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Cristina Calcagno, Mara Novero, Andrea Genre, Paola Bonfante and Luisa Lanfranco

Abstract
Nitric oxide (NO) is a signaling molecule involved in plant responses to abiotic and biotic stresses. While there is evidence for NO accumulation during legume nodulation, almost no information exists for arbuscular mycorrhizas (AM). Here, we investigated the occurrence of NO in the early stages of Medicago truncatula–Gigaspora margarita interaction, focusing on the plant response to fungal diffusible molecules. NO was visualized in root organ cultures and seedlings by confocal microscopy using the specific probe 4,5-diaminofluorescein diacetate. Five-minute treatment with the fungal exudate was sufficient to induce significant NO accumulation. The specificity of this response to AM fungi was confirmed by the lack of response in the AM nonhost Arabidopsis thaliana and by analyzing mutants impaired in mycorrhizal capacities. NO buildup resulted to be partially dependent on DMI1, DMI2, and DMI3 functions within the so-called common symbiotic signaling pathway which is shared between AM and nodulation. Significantly, NO accumulation was not induced by the application of purified Nod factor, while lipopolysaccharides from Escherichia coli, known to elicit defense-related NO production in plants, induced a significantly different response pattern. A slight upregulation of a nitrate reductase (NR) gene and the reduction of NO accumulation when the enzyme is inhibited by tungstate suggest NR as a possible source of NO. Genetic and cellular evidence, therefore, suggests that NO accumulation is a novel component in the signaling pathway that leads to AM symbiosis.

Keywords: Arbuscular mycorrhiza, Fungal diffusible molecules, Gigaspora margarita, Medicago truncatula, Nitric oxide.

Introduction

Nitric oxide (NO) is a highly reactive signal molecule common to animal and plant systems. In plants, NO is involved in a variety of morphogenetic and physiological processes that include seed germination, root growth, stomatal closing, and responses to biotic and abiotic stresses (Neill et al. 2002; Wendehenne et al. 2004; Besson-Bard et al. 2008a, b and references therein). NO is well known to participate in plant–pathogen interactions where it is rapidly produced during incompatible interactions with biotrophic pathogens as well as compatible interactions with necrotrophic pathogens (Delledonne et al. 1998; Van Baarlen et al. 2004). NO production is also induced by basal elicitors of plant responses such as bacterial lipopolysaccharides (LPS) (Zeidler et al. 2004). Finally, evidence of NO involvement in plant symbioses is emerging; the use of NO-specific fluorescent probes has shown the occurrence of NO accumulation in the early (Shimoda et al. 2005; Nagata et al. 2008; del Giudice et al. 2011) and late stages of the nitrogen-fixing symbiosis (NFS) that legumes establish with rhizobia (Baudouin et al. 2006; Pii et al. 2007; Horchani et al. 2011). In addition, the application of a specific NO scavenger to M. truncatula roots inoculated with rhizobia resulted in a significantly reduced number of nodules (Pii et al. 2007; del Giudice et al. 2011).

Several cellular and molecular features of NFS are also found in arbuscular mycorrhizal (AM) symbiosis and the plant genetic programs that lead to NFS or AM share a number of components, referred to as the "common SYM" pathway (Parniske 2008; Oldroyd et al. 2009). Four genes within this pathway have been described in M. truncatula through the analysis of mutant phenotypes. DMI2 (for Doesn't Make Infection) is a leucine-rich repeat receptor-like kinase (Endre et al. 2002), which is thought to transduce the perception of both rhizobial and AM fungal signals leading to the activation of DMI1, a putative cation channel localized at the nuclear envelope (Ané et al. 2004). This cascade leads to prolonged oscillations of calcium concentration in the nucleus (Chabaud et al. 2011) and perinuclear cytoplasm (Kosuta et al. 2008). The calcium- and calmodulin-dependent protein kinase DMI3 (Lévy et al. 2004) acts downstream of the calcium spiking and is believed to decode the calcium signal, inducing the transcription of symbiosis-related genes (Oldroyd and Downie 2006), likely through the action of its substrate IPD3 (Interacting Protein of DMI3; Messinese et al. 2007). Despite these analogies between NFS and AM, no information is currently available on the involvement of NO in AM establishment or functioning.

Interestingly, it has been shown that nitrate reductase (NR) and nitrite reductase (NiR) transcripts accumulate in the presence of germinated spores and/or upon hyphopodium formation of an AM fungus on wild-type but not DMI3-mutated M. truncatula roots (Weidmann et al. 2004; Gianinazzi-Pearson et al. 2008). Notwithstanding the crucial role of these genes in nitrate assimilation, they have also been described as potential sources of NO (Yamasaki and Sakihama 2000). Their activation during this early stage of the interaction suggests their involvement in NO metabolism rather than nitrogen assimilation (Gianinazzi-Pearson et al. 2008) and this encouraged us to investigate NO release during the early stages of AM development.

Root colonization by AM fungi requires the accomplishment of a series of events: precontact signaling mediating fungus/plant recognition, development of hyphopodia on the root surface, penetration of the outer root tissues, and colonization of the inner cortex with differentiation of highly branched arbuscules inside single cortical cells (Bonfante and Genre 2010).

Many insights into the early events of the interaction have been gained following the description of both plant and fungal responses to diffusible signaling molecules released by the reciprocal partner (Bonfante and Requena 2011). Root-exudated strigolactones are known to modify hyphal growth and metabolism (Akiyama et al. 2005, Besserer et al. 2006). Evidence is also accumulating for the secretion of bioactive fungal molecules (Gianinazzi-Pearson et al. 2008). In the presence of a permeable membrane preventing direct contact between the symbionts, a diffusible fungal factor was reported to induce the expression of the early nodulin gene MtENOD11 (Kosuta et al. 2003), as well as other genes normally expressed during AM colonization (Weidmann et al. 2004). Recently, the upregulation of a membrane steroid-binding protein was also shown to be induced in epidermal/subepidermal cells in the vicinity of AM fungal hyphae (Kuhn et al. 2010). Diffusible AM fungal signals were also shown to activate early events, such as changes in cytosolic (Navazio et al. 2007; Kosuta et al. 2008) and nuclear Ca2+ concentration (Chabaud et al. 2011), and delayed processes, such as root branching (Oláh et al. 2005) and starch accumulation in the root cortex (Gutjahr et al. 2009). Recently, fungal exudates from Glomus intraradices were shown to contain a mixture of sulfated and nonsulfated lipochitooligosaccharides (LCO), which are responsible for the activation of MtENOD11 gene expression and the induction of lateral roots formation (Maillet et al. 2011).

The accumulation of NO in plants can be rapid (Delledonne 2005) and preliminary experiments where NO could not be visualized in AM fungus-contacted roots suggested the necessity of investigating NO production in the first minutes that follow fungus perception (Calcagno et al., unpublished data). To this aim, we have used the specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) and a simplified experimental system where the exudate of an AM fungus was applied to root explants immediately before confocal imaging. This experimental setup provides a straightforward method to investigate rapid responses to diffusible fungal signals and was successfully used by Chabaud et al. (2011) to study nuclear calcium spiking in root tissues.

Our results provide evidence of NO accumulation in the roots of wild-type M. truncatula during the first minutes that follow fungal exudate treatment. The analysis of mutant lines defective in the AM colonization process allows the positioning of NO accumulation downstream of DMI1 and DMI2, but upstream of DMI3 activity. On this basis, we conclude that plant-produced NO is involved in the signaling pathway that mediates the early stages of AM interaction.

Materials and methods

Fungal material and elicitor

Spores of Gigaspora margarita Becker and Hall (BEG 34) were collected from Trifolium repens L. pot cultures. Aliquots of 100 spores were surface-sterilized twice for 10 min with 3% chloramine-T and 0.03% streptomycin sulfate, then rinsed several times with sterile distilled water. To induce germination, each aliquot was placed in 1 ml of sterile distilled water and incubated in the dark at 30°C for at least 5 days. The medium was then collected and the fungal exudate concentrated to one tenth of the initial volume by freeze-drying. We refer to the resulting solution as the fungal exudate.

Homogenates of G. margarita spore cell wall were obtained by disrupting 100 surface-sterilized spores using Mixer Mill (Retsch) with Retsch ball mill in 2 ml Eppendorf tube frozen in liquid nitrogen. Four hundred microliters of sterile water was added to this homogenate and then centrifuged at 20,000×g for 5 min. The pellet was resuspended in 50 µl of sterile water for roots treatment.
Nod factor was kindly provided by Allan Downie (JIC, Norwich, UK) and used at a final concentration of 10−8 M. The stock solution of LPS extract from Escherichia coli 0127:B8 (Sigma) was prepared by dissolving 1 mg/ml of LPS in buffer A (2.5 mM MgCl2, 1 mM CaCl2) and shaken for 3 h on a rotatory shaker. The working solution was obtained by diluting the stock solution in water to a final concentration of 100 µg/ml LPS.

Plant material

Seeds of Medicago truncatula Gaertn. cv. Jemalong were scarified using sandpaper P180-200, sterilized with 5% commercial bleach for 3 min, and rinsed three times for 10 min with sterile distilled water. Germination was induced under sterile conditions in 0.6% agar/water. Seedlings were transferred to minimal medium (Bécard and Fortin 1988) without sucrose and grown for 10 days in a growth chamber (16 h daylight at 21°C/8 h night at 18°C).
Agrobacterium rhizogenes transformed roots from M. truncatula wild-type and the three mutants, dmi1-1 (Ané et al. 2004), dmi2-2 (Endre et al. 2002), dmi3-1 (Lévy et al. 2004; Mitra et al. 2004) of M. truncatula Gaertn. cv. Jemalong (kindly provided by M. Chabaud and D. Barker, LIPM, Toulouse, France) were grown in sterile conditions in minimal medium at 25°C in the dark (Bécard and Fortin 1988).
Arabidopsis thaliana seeds were sterilized with 1 min wash in 70% ethanol followed by 20 min wash in 10% commercial bleach and rinsed three times for 10 min with sterile distilled water. Sterilized seeds were then placed in Petri plates filled with Murashige and Skoog growth medium (Murashige and Skoog 1962) and maintained in the dark for 24 h at +4°C and for 5 days at 25°C.

GUS assay

Experiments were performed on young entire roots of M. truncatula seedlings expressing the MtENOD11 promoter–GUS construct (kindly provided by M. Chabaud and D. Barker). Portions of lateral roots were treated in Petri dishes with the fungal exudate, sterile filtered water or 10−8 M aqueous solution of Sinorhizobium meliloti Nod factor. The treated root segments were covered with small squares (about 1×2 cm) of Biofolie 25 film (Sartorius) in order to limit liquid dispersion and evaporation during the following 6 h of incubation at room temperature.
Root segments were cut and then placed in single wells of a Multiwell plate and covered with freshly prepared GUS buffer (50 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Tween 20, 1.05 g/50 ml K4(FeII(CN6)), 0.83 g/50 ml K3(FeIII(CN)), 0.5 mg/ml x-Glc). To improve buffer penetration into the root segments, these were then placed under vacuum for 10 min, and the vacuum treatment was repeated three times. Finally, samples were incubated at 37°C for 24 h in the dark, destained with 70% ethanol, and observed with a light microscope (Nikon Eclipse E300).

Detection of nitric oxide

Excised segments of young root cultures (7- to 10-day-old subcultures) or of entire roots of M. truncatula or A. thaliana seedlings were incubated for 30 min in 2 ml of detection buffer (10 mM Tris–Hcl, pH 7.4, 10 mM KCl) containing 15 µM DAF-2DA (Sigma-Aldrich) with or without 1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) as an NO scavenger. Samples were then washed three times for 10 min with the detection buffer. Roots were placed on a microscope slide and fixed with a Secure-Seal™ 20-mm diameter, 0.8-mm deep hybridization chamber gasket (Invitrogen), immediately filled with detection buffer through the loading holes. The chambers were then examined in a Leica TCS-SP2 confocal microscope. The formation of DAF-2T following NO reaction with DAF-2DA was visualized using a ×40 long-distance water immersion objective. The 488-nm band of an Argon laser was used to excite the samples and fluorescence was detected using a 505- to 530-nm emission window. Images were processed and analyzed using the Leica Confocal Software application.

The detection buffer was then removed with a micropipette and immediately replaced by either the fungal exudate, the fungal exudate containing 1 mM cPTIO (Sigma), 10−8 M Nod factor, 100 µg/ml LPS extract (Sigma), or spore cell wall homogenate or sterile filtered water. To analyze the effect of NR inhibition, root fragments were incubated in 1 mM sodium tungstate dihydrate (Sigma) for 30 min and then incubated in DAF-2DA and treated with the fungal exudate as indicated above.

For each observation, ten optical sections were acquired over a distance of 100 µm along the z-axis in order to obtain a global image of about one third of the root thickness, including the epidermis and two layers of cortical cells. Images were acquired at 0, 3, 5, 10, and 15 min after the beginning of treatment. The Leica Confocal Software was then used to calculate the average fluorescence within 300 by 300 µm areas of the images. To normalize the data, the ratios of average fluorescence intensities at t3, t5, t10, and t15 to the average intensity at t0 were then calculated and used to analyze the changes in fluorescence over time.

At least five roots pieces were tested for each condition and three independent repeats were analyzed. Data were subjected to statistical analysis using the software Systat 10. The Kruskal–Wallis test for nonparametric data was used.

RNA extraction and real-time RT-PCR assays

M. truncatula root fragments were treated with fungal exudate or sterile water for 10 min or 6 h in square Petri dishes covered with Biofolie 25 film (Sartorius) as described above. Total RNA was isolated from about 20 mg root fragments using RNeasy Plant Mini Kit (Qiagen). Samples were also treated with TURBO™ DNase (Ambion) according to the manufacturer's instructions. The RNA samples were routinely checked for DNA contamination by reverse transcription polymerase chain reaction (RT-PCR) analysis, using primers MtTef-f 5 ′ -AAGCTAGGAGGTATTGACAAG-3 ′ and MtTef-r 5 ′ -ACTGTGCAGTAGTACTTGGTG-3′ for MtTefa (Vieweg et al. 2005) and the One-Step RT-PCR Kit (Qiagen).

For single-strand cDNA synthesis, about 100 ng of total RNA was denatured at 65°C for 5 min and then reverse-transcribed at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min in a final volume of 20 µl containing 10 µM random primers, 0.5 mM dNTPs, 4 µl 5X buffer, 2 µl 0.1 M DTT, and 1 µl Super-Script II (Invitrogen).

Real-time experiments were carried out in a final volume of 20 µl containing 10 µl of 23 Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µl of 3 µM primers, and 1 µl of cDNA. The primer pairs used were MtTef-f and MtTef-r 5′ for MtTefa (Vieweg et al. 2005); NRf 5′-CTTTAAACCCGCGTGAGAAA-3′ and NRr 5′-TCATCTACGCTGCTTGTTGG-3′ for NR (accession number AJ621867; Weidmann et al. 2004); and NiRf 5′-TATGTTGGAATTCACGTTCCAG-3′ and NiRr 5′-TGCCACAAGTGTTTTCATTAGG-3′ for NiR (accession number AJ621861; Weidmann et al. 2004). Real-time RT-PCR was performed on the StepOne Real-Time PCR System (Applied Biosystems) using the following program: 10 min preincubation at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each amplification was followed by melting curve analysis (60°C to 94°C) with a heating rate of 0.3°C every 15 s. All reactions were performed with three technical replicates and only Ct values with a standard deviation that did not exceed 0.3 were considered. Target gene expression was normalized with the MtTefa gene. The $2^{-\Delta\Delta Ct}$ method (Kenneth and Schmittgen 2001) was used to calculate the relative expression levels. The analyses were performed on three independent biological replicates. Statistical tests were carried out through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $p<0.05$.

Results

G. margarita exudate induces NO accumulation in the AM host plant M. truncatula

The biological activity of the fungal exudate obtained from germinating G. margarita spores has been recently demonstrated by Chabaud et al. (2011) with the induction of calcium spiking in host roots. To further show that the fungal exudate induces AM-related responses in our plant material, we first checked the expression of MtENOD11 by GUS histochemical reaction in seedlings of MtENOD11 pro-GUS transgenic lines. A 6-h incubation with G. margarita exudate induced GUS activity in patches of cortical and, occasionally, epidermal cells in the young differentiated zone of lateral roots (Supplementary Fig. S1), partially similar to the pattern described by Mukherjee and Ané (2011) in roots treated with G. intraradices exudates. The GUS staining of vascular tissues in the same area was also present in control roots treated with sterile water, as expected from previous data on nonsymbiotic roots (Journet et al. 2001). This was largely in agreement with the observations of Kosuta et al. (2003) showing ENOD11 activation in young lateral roots by AM fungal diffusible signals.

Our results confirm the presence of bioactive molecules in the exudate of G. margarita germinated spores and prove that root treatment with the fungal exudate can mimic—to some extent—the approach of AM hyphae during the presymbiotic phase of the interaction. The fungal exudate could, therefore, be confidently used to test whether diffusible fungal signals elicit NO accumulation in the host plant tissues.

The accumulation of NO in root tissues was analyzed in Agrobacterium rhizogenes transformed root organ cultures (ROC) and seedlings of M. truncatula using the cell-permeable NO-specific probe DAF-2DA which is converted into its fluorescent triazole derivate DAF-2T upon reaction with NO (Nakatsubo et al. 1998). The formation of DAF-2T was visualized by confocal microscopy. DAF-2T staining was limited to meristems and lateral root emergences in untreated roots (Supplementary Fig. S2a, b), as previously reported in tomato by Correa-Aragunde et al. (2004, 2006). The reliability of the detection system was confirmed by the strong signal reduction in the presence of the NO scavenger cPTIO (Supplementary Fig. S2c, d).

When roots were treated with the fungal exudate, a visible increase in DAF-2T fluorescence was detected in the epidermal and cortical tissues of both ROC (Fig. 1a) and seedling roots (Fig. 1b). The average fluorescence values recorded in ROC 5 min after the different treatments are reported in Fig. 2 as ratios of the respective fluorescence intensity at time 0. Notwithstanding a certain degree of variability, exudate treatment induced a marked and statistically significant increase in fluorescence intensity in ROC of M. truncatula, with an average ratio of 1.41. By contrast, when 1 mM cPTIO was added to the fungal exudate, no significant increase in fluorescence was induced, confirming the reliability of the experimental setup. The same lack of response was recorded when the fungal exudate was replaced by sterile water where a slight decrease in signal intensity was recorded, possibly due to DAF-2T fluorescence bleaching. The application of a suspension of homogenized spore walls instead of the fungal exudate did not induce any significant increase in fluorescence either (Fig. 2).

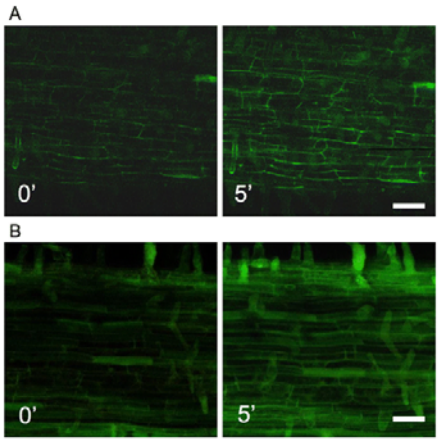

Fig. 1

Confocal imaging of DAF-2DA fluorescence in wild-type *M. truncatula* ROC segments (**a**) or seedling roots (**b**) at 0 and 5 min after treatment with the fungal exudate. Bar = 50 μm (**a**), 100 μm (**b**)

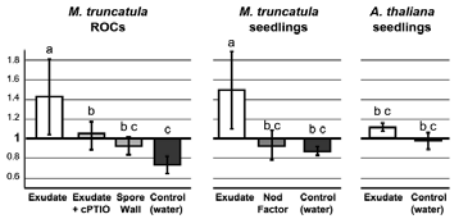

Fig. 2

DAF-2T fluorescence intensity values at 5 min after treatment of root segments from *M. truncatula* (ROC or seedlings) or from *A. thaliana* (seedlings) with the fungal exudate, fungal exudate together with the NO scavenger cPTIO, homogenate of spore walls, 10^{-8} M purified Nod factor, and sterile water. Average fluorescence values are reported as a ratio of the fluorescence intensity at 5 min to the fluorescence intensity at time 0. Different letters indicate statistically significant differences between samples ($p < 0.05$, Kruskal–Wallis test)

Since the signaling pathways leading to AM symbiosis and nodulation share common components (Parniske 2008), the effect of Nod factor application was also investigated. It is known, anyway, that root explants are not responsive to rhizobia (Akashi et al. 2003) and Nod factors (Chabaud et al. 2011); for this reason, only roots from M. truncatula seedlings were used in these experiments. No increase in fluorescence was observed in the root tissues in response to Nod factor application, whereas the fungal exudate induced a 1.51-fold increase in fluorescence, which was statistically comparable to the response observed in ROC.

Subsequently, fungal exudates were applied to root segments of A. thaliana, which is among the few plant species recalcitrant to AM colonization. No significant increase in fluorescence was detected in this plant (Fig. 2).

In brief, the fungal exudate induced a significant production of NO in the root tissues of the host plant. All our controls indicate this production to be related to a molecular dialogue occurring in the AM symbiosis: it cannot be elicited by Nod factor in M. truncatula nor by the fungal exudate in the AM nonhost plant Arabidopsis.

G. margarita exudate-induced NO accumulation is partially dependent on the SYM pathway

In order to examine the kinetics of the response, a further experiment was conducted where fluorescence intensity was recorded at different time points over 15 min of exposure to the fungal exudate. To understand where NO production positions along the common SYM pathway, three well-characterized nonsymbiotic mutants of M. truncatula (dmi1-1, dmi2-2, and dmi3-1), which are defective in both AM colonization and nodulation (Ané et al. 2004; Endre et al. 2002; Mitra et al. 2004), were tested. DAF-2T fluorescence progressively decreased over the observation time in all the samples treated with sterile water, as expected, due to the probe bleaching by prolonged laser exposure.

By contrast, fluorescence markedly increased in M. truncatula wild-type roots during the first 5 min of fungal exudate treatment. A further but slower increase in fluorescence was recorded for the remaining observation time (Fig. 3). Such an increase was not observed in dmi1-1 and dmi2-2 mutants where the average fluorescence remained basically unchanged. This suggests that the fungal exudate induced in these genotypes a limited NO production which was sufficient to compensate for DAF-2T bleaching, but markedly lower than the response observed in the wild type. By contrast, a fluorescence increase was recorded for dmi3-1. Its amplitude was significantly different from both the wild type and the first two mutants during the first 10 min. At 15 min, the statistical significance of this difference was lost due to the increased variance caused by DAF-2T bleaching by the laser (Supplementary Table S1). Thus, NO production resulted to be partly dependent on the activity of DMI1 and DMI2 and, to a smaller extent, DMI3.

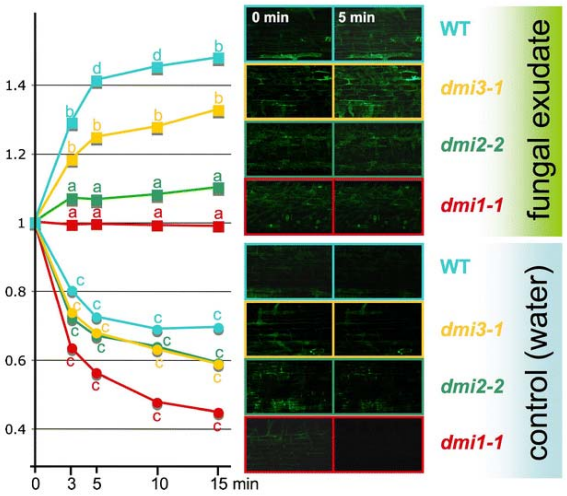

Fig. 3

Time course analysis of DAF-2T fluorescence intensity in wild-type (*cyan*), *dmi1-1* (*red*), *dmi2-2* (*green*), and *dmi3*-1 (*yellow*) *M. truncatula* ROC at 0, 3, 5, 10, and 15 min after treatment with the fungal exudate (*squares*) or with sterile water (*circles*) as control. Values indicate the ratio of fluorescence intensity at each time point to the respective intensity at time 0. Different letters indicate statistically significant differences ($p < 0.05$, Kruskal–Wallis test). Images of fluorescence at time 0 and after 5 min are shown on the *right*

As a further control, we also tested bacterial LPS preparations, which are general elicitor of plant defense responses known to induce a rapid NO burst in plant systems (Zeidler et al. 2004). LPS-induced NO accumulation could indeed be visualized in M. truncatula roots (Fig. 4). Nevertheless, the pattern of NO production under these conditions was markedly different from what was observed upon fungal exudate exposure. Fluorescence intensity in wild-type M. truncatula ROC peaked 15 min after

LPS treatment (with a ratio of 0.84), compared to the stronger and more rapid increase triggered by the fungal exudate (5 min; Figs. 2 and 3). Notably, this pattern of NO accumulation was also observed in the dmi1-1 genotype (ratio=0.89), indicating that LPS-induced NO production is independent of the SYM pathway.

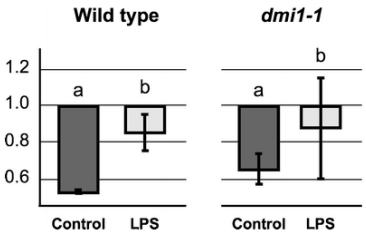

Fig. 4

DAF-2T fluorescence intensity values at 15 min after treatment of *M. truncatula* root segments (wild-type or *dmi1-1* genotype) with an LPS extract from *E. coli*. Average fluorescence values are reported as a ratio of the fluorescence intensity at 15 min to the fluorescence intensity at time 0. Different letters indicate statistically significant differences between samples ($p < 0.05$, Kruskal–Wallis test)

Nitrate reductase as a possible source of NO production

NR is a central enzyme in nitrogen assimilation that can also catalyze the reduction of nitrite, in high concentration, to NO (Yamasaki and Sakihama 2000). NiR is likewise suspected to participate in NO production (Gianinazzi-Pearson et al. 2008). Since NR and NiR expression is reported to be upregulated in response to hyphopodium formation (Weidmann et al. 2004; Gianinazzi-Pearson et al. 2008), we monitored the abundance of NR and NiR mRNA in wild-type M. truncatula roots during 10 min and 6 h exposure to the fungal exudate. Target gene expression was calibrated with the MtTefa housekeeping gene and the change in expression is given relative to the untreated samples. A slight but significant increase in NR and NiR transcript levels appeared after 10 min of treatment (Fig. 5a). Six hours exposure led to a weak decrease in NiR mRNA levels, while NR downregulation was not statistically significant. Interestingly, no gene regulation was observed in the roots of the dmi1-1 mutant at either time point (Fig. 5b).

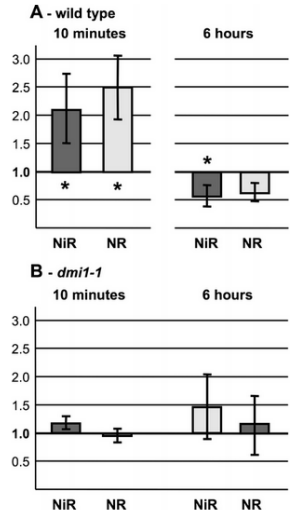

Fig. 5

NiR and NR relative expression in *M. truncatula* wild-type (a) or *dmi 1-1* (b) roots exposed to the fungal exudate for 10 min or 6 h. Gene expression was normalized to the *MtTefa* gene and reported as the amount of induction relative to control roots treated with sterile water. *Asterisks* indicate statistically significant differences in gene expression levels between roots treated with fungal exudate and roots treated with sterile water ($p < 0.05$, one-way ANOVA and Tukey's post hoc test)

To further test whether NR is involved in exudate-induced NO production, DAF-2T fluorescence was measured in roots where NR activity was inhibited by treatment with tungstate (Harper and Nicholas 1978; del Giudice et al. 2011) prior to fungal exudate application. The plots presented in Fig. 6 show that, in the presence of tungstate, the level of fluorescence remains approximately constant during the experiment, significantly different from the increase observed in the absence of tungstate. These results suggest that part of the NO accumulation in response to fungal exudates depends on the activity of NR.

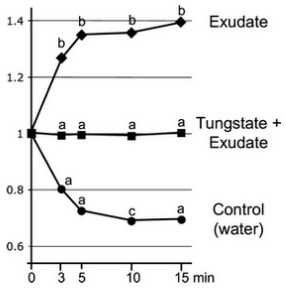

Fig. 6

Time course analysis of DAF-2T fluorescence intensity in wild-type *M. truncatula* ROC treated with sterile water (*circles*, control) or the fungal exudate with (*squares*) and without a preincubation in 1 mM tungstate (*diamonds*). Values indicate the ratio of fluorescence intensity at each time point to the respective intensity at time 0. Different letters indicate statistically significant differences ($p < 0.05$, Kruskal–Wallis test)

Discussion

Most studies on early events in the AM interaction have used simplified experimental systems where the plant was physically separated from the fungus (Weidmann et al. 2004; Kosuta et al. 2008; Gutjahr et al. 2009) or plant or fungal exudate was applied to the reciprocal symbiotic partner as a means of simulating the sudden appearance of diffusible signaling compounds (Buée et al. 2000; Navazio et al. 2007; Besserer et al. 2006; Tamasloukht et al. 2003; Chabaud et al. 2011; Mukherjee and Ané 2011; Maillet et al. 2011). In the present work, we followed the latter approach: a fungal exudate was obtained following the protocol that allowed Navazio et al. (2007) to detect a transient rise of cytosolic Ca2+ in cultured soybean cells and Chabaud et al. (2011) to highlight a nuclear Ca2+spiking in M. truncatula ROC.

NO generation is part of a general signaling and response system in different types of plant–pathogen interaction. NO buildup is relatively rapid, often taking place within minutes (Foissner et al. 2000; Lamotte et al. 2004; Zeidler et al. 2004). In these contexts, NO production is generally associated with plant cell death, hypersensitive response (Delledonne et al. 1998; van Baarlen et al. 2004), and the induction of defense genes (Durner et al. 1998; Zeidler et al. 2004).

The role of NO is much more obscure in symbiotic systems. On one hand, the transient NO generation observed in Lotus japonicus roots at 4 h after Mesorhizobium loti inoculation paralleled the enhanced expression of a nonsymbiotic hemoglobin gene LjHb1, possibly involved in NO detoxification (Shimoda et al. 2005; Nagata et al. 2008). These authors suggested the quick generation of NO to be the signal triggering NO-mediated defense responses in the host plant. LjHb1 would reduce the NO levels, thus limiting the defense responses and allowing the root colonization by rhizobia (Nagata et al. 2009). On the other hand, the application of an NO scavenger

to M. truncatula roots was shown to reduce the number of nodules (Pii et al. 2007; del Giudice et al. 2011) and to induce the downregulation of plant genes involved in nodule development (del Giudice et al. 2011). Remarkably, NO has also been detected in differentiated nodules (Baudouin et al. 2006; Pii et al. 2007; Horchani et al. 2011).

A 5-min treatment of seedling roots or ROC with the exudate of G. margarita was sufficient to induce significant NO accumulation in M. truncatula. On the contrary, parallel treatments with Nod factor never induced NO production in seedlings. These results, together with the comparable lack of response in the nonhost Arabidopsis, provide a first indication that this pattern of NO accumulation is specifically related to AM interactions. Moreover, NO production after the exposure of M. truncatula roots to a general elicitor (LPS extract) was markedly different from what was observed upon AM fungal exudate treatment and, more importantly, not dependent on the DMI1 function. Remarkably, our previous investigations did not show NO accumulation in the presence of fungal contact (Calcagno et al., unpublished data), suggesting that this response is mainly elicited during the presymbiotic phase, when fungal diffusible signals are perceived by the host root.

Our data from the mutants dmi1-1, dmi2-2, and dmi3-1 of three common SYM genes are of particular interest. The loss of DMI1 or DMI2 activity is sufficient to drastically decrease NO production in response to G. margarita hyphal exudates, while a significantly stronger accumulation of NO still occurred in the dmi3-1 mutant. Since the response in dmi3-1 is anyway weaker than in wild-type roots, it can be speculated that DMI3 activity is necessary to reinforce the AM-induced NO accumulation.

Our analyses, therefore, indicate that the NO response is partially dependent on the SYM pathway, with a major control by DMI1 and DMI2. Interestingly, the same DMI1/DMI2 dependency (although more clear-cut) is also observed for Ca2+ spiking responses induced by G. margarita exudate (Chabaud et al. 2011). This overlap between Ca2+ spiking and NO accumulation within the SYM pathway is intriguing. Evidence for an interplay between NO and Ca2+ is in fact emerging in both animals and plants (Lamotte et al. 2004; Besson-Bard et al. 2008a, b), stimulating the further investigation of a possible crosstalk between NO and Ca2+ in AM symbiosis.

NO accumulation is specifically induced by AM fungal but not rhizobial Nod factor. This suggests that early NO production is an AM-specific component of the plant response within the "common SYM pathway." Notably, Shimoda et al. (2005) described NO production in L. japonicus roots 4 h after rhizobium inoculation; however, they did not test the effect of purified Nod factor and rather proposed rhizobial LPS as the candidate inducers of NO production (Nagata et al. 2009). It will be interesting to evaluate whether the LCO recently identified as part of the fungal exudates of G. intraradices (Maillet et al. 2011) are responsible for the induction of NO production. This seems to be unlikely since these molecules are very similar to Nod factor which, in our case, did not induce NO accumulation.

While the production of NO is evident, its role in the early response of the host plants to AM fungal signals remains unclear. One possibility is that NO is active in the signaling pathway that stimulates lateral root formation (Oláh et al. 2005; Mukherjee and Ané 2011); both responses are in fact DMI1-/DMI2-dependent and DMI3-independent. This intriguing possibility, which requires further investigation, is also supported by the well-known presence of NO in root meristems and at sites of lateral root emergence (Correa-Aragunde et al. 2004, 2006).

Another clue to the possible role of NO in AM comes from the recent observation that variations in NO levels influence cellulose synthesis in roots (Correa-Aragunde et al. 2008); upregulation of a putative cellulose synthase has been observed at very early

stages of the AM interaction (Siciliano et al. 2007) and related to cell wall remodeling during the process of fungal accommodation within the epidermal cell.

A second open question concerns the routes of NO production. NO biosynthetic pathways in plants are only partially characterized and have been classified as oxidative or reductive. The routes via NR and mitochondrial and plasma membrane-associated NO production are all reductive reactions, while NO production from l-arginine or polyamine are oxidative routes (Gupta et al. 2011 and reference therein). The source of NO in plant responses to biotic stresses also remains largely unclear (Leitner et al. 2009). In a few plant–pathogen interactions, NO production has been related to a l-arginine-dependent pathway, which should involve a nitric oxide synthase (NOS), in analogy to what has been described in mammals (Foissner et al. 2000), while in others, NO production resulted to be NR-dependent (Yamamoto et al. 2003, 2004; Yamamoto-Katou et al. 2006; Horchani et al. 2011). In our experiments, the slight upregulation of M. truncatula NR and NiR genes upon fungal exudate treatment was consistent with previous studies showing an increase of their transcript levels in the presence of germinated spores and/or during hyphopodium formation (Weidmann et al. 2004; Gianinazzi-Pearson et al. 2008). Interestingly, we did not observe NR induction in the dmi1-1 mutant. This result confirms DMI1 control over NO production and, more importantly, suggests that NO production is mediated by NR. Remarkably, the experiments with tungstate also point at NR as a possible source of NO.

However, further investigation is surely needed to fully address this issue, in particular the evaluation of the involvement of other enzymatic systems such as the plasma membrane-bound nitrite/NO reductase (Stöhr and Stremlau 2006; Moche et al. 2010) or a NOS-like enzyme whose molecular identities are unfortunately still to be elucidated.

In conclusion, our cellular and genetic data provide the first evidence for the occurrence of NO in the plant's early response to AM fungal signals and suggest NO accumulation as a novel component of the AM signaling pathway.

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