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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 <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
MS	Murashige and Skoog
NBT	Nitroblue tetrazolium
O <sub>2</sub> <sup>·-</sup>	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 ( $\text{NO}_3^-$ ) (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 N<sub>2</sub>, 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 (O<sub>2</sub><sup>-</sup>), hydroxyl radical (·OH) and  
111 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), were demonstrated to either promote growth through cell  
112 division in the meristem zone (O<sub>2</sub><sup>-</sup>) and cell elongation in the elongation zone (·OH)

113 through cell wall loosening or, in contrast, restrict growth in the differentiation zone  
114 ( $\text{H}_2\text{O}_2$ ) 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 ( $\text{O}_2^{\cdot-}$ ,  $\text{H}_2\text{O}_2$ ,  $\cdot\text{OH}$ ) 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  $\text{O}_2^{\cdot-}$ ,  $\text{H}_2\text{O}_2$  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  $\text{NO}_3^-$ . For some experiments, 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or 100  $\mu\text{M}$  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 O<sub>2</sub><sup>•-</sup> detection and measurement

150 O<sub>2</sub><sup>•-</sup> was detected by nitroblue tetrazolium (NBT; Sigma-Aldrich), which is  
151 reduced by O<sub>2</sub><sup>•-</sup> 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 O<sub>2</sub><sup>•-</sup>  
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 O<sub>2</sub><sup>•-</sup> produced per g of fresh weight (FW).

### 168 2.2.2 H<sub>2</sub>O<sub>2</sub> detection and measurement

169 H<sub>2</sub>O<sub>2</sub> 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<sup>-1</sup> 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 H<sub>2</sub>O<sub>2</sub> 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  $\mu$ 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  $\mu$ L were incubated at 30°C for 15 min with 10  $\mu$ L of distilled water or 10  $\mu$ L of a  
181 solution containing 50 U of bovin liver catalase (SIGMA-Aldrich) for control  
182 experiments. After this incubation, 10  $\mu$ 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  $\mu$ L of reaction  
185 mixture solution composed of 65  $\mu$ M of luminol and 10  $\mu$ 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 H<sub>2</sub>O<sub>2</sub> was quantified  
188 using a standard curve realized with 0 to 50 pmol of H<sub>2</sub>O<sub>2</sub> prepared freshly by dilution  
189 of H<sub>2</sub>O<sub>2</sub> in trichloroacetic acid then sodium carbonate as above for the samples. Root  
190 specific H<sub>2</sub>O<sub>2</sub> content was determined by the difference between the values obtained  
191 with and without catalase and was expressed as  $\mu$ mol.g<sup>-1</sup> 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  $\mu$ 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 CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> 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 O<sub>2</sub><sup>•-</sup>. 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 O<sub>2</sub><sup>•-</sup> produced min<sup>-1</sup>.mg<sup>-1</sup> 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 O<sub>2</sub><sup>•-</sup> 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<sup>-1</sup> cellulase  
233 and 0.6 mg mL<sup>-1</sup> 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<sup>-1</sup> 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 H<sub>2</sub>O<sub>2</sub>  
242 or to convert H<sub>2</sub>O<sub>2</sub> in  $\cdot$ OH. For the capacity to remove H<sub>2</sub>O<sub>2</sub>, 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 H<sub>2</sub>O<sub>2</sub>. Absorbance was  
245 measured at 430 nm. POD activity was expressed in nmol of H<sub>2</sub>O<sub>2</sub> reduced min<sup>-1</sup>.mg<sup>-1</sup>  
246 FW. The capacity to convert H<sub>2</sub>O<sub>2</sub> in  $\cdot$ 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  $\cdot$ 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  $\mu$ L  
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  $\cdot$ 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  $\cdot$ 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  $\cdot$ 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**

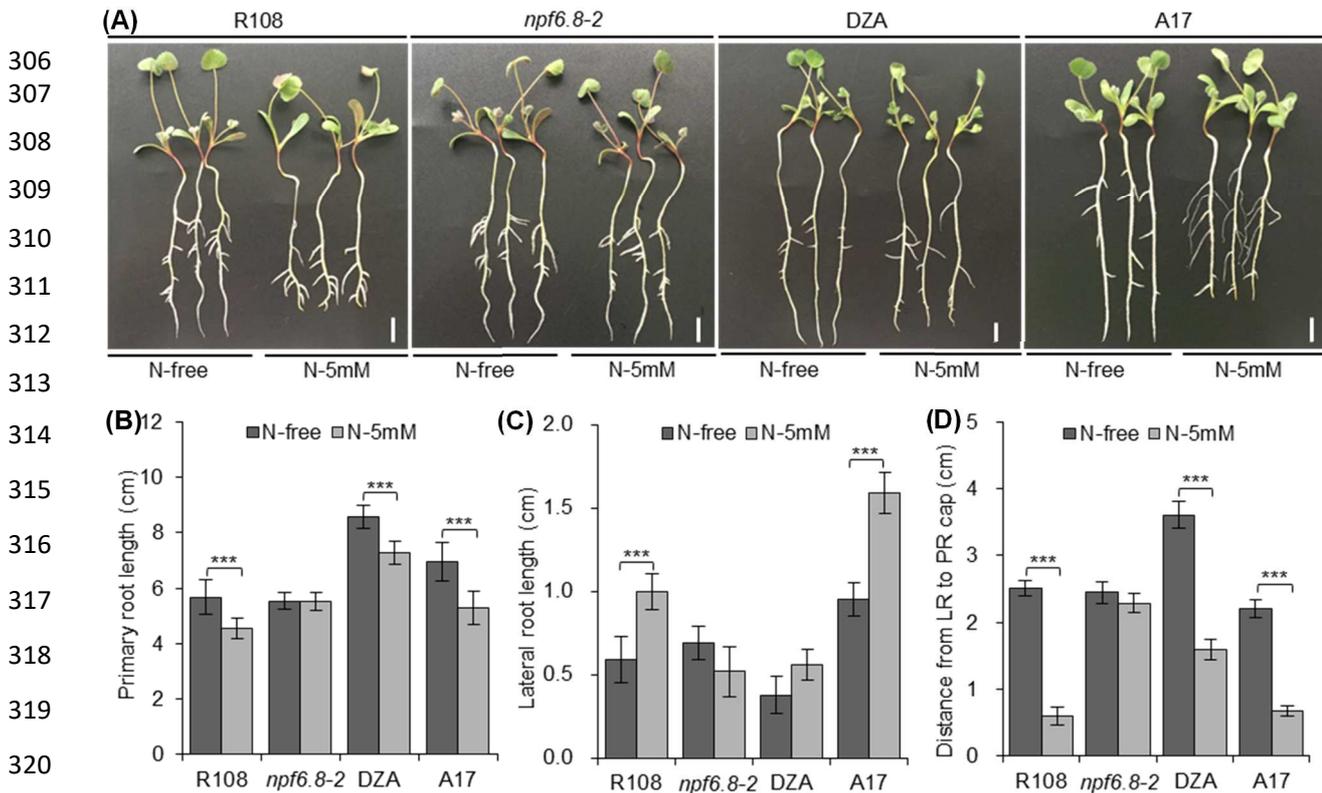
264 Each experiment was independently repeated at least three times at different  
265 dates. For root morphology characterization or root staining, at least 10 and often 20  
266 seedlings were used by repeat. Calculated means are represented  $\pm$  SD. For all data,  
267 relevant statistical tests 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 NO<sub>3</sub><sup>-</sup>, 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 NO<sub>3</sub><sup>-</sup>, 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.

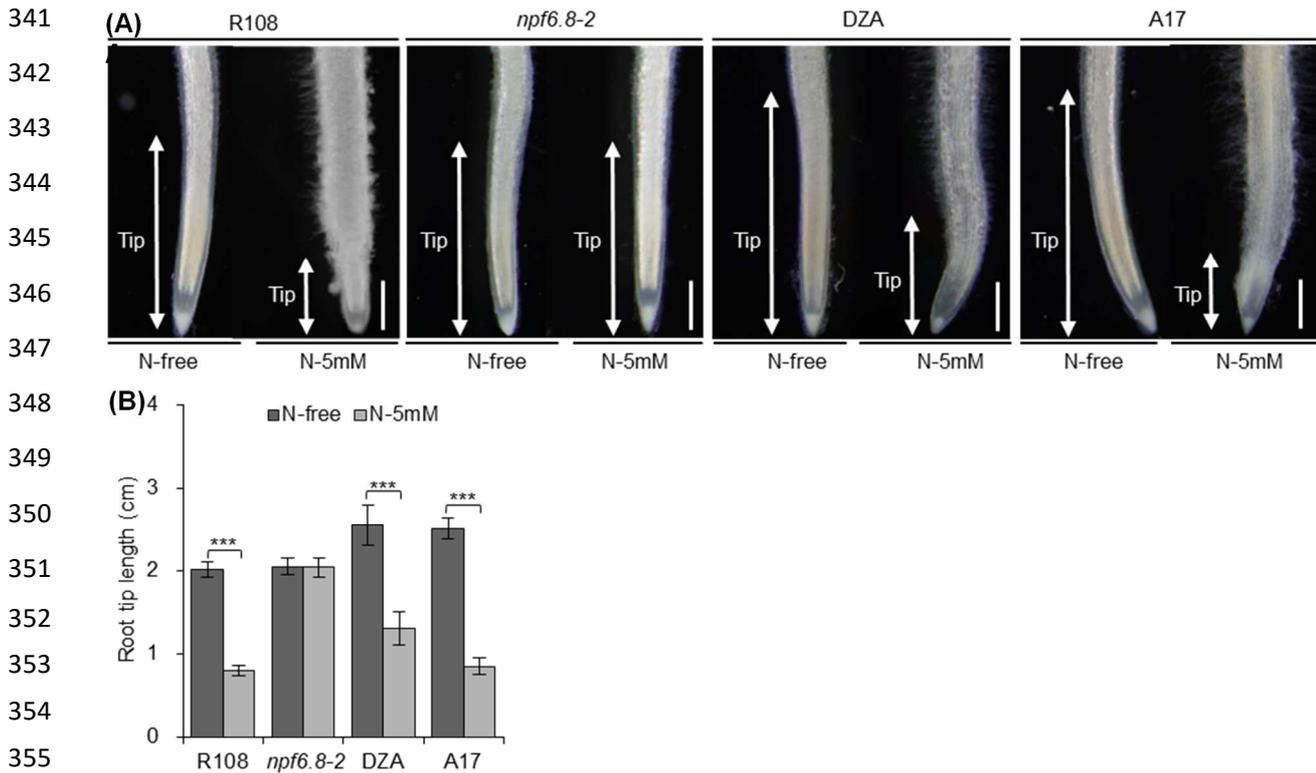


321 **Fig. 1 Nitrate modifies the architecture of the root system of the sensitive genotypes.** (A)  
322 Seedlings of R108, *npf6.8-2*, DZA 315-16 (DZA) and A17 grown in MS without nitrate (N-free) or with  
323 5 mM nitrate (N-5 mM) for 10 d. Scale bar = 1 cm. (B) Primary root length. (C) Lateral root 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  $\alpha = 5\%$ ; \*\*\*,  $p < 0.001$ .

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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  $\text{NO}_3^-$ , 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  $\mu$ 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  $\alpha = 5\%$ ; \*\*\*,  $p < 0.001$ .

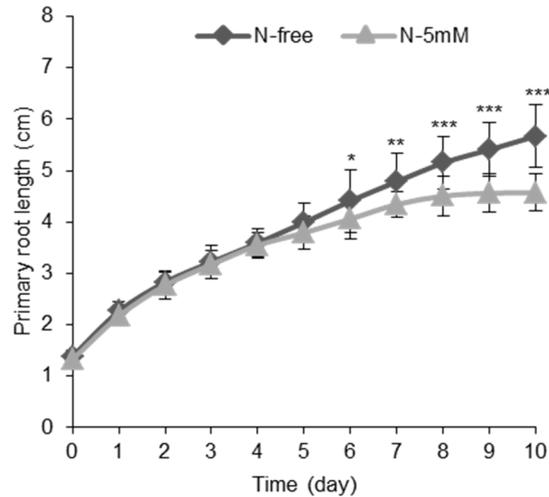
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### 362 3.2 Nitrate sensitivity takes place during early primary root growth

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  $\text{NO}_3^-$  (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  $\text{NO}_3^-$  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  $5.1 \pm 0.6$  cm. It is longer than that of the seedlings grown with 5 mM  
 372  $\text{NO}_3^-$  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.

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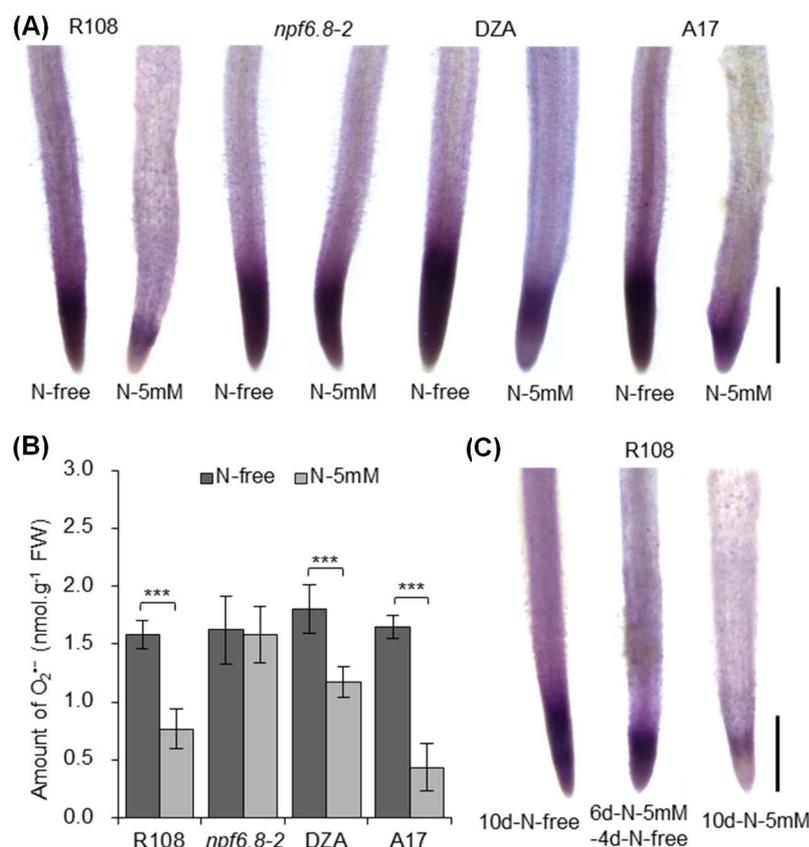
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431 **Fig. 4 Nitrate reduces  $O_2^{\cdot-}$  accumulation in the primary root tip of the sensitive genotypes.**

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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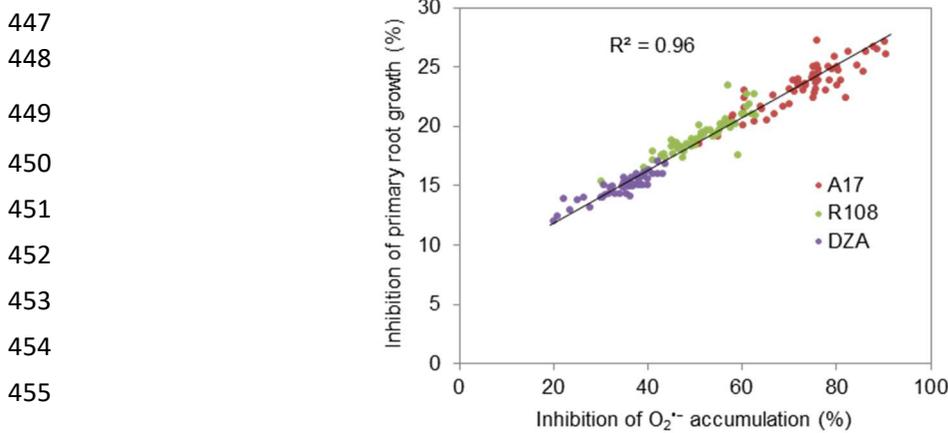
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These results raised the question whether the inhibition of the primary root  
 growth induced by nitrate could be linked to the removal of  $O_2^{\cdot-}$  in the tip. To test this  
 hypothesis, we plotted the percentage of growth inhibition as a 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.

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### 473 3.3.2 Nitrate reduces $H_2O_2$ accumulation

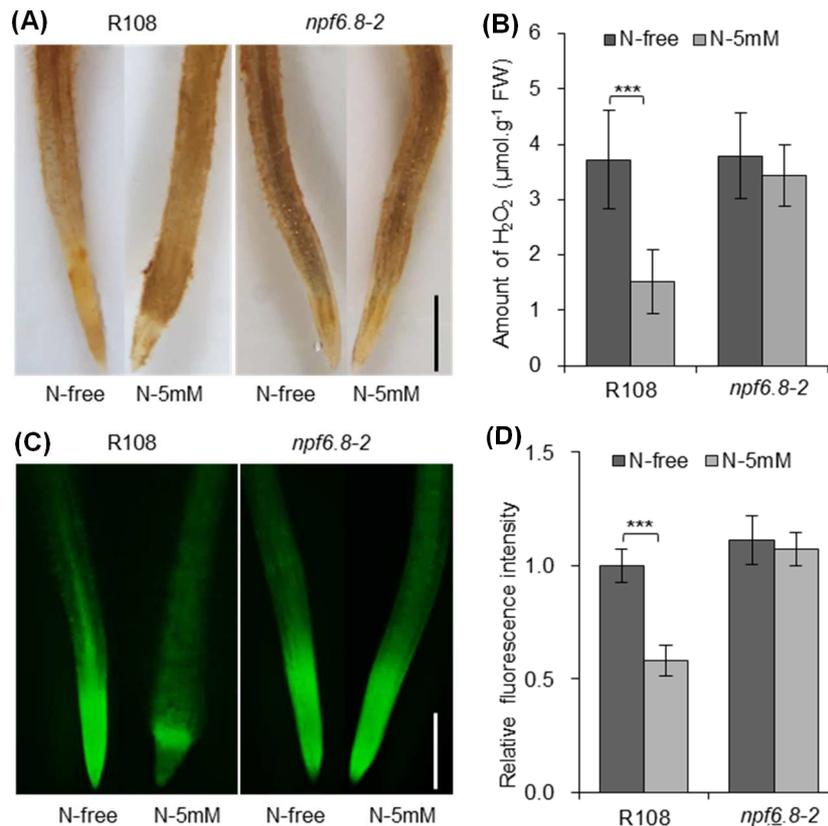
474 We used DAB to detect  $H_2O_2$  in the primary root of R108 and *npf6.8-2* (Fig. 6A),  
475 a product previously used for roots of *M. truncatula* (Zhang et al., 2014). In the  
476 absence of nitrate, DAB mainly stained the primary root tip. This was particularly  
477 obvious in younger seedlings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

478 accumulation of H<sub>2</sub>O<sub>2</sub> in the tip at 10 d was confirmed using luminol (Fig. 6B), a  
479 product recommended by Noctor et al. (2016). H<sub>2</sub>O<sub>2</sub> 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 NO<sub>3</sub><sup>-</sup>, the abundance of H<sub>2</sub>O<sub>2</sub>  
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 H<sub>2</sub>O<sub>2</sub> 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 <sup>•</sup>OH accumulation

487 We used the fluorescent probe DHR to detect <sup>•</sup>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, <sup>•</sup>OH was mainly distributed in the primary root  
490 tip (Fig. 6C and D), with a pattern similar to O<sub>2</sub><sup>•-</sup> (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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> in the primary root after 40 min of staining with 3,3'-diaminobenzidine (DAB). (B) Quantification of H<sub>2</sub>O<sub>2</sub> 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

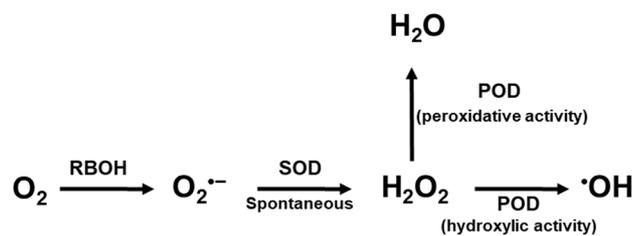
519 As shown above, nitrate had no impact on the phenotype of the primary root of  
520 2-d-old seedlings of the wild type genotypes R108, DZA and A17 (Fig. 3, Fig. S1 and  
521 S2). We wondered whether nitrate modifies the ROS accumulation pattern in the tip  
522 at this stage of primary root growth. 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 O<sub>2</sub><sup>·-</sup>,  
525 H<sub>2</sub>O<sub>2</sub> 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**

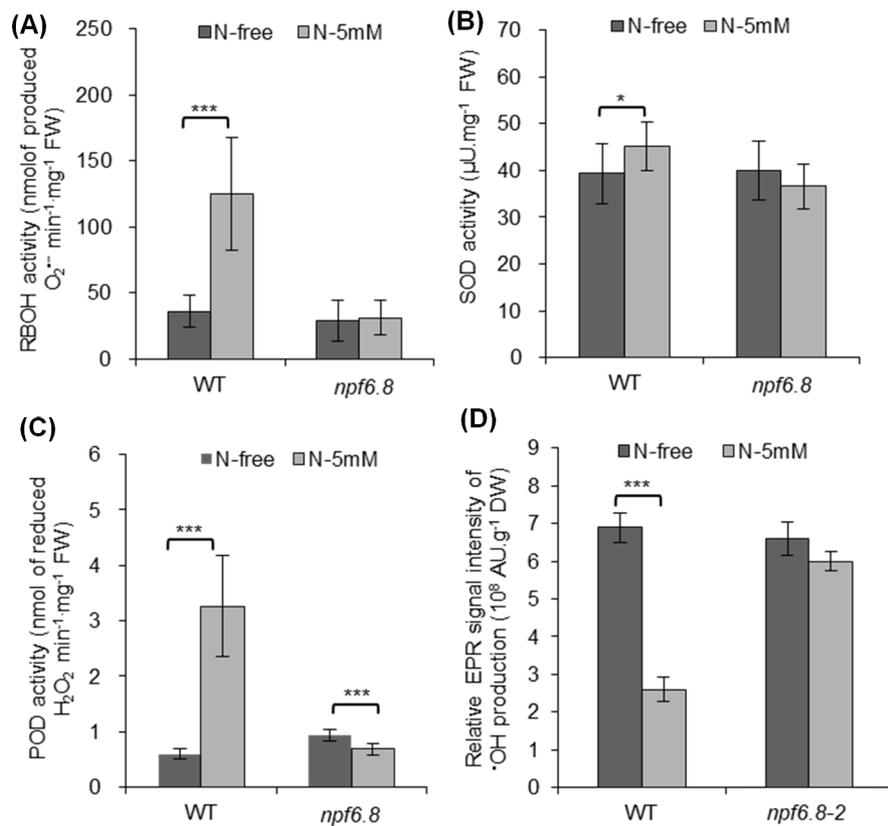
536 The decrease in ROS accumulation induced by nitrate could be due to a  
 537 decrease in ROS production and/or an increase in ROS removal. Thus, to find out  
 538 the origin of the decrease in ROS accumulation, we measured, in the primary root tip  
 539 of the wild type R108 and the *npf6.8-2* 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  $\mu\text{U}$  per mg of FW. POD capacity to remove  $\text{H}_2\text{O}_2$  increased 3.4 times  
 554 (from 0.60 to 3.27 nmol  $\text{H}_2\text{O}_2$  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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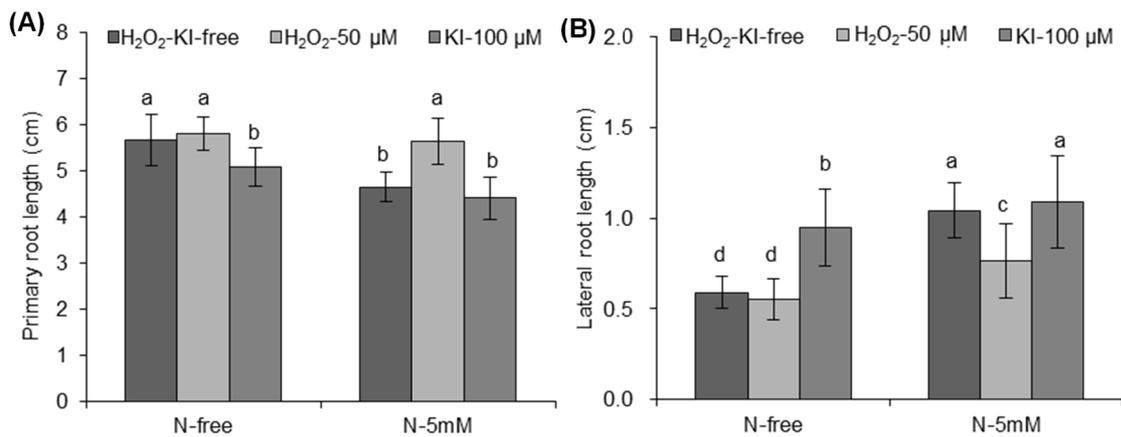
**Fig. 7 Nitrate alters RBOH, SOD and POD activities in the primary root tip of the R108 sensitive genotype.** Seedlings of R108 and *npf6.8-2* were grown for 10 d in MS without nitrate (N-free) or with 5 mM nitrate (N-5 mM). RBOH was measured in crude root tip homogenates while SOD and POD activities 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 <sup>•</sup>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 H<sub>2</sub>O<sub>2</sub> or mimicked 587 by KI, an H<sub>2</sub>O<sub>2</sub> scavenger

588 To address a possible central role of the regulation of H<sub>2</sub>O<sub>2</sub> abundance in the  
589 primary root tip by PODs in response to nitrate, we investigated the effect of a  
590 manipulation of H<sub>2</sub>O<sub>2</sub> abundance on this response by adding H<sub>2</sub>O<sub>2</sub> or KI, a H<sub>2</sub>O<sub>2</sub>  
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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> content may be important for the primary root  
 597 response to nitrate. This is also true for lateral roots (Fig. 8B). Indeed, H<sub>2</sub>O<sub>2</sub>  
 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 α =  
 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 H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> content is important for primary root  
772 response to nitrate. H<sub>2</sub>O<sub>2</sub> 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 (O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> measurement in root tip and H<sub>2</sub>O<sub>2</sub>  
811 measurement

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## 820 **Conflicts of interest**

821 The authors declare no conflict of interest.

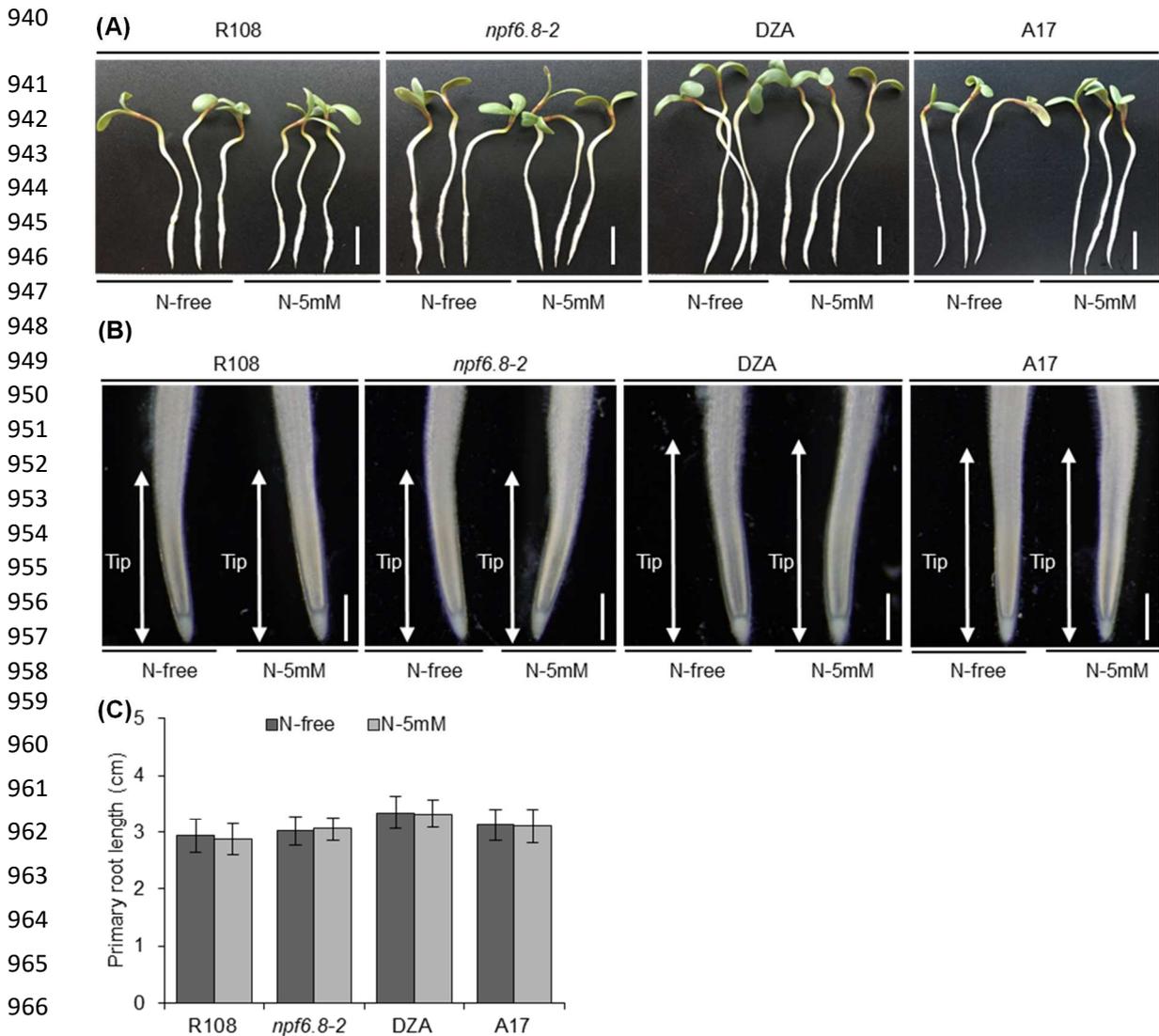
822 **References**

- 823 Bagchi, R., Salehin, M., Adeyemo, O.S., Salazar, C., Shulaev, V., Sherrier, D.J., Dickstein, R., 2012.  
824 Functional assessment of the *Medicago truncatula* NIP/LATD protein demonstrates that it is a  
825 high-affinity nitrate transporter. *Plant Physiol.* 160, 906–916.  
826 <https://doi.org/10.1104/pp.112.196444>
- 827 Baluška, F., Mancuso, S., 2013. Root apex transition zone as oscillatory zone. *Front. Plant Sci.* 4, 1–  
828 15. <https://doi.org/10.3389/fpls.2013.00354>
- 829 Dunand, C., Crèvecoeur, M., Penel, C., 2007. Distribution of superoxide and hydrogen peroxide in  
830 *Arabidopsis* root and their influence on root development: Possible interaction with peroxidases.  
831 *New Phytol.* 174, 332–341. <https://doi.org/10.1111/j.1469-8137.2007.01995.x>
- 832 Ferguson, B.J., Mens, C., Su, H., Jones, C.H., Zhang, M., Hastwell, A.H., Chu, X., Gresshoff, P.M.,  
833 2019. Legume nodulation: The host controls the party. *Plant. Cell Environ.* 42, 41–51.  
834 <https://doi.org/10.1111/pce.13348>
- 835 Foyer, C.H., Noctor, G., 2016. Stress-triggered redox signalling: What's in pROspect? *Plant Cell*  
836 *Environ.* 39, 951–964. <https://doi.org/10.1111/pce.12621>
- 837 Gill, R.A., Zang, L., Ali, B., Farooq, M.A., Cui, P., Yang, S., Ali, S., Zhou, W., 2015. Chromium-induced  
838 physio-chemical and ultrastructural changes in four cultivars of *Brassica napus* L. *Chemosphere*  
839 120, 154–164. <https://doi.org/10.1016/j.chemosphere.2014.06.029>
- 840 Junglee, S., Urban, L., Sallanon, H., Lopez-Lauri, F., 2014. Optimized assay for hydrogen peroxide  
841 determination in plant tissue using potassium iodide. *Am. J. Anal. Chem.* 05, 730–736.  
842 <https://doi.org/10.4236/ajac.2014.511081>
- 843 Kanno, Y., Hanada, A., Chiba, Y., Ichikawa, T., Nakazawa, M., Matsui, M., Koshihara, T., Kamiya, Y.,  
844 Seo, M., 2012. Identification of an abscisic acid transporter by functional screening using the  
845 receptor complex as a sensor. *Proc. Natl. Acad. Sci.* 109, 9653–9658.  
846 <https://doi.org/10.1073/pnas.1203567109>
- 847 Krapp, A., 2015. Plant nitrogen assimilation and its regulation: A complex puzzle with missing pieces.  
848 *Curr. Opin. Plant Biol.* 25, 115–122. <https://doi.org/10.1016/j.pbi.2015.05.010>
- 849 Kukavica, B.M., Veljović-Jovanović, S., Menckhoff, L., Lühje, S., 2012. Cell wall-bound cationic and  
850 anionic class III isoperoxidases of pea root: biochemical characterization and function in root  
851 growth. *J. Exp. Bot.* 63, 4631–4645. <https://doi.org/10.1093/jxb/err313>
- 852 Kwak, J.M., Mori, I.C., Pei, Z., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones,  
853 J.D.G., Schroeder, J.I., 2003. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-  
854 dependent ABA signaling in *Arabidopsis*. *EMBO J.* 22, 2623–2633.  
855 <https://doi.org/10.1093/emboj/cdg277>
- 856 Liskay, A., van der Zalm, E., Schopfer, P., 2004. Production of reactive oxygen intermediates (O<sub>2</sub><sup>-</sup>,  
857 H<sub>2</sub>O<sub>2</sub>, and ·OH) by maize roots and their role in wall loosening and elongation growth. *Plant*  
858 *Physiol.* 136, 3114–3123. <https://doi.org/10.1104/pp.104.044784.3114>
- 859 Lu, S., Song, J., Campbell-Palmer, L., 2009. A modified chemiluminescence method for hydrogen  
860 peroxide determination in apple fruit tissues. *Sci. Hortic.* 120, 336–341.  
861 <https://doi.org/10.1016/j.scienta.2008.11.003>
- 862 Manoli, A., Trevisan, S., Voigt, B., Yokawa, K., Baluška, F., Quaggiotti, S., 2016. Nitric oxide-mediated  
863 maize root apex responses to nitrate are regulated by auxin and strigolactones. *Front. Plant Sci.*  
864 6, 1–15. <https://doi.org/10.3389/fpls.2015.01269>
- 865 Maphosa, Y., Jideani, V.A., 2017. The role of legumes in human nutrition., in: Hueda, M.C. (Ed.),  
866 Functional food - Improve health through adequate food. InTech, pp. 103–121.  
867 <https://doi.org/10.5772/66263>
- 868 Mittler, R., 2017. ROS are good. *Trends Plant Sci.* 22, 11–19.  
869 <https://doi.org/10.1016/j.tplants.2016.08.002>
- 870 Mojović, M., Vuletić, M., Bačić, G.G., Vučinić, Ž., 2004. Oxygen radicals produced by plant plasma  
871 membranes: An EPR spin-trap study. *J. Exp. Bot.* 55, 2523–2531.  
872 <https://doi.org/10.1093/jxb/erh266>

- 873 Montiel, J., Arthikala, M.K., Quinto, C., 2013. *Phaseolus vulgaris RbohB* functions in lateral root  
874 development. *Plant Signal. Behav.* 8, 144–146. <https://doi.org/10.4161/psb.22694>
- 875 Morère-Le Paven, M.C., Viau, L., Hamon, A., Vandecasteele, C., Pellizzaro, A., Bourdin, C., Laffont,  
876 C., Laped, B., Lepetit, M., Frugier, F., Legros, C., Limami, A.M., 2011. Characterization of a  
877 dual-affinity nitrate transporter MtNRT1.3 in the model legume *Medicago truncatula*. *J. Exp. Bot.*  
878 62, 5595–5605. <https://doi.org/10.1093/jxb/err243>
- 879 Motte, H., Vanneste, S., Beeckman, T., 2019. Molecular and environmental regulation of root  
880 development. *Annu. Rev. Plant Biol.* 70, 465–488. <https://doi.org/10.1146/annurev-arplant-050718-100423>
- 882 Nacry, P., Bouguyon, E., Gojon, A., 2013. Nitrogen acquisition by roots: Physiological and  
883 developmental mechanisms ensuring plant adaptation to a fluctuating resource. *Plant Soil* 370,  
884 1–29. <https://doi.org/10.1007/s11104-013-1645-9>
- 885 Noctor, G., Mhamdi, A., Foyer, C.H., 2016. Oxidative stress and antioxidative systems: Recipes for  
886 successful data collection and interpretation. *Plant Cell Environ.* 39, 1140–1160.  
887 <https://doi.org/10.1111/pce.12726>
- 888 O'Brien, J.A., Vega, A., Bouguyon, E., Krouk, G., Gojon, A., Coruzzi, G., Gutiérrez, R.A., 2016. Nitrate  
889 transport, sensing, and responses in plants. *Mol. Plant* 9, 837–856.  
890 <https://doi.org/10.1016/j.molp.2016.05.004>
- 891 Pellizzaro, A., Alibert, B., Planchet, E., Limami, A.M., Morère-Le Paven, M.C., 2017. Nitrate  
892 transporters: an overview in legumes. *Planta* 246, 585–595. <https://doi.org/10.1007/s00425-017-2724-6>
- 894 Pellizzaro, A., Clochard, T., Cukier, C., Bourdin, C., Juchaux, M., Montrichard, F., Thany, S., Raymond,  
895 V., Planchet, E., Limami, A.M., Morère-Le Paven, M.C., 2014. The nitrate transporter MtNPF6.8  
896 (MtNRT1.3) transports abscisic acid and mediates nitrate regulation of primary root growth in  
897 *Medicago truncatula*. *Plant Physiol.* 166, 2152–2165. <https://doi.org/10.1104/pp.114.250811>
- 898 Porwol, T., Ehleben, W., Zierold, K., Fandrey, J., Acker, H., 1998. The influence of nickel and cobalt  
899 on putative members of the oxygen-sensing pathway of erythropoietin-producing HepG2 cells.  
900 *Eur. J. Biochem.* 256, 16–23. <https://doi.org/10.1046/j.1432-1327.1998.2560016.x>
- 901 Ramel, F., Sulmon, C., Bogard, M., Couée, I., Gouesbet, G., 2009. Differential patterns of reactive  
902 oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced  
903 tolerance in *Arabidopsis thaliana* plantlets. *BMC Plant Biol.* 9, 1–18.  
904 <https://doi.org/10.1186/1471-2229-9-28>
- 905 Sagi, M., Fluhr, R., 2001. Superoxide production by plant homologues of the gp91<sup>phox</sup> NADPH oxidase.  
906 Modulation of activity by calcium and by Tobacco Mosaic Virus infection. *Plant Physiol.* 126,  
907 1281–1290. <https://doi.org/10.1104/pp.126.3.1281>
- 908 Signora, L., De Smet, I., Foyer, C.H., Zhang, H., 2001. ABA plays a central role in mediating the  
909 regulatory effects of nitrate on root branching in *Arabidopsis*. *Plant J.* 28, 655–662.  
910 <https://doi.org/10.1046/j.1365-313x.2001.01185.x>
- 911 Singh, R., Parihar, P., Singh, S., Mishra, R.K., Singh, V.P., Prasad, S.M., 2017. Reactive oxygen  
912 species signaling and stomatal movement: Current updates and future perspectives. *Redox Biol.*  
913 11, 213–218. <https://doi.org/10.1016/j.redox.2016.11.006>
- 914 Trevisan, S., Trentin, A.R., Ghisi, R., Masi, A., Quaggiotti, S., 2018. Nitrate affects transcriptional  
915 regulation of UPBEAT1 and ROS localisation in roots of *Zea mays* L. *Physiol. Plant.* 166, 794–  
916 811. <https://doi.org/10.1111/ppl.12839>
- 917 Tsukagoshi, H., Busch, W., Benfey, P.N., 2010. Transcriptional regulation of ROS controls transition  
918 from proliferation to differentiation in the root. *Cell* 143, 606–616.  
919 <https://doi.org/10.1016/j.cell.2010.10.020>
- 920 Veljović-Jovanović, S., Kukavica, B., Vidović, M., Morina, F., Menckhoff, L., 2018. Class III  
921 peroxidases: functions, localization and redox regulation of isoenzymes., in: Gupta, D., Palma, J.,  
922 Corpas, F. (Eds.), *Antioxidants and Antioxidant Enzymes in Higher Plants*. Springer, Cham, pp.  
923 269–300. [https://doi.org/10.1007/978-3-319-75088-0\\_13](https://doi.org/10.1007/978-3-319-75088-0_13)
- 924 Walch-liu, P., Forde, B.G., 2008. Nitrate signalling mediated by the NRT1.1 nitrate transporter

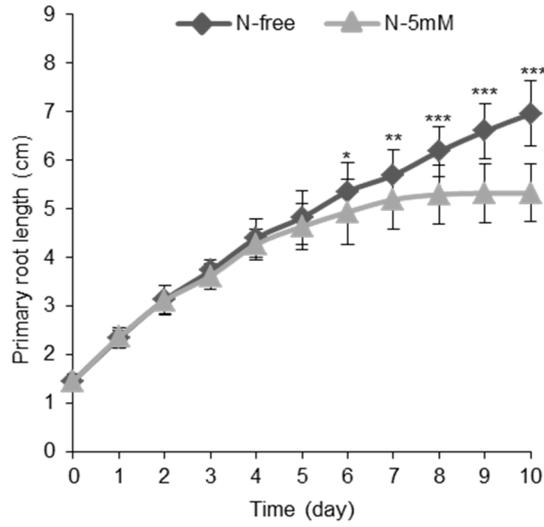
- 925 antagonises L-glutamate-induced changes in root architecture. *Plant J.* 54, 820–828.  
926 <https://doi.org/10.1111/j.1365-313X.2008.03443.x>
- 927 Yendrek, C.R., Lee, Y.C., Morris, V., Liang, Y., Pislariu, C.I., Burkart, G., Meckfessel, M.H., Salehin,  
928 M., Kessler, H., Wessler, H., Lloyd, M., Lutton, H., Teillet, A., Sherrier, D.J., Journet, E.P., Harris,  
929 J.M., Dickstein, R., 2010. A putative transporter is essential for integrating nutrient and hormone  
930 signaling with lateral root growth and nodule development in *Medicago truncatula*. *Plant J.* 62,  
931 100–112. <https://doi.org/10.1111/j.1365-313X.2010.04134.x>
- 932 Zhang, C., Bousquet, A., Harris, J.M., 2014. Abscisic acid and LATERAL ROOT ORGAN  
933 DEFECTIVE/NUMEROUS INFECTIONS AND POLYPHENOLICS modulate root elongation via  
934 reactive oxygen species in *Medicago truncatula*. *Plant Physiol.* 166, 644–658.  
935 <https://doi.org/10.1104/pp.114.248542>
- 936 Zhao, L., Liu, F., Crawford, N.M., Wang, Y., 2018. Molecular regulation of nitrate responses in plants.  
937 *Int. J. Mol. Sci.* 19, 2039. <https://doi.org/10.3390/ijms19072039>
- 938

939 **Supplemental figures:**



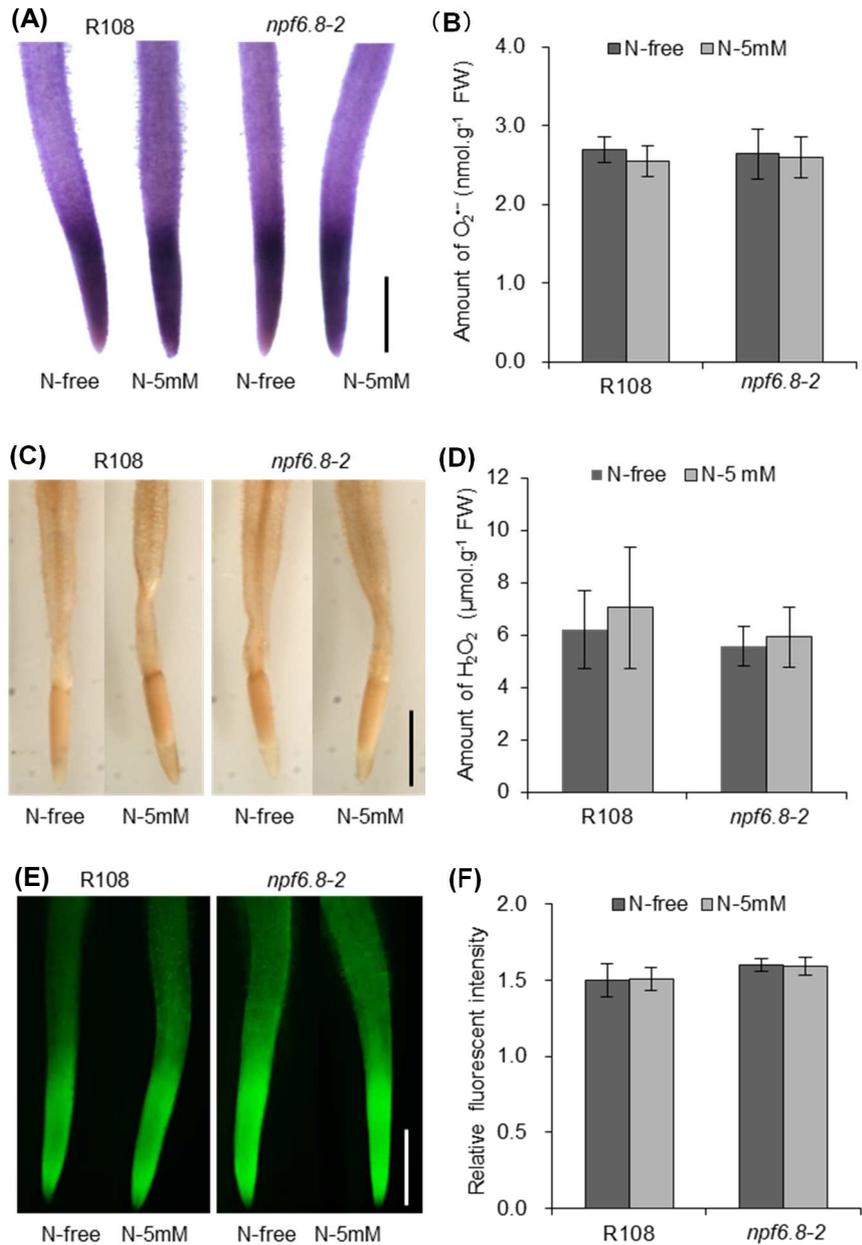
968 **Fig. S1 Nitrate has no effect on the primary root length and root tip morphology of 2-d-old**  
 969 **seedlings.** (A) Seedlings of R108, *npf6.8-2*, DZA and A17 were grown in MS without nitrate (N-free)  
 970 or with 5 mM nitrate (N-5 mM) for 2 d. Scale bar = 1 cm. (B) Primary root tip. Scale bar = 500  $\mu$ 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  $O_2^{\bullet-}$ ,  $H_2O_2$  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  $O_2^{\bullet-}$  after 10 min of staining with nitroblue tetrazolium (NBT). (B)  
 1013 Quantification of  $O_2^{\bullet-}$  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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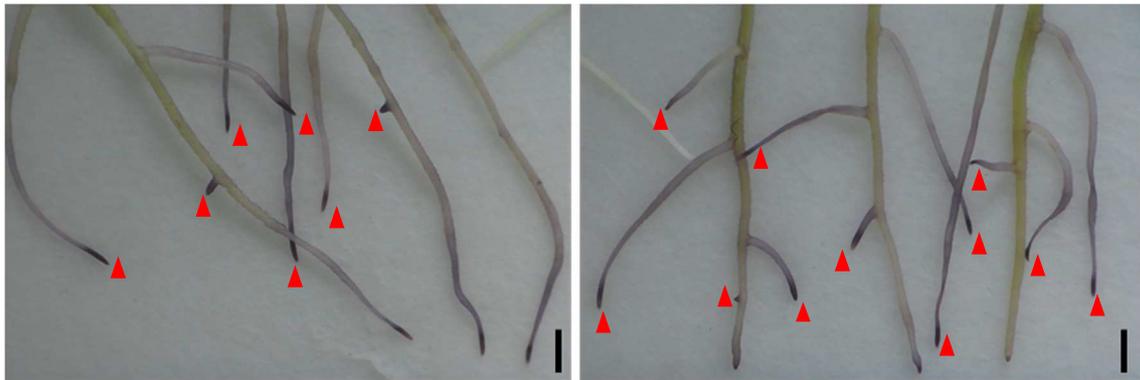
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N-free

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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