Nitrate inhibits primary root growth by reducing accumulation of reactive oxygen species in the root tip in Medicago truncatula

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Abstract

In *Medicago truncatula*, nitrate, acting as a signal perceived by NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER FAMILY 6.8 (MtNPF6.8), inhibits primary root growth through a reduction of root cell elongation. Since reactive oxygen species (ROS) produced and converted in root tip ($O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH$) have been reported to control cell elongation, the impact of nitrate on the distribution of these ROS in the primary root of *M. truncatula* was analyzed. We found that nitrate reduced the content of $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ in the root tip of three wild type genotypes, sensitive to nitrate (R108, DZA, A17), inhibition of root growth and $O_2^{\bullet-}$ accumulation being highly correlated. Nitrate also modified the capacity of R108 root tip to produce or remove ROS. The ROS content decrease observed in R108 in response to nitrate is linked to changes in peroxidase activity (EC1.11.1.7) with an increase in peroxidative activity that scavenge H_2O_2 and a decrease in hydroxylic activity that converts H_2O_2 into $^{\bullet}OH$. These changes impair the accumulation of H_2O_2 and then the accumulation of $^{\bullet}OH$, the species responsible for cell wall loosening and cell elongation. Accordingly, nitrate inhibitory effect was abolished by externally added H_2O_2 or mimicked by KI, an H_2O_2 scavenger. In contrast, nitrate has no effect on ROS production or removal capacities in *npf6.8-2,* a knockdown line insensitive to nitrate, affected in the nitrate transporter MtNPF6.8 (in R108 background) by RNAi. Altogether, our data show that ROS are mediators acting downstream of MtNPF6.8 in the nitrate signaling pathway.

Keywords: cell wall peroxidases, *Medicago truncatula*, NADPH oxidase (RBOH), nitrate signal, primary root, reactive oxygen species (ROS), superoxide dismutase

Highlights

1- Nitrate triggers a decrease in ROS content in primary root tip of sensitive genotypes

2- Nitrate inhibition of both root growth and $O_2^{\bullet -}$ accumulation are correlated

3- The decrease in ROS induced by nitrate is orchestrated by cell wall peroxidase

4- Nitrate has no effect on ROS content in primary root tip of *npf6.8-2* knockdown line

5- ROS mediate the nitrate signal downstream of the nitrate transporter MtNPF6.8

Abbreviations

ABA	Abscisic acid
DAB	Diaminobenzidine
DEPMPO	5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide
DHR	Dihydrorhodamine
DMSO	Dimethyl sulfoxide
DPI	Diphenyleneiodonium
DW	Dry weight
EPR	Electron paramagnetic resonance
FW	Fresh weight
H_2O_2	Hydrogen peroxide
MS	Murashige and Skoog
NBT	Nitroblue tetrazolium
$O_2^{\bullet -}$	Superoxide anion radical
$^{\bullet}OH$	Hydroxyl radical
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
XTT	2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

1. Introduction

Plants have the ability to respond to the changing environment through phenotypic plasticity. Notably, the root system is able to sense nutrient availability in soil and adapt its development accordingly (Motte et al., 2019). Nitrogen is an important nutrient for plants that is preferentially assimilated in the form of nitrate (NO_3^-) (Krapp, 2015). The response of the root system to nitrate has been mostly studied in *Arabidopsis thaliana*. In this species, nitrate was shown to have an effect on both primary and lateral root growth (Krapp, 2015; Zhao et al., 2018). However, this effect could be complex, leading to opposite results depending on the studies, performed with different nitrate concentrations and distributions in the medium, using different genotypes at different stages of development (Signora et al., 2001; Walch-liu and Forde, 2008).

In fact, nitrate was shown to act as a signal, sensed and transduced by nitrate transporters, as recently reviewed by Pellizzaro et al. (2017) and Zhao et al. (2018). How the nitrate signal is transduced downstream of the nitrate transporters acting as sensors is not totally understood. Pioneer works in *A. thaliana* have shown that hormone transport is among the events involved in response to nitrate (O'Brien et al., 2016). AtNPF6.3, a nitrate transporter belonging to the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY (NPF), is able to transport auxin in addition to nitrate, and to modify auxin distribution in lateral roots in a nitrate concentration dependent manner, thus affecting auxin gradient in the root, and consequently root growth. Furthermore, some NPF transporters such as AtNPF4.6 in *A. thaliana* have been shown to transport ABA as well (Kanno et al., 2012). ABA, which was proposed to play a role in mediating nitrate effects on lateral root development, was shown to relieve the inhibitory effect of nitrate on lateral root growth (Nacry et al., 2013). Besides hormones, transcription factors, protein kinases, or molecular components belonging to epigenetic mechanisms, have further also been identified as being involved in nitrate signaling in *A. thaliana* (Nacry et al., 2013).

The effect of nitrate on seedling establishment was less studied in legume than in non-legume species *e.g. A. thaliana.* However*,* legumes deserve such an attention because they play an important role in human and livestock alimentation due to their high level of proteins in seeds and aerial parts used as forage (Maphosa and Jideani, 2017). Through their capacity to establish symbiotic interaction with rhizobia to fix

atmospheric N_2, they also provide increasing ecosystemic services in cropping systems contributing to nitrogen enrichment of soils and thereby to sustainable agriculture. In legumes, not only root growth but also symbiosis interactions are controlled by nitrate. Nitrate has opposite effects on these two processes: while it favors seedling anchorage before the biological nitrogen fixation takes place through symbiosis, it impairs symbiotic interaction (Ferguson et al., 2019).

The fact that the root apex is a zone where different environmental stimuli, including nitrate, are perceived and integrated (Baluška and Mancuso, 2013) makes the root growth response to nitrate interesting to decipher in legumes. The sensitivity to nitrate of the early root development was reported for different genotypes of the model legume *Medicago truncatula* (Bagchi et al., 2012; Morère-Le Paven et al., 2011; Yendrek et al., 2010). Yendrek et al. (2010) also showed that the nitrate transporter MtNPF1.7, known as LATERAL ROOT ORGAN DEFECTIVE/NUMEROUS INFECTIONS and POLYPHENOLICS (LATD/NIP), is involved in the development of the root system in *M. truncatula*. In the same species we have shown that another nitrate transporter, MtNPF6.8, acts as a nitrate sensor involved in the reduction of the primary root growth due to a reduction of cell elongation, *npf6.8* knockdown lines generated by RNAi being insensitive to nitrate (Pellizzaro et al., 2014). Because exogenous ABA treatment restored the growth inhibitory effect of nitrate in *npf6.8* lines, we proposed that ABA acts downstream of MtNPF6.8 in this nitrate-signaling pathway. Moreover, ABA was shown to be transported by MtNPF6.8 (Pellizzaro et al., 2014). Whether the transduction of the nitrate signal requires such a transport is not known.

Our aim is to decipher the nitrate signaling pathway in *M. truncatula* primary root through the identification of mediators downstream of MtNPF6.8 and ABA. In the present work, we have addressed the possibility that reactive oxygen species (ROS) are among these mediators since ROS are not only able to transduce ABA signal in guard cells and roots (Kwak et al., 2003; Singh et al., 2017) but also govern several aspect of plant development including root growth (Foyer and Noctor, 2016; Mittler, 2017). Indeed, in the apoplast and cell wall of the roots of *A. thaliana* and maize, three main ROS, superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), were demonstrated to either promote growth through cell division in the meristem zone ($O_2^{\bullet -}$) and cell elongation in the elongation zone ($^{\bullet}OH$)

through cell wall loosening or, in contrast, restrict growth in the differentiation zone (H_2O_2) through cell wall stiffening (Dunand et al., 2007; Liszkay et al., 2004; Tsukagoshi et al., 2010).

To test whether ROS mediate the nitrate signal in the primary root of *M. truncatula*, we took advantage of the availability in this species of the *npf6.8* knockdown lines, which are not sensitive to nitrate. We determined the impact of nitrate on the abundance of ROS ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$) in the primary root of R108, DZA315-16 and A17, three wild type genotypes of *M. truncatula* as compared to *npf6.8-2*. We found that nitrate hampers the accumulation $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ in the root tip of the wild type genotypes having different sensitivities to nitrate whereas it does not modify the accumulation of ROS in the primary root of the insensitive *npf6.8-2*. The decrease in ROS abundance induced by nitrate in the wild type genotypes was accompanied by a modification of the capacity of the enzymes responsible for the production or the removal of ROS in the primary root tip.

2. Materials and methods

2.1 Plant materials and growth conditions

Three wild type genotypes of *M. truncatula* R108, DZA315.16 (DZA) and A17, and an RNAi knockdown line in the R108 background, *npf6.8*-2 (Pellizzaro et al., 2014), were used in this study.

Seeds scarified with an abrasive paper were surface-washed with sterilized water and then imbibed on a sterilized filter paper in a Petri dish (Φ= 90 mm) containing 3.5 mL of N-free Murashige and Skoog (MS) medium at 4°C in darkness for 4 d, as described by Pellizzaro et al. (2014). After this step of cold stratification to homogenize further seed germination, the Petri dish was incubated at 20°C for seed germination. Then, germinated seeds with a radicle of about 1 cm long were transferred for growth for 2 to 10 d on a sterilized filter paper in a square transparent plate (12.4 cm x 12.4 cm) containing 8 mL of either N-free MS solution or MS solution supplied with 5 mM NO_3^-. For some experiments, 50 µM H_2O_2 or 100 µM KI were also added in the MS solution. The plate was inclined at a 45° angle on a rack under a 16-h-light/8-h-dark photoperiod with a light intensity of 45 $\mu mol.m^{-2}.s^{-1}$ at 20°C in a growth chamber (the watering being regularly readjusted). For phenotypic analysis, primary root growth was scored by marking the plate cover every day after transfer.

Then, the plate covers were scanned into images (Scanner Sharp MX-4140) and primary root lengths were determined on the corresponding images using ImageJ software (Version 1.4.3.67).

2.2 ROS detection and measurement

2.2.1 $O_2^{\bullet-}$ detection and measurement

$O_2^{\bullet-}$ was detected by nitroblue tetrazolium (NBT; Sigma-Aldrich), which is reduced by $O_2^{\bullet-}$ in purple diformazan, using a protocol based on Zhang et al. (2014). Whole roots of seedlings grown for 2 to 10 d were stained in 20 mM phosphate buffer (pH 6.0) containing 3 mM NBT for 10 min in darkness. The reaction was stopped by transferring the seedlings in 80% (v/v) ethanol. The stained roots were then immediately observed with an Olympus stereomicroscope and images were taken with a HD camera. All the same setting parameters were kept for all the images in an experiment.

A method adapted from Ramel et al. (2009) was used for quantitative $O_2^{\bullet-}$ measurement, the tips of the roots stained in purple were collected in a 1.5 mL microtube containing 120 µL of 2 M KOH and then ground after addition of 140 µL of dimethyl sulfoxide (DMSO). After centrifugation for 10 min at 12,000 x g, the supernatant containing diformazan was introduced in a well of a microplate. A blank control was set with an extract of unstained root tips. Absorbance was measured at 630 nm with a spectrophotometer (Spectrostar, BMG LABTECH, Germany). The superoxide content was determined by comparison with a standard curve plotted with known quantities of NBT in a mix of 2 M KOH/DMSO (1/1.16, v/v). The values are expressed in nmol of $O_2^{\bullet-}$ produced per g of fresh weight (FW).

2.2.2 H_2O_2 detection and measurement

H_2O_2 was detected with 3, 3′-diaminobenzidine (DAB, Sigma-Aldrich) according to a method adapted from Zhang et al. (2014). Whole roots were incubated for 1 h in a solution of 1 $mg.mL^{-1}$ DAB in 50 mM citric acid (pH 3.5) prepared one day before, then washed with distilled water. Images were captured as previously described for NBT staining.

The abundance of H_2O_2 in the tip was measured using luminol (5-amino-2, 3-dihydro-1, 4-phthalazidone) with a method adapted from Lu et al. (2009) and Noctor

et al. (2016). Frozen root tips (20 mg) were ground in liquid nitrogen and homogenised in 500 µL of 5% trichloroacetic acid containing 5% of insoluble polyvinylpolypyrrolidone (w/v). The samples were centrifuged at 13,000 x g for 10 min. After a 100 times dilution in 0.1 M sodium carbonate buffer (pH 10.2), aliquots of 40 µL were incubated at 30°C for 15 min with 10 µL of distilled water or 10 µL of a solution containing 50 U of bovin liver catalase (SIGMA-Aldrich) for control experiments. After this incubation, 10 µL of each aliquot treated or not with catalase, were placed into wells of a microplate (white polypropylene MicroWell® plate, NUNC®, round bottom). The reaction started with addition of 250 µL of reaction mixture solution composed of 65 µM of luminol and 10 µM of cobalt chloride in 0.1 M sodium carbonate buffer (pH 10.2). The luminescence was measured over 5 s in a microplate reader (Fluostar OMEGA®, BMG Labtech®). The H_2O_2 was quantified using a standard curve realized with 0 to 50 pmol of H_2O_2 prepared freshly by dilution of H_2O_2 in trichloroacetic acid then sodium carbonate as above for the samples. Root specific H_2O_2 content was determined by the difference between the values obtained with and without catalase and was expressed as $\mu mol.g^{-1}$ FW.

2.2.3 Detection of $^{\bullet}OH$

Dihydrorhodamine 123 (DHR, Sigma-Aldrich) was used to detect $^{\bullet}OH$ (Porwol et al., 1998). DHR was first solubilized in DMSO and then diluted to a concentration of 25 µM in 20 mM phosphate buffer (pH 6.0). The whole roots were stained for 30 min, then washed three times with distilled water and observed with an Olympus stereomicroscope using the green filter with excitation and emission wavelengths of 500 nm and 536 nm, respectively. Images were immediately taken with the HD camera. ImageJ software (Version 1.4.3.67) was used to quantify in pixels the fluorescence intensity of DHR in root tips. The relative fluorescence intensity is expressed in an arbitrary unit, using a value of 1 for R108 grown in MS without nitrate. Four measures were performed for each root tip.

2.3 Enzyme activity measurement

2.3.1 NADPH oxidase activity

Total proteins from root tips were extracted in 1 mL of 50 mM Tris-HCl buffer (pH 7.5). After centrifugation at 15,000 x g at 4°C for 15 min to remove cell debris, the activity of NADPH oxidase (EC1.6.3.1), also known as respiratory burst oxidase

homolog (RBOH) was measured in the supernatant according to Sagi and Fluhr (2001) using XTT (2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, tetrazolium salt). The reaction mixture contained 0.3 mM XTT, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.3 mM NADPH supplemented or not with 50 µM diphenyleneiodonium (DPI) to inhibit NADPH oxidase or 1 KU of horse radish superoxide dismutase (SOD ; SIGMA-Aldrich) to remove $O_2^{\bullet-}$. Absorbance was measured at 470 nm. The difference in the rate of XTT oxidation measured in the absence or the presence of DPI, an inhibitor of NADPH oxidase, corresponds to NADPH oxidase activity that was expressed in nmol of $O_2^{\bullet-}$ produced $min^{-1}.mg^{-1}$ FW. This part of XTT oxidation sensitive to DPI, corresponding specifically to NADPH oxidase activity, was similar to the part abolished by the horse radish SOD. This indicates that peroxidase (POD) does not contribute to $O_2^{\bullet-}$ production through its oxidative activity in *M. truncatula* primary root tip as it was the case in maize (Liszkay et al., 2004).

2.3.2 SOD and POD activities

For SOD (EC1.15.1.1) and POD (EC1.11.1.7) activities, cell walls were first isolated from the root tips as previously described by Kukavica et al. (2012). Briefly, the root tips were ground in liquid nitrogen, then in a buffer solution containing 50 mM Tris-HCl (pH 7.2), 50 mM NaCl, 0.05% Tween-80 and 1 mM phenylmethylsulfonyl fluoride, and the extract was centrifuged at 1,000 x g for 20 min at 4°C. The fragments of the cell walls present in the pellet were washed four times by resuspension in 50 mM Tris-HCl (pH 7.2), and centrifugation at 4°C for 20 min at 1,000 x g or 15,000 x g for the final step of centrifugation. For some experiments, proteins bound to the cell walls were isolated by resuspension and solubilization of the cell wall fragments in 50 mM Tris-HCl, pH 7.2, containing 0.12 mg mL^{-1} cellulase and 0.6 mg mL^{-1} pectinase for 24 h at 4°C, and centrifugation at 15,000 x g for 15 min at 4°C. Then, enzyme activities were measured.

SOD activity was determined in the fraction of proteins isolated from the cell walls by the method of Gill et al. (2015) with some modifications. The reaction mixture contained 75 mM phosphate buffer (pH 7.8), 15.6 mM methionine, 15.6 µM riboflavin and 80 µM NBT. Absorbance was measured at 560 nm. SOD activity was determined in $\mu U.mg^{-1}$ FW by comparison with a standard curve realized with horse radish SOD.

Two different capacities of POD were measured, the capacities to remove H_2O_2 or to convert H_2O_2 in $^{\bullet}OH$. For the capacity to remove H_2O_2, the fraction of proteins isolated from the cell walls was used. The reaction mixture contained 200 mM phosphate buffer (pH 7.0), 50 mM o-diasinidine and 37.5 mM H_2O_2. Absorbance was measured at 430 nm. POD activity was expressed in nmol of H_2O_2 reduced $min^{-1}.mg^{-1}$ FW. The capacity to convert H_2O_2 in $^{\bullet}OH$ was determined by electron paramagnetic resonance (EPR) spectroscopy (Mojović et al., 2004) using the cell wall fraction and the spin-trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO; Alexis Biochemical, Lausen, Switzerland). To monitor $^{\bullet}OH$ production, each cell wall fraction was resuspended in 50 mM Tris–HCl (pH 7.2) with the ratio of 1.0 mg / 30 µL supplemented with 42.5 mM DEPMPO and placed into a gas-permeable Teflon tube (Zeus Industries, Inc.). The EPR signal of the DEPMPO/OH adduct (generated in the presence of $^{\bullet}OH$) was recorded using an X-band Bruker Elexsys II E540 EPR spectrometer at room temperature under the following parameters: center field 3483.30 G, sweep width 241.2 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave frequency 9.77 GHz, microwave power 10 mW and conversion time 58.59 ms, 20 scan accumulations. The reference DEPMPO/OH spin adduct was produced by $^{\bullet}OH$ generating system (Mojović et al., 2004). EPR signals of DEPMPO/OH adduct were analyzed using ELEANA software (v2.8). Spectral simulations of DEPMPO/OH adduct were performed with SciDAVis software (v1.23). At the end of the assays, the cell wall suspensions were dried and the $^{\bullet}OH$ production was expressed in arbitrary units per g (DW) of cell wall.

2.4 Number of replicates and statistical analysis of the data

Each experiment was independently repeated at least three times at different dates. For root morphology characterization or root staining, at least 10 and often 20 seedlings were used by repeat. Calculated means are represented ± SD. For all data, relevant statistical tests were performed and described in the legend of each figure. All statistical tests were carried out using R software (version 1.0.143). Shapiro and Bartlett tests were performed to verify the normality of the data and the homogeneity of the variances, respectively. Once these parameters verified, an ANOVA was performed to highlight significant differences between the mean values (*p* value lower than 0.05).

3. Results

3.1 Nitrate modifies root architecture and primary root tip morphology of sensitive seedlings

In a previous study performed with *M. truncatula*, we have shown that nitrate signal perceived by the nitrate transporter MtNPF6.8 restricts primary root growth of the R108 genotype but not of the *npf6.8* RNAi lines (*npf6.8-2*, *npf6.8-3*, *npf6.8-5*) affected in *MtNPF6.8* expression in the R108 background (Pellizzaro et al., 2014). In the present study, we confirmed the root sensitivity of R108 and the root insensitivity of *npf6.8-2* to nitrate, the root architecture of *npf6.8-2* being similar to that of R108 grown in absence of nitrate (Fig. 1). We also compared the phenotype of the wild type R108 with the phenotype of two other wild type genotypes, DZA 315-16 (DZA) and A17, to better characterize the nitrate sensitivity of the roots in *M. truncatula* (Fig. 1). The results highlight a significant variability in both root performance and root nitrate sensitivity in this species. At 10 d and in the absence of nitrate, the primary root length of R108, DZA and A17 were 5.7, 8.5 and 7.0 cm, respectively. Thus, regarding primary root growth in absence of nitrate, DZA appears as the most performing genotype while R108 is the least performing genotype. In presence of 5 mM NO_3^-, the primary root growth was reduced by 19.2%, 14.9% and 23.5% for R108, DZA and A17, respectively. Thus, A17 is the more sensitive genotype while DZA is the least sensitive genotype.

The impact of nitrate on the development and the growth of lateral roots was also characterized (Fig. 1C and D). At 10 d and in the absence of nitrate, the lateral roots had a cumulative length of 0.6, 0.4 and 0.9 cm and developed at a distance from the primary root cap of 2.5, 3.6 and 2.2 cm in R108, DZA and A17, respectively. Thus, DZA had the shortest lateral roots that developed at the longest distance from the primary root cap while A17 has the longest lateral roots that developed at a distance from the primary root cap similar to that of R108. In the presence of 5 mM NO_3^-, the cumulative lateral root length was not modified in DZA while it increased similarly in R108 (+ 40%) and A17 (+ 44%). The distance from the lateral roots to the primary root cap highly decreased in the three genotypes: 70% (R108), 68% (A17) and 56% (DZA). Therefore, A17 appears as the most performing genotype while DZA appears as the least performing genotype regarding the lateral root development and growth both in the absence or the presence of nitrate.

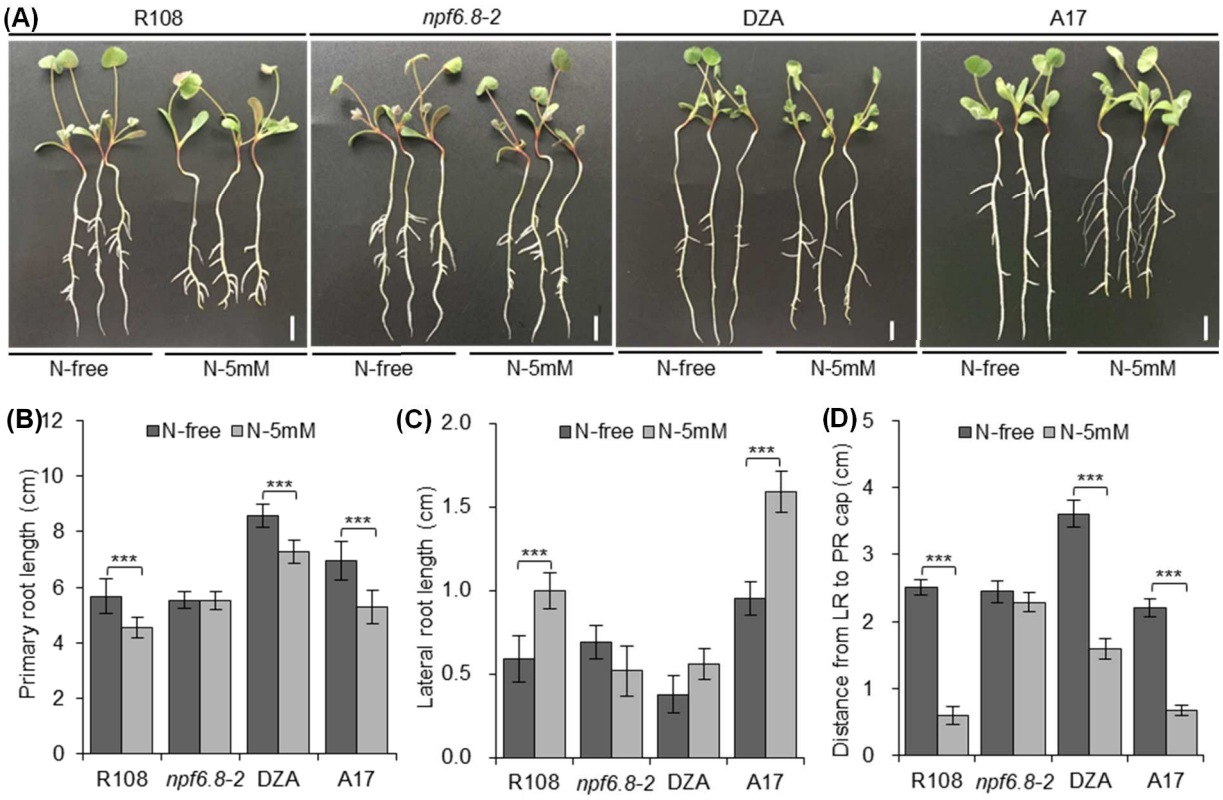


Fig. 1 Nitrate modifies the architecture of the root system of the sensitive genotypes. (A) Seedlings of R108, *npf6.8-2*, DZA 315-16 (DZA) and A17 grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 10 d. Scale bar = 1 cm. (B) Primary root length. (C) Lateral root length. (D) Distance from lateral root to primary root cap. Length and distance were measured using ImageJ software. Asterisks indicate a significant difference. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; ***, $p<0.001$.

We analyzed the morphology and determined the length of the primary root tip, comprising the zones of cell division and cell elongation, in the wild type genotypes and the *npf6.8-2* knockdown line. The tip was recognized by its shape and lack of hairs. In the absence of nitrate, the tip was thin and long, about 2 to 2.5 mm, the most performing wild type genotypes (DZA and A17, Fig. 1A and B) having interestingly the longest tip (Fig. 2) as compared with R108. In the presence of 5 mM NO_3^-, the tip was thicker and its length was reduced by about 50% for DZA, the least sensitive genotype, or by more than 60% for R108 and A17, the most sensitive genotypes. In the presence of nitrate, the mature part of the root was thicker as well (Fig. 2A). On the contrary, the morphology of the primary root tip in *npf6.8-2*, insensitive to nitrate, was not changed by nitrate, the tip remaining thin and long whatever the growth conditions, as for R108 cultivated in N-free medium (Fig. 2).

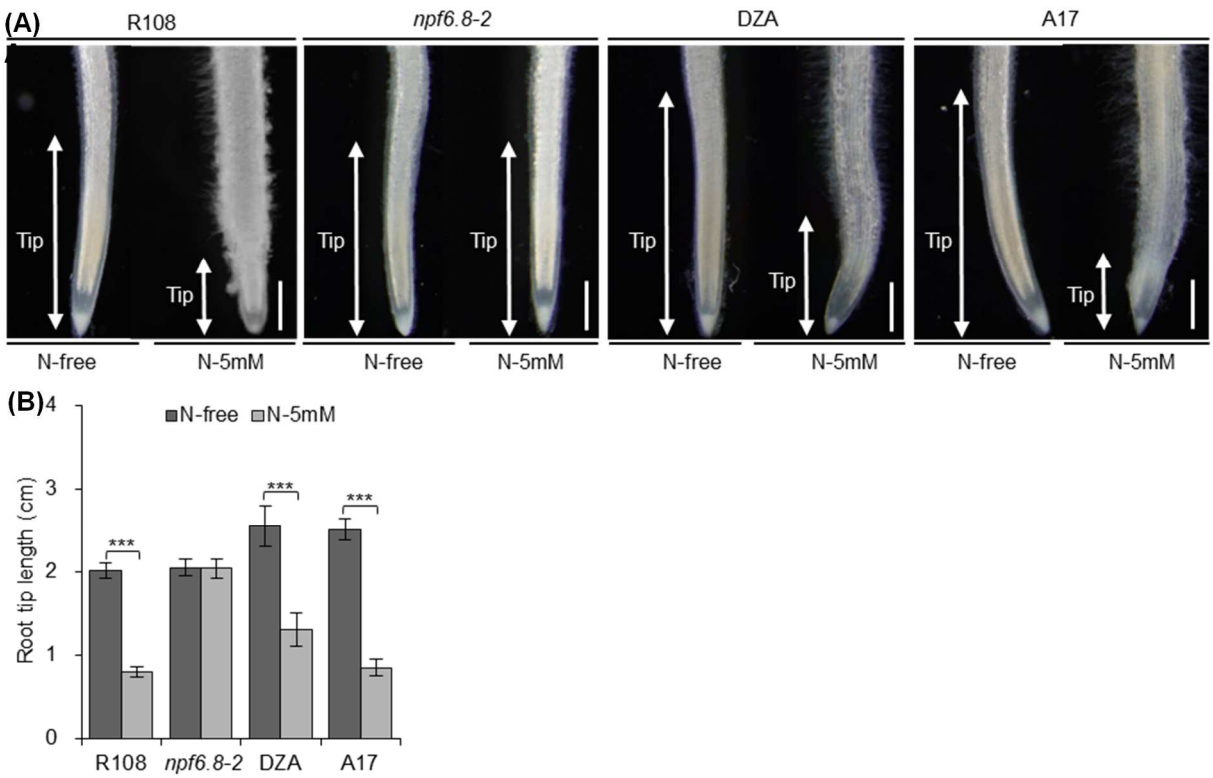


Fig. 2 Nitrate modifies the primary root tip morphology of the sensitive genotypes. Seedlings of R108, *npf6.8-2*, DZA and A17 were grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 10 d. **(**A) Primary root tip. Scale bar = 500 µm. (B) Primary root tip length. Asterisks indicate a significant difference. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; ***, $p<0.001$.

3.2 Nitrate sensitivity takes pla□e during early primary root growth

To determine when the nitrate sensitivity takes place in the root, we recorded the primary root length of R108 during the growth from 1 d to 10 d. No differences were observed in the length until 5 d of growth in the absence or the presence of 5 mM NO_3^- (Fig. 3). The inhibition of root growth induced by nitrate was significant only after 6 d. This is also true for A17 (Fig. S2).

To test whether the growth inhibition induced by nitrate is reversible, seedlings grown with 5 mM NO_3^- for 6 d were transferred to a medium without nitrate and grown until 10 d. In these conditions, the primary root length of R108 measured at 10 d was about 5.1 ± 0.6 cm. It is longer than that of the seedlings grown with 5 mM NO_3^- for 10 d (4.6 cm ± 0.3 cm) and shorter than that of the seedlings grown without nitrate for 10 d (5.7 cm ± 0.6 cm), showing a recovery of a higher growth rate after the removal of nitrate. Similar observations were made for A17 (Fig. S2). These results clearly show that the inhibition exerted by nitrate is reversible.

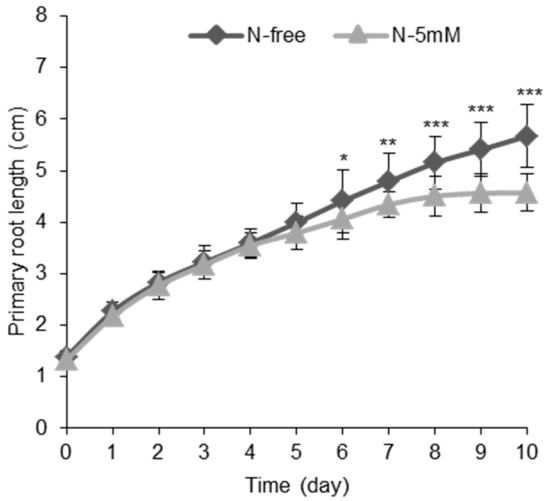


Fig. 3 Nitrate sensitivity of R108 primary root growth takes place during early growth. Seedlings of R108 were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) and the primary root length was recorded from 1 to 10 d. Asterisks indicate when the root length determined at a given day is significantly different between the two conditions of growth. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$.

3.3 Nitrate reduces ROS accumulation in the primary root tip of 10-d-old sensitive seedlings

We wondered whether the nitrate sensitivity is mediated by ROS in the tip of *M. truncatula*. To test this hypothesis, we took advantage of the availability of our *npf6.8* knockdown lines which are not sensitive to nitrate. We analyzed ROS accumulation in the primary root tip of the sensitive genotypes R108, DZA, A17 and compared the results with those obtained with the *npf6.8-2* line. For this purpose, we used methods either recommended by researchers well known in the field such as the use of luminol for H_2O_2 measurement (Noctor et al., 2016) or EPR for $^{\cdot}OH$ production (Liszkay et al., 2004; Mojović et al., 2004) or previously used with *A. thaliana* (Dunand et al., 2007) and *M. truncatula* (Zhang et al., 2014) roots for comparison.

3.3.1 Nitrate reduces $O_2^{\bullet-}$ accumulation

We used NBT to detect $O_2^{\bullet-}$ in the primary root of R108, DZA, A17 and *npf6.8-2*. In the absence of nitrate, NBT (reduced in purple diformazan by $O_2^{\bullet-}$) mainly stained the tip (Fig. 4A). The stained zone seems slightly longer in DZA than in the other genotypes suggesting that DZA is able to produce more $O_2^{\bullet-}$ than the other

genotypes. Consistently, the amount of $O_2^{\bullet-}$ quantified after extraction of diformazan from the tips was about 1.6 nmol.g^{-1} FW for R108, *npf6.8-2* and A17, and 1.8 nmol.g^{-1} FW for DZA (Fig. 4B). Interestingly, in the presence of nitrate, $O_2^{\bullet-}$ accumulation in the tip was reduced (Fig. 4A and B) either strongly for A17 (-74%), and R108 (-51%) or to a lesser extent for DZA (-35%) but not in *npf6.8-2*.

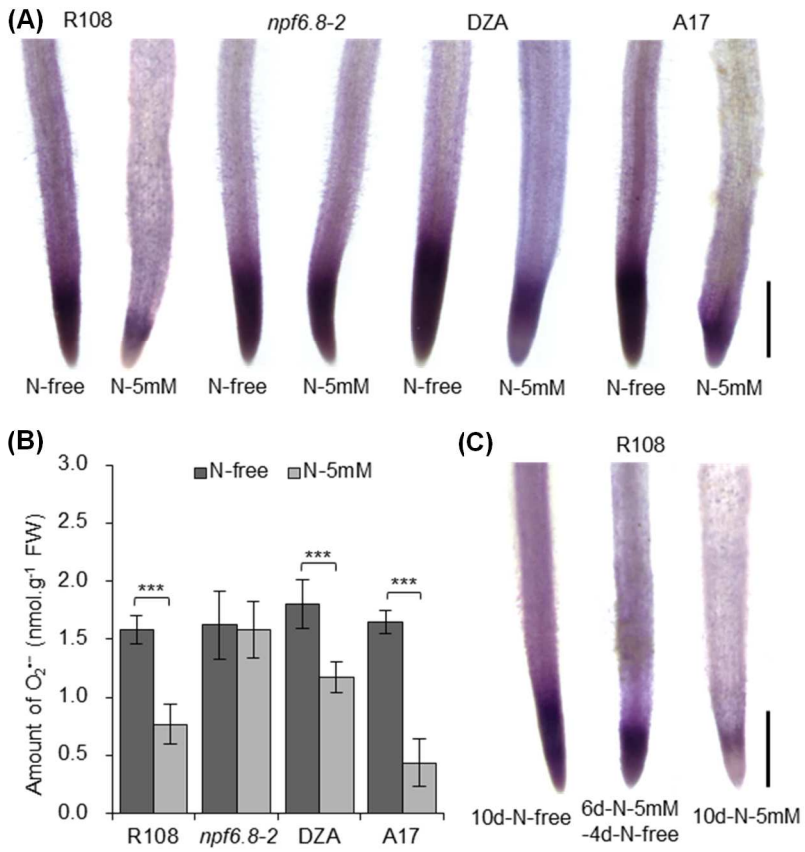


Fig. 4 Nitrate reduces $O_2^{\bullet-}$ accumulation in the primary root tip of the sensitive genotypes. Seedlings of R108, *npf6.8-2*, DZA and A17 were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM). (A) Detection of $O_2^{\bullet-}$ in the primary root tip after 10 min of staining with nitroblue tetrazolium (NBT). (B) Quantification of $O_2^{\bullet-}$ in the root tip. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; ***, p<0.001. (C) Detection of $O_2^{\bullet-}$ in seedlings of *M. truncatula* R108, grown in MS without nitrate (10d-N-free), MS with 5 mM nitrate (10d-N-5mM) for 10 d or in MS with 5 mM nitrate for 6 d then and in MS without nitrate for 4 d (6d-N-5mM-4d-N-free), stained with NBT. Scale bar = 1 mm.

These results raised the question whether the inhibition of the primary root growth induced by nitrate could be linked to the removal of $O_2^{\bullet-}$ in the tip. To test this hypothesis, we plotted the percentage of growth inhibition as a function of the percentage of inhibition of $O_2^{\bullet-}$ accumulation induced by nitrate. Figure 5 clearly demonstrates that the genotype which was most sensitive to nitrate (A17) showed the highest $O_2^{\bullet-}$ reduction and conversely. The extent of nitrate inhibition on primary

root growth is tightly linked to the extent of inhibition of $O_2^{\bullet-}$ accumulation induced by nitrate (R^2 = 0.96; Fig. 5).

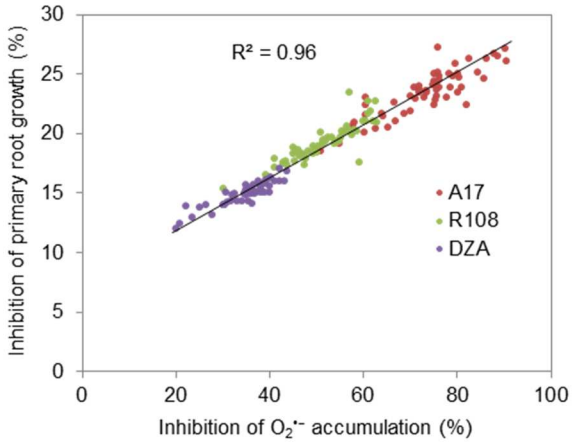


Fig. 5 Inhibition of primary root growth and $O_2^{\bullet-}$ accumulation induced by nitrate are correlated in the sensitive genotypes. Percentage of inhibition of root growth is plotted as a function of percentage of inhibition of $O_2^{\bullet-}$accumulation.

We further addressed the question whether the resume of the primary root growth of R108 in the recovery experiment (Fig. 3) was accompanied by a resume of $O_2^{\bullet-}$ accumulation. Interestingly, the recovery of a thin and long root tip was associated with an increase in $O_2^{\bullet-}$ detected in the tip using NBT (Fig. 4C). The increase was quantified as above after diformazan extraction from the tip. $O_2^{\bullet-}$ amount reached 1.2 nmol.g^{-1} FW after 6 d of growth with nitrate then 4 d without nitrate, a value intermediary between 1.6 and 0.8 nmol.g^{-1} FW after 10 d of growth without or with nitrate, respectively.

Because the three wild types seemed to respond similarly to nitrate, the sensitivity of the primary root taking place after 6 d of growth (Fig. 3 and S2) and being associated with a reduction of $O_2^{\bullet-}$ accumulation (Fig. 4 and 5), we further focused our study on R108 and *npf6.8-2* that have the same genetic background.

3.3.2 Nitrate reduces H_2O_2 accumulation

We used DAB to detect H_2O_2 in the primary root of R108 and *npf6.8-2* (Fig. 6A), a product previously used for roots of *M. truncatula* (Zhang et al., 2014). In the absence of nitrate, DAB mainly stained the primary root tip. This was particularly obvious in younger seedlings (see below 2-d-old seedlings, Fig. S3). The

accumulation of H_2O_2 in the tip at 10 d was confirmed using luminol (Fig. 6B), a product recommended by Noctor et al. (2016). H_2O_2 was also found to be present but to a lesser extent in the mature part of the root (Fig. 6A), the root hairs being notably well stained with DAB. In the presence of 5 mM NO_3^-, the abundance of H_2O_2 decreased in the tip (Fig. 6A and B) while it increased in the mature part of the root of R108 (Fig. 6A). In comparison, nitrate had no impact on H_2O_2 distribution in the primary root of *npf6.8-2* whatever the zone (Fig. 6A and B) which is similar to that in the root of R108 grown without nitrate.

3.3.3 Nitrate decreases $^{\bullet}OH$ accumulation

We used the fluorescent probe DHR to detect $^{\bullet}OH$ in the primary root of R108 and *npf6.8-2*, a product previously used with animal cells (Porwol et al., 1998). In the absence of nitrate, in both genotypes, $^{\bullet}OH$ was mainly distributed in the primary root tip (Fig. 6C and D), with a pattern similar to $O_2^{\bullet-}$ (Fig. 4A). In the presence of nitrate, this zone was highly reduced for R108 but not for *npf6.8-2* (Fig. 6C). Quantification of DHR fluorescence intensity in the primary root tip confirmed these observations (Fig. 6D).

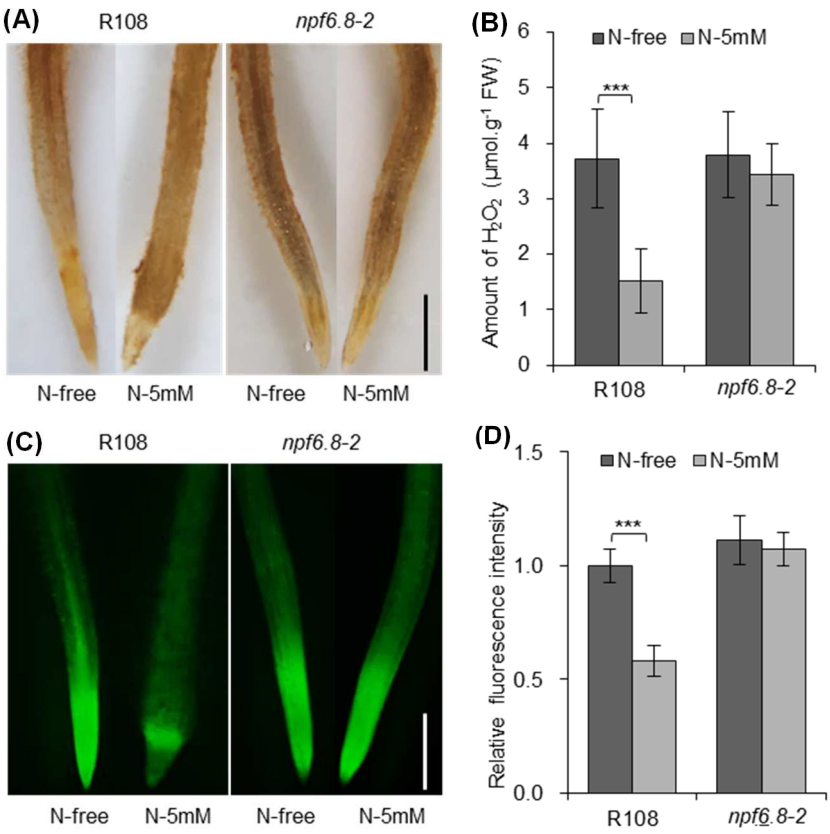


Fig. 6 Nitrate reduces the accumulation of H_2O_2 and $^{\bullet}OH$ in the primary root tip of the R108 sensitive genotype. Seedlings of R108 and *npf6.8-2* were grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 10 d. (A) Detection of H_2O_2 in the primary root after 40 min of staining with 3,3′-diaminobenzidine (DAB). (B) Quantification of H_2O_2 in the primary root tip with luminol. (C) Detection of $^{\bullet}OH$ in the primary root after 30 min of staining using dihydrorhodamine (DHR). (D) Quantification of DHR fluorescence intensity in the primary root tip. Quantification was realized using high resolution images and ImageJ software. Asterisks indicate a significant difference. Scale bar = 1 mm. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; ***, $p<0.001$.

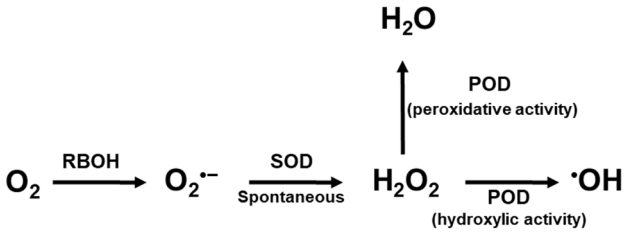
3.4 ROS pattern is not modified by nitrate in the tip of primary root at 2 d or lateral roots at 10 d

As shown above, nitrate had no impact on the phenotype of the primary root of 2-d-old seedlings of the wild type genotypes R108, DZA and A17 (Fig. 3, Fig. S1 and S2). We wondered whether nitrate modifies the ROS accumulation pattern in the tip at this stage of primary root growth. We used R108 and *npf6.8-2* to address this question. Although no difference between the two genotypes and no change in ROS abundance could be observed in response to nitrate (Fig. S3), the amounts of $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ determined in the tip at 2 d were higher (about 1.5 to 2 times higher) than at 10 d (Fig. 4 and Fig. 6). In contrast, it was lower in the mature part of the root at 2 d as compared with 10 d, according to the DAB staining (Fig. 6; Fig. S3).

The pattern of ROS was also observed at 10 d in the lateral roots of R108, DZA and A17, whose number and/or length increased in the presence of nitrate (Fig. 1). This pattern was similar to that reported for the primary root grown in absence of nitrate but was not altered by the presence of nitrate as it is well visible for R108 roots stained with NBT (Fig. S4).

3.5- Nitrate modifies the capacity of the primary root tip to produce or remove ROS

The decrease in ROS accumulation induced by nitrate could be due to a decrease in ROS production and/or an increase in ROS removal. Thus, to find out the origin of the decrease in ROS accumulation, we measured, in the primary root tip of the wild type R108 and the *npf6.8-2* knockdown line both grown with or without nitrate, the activity of the enzymes responsible for the production or the removal of ROS in the apoplast and the cell wall, namely NADPH oxidase also known as RBOH, SOD and PODs according to the following scheme:



NADPH oxidase activity, sensitive to DPI, was measured in crude homogenates of root tips while SOD and POD activities were measured in cell wall fractions isolated from the root tips. NADPH oxidase activity was monitored by the oxidation of XTT. In the absence of nitrate, the levels of these enzyme activities interfering with ROS accumulation were found to be similar for the wild type R108 and the *npf6.8-2* knockdown line (Fig. 7). However, in the presence of nitrate, they were altered in R108 but almost unchanged in *npf6.8-2*. In R108, NADPH oxidase activity increased 3.5 times (Fig. 7A), from 35.7 to 124.8 nmol of $O_2^{\bullet-}$ produced per min and mg of FW. SOD activity also significantly increased but to a lesser extend (x 1.15; Fig. 7B), from 39.3 to 45.3 U per mg of FW. POD capacity to remove H_2O_2 increased 3.4 times (from 0.60 to 3.27 nmol H_2O_2 reduced per min and mg of FW) while POD capacity to produce $^{\bullet}OH$ decreased 2.7 times (from 6.9 to 2.6 10^5 arbitrary units per mg of DW).

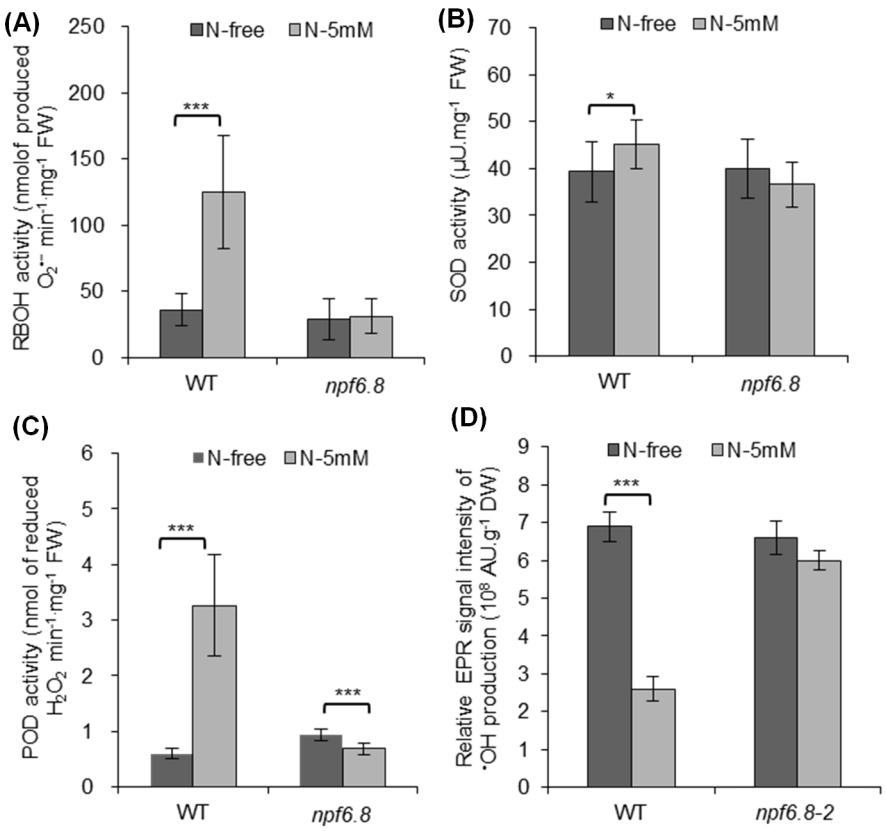


Fig. 7 Nitrate alters RBOH, SOD and POD activities in the primary root tip of the R108 sensitive genotype. Seedlings of R108 and *npf6.8-2* were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM). RBOH was measured in crude root tip homogenates while SOD and POD activities were measured in root tip cell wall isolates. (A) RBOH activity as measured with XTT. (B) Superoxide dismutase (SOD) activity as measured with NBT. (C) Peroxidative activity of class III peroxidases (POD) as measured with o-diasinidine (D) Hydroxylic activity of POD as measured with the level of •OH production by EPR spectroscopy. Asterisks indicate a significant difference. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; *, p<0.05, ***, p<0.001.

3.6 Nitrate inhibitory effe□t is abolished by externally added H_2O_2 or mimi□ked by KI, an H_2O_2 s□avenger

To address a possible central role of the regulation of H_2O_2 abundance in the primary root tip by PODs in response to nitrate, we investigated the effect of a manipulation of H_2O_2 abundance on this response by adding H_2O_2 or KI, a H_2O_2 scavenger (Junglee et al., 2014), to the culture medium, as it was done in the case of *A. thaliana* (Dunand et al., 2007). The addition of 50 □M H_2O_2 completely abolished the inhibitory effect of nitrate on the primary root growth while it had nearly no impact in absence of nitrate (Fig. 8A). Conversely, the addition of 100 □M KI restricted the primary root growth similarly to nitrate (Fig. 8A). These results were in favor of the

hypothesis that the regulation of H_2O_2 content may be important for the primary root response to nitrate. This is also true for lateral roots (Fig. 8B). Indeed, H_2O_2 externally added counteracted the nitrate effect on the lateral roots that did not grow as in absence of nitrate (Fig. 1C) while it had nearly no impact in absence of nitrate (Fig. 8B). In contrast, the addition of KI was accompanied with the development and the growth of lateral roots (Fig. 8B), as in presence of nitrate (Fig. 1C).

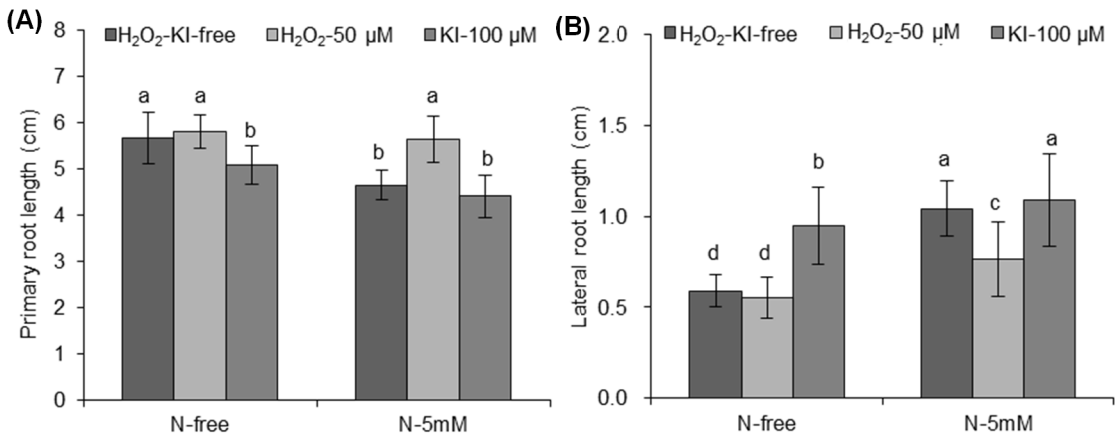


Fig. 8 Manipulation of H_2O_2 abundance can abolish or mimic the nitrate effect on root growth of the R108 sensitive genotype. (A) Primary root length. (B) Lateral root length. Seedlings from R108 were grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) ± 50 µM H_2O_2 or 100 µM KI for 10 d. Primary and lateral root lengths were measured on highly resolved images using ImageJ software. Letters indicate significant differences between the six different conditions compared in A and B. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%.

4. Discussion

4.1 The nitrate sensitivity of the primary root shows a natural variability and is acquired during early growth in *M. truncatula*

Studying how nitrate regulates root growth and nodulation is important in order to be implemented either into genetic or agronomic programs dealing with the interaction of crop legume implantation and nitrate concentration in the fields.

Here, we characterized and compared the sensitivity to nitrate of the root systems of three different wild type genotypes of the model legume *M. truncatula*: R108, DZA and A17 (Fig. 1 to Fig. 3). At 5 mM, nitrate reduced the tip length and the elongation of the primary root of these sensitive genotypes, but in a genotype dependent manner, the most sensitive genotype being A17 and the least sensitive

637 genotype being DZA. Nitrate had an opposite action on the lateral roots, promoting
638 their development and/or growth in a genotype dependent manner, as well, but the
639 most performing genotype was A17 and the least performing genotype was DZA.
640 R108 appeared to have an intermediary sensitivity to nitrate regarding both primary
641 and lateral root growth.

642 Thus, among these three genotypes, DZA appears as a genotype whose root
643 system development is the least affected by the variation of nitrate concentration in
644 the culture medium. Such a low sensitivity to nitrate would be an advantage for a
645 legume crop, leading to a homogenous plant establishment in fields in which nitrate
646 could be extremely variable in space and time. A deeper root system is also favorable
647 to acquire more nitrate, a rather mobile nutrient, in case of nitrate deficiency (Motte et
648 al., 2019). Whether such a low sensitivity of root development to nitrate is
649 accompanied by a low sensitivity of nodulation to nitrate remains to be determined.

650 We further showed that the nitrate sensitivity takes place in the primary root after
651 5 d of growth. Consistently, the tip length and morphology in 2-d-old seedlings were
652 not modified by nitrate (Fig. S1). The growth reduction induced by nitrate is
653 nonetheless reversible by removing nitrate from the growth medium.

654

655 **4.2 Nitrate triggers a decrease in ROS content in the tip of the primary root of**
656 **the sensitive genotypes**

657 ROS production and conversion play a crucial role in root growth (Dunand et al.,
658 2007; Liszkay et al., 2004; Tsukagoshi et al., 2010). Therefore we have characterized
659 the patterns of ROS accumulation in the primary root of *M. truncatula* to determine
660 whether nitrate would have an impact on these patterns. We found that in the wild
661 type genotypes, $O_2^{\bullet -}$ and $^{\bullet}OH$ accumulated in the tip (Fig. 4 and Fig. 6) while H_2O_2
662 accumulated both in the tip and in the mature part of the root, but to a lower extent.
663 When the seedlings were supplied with nitrate (5 mM), the levels of all three ROS
664 dramatically decreased in the root tip of the sensitive genotypes. A strong link was
665 further found to exist between the extent of inhibition of primary root growth and the
666 extent of inhibition of $O_2^{\bullet -}$ accumulation induced by nitrate comparing the three
667 different wild type genotypes (R108, DZA and A17).

On the other hand, nitrate had no impact on the primary root growth or the ROS content of the root tip of the *npf6.8-2* knockdown line. It did not modify the ROS accumulation in the lateral root tip, irrespective to the genotype. This is not surprising since lateral root development and/or growth is favored by nitrate. These results suggest that a functional MtNPF6.8 is necessary for triggering the nitrate-induced decrease in ROS content in the primary root tip and reinforce the hypothesis that ROS contribute to nitrate signaling pathway that control the primary but not the lateral root growth.

Studies on ROS detection in the primary root tip have been carried out with several species such as maize (Liszkay et al., 2004; Trevisan et al., 2018), *A. thaliana* (Dunand et al., 2007; Tsukagoshi et al., 2010), and legumes, the model legume *M. truncatula* (Zhang et al., 2014) and the crop legume *Phaseolus vulgaris* (Montiel et al., 2013). The results regarding $O_2^{\bullet-}$ and H_2O_2 abundance in the tip of growing roots we have obtained here for *M. truncatula* are similar to those previously reported for maize and legumes, including *M. truncatula.* However, they are different from those reported for *A. thaliana.* In the growing primary root of *A. thaliana*, $O_2^{\bullet-}$ mainly accumulated in the root tip while H_2O_2 accumulated in the mature part of the root but not in the tip. Although the difference regarding H_2O_2 accumulation may be linked to the probes used to detect this ROS that were not the same, a difference in H_2O_2 production, conversion or function may exist between species. This idea is supported by the results of the experiments of manipulation of H_2O_2 in which H_2O_2 proved to have opposite effects in *A. thaliana* (Dunand et al., 2007; Tsukagoshi et al., 2010) and in *M. truncatula* (Fig. 8).

In addition, in *M. truncatula*, a tight correlation was found between the inhibition of the primary root growth and the inhibition of $O_2^{\bullet-}$ accumulation in the root tip (Fig. 5). This strengthens the early idea that the root growth performance and the level of $O_2^{\bullet-}$ accumulation in the root tip might be linked (Liszkay et al., 2004). However, in the work performed by Zhang et al. (2014), using the *npf1.7* mutant of *M. truncatula* deficient in the MtNPF1.7 nitrate transporter, the authors made an unexpected observation. As compared with the corresponding wild type A17, this mutant is characterized by a reduction of the primary root growth performance which is associated to an overaccumulation of $O_2^{\bullet-}$ in the root tip, probably due to a debrided NADPH oxidase activity. As an explanation of this apparent negative link between $O_2^{\bullet-}$ accumulation and root growth performance in this mutant, the authors proposed

that a high level of $O_2^{\bullet-}$ would be toxic for root cells and thus detrimental for the primary root growth. They suggested that an optimal level of $O_2^{\bullet-}$ might exist for the performance of the primary root growth. On the basis of the existence of an optimal $O_2^{\bullet-}$ level, we propose that DZA - that produces more $O_2^{\bullet-}$ than A17 and R108 and has longer roots - may produce $O_2^{\bullet-}$ at a level close to the optimal level for *M. truncatula* species while A17 and R108 may produce suboptimal levels of $O_2^{\bullet-}$ (Fig. 4). These results obtained with two different mutants deficient in two different nitrate transporters also show a link between NADPH oxidase activity and nitrate transporter integrity, the activity being debrided in *npf1.7* or no more responding to nitrate in *npf6.8-2*.

4.3 The decrease in ROS induced by nitrate is orchestrated by PODs

To determine the origin of the decrease in ROS content observed in the sensitive seedlings of *M. truncatula* in response to nitrate, we determined the impact of nitrate on the capacity of the primary root tip of R108 and *npf6.8-2* to produce or remove ROS in the apoplast and the cell wall. We found that, in the absence of nitrate, these capacities were similar for the wild type and the *npf6.8-2* knockdown line (Fig. 7). However, in the presence of nitrate, they were altered in R108 but almost not affected in *npf6.8-2.* A determinant change in the capacities of NADPH oxidase and PODs was notably observed.

There is an apparent accordance between the decrease in abundance of H_2O_2 and $^{\bullet}OH$ (Fig. 6) and the change in the capacity of PODs in response to nitrate in the root tip of R108. Thereby the decrease observed in H_2O_2 induced by nitrate could be due to a more efficient removal of this ROS by the POD peroxidative activity which increased while the decrease in $^{\bullet}OH$ could be explained both by a lack of its H_2O_2 precursor and a less efficient conversion of H_2O_2 in $^{\bullet}OH$ by the POD hydroxylic activity which decreased (Fig. 7D). POD isoforms present in the elongation zone of the primary root in other species were shown to play a crucial role in the root growth, the expression of the corresponding genes being under the control of a helix-loop-helix transcription factor named UPBEAT1 (Liszkay et al., 2004; Trevisan et al., 2018; Tsukagoshi et al., 2010). Whether a possible ortholog of UPBEAT1 in *M. truncatula* is involved in the regulation of *POD* gene expression in response to nitrate remains to be addressed.

In contrast, there is no accordance between $O_2^{\bullet-}$ abundance and NADPH oxidase or SOD activities. Indeed, $O_2^{\bullet-}$ abundance decreased in the root tip in response to nitrate whereas the capacity to produce $O_2^{\bullet-}$ (NADPH oxidase activity) highly increased and the capacity to remove of $O_2^{\bullet-}$ (SOD activity) only slightly increased (Fig. 7). Nonetheless, the high increase in the capacity of PODs to remove H_2O_2 could be at the origin of the decrease of $O_2^{\bullet-}$. Indeed, the non-enzymatic and/or the enzymatic superoxide dismutation, driven by the H_2O_2 disappearance, may be accelerated, removing $O_2^{\bullet-}$ even in the case of a high capacity of $O_2^{\bullet-}$ production. Therefore, the regulation of H_2O_2 abundance in the primary root tip by PODs could play a central role in the nitrate sensitivity.

Therefore, we suggest that the nitrate-induced decrease in ROS content is due to a removal of H_2O_2 that drives the removal of $O_2^{\bullet-}$, together with a poor conversion of H_2O_2 into $^{\bullet}OH$, both changes contributing to limit the accumulation of $^{\bullet}OH$. The decrease in $^{\bullet}OH$ results in turn in the reduction of primary root growth. This highlighted the central role of the regulation of H_2O_2 accumulation in the control of the primary root growth by nitrate in legume, further supported by experiments of the externally manipulation of H_2O_2 concentration. Because almost no change in enzyme capacities were observed in *npf6.8-2*, the nitrate-induced decrease in $^{\bullet}OH$ orchestrated by PODs in the primary root tip in R108 necessitates a functional MtNPF6.8.

According to our knowledge, the impact of nitrate on the accumulation of ROS in the primary root tip was analyzed only in two studies (Trevisan et al., 2018; Zhang et al., 2014). In contradiction with our results, Zhang et al. (2014) reported that nitrate has no significant effect on the growth or on ROS accumulation in the primary root of *M. truncatula* A17. A possible explanation for this discrepancy is that they worked with 5-d-old seedlings that are poorly sensitive to nitrate according to our observation (Fig. S2). In accordance with our results, Trevisan et al. (2018) observed that nitrate modifies the patterns of ROS accumulation in the sensitive primary root of maize but in a different way as compared with *M. truncatula*. Indeed, a treatment of N-deprived seedlings with nitrate (longer than 24 h) that resulted in a decrease of primary root growth was associated with an increase in $O_2^{\bullet-}$ and a decrease in H_2O_2. Thus, ROS response to nitrate may depend on the species, reinforcing the interest to conduct such studies with different species. Nevertheless, in maize, the authors suggested

768 that the reduction of the root growth in response to nitrate was due to the decrease in
769 H_2O_2 under the action of a specific POD isoform, the gene of which being
770 upregulated (Trevisan et al., 2018).

771 Altogether these results suggest that H_2O_2 content is important for primary root
772 response to nitrate. H_2O_2 is a rather stable ROS able to cross cell membranes and
773 act as a secondary messenger signal in signaling pathways. In the root elongation
774 context, one can imagine that it may accumulate above a given threshold in the
775 apoplast, enter the adjacent cells and trigger a cascade of events to control POD
776 activities. These results highlight the importance of the role of class III PODs not only
777 in root elongation as demonstrated in maize and *A. thaliana* (Dunand et al., 2007;
778 Liszkay et al., 2004; Tsukagoshi et al., 2010) but also in the regulation of root
779 elongation in response to nitrate (Trevisan et al. (2018) and this study). They also
780 suggest that among the great number of isoforms of PODs of class III located in the
781 apoplast or the cell wall in root tips, some isoforms could have a dedicated activity
782 either peroxidative or hydroxylic (Kukavica et al., 2012; Veljović-Jovanović et al.,
783 2018).

784 In maize, nitrate sensing at the level of the root apex was also reported to be
785 coordinated by auxin and nitric oxide ($^{\bullet}NO$) (Manoli et al., 2016) suggesting a
786 possible link between auxin/$^{\bullet}NO$ regulation and ROS regulation of the primary root
787 growth in this species (Trevisan et al., 2018). It would be therefore interesting to see
788 whether $^{\bullet}NO$ and auxin could be actors in the nitrate signaling pathway mediated by
789 ROS in legume species.

790

791 ## 5. Conclusions

792 Altogether the results gained in *M. truncatula* highlight the link between the
793 growth performance of the primary root and the ROS accumulation in the root tip as
794 well as the control that nitrate exerts on this ROS accumulation through the control of
795 POD activity. Our study extends the understanding of the role of ROS in root growth
796 performance in legume, also highlighting some differences with *A. thaliana*. It also
797 showed how nitrate restricts the primary root growth, but not the lateral root growth,
798 by controlling ROS accumulation, adding 7 novel actors in the nitrate signaling
799 pathway downstream of MtNPF6.8 and ABA: three ROS ($O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$), and
800 four enzyme activities (NADPH oxidase, SOD, peroxidative and hydroxylic POD

activities).

Contributions

L.Z.: seedling growth, ROS detection, acquisition, analysis, and interpretation of data for the work AND writing of the manuscript.

F.M., M-C.M-L.P, A.M.L.: conception and design of the work AND acquisition, analysis, and interpretation of data for the work AND writing of the manuscript.

T.C., P.S.: seedlings growth and enzyme activity measurement

M.V.: interpretation of data AND, discussion and writing of the manuscript.

M. M.: acquisition, analysis, and interpretation of EPR data

A.P.: set up a method adapted for H_2O_2 measurement in root tip and H_2O_2 measurement

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Conflicts of interest

The authors declare no conflict of interest.

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Supplemental figures:

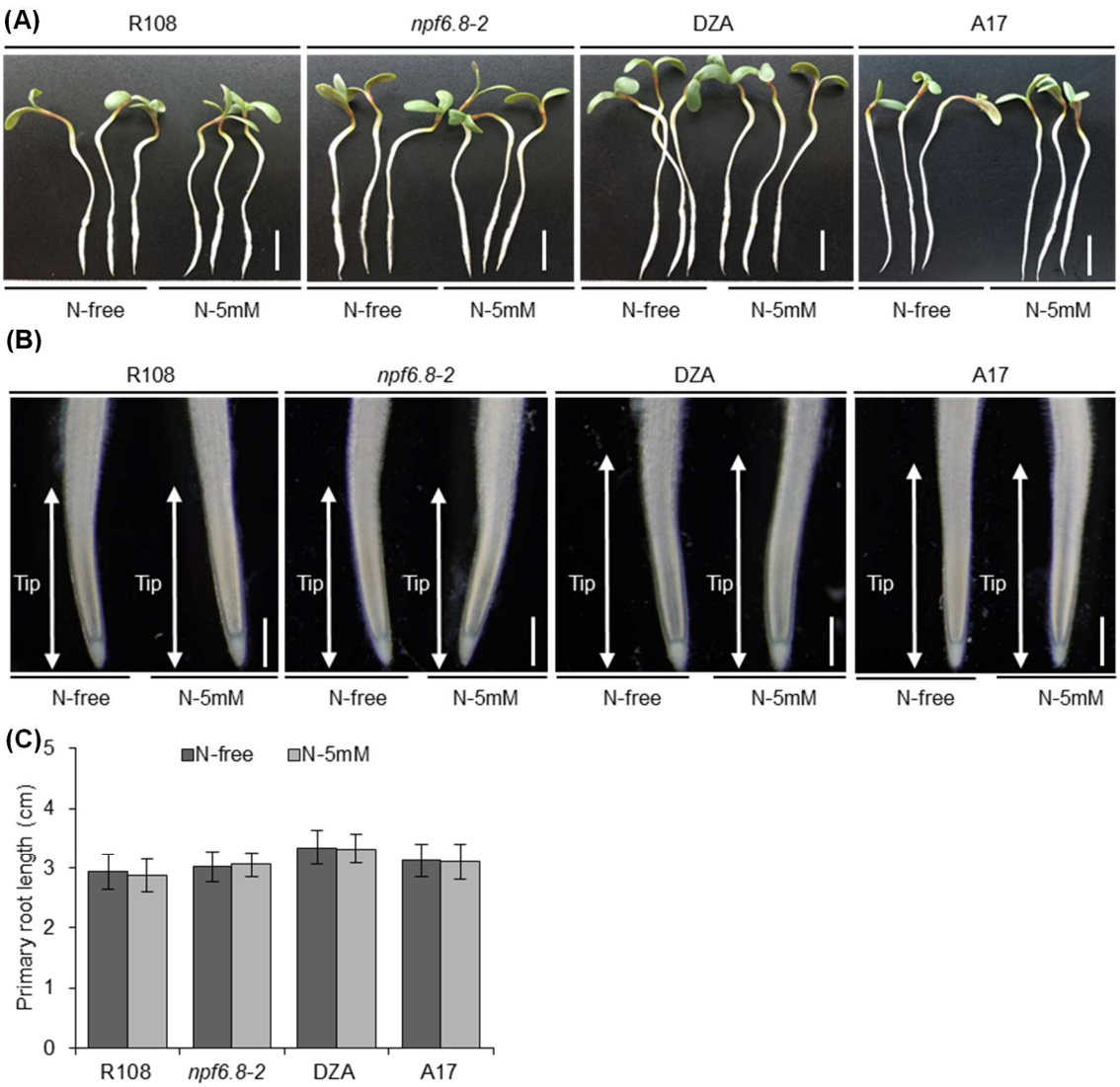


Fig. S1 Nitrate has no effect on the primary root length and root tip morphology of 2-d-old seedlings. (A) Seedlings of R108, *npf6.8-2*, DZA and A17 were grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 2 d. Scale bar = 1 cm. (B) Primary root tip. Scale bar = 500 µm. (C) Primary root length. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%.

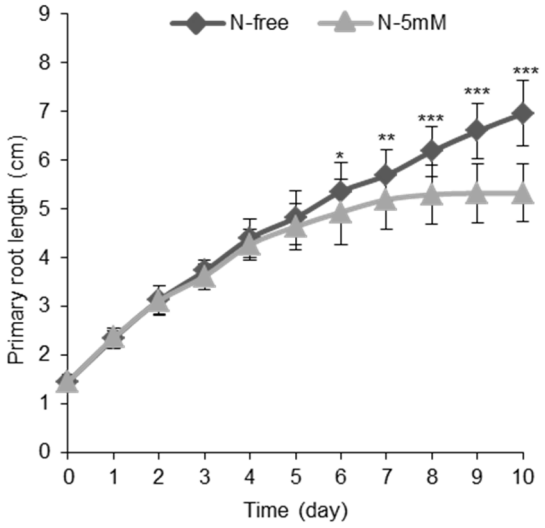


Fig. S2 Nitrate sensitivity of A17 primary root growth takes place during early growth. Seedlings of A17 were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM). Asterisks indicate when the root length determined at a given day is significantly different between the two conditions of growth. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%; *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$.

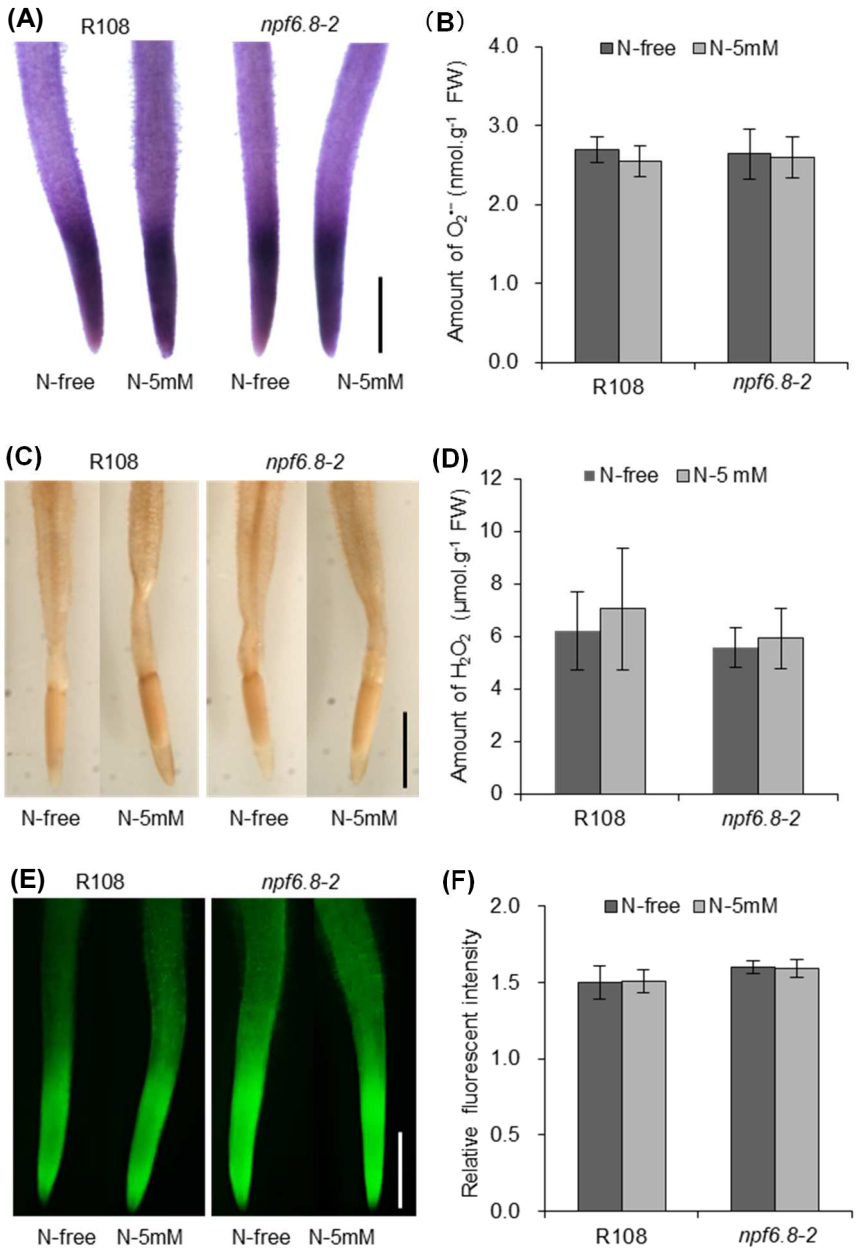
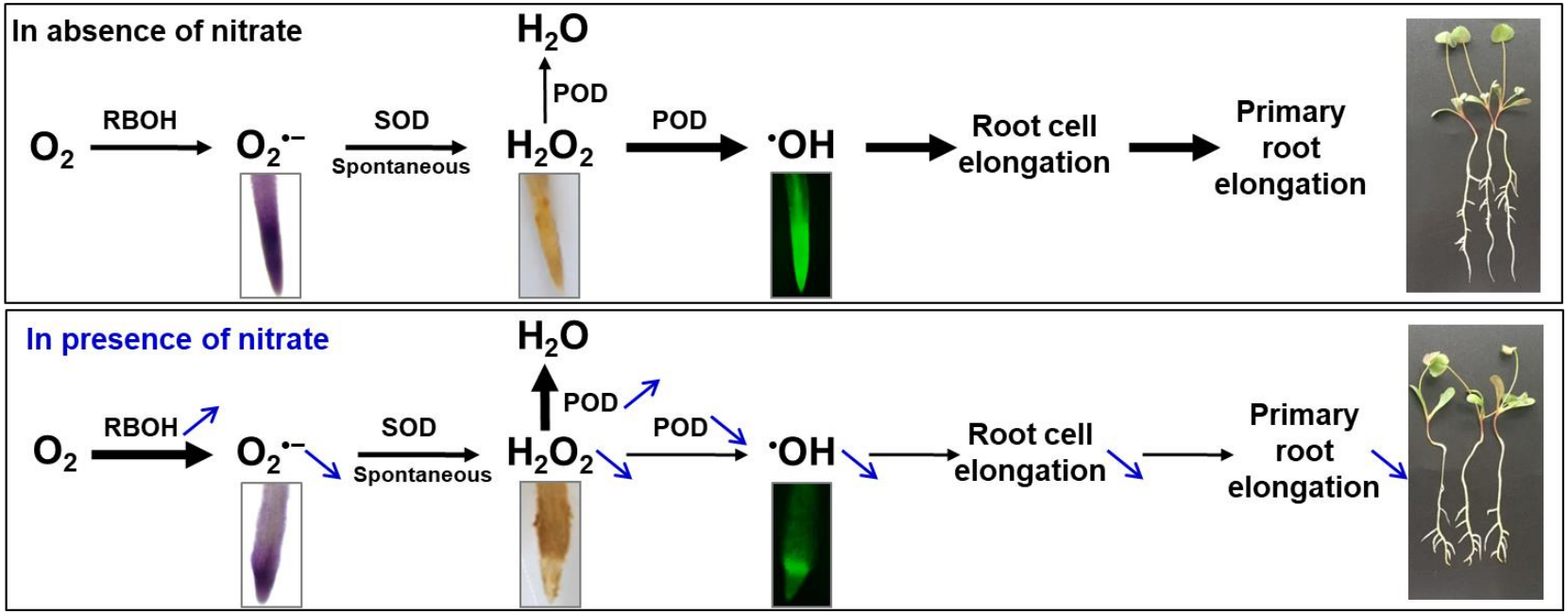


Fig. S3 Nitrate has no effect on $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ accumulation in the primary root of 2-d-old seedlings. Seedlings of R108 and *npf6.8-2* were grown without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 2 d. (A) Detection of $O_2^{\bullet-}$ after 10 min of staining with nitroblue tetrazolium (NBT). (B) Quantification of $O_2^{\bullet-}$ in the root tip. (C) Detection of H_2O_2 after 40 min of staining with diaminobenzidine (DAB). (D) Quantification of H_2O_2 in the primary root tip with luminol. (E) Detection of $^{\bullet}OH$ after 30 min of staining using dihydrohodamine (DHR). (F) Quantification of DHR fluorescence intensity. Scale bar = 1 mm. Quantification was realized using high resolution images and ImageJ software. The statistical test used is an ANOVA and the significance is determined at the threshold α = 5%.

Fig. S4 Nitrate has no effect on $O_2^{\bullet-}$ accumulation in the lateral root of 10-d-old seedlings in R108. Seedlings were grown without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 10 d. Detection of $O_2^{\bullet-}$ after 10 min of staining with nitroblue tetrazolium (NBT). Lateral root tips are indicated with red triangles. Scale bar = 2 mm.


In absence of nitrate
H_2O
POD
O_2
RBOH
$O_2^{\bullet-}$
SOD
Spontaneous
H_2O_2
POD
$^{\bullet}OH$
Root cell elongation
Primary root elongation
In presence of nitrate
H_2O
POD
O_2
RBOH
$O_2^{\bullet-}$
SOD
Spontaneous
H_2O_2
POD
$^{\bullet}OH$
Root cell elongation
Primary root elongation