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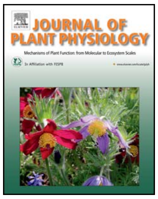

Effect of short-term aluminum stress and mycorrhizal inoculation on nitric oxide metabolism in *Medicago truncatula* roots

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ABSTRACT

Aluminum (Al) toxicity can induce oxidative and nitrosative stress, which limits growth and yield of crop plants. Nevertheless, plant tolerance to stress may be improved by symbiotic associations including arbuscular mycorrhiza (AM). Nitric oxide (NO) is a signaling molecule involved in physiological processes and plant responses to abiotic and biotic stresses. However, almost no information about the NO metabolism has been gathered about AM. In the present work, *Medicago truncatula* seedlings were inoculated with *Rhizophagus irregularis*, and 7-week-old plants were treated with 50 μM $AlCl_3$ for 3 h. Cytochemical and molecular techniques were used to measure the components of the NO metabolism, including NO content and localization, expression of genes encoding NO-synthesis (*MtNR1, MtNR2* and *MtNIR1*) and NO-scavenging (*MtGSNOR1, MtGSNOR2, MtHB1* and *MtHB2*) enzymes and the profile of protein tyrosine nitration (NO_2-Tyr) in *Medicago* roots.

For the first time, NO and NO_2-Tyr accumulation was connected with fungal structures (arbuscules, vesicles and intercellular hyphae). Expression analysis of genes encoding NO-synthesis enzymes indicated that AM symbiosis results in lower production of NO in Al-treated roots in comparison to non-mycorrhizal roots. Elevated levels of transcription of genes encoding NO-scavenging enzymes indicated more active NO scavenging in AMF-inoculated Al-treated roots compared to non-inoculated roots. These results were confirmed by less NO accumulation and lower protein nitration in Al-stressed mycorrhizal roots in comparison to non-mycorrhizal roots.

This study provides a new insight in NO metabolism in response to arbuscular mycorrhiza under normal and metal stress conditions. Our results suggest that mycorrhizal fungi decrease NO and tyrosine nitrated proteins content in Al-treated *Medicago* roots, probably via active NO scavenging system.

1. Introduction

Aluminum (Al) is one of the most toxic metals that negatively affects root growth (Kochian et al., 2005). Nevertheless, plants have developed different mechanisms of Al tolerance. One of them is based on the exclusion of Al from the root symplasm by binding Al in the cell wall (Kochian et al., 2004; Horst et al., 2010). The apoplast of peripheral root cells is the main barrier for toxic ions and, at the same time, the main place of Al toxicity, where it affects cell wall modification, cytoskeleton dynamics, plasma membrane and plasmodesmata properties (Kochian, 1995; Barlow and Baluška, 2000; Silva et al., 2000; Horst et al., 2010; Yang et al., 2010; Kochian et al., 2015). Al toxicity can induce oxidative and nitrosative stress in plant cells (Yamamoto et al., 2002). On the other hand, heavy metal uptake can be modulated by soil microorganisms, including plant growth-promoting bacteria (Burd et al., 2000), rhizobia (Pajuelo et al., 2011) and arbuscular mycorrhizal (AM) fungi (Cumming and Ning, 2003).

Arbuscular mycorrhiza (AM) is the symbiosis between fungi from *Glomeromycota* division and the majority of plant species (Wang and Qiu, 2006). Through extraradical hyphae, AM fungi absorb inorganic materials and water from the rhizosphere, which is transferred into the host root. In return, the fungi acquire photosynthate from the plant to support their growth and reproduction (Harrison, 2005). Colonization of roots by AM fungi occurs in several stages: 1. pre-symbiotic phase (signal transduction between symbiotic partners); 2. early symbiotic phase (hyphopodia development on the root surface); 3. mature symbiotic phase (arbuscules development in a root cortex cells) (Bonfante and Genre, 2010). Arbuscules are specific structures that are separated from the host root cells cytoplasm by the periarbuscular membrane (PM). Through the changes in PM integrity, plants can regulate fungi

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colonization (Bonfante and Perotto, 1995; Bonfante and Genre, 2010). Importantly, AM symbiosis is essential for the plants survival under unfavorable conditions such as drought and heat stress, soil salinity or heavy metal contamination (Borie and Rubio, 1999; Cumming and Ning, 2003; Smith and Read, 2008; Bano and Ashfag, 2013; Liu et al., 2015). AM fungi may also play a role in conferring plant resistance to Al (Rufyikiri et al., 2000; Lux and Cumming, 2001; Cumming and Ning, 2003). Protective role of AM can be performed directly, through Al immobilization in fungal structures or compounds produced by AM fungi, or indirectly, by plant better nutrient acquisition (Lux and Cumming, 2001; Aguilera et al., 2011; Liu et al., 2015).

Nitric oxide (NO) is a highly reactive signal molecule common in animal and plant kingdoms. In plants, it regulates development and responses to biotic and abiotic stresses (Corpas and Barroso, 2013; Scheler et al., 2013; Corpas and Barroso, 2015). However, the knowledge concerning a role of NO in mycorrhiza in metal toxicity is quite limited (Sahay and Gupta, 2017 and references therein). There are some studies concentrating on a role of NO in Al toxicity and Al-tolerant reactions that are based on the nitric oxide synthase (NOS) inhibitor, nitrate reductase (NR)/NOS mutants and utilization of NO scavengers (He et al., 2012). It has been shown that NO is involved in cell wall modification (Sun et al., 2016), which is the main site of Al toxicity (Kochian, 1995; Horst et al., 2010). There are also data indicating that Al toxicity may disrupt NO homeostasis, leading to insufficient NO concentration for root elongation (Sun et al., 2016). Furthermore, NO is also involved in plant symbiotic interactions, during early and late stages of legume-Rhizobium symbiosis (Shimoda et al., 2005; Horchani et al., 2011; Corpas and Barroso, 2015), and lichen symbiosis (Weissman et al., 2005). However, there is little information concerning AM. Few studies have demonstrated NO participation in the early stage of AM symbiotic associations (Calcagno et al., 2012; Espinosa et al., 2014) and in mature AM symbiosis (Li et al., 2013; Zhang et al., 2013). Nevertheless, for arbuscular mycorrhiza scarce information is available on the relation between NO production and AMF colonization.

In plants, NO can be generated by enzymatic and non-enzymatic pathways (Corpas et al., 2004, Wilson et al., 2008). The best-characterized pathway for NO production in plants is the one employing nitrate reductase (NR) (Planchet and Kaiser, 2006). In *Arabidopsis thaliana*, NR is encoded by two homologous genes, *NIA1* (AT1G77760) and *NIA2* (AT1G37130) (Wilkinson and Crawford, 1993). NR activity leads to the reduction of nitrate to nitrite at the expense of NAD(P)H. However, about 1% of NR activity catalyzes also an electron transfer from NAD(P)H to NO_2, resulting in NO formation (Rockel et al., 2002). The importance of NR as a NO source was demonstrated in NR-deficient plants producing significantly lower amounts of NO (Planchet et al., 2005). Two NR genes have been identified in *M. truncatula*, NR1 (MTR_5g059820) and NR2 (MTR_3g073180) (Horchani et al., 2011). Interestingly, it has been shown that NR and nitrite reductase (NiR) transcripts accumulate in *M. truncatula* roots in the presence of germinated spores of *G. margarita* (Gianinazzi-Pearson et al., 2008; Calcagno et al., 2012). Weidmann (2004) observed an activation of the NiR gene before establishment of symbiosis between *M. truncatula* and *Glomus mosseae*. Another source of NO in plant tissue is nitrite:NO reductase (NiNOR, AT2G15620), converting nitrite to NO (Moreau et al., 2010; Stöhr and Stremlau, 2006). Interestingly, NiNOR-mediated NO production was shown to play a role in the regulation of root infection by mycorrhizal fungi (Moche et al., 2010). Considering the NO scavenging, two pathways were distinguished in *A. thaliana*, engaging *S*-nitrosoglutathione reductase GSNOR1 (AT5G43940) (Mur et al., 2013) or non-symbiotic hemoglobin HB1 (AT2G16060) (Hill, 2012).

Once generated NO can interact with amino acids such as tyrosine, leading to post-translation modifications of proteins (Corpas et al., 2013). Tyrosine nitration is believed to be a biomarker of nitrosative stress (Corpas et al., 2011, 2013) and, at the same time, some studies have also indicated the involvement of this process in proteins function modifications and signal transduction during normal plant development (Gow et al., 2004) and rhizobial symbiosis (Melo et al., 2011). However, no information about tyrosine nitration is available in relation to mycorrhiza symbiosis.

Since NO is produced under abiotic stresses and in symbiotic interactions such as rhizobial and lichen symbioses, the goal of this study was to investigate the effect of mycorrhizal inoculation under short-term Al stress on NO metabolism in *M. truncatula* roots. Since the effects of long-term stress, may be caused by secondary Al-related physiological and biochemical changes, we applied short-term treatment to determine primary toxic effect of Al. Such short-term Al treatment was successfully tested in our previous study for M. *truncatula-Ensifer medicae* symbiosis (Sujkowska-Rybkowska et al., 2012). We determined the NO content and localization, the expression of genes encoding NO-scavenging (*MtGSNOR1, MtGSNOR2, MtHB1* and *MtHB2*) and NO-synthesis (*MtNR1, MtNR2* and *MtNIR1*) enzymes and the profile of protein tyrosine nitration (NO_2-Tyr) in *Medicago* roots. This study provides a new insight in NO metabolism in response to mycorrhiza under normal and metal stress conditions.

2. Materials and Methods

2.1. Plant and fungal material, and growth conditions

Medicago truncatula Gaertn. cv Jemalong line J5 seeds were surface-sterilized with 98% sulphuric acid for 6 min, rinsed with distilled water and germinated on 0.8% sterile agar at 27 °C in the dark, and transplanted into 400 ml plastic pots containing a mix (1:2 v/v) of soil and perlite. A total of 20 pots were prepared (10 pots with mycorrhiza and 10 pots without mycorrhiza, respectively). *Rhizophagus irregularis* inoculum, containing ca. 20 spores per gram of growth medium, was produced in pot cultures according to Jurkiewicz et al. (2010). AM fungal inoculum (2 g per pot, including spores, roots and hyphae) was added prior to seedlings transfer. Non-mycorrhizal plants received an equivalent amount of autoclaved inoculum. Plants were grown for 7 weeks under controlled conditions (355 $\mu E/m^2/s$, 16 h/8 h photoperiod and 24/21 °C day/night, and 70% humidity). Each plant was fertilized with 50 ml of modified Long Ashton solution (Seddas-Dozolme et al., 2010) without phosphorus and a double amount of nitrogen to inhibit the nodulation, once a week and watered with distilled water on the other days. Half of control and *R. irregularis*-inoculated plants were watered with 50 µM $AlCl_3$ at pH 4.3 for 3 h. Non Al-treated counterparts were watered with distilled water at pH 4.3. At harvest, roots were removed from pots, gently rinsed with tap water and then with distilled water. Before RNA extraction and microscopy analyses, ~1 cm roots fragments randomly collected were stained according to Vierheilig et al. (1998) and mycorrhizal infection was quantified according to the procedure by Trouvelot et al. (1986). The intensity of infection (M%) and arbuscular development in the infected regions of the roots (A%) were estimated in stained roots (Trouvelot et al., 1986). Only fully colonized plants (M ≥ 80% and A ≥ 80%) were used as mycorrhizal.

2.2. Sequence alignment

In order to define the phylogenetic relationship between *M. truncatula* and *A. thaliana* orthologs, we used the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) within NCBI database. BLAST alignment was performed for each *A. thaliana* full protein sequence NIA1 (AT1G77760), NIA2 (AT1G37130), NiNOR (AT2G15620), GSNOR1 (AT5G43940) and HB1 (AT2G16060) with *M. trucatula* protein database, using the BLASTP algorithm. Orthologs were selected according to the highest query cover score. On the basis of this comparison, we identified *M. trucatula* orthologs that are most closely related to the corresponding NO-metabolism proteins from *A. thaliana*.