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Lili Zang, Marie-Christine Morère-Le Paven, Thibault Clochard, Alexis Porcher, Pascale Satour, et al.. Nitrate inhibits primary root growth by reducing accumulation of reactive oxygen species in the root tip in *Medicago truncatula*. *Plant Physiology and Biochemistry*, 2020, 146, pp.363-373. 10.1016/j.plaphy.2019.11.006 . hal-02499253

HAL Id: hal-02499253

<https://univ-angers.hal.science/hal-02499253>

Submitted on 21 Jul 2022

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

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16 **Abstract**

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

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

39 **Highlights**

40 1- Nitrate triggers a decrease in ROS content in primary root tip of sensitive
41 genotypes

42 2- Nitrate inhibition of both root growth and $O_2^{\cdot-}$ accumulation are correlated

43 3- The decrease in ROS induced by nitrate is orchestrated by cell wall peroxidase

44 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

45

46 **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 ₂ O ₂	Hydrogen peroxide
MS	Murashige and Skoog
NBT	Nitroblue tetrazolium
O ₂ ^{·-}	Superoxide anion radical
·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

47 **1. Introduction**

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

59 In fact, nitrate was shown to act as a signal, sensed and transduced by nitrate
60 transporters, as recently reviewed by Pellizzaro et al. (2017) and Zhao et al. (2018).
61 How the nitrate signal is transduced downstream of the nitrate transporters acting as
62 sensors is not totally understood. Pioneer works in *A. thaliana* have shown that
63 hormone transport is among the events involved in response to nitrate (O'Brien et al.,
64 2016). AtNPF6.3, a nitrate transporter belonging to the NITRATE TRANSPORTER
65 1/PEPTIDE TRANSPORTER FAMILY (NPF), is able to transport auxin in addition to
66 nitrate, and to modify auxin distribution in lateral roots in a nitrate concentration
67 dependent manner, thus affecting auxin gradient in the root, and consequently root
68 growth. Furthermore, some NPF transporters such as AtNPF4.6 in *A. thaliana* have
69 been shown to transport ABA as well (Kanno et al., 2012). ABA, which was proposed
70 to play a role in mediating nitrate effects on lateral root development, was shown to
71 relieve the inhibitory effect of nitrate on lateral root growth (Nacry et al., 2013).
72 Besides hormones, transcription factors, protein kinases, or molecular components
73 belonging to epigenetic mechanisms, have further also been identified as being
74 involved in nitrate signaling in *A. thaliana* (Nacry et al., 2013).

75 The effect of nitrate on seedling establishment was less studied in legume than
76 in non-legume species *e.g.* *A. thaliana*. However, legumes deserve such an attention
77 because they play an important role in human and livestock alimentation due to their
78 high level of proteins in seeds and aerial parts used as forage (Maphosa and Jideani,
79 2017). Through their capacity to establish symbiotic interaction with rhizobia to fix

80 atmospheric N₂, they also provide increasing ecosystemic services in cropping
81 systems contributing to nitrogen enrichment of soils and thereby to sustainable
82 agriculture. In legumes, not only root growth but also symbiosis interactions are
83 controlled by nitrate. Nitrate has opposite effects on these two processes: while it
84 favors seedling anchorage before the biological nitrogen fixation takes place through
85 symbiosis, it impairs symbiotic interaction (Ferguson et al., 2019).

86 The fact that the root apex is a zone where different environmental stimuli,
87 including nitrate, are perceived and integrated (Baluška and Mancuso, 2013) makes
88 the root growth response to nitrate interesting to decipher in legumes. The sensitivity
89 to nitrate of the early root development was reported for different genotypes of the
90 model legume *Medicago truncatula* (Bagchi et al., 2012; Morère-Le Paven et al.,
91 2011; Yendrek et al., 2010). Yendrek et al. (2010) also showed that the nitrate
92 transporter MtNPF1.7, known as LATERAL ROOT ORGAN
93 DEFECTIVE/NUMEROUS INFECTIONS and POLYPHENOLICS (LATD/NIP), is
94 involved in the development of the root system in *M. truncatula*. In the same species
95 we have shown that another nitrate transporter, MtNPF6.8, acts as a nitrate sensor
96 involved in the reduction of the primary root growth due to a reduction of cell
97 elongation, *npf6.8* knockdown lines generated by RNAi being insensitive to nitrate
98 (Pellizzaro et al., 2014). Because exogenous ABA treatment restored the growth
99 inhibitory effect of nitrate in *npf6.8* lines, we proposed that ABA acts downstream of
100 MtNPF6.8 in this nitrate-signaling pathway. Moreover, ABA was shown to be
101 transported by MtNPF6.8 (Pellizzaro et al., 2014). Whether the transduction of the
102 nitrate signal requires such a transport is not known.

103 Our aim is to decipher the nitrate signaling pathway in *M. truncatula* primary root
104 through the identification of mediators downstream of MtNPF6.8 and ABA. In the
105 present work, we have addressed the possibility that reactive oxygen species (ROS)
106 are among these mediators since ROS are not only able to transduce ABA signal in
107 guard cells and roots (Kwak et al., 2003; Singh et al., 2017) but also govern several
108 aspect of plant development including root growth (Foyer and Noctor, 2016; Mittler,
109 2017). Indeed, in the apoplast and cell wall of the roots of *A. thaliana* and maize,
110 three main ROS, superoxide anion radical (O₂⁻), hydroxyl radical (·OH) and
111 hydrogen peroxide (H₂O₂), were demonstrated to either promote growth through cell
112 division in the meristem zone (O₂⁻) and cell elongation in the elongation zone (·OH)

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

116 To test whether ROS mediate the nitrate signal in the primary root of *M.*
117 *truncatula*, we took advantage of the availability in this species of the *npf6.8*
118 knockdown lines, which are not sensitive to nitrate. We determined the impact of
119 nitrate on the abundance of ROS ($\text{O}_2^{\cdot-}$, H_2O_2 , $\cdot\text{OH}$) in the primary root of R108,
120 DZA315-16 and A17, three wild type genotypes of *M. truncatula* as compared to
121 *npf6.8-2*. We found that nitrate hampers the accumulation $\text{O}_2^{\cdot-}$, H_2O_2 and $\cdot\text{OH}$ in the
122 root tip of the wild type genotypes having different sensitivities to nitrate whereas it
123 does not modify the accumulation of ROS in the primary root of the insensitive
124 *npf6.8-2*. The decrease in ROS abundance induced by nitrate in the wild type
125 genotypes was accompanied by a modification of the capacity of the enzymes
126 responsible for the production or the removal of ROS in the primary root tip.

127 **2. Materials and methods**

128 **2.1 Plant materials and growth conditions**

129 Three wild type genotypes of *M. truncatula* R108, DZA315.16 (DZA) and A17,
130 and an RNAi knockdown line in the R108 background, *npf6.8-2* (Pellizzaro et al.,
131 2014), were used in this study.

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

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

148 **2.2 ROS detection and measurement**

149 2.2.1 O₂^{•-} detection and measurement

150 O₂^{•-} was detected by nitroblue tetrazolium (NBT; Sigma-Aldrich), which is
151 reduced by O₂^{•-} in purple diformazan, using a protocol based on Zhang et al. (2014).
152 Whole roots of seedlings grown for 2 to 10 d were stained in 20 mM phosphate buffer
153 (pH 6.0) containing 3 mM NBT for 10 min in darkness. The reaction was stopped by
154 transferring the seedlings in 80% (v/v) ethanol. The stained roots were then
155 immediately observed with an Olympus stereomicroscope and images were taken
156 with a HD camera. All the same setting parameters were kept for all the images in an
157 experiment.

158 A method adapted from Ramel et al. (2009) was used for quantitative O₂^{•-}
159 measurement, the tips of the roots stained in purple were collected in a 1.5 mL
160 microtube containing 120 µL of 2 M KOH and then ground after addition of 140 µL of
161 dimethyl sulfoxide (DMSO). After centrifugation for 10 min at 12,000 x g, the
162 supernatant containing diformazan was introduced in a well of a microplate. A blank
163 control was set with an extract of unstained root tips. Absorbance was measured at
164 630 nm with a spectrophotometer (Spectrostar, BMG LABTECH, Germany). The
165 superoxide content was determined by comparison with a standard curve plotted with
166 known quantities of NBT in a mix of 2 M KOH/DMSO (1/1.16, v/v). The values are
167 expressed in nmol of O₂^{•-} produced per g of fresh weight (FW).

168 2.2.2 H₂O₂ detection and measurement

169 H₂O₂ was detected with 3, 3'-diaminobenzidine (DAB, Sigma-Aldrich) according
170 to a method adapted from Zhang et al. (2014). Whole roots were incubated for 1 h in
171 a solution of 1 mg.mL⁻¹ DAB in 50 mM citric acid (pH 3.5) prepared one day before,
172 then washed with distilled water. Images were captured as previously described for
173 NBT staining.

174 The abundance of H₂O₂ in the tip was measured using luminol (5-amino-2, 3-
175 dihydro-1, 4-phthalazidone) with a method adapted from Lu et al. (2009) and Noctor

176 et al. (2016). Frozen root tips (20 mg) were ground in liquid nitrogen and
177 homogenised in 500 μ L of 5% trichloroacetic acid containing 5% of insoluble
178 polyvinylpolypyrrolidone (w/v). The samples were centrifuged at 13,000 x g for 10
179 min. After a 100 times dilution in 0.1 M sodium carbonate buffer (pH 10.2), aliquots of
180 40 μ L were incubated at 30°C for 15 min with 10 μ L of distilled water or 10 μ L of a
181 solution containing 50 U of bovin liver catalase (SIGMA-Aldrich) for control
182 experiments. After this incubation, 10 μ L of each aliquot treated or not with catalase,
183 were placed into wells of a microplate (white polypropylene MicroWell® plate,
184 NUNC®, round bottom). The reaction started with addition of 250 μ L of reaction
185 mixture solution composed of 65 μ M of luminol and 10 μ M of cobalt chloride in 0.1 M
186 sodium carbonate buffer (pH 10.2). The luminescence was measured over 5 s in a
187 microplate reader (Fluostar OMEGA®, BMG Labtech®). The H₂O₂ was quantified
188 using a standard curve realized with 0 to 50 pmol of H₂O₂ prepared freshly by dilution
189 of H₂O₂ in trichloroacetic acid then sodium carbonate as above for the samples. Root
190 specific H₂O₂ content was determined by the difference between the values obtained
191 with and without catalase and was expressed as μ mol.g⁻¹ FW.

192 2.2.3 Detection of \cdot OH

193 Dihydrorhodamine 123 (DHR, Sigma-Aldrich) was used to detect \cdot OH (Porwol et
194 al., 1998). DHR was first solubilized in DMSO and then diluted to a concentration of
195 25 μ M in 20 mM phosphate buffer (pH 6.0). The whole roots were stained for 30 min,
196 then washed three times with distilled water and observed with an Olympus
197 stereomicroscope using the green filter with excitation and emission wavelengths of
198 500 nm and 536 nm, respectively. Images were immediately taken with the HD
199 camera. ImageJ software (Version 1.4.3.67) was used to quantify in pixels the
200 fluorescence intensity of DHR in root tips. The relative fluorescence intensity is
201 expressed in an arbitrary unit, using a value of 1 for R108 grown in MS without
202 nitrate. Four measures were performed for each root tip.

203 2.3 Enzyme activity measurement

204 2.3.1 NADPH oxidase activity

205 Total proteins from root tips were extracted in 1 mL of 50 mM Tris-HCl buffer (pH
206 7.5). After centrifugation at 15,000 x g at 4°C for 15 min to remove cell debris, the
207 activity of NADPH oxidase (EC1.6.3.1), also known as respiratory burst oxidase

208 homolog (RBOH) was measured in the supernatant according to Sagi and Fluhr
209 (2001) using XTT (2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-
210 carboxanilide, tetrazolium salt). The reaction mixture contained 0.3 mM XTT, 2 mM
211 CaCl₂, 1 mM MgCl₂ and 0.3 mM NADPH supplemented or not with 50 μM
212 diphenyleneiodonium (DPI) to inhibit NADPH oxidase or 1 KU of horse radish
213 superoxide dismutase (SOD ; SIGMA-Aldrich) to remove O₂^{•-}. Absorbance was
214 measured at 470 nm. The difference in the rate of XTT oxidation measured in the
215 absence or the presence of DPI, an inhibitor of NADPH oxidase, corresponds to
216 NADPH oxidase activity that was expressed in nmol of O₂^{•-} produced min⁻¹.mg⁻¹ FW.
217 This part of XTT oxidation sensitive to DPI, corresponding specifically to NADPH
218 oxidase activity, was similar to the part abolished by the horse radish SOD. This
219 indicates that peroxidase (POD) does not contribute to O₂^{•-} production through its
220 oxidative activity in *M. truncatula* primary root tip as it was the case in maize (Liszskay
221 et al., 2004).

222 2.3.2 SOD and POD activities

223 For SOD (EC1.15.1.1) and POD (EC1.11.1.7) activities, cell walls were first
224 isolated from the root tips as previously described by Kukavica et al. (2012). Briefly,
225 the root tips were ground in liquid nitrogen, then in a buffer solution containing 50 mM
226 Tris-HCl (pH 7.2), 50 mM NaCl, 0.05% Tween-80 and 1 mM phenylmethylsulfonyl
227 fluoride, and the extract was centrifuged at 1,000 x g for 20 min at 4°C. The
228 fragments of the cell walls present in the pellet were washed four times by
229 resuspension in 50 mM Tris-HCl (pH 7.2), and centrifugation at 4°C for 20 min at
230 1,000 x g or 15,000 x g for the final step of centrifugation. For some experiments,
231 proteins bound to the cell walls were isolated by resuspension and solubilization of
232 the cell wall fragments in 50 mM Tris-HCl, pH 7.2, containing 0.12 mg mL⁻¹ cellulase
233 and 0.6 mg mL⁻¹ pectinase for 24 h at 4°C, and centrifugation at 15,000 x g for 15
234 min at 4°C. Then, enzyme activities were measured.

235 SOD activity was determined in the fraction of proteins isolated from the cell
236 walls by the method of Gill et al. (2015) with some modifications. The reaction
237 mixture contained 75 mM phosphate buffer (pH 7.8), 15.6 mM methionine, 15.6 μM
238 riboflavin and 80 μM NBT. Absorbance was measured at 560 nm. SOD activity was
239 determined in μU.mg⁻¹ FW by comparison with a standard curve realized with horse
240 radish SOD.

241 Two different capacities of POD were measured, the capacities to remove H₂O₂
242 or to convert H₂O₂ in ·OH. For the capacity to remove H₂O₂, the fraction of proteins
243 isolated from the cell walls was used. The reaction mixture contained 200 mM
244 phosphate buffer (pH 7.0), 50 mM o-diasinidine and 37.5 mM H₂O₂. Absorbance was
245 measured at 430 nm. POD activity was expressed in nmol of H₂O₂ reduced min⁻¹.mg⁻¹
246 FW. The capacity to convert H₂O₂ in ·OH was determined by electron paramagnetic
247 resonance (EPR) spectroscopy (Mojović et al., 2004) using the cell wall fraction and
248 the spin-trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO; Alexis
249 Biochemical, Lausen, Switzerland). To monitor ·OH production, each cell wall fraction
250 was resuspended in 50 mM Tris–HCl (pH 7.2) with the ratio of 1.0 mg / 30 µL
251 supplemented with 42.5 mM DEPMPO and placed into a gas-permeable Teflon tube
252 (Zeus Industries, Inc.). The EPR signal of the DEPMPO/OH adduct (generated in the
253 presence of ·OH) was recorded using an X-band Bruker Elexsys II E540 EPR
254 spectrometer at room temperature under the following parameters: center field
255 3483.30 G, sweep width 241.2 G, modulation frequency 100 kHz, modulation
256 amplitude 2 G, microwave frequency 9.77 GHz, microwave power 10 mW and
257 conversion time 58.59 ms, 20 scan accumulations. The reference DEPMPO/OH spin
258 adduct was produced by ·OH generating system (Mojović et al., 2004). EPR signals
259 of DEPMPO/OH adduct were analyzed using ELEANA software (v2.8). Spectral
260 simulations of DEPMPO/OH adduct were performed with SciDAVis software (v1.23).
261 At the end of the assays, the cell wall suspensions were dried and the ·OH
262 production was expressed in arbitrary units per g (DW) of cell wall.

263 **2.4 Number of replicates and statistical analysis of the data**

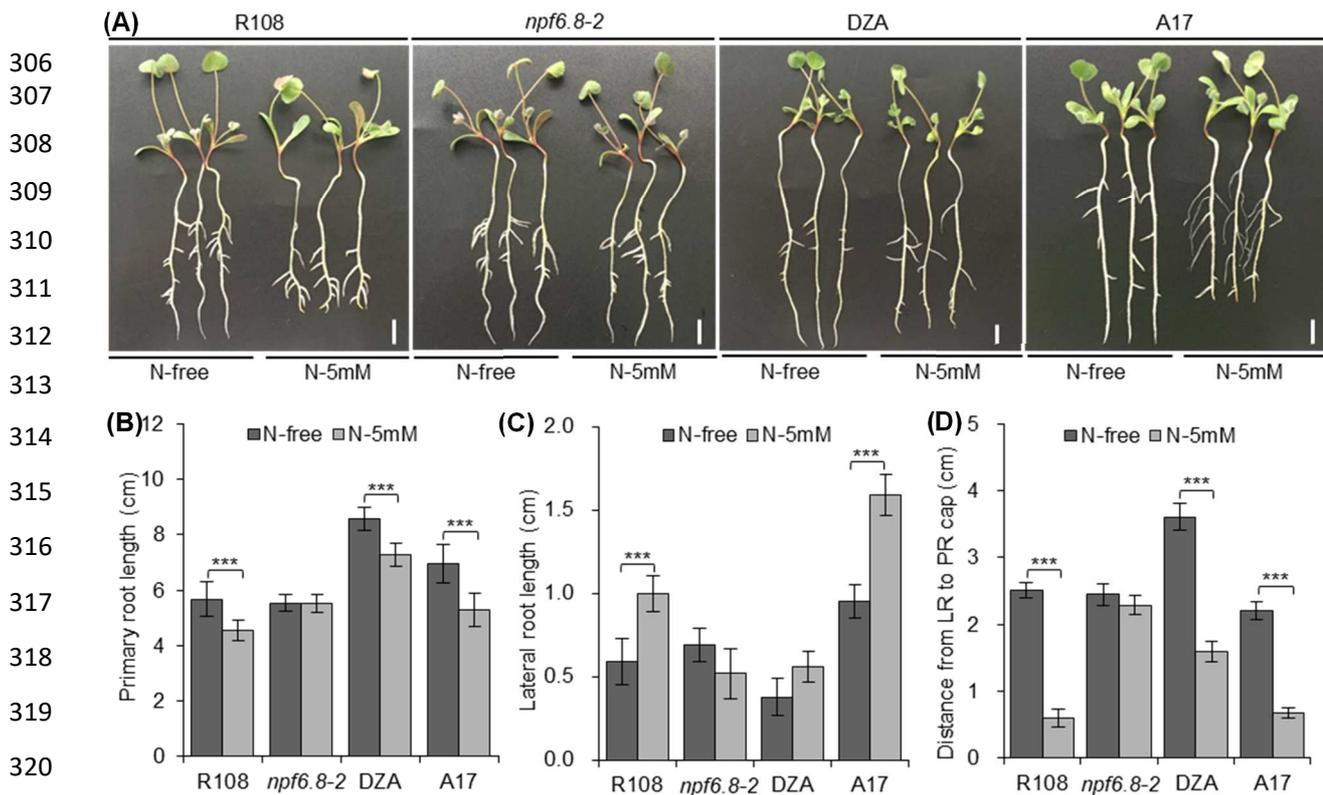
264 Each experiment was independently repeated at least three times at different
265 dates. For root morphology characterization or root staining, at least 10 and often 20
266 seedlings were used by repeat. Calculated means are represented ± SD. For all data,
267 relevant statistical tests were performed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

273 3. Results

274 3.1 Nitrate modifies root architecture and primary root tip morphology of 275 sensitive seedlings

276 In a previous study performed with *M. truncatula*, we have shown that nitrate
277 signal perceived by the nitrate transporter MtNPF6.8 restricts primary root growth of
278 the R108 genotype but not of the *npf6.8* RNAi lines (*npf6.8-2*, *npf6.8-3*, *npf6.8-5*)
279 affected in *MtNPF6.8* expression in the R108 background (Pellizzaro et al., 2014). In
280 the present study, we confirmed the root sensitivity of R108 and the root insensitivity
281 of *npf6.8-2* to nitrate, the root architecture of *npf6.8-2* being similar to that of R108
282 grown in absence of nitrate (Fig. 1). We also compared the phenotype of the wild
283 type R108 with the phenotype of two other wild type genotypes, DZA 315-16 (DZA)
284 and A17, to better characterize the nitrate sensitivity of the roots in *M. truncatula* (Fig.
285 1). The results highlight a significant variability in both root performance and root
286 nitrate sensitivity in this species. At 10 d and in the absence of nitrate, the primary
287 root length of R108, DZA and A17 were 5.7, 8.5 and 7.0 cm, respectively. Thus,
288 regarding primary root growth in absence of nitrate, DZA appears as the most
289 performing genotype while R108 is the least performing genotype. In presence of 5
290 mM NO₃⁻, the primary root growth was reduced by 19.2%, 14.9% and 23.5% for
291 R108, DZA and A17, respectively. Thus, A17 is the more sensitive genotype while
292 DZA is the least sensitive genotype.

293 The impact of nitrate on the development and the growth of lateral roots was
294 also characterized (Fig. 1C and D). At 10 d and in the absence of nitrate, the lateral
295 roots had a cumulative length of 0.6, 0.4 and 0.9 cm and developed at a distance
296 from the primary root cap of 2.5, 3.6 and 2.2 cm in R108, DZA and A17, respectively.
297 Thus, DZA had the shortest lateral roots that developed at the longest distance from
298 the primary root cap while A17 has the longest lateral roots that developed at a
299 distance from the primary root cap similar to that of R108. In the presence of 5 mM
300 NO₃⁻, the cumulative lateral root length was not modified in DZA while it increased
301 similarly in R108 (+ 40%) and A17 (+ 44%). The distance from the lateral roots to the
302 primary root cap highly decreased in the three genotypes: 70% (R108), 68% (A17)
303 and 56% (DZA). Therefore, A17 appears as the most performing genotype while DZA
304 appears as the least performing genotype regarding the lateral root development and
305 growth both in the absence or the presence of nitrate.

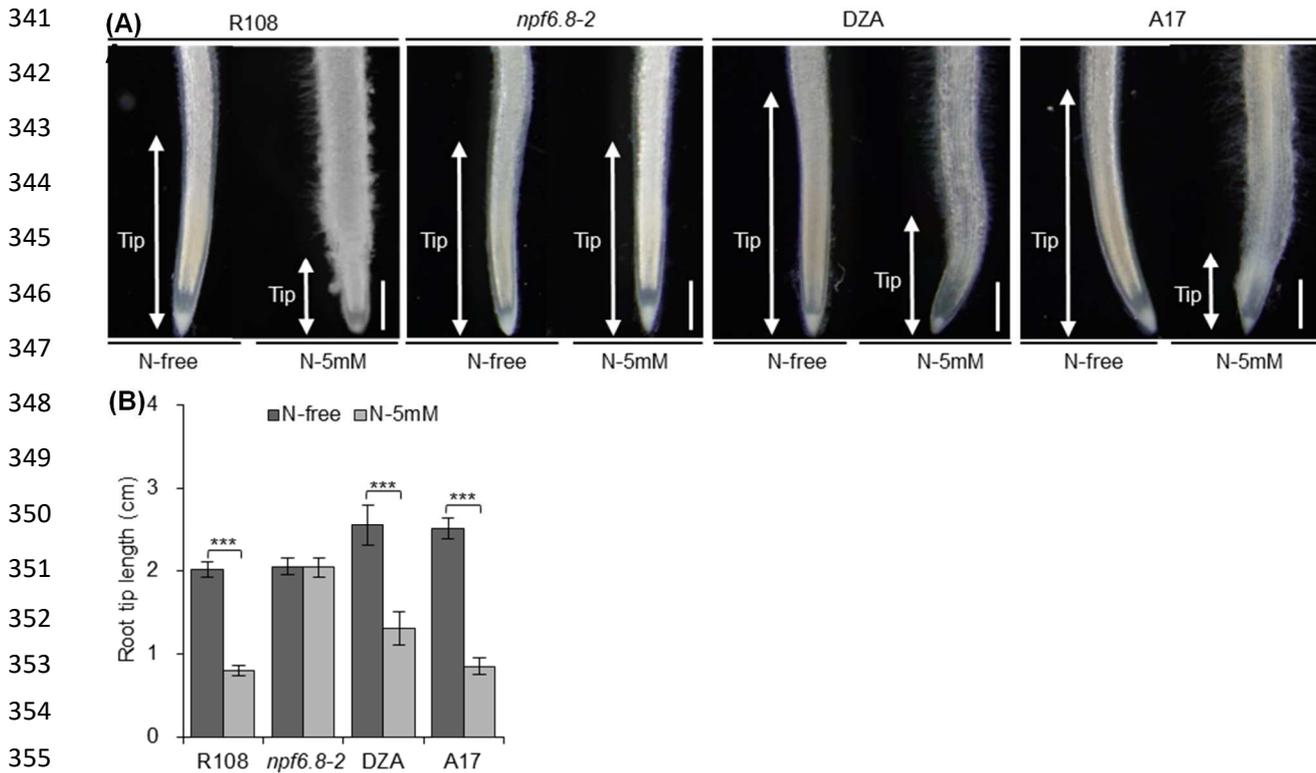


321 **Fig. 1 Nitrate modifies the architecture of the root system of the sensitive genotypes.** (A)
322 Seedlings of R108, *npf6.8-2*, DZA 315-16 (DZA) and A17 grown in MS without nitrate (N-free) or with
323 5 mM nitrate (N-5 mM) for 10 d. Scale bar = 1 cm. (B) Primary root length. (C) Lateral root length. (D)
324 Distance from lateral root to primary root cap. Length and distance were measured using ImageJ
325 software. Asterisks indicate a significant difference. The statistical test used is an ANOVA and the
326 significance is determined at the threshold $\alpha = 5\%$; ***, $p < 0.001$.

327

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

340



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

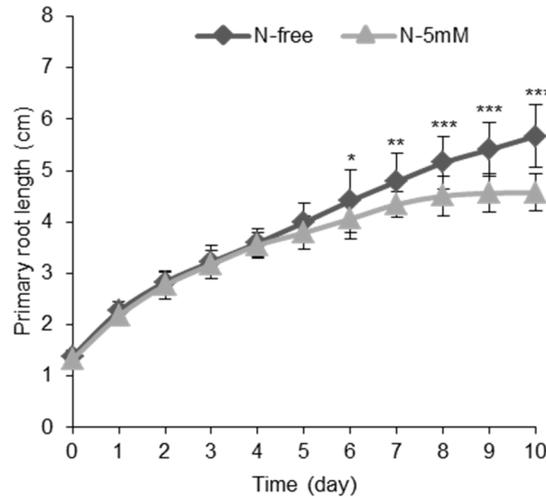
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362 3.2 Nitrate sensitivity takes place during early primary root growth

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

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

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386 **Fig. 3 Nitrate sensitivity of R108 primary root growth takes place during early growth.** Seedlings
387 of R108 were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) and the
388 primary root length was recorded from 1 to 10 d. Asterisks indicate when the root length determined at
389 a given day is significantly different between the two conditions of growth. The statistical test used is
390 an ANOVA and the significance is determined at the threshold $\alpha = 5\%$; *, $p < 0.05$, **, $p < 0.01$; ***,
391 $p < 0.001$.

392 **3.3 Nitrate reduces ROS accumulation in the primary root tip of 10-d-old** 393 **sensitive seedlings**

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

404

405 **3.3.1 Nitrate reduces $O_2^{\cdot-}$ accumulation**

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

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

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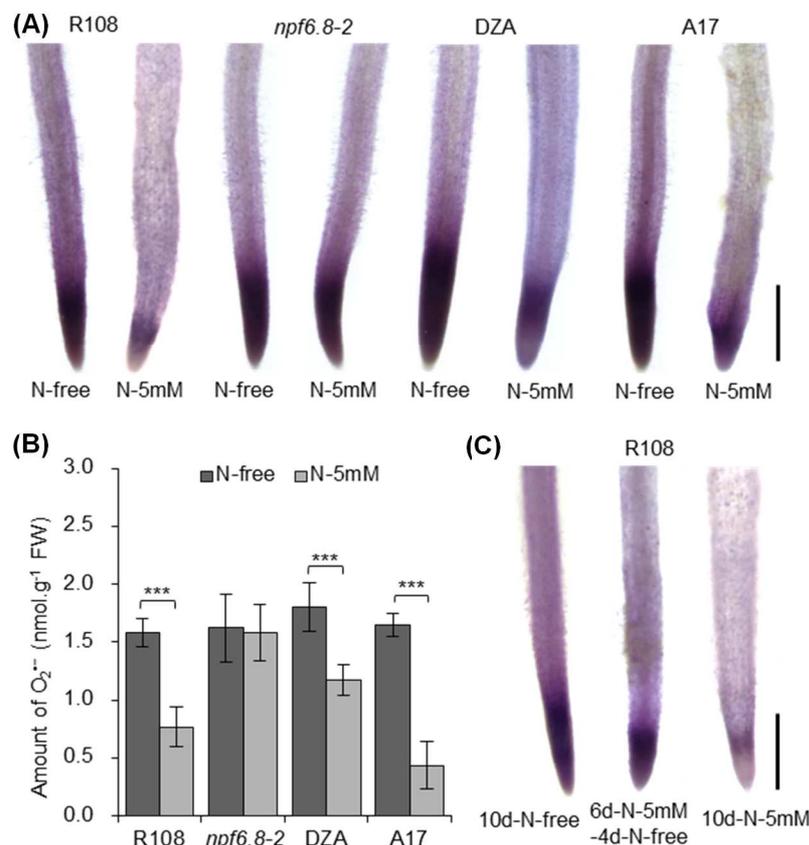
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431 **Fig. 4 Nitrate reduces $O_2^{\cdot-}$ accumulation in the primary root tip of the sensitive genotypes.**

432 Seedlings of R108, *npf6.8-2*, DZA and A17 were grown for 10 d in MS without nitrate (N-free) or with 5
 433 mM nitrate (N-5 mM). (A) Detection of $O_2^{\cdot-}$ in the primary root tip after 10 min of staining with nitroblue
 434 tetrazolium (NBT). (B) Quantification of $O_2^{\cdot-}$ in the root tip. The statistical test used is an ANOVA and
 435 the significance is determined at the threshold $\alpha = 5\%$; ***, $p < 0.001$. (C) Detection of $O_2^{\cdot-}$ in seedlings
 436 of *M. truncatula* R108, grown in MS without nitrate (10d-N-free), MS with 5 mM nitrate (10d-N-5mM)
 437 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),
 438 stained with NBT. Scale bar = 1 mm.

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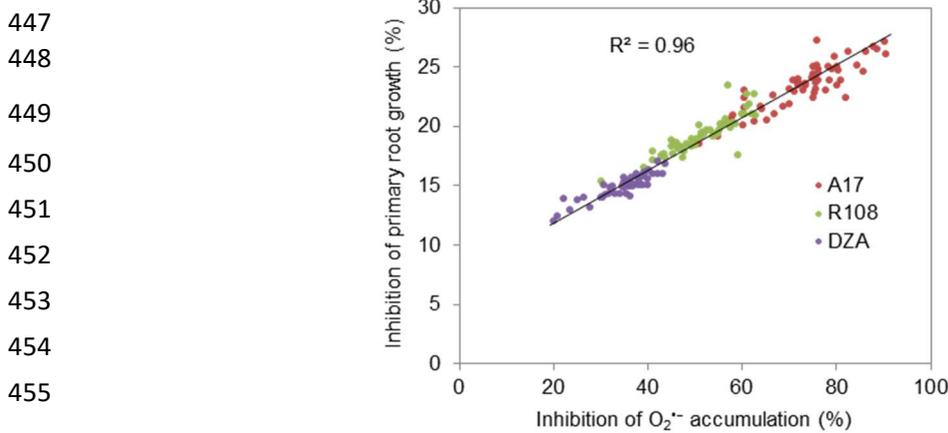
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These results raised the question whether the inhibition of the primary root
 growth induced by nitrate could be linked to the removal of $O_2^{\cdot-}$ in the tip. To test this
 hypothesis, we plotted the percentage of growth inhibition as a function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

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



456 **Fig. 5 Inhibition of primary root growth and $O_2^{\cdot-}$ accumulation induced by nitrate are correlated**
457 **in the sensitive genotypes.** Percentage of inhibition of root growth is plotted as a function of
458 percentage of inhibition of $O_2^{\cdot-}$ accumulation.
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460 We further addressed the question whether the resume of the primary root
461 growth of R108 in the recovery experiment (Fig. 3) was accompanied by a resume of
462 $O_2^{\cdot-}$ accumulation. Interestingly, the recovery of a thin and long root tip was
463 associated with an increase in $O_2^{\cdot-}$ detected in the tip using NBT (Fig. 4C). The
464 increase was quantified as above after diformazan extraction from the tip. $O_2^{\cdot-}$
465 amount reached $1.2 \text{ nmol.g}^{-1} \text{ FW}$ after 6 d of growth with nitrate then 4 d without
466 nitrate, a value intermediary between 1.6 and $0.8 \text{ nmol.g}^{-1} \text{ FW}$ after 10 d of growth
467 without or with nitrate, respectively.

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

472

473 3.3.2 Nitrate reduces H_2O_2 accumulation

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

478 accumulation of H₂O₂ in the tip at 10 d was confirmed using luminol (Fig. 6B), a
479 product recommended by Noctor et al. (2016). H₂O₂ was also found to be present but
480 to a lesser extent in the mature part of the root (Fig. 6A), the root hairs being notably
481 well stained with DAB. In the presence of 5 mM NO₃⁻, the abundance of H₂O₂
482 decreased in the tip (Fig. 6A and B) while it increased in the mature part of the root of
483 R108 (Fig. 6A). In comparison, nitrate had no impact on H₂O₂ distribution in the
484 primary root of *npf6.8-2* whatever the zone (Fig. 6A and B) which is similar to that in
485 the root of R108 grown without nitrate.

486 3.3.3 Nitrate decreases [•]OH accumulation

487 We used the fluorescent probe DHR to detect [•]OH in the primary root of R108
488 and *npf6.8-2*, a product previously used with animal cells (Porwol et al., 1998). In the
489 absence of nitrate, in both genotypes, [•]OH was mainly distributed in the primary root
490 tip (Fig. 6C and D), with a pattern similar to O₂^{•-} (Fig. 4A). In the presence of nitrate,
491 this zone was highly reduced for R108 but not for *npf6.8-2* (Fig. 6C). Quantification of
492 DHR fluorescence intensity in the primary root tip confirmed these observations (Fig.
493 6D).

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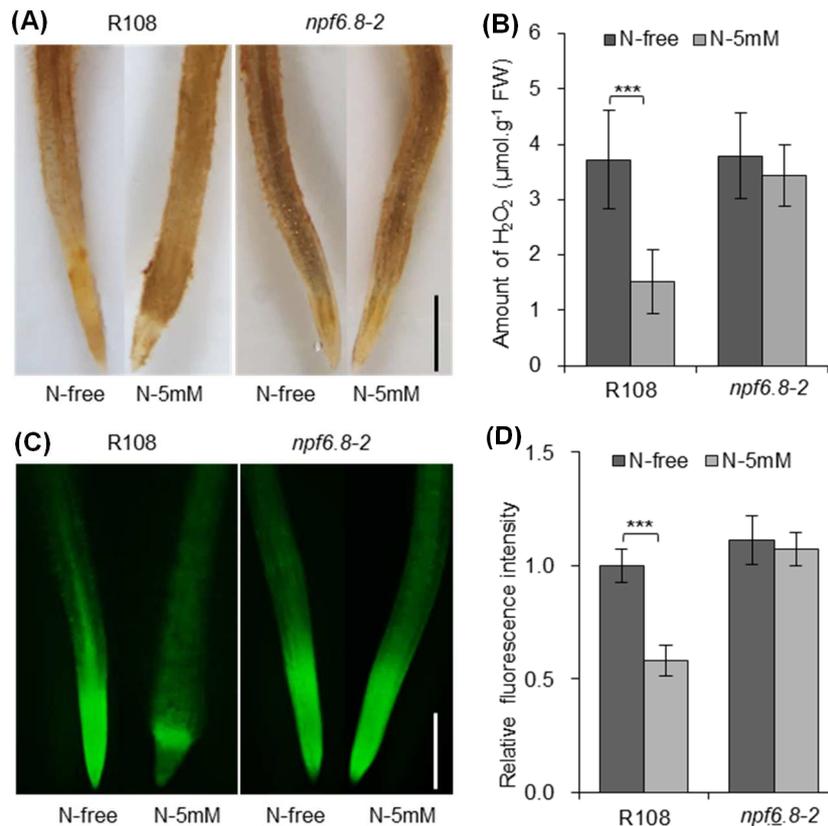


Fig. 6 Nitrate reduces the accumulation of H₂O₂ and ·OH in the primary root tip of the R108 sensitive genotype. Seedlings of R108 and *npf6.8-2* were grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 10 d. (A) Detection of H₂O₂ in the primary root after 40 min of staining with 3,3'-diaminobenzidine (DAB). (B) Quantification of H₂O₂ in the primary root tip with luminol. (C) Detection of ·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 $\alpha = 5\%$; ***, $p < 0.001$.

517 3.4 ROS pattern is not modified by nitrate in the tip of primary root at 2 d or 518 lateral roots at 10 d

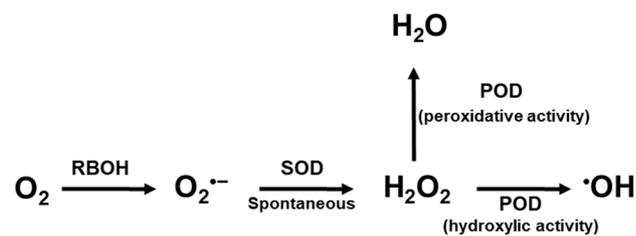
519 As shown above, nitrate had no impact on the phenotype of the primary root of
520 2-d-old seedlings of the wild type genotypes R108, DZA and A17 (Fig. 3, Fig. S1 and
521 S2). We wondered whether nitrate modifies the ROS accumulation pattern in the tip
522 at this stage of primary root growth. We used R108 and *npf6.8-2* to address this
523 question. Although no difference between the two genotypes and no change in ROS
524 abundance could be observed in response to nitrate (Fig. S3), the amounts of O₂^{·-},
525 H₂O₂ and ·OH determined in the tip at 2 d were higher (about 1.5 to 2 times higher)
526 than at 10 d (Fig. 4 and Fig. 6). In contrast, it was lower in the mature part of the root
527 at 2 d as compared with 10 d, according to the DAB staining (Fig. 6; Fig. S3).

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

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534 **3.5- Nitrate modifies the capacity of the primary root tip to produce or remove**
 535 **ROS**

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



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544 NADPH oxidase activity, sensitive to DPI, was measured in crude homogenates
 545 of root tips while SOD and POD activities were measured in cell wall fractions
 546 isolated from the root tips. NADPH oxidase activity was monitored by the oxidation of
 547 XTT. In the absence of nitrate, the levels of these enzyme activities interfering with
 548 ROS accumulation were found to be similar for the wild type R108 and the *npf6.8-2*
 549 knockdown line (Fig. 7). However, in the presence of nitrate, they were altered in
 550 R108 but almost unchanged in *npf6.8-2*. In R108, NADPH oxidase activity increased
 551 3.5 times (Fig. 7A), from 35.7 to 124.8 nmol of $\text{O}_2^{\cdot-}$ produced per min and mg of FW.
 552 SOD activity also significantly increased but to a lesser extend (x 1.15; Fig. 7B), from
 553 39.3 to 45.3 μU per mg of FW. POD capacity to remove H_2O_2 increased 3.4 times
 554 (from 0.60 to 3.27 nmol H_2O_2 reduced per min and mg of FW) while POD capacity to
 555 produce $\cdot\text{OH}$ decreased 2.7 times (from 6.9 to 2.6 10^5 arbitrary units per mg of DW).

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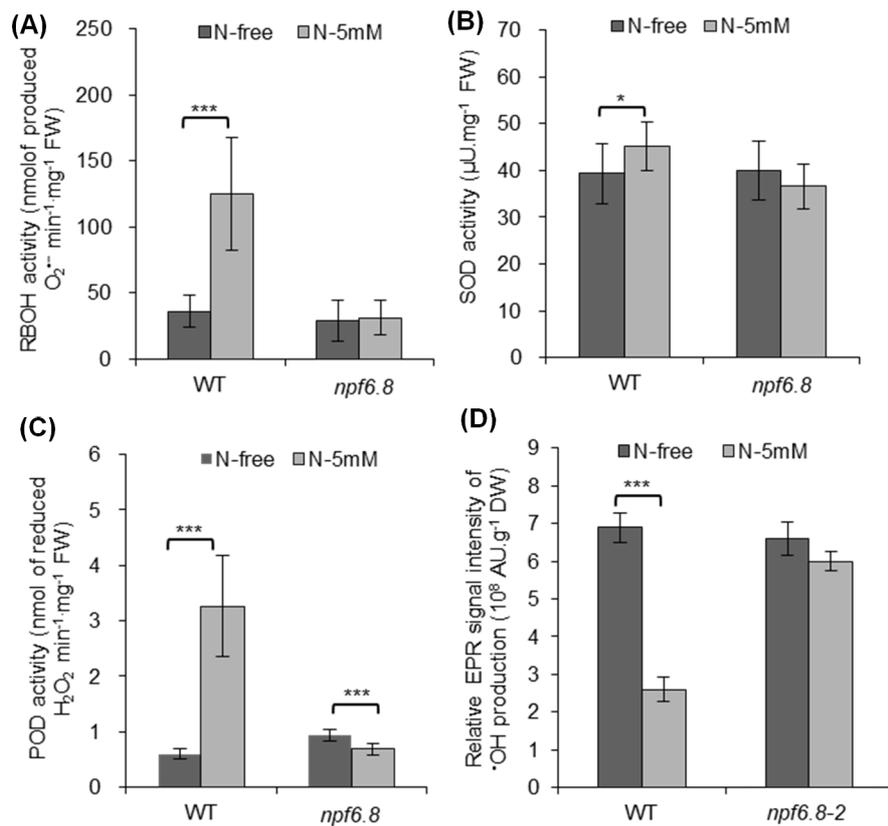


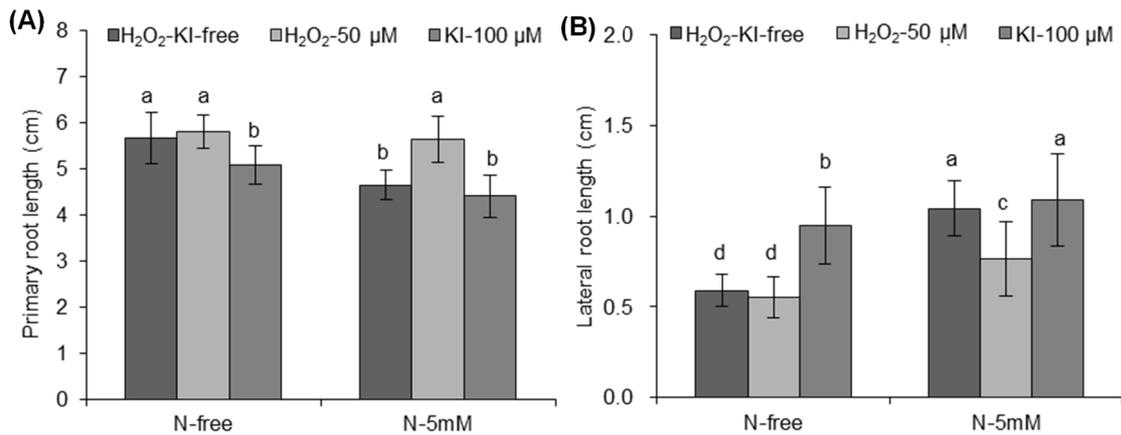
Fig. 7 Nitrate alters RBOH, SOD and POD activities in the primary root tip of the R108 sensitive genotype. Seedlings of R108 and *npf6.8-2* were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM). RBOH was measured in crude root tip homogenates while SOD and POD activities were 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 $\alpha = 5\%$; *, $p < 0.05$, ***, $p < 0.001$.

586 3.6 Nitrate inhibitory effect is abolished by externally added H₂O₂ or mimicked 587 by KI, an H₂O₂ scavenger

588 To address a possible central role of the regulation of H₂O₂ abundance in the
589 primary root tip by PODs in response to nitrate, we investigated the effect of a
590 manipulation of H₂O₂ abundance on this response by adding H₂O₂ or KI, a H₂O₂
591 scavenger (Junglee et al., 2014), to the culture medium, as it was done in the case of
592 *A. thaliana* (Dunand et al., 2007). The addition of 50 μM H₂O₂ completely abolished
593 the inhibitory effect of nitrate on the primary root growth while it had nearly no impact
594 in absence of nitrate (Fig. 8A). Conversely, the addition of 100 μM KI restricted the
595 primary root growth similarly to nitrate (Fig. 8A). These results were in favor of the

596 hypothesis that the regulation of H₂O₂ content may be important for the primary root
 597 response to nitrate. This is also true for lateral roots (Fig. 8B). Indeed, H₂O₂
 598 externally added counteracted the nitrate effect on the lateral roots that did not grow
 599 as in absence of nitrate (Fig. 1C) while it had nearly no impact in absence of nitrate
 600 (Fig. 8B). In contrast, the addition of KI was accompanied with the development and
 601 the growth of lateral roots (Fig. 8B), as in presence of nitrate (Fig. 1C).

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 619 **Fig. 8 Manipulation of H₂O₂ abundance can abolish or mimic the nitrate effect on root growth of**
 620 **the R108 sensitive genotype.** (A) Primary root length. (B) Lateral root length. Seedlings from R108
 621 were grown in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM) ± 50 μM H₂O₂ or 100 μM KI
 622 for 10 d. Primary and lateral root lengths were measured on highly resolved images using ImageJ
 623 software. Letters indicate significant differences between the six different conditions compared in A
 624 and B. The statistical test used is an ANOVA and the significance is determined at the threshold $\alpha =$
 625 5%.

626 4. Discussion

627 4.1 The nitrate sensitivity of the primary root shows a natural variability and is 628 acquired during early growth in *M. truncatula*

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

632 Here, we characterized and compared the sensitivity to nitrate of the root
 633 systems of three different wild type genotypes of the model legume *M. truncatula*:
 634 R108, DZA and A17 (Fig. 1 to Fig. 3). At 5 mM, nitrate reduced the tip length and the
 635 elongation of the primary root of these sensitive genotypes, but in a genotype
 636 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.

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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; Liskay 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, $O_2^{\cdot-}$ and $\cdot OH$ 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 $O_2^{\cdot-}$ accumulation induced by nitrate comparing the three
667 different wild type genotypes (R108, DZA and A17).

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

676 Studies on ROS detection in the primary root tip have been carried out with
677 several species such as maize (Liszkay et al., 2004; Trevisan et al., 2018), *A.*
678 *thaliana* (Dunand et al., 2007; Tsukagoshi et al., 2010), and legumes, the model
679 legume *M. truncatula* (Zhang et al., 2014) and the crop legume *Phaseolus vulgaris*
680 (Montiel et al., 2013). The results regarding $O_2^{\cdot-}$ and H_2O_2 abundance in the tip of
681 growing roots we have obtained here for *M. truncatula* are similar to those previously
682 reported for maize and legumes, including *M. truncatula*. However, they are different
683 from those reported for *A. thaliana*. In the growing primary root of *A. thaliana*, $O_2^{\cdot-}$
684 mainly accumulated in the root tip while H_2O_2 accumulated in the mature part of the
685 root but not in the tip. Although the difference regarding H_2O_2 accumulation may be
686 linked to the probes used to detect this ROS that were not the same, a difference in
687 H_2O_2 production, conversion or function may exist between species. This idea is
688 supported by the results of the experiments of manipulation of H_2O_2 in which H_2O_2
689 proved to have opposite effects in *A. thaliana* (Dunand et al., 2007; Tsukagoshi et al.,
690 2010) and in *M. truncatula* (Fig. 8).

691 In addition, in *M. truncatula*, a tight correlation was found between the inhibition
692 of the primary root growth and the inhibition of $O_2^{\cdot-}$ accumulation in the root tip (Fig.
693 5). This strengthens the early idea that the root growth performance and the level of
694 $O_2^{\cdot-}$ accumulation in the root tip might be linked (Liszkay et al., 2004). However, in
695 the work performed by Zhang et al. (2014), using the *npf1.7* mutant of *M. truncatula*
696 deficient in the MtNPF1.7 nitrate transporter, the authors made an unexpected
697 observation. As compared with the corresponding wild type A17, this mutant is
698 characterized by a reduction of the primary root growth performance which is
699 associated to an overaccumulation of $O_2^{\cdot-}$ in the root tip, probably due to a debrided
700 NADPH oxidase activity. As an explanation of this apparent negative link between
701 $O_2^{\cdot-}$ accumulation and root growth performance in this mutant, the authors proposed

702 that a high level of $O_2^{\cdot-}$ would be toxic for root cells and thus detrimental for the
703 primary root growth. They suggested that an optimal level of $O_2^{\cdot-}$ might exist for the
704 performance of the primary root growth. On the basis of the existence of an optimal
705 $O_2^{\cdot-}$ level, we propose that DZA - that produces more $O_2^{\cdot-}$ than A17 and R108 and
706 has longer roots - may produce $O_2^{\cdot-}$ at a level close to the optimal level for *M.*
707 *truncatula* species while A17 and R108 may produce suboptimal levels of $O_2^{\cdot-}$ (Fig.
708 4). These results obtained with two different mutants deficient in two different nitrate
709 transporters also show a link between NADPH oxidase activity and nitrate transporter
710 integrity, the activity being debrided in *npf1.7* or no more responding to nitrate in
711 *npf6.8-2*.

712

713 **4.3 The decrease in ROS induced by nitrate is orchestrated by PODs**

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

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

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

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

755 According to our knowledge, the impact of nitrate on the accumulation of ROS in
756 the primary root tip was analyzed only in two studies (Trevisan et al., 2018; Zhang et
757 al., 2014). In contradiction with our results, Zhang et al. (2014) reported that nitrate
758 has no significant effect on the growth or on ROS accumulation in the primary root of
759 *M. truncatula* A17. A possible explanation for this discrepancy is that they worked
760 with 5-d-old seedlings that are poorly sensitive to nitrate according to our observation
761 (Fig. S2). In accordance with our results, Trevisan et al. (2018) observed that nitrate
762 modifies the patterns of ROS accumulation in the sensitive primary root of maize but
763 in a different way as compared with *M. truncatula*. Indeed, a treatment of N-deprived
764 seedlings with nitrate (longer than 24 h) that resulted in a decrease of primary root
765 growth was associated with an increase in $O_2^{\cdot-}$ and a decrease in H_2O_2 . Thus, ROS
766 response to nitrate may depend on the species, reinforcing the interest to conduct
767 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 H₂O₂ 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 H₂O₂ content is important for primary root
772 response to nitrate. H₂O₂ 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 Liskay 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 (•NO) (Manoli et al., 2016) suggesting a
786 possible link between auxin/•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 •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 (O₂^{•-}, H₂O₂ and •OH), and
800 four enzyme activities (NADPH oxidase, SOD, peroxidative and hydroxylic POD

801 activities).

802 **Contributions**

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

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

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

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

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

810 A.P.: set up a method adapted for H₂O₂ measurement in root tip and H₂O₂
811 measurement

812 **Acknowledgements**

813 We acknowledge Aurélia Rolland and Fabienne Simonneau for their
814 technological assistance at the IMAC/SFR QUASAV facility. This research supported
815 by the "IONIS Project 2017-2020" was conducted in the framework of the regional
816 program "Objectif Végétal, Research, Education and Innovation in Pays de la Loire",
817 supported by the French Region Pays de la Loire, Angers Loire Métropole and the
818 European Regional Development Fund AND by the Lili Zang PhD fellowship from the
819 China Scholarship Council (No. 201609370043).

820 **Conflicts of interest**

821 The authors declare no conflict of interest.

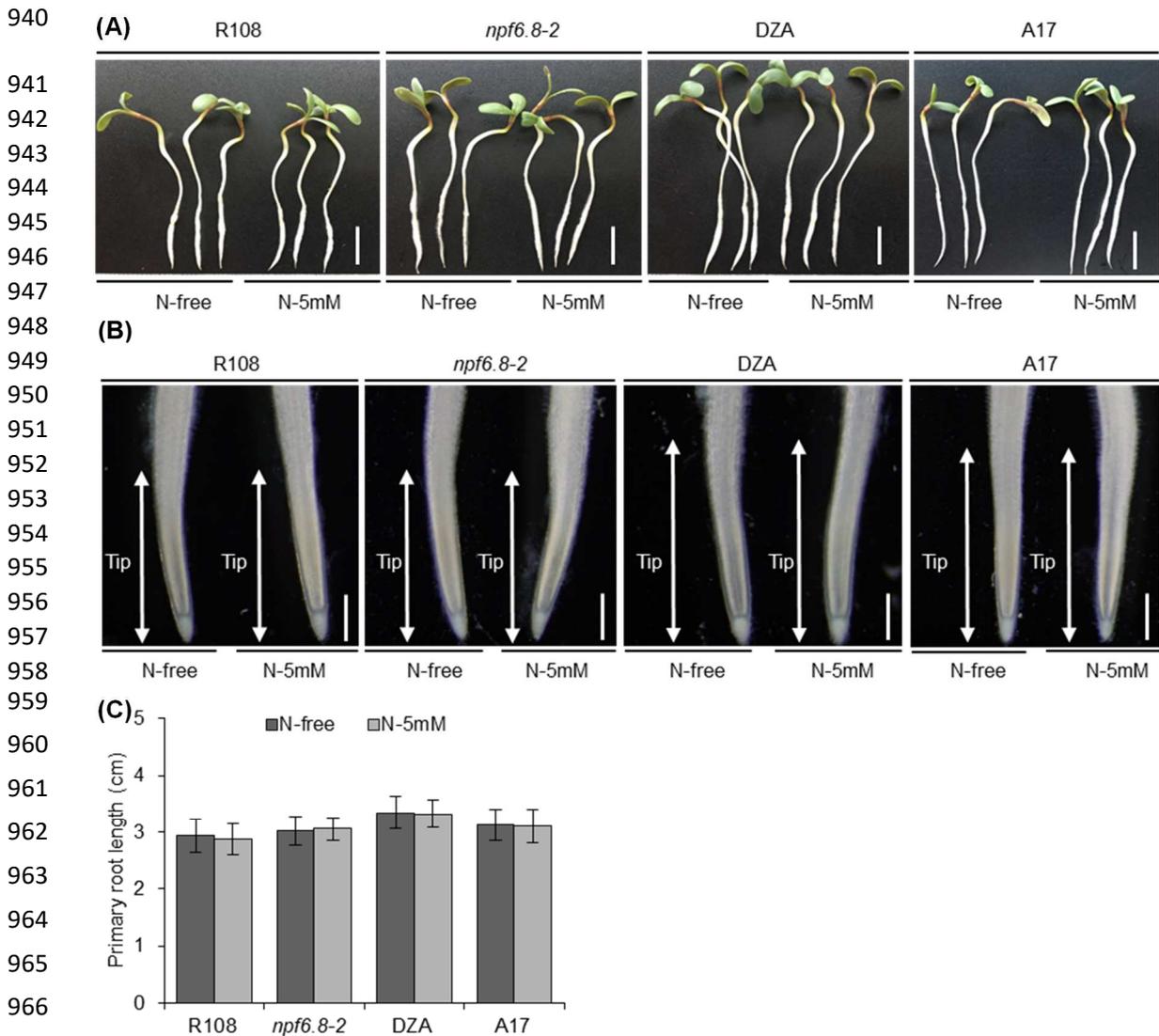
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939 **Supplemental figures:**



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

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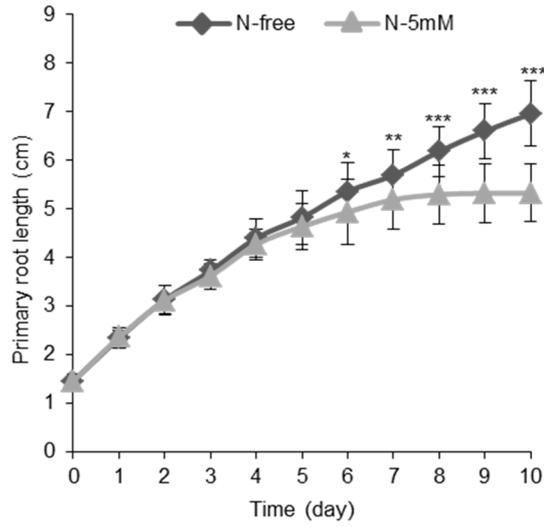
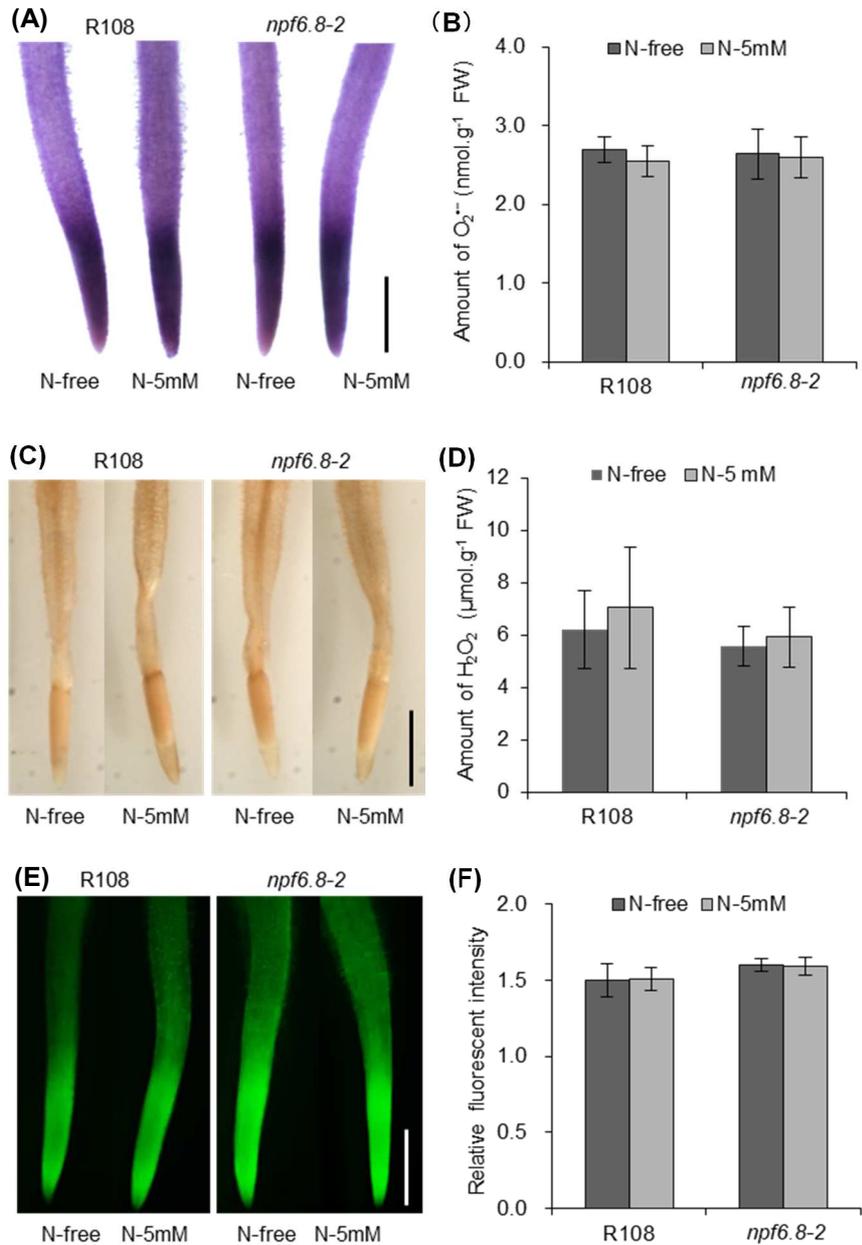


Fig. S2 Nitrate sensitivity of A17 primary root growth takes place during early growth. Seedlings of A17 were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM). Asterisks indicate when the root length determined at a given day is significantly different between the two conditions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

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1010 **Fig. S3 Nitrate has no effect on $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$ accumulation in the primary root of 2-d-old**
 1011 **seedlings.** Seedlings of R108 and *npf6.8-2* were grown without nitrate (N-free) or with 5 mM nitrate
 1012 (N-5 mM) for 2 d. (A) Detection of $O_2^{\cdot-}$ after 10 min of staining with nitroblue tetrazolium (NBT). (B)
 1013 Quantification of $O_2^{\cdot-}$ in the root tip. (C) Detection of H_2O_2 after 40 min of staining with
 1014 diaminobenzidine (DAB). (D) Quantification of H_2O_2 in the primary root tip with luminol. (E) Detection of
 1015 $\cdot OH$ after 30 min of staining using dihydrohodamine (DHR). (F) Quantification of DHR fluorescence
 1016 intensity. Scale bar = 1 mm. Quantification was realized using high resolution images and ImageJ
 1017 software. The statistical test used is an ANOVA and the significance is determined at the threshold $\alpha =$
 1018 5%.

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N-free

N-5 mM

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Fig. S4 Nitrate has no effect on $O_2^{\cdot-}$ accumulation in the lateral root of 10-d-old seedlings in

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R108. Seedlings were grown without nitrate (N-free) or with 5 mM nitrate (N-5 mM) for 10 d. Detection

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of $O_2^{\cdot-}$ after 10 min of staining with nitroblue tetrazolium (NBT). Lateral root tips are indicated with red

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triangles. Scale bar = 2 mm.

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