NO homeostasis is a key regulator of early nitrate perception and root elongation in maize

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* This work is dedicated to our friend and master Angelo Ramina.
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Abstract

Crop plant development is strongly dependent on nitrogen availability in the soil and on the efficiency of its recruitment by roots. For this reason, the understanding of the molecular events underlying root adaptation to nitrogen fluctuations is a primary goal to develop biotechnological tools for sustainable agriculture. However, knowledge about molecular responses to nitrogen availability is derived mainly from the study of model species. Nitric oxide (NO) has been recently proposed to be implicated in plant responses to environmental stresses, but its exact role in the response of plants to nutritional stress is still under evaluation. In this work, the role of NO production by maize roots after nitrate perception was investigated by focusing on the regulation of transcription of genes involved in NO homeostasis and by measuring NO production in roots. Moreover, its involvement in the root growth response to nitrate was also investigated. The results provide evidence that NO is produced by nitrate reductase as an early response to nitrate supply and that the coordinated induction of non-symbiotic haemoglobins (nsHbs) could finely regulate the NO steady state. This mechanism seems to be implicated on the modulation of the root elongation in response to nitrate perception. Moreover, an improved agar-plate system for growing maize seedlings was developed. This system, which allows localized treatments to be performed on specific root portions, gave the opportunity to discern between localized and systemic effects of nitrate supply to roots.

Key words: Maize, nitrate, nitrate reductase, nitric oxide, non-symbiotic haemoglobin, root, transition zone.

Introduction

Soil nutrient acquisition intensely affects global crop production (Forde and Clarkson, 1999; Robertson and Vitousek, 2009). In poor nations drought and low soil fertility cause low yields and food insecurity, while in rich nations intensive fertilization leads to leaching of nutrients and/or greenhouse gas emission (Donner and Kucharik, 2008). The development of new crop cultivars with enhanced soil resource acquisition is therefore an important strategic goal for modern agriculture (Lynch, 1998, 2007; Vance et al., 2003). Understanding nutrient responses at the organism level will be useful to modify plant metabolism, physiology, growth, and developmental programmes to improve nutrient use efficiency and productivity in crops.

The macronutrient nitrogen is essential for plant growth and development as it is a component of proteins, nucleic acids, and many cofactors and secondary metabolites. In aerobic soils, nitrate is the major source of nitrogen for most plant species (Ahmad et al., 2007; Nischal et al., 2012).

Plants have the potential for adaptation to dramatic fluctuations of nitrogen availability by modulating their capacity for nutrient acquisition and by altering whole-plant morphology and metabolism, such as increasing the root/shoot ratio (Rubio et al., 2009). Developmental adaptive mechanisms stimulate growth in organs that directly participate in nutrient acquisition, such as primary roots (Walch-Liu and Forde, 2008). A dual effect of external nitrate on root system
architecture development has been depicted in the model species Arabidopsis thaliana: (i) a systemic inhibition of lateral primordia by uniformly high nitrate concentrations at a post-emergence stage; and (ii) a localized stimulation of elongation in N-starved roots at the site of contact with a nitrate-rich supply, known as the foraging capacity (Zhang and Forde, 1998; Zhang et al., 1999, 2007; Linkohr et al., 2002; Ruffel et al., 2011). Apart from a few known pathways that involve transpiration factors, micro-RNAs, hormonal signals, and, more recently, nitrate transporters with dual affinity for nitrate and auxin (Little et al., 2005; Remans et al., 2006; Chiou, 2007; Miller et al., 2007; Gifford et al., 2008; Krouk et al., 2010, 2011; Vidal et al., 2010; Castaing et al., 2011; Rubio-Somoza and Weigel, 2011; Ruffel et al., 2011; Xu et al., 2011; Trevisan et al., 2012), the understanding of the sensing of external nitrate conditions and of the signal transduction system that leads to an altered development of roots is still poor.

To trigger adaptive responses and to induce fast switching from starvation metabolism to nutrient assimilation, nitrate itself or its primary assimilation products serve as signalling molecules (Schulze et al., 1994; Crawford, 1995; Scheible et al., 1997; Stitt, 1999; Gojon et al., 2010). Significant advances have been made recently concerning the molecular mechanisms of NO$_2$ sensing and signalling in Arabidopsis, and the striking action of NO$_3$ as a signal in regulating genome expression has been unravelled (Bouguyon et al., 2012). Prolonged nitrate starvation has been demonstrated to largely affect gene transcription, which produces effects on the early nitrate signalling mechanisms. Transcriptomic analyses have evidenced coregulated transcriptional patterns in maize root epidermal cells for genes putatively involved in nitric oxide (NO) synthesis/scavenging (Trevisan et al., 2012).

Nitric oxide is a free radical that is considered to be a general plant signal, since it regulates both normal developmental processes and biotic or abiotic stress responses involving cross-talk with phytohormones (for reviews, see Durner and Klessig, 1999; Wojtaszek, 2000; Beligni and Lamattina, 2001; Lamattina et al., 2003). NO has been reported to be required for root organogenesis (Pagnussat et al., 2002), formation of adventitious roots (Pagnussat et al., 2003), lateral root development (Correa-Aragunde et al., 2004), and root hair formation (Lombardo et al., 2006). Recently, Correa-Aragunde et al. (2004) suggested the possibility that auxin and NO might be on a linear signalling pathway in the process of lateral root formation in tomato. However, knowledge of the molecular mechanisms by which NO regulates growth and development is still fragmentary.

NO is produced in plant tissues by two major pathways, one enzymic and the other non-enzymic (Wendehenne et al., 2004). The NO-producing enzymes identified in plants are nitrate reductase (NR) and several NO synthase-like proteins, including one localized in peroxisomes which has been biochemically characterized (del Rio et al., 2004). Interestingly, it was recently shown that non-symbiotic haemoglobin nsHb 1 could reduce NO$_2$ to NO at a constant rate that was far in excess of that reported for other haemoglobins (Sturms et al., 2011). Plant Hbs are able to regulate several NO effects, as recently reviewed by Hill (2012). Class II nsHbs contribute to NO removal when overexpressed (Hebelstrup et al., 2006, 2012). Moreover, several studies have demonstrated a role for plant Hbs in catalysing the turnover of NO to nitrate (Dordas et al., 2003a, 2003b, 2004; Perazzoli et al., 2004; Hebelstrup et al., 2006, 2012).

The nitrate-regulated expression and the spatial distribution of NR and Hb transcripts in epidermal cells that have been recently evidenced in maize roots strongly suggest that they could play an important role during the early perception and signalling of nitrate in the rhizosphere (Trevisan et al., 2011). Moreover the colocalization of mRNAs for NR and Hb observed in the root apex matches with the major sites of NO accumulation, as shown in Arabidopsis (Stöhr and Stremlau, 2006), suggesting that these two genes may represent the pivotal elements of a finely tuned system for NO homeostasis and signalling.

The involvement of NO in the nitrate signalling pathway opens a wide field of research. This report evaluated the contribution of NO in the nitrate-regulated pathway that directs root system architecture, unravelling the role of NO as a nitrate-related signal. The study focused on both the characterization of the expression profiles of selected genes putatively involved in NO homeostasis and the determination of NO production by roots in response to different N treatments. In addition, since the genes therein selected have proved to be very good candidates for monitoring nitrate sensing in maize roots, they are proposed to be early physiological molecular markers for the response to this anion. Furthermore, this study also deepened the knowledge of the effect of nitrate on root growth and especially on root elongation. Finally, an improved agar-plate culture system for studying the Zea mays L. root response to nutrients has been developed. Thanks to this system, it has been possible to discriminate between localized and systemic effects of nitrate supply to roots. Overall, the data provide evidence that, in maize roots, NO is produced by NR as an early response to nitrate supply. Moreover, the coordinated induction of nsHbs finely regulate its steady-state level. The control of the NO production by the systemic response of NR and nsHbs would seem, moreover, to participate to the complex signalling network involved in the modulation of root growth in response to nitrate.

Materials and methods

Maize growth and experimental design

Seeds of maize inbred line B73 were sown and then transferred to nutrient solution as described by Quaggiotti et al. (2003). For a first set of expression analyses, seedlings were grown in different nutrient solutions for 5 days and then treated for a few hours, as described in Fig. 1. Nitrate, ammonium, and ammonium nitrate were supplied at a concentration of 1 mM. In the nitrogen-depleted nutrient solution, KNO$_3$ was replaced by 1 mM KCl and NH$_4$SO$_4$ by MgSO$_4$.

For NO content measurement, for subsequent expression analyses, and for the analysis of root elongation rate, seedlings were grown for 24 h in the nutrient solution to allow the manipulation of younger roots. To deepen the understanding of the role of NO in maize root response to nitrate, 1 mM sodium tungstate dihydrate (Na$_2$WO$_4$·2H$_2$O), 1 mM 2-(4-carboxyphenyl)-
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4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 0.2 mM l-NG-nitroarginine methyl ester (l-NAME), 0.01 mM sodium nitroprusside (SNP), or 1 mM (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR) were supplied to the nutrient solution (either NO\textsubscript{3}−-supplied or NO\textsubscript{3}−-deprived).

Seeds of the same age were also utilized to evaluate the expression of selected genes in four different portions of roots, as indicated by Baluška et al. (2010), after nitrate supply. The four zones sampled were the root meristem (4 mm from the root tip), the transition zone (the next 1 cm), the elongation zone (the next 1 cm), and the matura

Growth of maize seedlings in agar medium

An improved method was developed to grow maize seedlings on agar. To this aim, plastic boxes (17.9 × 12.9 × 2.6 cm) modified with suitable holes on one side were utilized (Supplementary Fig. S1, available at JXB online). This system permitted the insertion of young roots, which can grow vertically along the agar medium allowing the shoot to develop outside the box and enabled us to perform localized treatments on single root portions, as described in Fig. 2. The agar concentration utilized was 1% after a preliminary test with concentrations ranging from 0.8 to 1.2%. The nutrients were supplied as indicated for hydroponics.

Roots of seedlings grown for 24 h in a nitrate-depleted agar plate were transferred on an identical medium to which, in correspondence to specific root regions, round slices (about 1–1.5 cm in diameter) of agar were removed and substituted with new ones containing 1 mM nitrate. For the negative control, the slices were substituted with new nitrate-depleted ones to subject roots to a similar mechanical stress, thus avoiding false positives due to the perception of the discontinuance of agar and not to the nitrate presence.

Morphological analyses

For the analysis with WinRhizo, germinated seeds of maize inbred line B73 were transferred to 2-L tanks containing five different aerated nutrient solutions (changed every 2 days) according to the treatments—(a) +NO\textsubscript{3}−, (b) −NO\textsubscript{3}−, (c) +NH\textsubscript{4}+ (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR) were supplied to the nutrient solution (either NO\textsubscript{3}−-supplied or NO\textsubscript{3}−-deprived). Seeds of maize inbred line B73 were sown on paper and then seedlings were transferred to a vertical plate system. Plate prepared with N-depleted solution and 1% agar were either supplied with 1 mM nitrate (+N) or depleted (−N) by cutting and replacing a rounded portion of the agar, thus only apical portion of the root system could perceive the change of treatment. Seedlings continued to grow after the replacement of the rounded portion of agar, and at the end of the treatment they were removed from the system and harvested. M, meristem; TZ, transition zone; EZ, elongation zone; MZ, maturation zone.

Fig. 2. Design of the split-root system used to investigate the localized effects of nitrate on the intact root apex of maize seedlings. Seeds of maize inbred line B73 were sown on paper and then seedlings were transferred to a vertical plate system. Plate prepared with N-depleted solution and 1% agar were either supplied with 1 mM nitrate (+N) or depleted (−N) by cutting and replacing a rounded portion of the agar, thus only apical portion of the root system could perceive the change of treatment. Seedlings continued to grow after the replacement of the rounded portion of agar, and at the end of the treatment they were removed from the system and harvested. M, meristem; TZ, transition zone; EZ, elongation zone; MZ, maturation zone.

RNA extraction and cDNA synthesis

Tissues used for gene expression analyses were collected and immediately frozen in liquid nitrogen and kept at −80 °C for subsequent RNA extraction.
Total RNA was extracted from 250 mg frozen tissue as described by Trevisan et al. (2011) and using the TRIzol method (Invitrogen, San Giuliano Milanese, Italy). An aliquot of total RNA was treated with RQ1 RNase-free DNase (Promega, Milano, Italy) as described by Falchi et al. (2010). Total RNA (1 μl) was quantified using a NanoDrop (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA). cDNA was synthesized from 500 ng of total RNA mixed with 1 μl of 10 mM oligo-dT, as described by Manoli et al. (2012).

Selection of genes to be evaluated, maize sequences identification, and primer design

The list of genes analysed is reported in Supplementary Table S1, together with the primers utilized for real-time quantitative PCR (RT-qPCR) expression analysis. They were chosen according to previously published results (Trevisan et al., 2011, 2012). Hb (NCBI accession AF236080.1) and NRI (AF153448.1) were then chosen for further more detailed analysis and the analysis was extended to the expression of Ha2 (NM_001112349.1), NiR (ACG29734.1), and NOA1 (NM_001174573), which were selected by screening the B73 genome database (http://www.maizesequence.org/index.html), and NRT2.1 (AY129953.1, Quaggiotti et al., 2003), which was used as a positive control for nitrate perception. The NOA1 sequence was identified based on its similarity with AtNOA1 (At3g47540.1). Primers were designed with Primer3 web tool (version 0.4.0; http://frodo.wi.mit.edu/primer3/) and further verified with the PRATO web tool (Nonis et al., 2011; http://prato.daapv.unipd.it).

Real-time quantitative PCR

Relative quantification of transcripts by RT-qPCR was performed in a StepOne Real-Time PCR System (Applied Biosystems, Monza, Italy) as described by Nonis et al. (2007). Experiments were conducted using SYBR Green chemistry (Applied Biosystems), according to the manufacturer’s instructions. For each reaction, 2.5 ng retrotranscribed RNA was used as template. Three technical replicates were performed on six independent biological replicates using the conditions described by Trevisan et al. (2011). Melting curve analysis was performed to confirm the absence of multiple products and primer dimers. Data were exported and analysed according to the Livak and Schmittgen (2001) method using as reference genes LUC (leucine primers, forward 5′-TCAATGTGCTACAGGAAAGT-3′ and reverse 5′-GTGGATTTCTTGAACCGC-3′) and MEF (membrane protein PBIA10.07c, primers: forward 5′-TGATCTCGGGAATCTCCTTG-3′ and reverse 5′-TTGATATGTCCAGGGCTACC-3′), according to Manoli et al. (2012). For each transcript, the ratio between the expression measured for a given treatment and of its own control was used to estimate up- or downregulation of genes. The ratios obtained were then expressed as base-2 logarithm to build the graphs.

NO detection

Germinated seeds were transferred to a nitrogen-depleted nutrient solution, and after 24 h root apices about 2 cm long were excised and incubated for 30 min in 2 ml detection buffer (10 mM Tris-HCl, pH 7.4) with 15 μM of 4,5-diaminofluorescein diacetate (DAF-2DA). Subsequently, the apices were washed twice for 5 min with fresh detection buffer and placed on a microscope slide fixed with a Secure-Seal hybridization chamber gasket (Life Technologies, Carlsbad, CA, USA) (20-mm diameter, 0.8-mm deep) and analysed for NO production by stereo- and confocal microscopy. For each chamber, one apex was incubated (as was was described in detail).

For stereomicroscopy, the chambers were immediately filled with fresh detection buffer and placed on a microscope slide fixed with a Secure-Seal hybridization chamber gasket (Life Technologies, Carlsbad, CA, USA) (20-mm diameter, 0.8-mm deep) and analysed for NO production by stereo- and confocal microscopy. For each chamber, one apex was incubated (as was was described in detail).

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Results

Nitrates exerts specific effects on genes involved in NO homeostatic control

The expression of a number of previously identified genes (Quaggiotti et al., 2003; Trevisan et al., 2011, 2012), together with that of some new ones (Supplementary Table S1), was measured in roots and leaves of seedlings grown for 5 days in a nutrient solution containing 1 mM nitrate (+NO3), 1 mM ammonium (+NH4), or N-deprived (both –NO3 and –NH4) (Fig. 3). The transcriptional response of five genes (NRI, Hb, Hb2, NRT2.1, NiR) evidenced a very strong nitrate responsiveness in roots. A similar behaviour was observed in leaves, even if to a lower extent. The rest of genes selected, on the contrary, did not evidence a specific nitrate responsiveness.

The expression of the same set of genes was also assessed on root and leaf tissues of 5-day-old seedlings, but after only

Fig. 3. Heat map showing gene expression of 14 genes significantly regulated by long-term nitrate or ammonium supply and depletion in Zea mays L. roots and leaves. Seedlings were grown for 5 days in a nutrient solution containing 1 mM nitrate (+NO3) or 1 mM ammonium (+NH4) or N-deprived (–NO3 and –NH4). At the end of the treatment, seedlings were harvested and roots were separated from leaves. The grey scale represents the level of gene expression. Values are reported as arbitrary units and are the means of three technical repetitions performed on six independent biological replicates.
30 min, 2 h, and 6 h of nitrate/ammonium provision or depletion. The time course of the expression of the five nitrate-specific targets in both roots and leaves after a few hours of nitrate/ammonium supply/starvation is shown in Fig. 4 (the expression patterns of the other genes tested is reported in Supplementary Fig. S2).

The nitrate supply induced a significant increase of transcript accumulation for all five genes in both roots and leaves (Fig. 4A).

**Fig. 4.** Time course of the expression of five genes significantly regulated by short-term nitrate/ammonium treatments in *Zea mays* L. roots (A) and leaves (B). Data are reported as base-2 logarithm of the ratio between the expression levels measured for samples subjected to the treatments, as described in Fig. 1 (short-term nitrogen starvation in seedlings grown in +N conditions and short-term nitrogen provision in seedlings grown in –N conditions) and of its own control. The left column (−) shows the differences in gene expression in seedlings supplied for 5 days with NO$_3^-$ (upper part) or NH$_4^+$ (lower part) and then deprived for 30 min, 2 h, and 6 h; the right column (+) shows the differences of expression measured after resupply with NO$_3^-$ (upper panels) or NH$_4^+$ (lower panels) for 30 min, 2 h, and 6 h in seedlings grown 5 days in a N-deprived medium.
and 4B, upper panels), even if in roots it was much more noticeable (from 4–16-fold after 30 min of NO$_3^-$ supply to 8–>100-fold after 6 h). Conversely, the transcription of the five genes did not show a similar increase when ammonium was supplied as the form of nitrogen, neither in roots nor in leaves (Fig. 4A, lower panels), confirming the specificity of responsiveness to nitrate. Also in the case of N-deprivation, all five genes displayed a more evident response (decrease of expression) to nitrate deprivation in comparison to that measured for ammonium removal (Fig. 4A, B, left panels), both in leaves and in roots. These five specifically nitrate-inducible genes were thus selected for the subsequent and more detailed expression analyses.

Root growth responds specifically to nitrate availability

The effect of nitrate supply on root development was evaluated in comparison to that of both ammonium and NO$_3^-$NH$_4$ in plants grown in nutrient solution for 5 days (Table 1 and Supplementary Fig. S4). The analysis of root length, root surface area, and number of tips evidenced a similar pattern, showing the strongest root growth stimulation in seedlings grown with 1 mM nitrate. Values measured for these three parameters in plants grown with ammonium were significantly lower (50–60%) than those observed for nitrate-supplied roots and were closest to rates observed for NO$_3^-$-depleted roots (nitrate-negative control). Furthermore, an inhibitory effect of ammonium supply was visible for both root length and tip number, which showed values even lower with respect to the negative control. The supply of NO$_3^-$NH$_4$ slightly stimulated these three parameters, even if to a significantly lower extent with respect to nitrate.

The mean root diameter showed an opposite trend with the maximum rate observed for ammonium-treated roots and the lowest one for nitrate-supplied plants, which evidenced values even lower than those observed for nitrate-depleted roots. These observations, besides suggesting a compensatory mechanism between the growth in length and in thickness in maize root, highlighted the specificity of nitrate in affecting the root growth, which conversely did not show any similar response when nitrogen was supplied as ammonium.

NR-dependent NO production after nitrate supply

To better understand the role of NO in nitrate signalling, its production was monitored by measuring the DAF-2T fluorescence in stereomicroscopy. Seedlings grown for 24 h without nitrate were supplied with 1 mM nitrate and the fluorescence produced was observed (Fig. 5A, panels I–P) in comparison with that measured in negative controls (Fig. 5A, panels A–H). The nitrate supply caused a slight but consistent increase in DAF fluorescence in the first minutes after treatment (panels J and K). No fluorescence increase was induced by NO$_3^-$-deprived control treatments, whereby in contrast a signal decrease was observed after 10 min, probably due to the decay of the probe (panels A–H). Based on these observations, the increment of fluorescence was mainly localized immediately above the meristematic apex and more precisely in the transition zone, as defined by Verbelen et al. (2006) and Mugnai et al. (2012).

In order to get a more detailed imaging and quantification of DAF fluorescence, the experiment was repeated at the confocal microscope and the effects of a NO donor (NOR), a NO scavenger (cPTIO), and a NR inhibitor (tungstate) were evaluated. Results for both –NO$_3^-$ and +NO$_3^-$ treatments at T$_0$ and after 30 min of observation are shown in Fig. 5B. There is a clear difference between the two treatments, with a strong increase in the DAF fluorescence in response to nitrate provision (panel D) that was not observed in negative controls (–NO$_3^-$ roots, panel C). Moreover, higher-magnification analyses (Supplementary Fig. S3) revealed a few cytological details on the different cell types observed, which typically distinguish the transition zone. In the distal part of the portion of root examined, nuclei were positioned in the centre of the cell, similarly to the meristem, whereas the more distal zone cells resembled those of the elongation zone with large central vacuoles and nuclei pushed to the side cell walls. The same observations were performed in the presence of tungstate, NOR, and cPTIO. The results obtained with five biological repetitions are reported in Fig. 5C. Data were expressed as relative fluorescence increase/decrease after 30 min of observation. Results showed a significant increase of fluorescence for nitrate-supplied and NOR-treated roots. On the contrary, when seedlings were supplied with –NO$_3^-$-solution (negative control), with nitrate plus tungstate, or nitrate plus cPTIO, the fluorescence did not increase throughout the experiment. These results suggest that a NR-dependent NO burst occurred immediately after nitrate supply to roots.

Table 1. Effects of nitrate supply on root development after 5 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length (cm)</th>
<th>Surface area (cm$^2$)</th>
<th>Diameter (mm)</th>
<th>Tips (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3^-$ supply</td>
<td>79.48 ± 3.98$^a$</td>
<td>12.58 ± 0.55$^a$</td>
<td>0.50 ± 0.01$^d$</td>
<td>91.20 ± 5.07$^a$</td>
</tr>
<tr>
<td>NO$_3^-$ depletion</td>
<td>51.61 ± 2.96$^c$</td>
<td>8.60 ± 0.46$^c$</td>
<td>0.55 ± 0.01$^c$</td>
<td>67.40 ± 4.57$^b$</td>
</tr>
<tr>
<td>NH$_4^+$ supply</td>
<td>36.59 ± 1.39$^d$</td>
<td>8.18 ± 0.28$^d$</td>
<td>0.70 ± 0.01$^d$</td>
<td>55.43 ± 3.34$^c$</td>
</tr>
<tr>
<td>NH$_4^+$ depletion</td>
<td>51.34 ± 2.15$^c$</td>
<td>9.03 ± 0.43$^c$</td>
<td>0.52 ± 0.01$^d$</td>
<td>72.90 ± 2.75$^b$</td>
</tr>
<tr>
<td>NO$_3^-$NH$_4^+$ supply</td>
<td>60.08 ± 3.56$^b$</td>
<td>10.42 ± 0.55$^b$</td>
<td>0.61 ± 0.01$^b$</td>
<td>64.23 ± 2.79$^{bc}$</td>
</tr>
</tbody>
</table>

Different letters indicate statistically significant differences among samples (P < 0.05, ANOVA).

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Nitrate induces nitric oxide production in maize roots

Due to the size of the mini-chamber utilized for both stereo- and confocal microscopy, it was necessary to work with roots sampled from younger seedlings. For this reason, we shifted the experimental plan to younger seedlings also for the following expression analyses, focusing only on the early events after nitrate provision. Plants were, thus, grown for 24 h in –NO\textsubscript{3} solution and then transferred to +NO\textsubscript{3} medium for 2 h. The transcript accumulation of the previously selected genes (NR1, Hb, Hb2, NiR) together with a new one (NOA1) encoding a putative AtNOA1 orthologue was observed after nitrate supply and in the presence of cPTIO, tungstate, or l-NAME. The expression of NRT2.1 was also included among the analyses as a positive control of the nitrate perception.

Nitrate addition induced strong increments of transcription for all genes analysed except for NOA1 (Fig. 6, first two columns for each gene). The expression of NR gene was 6–9-fold higher in comparison to that measured in –NO\textsubscript{3} roots, whereas the two isoforms of nsHb increased their transcription by 27–72-fold. NiR and NRT2.1 showed an induction of their expression of 21- and 6-fold, respectively. When cPTIO was given together with nitrate (third column), the expression of both Hb and Hb2 was very strongly inhibited, whereas the other genes analysed did not evidence significant differences of expression in comparison to the positive control (+NO\textsubscript{3}). Similarly, the addition of tungstate (fourth column), led to an inhibition of 75–90% of transcription of all genes, with the exception of NOA1. Conversely, the provision of l-NAME, an inhibitor of nitric oxide synthase (NOS), induced only slight and rarely significant decreases of the expression of these genes.
seedlings grown without nitrate were treated with the anion
five genes evidenced a significant change of localization when
nitrate-depleted roots and after 2
zone, maturation zone, as schematized in
different root portions (meristem, transition zone, elongation
nitrite reductase and of
the genes encoding nitrate reductases, haemoglobins, and
immediately above the meristematic apex, and more precisely
this molecule after nitrate provision is preferably localized
Results on NO measurements suggest that the production of
in the transition and elongation zones. The expression of
involvement of NR-derived NO as a key signal in nitrate sig
biosynthesis and scavenging provided further evidence of the
Fig. 6. Effects of five different chemicals interfering with NO
biosynthesis and scavenging on the expression profile of six genes
differentially regulated by nitrate supply/depletion. Plants were
grown for 24 h in –NO₃⁻ solution and then transferred to +NO₃⁻
medium for 2 h. The transcript accumulation of six genes (NR1,
Hb, Hb2, NiR, NRT2.1, and NOA1) was examined after nitrate
supply and in the presence of 1 mM cPTIO, 1 mM tungstate (W),
and 0.2 mM L-NAME. a.u., arbitrary units.

These results confirmed the role of the regulation of NR1,
Hb, and NiR in the early response to nitrate even in younger
roots. Moreover, the use of chemicals interfering with NO
biosynthesis and scavenging provided further evidence of the
involvement of NR-derived NO as a key signal in nitrate sig-
alling in roots of maize.

Transcription of genes involved in NO production and
scavenging is maximally induced in root transition
zones after nitrate induction

Results on NO measurements suggest that the production of
this molecule after nitrate provision is preferably localized
immediately above the meristematic apex, and more precisely
at in the transition and elongation zones. The expression of
the genes encoding nitrate reductases, haemoglobins, and
nitrite reductase and of NRT2.1 was, therefore, studied in four
different root portions (meristem, transition zone, elongation
zone, maturation zone, as schematized in Fig. 7A), both in
nitrate-depleted roots and after 2 h of nitrate provision. All
five genes evidenced a significant change of localization when
seedlings grown without nitrate were treated with the anion
(Fig. 7B). In fact, in nitrate-starved roots, 70–80% of
mRNA was concentrated in the meristematic cells for all
five genes, with the remaining 20–30% of mRNA preva-
ently localized in the elongation and maturation zones.
In these conditions, the amount of transcript detected at
the transition zone level was extremely low or even neg-
ligible. On the contrary, in seedlings supplied with 1 mM
nitrate for 2 h (after being grown 24 h in –NO₃⁻ solution),
the transcripts of all five genes were more equally distrib-
buted between the apical meristem and the transition
zone, with a significant increase of accumulation in the
transition zone which showed an amount of mRNA for
each gene ranging from 20 to 40% of the total. Moreover,
after nitrate supply the maturation zone also evidenced
an increase in terms of gene expression compared with
nitrate-depleted roots.

Fig. 7C describes the increases of transcription for each gene
in each of the four portions, independently from their relative
abundance. All five genes evidenced an induction of their expres-
sion in all the four portions sampled, with the maximum in the
transition zone which showed a transcription rate more than
30-times higher if compared with that measured in the same
portion of nitrate-depleted roots (except the case of NRT2.1,
which increased more than 10-times). In the elongation and
maturation zones of nitrate-supplied roots, the amount of
mRNA increased by around 4–8 and 8–20 times, respectively.
On the contrary, in the meristematic cells the increase of gene
transcription measured was very low or insignificant.

In general, it would seem that nitrate supply induces a
redistribution of transcripts in zones of roots different from
the meristem, which in turns appears to be the main site of
their accumulation in conditions of nitrate starvation.

The nitrate-induced root length increase is dependent
on a NO signalling pathway

After germination, seedlings were transferred to seven differ-
ent nutrient solutions (+NO₃⁻, –NO₃⁻, +NH₄⁺, +NO₃⁻+tung-
state, –NO₃⁻+SNP, +NO₃⁻+cPTIO, +NO₃⁻+L-NAME) and
and the growth of primary root was monitored for 24 h (Fig. 8).
Nitrate-supplied seedlings and –NO₃⁻+SNP seedlings showed
a more elevated rate of root elongation, with values signifi-
cantly higher in comparison to all the remaining treatments.
The supply of ammonium did not produce any increase in the
elongation rate, which was similar to that measured for nega-
tive control plants, as already observed also for older seedlings.
The provision of tungstate together with nitrate inhibited even
more significantly the root growth in comparison with nitrate-
depleted roots. A similar decrease was also observed in roots
supplied with nitrate plus cPTIO. On the contrary, the addi-
tion of SNP to nitrate-depleted roots stimulated root growth
to levels comparable to those measured for positive control.
Conversely, as also observed in the case of gene transcription,
the supply of L-NAME, which inhibits NOS activity, did not
produce significant effects on root lengthening.

These results, besides suggesting the involvement of NO
in the regulation of nitrate-induced root elongation, clearly
confirm the key role of NR for this signalling pathway.
Nitrate induces nitric oxide production in maize roots

The fresh weight of both roots and shoots was also determined to exclude toxicity effects of chemicals utilized (Supplementary Table S2).

The nitrate-induced NO signalling pathway is a localized effect

The set up of a method to grow maize seedlings on a semi-solid agar medium allowed to perform targeted treatments to single zone of root (Fig. 2). This system permits the treatment of only specific zones of root, thus enabling the discrimination between local and systemic effects on gene expression.

As a preliminary experiment, to test the validity of this method as an alternative to hydroponics, seedlings were grown on nitrate-supplied or nitrate-deprived agar, using the same timing and concentrations as for the experiments in hydroponics, and the expression of the previously selected genes was evaluated. RT-qPCR were carried out on both roots and shoots and for all the genes and the nutritional conditions described in the first paragraph of the Results (data not shown). Figure 9A shows only the genes closely related to the induction of NO pathway in roots after nitrate supply. The results fully confirmed those obtained for seedlings grown in hydroponics. Furthermore, the root growth, analysed by

Fig. 7. Spatial distribution of five genes differentially regulated by supply/depletion of nitrate. (A) Graphical representation of the different parts of primary root analysed; M, meristem; TZ, transition zone; EZ, elongation zone; MZ, maturation zone. (B) Gene expression in the different zones reported as percentage in both nitrate-starved and -supplied roots; percentages are expressed as the ratio between the mRNA abundance measured in each specific root zone and the global amount of transcript in the overall root. (C) Increase of transcription for each gene in each of the four portions; data are as log₂ of the ratio of +N/–N.

Fig. 8. Effect of different nitrate treatments on primary root growth. After germination seedlings were transferred to different nutrient solutions (+NO₃⁻, –NO₃⁻, +NH₄⁺, +NO₃⁻ + W, –NO₃⁻ + W, –NO₃⁻ + l-NAME) and the growth of primary root was measured for 24h with a ruler on 16 seedlings for each group. Values represent the mean of four independent biological repetitions. Different letters indicate significantly different means (P < 0.05).
Mean of WinRhizo software, evidenced the same behaviour of plants grown in nutrient solution (Supplementary Fig. S4), further confirming the validity of this method.

Seedlings were then submitted to a treatment with nitrate localized only to the meristematic apex (4 mm). The transcription of the five genes previously chosen was evaluated independently in the four root zones (meristem, transition zone, elongation zone, maturation zone) in both seedlings whose tips was treated with 1 mM nitrate and negative control (Fig. 9B). NR1, Hb1, and Hb2 strongly increased their expression in the apices of nitrate-supplied roots, whereas NiR transcription increased to a lesser extent. On the contrary, abundance of NRT2.1 mRNA did not evidence any increase, indicating that this high-affinity nitrate transporter is not involved in the influx of nitrate by the root meristem. Furthermore, in the three other root zones, no differences in terms of transcript accumulation were detected after local nitrate provision to the root apex, suggesting that NO signalling activation by nitrate should represent a localized effect of nitrate.

Discussion

Nitrogen is a major element for plant life, and crops strongly depend on intense fertilization programmes throughout the world, thus affecting environment quality. The identification of crop cultivars with improved nutrient acquisition efficiency in low-input farming systems continues to be a real priority for plant scientists (Robertson and Vitousek, 2009; Xu et al., 2012). Nitrate is the main nitrogen source for plants in regular agricultural systems and, acting also as a signal, triggers a number of molecular and physiological events leading to the overall plant’s response to its availability (Gojon et al., 2010 and references therein).

The control of NO homeostasis through the spatiotemporal coordination of nitrate reductase and haemoglobin gene expression has been recently hypothesized to participate to nitrate sensing in maize roots (Trevisan et al., 2011). The present work tried to more deeply characterize the role of NO in the maize root response and adaptation to nitrate fluctuations.

To better discriminate nitrate-specific effects from those more generally N-dependent, the expression of previously selected genes (Quaggiotti et al., 2003; Trevisan et al., 2011, 2012) was evaluated in response to nitrate or ammonium supply and deprivation (Fig. 3). This first screening allowed the current work to focus later only on genes responding exclusively to nitrate (and not to ammonium), which coincided with those involved in the control of NO biosynthesis and scavenging. In particular, genes encoding the cytosolic NR and two different nsHbs, together with a gene encoding nitrite reductase, evidenced both in short-term and long-term experiments a clear and noticeable responsiveness to nitrate supply or starvation, but did not change their expression in response to ammonium (Figs 3 and 4). A gene encoding a high-affinity root nitrate transporter was also used as internal control, in light of its putative role in nitrate influx.
and its transcriptional inducibility during the first phases of nitrate supply (Quaggiotti et al., 2003). The expression profile recovered for this gene provided indirect evidence of the entry of nitrate into root epidermal cells, hence enabling the activation of the signalling pathways in which nitrate is involved.

Besides being the first enzyme of nitrate assimilation, NR represents also one of the most important sources of NO in plants (Mur et al., 2012). It is a cytosolic enzyme that can reduce both nitrate to nitrite and nitrite to NO, even if it shows a better affinity for nitrate than for nitrite. However, NR seems to be switched to the latter reaction when high nitrite levels are produced (Gupta et al., 2011; Mur et al., 2012). This occurs, for example, when the external nitrate rapidly increases after a nitrate starvation, as a consequence leading to a strong increase of NO influx inside cells, as it might have happened in the current study. Once inside the root cells, nitrate is promptly converted to nitrite by NR, leading to nitrite accumulation. Nitrite accumulation could in turn shift the NR equilibrium toward its second mode of action, thus promoting the biosynthesis of NO in response to nitrate. This scenario seems consistent with the main findings of this paper. Nitrate reductase is involved in NO production during bacteria-induced defence (Modolo et al., 2005), disease development in certain pathogenic interactions (Shi and Li, 2008), drought (Freschi et al., 2010), cold (Zhao et al., 2009), osmotic stress response in roots of Arabidopsis (Kolbert et al., 2010), stomatal regulation (Srivastava et al., 2009), and many developmental processes such as the initiation of flowering (Seligman et al., 2008).

The strong increase of the expression of both of the nsHbs genes observed after 30 min of nitrate supply is not surprising if considering the high reactivity of NO which, as well as serving as a signal in regulating several physiological events, must also be kept at a steady level to avoid damage due to its toxicity. Recently, several studies have indicated a role for haemoglobins in detoxification of high intracellular NO concentrations (Dordas et al., 2003a,b; Perazzolli et al., 2004; Vieweg et al., 2005). The patterns of expression of non-symbiotic haemoglobins vary depending on tissues and in response to different types of stress (Hunt et al., 2001). Perazzolli et al. (2004) provided evidence that Arabidopsis non-symbiotic haemoglobin AtHb1 functions as a NO-dioxygenase, metabolizing NO to nitrate. Moreover, plant haemoglobins seem to be involved in the control of NO accumulation during rhizobial and mycorrhizal symbioses (Vieweg et al., 2005) and in the response to hypoxia in different tissues such as seeds, roots, and stem tissue (Dordas et al., 2003a,b). Plant haemoglobins can control developmental and physiological reactions by modulating cellular NO levels (Hill, 2012) and should be considered to be as important as NO generation in regulating in planta NO signalling (Mur et al., 2012).

These results, besides confirming the already hypothesized involvement of NO homeostasis control in the maize root response to nitrogen (Trevisan et al., 2011), demonstrate also that this is an exclusive prerogative of NO3 signalling. In fact, when ammonium was supplied to nutrient solution as the sole nitrogen source, no significant effects were measured on the transcription of genes involved in NO production and scavenging. On the contrary, the expression of the other genes here analysed did not show a similar nitrate-specific responsiveness. In addition, data obtained by analysing root morphological parameters by the WinRhizo software highlighted the same specificity of nitrate, which significantly affected root growth when supplied to N-deprived roots, in contrast to what happened when the same concentration of ammonium was given to roots (Table 1).

Thus, it would seem that NO may be produced by roots as an early signal of nitrate perception. To deepen this hypothesis, in vivo NO detection was carried out. Results obtained using the DAF-2DA probe (Kojima et al., 1998) and stereo- and confocal microscopy evidenced a clear induction of fluorescence after nitrate provision (Fig. 5). The main zone of NO production seemed to be located immediately above the meristematic apex. A similar localization has been recently observed in the same species by Mugnai et al. (2012) as a response to hypoxic conditions. The fluorescence detected after nitrate supply was not relieved in the presence of tungstate, giving support to the role of NR in this process. Moreover, the addition of cPTIO suppressed the development of fluorescence, confirming the specificity of NO detection by the probe. This was also corroborated by the strong increase of fluorescence when NOR was supplied to nitrate-depleted roots.

To give more strength to these results, this work tried to operate by following the steps indicated by Mur et al. (2012), being well conscious that it should be always preferable to employ parallel approaches for NO measurements. The NR-dependent NO production observed after nitrate supply was then corroborated by the expression analyses performed on roots of 1-day-old seedlings (Fig. 6). In particular, the results proved the strong induction of NR, NiR, and nsHbs transcription in the early phases of nitrate perception. As also observed in the case of NO production, the transcription of all genes was significantly inhibited after tungstate and cPTIO addition, confirming the cooperation between nitrate reductase and haemoglobin activities in the finely tuned control of NO homeostasis. However, to exclude the possible involvement of sources of NO other than NR, the study was extended also to the orthologous gene of Arabidopsis, AtNOA1 (Guo et al., 2003), encoding the nitric oxide associated 1 protein (Zemojtel et al., 2006). NOA1 was previously named AtNOS1 and it has been described as a potential NOS in A. thaliana, despite lack of sequence similarity to animal NOSs. It has been successively established to be a GTPase (Moreau et al., 2008) and not to possess NOS activity, and for this reason it has been renamed AtNOA1. Previous studies have shown that NOA1-dependent NO synthesis is involved in hormonal signalling, stomatal movement, flowering, pathogen defence, and oxidative stress (Guo et al., 2003; He et al., 2004; Zeidler et al., 2004; Zhao et al., 2007). The transcription of the orthologous AtNOA1 in maize did not evidence any alteration neither in response to nitrate nor to the other chemicals utilized.

Moreover, to exclude the involvement of a more generic NOS activity, nitrate-supplied seedlings were also treated...
with l-NAME, which is commonly used to inhibit NOS activity in mammals and also in plants. No effects, neither on transcription of nitrate-responsive genes (especially with regards to nsHbs) nor on the nitrate-induced root lengthening, were observed (Figs 6 and 8), giving more strength to the idea that NO production after nitrate provision is predominantly dependent on the activity of NR.

To deepen the spatial regulation of NO homeostasis balance, the expression of the five genes was analysed in four different root zones (meristem, transition zone, elongation zone, maturation zone) both in nitrate-depleted and in nitrate-treated (1 mM) seedlings (Fig. 7). In N-starved roots, all five transcripts evidenced their maximum accumulation at the meristem level. This pattern radically changed when nitrate was furnished to roots with a very significant increase of transcript abundance in the transition zone, located between the meristem and the region of fast cell elongation. Cells of the transition zone undergo a series of fundamental changes in their cytoarchitecture and physiology and they accomplish dramatic rearrangements of the actin cytoskeleton (Baluška et al., 1997, 2001). This is essential for the developmental switch into rapidly elongating root cells which expand strictly uniaxially (Baluška et al., 1997). The distal part of this zone in characterized by a prevalence of cells that optionally can re-enter the cell cycle, whereas the proximal part is equipped with cells competent to rapidly enter the fast cell elongation zone. As this developmental passage of cells can be differentially regulated at the opposite root flanks, this unique zone provides the root apices with an effective mechanism to reorientate growth in response to environmental stimuli (Verbel et al., 2006). A number of experimental proofs suggest that the transition zone should be considered as a sort of sensory and information processing zone, enabling the growing root apex to monitor environmental parameters continuously and to trigger appropriate

responses (Mugnai et al., 2012). If this is true and if a role for NO homeostasis control through the combined action of NR and nsHb in the early perception of nitrate by roots is hypothesized, the current results on transcript accumulation redistribution along root apex are not surprising. Based on this finding, it would seem that nitrate supply could activate its own sensing by stimulating NO production by the transition zone cells, thus initiating a signalling pathway contributing to the physiological adaptation (e.g. root growth) to nitrate fluctuations.

The most important example of the plasticity that plants express to fit with nutrient withdrawal in soil is, in fact, represented by the capability of rearranging root architecture to maximize their capture (López-Bucio et al., 2003; Hermans et al., 2006; Zhang et al., 2007; Zolla et al., 2010; Giehl et al., 2012; De Pessemier et al., 2013). Nitrate affects root development by finely regulating the growth of lateral roots depending on its external concentration and localization, as aforementioned (Péret et al., 2009; Mounier et al., 2013; Yu et al., 2013) and as also showed by the current findings obtained with the WinRhizo software (Table 1). Based on the preliminary results showing the preferential localization of NO production at the level of the transition zone, the attention was focused on nitrate effects on root elongation, which takes place in the zone immediately above and neighbouring the transition zone (Fig. 8). The results evidenced a strong and specific induction of root elongation of young maize seedlings supplied with 1 mM nitrate and a drastic inhibition in the presence of ammonium, cPTIO, and tungstate. No effects were recorded in the presence of l-NAME. On the contrary, when the negative control (–NO3−) was supplied with a NO donor (SNP) the root length increased significantly. These results strongly suggest that the NO generated through NR should significantly contribute to the root lengthening noticed after nitrate provision.
The involvement of NO in root development has been observed in numerous studies, such as those published by the Lamattina group (Pagnussat et al., 2002, 2003; Correa-Aragunde et al., 2004; Lombardo et al., 2006), but it was already being hypothesized in 1997 by Gouêva et al., who found that NO was able to induce cell elongation in a way similar to auxin. Moreover, a recent study suggested that class-2 non-symbiotic haemoglobin plays a role in regulating the synthesis and transport of auxins by altering the level of the signal molecule, NO, in specific cells (Elhiti et al., 2013). Besides this, NO is involved in the regulation of actin cytoskeleton, endocytosis, vesicle trafficking, and the polarity of growing tip cells (Prado et al., 2004; Lombardo et al., 2006; Salmi et al., 2007; Prado et al., 2008; Kasprowicz et al., 2009; Wang et al., 2009), which are all prerequisites to acquire competence for cells to elongate. Considering also that NO is widely implicated in the plant response to environmental stresses (Beligni and Lamattina, 2001; Dat et al., 2004), it seems to play crucial functions at the crossroads between developmental and abiotic stress tolerance. For this reason, it should also represent a very good molecular candidate to regulate root development in response to abiotic stresses, such as nutrient or oxygen deprivation (Mugnai et al., 2012), but also an early player in symbiotic interactions establishment, which also need root architecture to be adapted to the environment.

In the present research, thanks to a method allowing the growth of maize seedlings in vertical plates with an agar medium, some major details on NO-mediated nitrate signaling have been attained. The results suggest that the mechanism underling the root response to nitrate and involving NO signaling is directly activated on cells which enter in contact with external nitrate (Fig. 9). Moreover, this alert system does not seem to be turned on by some nitrate-derived compounds or by the nitrate that moves up through the root. In fact, when only the meristematic apex was treated with nitrate, the induction of the transcription of \( NRI \) and \( Hbs \) was exclusively restricted to the apex itself, whereas in the upper zone of the roots no differences were detected in comparison with the negative control. This is even more remarkable considering that, conversely, when the entire root is in contact with nitrate, the apex is the portion that shows the lower responsiveness to this anion in terms of induction of gene expression, the transition zone being the most receptive. Moreover, these results indicate that nitrate transporters other than \( NRT2.1 \) should be implicated in nitrate perception at the root meristem, since the transcription of \( NRT2.1 \) is not activated at all by nitrate, in contrast to what was observed in all the other three root zones when the whole root was supplied with nitrate.

Based on these data, it would seem that the NO-mediated pathway here described represents an early alert system for external nitrate sensing by root cells, which seem to individually possess the competence to activate this pathway when external nitrate is perceived. But also, since root growth is modulated by the convergence of multiple environmental inputs which are integrated by specific signal pathways to decide how to explore the surrounding environment, additional experiments will be needed to better understand the functioning of this NO-mediated pathway and to identify the downstream events that link the NO burst with the physiological redirection of root growth. Even if a high number of specific and comprehensive issues on the NO role in the complicated cross point between root and nitrate (and root and abiotic stress perception in general) need to be further deepened, the current findings suggest that the triggering of a NO burst is a direct response to the rapid increase of nitrate availability and that it could mediate the root elongation observed after nitrate provision (Fig. 10).

### Supplementary material

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** The improved agar-plate culture system for studying the *Zea mays* L. root response to different nutrient availability.

**Supplementary Fig. S2.** Time course of the expression of genes following short-term nitrate/ammonium treatments in maize roots and leaves.

**Supplementary Fig. S3.** Confocal detection of DAF-2T in the transition zone of nitrate-treated apices.

**Supplementary Fig. S4.** Root and leaf fresh weight and relative root/shoot ratio in seedlings grown in nutrient solution for 5 days and total root length, total surface area, mean diameter, number of root tips, and leaf fresh weight in seedlings grown for 5 days in agar with or without 1 mM NO\(_3^-\).

**Supplementary Table S1.** List of genes analysed by real-time quantitative PCR.

**Supplementary Table S2.** Merged effects of different chemicals interfering with NO biosynthesis/scavenging and nitrate supply/depletion on root and leaf fresh weight.

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### References


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*Supplementary Fig. S1.* The improved agar-plate culture system for studying the *Zea mays* L. root response to different nutrient availability. *Supplementary Fig. S2.* Time course of the expression of genes following short-term nitrate/ammonium treatments in maize roots and leaves. *Supplementary Fig. S3.* Confocal detection of DAF-2T in the transition zone of nitrate-treated apices. *Supplementary Fig. S4.* Root and leaf fresh weight and relative root/shoot ratio in seedlings grown in nutrient solution for 5 days and total root length, total surface area, mean diameter, number of root tips, and leaf fresh weight in seedlings grown for 5 days in agar with or without 1 mM NO\(_3^-\). *Supplementary Table S1.* List of genes analysed by real-time quantitative PCR. *Supplementary Table S2.* Merged effects of different chemicals interfering with NO biosynthesis/scavenging and nitrate supply/depletion on root and leaf fresh weight.
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