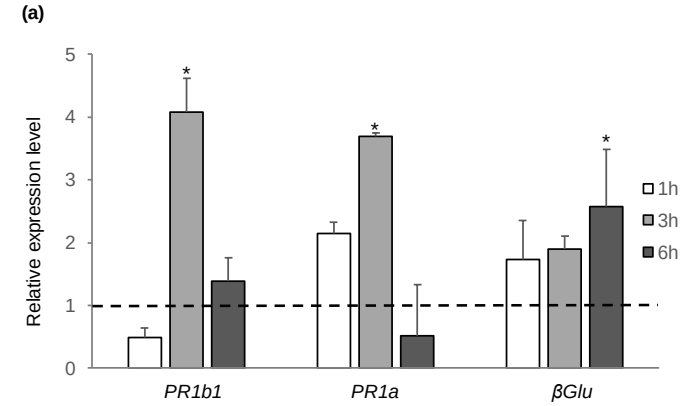
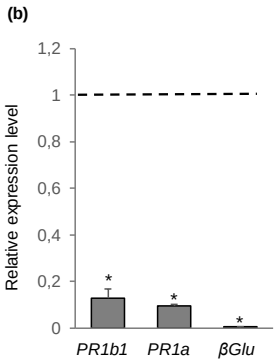


Fig. S4. Effect of exudates from germinating spores (GSE) from *Rhizophagus irregularis* and *Fusarium oxysporum* on the expression of *PHYTOGB1* gene. Expression level was analyzed in roots of tomato plants at 3, 6 and 24 hours after treatment with the GSE. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the control plants not treated with the GSE at each time point ± SE (n = 4 biological replicates). Data not sharing a letter in common in each time point differ significantly according to Tukey's HSD test (P < 0.05). ns, not significant.


(a)
Relative expression level
5
4
3
2
1
0
1h
3h
6h
PR1b1
PR1a
βGlu


(b)
Relative expression level
1,2
1
0,8
0,6
0,4
0,2
0
PR1b1
PR1a
βGlu

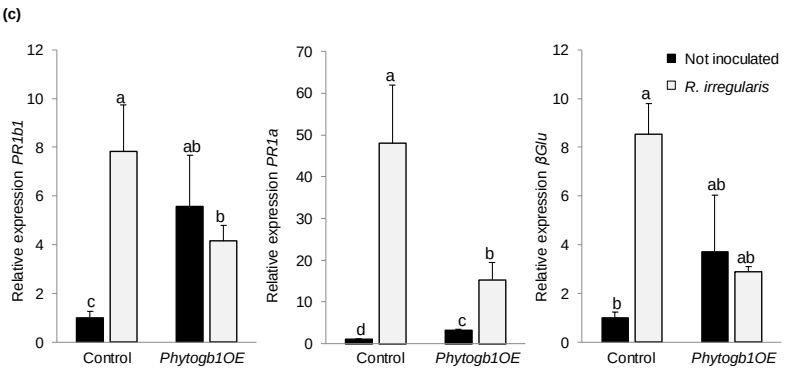
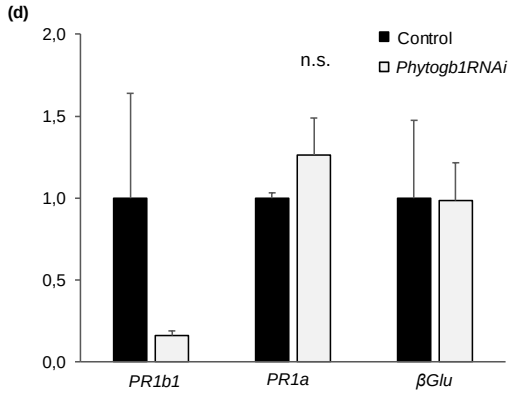

(c)
Relative expression PR1b1
Relative expression PR1a
Relative expression βGlu
Not inoculated
R. irregularis
Control
Phytogb1OE

Fig. S5. Impact of nitric oxide (NO) on tomato plant defenses. (a) Tomato roots were treated with the NO donor GSNO, and expression of the defense related genes *PR1b1* (Pathogenesis related protein 1b), *PR1a* (Pathogenesis related protein 1a) and *βGlu* (β–1,3–glucanase) were analyzed at 1, 3 and 6 hours after treatment. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that on non–treated plants ± SE (n = 4 biological replicates). **(b)** Tomato roots were treated with the NOS–like inhibitor aminoguanidine, and expression of the defense related genes *PRb1*, *PR1a* and *βGlu* were analyzed at 24 hours after treatment. The expression levels are reported as the fold change relative to that on non–treated plants ± SE (n = 4 biological replicates). **(c)** The relative expression of the defense related genes *PRb1*, *PR1a* and *βGlu* were analyzed in roots of *PHYTOGB1* overexpressing lines (*PHYTOGB1*–OE) and in root of plants transformed with the empty vector (control), 6 weeks after inoculation with *Rhizophagus irregularis* and in not inoculated plants. The expression levels are reported as the fold change relative to that on non–treated plants ± SE (n = 4 biological replicates). **(d)** The relative expression of the defense related genes *PRb1*, *PR1a* and *βGlu* were analyzed in roots of *PHYTOGB1* silenced lines (*PHYTOGB1*–RNAi) and in root of plants transformed with the empty vector (control), 6 weeks after inoculation with *Rhizophagus irregularis*. The expression levels are reported as the fold change relative to that on non–treated plants ± SE (n = 4 biological replicates). The asterisks in (a) and (b) indicate a statistically significant differences in every analyzed gene in comparison to not treated control according to Student's t –test ($P < 0.05$). In (c) data not sharing a letter in common differ significantly according to Tukey's HSD test ($P < 0.05$); ns, not significant. Primers used: *PR1b1*–Fw: TGGTATTAGCCATATTTCACTC and *PR1b1*–Rv: CACATTGGTTGGTAGCGTAG (Yan *et al*., 2013); *PR1a*–Fw: TATCTTAACGCTCACAATGCAG and *PR1a*–Rv: GTTTTCACCGTAAGGTCCAC; *βGlu*–Fw: CCATCACAGGGTTCATTTAGG and *βGlu*–Rv: CCATCCACTCTCTGACACAACT (Martínez–Medina *et al*., 2013).



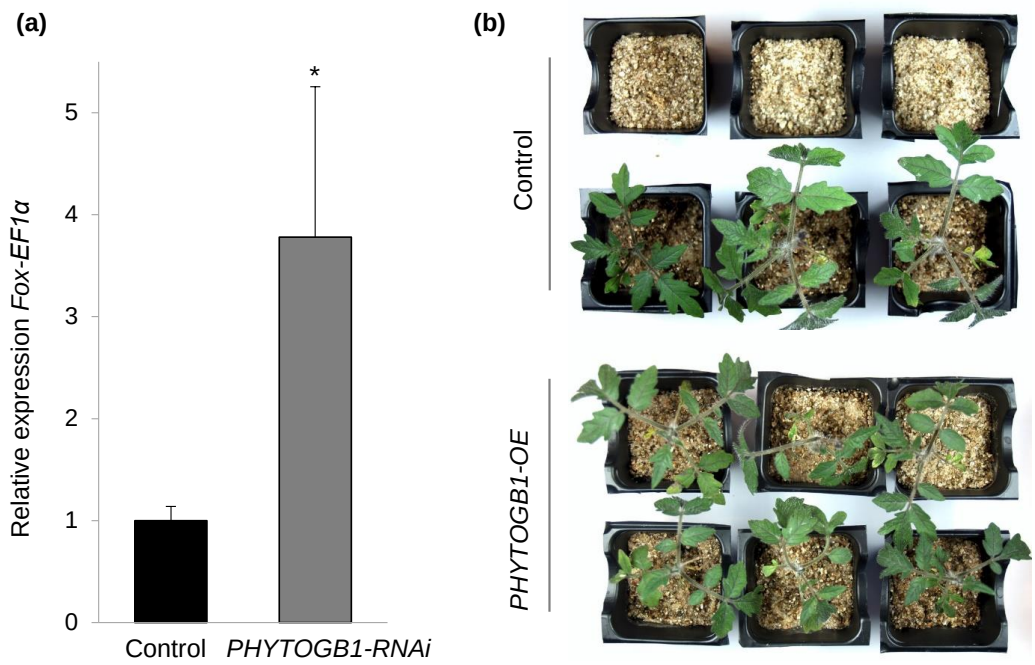


Fig. S6. Effect of altered *PHYTOGB1* levels on *Fusarium oxysporum* infection. **(a)** Relative expression of the *F. oxysporum* constitutive gene *Fox–EF1α* in empty–vectors controls and *PHYTOGB1*–RNAi transformed hairy roots 72 hours after contact with *F. oxysporum*. *In vitro*–grown cultures of *F. oxysporum* were used in the experiment. Expression levels are reported as the fold increase relative to that of the empty–vector control root ± SE (n = 6 biological replicates). The asterisk indicates a statistically significant difference in comparison to the empty–vector control root according to Student's t –test ($P < 0.05$). **(b)** Overview of the plants carrying the empty vectors controls and *PHYTOGB1*–OE constructs, 2 weeks after infection with *F. oxysporum*. The figure is a representative picture showing lower plant mortality in *PHYTOGB1*–OE lines. Mortality in plants carrying the empty vectors was about 33% while mortality in *PHYTOGB1*–OE lines was 0%. Primers used for *F. oxysporum* quantification: *Fox–EF1α*–Fw: CGGTAAGGGTTCCTTCAAGT and *Fox–EF1α*–Rv: TGACCGGGAGCGTCGATGA (Van der Does *et al.*, 2008).

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CHAPTER II

Trichoderma harzianum triggers an early and transient burst of nitric oxide and the upregulation of *PHYTOGB1* in tomato roots

Adapted from:

Martínez–Medina A, Fernández I, **Pescador L**, Romero–Puertas MC, Pozo MJ. 2019. *Trichoderma harzianum* triggers an early and transient burst of nitric oxide and the upregulation of *PHYTOGB1* in tomato roots . Plant Signalling & Behavior. doi:10.1080/1 5592324.2019.1640564

Abstract

We recently demonstrated that nitric oxide (NO) accumulation and *PHYTOGB1* transcriptional regulation are early components of the regulatory pathway that is activated in tomato roots during the onset of the mycorrhizal symbiosis between *Rhizophagus irregularis* and tomato roots. We further showed that the mycorrhizal interaction was associated with a specific NO–related signature, different from that triggered by the pathogen *Fusarium oxysporum*. Here, we extend our investigation by exploring the NO– and *PHYTOGB1*–related root responses elicited by another root mutualistic endosymbiotic fungus: *Trichoderma harzianum* T–78. By using T–78 *in vitro*–grown cultures, we found that T–78 triggered an early and transient burst of NO in tomato roots during the first hours after the interaction. T–78 also elicited the early upregulation of *PHYTOGB1*, which was maintained during the analyzed timespan. By using glass–house bioassays, we found that in a well–established tomato–T–78 symbiosis, NO root levels were maintained at basal level while *PHYTOGB1* expression remained upregulated. Our results demonstrate that the T–78 symbiosis is associated with a rapid and transient burst of NO in the host roots and the transcriptional activation of *PHYTOGB1* from early stages of the interaction until the establishment of the symbiosis, most likely to control NO levels and favor the mutualistic symbiosis.

Introduction

Nitric oxide (NO) is a diffusible reactive gaseous molecule involved in the regulation of a wide range of plant developmental processes and defense against biotic and abiotic stresses (Besson–Bard *et al*., 2008; Domingos *et al*., 2015). During plant immune responses, NO triggers a reprograming of the expression of defense–related genes, the production of secondary metabolites with antimicrobial properties, and the hypersensitive response (Mur *et al*., 2006). More recent evidences further indicate a role of NO in the establishment of plant–microbe mutualistic associations as the rhizobial and mycorrhizal symbioses (Hichri *et al*., 2015; Martínez–Medina *et al*., 2019b). Although the specific role(s) of NO in plant–

microbe mutualistic interactions remains largely unexplored, experimental data support a different regulation pattern and functions of NO in plant interaction with beneficial and pathogenic microbes (Martínez–Medina *et al*., 2019a, b; Nagata *et al*., 2008). NO plant accumulation can be regulated by the activity of plant phytoglobins that may function as NO dioxygenases that catalytically metabolize NO to nitrate (Perazzolli *et al*., 2004; Hill 2012; Hill *et al*., 2014). We recently found that the onset of the arbuscular mycorrhizal (AM) symbiosis between *Rhizophagus irregularis* and tomato roots is associated with a specific NO–related signalling in the host root, and the transcriptional activation of the tomato NO–inducible phytoglobin gene *PHYTOGB1* (Martínez–Medina *et al*., 2019a). Here, we extend our investigation by exploring the NO– and *PHYTOGB1*–related root responses elicited by another root mutualistic endosymbiotic fungus: *Trichoderma harzianum* T–78 (hereafter T–78) (Martínez**–**Medina *et al*., 2017).

Material and methods

Plant and fungal material

Tomato (*Solanum lycopersicum* cv Moneymaker) seeds were surface–sterilized in 4% sodium hypochlorite and germinated in sterile water at 25°C in darkness. After 1 week, seedlings were transferred to hydroponic conditions in 3–l tanks containing Long Ashton nutrient solution (Hewitt, 1966) with constant aeration. Plants were grown in the hydroponic tanks (six plants per tank) at 16 h: 8 h, light (24°C): dark (16°C) cycle at 70% relative humidity for two weeks before use. For the early interaction experiment, *Trichoderma harzianum* T–78 (CECT 20714, Spanish collection of type cultures) was grown on potato dextrose agar (PDA) at 28°C in dark conditions for 5 days. For treatments including T–78 inoculation in pots in the glass–house bioassays, we prepared a T–78 solid inoculum containing commercial oat, bentonite and vermiculite, according to Martínez–Medina *et al.* (2009).

Early interaction experiment set–up

A small orifice (3–mm diameter) was made in the side and the lid of the Petri dishes containing the Trichoderma cultures. Two–week–old tomato plants, grown in the hydroponic tanks were transferred to the Petri plates, one plant per plate, placing the roots on the surface of the culture and the stem in the hole, letting the shoot expand outside the plate, in open air conditions as described by Voets *et al*. (2005). Petri plates were closed and covered to keep the root system in the dark, and plants were kept in a growth chamber at 16 h: 8 h, light (24°C): dark (16°C) cycle at 70% relative humidity. At 4, 8, 24, and 48 h after setting up the experiment, plants were harvested and root material was collected.

Colonization bioassays

Tomato plants were transferred to 250 ml pots with a sterile sand: soil (4:1) mixture containing the Trichoderma inoculum. *T. harzianum* inoculum was mixed through the soil to a final density of 1×10^6 conidia per g of soil before transplanting the plants. The same amount of sand: soil mixture without *T. harzianum* was added to control plants. For each treatment a total of four plants were used. Plants were randomly distributed and grown in a glass–house at 16h: 8 h, light (24°C): dark (16°C) cycle at 70% relative humidity. Plants were watered three times a week with nutrient solution (Hewitt, 1966). Five weeks after transplanting into pots, plants were harvested and root material was collected for NO measurements and gene expression analyses. Trichoderma root colonization was checked by incubation of surface–sterilized tomato roots in PDA plates supplemented with 50 mgL^{-1} rose bengal and 100 mgL^{-1} streptomycin sulphate, according to Martínez–Medina *et al*. (2011). Plates were incubated at 28°C and coloni forming units (cfu) were quantified after 5 days by a plate count technique.

NO detection and quantification

Quantitative NO determination was performed through spectrofluorometry as described previously (Nakatsubo *et al*., 1998; Besson–Bard *et al*., 2009). Briefly, 0.2 g of fresh root samples were ground in 0.8 ml extraction buffer (50mM Tris–HCl, pH 7.8; 0.1 mM EDTA; 0.2% triton X–100; 10% glycerol; 2% PVPP) with a mortar. Homogenates were centrifuged at 11300 g for 30 min. Aliquots of supernatants were immediately diluted 50–fold in HEPES buffer (50 mM, pH 7.5). DAF–2 (Merck Biosciences) was added at 2 μM final concentration and the reaction mixtures were incubated at 37°C in the dark for 2 h. Fluorescence was measured in a RF–540 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 485 and 515 nm, respectively.

Real–time quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Extraction of total RNA from plant roots and synthesis of cDNA was performed according to Martínez–Medina *et al*. (2013). Real–time qRT–PCR reactions and relative quantification of specific mRNA levels were performed according to Martínez–Medina *et al*. (2013) and by using the gene–specific primers described in Table S1. The data were normalized using the housekeeping gene *SlEF* (X14449) encoding for the tomato translation elongation factor–1α, whose expression remained stable in the different lines and conditions. mRNA sequences of the tomato phytoglobin genes *PHYTOGB1* (AY026343), *PHYTOGB2* (AY026344) and *PHYTOGB3* (AW036344) were found in the online database NCBI. Gen structure information was obtained using the on–line database SOL Genomics Network (http://solgenomics.net/).

Results and discussion

T–78 triggers a transient burst of NO accumulation and the upregulation of *PHYTOGB1* in the host root during early steps of the symbiotic interaction

We first determined whether NO is an early signalling component of the interaction between T–78 and tomato roots. To this aim, we analysed NO levels in tomato roots by using T–78 *in vitro*–grown cultures and the fluorescent indicator for the detection of NO DAF–2. We monitored the NO accumulation at 4, 8, 24, and 48 h after setting up the experiment. This time span was selected according to our previous findings which evidenced two different peaks of NO plant production in *R. irregularis* roots during the first 48 h after the contact (Martínez–Medina *et al*., 2019a). We detected a transient burst of NO in tomato roots 4 h after the contact with T–78 (Fig. 1A). After this NO peak, NO levels returned to basal levels and no differences on NO accumulation were found between control and T–78 roots at 8, 24, and 48 h after the contact. This result indicates that root interaction with T–78 is associated with an early and transient burst of NO. In analogy to our observations, a rapid and transient increase of NO was previously detected in roots of *Arabidopsis thaliana* following the contact with the mutualistic endosymbiont *Trichoderma asperelloides* (Gupta *et al*., 2014). It is remarkable that the root interaction with the AM fungus *R. irregularis* and with the pathogenic fungus *F. oxysporum* is similarly associated with an early burst of NO in the host roots (Martínez–Medina *et al*., 2019a). These results might suggest that the rapid NO burst triggered by T–78 is part of an unspecific early plant response to different fungi, probably as a response to general fungal microbe–associated molecular patterns. However, after this early NO burst, the transient character of the NO burst triggered by T–78 contrasts with the oscillatory pattern of NO accumulation observed during the AM interaction (Martínez–Medina *et al*., 2019a). Such differences in the patterns of NO–related signalling might highlight the different colonization strategies followed by these different mutualistic fungal symbionts. The AM symbiosis establishment relies on a continual signalling between the symbionts and on the activation of an extensive

genetic and developmental program in both partners (MacLean *et al*., 2019). In contrast, the strategy followed by T–78 and *T. asperelloides* to colonize roots is mostly based on the early repression of plant immune responses to scape plant defenses (Martínez–Medina *et al*., 2017; Brotman *et al*., 2013).

We next studied whether T–78 regulates the tomato phytoglobin genes during early stages of the interaction. To this aim, we analyzed the expression of the tomato phytoglobin genes *PHYTOGB1*, *PHYTOGB2* and *PHYTOGB3* in tomato roots at 4, 8, 24, and 48 h after the contact. T–78 triggered the transcriptional activation of the gene *PHYTOGB1* in tomato roots already at 4 h after the contact (Fig. 1B). *PHYTOGB1* remained upregulated in T–78 roots during the entire monitored timespan. By contrast, transcript levels of *PHYTOGB2* and *PHYTOGB3* were not (or just marginally) affected by T–78. These results indicate that T–78 triggers the transcriptional activation of the phytoglobin gene *PHYTOGB1* in the host roots during the early stages of the interaction. Together, our results indicate that during the early stages of the T–78–tomato symbiosis, NO is rapidly and transiently accumulated in the host roots. Moreover, due to the role of *PHYTOGB1* on the regulation of NO accumulation in tomato roots (Martínez–Medina *et al*., 2019a) our findings further suggest that T–78 induces the upregulation of *PHYTOGB1* to control NO levels and evade the activation of plant defenses in order to successfully colonize the host root.

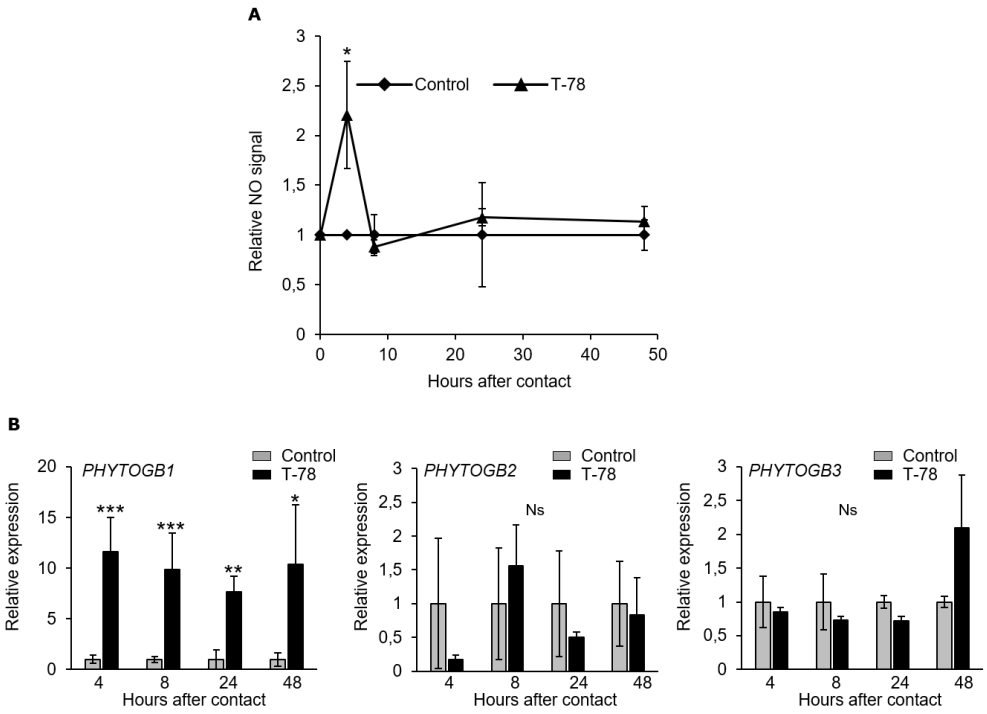

Fig. 1. Nitric oxide (NO) accumulation and expression of the tomato phytoglobin genes in tomato roots after early stages of the interaction with *Trichoderma harzianum* T–78. (A) NO was detected in tomato roots by fluorimetry by using the specific NO detector DAF–2 at 4, 8, 24, and 48 h after the contact with T–78 and in control roots. *In vitro*–grown cultures of T–78 were used in the experiments. NO levels are reported as the fold increased relative to that of the control roots at each time point ± SE (n = 4 biological replicates). The asterisk indicates a statistically significant difference in comparison to control roots according to Student's t–test ($P < 0.05$). **(B)** Expression level of *PHYTOGB1*, *PHYTOGB2*, and *PHYTOGB3* was analyzed in roots of tomato plants at 4, 8, 24, and 48 h after the contact with T–78 and in control roots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the control plants at each time point ± SE (n = 4 biological replicates). At each time point asterisks indicate significant differences compared to control plants at $^{***}P < 0.001$, $^{**}P < 0.01$ and, $^{*}P < 0.05$ according to Student's t–test. Ns: not significant.

The well–established symbiosis between T–78 and tomato roots is associated with basal root NO levels and the transcriptional activation of the *PHYTOGB1* gene

After the initial recognition, T–78 grows externally on the root surface and ingresses into the root cortex where it remains accommodated by the plant as an avirulent symbiont (Martínez–Medina *et al*., 2013, 2017). The T–78 symbiosis should be finely regulated, assuring benefits to both partners, with the plant receiving protection and more available nutrients and the fungus obtaining organic compounds and a niche for growth (Martínez–Medina *et al*., 2016). In order to investigate whether a well–established T–78 symbiosis is associated with changes on NO accumulation in the host root, we established a glass–house bioassay and after 5 weeks we measured NO levels in tomato roots. As shown in Fig. 2A, NO levels in roots of T–78–inoculated plants were similar to that observed in roots of control plants. By using qPCR, we found that *PHYTOGB1* was strongly upregulated in T–78–colonized roots compared to roots of control plants (Fig. 2B). These results indicate that the well–established T–78–tomato symbiosis is associated with the transcriptional activation of *PHYTOGB1* in the host root, most likely to control NO levels and maintain the symbiosis. Although to a lesser extent, *PHYTOGB2* was also upregulated in roots of T–78 colonized plants, while T–78 colonization did not affect significantly the expression of *PHYTOGB3*. Though *PHYTOGB2* is not an NO–inducible gene in tomato roots (Martínez–Medina *et al*., 2019a) several studies indicate that it is involved in stress–related responses and in hormonal signalling (Trevaskis *et al*., 1997; Hunt *et al*., 2001; Bustos–Sanmamed *et al*., 2011). However, its specific role on plant–microbe interactions remains largely unknown. Altogether, our results show that the T–78–tomato symbiosis is associated with a rapid and transient burst of NO in the host roots, and the transcriptional activation of *PHYTOGB1* from early stages of the interaction until the establishment of the symbiosis, most likely to control NO levels and favor the mutualistic interaction. Our results further demonstrate that the NO– and *PHYTOGB1*–related responses elicited by T–78 are different to the the ones triggered by the AM fungus *R. irregularis* and

the pathogenic fungus *F. oxysporum*, suggesting a specificity of the NO–related plant responses according to the specific plant interaction.

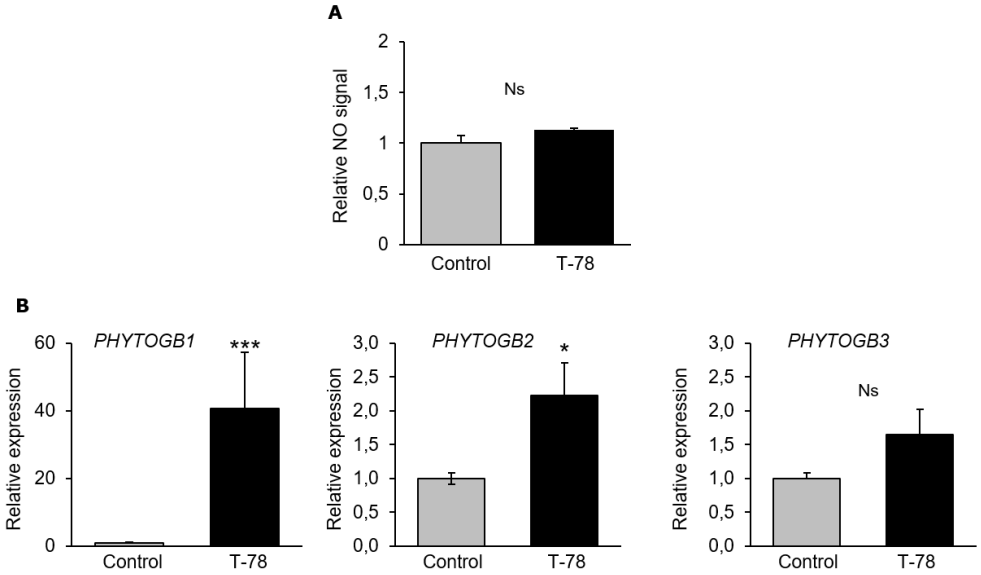

Fig. 2. Nitric oxide (NO) accumulation and expression of the tomato phytoglobin genes in tomato roots after the establishment of the plant–*Trichoderma harzianum* T–78 symbiosis. (A) NO was detected in tomato roots by fluorimetry by using the specific NO detector DAF–2, 5 weeks after the inoculation with T–78. Plants were grown in pots in a glass–house. NO level is reported as the fold increase relative to that of the control roots ± SE (n = 4 biological replicates). Ns: not significant. **(B)** Expression level of *PHYTOGB1*, *PHYTOGB2*, and *PHYTOGB3* was analyzed in roots of tomato plants 5 weeks after the inoculation with T–78 and in control roots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold changes relative to that of the control plants ± SE (n = 4 biological replicates). The asterisks indicate a statistically significant difference in comparison to the control roots at ***P < 0.001 and *P < 0.05 according to Student's t–test. Ns: not significant.

Supplementary data

Table S1: List of primers used in the analyses.

ID	Target gene	Sequence (5' → 3')
AY026343	*PHYTOGB1*	ATGCTGGTGAATGGGGTCTC TCCCTCACCACAACCTTTCC
AY026344	*PHYTOGB2*	GGACTCTGATGAACTTCCTGAGAATAAT TCGTTTCTGAAGATGGATGGAT
AW036344	*PHYTOGB3*	GTAAAGAACATGCCATTAGGAATC ATGGCGTCCAATTAATGGTGG
X14449	*SlEF* (*Elongation factor* 1α)[1]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC

[1]Rotenberg *et al*. (2006).

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CHAPTER III

Nitric oxide in plant–fungal interactions

Adapted from:

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Abstract

Whilst many interactions with fungi are detrimental for plants, others are beneficial and result in improved growth and stress tolerance. Thus, plants have evolved sophisticated mechanisms to restrict pathogenic interactions while promoting mutualistic relationships. Numerous studies have demonstrated the importance of nitric oxide (NO) in the regulation of plant defense against fungal pathogens. NO triggers a reprograming of defense–related gene expression, the production of secondary metabolites with antimicrobial properties, and the hypersensitive response. More recent studies have shown a regulatory role of NO during the establishment of plant–fungal mutualistic associations from the early stages of the interaction. Indeed, NO has been recently shown to be produced by the plant after the recognition of root fungal symbionts, and to be required for the optimal control of mycorrhizal symbiosis. Although studies dealing with the function of NO in plant–fungal mutualistic associations are still scarce, experimental data indicate that different regulation patterns and functions for NO exist between plant interactions with pathogenic and mutualistic fungi. Here, we review recent progress in determining the functions of NO in plant–fungal interactions, and try to identify common and differential patterns related to pathogenic and mutualistic associations, and their impacts on plant health.

Introduction

Fungi play a major role in natural and agricultural ecosystems. They are important decomposers and recyclers of organic materials and they can interact with plant roots in the rhizosphere or with above–ground tissues (Zeilinger *et al*., 2016). The interactions between plants and their associated fungi are complex and the outcomes are diverse, ranging from parasitism to mutualism. Fungal plant pathogens are of huge economic importance because they threaten crop production, not only when plants are growing in the field but also in the form of post–harvest diseases. Most of the major economically relevant plant pathogens are fungi such as *Botrytis cinerea*, and species of *Fusarium*, *Rhizoctonia*, and *Magnaporthe* (Dean *et al*.,

2012). On the other hand, mutualistic associations between fungi and plants are common in nature and can improve the productivity of crop plants. For instance, it is estimated that about 90% of plants form mycorrhizal symbioses, in which photosynthates from the host are exchanged for mineral resources acquired by the fungus from the soil (Ferlian *et al*., 2018). To cope with pathogenic fungi, plants are able to activate defense mechanisms, and are generally at least partially resistant to most fungal pathogens. Hence, mutualistic and neutral associations are most prevalent and parasitic associations are considered to be the exception (Staskawicz, 2001).

The interactions of plants with fungi are characterized by a series of sequential events beginning with the initial contact with the host plant, and including the fungal attachment to the host structures, the entry and colonization of the plant tissues, and the fungal reproduction (Lo Presti *et al*., 2015). Depending on the nature of the interaction (pathogenic, neutral, or mutualistic) and the life cycle of the fungus (necrotrophic or biotrophic), plants may respond to fungal colonization with an immune response in which several plant signalling compounds play pivotal roles, including intracellular calcium (Ca^{2+}) and other ions, reactive oxygen and nitrogen species (ROS/RNS), phytohormones, and small RNAs (Mur *et al*., 2006; Pieterse *et al*., 2012; Weiberg *et al*., 2014; Pozo *et al*., 2015; Waszczak *et al*., 2018). It is notable that the signalling networks and key regulatory elements that are involved in the plant responses to pathogenic and mutualistic fungi overlap (Pozo *et al*., 2015). This indicates that the regulation of the adaptive response of the plant is finely balanced between protection against aggressors and acquisition of benefits from mutualistic associations (Pieterse *et al*., 2014). Achieving this balance requires the perception of potential invading fungi, followed by rapid and tight regulation of immune responses to promote or contain the fungal colonization of plant tissues (Zamioudis and Pieterse, 2012; Zipfel and Oldroyd, 2017; Plett and Martin, 2018).

Nitric oxide (NO) is a diffusible, reactive free–radical gaseous molecule that is involved in the regulation of a wide range of plant developmental processes, such as seed germination (Gibbs *et al*., 2014; Albertos *et al*., 2015; Del Castello *et al*.,

2019), root development (Sanz *et al*., 2015; Castillo *et al*., 2018), flowering (He *et al*., 2004; Prado *et al*., 2004; Serrano *et al*., 2012), and fruit development (Manjunatha *et al*., 2012; Du *et al*., 2014). NO also regulates plant responses to several abiotic stresses such as hypoxia, salinity, and heavy metals (Gupta *et al*., 2016; Romero–Puertas *et al*., 2018), and it is involved in defense responses against microbial pathogens, including bacteria and fungi (Trapet *et al*., 2015). Indeed, during plant immune responses against fungal pathogens, NO triggers a global reprograming of gene expression, the production of secondary metabolites with antimicrobial properties, and the hypersensitive response (Mur *et al*., 2017). There is a growing body of evidence that indicates that NO is also produced during the establishment of mutualistic interactions between plants and fungi (Calcagno *et al*., 2012; Espinosa *et al*., 2014; Gupta *et al*., 2014; Martínez–Medina *et al*., 2019a). Although the specific role(s) of NO in plant–fungal mutualisms remains unclear, recent evidence suggests that tight control of NO levels is required for the control of the mycorrhizal symbiosis (Martínez–Medina *et al*., 2019a).

The diverse roles of NO during detrimental and mutualistic plant–fungal interactions might seem contradictory but can be explained by its versatile properties. As a signalling molecule, NO function depends on the rate and location of its production, and its concentration is critical: it acts as a signal at low concentrations but displays toxic effect when present at high concentrations (Hancock and Neill, 2019). Moreover, the highly reactive nature of NO facilitates its different regulatory roles as it reacts directly with other free radicals, metals, and proteins, leading to post–translational modifications that regulate protein activity and stability, and gene expression (Abello *et al*., 2009; Martínez–Ruiz *et al*., 2013; Lamotte *et al*., 2014; Yu *et al*., 2014; Romero–Puertas and Sandalio, 2016). Here, we review and synthesize recent and relevant information dealing with the role(s) of NO in the interaction of plants with pathogenic and beneficial fungi, highlighting recent advances and identifying the major gaps in our knowledge. We acknowledge that both the plant and the fungal partners are potential sources and regulators of NO during interactions; however, several excellent reviews have recently been published on fungal NO (Arasimowicz–Jelonek and Floryszak–Wieczorek, 2016;

Cánovas *et al*., 2016), so here we focus on the NO produced by plants during their interactions with diverse fungi.

Roles and metabolism of NO in plant immunity

Plants can be considered as unexpectedly healthy given the enormous number of potential pathogens in their environments (Dangl, 2013), and this is mainly due to the plant immune system. After the recognition of potential aggressors through the perception of pathogen–associated molecular patterns (the so called PAMPs; MAMPs when it is associated with non–pathogenic microbes) or from signals related to self–damage (damage–associated molecular patterns, DAMPs), the plant activates a defense response termed the basal or PAMP–triggered immunity (PTI). Some pathogens are able to avoid PTI by evading recognition or by the blocking defense response through small molecules called effectors, which promote infection (Couto and Zipfel, 2016). Plants can, however, possess a second layer of perception involving intracellular receptors with nucleotide binding–site leucine–rich repeats (NBS–LRRs, also termed NLRs) through which they are able to recognize microbe effectors, thus inducing effector–triggered immunity (ETI; Couto and Zipfel, 2016). Although both PTI and ETI activate similar mechanisms, ETI is stronger and faster, and leads to programmed cell death of the invaded area in order to restrain dispersion of the pathogen, a process known as the hypersensitive response (HR; Dodds and Rathjen, 2010).

One of the first biological functions assigned for NO in plants was related to plant immunity (Yu *et al*., 2014). The occurrence of a peak of NO has been observed during both PTI and ETI responses. However, most studies have dealt with the role of NO in ETI and HR, and less attention has been paid to NO production and function during PTI. Different MAMPs or DAMPs such as cryptogein, lipopolysaccharides, and oligogalacturonides have also been shown to induce NO production (Trapet *et al*., 2015), and show a feedback interaction with Ca^{2+} (Courtois *et al*., 2008). In this context, NO is able to regulate a wide variety of different plant immune responses (Yu *et al*., 2012; Bellin *et al*., 2013). Indeed, it is well known that NO produced after

microbe recognition triggers a global reprograming of gene expression, the production of secondary metabolites with antimicrobial properties, and ultimately, the HR and systemic acquired resistance (Bellin *et al*., 2013; Wendehenne *et al*., 2014). NO and related RNS perform their bioactivity mainly via chemical reactions with specific target proteins, leading to NO–dependent post–translational modification (PTMs), namely *S*–nitrosylation, nitration, or nitrosylation. Comprehensive reviews on this topic have been published by Scheler *et al*. (2013) and Yu *et al*. (2014). The levels of nitrosothiols are very important in the evolution of plant defense responses, as mutants with altered *S*–nitrosoglutathione reductase levels show impaired pathogen resistance (Feechan *et al*., 2005; Rustérucci *et al*., 2007). Furthermore, proteomic analyses of plants undergoing HR show that there are changes in *S*–nitrosylated proteins related to intermediary metabolism, hormone–dependent signalling, and ROS–producing enzymes, and in proteins related to antioxidant defenses and programmed cell death (Feechan *et al*., 2005; Romero–Puertas *et al*., 2007, 2008). Different transcription factors have also been shown to be targets of *S*–nitrosylation. This fact could explain how NO can coordinate gene expression changes. For example, in Arabidopsis NO has been proposed to switch the translocation of NPR1, a transcriptional coactivator involved in the induction of pathogenesis related genes (PR), into the nucleus, and to regulate the specific DNA–binding of its transcription–factor interactor, TGA1 (Tada *et al*., 2008; Lindermayr *et al*., 2010). Recently, it has been shown that the zinc finger trascription factor SRG1, which functions as a positive regulator of plant immunity, is a central target of NO bioactivity. When SRG1 is *S*–nitrosylated (represented as SRG1–SNO) it contributes to a negative feedback loop that decreases the plant immune responses (Cui *et al*., 2018). Proteomic analyses during plant defense responses have also shown protein targets of nitration that are involved in different cellular processes such as photosynthesis, glycolysis, and nitrate assimilation (Cecconi *et al*., 2009). Analysis in tobacco has suggested that tyrosine nitration may regulate MAPKK signalling and therefore phosphorylation cascades during the defense response (Vandelle and Delledonne, 2011). Despite an increasing body of literature on the roles of NO in plants, there are still many unknowns regarding the

sources of NO as well as the proteins/molecules that regulate NO levels in the cell. Several mechanisms have been reported with respect to NO production in plants. The best–characterized enzymatic route of NO production is the nitrate reductase (NR) pathway, in which nitrate is reduced to nitrite. The oxidative pathway and nitric oxide synthase (NOS)–like activity have also been shown to be involved in NO production during plant defense. Readers are referred to several excellent reviews for additional information on this topic (Baudouin and Hancock, 2013; Mur *et al*., 2013; Yu *et al*., 2014; Jeandroz *et al*., 2016; Astier *et al*., 2018). Our knowledge of NO catabolism is also very incomplete. NO can quickly react with glutathione (GSH) to form *S*–nitrosoglutathione (GSNO), with O_2 and $O_2^{\cdot-}$ to form nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), which is involved in NO–dependent PTMs as described above (Neill *et al*., 2008). Phytoglobins (previously known as non–symbiotic haemoglobins), which are able to modulate NO levels through their NO dioxygenase activity, have also been shown to be involved in NO modulation in plant immunity (Hebelstrup *et al*., 2014). Overall, the complex regulation of NO has slowed down the identification of downstream NO–regulated processes because it makes the generation of null NO–producing mutants difficult (Bruand and Meilhoc, 2019). However, thanks to the use of NO donors and scavengers, and mutants impaired in NO metabolism, the regulatory role of NO in numerous plant processes including plant immunity is now well established.

Although our knowledge of the molecular mechanisms mediating the role of NO in plant immunity has increased considerably over recent decades, most of the studies have been performed on model plants (mostly Arabidopsis) interacting with bacteria. Despite the importance of both beneficial and pathogenic fungi on plant health, the roles of NO in plant–fungal interactions have been far less well explored. In the following sections we attempt to compile and summarize the available information on these interactions, and to highlight common and differential patterns and functions during interactions with beneficial and pathogenic fungi.

NO in plant–fungal pathogenic interactions

Pathogenic fungi can use diverse strategies to colonize plants and cause disease. Necrotrophic fungal pathogens, which often show a broad host range, kill their hosts and take up nutrients released from the dead tissues. Several compounds including cell wall–degrading enzymes, ROS, and/or toxins have been implicated in the degradation of host cells by necrotrophic fungi (Wolpert *et al*., 2002). In contrast, biotrophic fungal pathogens, which show host specificity, do not produce toxins but often secrete effectors to suppress the host immune system (Perfect and Green, 2001). Hemibiotrophic fungal pathogens are intermediate between the necrotrophic and the biotrophic life styles, initially growing as biotrophs and later switching to being necrotrophic (Koeck *et al*., 2011). In agreement with the essential role of NO in plant immunity (see above), several studies have indicated that NO is an early component of the defense response triggered by plants to combat fungal infections (Table 1, and references therein). However, the specific role(s) of NO during the interaction of plants with pathogenic fungi seems to be influenced by the necrotrophic/biotrophic character of the pathogen, which dictates the concentration and the spatio–temporal patterns of NO accumulation in the plant tissues. Strikingly, in plant–fungal pathogenic interactions, the fungi may also participate in the production and metabolism of NO (Arasimowicz–Jelonek and Floryszak–Wieczorek, 2016; Cánovas *et al*., 2016). Several studies have indicated that NO plays an important role in fungal development (Wang and Higgins, 2005; Prats *et al*., 2008; Baidya *et al*., 2011). Moreover, fungal pathogens may use NO to their own benefit to accelerate the spread of infection, especially in interactions involving necrotrophic and hemibiotrophic pathogens (van Baarlen *et al*., 2004; Sarkar *et al*., 2014; Arasimowicz–Jelonek and Floryszak–Wieczorek, 2016). Indeed, NO has been found to be produced by several necrotrophic pathogens, including *B. cinerea*, *Aspergillus nidulans*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum coccodes* (Conrath *et al*., 2004; Wang and Higgins, 2005; Floryszak–Wieczorek *et al*., 2007; Turrion–Gomez and Benito, 2011; Sarkar *et al*., 2014). Thus, fungus–produced NO can also be considered as a virulence factor that determines

the success of the aggressor. As noted in the Introduction, excellent recent reviews focused on fungal–produced NO during pathogenesis are available (Arasimowicz–Jelonek and Floryszak–Wieczorek, 2016; Cánovas *et al*., 2016).

Necrotrophic fungi

The well–characterized necrotrophic foliar pathogen *Botrytis cinerea* has been used to demonstrate the importance of NO in the onset of the plant immune response against shoot–associated necrotrophic fungi in different plant species. For example, *B. cinerea* infection of tobacco (*Nicotiana benthamiana*) triggers an increase in NO levels in cells adjacent to invaded areas, which is concomitant with the activation of the salicylic acid–regulated defense pathway (Asai and Yoshioka, 2009). By using a pharmacological approach, these authors also showed that NO plays a pivotal role in the basal defense against *B. cinerea*, and in pathogen–triggered expression of *PR1*. Similarly, an increase in NO was observed in cells infected with *B. cinerea* and in surrounding uninfected cells in Arabidopsis (van Baarlen *et al*., 2007). The critical role of NO in Arabidopsis resistance to *B. cinerea* was later confirmed by manipulation of NO levels through a genetic approach (Mur *et al*., 2012), with mutant lines that displayed increased NO levels (due to a mutation in the *PHYTOGB1* gene) showing increased levels of the defense related plant hormones jasmonic acid and ethylene together with an increased resistance to *B. cinerea* infection, whilst decreased NO levels in *PHYTOGB1*–overexpressing lines resulted in an opposite phenotype. Pharmacological approaches have also revealed the importance of the NO burst in plant resistance against *B. cinerea* in tomato (*Solanum lycopersicum*; Sivakumaran *et al*., 2016). Taken together, these studies demonstrate a key role of pathogen–triggered NO in plant immunity against *B. cinerea* in different plant species. A similar role for NO has been suggested for plant immune responses against other leaf–associated necrotrophic fungi such as *Colletotrichum orbiculare* (Asai *et al*., 2008) and *Sclerotinia sclerotiorum* (Perchepied *et al*., 2010).

Strikingly, a study by Turrion–Gomez and Benito (2011) indicated that *B. cinerea* may use NO signalling to enhance its spreading within plant cells. Although

the authors mostly focused on NO produced by the fungus, they hypothesized that the plant cell death mediated by the NO–triggered HR might favour the growth of the necrotrophic fungus within plant tissues. It is notable that we recently found that *B. cinerea* triggered the down–regulation of *PHYTOGB1* in tomato leaves, most likely to increase NO levels and to enhance cell death (Martínez–Medina *et al*., 2019a). This offers an apparently contradictory scenario where NO is being used by the host plant for defense and by the pathogenic fungus to promote virulence. Understanding this disparate data will require careful spatio–temporal measurement of NO concentrations (Box 1), as the relative concentration of NO during the different stages of the infection process could play a key role in governing its action. Indeed, Turrion–Gomez and Benito (2011) hypothesized that above a certain threshold NO triggers plant cell death, which would favour the infection, while below this threshold NO would act as a key signalling molecule in the onset of the plant immune response. In line with this hypothesis, Floryszak–Wieczorek *et al*. (2007) found uncontrolled generation of NO in tissues of susceptible *Pelargonium peltatum* infected with *B. cinerea*. This was accompanied by very intensive synthesis of H_2O_2 and ethylene. Moreover, when the pathogen colonized susceptible cells it further produced considerable amounts of NO, which enhanced the nitrosative and oxidative stress in host tissues. By contrast, a more controlled burst of NO was observed in the incompatible interaction of *B. cinerea* with a resistant *Pelargonium* genotype. In this case, the resistance response was accompanied by a strong first burst of NO followed by a controlled secondary wave of NO generation, which was co–expressed with the activation of plant defenses. This response triggered a resistance that was not associated with cell death but which did have an enhanced pool of antioxidants, which ultimately favoured the maintenance of homeostasis of the surrounding cells. According to these findings, in susceptible interactions, necrotrophic fungi may exploit the NO–related plant defense system in order to expand the infection. However, in incompatible interactions, NO would be mostly acting as a key signal in the onset of the plant immune response.

Biotrophic fungi

In contrast to necrotrophic pathogens that feed on dead tissue and are thus not deterred by plant cell death, biotrophs require compounds from living host cells. Thus, HR–triggered cell death is probably one of the most important strategies in impeding the growth of biotrophic fungi (Govrin and Levine, 2000) and, accordingly, it is a likely hypothesis that NO–triggered HR would restrict the spread of biotrophic fungi. Indeed, Prats *et al*. (2005) found NO as one of the first responses of barley epidermal cells against *Blumeria graminis*. However, the role of NO in plant interactions with biotrophic fungal pathogens has not been thoroughly studied. It appears to have an important role in plant resistance to powdery mildew, as Schlicht and Kombrink (2013) found that Arabidopsis responded to both compatible (*Golovinomyces orontii*) and incompatible (*Erysiphe pisi*) interactions with powdery mildew with a rapid and transient accumulation of NO; however, there were significant differences in the patterns of NO accumulation. In leaves infected with *G. orontii*, the NO level rapidly declined after the initial burst and the authors suggested that this was most likely a consequence of an active effector–mediated defense suppression by the fungus. In contrast, NO levels remained high for an extended period of time during the incompatible interaction with *E. pisi*, indicating a correlation between the resistance phenotype and the amount and duration of NO production. Piterková *et al*. (2009) also found significant differences in the extent and timing of the increase in NO production triggered by *Oidium neolycopersici* between susceptible and resistant tomato genotypes. In the susceptible genotype, elevated NO production was observed only during the early moments following inoculation, whilst a two–phase increase in production was detected in the resistant genotypes. Similarly, a study by Qiao *et al*. (2015) suggested the importance of the intensity and duration of the NO burst in wheat immunity against the biotrophic fungus *Puccinia triticina*. In the incompatible plant–fungal interaction, a continuous and sustained increase in NO was found in the stomatal guard cells at the infection site. This burst primarily occurred in the cells undergoing a hypersensitive response. For the compatible interaction, a smaller and transient accumulation of NO was found. Taken together, these data

suggest that the ability of the plant to rapidly and continuously increase NO production forms part of the molecular basis of plant resistance to biotrophic fungi.

Root fungal pathogens

The role of NO in plant interactions with root fungal pathogens has been relatively poorly studied, most likely because of the challenges involved in examining interactions belowground (Shelef *et al*., 2019). By using an *in vitro* system, we recently found that the compatible interaction of tomato with the necrotrophic pathogen *F. oxysporum* is associated with an early strong and transient burst of NO in tomato roots (Martínez–Medina *et al*., 2019a). This first burst is followed by a sustained and uncontrolled accumulation of NO that is concomitant with cell death. Moreover, as the infection progressed a down–regulation of *PHYTOGB1* in infected tomato roots occurred, most likely in order to further increase NO levels and to promote cell death. By manipulating NO levels through a genetic approach, we were able to demonstrate the important role of NO in tomato susceptibility to *F. oxysporum*. Higher biomass of *F. oxysporum* and greater host cell death were observed in tomato lines displaying increased NO levels. By contrast, a decreased susceptibility to the pathogen was found in *PHYTOGB1*–overexpressing plants that displayed decreased NO levels. An increase in NO levels has also been found within the first hour after *F. oxysporum* infection of Arabidopsis roots (Gupta *et al*., 2014). Furthermore, Espinosa *et al*. (2014) found a strong increase in NO in roots of olive seedlings 1 h after contact with the necrotrophic fungus *Verticillium dahliae*. NO was spread across cell walls and in the cytoplasm of epidermal and cortical cells, and a concomitant increase in phenolic compounds was observed. Although the authors did not study the temporal dynamics of the NO burst and of the infection, they suggested that the burst was related to the activation of the plant immune response to the pathogen. Application of the NO–donor sodium nitroprusside (SNP) reduces the disease caused by *Rhizoctonia solani* in resistant and susceptible tomato cultivars via involvement of both the octadecanoid and phenylpropanoid pathways (Noorbakhsh and Taheri, 2016). These studies may suggest that, similar to the observations from above–ground plant parts, NO might

play a dual role in root interactions with necrotrophic fungi. NO might act as a signal to initiate a defense response in incompatible interactions, while the NO signal might also be exploited by the pathogen to spread lesions in compatible interactions.

The rapid induction kinetics of the first NO burst and the lack of specificity of this early response during plant–fungal pathogenic interactions may indicate that NO accumulation is part of the plant response to fungal PAMPs. Indeed, the application of chitosan, a mycelial fungal elicitor of cell walls from *F. oxysporum*, triggers a rapid burst of NO (Wang and Wu, 2004; Srivastava *et al*., 2009; Martínez–Medina *et al*., 2019a). In accordance with this, we propose the following model. The interaction of the plant with necrotrophic pathogenic fungi results in a rapid and unspecific PAMP–triggered burst of NO that activates the plant response at the early stages. NO is massively produced after the first peak with the advance of the infection, and the associated cell death can be exploited by the pathogen to further expand lesions at later stages (Fig. 1A). In the case of plant interactions with biotrophic fungal pathogens, it seems that there is a correlation between the concentration and duration of the NO burst with plant resistance (Fig. 1B), although experimental data are scarce.

NO in plant–fungal mutualistic interactions

Interactions between plants and mutualistic fungi are ubiquitous and diverse, and often result in the improvement of plant growth and stress tolerance. In return, plants deliver carbohydrates and an ecological niche to their fungal associates, thus contributing to a stable association between the interacting partners (Zeilinger *et al*., 2016). Intimate mutualistic plant–fungal interactions include those between plants and foliar and root mutualistic endophytes, and mycorrhizal symbioses. The establishment and maintenance of intimate mutualistic interactions requires mutual recognition and substantial coordination of the plant and fungal responses. This coordination is based on finely regulated molecular dialogue between the partners, in which the host immune responses are tightly

controlled to enable successful colonization and to maintain the balance of mutual benefits (Zipfel and Oldroyd, 2017; Plett and Martin, 2018). Given the crucial role of NO in plant immunity (as discussed above), it might be speculated that NO operates in the establishment and maintenance of mutualistic plant–fungal interactions. Remarkably, we could not find any reports related to NO signalling during plant interactions with fungal endophytes in leaves, despite their well–recognized benefits in plant health (Porras–Alfaro and Bayman, 2011). However, we found several studies on the specific roles of NO in endophyte–induced secondary metabolites in plants (Ren and Dai, 2013; Fan *et al*., 2014; Cui *et al*., 2017). The only reports regarding plant–produced NO during beneficial plant–fungal interactions concern root colonizers. A few recent studies report the occurrence of a burst of NO during the early steps of arbuscular mycorrhizal (AM) symbiosis and during the early interaction of roots with mutualistic fungal endophytes (Calcagno *et al*., 2012; Espinosa *et al*., 2014; Gupta *et al*., 2014; Zou *et al*., 2017; Martínez–Medina *et al*., 2019a). However, the specific role(s) of NO in plant–fungal mutualistic interactions remain unknown.

The first experimental data demonstrating the occurrence of a NO burst in mycorrhizal symbiosis were reported by Calcagno *et al*. (2012), who found that NO increased in the roots of *Medicago truncatula* within minutes following treatment with exudates of germinating spores of the AM fungus *Gigaspora margarita*. The authors suggested that this increase was mediated by the activity of nitrate reductase, and that was associated with the activation of the symbiotic regulatory (SYM) pathway. In agreement with these findings, we recently found a similar response in roots of tomato after treatment with exudates from germinating spores of the AM fungus *Rhizophagus irregularis* (Martínez–Medina *et al*., 2019a). This response was specific for the AM fungus, as exudates from germinating spores of pathogenic *F. oxysporum* did not trigger NO accumulation. These findings indicate that the perception by the plant of bioactive molecules present in the exudates of germinating AM fungal spores triggers a NO–related response. It is notable that the chemical communication between the host plant and the AM fungus is initiated prior to physical contact between the symbionts (Buee *et al*., 2000; Chabaud *et al*.,

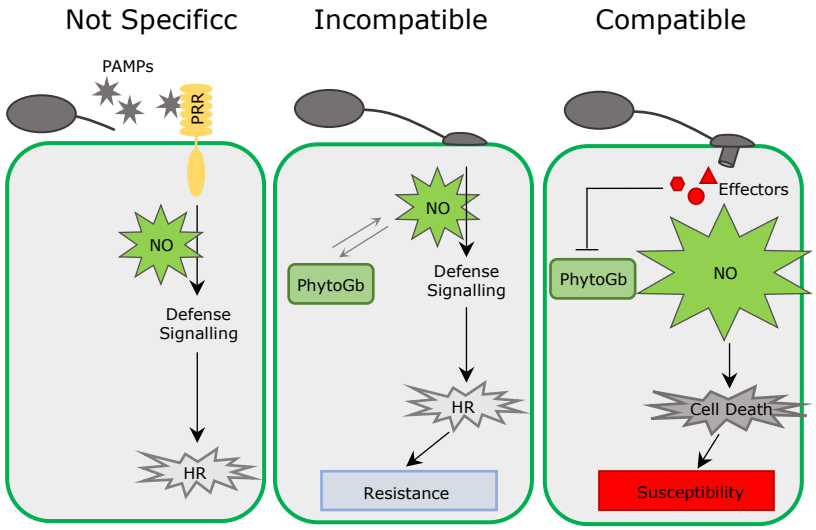
2011). Plant perception of diffusible fungal signals (termed Myc factors) is translated into a transcriptional response that prepares the plant for the forthcoming fungal colonization (Maillet *et al*., 2011; Genre *et al*., 2013). It seems that NO is a component of the SYM pathway that is triggered in the host plants after the perception of Myc factors during the pre–symbiotic stage of the AM symbiosis. The first experimental data demonstrating the occurrence of a NO burst in mycorrhizal symbiosis were reported by Calcagno *et al*. (2012), who found that NO increased in the roots of *Medicago truncatula* within minutes following treatment

Besides the pre–symbiotic stage, NO also accumulates in root cells shortly after contact with the mycelium of AM fungi. For example, NO increases in roots of olive seedlings (Espinosa *et al*., 2014) and tomato plants (Martínez–Medina *et al*., 2019a) within hours of contact with the mycelium of *R. irregularis*. Both sets of authors suggested that NO may function as a signalling component in regulating some key processes in the early stages of the AM interaction, such as cell wall remodelling, lateral root development, and controlling host defense. In addition, an increased NO level is observed in roots of seedlings of trifoliate orange (*Citrus trifoliata*) 21 day after inoculation with the AM fungus *Diversispora versiformis* (Zou *et al*., 2017), suggesting that NO might further function as a regulatory component in the maintenance of well–established AM symbioses (Fig. 1C). By manipulating the levels of NO in tomato roots using a genetic approach we have shown that NO appears to be a regulatory component of the establishment of AM symbiosis (Martínez–Medina *et al*., 2019a). Tomato roots displaying increased NO levels (through silencing of *PHYTOGB1*) or decreased NO levels (through the overexpression of *PHYTOGB1*) display increased mycorrhizal colonization, suggesting a role for NO in the tight regulation of the symbiosis.

Similar to mycorrhizal symbiosis, an increase of NO is observed in roots of Arabidopsis within minutes following contact with the mycelium of the mutualistic endosymbiotic fungus *Trichoderma asperelloides* (Gupta *et al*., 2014). The increase of NO is mediated by the activity of nitrate reductase, and is restricted to discrete root cells. *Trichoderma harzianum* also induced an early and transient burst of NO in tomato roots during the first hours after the interaction in parallel with the

upregulation of *PHYTOGB1*, which was then maintained (Martínez–Medina *et al*., 2019b). These findings may suggest that NO is a common component of the plant signalling pathways that regulate the establishment of different plant–fungal mutualistic symbioses. It is notable that in the case of the *Trichoderma* symbiosis the increase in NO triggered by the fungus is limited to the early steps of the interaction (Gupta *et al*., 2014; Martínez–Medina *et al*., 2019b). This contrasts with the temporal organization displayed by NO accumulation during the AM interaction, in which NO levels peak in the host roots during the first days following contact with the AM fungal mycelia (Martínez–Medina *et al*., 2019a). These differences in the patterns of NO accumulation may highlight the different colonization strategies followed by these different mutualistic fungal symbionts. In the case of AM symbiosis, the plant actively accommodates the fungal partner in specialized host–membrane compartments in the root cortical cells to form arbuscules (Bonfante and Genre, 2010). This relies on continual signalling between the symbionts and in the activation of an extensive genetic and developmental program in both partners during the entire colonization process (MacLean *et al*., 2017). In contrast, the strategy followed by *Trichoderma* to colonize roots is mostly based on the early repression of plant immune responses to escape the plant defenses (Brotman *et al*., 2013). These findings suggest that although NO is a common component of the plant signalling pathways that regulate the establishment of different plant–fungal mutualistic interactions, the patterns of NO and possibly its particular role(s) might be specific for every type of mutualistic association. However, experimental data on NO signalling during mutualistic plant–fungal interactions are still too scarce to develop accurate models.

A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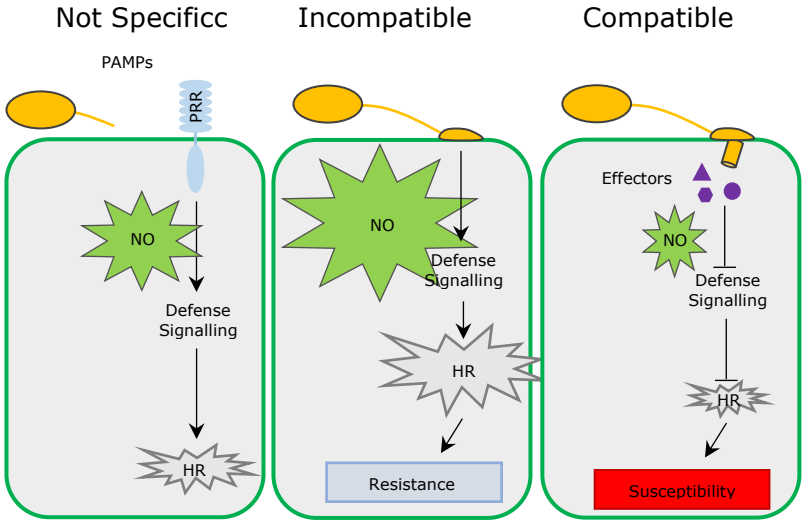
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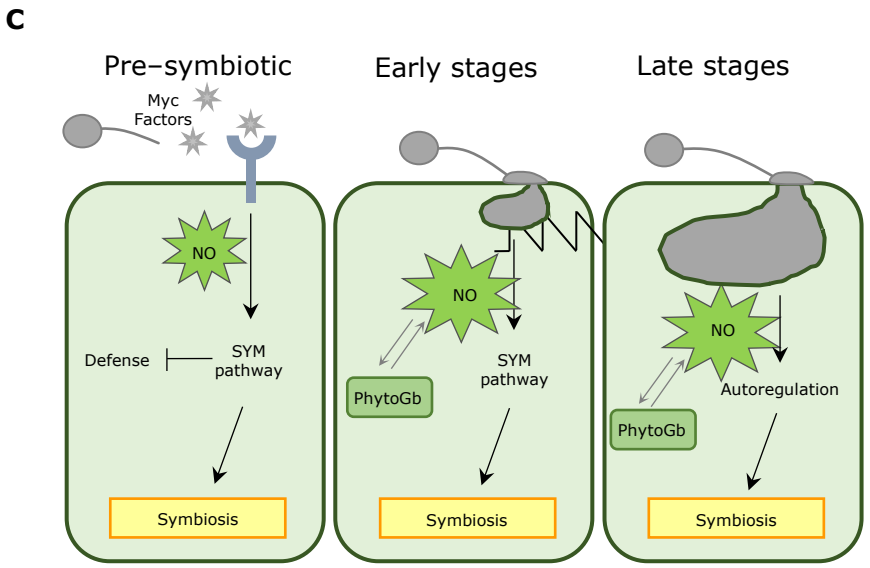

Fig. 1. Model of NO functioning in plant–fungal interactions. (A) During interactions with necrotrophic fungi, the perception of fungal pathogen–associated molecular patterns (PAMPs) by plant pattern–recognition receptors (PRRs) triggers a rapid and unspecific burst of NO, which activates the plant response at the early stages. At later stages, NO is massively produced with the advance of the infection, and the associated cell death can be exploited by the pathogen to further expand the lesions (Floryszak–Wieczorek *et al*., 2007; Turrion–Gomez and Benito, 2011; Martínez–Medina *et al*., 2019a). **(B)** In interactions with biotrophic pathogens, plant perception of fungal PAMPs also triggers a rapid and unspecific burst of NO to activate the plant response. During incompatible interactions a second burst of NO leads to the hypersensitive response (HR) and cell death, which prevents the pathogen from spreading along the tissue as biotrophs only thrive in living cells. By contrast, in compatible interactions the NO level rapidly decreases after the initial burst, most likely due to active effector–mediated suppression of defenses by the fungus, which leads to susceptibility (Piterková *et al*., 2009; Schlicht and Kombrink, 2013; Qiao *et al*., 2015). **(C)** During the pre–symbiotic stages of mycorrhizal symbiosis, Myc factors released by the fungus are perceived by plant receptors, triggering a burst of NO that is linked with the activation of the symbiotic regulatory (SYM) pathway. The activation of this pathway partially suppresses the host immune responses and prepares the plant for the forthcoming fungal colonization. After hyphal contact, the level of NO in the root cells spikes in a controlled manner thanks to the action of phytoglobins. This specific NO pattern may function as a regulatory element in the establishment of the symbiosis. In later stages, when the symbiosis is well established, NO is further controlled by the action of the phytoglobins, and is involved in the autoregulation of the symbiosis (Calcagno *et al*., 2012; Espinosa *et al*., 2014; Zou *et al*., 2017; Martínez–Medina *et al*., 2019a).

Differential roles of NO in pathogenic and mutualistic plant–fungal interactions

As discussed above, it seems that NO is a common component of the plant signalling pathways that control both immunity against fungal pathogens and the establishment of symbioses with fungal mutualists. However, the spatio–temporal kinetics of NO accumulation in the two scenarios seem to differ widely. We found remarkable differences when comparing NO accumulation triggered in tomato roots by the AM fungus *R. irregularis* and triggered by the necrotrophic pathogen *F. oxysporum* (Martínez–Medina *et al*., 2019a). After an initial rapid and unspecific burst of NO, the pathogen triggered a massive accumulation of NO through the entire root, which was concomitant with a strong down–regulation of *PHYTOGB1* and with the progression of cell death. In contrast, the AM mutualistic interaction triggered a series of more controlled oscillations of NO accumulation, which overlapped with the regulation of *PHYTOGB1*. In the case of the mutualistic association, the accumulation of NO was further restricted to the outer cell layers and root hairs. It is notable that these specific root zones are associated with Ca^{2+} signalling during early stages of the mycorrhizal process (Genre *et al*., 2013), perhaps suggesting an interplay between Ca^{2+} and NO in the onset of the AM symbiosis. Similarly, Espinosa *et al*. (2014) found that *R. irregularis* triggers a controlled burst of NO that is localized in the external cell layers. By contrast, the NO burst triggered by the pathogen *V. dahliae* is stronger and spreads not only to external external cell layers, but also to cortical cells. A similar pattern has been observed when comparing NO accumulation triggered by *T. asperelloides* and *F. oxysporum* in Arabidopsis roots (Gupta *et al*., 2014): while the accumulation of NO that is triggered during the mutualistic interaction is weak and restricted to discrete root cells, accumulation triggered by the pathogen is stronger and is spread over wide portions of the roots. Thus, it seems that although NO–related signalling is a common regulatory component in mutualistic and pathogenic plant–fungal interactions, the NO–related signature that is triggered and probably also the

specific NO functions differ widely. We envisage that future studies that compare pathogenic and mutualistic interactions within the same plant system will allow the specific regulatory role(s) of NO to be deciphered.

Concluding remarks

The information currently available on NO regulation during plant–fungal interactions allows to conclude that it is a key signal in the establishment and the fine–tuning of both mutualistic and pathogenic interactions. Although NO production is a common feature of both, the signature that is triggered seems to differ quantitatively and in its spatio–temporal distribution in the two types of interactions. These differences most likely determine the specific NO functions that can shape the final outcome of the interaction. Based on our current knowledge, we propose a model for NO regulation and function in the different types of interactions (Fig. 1), but this identifies important gaps in the available information. Comparative studies among different mutualistic and pathogenic interactions, using similar methodologies and across multiple plant systems are required in order to identify common patterns and major regulatory nodes. Moreover, studies devoted to examining the role of NO as a cue in the plant defense signalling network are required to explore the specific functions of NO in mutualistic and pathogenic interactions. This review highlights the importance of the spatio–temporal dynamics in NO production, and the need of precise and sensitive methods to measure it and to determine its sources and metabolism. Thus, important technical challenges remain ahead, as described in Box 1, but carefully designed new experiments together with the technical progress already taking place should result in great advances being made in the coming years. Such research will boost our knowledge of NO functions and the regulation of plant–fungal interactions, and potentially lead to biotechnological applications for plant health in agricultural systems.

Table 1. A summary of studies where NO production in plant–fungal interactions have been demonstrated, together with its proposed role.

Fungus	Plant	Type of interaction*	NO level (technique)	Time scale	NO source	Gene expression	Pharmacological approach	Genetic approach	Suggested function	Reference
Blumeria graminis	*Hordeum vulgare* (leaf)	Path	DAF-2DA	6-24 h	-	-	cPTIO (0.25 mM) SNP (0.05 mM) L-NAME (1 mM)	-	NO contributes to HR and cell death, leading to the stop of the infection. NO also contributes to papilla formation.	Prats *et al.*, 2005
Botrytis cinerea	*Arabidopsis. thaliana* (leaf)	Path	DAF-2DA	6 d	-	*PR1/ LOX2/ LOX3/ AOS/ OPR3/ VSP2/ GDSL/ ERF2* + array	N-isobutyl decanamide (60 µM)	*Jar1/ Coi1/ Eds16/ Mpk6*	Alkamides are involved in plant immunity induction and change NO levels.	Méndez-Bravo *et al.*, 2011
B. cinerea	*A. thaliana* (leaf)	Path	DAF-2DA	30 min-6 h	NR Arg	-	OG L-NAME (5 mM) cPTIO (500 µM)	*nia1nia2/ cngc2/ per4-1/ per4-2/ glu/ RBOH-D*	NO participates in the regulation of OG-responsive genes (*PER4*/ a b-1,3-glucanase). Plants treated with cPTIO, were more susceptible to *B. cinerea.*	Rasul *et al.*, 2012
***B. cinerea* (PebC1)**	*A. thaliana* (leaf/ cells)	Path	Griess reagent	3-6 h	-	*PR1/ BGL-2/ PR4/ PDF1.2/ This2.1*	-	*Ein2/ Coi1/ Npr1/ NahG*	PebC1 protein promotes Arabidopsis resistance to infection by rapid increase of NO.	Zhang *et al.*, 2014
B. cinerea	*Nicotiana benthamiana* (leaf)	Path	DAF-2DA	2-12 d	NOS NR	*NbPR-1/ NbLOX/ NbGST/ NbCAT1*	DPI (50 µM) L-NAME (5 mM) D-NAME (50 µM) cPTIO (500 µM)	*NbNOA1/ NbRBOHB* VIGS	NO contributes to disease resistance against *B. cinerea.*	Asai and Yoshioka, 2009
B. cinerea	*Pelargonium peltatum* (leaf)	Path	DAF-2DA/ PGSTAT 30	5 min-3 d	-	-	-	-	An early NO burst and a later wave of NO generation enhance the resistance of *P. peltatum* to *B. cinerea.*	Floryszak-Wieczorek *et al.*, 2007
B. cinerea	*Solanum lycopersicum N. tabacum, A. thaliana* (leaf)	Path	DAF-2DA	1-4 d	-	-	c-PTIO (0.25 mM) L-NAME (5 mM)	-	A NO concentration threshold will trigger plant cell death. Below this threshold, NO acts as a signalling molecule to activate diverse plant defense systems against the fungus.	Turrion-Gomez and Benito, 2011

Fungus	Plant	Type of interaction*	NO level (technique)	Time scale	NO source	Gene expression	Pharmacological approach	Genetic approach	Suggested function	Reference
B. cinerea	*S. lycopersicum* (leaf)	Path	Quantum cascade laser	30 min-24 h	NR	-	L-NAME (5 mM) SNP (0.1 mM)	ABA mutant *sitiens*	ABA can decreases resistance to *B. cinerea* via reduction of NO production.	Sivakumaran *et al.*, 2016
B. cinerea	*S. tuberosum* cv. Bintje/ Bzura (leaf)	Path	Electrochemical method	0-24 h	-	*PR-1/ PR-2/ PR-3*	-	-	*B. cinerea* triggered huge NO overproduction.	Floryszak-Wieczorek and Arasimowicz-Jelonek, 2016
Colletotrichum orbiculare	*N. benthamiana* (leaf)	Path	DAF-2DA	4-6 d	NR NOS Non enz.	-	Tungstate (100 mM)	*NOA1*-silenced plants (VIGS)	NO helps to defend the plant against *C. orbiculare*. Posttranscriptional control of *NOA1*-influenced NO production and is affected through the *MEK2 SIPK/ NTF4* cascade.	Asai and Yoshioka, 2008
Chitiosan (fungal elicitor)	*Pisum sativum* (leaf)	Path	DAF-2DA	10-20 min	NR NOS	-	cPTIO (0.2 mM) L-NAME (0.1 mM) Tungstate (0.1 mM)	-	NO production may be responsive to fungal PAMPs.	Srivastava *et al.*, 2009
***Funneliformis mosseae* (AMF)**	*Trifolium. repense* (root)	Benef	DAF-FM DA	5-9 weeks	-	*PAL/ CHS*	-	-	AMF increases NO levels in roots, independently of the mycorrhization week.	Zhang *et al.*, 2013
***F. mosseae* (AMF)**	*T. repense* (root)	Benef	DAF-FM DA	5-9 weeks	-	*PAL/ CHS*	-	-	AMF increases NO in roots, but not systemically to non-mycorrhizal roots in the split root system.	Zhu *et al.*, 2015
Fusarium oxysporum* (*Fox*) *Trichoderma asperelloides	*A. thaliana* (root)	Path Benef	DAF-2DA	10-120 min	-	78 NO-modulated genes	cPTIO (100 µM) L-NAME (2.5 mM)	*nia1nia2*	*T. asperelloides* suppresses NO generation elicited by *Fox*.	Gupta *et al.*, 2014
Fox **(Fusaric acid)**	*N. tabacum* (cells)	Path	DAF-2 DAF-FM DA	15-90 min	-	*PAL/ Hsr203J*	cPTIO (100 mM) L-NMMA (100 mM)	-	FA can induce PCD in tobacco suspension cells in a NO-dependent way.	Jiao *et al.*, 2013

Fungus	Plant	Type of interaction*	NO level (technique)	Time scale	NO source	Gene expression	Pharmacological approach	Genetic approach	Suggested function	Reference
Fox	*S. lycopersicum* (root)	Path	DAF-2DA Haemoglobin assay	48 h	NR	*PRs/ PAL/ ProtIn/ PO/ GST/ CAM/ NR*	SNP (100 µM) cPTIO (100 µM) L-NAME (10 µM)	-	Ca-treated plants showed higher NO production *vs* control. Disease incidence was reduced in Ca treated plants, may be due to the higher NO concentration.	Chakraborty *et al.*, 2017
Fox **(fungal elicitor)**	*Taxus chinensis* (cells)	Path	DAF-2 DA	0-12 h	NOS	*PAL*	SNP (10 µM) L-NNA (100 µM) PTIO (100 µM)	-	NO activates fungal elicitor-induced responses involving secondary metabolism.	Wang and Wu, 2004
Gigaspora margarita **(exudates)**	*Medicago truncatula* (root)	Benef (symb)	DAF-2DA	0-15 min	NR	*NR/ NiR*	cPTIO (1 mM)	Trans. roots (*DMI1-1*, *DMI2-2*, and *DMI3-1*)	There is a NO specific signature related to AM-interactions and a different NO signature when plants were exposed to a general elicitor like bacterial LPS extract.	Calcagno *et al.*, 2012
Magnaporthe grisea **(cell wall)**	*Oriza sativa* (leaf/ cells)	Path	Spectrophotometry	30 min; 12 h	NOS	*PAL/ PR-1/ CHI*			NO acts as a signal mediating the HR induced by the fungus and it is also necessary for the induction of cell death in combination with H_2O_2.	Hu *et al.*, 2003
M. oryzae **(Nep1Mo)**	*A. thaliana* (leaf)	Path	DAF-2DA	3 h	-	*AtERF1/ AtLOX3*	SNP (25 mM) cPTIO (400 µM)	At*ALY4*	*AtAlY4*–H_2O_2–NO pathway mediates multiple Nep1Mo-triggered responses, including stomatal closure, HCD, and defense-related gene expression.	Teng *et al.*, 2014
M. oryzae	*Hordeum vulgare O. sativa* (leaf)	Path	-	-	-	-	PTIO (250-500 µM)	-	Removal of NO delays germination development and reduces disease lesion numbers.	Samalova *et al.*, 2013
Macrophomina phaseolina **and xylanase**	*Corchorus capsularis* (leaf)	Path	DAF-FM DA	8 h	-	-	cPTIO (200 mM)	-	Low NO concentration functions as a signalling molecule. High NO concentrations facilitate	Sarkar *et al.*, 2014

Fungus	Plant	Type of interaction*	NO level (technique)	Time scale	NO source	Gene expression	Pharmacological approach	Genetic approach	Suggested function	Reference
									fungal infection by triggering PCD. *M. phaseolina* could enhance the infection of plant cells through its own production of NO.	
Oidium neolycopersici	*S. lycopersicum* cv. Amateur/ *chmielewskii/ hirsutum* f.sp. *glabratum* (leaf)	Path	Oxyhaemoglobin method DAF-FM DA	0-216 h	NOS	-	cPTIO (0.1 mM) L-NAME (10 mM) AMG (10 mM)	-	NO levels are higher in resistant varieties leading to plant resistance.	Piterkova *et al.*, 2009
O. neolycopersici	*S. lycoper/ chmielewskii/ habrochaites* f.sp. *glabratum* (leaf/disc)	Path	DAF-FM DA	8-72 h	NOS	-	SNP (0.1 mM) L-NAME (1 mM) PTIO (0.1 mM)	-	In moderate susceptible genotype the disease rate is diminished if NO production by NOS is reduced. NO activates defenses in resistant genotype. With cPTIO, the fungus germinates better on the leaves.	Piterková *et al.*, 2011
***Puccinia striicformis* CY22-2/ CY29-1**	*T. aestivum* cv. Lovrin10 (leaf)	Path	Electron spin resonance	0-120 h	-	-	SNP (0.5; 2.5 mM)	-	There is a general correlation of NO formation and race-specific resistance.	Guo *et al.*, 2004
P. coronata* f.sp. *avenae	*A. sativa* (leaf)	Path	DAF	12-60 h	-	-	cPTIO (500 µM)	-	The simultaneous generation of NO and H_2O_2 might be associated with the death of adjacent cells of those infected by an avirulent isolate of *P. coronata*.	Tada *et al.*, 2004
P. triticina	*A. thaliana Triticum aestivum* (leaf)	Path	DAF-DA	24 h	-	-	-	*atrbohD/ atrbohF/ atrbohD+F / A. thaliana* (natural variation)	Identification of loci controlling non-host disease resistance and changes in NO levels.	Shafiei *et al.*, 2007
P. triticina	*T. aestivum* (leaf)	Path	DAF-FM DA	4-72 h	NR NOS	-	Na_2WO_4 (100 µM) c-PTIO (200 µM) L-NAME (100 µM)	-	In the incompatible combination NO acts as an important signalling	Qiao *et al.*, 2015

Fungus	Plant	Type of interaction*	NO level (technique)	Time scale	NO source	Gene expression	Pharmacological approach	Genetic approach	Suggested function	Reference
									molecule and mediates HR.	
Trichoderma brevicompactum	*A. thaliana* (leaf)	Path	DAF-DA	2h	-	-	Alamethicin (50 µM)	-	rRNA cleavage was suppressed by NO.	Rippa *et al.*, 2007
Verticillium dahliae **(VD-toxins)**	*A. thaliana* (leaf)	Path	DAF-2-DA	45min	-	*PR-1*	Tungstate (100 µM) cPTIO (100 µM)	*Atnoa1*	Cortical microtubule dynamics are mediated by NO-dependent signalling.	Shi *et al.*, 2009
V. dahliae **(VD-toxins)**	*A. thaliana* (leaf)	Path	DAF-2-DA	60min	NR	-	Tungstate cPTIO	*nia1nia2*	VD-toxin-induced NO accumulation H_2O_2-dependent and that H_2O_2 acted synergistically with NO to modulate the dynamic microtubule cytoskeleton responses to VD-toxins.	Yao *et al.*, 2014
V. dahliae/ Rhizophagus irregularis	*Olea europaea* (root)	Path Benef	DAF-2DA	1-24h	-	-	cPTIO (400 mM)	-	NO may be a key in the symbiosis establishment and the defense response to pathogens.	Espinosa *et al.*, 2014
V. dahliae	*A. thaliana* (leaf)	Path	DAF2-DA	60min	-	-	SNP (400 µM)	GhHb1-trans. *Arabidopsis*	GhHb1 proteins play a role in the defense responses against pathogenic invasions, possibly by modulating the NO level and the ratio of H_2O_2/NO in the defense process.	Qu *et al.*, 2006
V. dahliae	*A. thaliana* (leaf)	Path	DAF-2-DA	50-60 min	NR	*NIA1*	Tungstate (100 µM) L-NNA (100 µM) cPTIO (100 µM)	*Atnoa1/ nia1/ nia2*	NO was induced in response to VD-toxins in Arabidopsis.	Shi and Li, 2008
V. dahliae	*Helianthus annuus* (root)	Path	-	-	-	-	SNP (20 µM) GSNO (50 µM)	-	NO pre-treatments could not reduce *Verticillium* wilt. NO donors appear to promote fungal infection.	Monzón *et al.*, 2015
V. longisporum	*A. thaliana* (root/leaf)	Path	DAF-2	50-80 min	-	Genes analysis at NO peak	-	-	732 genes in the roots and 474 genes in the shoot may be regulated by NO.	Tischner *et al.*, 2010

Box 1. Future challenges for NO studies in plant–fungal interactions

The role of NO in plant–fungal interactions is of considerable complexity, as it has a regulatory role in both plant defense responses and in the process of pathogenicity and/or the proper establishment of beneficial interactions. Accordingly, we need a more accurate understanding of NO dynamics, distribution, and function in specific plant–fungal interactions. Such knowledge should contribute to the improvement of biotechnological applications for crop resistance through the identification of key regulation points that determine pathogenicity or beneficial effects of microbial inoculants. We consider that the following technical and experimental challenges need to be addressed.

• Development of appropriate NO sensors to allow monitoring of levels *in vivo* in order to follow the spatial and temporal dynamics and to identify the source of NO production during plant–fungal interactions.

• Functional studies need to be conducted in which plant or fungal NO levels are manipulated at specific sites or time–points in order to determine their impact on the interaction and on plant health (for example, overexpression of phytoglobins in an inducible way, within specific tissues by using appropriate promoters).

• Identification of targets of NO bioactivity during plant–fungal interactions would help to reveal the molecular mechanisms that underly NO functioning in these interactions.

• Further studies are required that include plant species from diverse families in order to identify possible general patterns in NO regulation and potential family– or species–specific aspects of the plant responses and their impact on pathogenic and beneficial interactions.

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CHAPTER IV

Nitric oxide signalling is required for MYB72–dependent induced disease resistance by *Trichoderma* volatiles

Adapted from:

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Abstract

The activation of induced systemic resistance (ISR) by root–colonizing *Trichoderma* fungi in Arabidopsis is dependent on the transcription factor MYB72. *Trichoderma* volatile compounds (VCs) are important elicitors of *MYB72* and determinants for ISR. Nitric oxide (NO) is involved in the regulation of plant defense responses and MYB72–dependent iron deficiency responses. Here we investigated the role of NO signalling in *Trichoderma* VCs–mediated regulation of *MYB72* and ISR. Using *in vitro* bioassays, VCs from *Trichoderma harzianum* and *Trichoderma asperellum* were applied to Arabidopsis seedlings. Plant perception of *Trichoderma* VCs triggered a burst of NO in Arabidopsis roots. By using a NO scavenger, we show the involvement of NO signalling in *Trichoderma* VCs–mediated regulation of *MYB72*. Moreover, different *in planta* bioassays including the *myb72* Arabidopsis line and a NO scavenger demonstrate the requirement of MYB72 and NO signalling in *Trichoderma* VCs–mediated ISR against the pathogen *Botrytis cinerea*. Expression analysis of the defense genes *PR1* and *PDF1.2* point to the involvement of NO signalling in the priming of plant defenses triggered by *Trichoderma* VCs leading to enhanced protection against *B. cinerea*. Our results support a key role of NO signalling upstream of MYB72 during the onset of ISR mediated by *Trichoderma* VCs.

Introduction

Plant roots host a plethora of soil microbes that can establish beneficial interactions (Berendsen *et al.*, 2012). Among them, plant interaction with fungi from the genus *Trichoderma* (hereafter Trichoderma) provides essential services to the plant, improving plant nutrition and protection against soil–borne pathogens (Harman *et al.*, 2004; Harman, 2011; Viterbo and Horwitz, 2010; Hermosa *et al.*, 2012; Martínez–Medina *et al.*, 2016a). Moreover, selected Trichoderma isolates can confer a form of systemic immunity in their host that is effective against a broad spectrum of shoot and root pathogens, a phenomenon known as induced systemic resistance (ISR) (Pieterse *et al.*, 2014; Martínez–Medina *et al.*, 2013; 2017b). ISR can

also be conferred by other beneficial microbes, such as plant growth–promoting rhizobacteria and mycorrhizal fungi (Van Wees *et al.*, 2008; Shoresh *et al.*, 2010; Jung *et al.*, 2012; Pieterse *et al.*, 2014). Typically, ISR triggered by beneficial microbes, including Trichoderma fungi, is associated with priming of the plant immune system, resulting in an enhanced and/or faster activation of plant defenses upon pathogen attack (Van Wees *et al.*, 2008; Martínez–Medina *et al.*, 2013, 2016b, 2017b; Mauch–Mani *et al.*, 2017). Defense priming by beneficial microbes provides the plant with a cost–effective mechanism of protection against shoot and root attackers (Martínez–Medina *et al.*, 2016b; Mauch–Mani *et al.*, 2017).

The Arabidopsis (*Arabidopsis thaliana*) root R2R3–type MYB transcription factor MYB72 is an essential regulator for the initiation of ISR mediated by beneficial microbes, including Trichoderma fungi. Indeed, Arabidopsis *myb72* mutant plants are impaired in their ability to express ISR triggered by *Trichoderma asperellum* root colonization (Segarra *et al.*, 2009). Interestingly, ISR mediated by *Pseudomonas simiae* WCS417 (formerly known as *Pseudomonas fluorescens*; Berendsen *et al.*, 2015) is also dependent on MYB72 (Van der Ent *et al.*, 2008), indicating that this transcription factor is a node of convergence in the ISR signalling pathways triggered by different beneficial microbes. Besides regulating the onset of ISR in roots, MYB72 has been shown to control the biosynthesis and excretion of iron–mobilizing coumarins in the root environment (Zamioudis *et al*., 2014; Stringlis *et al*., 2018). Specific MYB72–dependent coumarins have selective anti–microbial activity and play a role in shaping root microbiome assembly to promote plant growth and health (Stringlis *et al.* 2018). In addition to MYB72, signalling molecules, such as the hormones jasmonic acid (JA), salicylic acid (SA), ethylene and abscisic acid, have been implicated in Trichoderma–mediated ISR (Martínez–Medina *et al.*, 2013, 2017a; Saravanakumar *et al.*, 2016; Alkooranee *et al.*, 2017; Agostini *et al.*, 2019). More recently, the signalling molecule nitric oxide (NO) has been suggested to be further involved in ISR mediated by Trichoderma in cucumber plants (Nawrocka *et al.*, 2019), although its specific role in Trichoderma–mediated ISR remains obscure.

NO is a highly reactive free radical that can diffuse across biological membranes due to its gaseous and lipophilic nature and can be a counterpart of cell–to–cell signalling in short periods of time (Beligni and Lamattina, 2001; Brouquisse, 2019; León and Costa–Broseta, 2019). NO is involved in a wide range of plant processes, such as seed germination (Arc *et al.*, 2013; Gibbs *et al.*, 2014; Albertos *et al.*, 2015; Del Castello *et al.*, 2019), root development (Sanz *et al.*, 2015; Castillo *et al.*, 2018) and plant reproduction (Hiscock *et al.*, 2007; Kwon *et al.*, 2012; Du *et al.*, 2014). NO is also implicated in plant responses to several abiotic stresses, including plant adaptation to low iron availability (Graziano and Lamattina, 2007; Chen *et al.*, 2010; García *et al.*, 2010; Meiser *et al*., 2011; Gupta and Igamberdiev, 2016; Romero–Puertas *et al.*, 2018; Sami *et al.*, 2018; Sánchez–Vicente *et al.*, 2019); and biotic stresses (Molina–Moya *et al.*, 2019; Martínez–Medina *et al.*, 2019b; Sánchez–Vicente *et al.*, 2019). NO has been shown to be further involved in plant interaction with beneficial microbes (Meilhoc *et al.*, 2013; Berger *et al.*, 2019, 2020; Martínez–Medina *et al.*, 2019a, b, c). During plant–interactions with Trichoderma fungi, NO rapidly accumulates in roots of Arabidopsis and tomato plants, suggesting a role for NO in the establishment of the plant–Trichoderma symbiosis (Gupta *et al.*, 2014; Martínez–Medina *et al.*, 2019a).

We recently found that volatile compounds (VCs) from the ISR–inducing Trichoderma fungi *Trichoderma harzianum* T–78 and *Trichoderma asperellum* T–34 act as determinants for the elicitation of ISR against the necrotrophic fungus *Botrytis cinerea* (Martínez–Medina *et al.*, 2017c). Moreover, we demonstrated that root perception of Trichoderma–VCs triggered the induction of *MYB72*, as a part of the activation of the strategy I iron–deficiency response in Arabidopsis roots. Similarly, VCs released by the ISR–inducing rhizobacteria *P. simiae* WCS417 triggered the expression of *MYB72* in Arabidopsis roots (Zamioudis *et al.*, 2015), indicating that elicitation of *MYB72* and activation of the strategy I iron uptake response is a feature conserved among different root–associated mutualists. Interestingly, root elicitation of *MYB72* by rhizobacteria VCs was found to be associated with NO signalling (Zamioudis *et al.* 2015), indicating that NO might act

upstream of MYB72 in the activation of the strategy I iron–deficiency response mediated by microbial VCs.

Here we hypothesise that NO is an early key component for the onset of ISR mediated by Trichoderma VCs, acting upstream of MYB72. To test this hypothesis, we first monitored the NO accumulation elicited by Trichoderma VCs in Arabidopsis roots. We found that plant perception of Trichoderma VCs triggered an early accumulation of NO in Arabidopsis roots. By using a NO scavenger, we showed that NO signalling is essential for Trichoderma VCs–mediated induction of *MYB72*. Moreover, by performing different bioassays including the *myb72* Arabidopsis mutant line and a NO scavenger, we demonstrated the requirement of MYB72 and NO signalling in induced resistance mediated by Trichoderma VCs against the leaf pathogen *Botrytis cinerea*. Our results indicate a pivotal role for NO signalling upstream of MYB72 during the initiation of ISR triggered by Trichoderma VCs.

Materials and methods

Plant and fungal material

We used *Arabidopsis thaliana* wild–type (WT) accession Col–0, the Arabidopsis *myb72–2* mutant line (Van der Ent *et al*., 2008) and the Arabidopsis reporter line *pMYB72:GFP–GUS* (Zamioudis *et al.*, 2015). Arabidopsis seeds were surface disinfected and stratified for 2 days at 4°C. *Trichoderma harzianum* T–78 (T–78; Martínez–Medina *et al.*, 2009) and *Trichoderma asperellum* T–34 (T–34; Segarra *et al.*, 2009) were cultured on potato dextrose agar plates during 5 days at 28°C in dark as described by Martínez–Medina *et al.* (2014). For treatments including T–78 inoculation in pots, we prepared a T–78 solid inoculum containing commercial oat, bentonite and vermiculite, according to Martínez–Medina *et al.* (2009). *Botrytis cinerea* strain B05.10 (Van Kan *et al.*, 1997) was cultivated on half–strength potato dextrose agar plates for 10 days at 22°C.

Bioassays in plates

Surface–sterilized Arabidopsis seeds were sown on Murashige and Skoog (MS) agar–solidified medium supplemented with vitamins and 0.5% of sucrose, pH 6, in one of the compartments of two–compartment circular plates (120 mm diameter), according to Zamioudis *et al.* (2015) and Martínez–Medina *et al.* (2017c). The plates were positioned vertically and placed in a growth chamber (22°C, 10 h: 14 h, light: dark; light intensity 100 μmol m^{-2} s^{-1}) (Fig. S1). After 12 days, a 7–mm diameter plug of each Trichoderma strain from the actively growing margins of cultures was transferred into the plant–free compartment containing MS agar–solidified medium. The plates were sealed with one layer of gas–permeable Parafilm (Sigma) and placed in a vertical position in the growth chamber for 1, 2 or 3 days. In the two–compartment plates, seedlings and microbes were physically separated, but the gas exchange was allowed between the compartments. It was recently demonstrated that respiratory CO_2 only plays a minor role in plant responses to microbial VCs, indicating the suitability of sealed co–cultivation systems for testing the impact of microbial VCs on plant defense responses (Sánchez–López *et al*., 2016; García–Gómez *et al*., 2019).

Bioassays in pots

Individual seedlings that were growing in the plates for 15 days were transferred to 50–mL pots (Fig. S1) containing sterile sand: soil mixture (5:12, v:v). For those pots containing the T–78 inoculum, T–78 inoculation was achieved by mixing the inoculum through the soil: sand mixture to a final density of 1×10^6 conidia per g of soil. Plants were then randomly distributed and cultivated in a growth chamber with an 8 h light (24 °C, light intensity 100 μmol m^{-2} s^{-1}) and 16 h dark (20°C) cycle at 70% relative humidity. Plants were watered every other day and received half–strength Hoagland solution (Hoagland and Arnon, 1938) containing 10 μM sequestreen (CIBA–Geigy) once a week.

Botrytis cinerea bioassays

Five–week–old Arabidopsis plants were inoculated with *B. cinerea* strain B05.10 (Van Kan *et al.*, 1997) according to Van Wees *et al.* (2013). A 5–µL droplet of a suspension of 5×10^5 spores mL^{-1} was applied to six leaves per plant. Thereafter, plants were placed under a lid to increase relative humidity to 100% to promote the infection. Disease symptoms were scored 3 days after *B. cinerea* inoculation by visual inspection. Disease ratings were assigned on each leaf according to van der Ent *et al.* (2008). Percentage of leaves in each class was calculated per plant. Shoot samples for quantification of *B. cinerea TUBULIN* mRNA levels were harvested 24 h after *B. cinerea* inoculation (Fig. S1).

Fluorescence microscopy

The presence of NO in Arabidopsis roots was analysed using the cell–permeable NO–specific probe 4,5–diaminofluorescein diacetate (DAF–2DA), which is converted to its fluorescent triazole derivative DAF–2T upon reaction with NO (Nakatsubo *et al.,* 1998). Segments of plant primary roots from the apex were incubated for 1 h in darkness with 10 µM DAF–2DA (Merck Biosciences), prepared in 10 mM Tris–HCl (pH 7.4) as described by Sandalio *et al.* (2008). As a negative control, root segments were similarly incubated with the NO scavenger 2–(4 carboxyphenyl)–4,4,5,5–tetramethylimidazoline–1–oxyl–3–oxide (cPTIO; Sigma) at a final concentration of 500 µM. Subsequently, the segments were washed three times for 15 min each in 10 mM Tris–HCl (pH 7.4). The fluorescence emitted by DAF–2T was detected by excitation at 495 nm and emission at 515 nm (Sandalio *et al.*, 2008). Fluorescence intensity was quantified by using ZEN Lite software from Zeiss. As counterstain, roots were stained with 10 µg mL^{-1} propidium iodide solution for 2 min. GFP fluorescence in *pMYB72:GFP–GUS* was examined on a Leica MZ16FA fluorescence stereomicroscope equipped with GFP3 filter.

Chemical treatment

To study whether NO is required for Trichoderma VCs–triggered *MYB72* expression, inhibition of NO signalling was achieved by transferring seedlings to plates containing MS agar–solidified medium supplemented with the NO scavenger cPTIO at the final concentration of 500 μM (Sandalio *et al.*, 2008). To study whether NO is required for Trichoderma VCs–mediated ISR against the pathogen, roots of Arabidopsis plants growing in the split–plates were treated with 500 μL of cPTIO 500 mM. cPTIO was applied every 8 h during the duration of the split–plate bioassay. For control plates, the same procedure was done with sterile water.

Real–time quantitative RT–PCR

Total RNA from Arabidopsis leaves was extracted using Tri–sure (Bioline), and was subsequently purified by column using the RNA Clean and Concentrator–5 kit (Zymo Research). RNA samples were treated with NZY DNase I (NZYTech). First–strand cDNA was synthesized from 1 μg of purified total RNA by using the PrimeScript RT Master Mix (Takara). Real–time quantitative RT–qPCR reactions were performed using SYBR® Premix Ex Taq™ (Takara) and an iCycler 5 (Bio–Rad). All kits were used according to manufacturer´s instructions. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak and Schmittgen, 2001) by using the gene–specific primers described in Table S1. Expression values were normalized using the Arabidopsis housekeeping genes *TUBULIN–4* (At5g44340) or *ACTIN7* (At5g09810). Fungal infection was measured by analysing *B. cinerea β–TUBULIN* gene (XM_001560987.1) relative to the Arabidopsis *TUBULIN–4* gene.

Results

MYB72 is required for Trichoderma VCs–mediated ISR

We previously found that VCs released by T–78 and T–34 trigger ISR against the shoot pathogen *B. cinerea* (Martínez–Medina *et al.*, 2017c). The transcription factor MYB72 is essential for the onset of the ISR mediated by root colonization by

beneficial rhizobacteria and rhizofungi (Van der Ent *et al.*, 2008; Segarra *et al.*, 2009). Here, we first aimed to investigate whether MYB72 is also required for Trichoderma VCs–mediated ISR. To this end, Arabidopsis seedlings from WT and *myb72* lines were treated or not with VCs from T–78 or T–34 during 3 days in split–plate assays. Subsequently, WT and *myb72* seedlings were transplanted into pots, and 3 weeks later the plants were challenged with the necrotrophic pathogen *B. cinerea*. As a control for ISR we used WT and *myb72* plants that were root inoculated with T–78. As expected, T–78 root–inoculated plants developed significantly less–severe disease symptoms than non–treated control plants. Similarly, WT plants that were previously exposed to T–78 and T–34 VCs also developed significantly less–severe disease symptoms compared to control plants (Fig. 1A), indicating that VCs from T–78 and T–34 induce resistance against *B. cinerea* to a similar extend as root inoculation with T–78. As expected, ISR triggered by T–78 root inoculation was absent in *myb72* mutant plants (Fig. 1B). Mutant *myb72* plants developed even more–severe disease symptoms when the roots were inoculated with T–78 compared to non–treated plants. Interestingly, *myb72* plants also failed to mount VCs–mediated ISR (Fig. 1B), as the fungal VCs–treated *myb72* plants developed more–severe symptoms than the non–treated *myb72* plants. These results evidence that MYB72 is required for Trichoderma VCs–mediated ISR against *B. cinerea*.

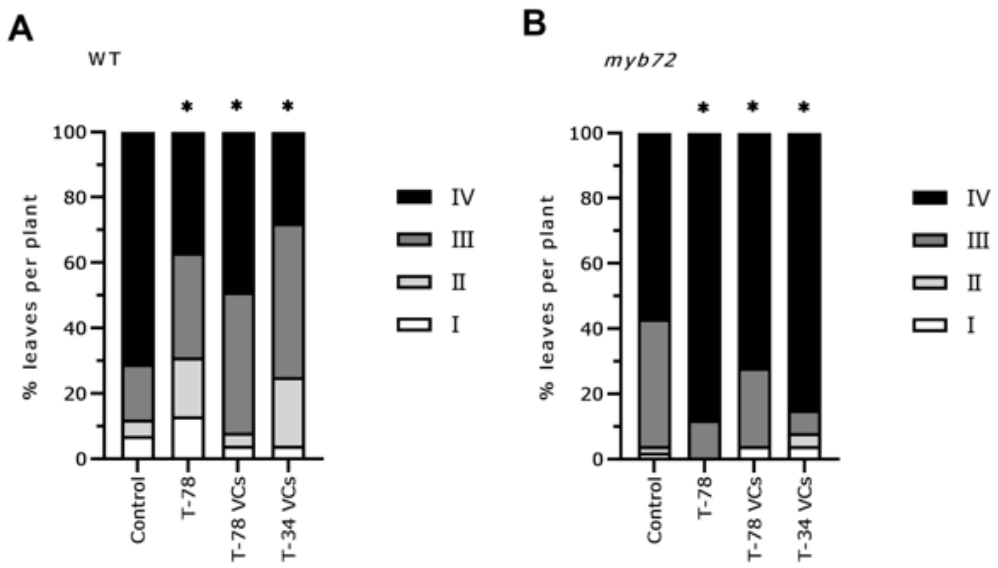
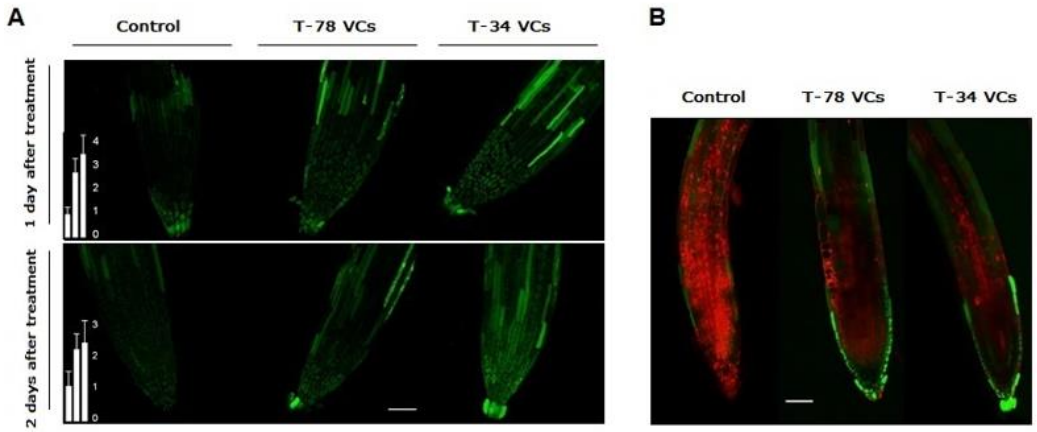


Fig. 1. MYB72 is required for Trichoderma VCs–triggered systemic immunity. Quantification of disease symptoms in leaves of Arabidopsi*s* WT **(A)** and *myb72* mutant **(B)** lines after inoculation with *B. cinerea*. Seedlings were mock–treated (control) or treated with VCs from *T. harzianum* T–78 (T–78 VCs) or *T. asperellum* T–34 (T–34 VCs) for 3 days in split–plate assays before transplanting them into pots. A set of mock–treated seedlings were also inoculated with *T. harzianum* T–78 in pots (T–78). Three weeks after transplanting, seedlings were challenged with *B. cinerea*. Disease severity was scored 3 days after inoculation by using four disease severity classes: I, no visible disease symptoms; II, non–spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen. Percentage of leaves in each class was calculated per plant. The asterisks indicate statistically significant differences compared with mock–treated control plants (χ^2 test; n = 50 plants). These results are representative of two independent experiments.

Trichoderma VCs trigger the accumulation of NO in Arabidopsis roots

NO accumulates in plant roots during the early stages of Trichoderma interaction with Arabidopsis and tomato plants (Gupta *et al.*, 2014; Martínez–Medina *et al.*, 2019a). To study whether Trichoderma VCs trigger the accumulation of NO in Arabidopsis roots, endogenous NO accumulation was monitored in roots of Arabidopsis seedlings in the split–plate assays, by using the NO fluorescent probe DAF–2DA. Roots of non–treated control plants contained basal levels of NO along the root (Fig. 2A, B, control). However, roots of VCs–treated seedlings displayed a more intense NO fluorescence at 1 and 2 days after treatment (Fig. 2A, B). NO fluorescence in VCs–treated seedlings was more intense in the apical and subapical root zones and mostly confined to the outermost cell layers (Fig. 2B). According to this observation, the NO–responsive gene *PHYTOGB1* (Perazzolli *et al.*, 2004), which codes for Phytoglobin 1, was upregulated in Arabidopsis roots in response to Trichoderma VCs treatment (Fig. 2C). These observations strongly support that Trichoderma VCs trigger an early burst of NO in Arabidopsis roots.



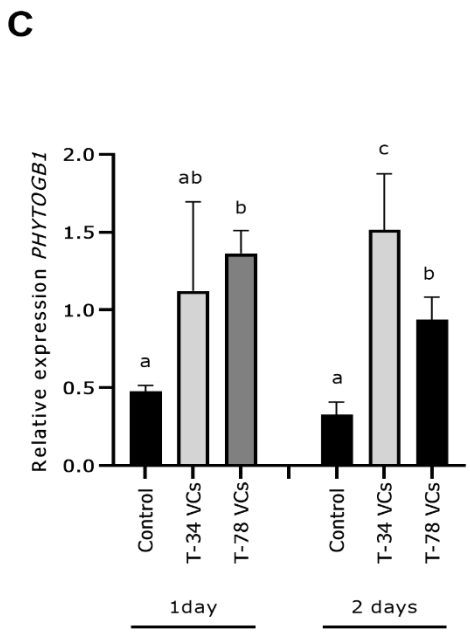


Fig. 2. Trichoderma VCs trigger NO accumulation in Arabidopsis roots. **(A)** Imaging of NO production in roots of Arabidopsis seedlings by confocal laser scanning fluorescence microscopy. Images are projections of several optical sections collected by confocal microscopy showing NO–dependent DAF–2DA fluorescence (green; excitation at 495 nm, emission at 515 nm) from mock–treated (control) seedlings, or seedlings treated with VCs from *T. harzianum* T–78 (T–78 VCs) or *T. asperellum* T–34 (T–34 VCs) for 1 or 2 days using split–plate assays. Scale bar = 50 μm. Bars show the relative fluorescence intensities corresponding to NO, quantified in arbitrary units in mock–treated controls (left bar), T–78 VCs (middle bar) and T–34 VCs (right bar) treated seedlings. **(B)** Representative confocal images of NO accumulation in roots of Arabidopsis seedlings mock–treated (control) or treated with VCs from T–78 or T–34 for 1 day. The images show DAF–2DA fluorescence as in (A). Cell walls were counterstained with propidium iodide (red signal). Scale bar = 50 μm. These results are representative of two independent experiments. **(C)** Expression of the NO responsive gene *PHYTOGB1* in Arabidopsis roots. Seedlings were either mock–treated (control) or treated with VCs from T–78 or T–34 for 1 or 2 days. Results were normalized to the *ACTIN7* gene expression in the same samples. Values are means ± SE of five biological replicates. Each biological replicate consisted of pooled root material from 4 plates, each containing 12–15 Arabidopsis seedlings. For each time point, different letters indicate statistically significant differences between treatments (Tukey's HSD test; $P < 0.05$).

NO is required for Trichoderma VCs–mediated induction of *MYB72*

We previously found that Trichoderma VCs trigger the expression of *MYB72* in Arabidopsis roots (Martínez–Medina *et al.*, 2017c). To determine whether NO signalling is required for Trichoderma VCs–mediated induction of *MYB7*2, we used the Arabidopsis transgenic line *pMYB72:GFP–GUS* expressing the GFP–GUS fusion protein under the control of the *MYB72* promoter (Zamioudis *et al.*, 2015), and the NO scavenger cPTIO. We first confirmed that cPTIO was effective in reducing NO accumulation in Arabidopsis roots in the split–plate assays (Fig. S2). Indeed, the cPTIO treatment reduced significantly the NO levels in Arabidopsis roots and further prevented the promotion of lateral roots triggered by Trichoderma VCs (Martínez–Medina *et al.*, 2017c). As shown in Fig. 3A, a stronger accumulation of the GFP fluorophore was observed in roots of *pMYB72:GFP–GUS* plants that were treated with T–78 and T–34 VCs, compared to control roots, confirming that fungal VCs trigger the expression of *MYB72*. Interestingly, the induction of *MYB72* by fungal VCs was abolished in seedlings that were also treated with the NO scavenger (Fig. 3B). Thus, our results demonstrate the requirement of NO signalling for Trichoderma VCs–mediated activation of *MYB72*.

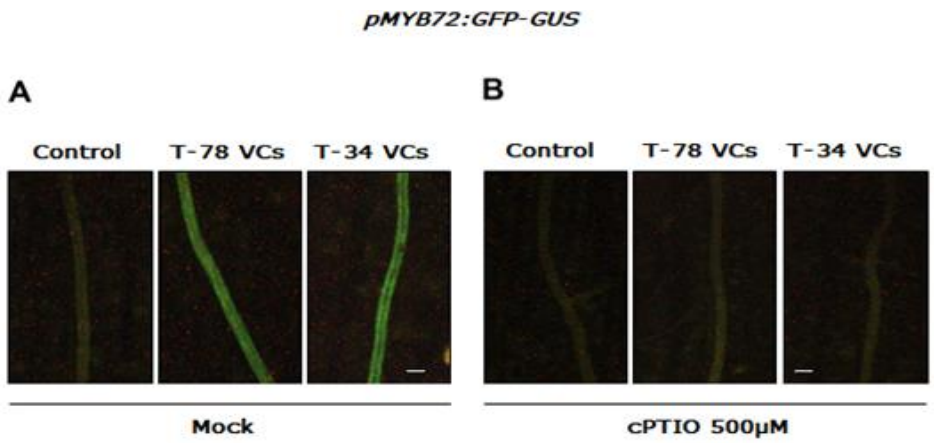


Fig. 3. NO accumulation is required for *MYB72* induction by Trichoderma VCs. Representative images showing the accumulation of GFP fluorescent protein (green signal) from p*MYB72:GFP–GUS* roots by using fluorescence stereo microscopy. Seedlings were mock–treated (control) or treated with VCs from *T. harzianum* T–78 (T–78 VCs) or *T. asperellum* T–34 (T–34 VCs) for 2 days in split–plate assays in the absence (mock) or presence (cPTIO 500 μM) of the NO scavenger cPTIO. Scale bar = 200 μm. These results are representative of two independent experiments.

NO signalling is required for ISR mediated by Trichoderma VCs

Given the critical role of *MYB72* in Trichoderma VCs–mediated ISR (Fig. 1), and the importance of NO signalling in the activation of *MYB72* (Fig. 3), we next aimed to discern whether NO functions as a key signalling molecule in the onset of VCs–mediated ISR. To test this, we focused specifically on VCs from T–78. Seedlings were treated with T–78 VCs for 3 days in the split–plate assays that were supplemented or not with cPTIO. Subsequently, seedlings were transplanted into pots, and 3 weeks later, the plants were challenged with *B. cinerea* (Fig. S1). As in the previous experiments, plants exposed to T–78 VCs developed significantly less severe disease symptoms (Fig. 4A), and contained less pathogen, as determined by *B. cinerea β–TUBULIN*–relative quantification (Fig. 4B). Remarkably, in cPTIO root–treated plants, T–78 VCs–mediated ISR was completely blocked. Plants exposed to T–78 VCs that were root–treated with cPTIO developed a similar disease severity and contained similar amounts of *B. cinerea* than plants that were not exposed to T–78 VCs (Fig. 4A, B). Taken together, these observations demonstrate that NO signalling is essential for the ISR triggered by T–78 VCs.

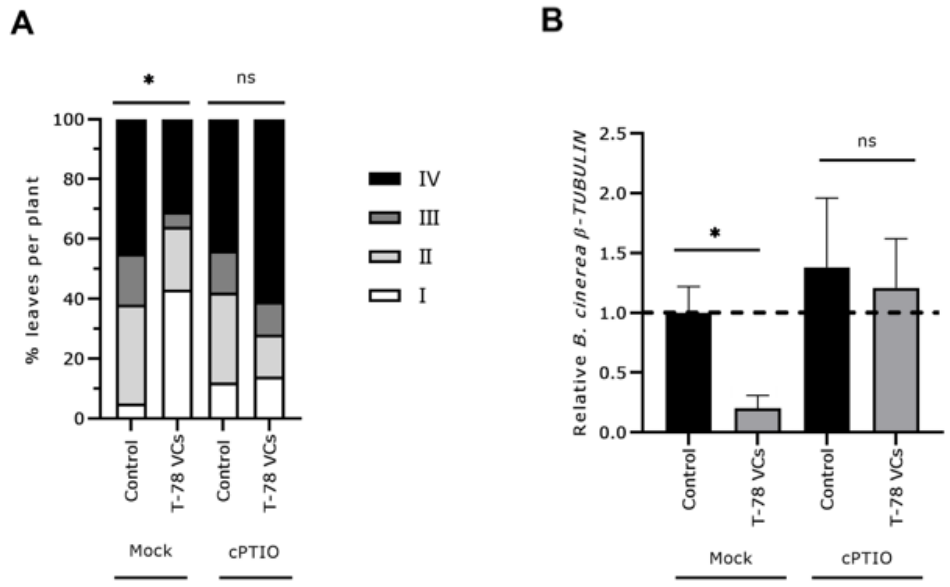


Fig. 4. NO signalling is required for Trichoderma VCs–mediated ISR against *Botrytis cinerea*. (A) Quantification of disease symptoms in Arabidopsis leaves after inoculation with *B. cinerea*. Seedlings were mock–treated (control) or treated with VCs from *Trichoderma harzianum* T–78 (T–78 VCs) for 3 days in split–plate assays that were mock–treated or supplemented with cPTIO. Subsequently, seedlings were transplanted into pots, and 3 weeks later inoculated with *B. cinerea*. Disease severity was scored 3 days after inoculation by using four disease severity classes: I, no visible disease symptoms; II, non–spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen. Percentage of leaves in each class was calculated per plant. The asterisks indicate statistically significant differences compared with mock–treated control plants (χ^2 test; n = 50 plants; ns, not significant). **(B)** Quantification of *B. cinerea* in Arabidopsis leaves. The relative amount of *B. cinerea* was determined 1 day after inoculation by quantitative RT–PCR analysis of the *B. cinerea β–TUBULIN* gene relative to the Arabidopsis *TUBULIN–4* gene. Values are means ± SE of five biological replicates. The asterisks indicate statistically significant differences compared to control plants according to Student's *t*–test ($P < 0.05$), ns, not significant. These results are representative of two independent experiments.

Depletion of NO compromises the boost of plant defense–related genes triggered by Trichoderma VCs against *B. cinerea*

We next aimed to gain further insight into the role of NO in the protective ability mediated by Trichoderma VCs against *B. cinerea*. With this purpose, we assessed the expression of the SA/NO–responsive marker gene *PR1* (*PATHOGENESIS–RELATED PROTEIN 1*; Uknes *et al.*, 1992; Durner *et al.*, 1998) and

the JA–responsive marker gene *PDF1.2* (*PLANT DEFENSIN1.2*; Penninckx *et al.*, 1998) in plants that were challenged with the pathogen *B. cinerea*. Plants were first exposed to T–78 VCs in the split–plate assay supplemented or not with the NO scavenger cPTIO. Seedlings were subsequently transplanted into pots and 3 weeks later challenged with *B. cinerea*. One day after challenging the plants with the pathogen, we assessed the expression of *PR1* and *PDF1.2*. A higher expression of *PR1* and *PDF1.2* was observed in *B. cinerea* challenged plants that were previously exposed to T–78 VCs as compared to plants not exposed to T–78 VCs (Fig. 5A, B). By contrast, the enhanced *PR1* and *PDF1.2* expression triggered by T–78 VCs was abolished by cPTIO treatment (Fig. 5A, B). These results support a role of NO signalling in the induction of priming for enhanced defenses triggered by T–78 VCs against *B. cinerea* infection.

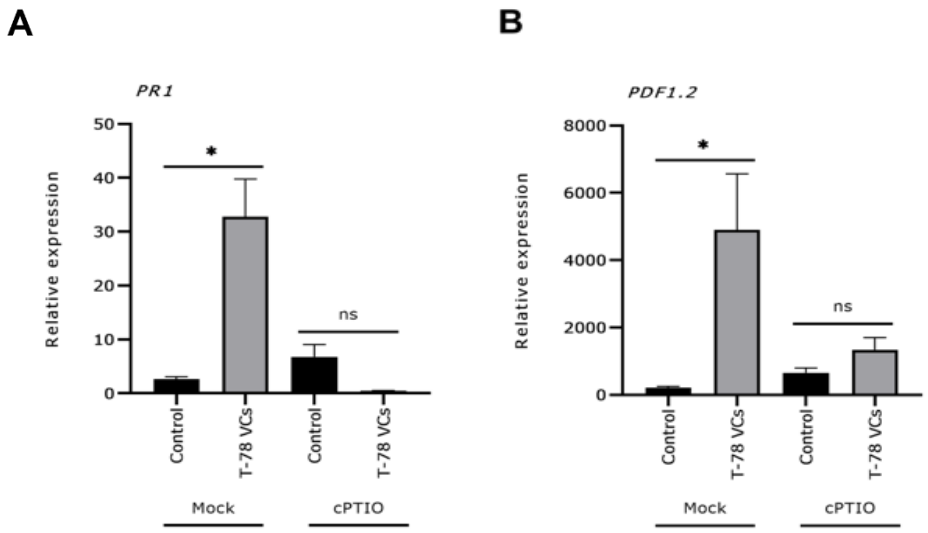


Fig. 5. Depletion of NO impairs the enhanced expression of defense genes triggered by Trichoderma VCs against *Botrytis cinerea*. Relative expression of the defense marker genes *PR1* **(A)** and *PDF1.2* **(B)** in Arabidopsis leaves 1 day after inoculation with *B. cinerea*. Seedlings were mock–treated (control) or treated with VCs from *Trichoderma harzianum* T–78 (T–78 VCs) for 3 days in split–plate assays that were mock–treated or supplemented with cPTIO. Subsequently, seedlings were transplanted into pots, and 3 weeks later inoculated with *B. cinerea*. Expression was normalized to that of *TUBULIN–4* gene. The expression levels are reported as the fold increase relative to that of the plants not inoculated with *B. cinerea* in each treatment. Values are means ± SE of at least four biological replicates. The asterisks indicate statistically significant differences between treatments, according to Student's *t*–test ($P < 0.05$), ns, not significant. These results are representative of two independent experiments.

Discussion

Selected Trichoderma strains can improve plant health by triggering a broad–spectrum ISR (Segarra *et al.*, 2009; Shoresh *et al.*, 2010; Mathys *et al.*, 2012; Martínez–Medina *et al.*, 2013, 2017b; Saravanakumar *et al.*, 2016; Alkooranee *et al.*, 2017; Nawrocka *et al.*, 2018; Herrera–Téllez *et al.*, 2019). In Arabidopsis, the root–specific transcription factor MYB72 is essential for the initiation of ISR after root colonization by Trichoderma (Segarra *et al.*, 2009). More recently, it was demonstrated that Trichoderma VCs may act as determinants for the elicitation of *MYB72* and ISR via root–to–shoot signalling (Kottb *et al.,* 2015; Martínez–Medina *et al.*, 2017c), which is in line with observations obtained with VCs from ISR–eliciting *Pseudomonas* spp. strains (Zamioudis *et al.,* 2015). Here, we first analysed whether MYB72 is also required for the initiation of ISR triggered by VCs released by the ISR–inducing Trichoderma strains *T. harzianum* T–78 and *T. asperellum* T–34. We found that VCs–mediated ISR against the shoot pathogen *B. cinerea* was completely abolished in *myb72* plants. These findings demonstrate that MYB72 is also essential for the activation of ISR by Trichoderma VCs, and further reinforce the central role of MYB72 in the onset of ISR triggered by different microbes and elicitors.

In Arabidopsis roots, the initiation of the MYB72–dependent iron deficiency response triggered by *Pseudomonas* spp. is associated with NO signalling (Zamioudis *et al.*, 2015). Indeed, NO is a well–established key component of the regulatory mechanisms that orchestrate iron uptake in plants (Graziano and Lamattina, 2007; Chen *et al.*, 2010; García *et al.*, 2010; Meiser *et al*., 2011). We found that plant perception of Trichoderma VCs triggered the expression of *MYB72*, as previously described by Martínez–Medina *et al.* (2017c). Interestingly, Trichoderma VCs also triggered a strong accumulation of NO in Arabidopsis roots, suggesting that NO signalling is an early component of the plant response to Trichoderma VCs. It was previously observed that the interaction of Arabidopsis and tomato roots with Trichoderma fungi is associated with an early burst of NO (Gupta *et al.*, 2014; Martínez–Medina *et al.*, 2019a). Similarly, root perception of rhizobacterial VCs and root interaction with the beneficial fungus *Rhizophagus irregularis* and the

pathogenic fungus *Fusarium oxysporum* is also associated with an early burst of NO in roots (Gupta *et al*., 2014; Zamioudis *et al*., 2015; Martínez–Medina *et al.*, 2019c). These findings suggest that the rapid burst of NO triggered by Trichoderma VCs is part of common early plant response to different microbial elicitors. Remarkably, the NO burst triggered by Trichoderma VCs was mainly restricted to the root epidermis and cortical cells. Similarly, *MYB72* upregulation by Trichoderma and rhizobacteria VCs was found to be restricted to the epidermal and cortical cells (Zamioudis *et al.*, 2015; Martínez–Medina *et al.*, 2017c). This indicates that NO–signalling triggered by Trichoderma VCs is activated in root cell types associated with MYB72–related root responses, and suggest a link between both components in the root responses to Trichoderma VCs. Indeed, by using the NO scavenger cPTIO, we demonstrated that NO signalling is required for Trichoderma VCs–mediated induction of *MYB72*. Similarly, NO signalling was reported to be essential for rhizobacteria VCs–mediated induction of *MYB72* during the onset of the iron deficiency response (Zamioudis *et al.*, 2015). Our findings demonstrate that NO is involved in the activation of *MYB72* triggered by VCs from different microbes, including bacteria and fungi.

We found that MYB72 is essential for Trichoderma VCs–mediated ISR. We further demonstrated that the activation of *MYB72* by Trichoderma VCs was dependent on NO signalling. To analyse the involvement of NO signalling on MYB72–dependent ISR elicited by Trichoderma VCs, we performed bioassays in which NO signalling was compromised specifically during treatment of the plants with Trichoderma VCs. We found that depletion of NO blocked Trichoderma VCs–mediated ISR against *B. cinerea*. Thus, our findings demonstrate that NO signalling is required for the elicitation of ISR mediated by Trichoderma VCs. NO has been previously associated with induced resistance against biotic stresses (Manjunatha *et al.*, 2009; Fu *et al.*, 2010; Acharya *et al.*, 2011; Keshavarz–Tohid *et al.*, 2016). Indeed, NO has been proposed to be involved in the accumulation of defense–related enzymes and total phenolics during rhizobacteria–mediated ISR (Acharya *et al.*, 2011). More recently, NO has been further proposed to be involved in the ISR

mediated by Trichoderma fungi (Nawrocka *et al.*, 2019). Here, we demonstrate that NO signalling is essential for the activation of ISR by Trichoderma VCs.

Trichoderma–ISR is generally associated with priming for enhanced activation of defenses upon pathogen attack (Segarra *et al.*, 2009; Brotman *et al.*, 2012; Perazzolli *et al.*, 2012; Martínez–Medina *et al.*, 2013, 2017b; Coppola *et al.*, 2019). We found that treatment with Trichoderma VCs resulted in an enhanced induction of the defense–related genes *PR1* (a marker of the SA/NO–related pathway) and *PDF1.2* (a marker for the JA–related pathway) in response to *B. cinerea* infection. In previous studies, we demonstrated that treatment with VCs from T–78 led to an accelerated and augmented induction of *PDF1.2* upon treatment with MeJA (Martínez–Medina *et al.*, 2017c). Moreover, we found here that treatment with T–78 and T–34 VCs led to a boosted expression of *PR1* upon treatment with SA (Fig. S3). Interestingly, it was recently demonstrated that both the SA– and JA–related pathway provide resistance to *B. cinerea* in Arabidopsis plants (Zhang *et al.*, 2017). Our data thus suggest that Trichoderma VCs might confer plant protection against *B. cinerea* by priming the JA– and SA–related defense. In the same line, VCs from the beneficial bacteria *Bacillus subtilis* also potentiated the expression of *PR1* and *PDF1.2* and protected plants against *B. cinerea* attack (Sharifi and Ryu, 2016). Remarkably, here we show that Trichoderma VCs treatment failed totally or partially in enhancing *PR1* and *PDF1.2* expression when NO signalling was impaired, supporting the involvement of NO signalling in priming of plant defenses mediated by Trichoderma VCs. Similarly, Manjunatha *et al.* (2009) suggested a role for NO in chitosan–triggered defense priming against *Sclerospora graminicola*. Some studies have indicated that defense priming could involve NO participation, as priming implies the reversible *S*–nitrosylation of proteins and other epigenetic changes sensitized by NO (Floryszak–Wieczorek *et al.*, 2012; Lindermayr *et al.*, 2020). Altogether, our study demonstrates that plant perception of Trichoderma VCs triggers a burst of NO in plant roots, which is involved in MYB72 activation that leads to ISR. Our study further indicates a key role of NO signalling upstream of MYB72 during the onset of ISR mediated by Trichoderma VCs.

Supplementary data

Table S1. List of primers used in the analyses.

ID	Target gene	Sequence (5' → 3')
At5g44420	*PDF1.2*[1]	TTTGCTGCTTTCGACGCAC
		CGCAAACCCCTGACCATG
At5g24770	*VSP2*[2]	CGGGTCGGTCTTCTCTGTTC
		CCAAAGGACTTGCCCTA
At2g14610	*PR1*[3]	GGAGCTACGCAGAACAACTAAGA
		CCCACGAGGATCATAGTTGCAACTGA
XM_001560987.1	*β–TUBULIN B. cinerea*[4]	CCGTCATGTCCGGTGTTACCAC
		CGACCGTTACGGAAATCGGAA
At5g44340	*TUBULIN–4*[5]	GAGGGAGCCATTGACAACATCTT
		GCGAACAGTTCACAGCTATGTTCA
At5g09810	*ACTIN7*[6]	AGTGGTCGTACAACCGGTATTGT
		GATGGCATGAGGAAGAGAGAAAC

[1]Vos *et al*. (2015); [2]Martínez–Medina *et al*. (2017); [3]Journot–Catalino *et al*. (2006); [4]Brouwer *et al*. (2003); [5]Terrón–Camero *et al*. (2020); [6]Zamioudis *et al*. (2015).

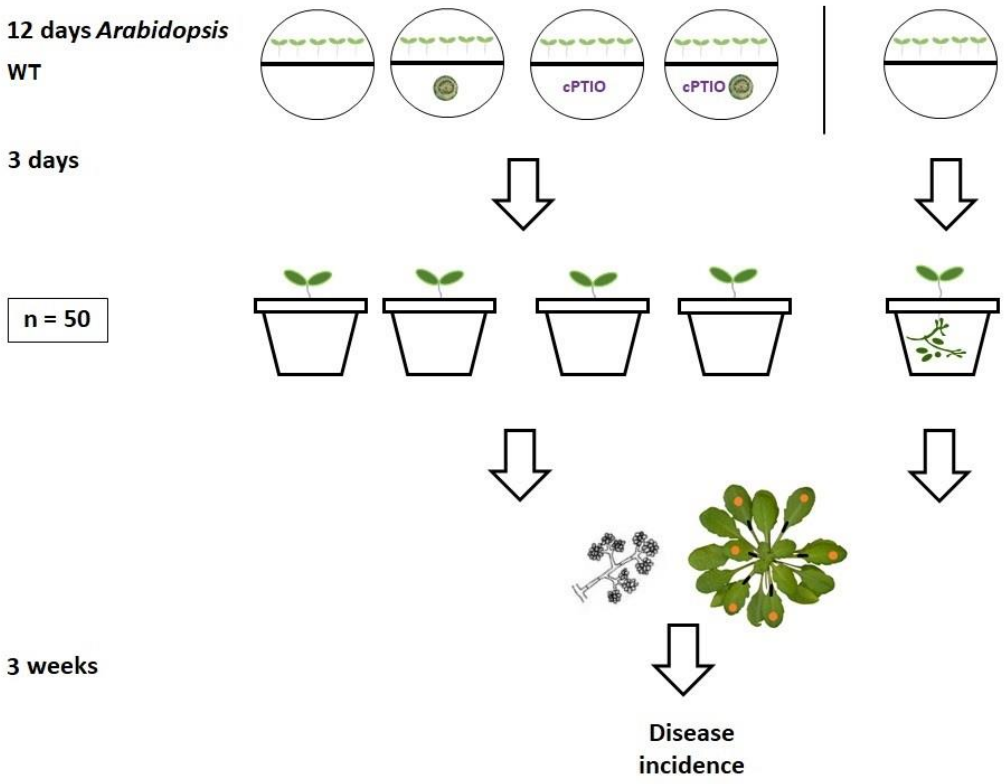


Fig. S1. Scheme of the experimental design performed in this research. Arabidopsis seedlings were grown on the split–plates for 12 d. Then, seedlings were untreated (control) or treated with VCs from each Trichoderma strain, and also were mock–treated or supplemented with cPTIO in the split–plates. After 3 days, seedlings were transplanted into pots. For *Trichoderma harzianum* T–78 treatment (right part), a set of untreated seedlings were grown in plates for 15 days and then transferred into pots containing the T–78 inoculum. 3 weeks later, all plants were inoculated with *B. cinerea* with a droplet of spores suspension to six leaves per plant. Disease symptoms were scored 3 days after *B. cinerea* inoculation by visual inspection.

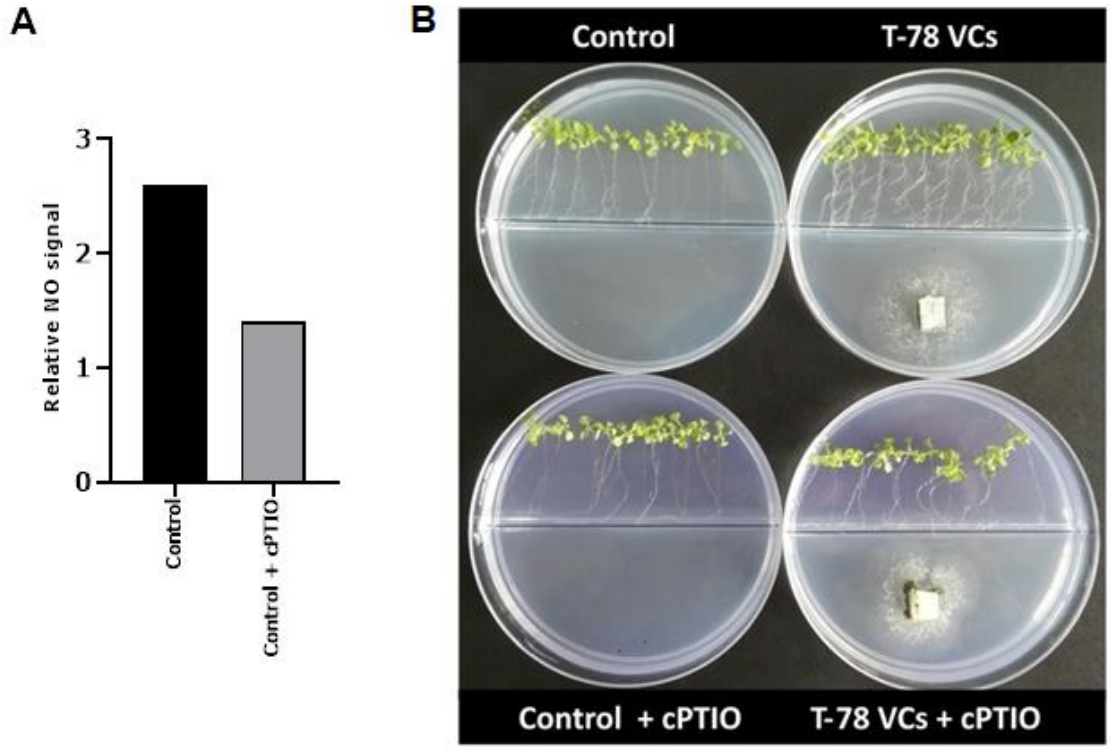


Fig. S2. cPTIO treatment reduces the NO content in Arabidopsis roots. (A) NO production in untreated Arabidopsis roots mock–treated (control) or treated with cPTIO (control + cPTIO) for 2 days in split–plate assays. NO accumulation was detected by fluorimetry using the specific NO detector DAF–2 according to Martínez–Medina *et al*. (2019). Each bar corresponds to the mean of five fluorimetry measures of a pool of 30 roots. **(B)** Arabidopsis seedlings untreated (control) or treated with VCs from *Trichoderma harzianum* T–78 (T–78 VCs) for 3 days in split–plate assays that were supplemented or not with cPTIO. T–78 VCs treatment induced the development of secondary roots according to Martínez–Medina *et al*. (2017). This effect was abolished with cPTIO treatment.

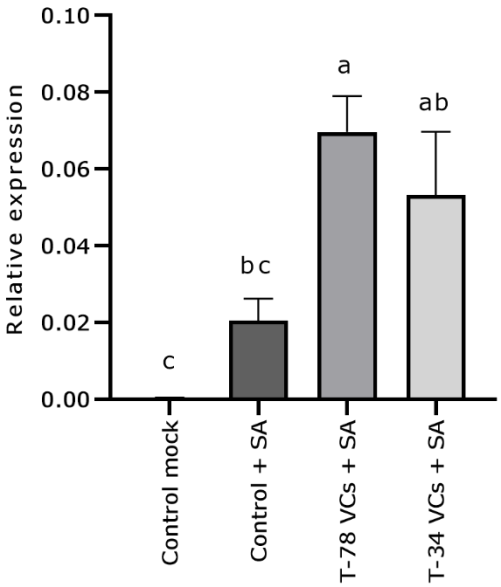


Fig. S3. Volatile compounds from *Trichoderma harzianum* T–78 and *Trichoderma asperellum* T–34 led to a boosted expression of *PR1* gene upon treatment with salicylic acid. Relative expression of the defense gene *PR1*, in shoots of untreated (control) Arabidopsis plants or plants exposed to VCs from *Trichoderma harzianum* T–78 (T–78 VCs) or *T. asperellum* T–34 (T–34 VCs) for 3 days in split–plate assays. Plants were subsequently mock–treated or treated with salicylic acid (SA) 0.5 mM. Samples were collected 5 h after the SA treatment. Expression was normalized to that of *ACTIN7* gene. For each point, different letters indicate statistically significant differences between treatments (Tukey's HSD test; $P < 0.05$).

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GENERAL DISCUSSION

General Discussion

The interactions between plants and fungi are complex and the outcome of these interactions could be detrimental or beneficial to plant health, as fungi can act as pathogens or as biostimulants and/or bioprotectors, respectively. Understanding these interactions and unravelling the control that the plant exerts will contribute to improve pathogen control and promote beneficial fungi use.

Plants recognize MAMPS and finely tune the response to fungal interactions triggering a defense response adapted to the nature of the interplay.

NO is a signalling molecule that exerts a plethora of functions in plants. Among those functions, NO is responsible for regulating the early events of the plant immune response. Despite this, almost all the literature regarding the role of NO in plant–microbe interactions has focused on bacteria. Indeed, few studies accomplish plant–fungal interactions, although they play a major role in natural and agricultural ecosystems.

With this background, the present Thesis is demarcated in plant–fungal interactions. First, using tomato and Arabidopsis as model plants, *Rhizophagus irregularis* and *Trichoderma harzianum* as beneficial fungi and *Fusarium oxysporum* and *Botrytis cinerea* as pathogenic fungal species, we have explored the role of NO in plant fungal–interactions. We have studied the function of NO in the early stages of plant–fungal interactions (Chapters I and II). Then, based on our results and reviewing and compiling the existing literature we have tried to elaborate models on NO functions in plant–fungal interactions (Chapter III). Finally, we tried to expand the knowledge on NO functions in plant–fungal interactions to some of the benefits triggered by beneficial fungi, as ISR (Chapter IV).

Beneficial and pathogenic root fungal interactions trigger different NO patterns

Several studies have reported the accumulation of NO in the interaction of plant roots with pathogenic or beneficial fungi. Despite the different experimental approaches used, timings and plant systems employed in the different studies, some patterns seemed to arise. As we discuss in Chapter III, we found different patterns for necrotrophic and biotrophic pathogens. While NO accumulation mostly benefits necrotrophic pathogens; for biotrophic ones, it depends on the nature of the interaction. During incompatible interactions, NO helps to restrict the spread of the pathogen, whereas for compatible interactions NO accumulation decreases most likely due to active suppression by the fungus. For beneficial interactions, in the case of AM symbiosis, we proposed that NO plays a regulatory role in the establishment of the symbiosis, displaying a specific NO pattern.

However, testing these models require comparative studies using similar plant systems and tools, but very few studies had compared the plant response to both pathogenic and beneficial fungus. Before this PhD, the data available in this regard were mainly those provided by Espinosa *et al*. (2014) and Gupta *et al*., (2014).

Espinosa and collaborators (2014) examined the NO accumulation 1 h after the contact of the pathogenic fungus *Verticillium dahliae* or the AMF *R. irregularis* with olive seedlings. They found a higher accumulation of NO in roots in contact with the pathogen than with the AMF. Therefore, they suggested that the roots were able to differentiate between the two fungi, attenuating defense reactions to facilitate the establishment of the AM interaction and, in contrast, inducing a higher defense reaction against the pathogen. With a similar approach, Gupta *et al*. (2014) compared the NO accumulation elicited by *F. oxysporum* or *T. asperelloides* in Arabidopsis roots at different time points during 2 h after the initial contact. They reported a weak accumulation of NO in the case of Trichoderma, just during the first minutes, while with the pathogen the NO accumulation was stronger.

In contrast with these previous studies, focussed in a very early and short time span, we have monitored the NO accumulation at several time points during a longer period (100 h after the contact with *F. oxysporum* or *R. irregularis* and 50 h in the case of *T. harzianum*). This longer time frame allowed us to monitor NO production not only in the very initial contact when the plant perceives MAMPs from the fungi, but also when after recognition the plant try to control NO levels and modulate the defense responses. Therefore, we could get a better picture of the NO signature characteristic to each interaction.

Recently, Chen and collaborators (2019) also measured the NO production by the contact of *Trichoderma harzianum* and *F. oxysporum* in cucumber plants. Nevertheless, this NO measurement was not in the first stages of the interaction but at a later stage, 9 days after the fungal inoculation. They found a huge NO production in the case of *F. oxysporum* interaction, while the Trichoderma inoculated plants presented lower NO levels than the control plants.

Our findings in Chapters I and II corroborate that the initial burst of NO elicited by a root fungal pathogen is greater than that elicited by a beneficial fungus such as an AMF or Trichoderma, reinforcing the findings made in different plant models by Gupta *et al*. (2014) or Espinosa *et al*. (2014). Regarding later stages, we also found low NO concentration in the interaction with Trichoderma, as found by Chen *et al*. (2019).

Remarkably, not only the NO signatures are different just between a beneficial and a pathogenic fungus, but also differ between beneficial fungi with different lifestyles and colonizing strategies, as the obligate biotroph *R. irregularis,* and the free–living fungus *T. harzianum*. While the NO concentration spikes in the case of the AMF, for the Trichoderma interaction there is an initial NO pike and then the concentration remains low during all the time points examined.

In Chapters I and II we demonstrate that all the studied fungal interactions trigger an initial common NO burst, probably as a consequence of the recognition of common fungal associated molecular patterns such as chitin or chitosan. This peak was more intense in the case of the pathogen, supporting the existence of potential

mechanisms adapted by beneficial pathogens to avoid the plant defense response (Teixeira *et al*., 2019). After this common peak, the interaction with the AMF triggers a specific temporal pattern with several controlled NO spikes during the first days following the initial contact of the roots with the AMF mycelia. In contrast, Trichoderma did not elicit the later NO accumulation, being the NO burst then limited to the early steps of the interaction. We argued that these differences in NO accumulation patterns between the two beneficial fungi might be due to the different colonization strategies they follow. The AMF is actively accommodated in the root specialized host–membrane compartments, and this process is finely controlled by the plant (Pozo *et al*., 2015) and relies on a constant signalling dialogue that leads to the root colonization (MacLean *et al*., 2017). In contrast, Trichoderma bases its colonization strategy in the early repression of plant defenses (Brotman *et al*., 2013). Anyway, these observations suggest that the infection of the plant requires a low NO concentration and a decrease in plant–defense responses.

Interestingly, not only beneficial and pathogenic root fungal interactions trigger different temporal signatures of NO accumulation, but also have a different spatial pattern. We have checked that the NO accumulation in the AMF interaction (Chapter I), as well as the NO accumulation produced by Trichoderma VCs (Chapter IV) are restricted to the external root cell layers. Previously, it was also reported that direct contact with Trichoderma also produced the same NO accumulation spatial pattern (Gupta *et al*., 2014). Moreover, this restricted NO accumulation to the outermost root cell layers has also been found in bacterial beneficial interactions as showed by Nagata *et al*. (2008) with the interaction between *Lotus japonicus* and the Rhizobium *Mesorhizobium loti*. On the contrary, we found that pathogenic fungal interaction with *F. oxysporum* does not show that NO restriction pattern. *F. oxysporum* triggers a non–restricted NO accumulation along wider root sections, as also found by Gupta *et al*. (2014). This pattern is also shared by other fungal pathogens as *V. dahliae* (Espinosa *et al*., 2014). Indeed, the same pattern was found in bacterial pathogenic interactions as shown by Nagata *et al*. (2008) with the interaction of *L. japonicus* with the root pathogens *Ralstonia solanacearum* or

Pseudomonas syringae. These findings reinforce the idea that spatio–temporal NO accumulation patterns are dependent on the nature of the interaction (Chapter III).

Other authors as Calcagno and collaborators (2012) did not study NO accumulation during interactions with beneficial fungus *per se,* but with exudates from it. Thus, they compared the NO accumulation produced in the plant upon the perception of the exudates from the AMF *Gigaspora margarita*, and the extract of a general elicitor as bacterial lipopolysaccharides (LPS) in *Medicago truncatula* roots. They found that NO increased in roots 5 min after the treatment with exudates of germinating spores of the AMF. They also confirmed that the NO increase was specifically associated with the SYM pathway. Besides, they verify that the NO signature elicited by the AMF fungus differs considerably from that induced by the LPS extract. Likewise, we bear out those results in Chapter I, where we also reported that exudates from germinating spores of the AMF *R. irregularis* did elicit the NO accumulation in tomato roots, whilst the exudates from spores of the pathogen *F. oxysporum* did not. In contrast, we found a NO peak when roots were treated with a suspension of *F. oxysporum* cell walls, whereas a suspension of the AMF cell walls did not elicit this NO response. These results suggest that recognition of MAMPs were not integral to this response and therefore reinforce the idea of Calcagno and collaborators (2012) that NO signalling is a component of the early plant response to diffusible factors in the exudates from AM fungal germinating spores. Our results also corroborate the NO peak observed by other authors in different plants genera after the application of chitosan, a fungal elicitor of cell walls from *F. oxysporum* (Wang and Wu, 2004; Srivastava *et al*., 2009). As discussed in Chapter I, this might be a rapid and unspecific PAMPs–triggered burst of NO that activates the plant responses at the early stages of the interaction.

Although the scope of this Thesis is the NO production in the plant, it is important to note that also the microorganisms that interact with plants can produce NO. In Chapter III we highlight some excellent reviews regarding fungal NO. However, it is important to remark that a recent study carried out by Ding *et al*. (2020) found that also pathogens, as *F. graminearum,* can produce NO in response

to plant–derived signals. Thus, fungal sensing of a host plant can occur without physical contact between the pathogen and plant roots. These results point out the complexity of NO signalling during plant–fungal interactions. For example, while NO accumulation can be triggered in the pathogen before the contact with plants roots, according to our results the plant would not be able to detect the pathogen till the contact, as exudates from *F. oxysporum* did not trigger the accumulation of NO in plants roots, whereas fungal cell walls did (Chapter I).

NO peaks are concomitant with *PHYTOGB1* oscillations in different plant–microbe models

As some phytoglobins have been related to control NO levels in plants (Perazzolli *et al*., 2004; Hill, 2012) we explored how tomato *PHYTOGB* genes were regulated during beneficial and pathogenic fungal interactions (Chapters I and II). Our results support that only *PHYTOGB1* responds to NO, as *PHYTOGB1* is the only tomato *PHYTOGB* gene that is regulated by NO.

We further demonstrate that beneficial plant–fungal interactions do not only display a characteristic pattern of NO production, but also a specific transcriptional profile of *PHYTOGB1* that seems to be responsible for such NO accumulation patterns (Chapters I and II). We have shown that *R. irregularis* induced NO signature also triggered the concomitant oscillations in the expression pattern of the NO–inducible gene *PHYTOGB1*. Furthermore, we prove that this pattern differs from that found for interactions with *T. harzianum*, which only triggers an initial NO burst, and *PHYTOGB1* remains upregulated after this peak. In contrast, in the pathogenic interaction with *F. oxysporum*, the NO level increased over time at later stages, while *PHYTOGB1* was repressed, despite being *PHYTOGB1* a NO inducible gene. The results suggest that this response culd be driven by the pathogen to promote the infection, as high NO levels favour *F. oxysporum* invasion (Chapter III). Thus, we propose that effectors from the pathogen may target *PHYTOGB1* regulation to repress it, then blocking its action as NO scavenger to promote pathogenicity.

To further explore this possibility, we tested *PHYTOGB1* regulation in tomato during the interaction with other pathogens. It is noteworthy that we also found a down–regulation of *PHYTOGB1* in tomato plants infected with the root pathogen *Phytophthora parasitica* and the foliar pathogen *B. cinerea* at late stages of the interaction (Chapter I). These results are also consistent with previous studies on interactions with necrotrophic or hemibiotrophic fungi, where it was proposed that in late stages, NO might be used in the benefit of the pathogen to kill cells and expand the infection. This behaviour is described in the model proposed for necrotrophic fungi in Chapter III. Indeed, by manipulating NO levels through a genetic approach silencing or overexpressing *PHYTOGB1* in roots, we demonstrate the importance of NO role in plant susceptibility to *F. oxysporum*: lines displaying increased NO levels presented both higher *F. oxysporum* biomass and greater host cell death. On the contrary, in lines with decreased NO levels we found reduced susceptibility to the pathogen. Thus, our results confirm that greater NO concentration favours *F. oxysporum* infection and support the hypothesis of an actual suppression of *PHYTOBG1* by the pathogen.

Later on, we found that root perception of Trichoderma–VCs is also associated with an early burst of NO in Arabidopsis roots, with a concomitant *PHYTOGB1* gene upregulation (Chapter IV). Similarly, root perception of rhizobacterial VCs was previously shown to be associated with an early peak of NO in roots (Zamioudis *et al*., 2015), suggesting that this initial NO signalling is part of common early plant response to different microbial elicitors. In fact, characteristic patterns of NO regulation by *PHYTOGB1* are not exclusive of fungal interactions but also have been reported in bacterial interplays. Nagata *et al*. (2008) showed different NO accumulation and *PHYTOGB1* regulation patterns in *L. japonicus* after the inoculation by the symbiotic rhizobium *M. loti* and the bacterial pathogens *R. solanacearum* and *P. syringae*. In line with our results with pathogenic fungi (Chapter I), they showed that *PHYTOGB1* was not induced by root nor shoot pathogens, reinforcing the idea that repressing *PHYTOGB1* expression (or avoiding its induction) is a common strategy among pathogens (fungi and bacteria).

It is noteworthy that the research presented in Chapters I and II have led to subsequent similar investigations in other symbiotic models. This is the case of the study of Berger and collaborators (2020) on the Rhizobium–legume symbiosis established between *Medicago truncatula* roots and *Sinorhizobium meliloti*. In their study, the authors further support our results on the regulatory role played by *PHYTOGB1* and NO in symbiotic interactions. They found several NO accumulation peaks that were concomitant with *PHYTOGB1* expression during the symbiotic interaction. They argued that the first NO peak may be related to the defense mechanisms in response to the rhizobial interaction, thus supporting our idea that the initial NO burst is related to plant defense responses. In fact, they found that *PHYTOGB1* reduced NO to basal levels in the first days of the interaction, which, in turn, lowered the defense reactions, allowing the colonization by *S. meliloti*. Those results corroborate that the first steps of the interaction between plants and different beneficial microbes as AMF, Trichoderma or Rhizobium bacteria are controlled by the *PHYTOGB1*–NO couple. In line with our study, Berger *et al*. (2020) also developed *M. truncatula* lines with altered NO levels in roots by silencing or overexpressing *PHYTOGB1*. They found altered nodulation phenotypes, as both higher and lower NO concentrations inhibit the nodulation in *M. truncatula* roots. These results reinforce our hypothesis that precise fine–tuning of NO levels is required for the control of the symbiosis. In our case, a fine–tunning of NO is necessary for AM symbiosis establishment and extension, as we also found altered mycorrhization patterns in our *PHYTOGB1* root silenced and overexpressing lines, displaying increased mycorrhizal colonization in both cases. Nevertheless, the deregulation of NO levels did not alter the abundance of arbuscles, pointing out that in our case the role of *PHYTOGB1* is important in the regulation of the extension of colonization, but not in the arbuscles formation.

Notably, our results have been commented by Kumari *et al*. (2019) where the authors tried to discuss potential explanations for the mycorrhizal phenotype of our *PHYTOGB1* root overexpressor line. They propose that although the enzyme responsive for NO production during AM interaction has not been already elucidated, the reductive pathway enzymes nitrite reductase and plasma–

membrane nitrite–NO reductase may be involved. These enzymes work under low oxygen concentrations, like hypoxia, a situation that often plants roots experience. Then, hypoxia may trigger NO signalling and in consequence, enhance mycorrhization. Nevertheless, this explanation assumes that *PHYTOGB1* overexpression will not be enough to reduce the NO produced in hypoxia. However, Perazzolli and collaborators (2004) already probed that overexpressing *PHYTOGB1* in Arabidopsis plants was sufficient to reduce the NO burst under hypoxic conditions. Moreover, Kumary *et al*. (2019) also propose that the *PHYTOGB1* root silenced line present higher mycorrhization levels due to the higher NO concentration. Notwithstanding, we hypothesise that the observed phenotype is due to the deregulation of NO levels because a fine NO–tunning is mandatory for the symbiosis establishment. Therefore, more studies are needed to test these hypotheses.

NO is also involved in defense responses triggered by beneficial fungi

Taking into consideration the role of NO in the regulation of plant defenses that we show in Chapter I, we were then interested in exploring the participation of NO in the potential boosting of plant immunity during plant interaction with beneficial microbes. The direct interaction of plants with some beneficial soil fungi can lead to ISR (Pieterse *et al*., 2014; Martínez–Medina *et al*., 2017a). As discussed above, NO was rapidly accumulated in roots during the interaction of tomato and Arabidopsis plants with the ISR inducing fungus Trichoderma (Chapter II; Gupta *et al*., 2014), thus suggesting a role for NO in the establishment of the plant–Trichoderma symbiosis. Recently, it has been discovered that not only direct contact with this beneficial fungus triggers ISR. Indeed, Trichoderma VCs have been shown to lead to ISR against the shoot pathogen *B. cinerea* in Arabidopsis plants (Martínez–Medina *et al*., 2017b). For that reason, in Chapter IV we aim to decipher the potential role of NO in the ISR mediated by Trichoderma VCs. We discovered that NO not only has a role in the first stages of the interaction with beneficial microbes (Chapters I and II) but also it is crucial in some of the benefits that the interaction with these organisms entails, as the ISR response (Chapter IV).

We found that NO signalling is critical for the ISR triggered by Trichoderma VCs trough the activation of *MYB72* gene. Indeed, our results support the notion of MYB72 being a node of convergence in the ISR signalling pathways triggered by different beneficial microbes. Most previous data regarding MYB72 and ISR have been obtained using Arabidopsis as a model plant, while the study by Martínez–Medina *et al*. (2017b) was carried out using Arabidopsis and tomato plants. In this study, the authors found that Trichoderma VCs primed both Arabidopsis and tomato plants for enhanced resistance against *B. cinerea*. Also, they reported that Trichoderma VCs lead to a similar iron deficiency response in Arabidopsis and tomato roots, involving homologous genes. Thus, Martínez–Medina *et al*. (2017b) evidenced similar responses to Trichoderma VCs regarding iron acquisition mechanisms and host immunity in both Arabidopsis and tomato. Moreover, MYB72 has also been linked to iron uptake responses in tomato (Asins *et al*., 2020). Taking into consideration that iron uptake mechanisms seem to be a common trait in different plants families, it is plausible that the mechanisms underlying Trichoderma VCs–mediated ISR are common. Albeit more studies are needed to check the implication of *MYB72* and NO levels in Trichoderma VCs mediated ISR in other plants genera, it is tempting to speculate that our results in Arabidopsis (Chapter IV) can be extrapolated to other plant families including crop species as tomato.

MYB72 involvement in the iron deficiency responses is related to its implication in the biosynthesis and excretion of iron–mobilizing coumarins in the rhizosphere (Zamioudis *et al*., 2014; Stringlis *et al*., 2018). Remarkably, those coumarins have selective anti–microbial properties and have a role in assembling root microbiome to promote plant growth and health (Stringlis *et al*. 2018). These facts highlight the close bond between plant defense responses and responses to nutrient availability. It is well–known that plants have to deal with growth–defense tradeoffs when facing different stress conditions. Nobori and Tsuda (2019) emphasize in a recent review that the link between these two responses (defense and nutrition), might be an adaptive mechanism that makes possible for the plant to integrate several external cues. The results presented in Chapter IV reinforce the

idea of common signalling components shared by both nutrition and defense responses, and pinpoint NO as one of the key molecules that orchestrate this growth–defense cross–regulation.

Future challenges

The fact that NO can have a dual role both regulating beneficial and pathogenic interactions is an exciting discovery but also illustrate the extreme complexity of the regulation of plant immune responses. Despite being a common regulatory element in both type of interactions, the spatio–temporal NO signature differs considerably between the response to beneficial and pathogenic organisms. Although several studies have addressed the role of NO in pathogenic interactions, not so many have focussed on interactions with mutualistic fungi, and there are really few studies comparing both of them. We tried to partially fill this gap by performing comparative studies checking NO accumulation with different beneficial fungi, and also with a pathogen, at different time points and using diverse methodologies. However, to be able to draw more general conclusions, it is necessary to accomplish similar studies in other plant–fungal systems. This will allow identifying common patterns and major regulatory nodes. Such advance in our knowledge on the regulation of plant–fungal interactions could have promising biotechnological applications in agriculture. Identifying key regulation points that determine the pathogenicity of deleterious fungi and beneficial effects of microbial inoculants could give rise to improved crop management strategies by favouring beneficial interactions while restricting deleterious ones.

- We show that the root NO–*PHYTOGB1* signatures in response to the beneficial fungi differ considerably to that of the pathogen *F. oxysporum*. It is important to highlight that this NO signature corresponds to the compatible interaction of the plant with the pathogen. It would be interesting to analyse how NO is regulated during an incompatible interaction, using a tomato variety resistant to *F. oxysporum*. It also would be stimulating to check the NO response and resistance phenotype in the incompatible interactions using *PHYTOGB1* root

overexpressing and silenced lines. This will allow to determine the potential contribution of NO to ETI.

- Some studies have monitored NO accumulation in the combined interaction of a beneficial and a pathogenic fungus (Gupta *et al*., 2014). In this line, it would be challenging to investigate the NO signature of a plant inoculated with beneficial fungi and then challenged with the pathogen. This research will shed new light on the signalling roles of NO when the plant has to cope with simultaneous interactions with pathogenic and beneficial microorganisms, a situation that is closer to what plants face under field conditions.

- It also would be interesting to deepen in the defense regulation during AM interactions. We suggest that NO might be involved in the regulation of the degree of mycorrhizal colonization by regulating plant defenses (Chapter I). However, we did not perform a time–course analysis of defense mechanisms. This could shed light on the impact of NO on plant defenses during the interactions.

- Moreover, it would be challenging to perform the experiments carried out in Chapter IV with Arabidopsis NO mutants. This could provide an insight into the source of NO that is governing Trichoderma VCs–mediated ISR.

- It also would be ambitious to investigate which VCs are implicated in Trichoderma VCs–mediated ISR response. This might allow to develop new strategies in crop protection.

- It would be of great interest to explore the role of NO in Trichoderma VCs–mediated ISR using crop plants. This approach may contribute to identifying general patterns in NO signalling during ISR response, making easier to develop breeding programmes for more resistant plants.

- Also, a promising future challenge for breeding programmes could be developing plants with altered NO levels. As shown in Chapter I, plants overexpressing *PHYTOGB1* in roots not only had increased mycorrhization level but also were less susceptible to *F. oxysporum* attack. However, we explored the effects of altering *PHYTOGB1* levels only in the roots. The impact of altered levels in shoots needs to be explored by using stable transformation methods. Developing plants with transformed hairy roots is not a feasible approach for agriculture applications since it is a costly, high time and effort demanding process. In addition, more studies are needed to understand the impact of NO altered levels on the behaviour of the plant along its life cycle, and how it may impact yield and product quality, as they are the major goals in the market. Nevertheless, this could not be possible in the European Union due to the strict restrictions on the use of transgenic plants for human consumption. As an alternative, searching for cultivars/varieties with different phytoglobins levels could be a tool to identify plants with different NO accumulation patterns and potentially different resistance phenotypes.

CONCLUSIONS

1. NO accumulation and its regulation by *PHYTOGB1* in roots are early components of the regulatory pathways that are activated in plants during the onset of plant–fungal interactions. Although NO is a common signal component in mutualistic and pathogenic plant–fungal interactions, the NO-related signature and *PHYTOGB1* regulation are different.

2. Fine–tuned NO accumulation by *PHYTOGB1* is required for the proper establishment of symbiotic interactions and for the control of the extension of the fungal colonization as showed for the beneficial fungi *R. irregularis* and for *T. harzianum*. In a similar way, control of NO levels is required for a restriction of the pathogen (*F. oxysporum*) infection.

3. Analysis of different reports demonstrate the complexity of NO as a key signal in controlling plantCfungal interactions, where the spatiotemporal dynamics of NO accumulation seems to be key in their regulation:

   a. In biotrophic plant–fungal interactions NO triggers plant defenses at early stages. In incompatible interactions NO triggers HR and prevents the spread of the pathogen. In compatible interactions NO levels are most likely decrease by the pathogen to spread the invasion.
   b. In necrotrophic interactions NO activates plant defenses at early stages, while later NO is massively produced and exploited for the pathogen to expand the infection.

4. NO is an early signalling component in the plant response after plant perception of Trichoderma VCs upstream of *MYB72* that leads to the onset of ISR, rendering the plant more resistant against *B. cinerea* through priming of plant defense responses.

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