Trichoderma asperelloides enhances local and systemic acquired resistance response under low nitrate nutrition in Arabidopsis

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Author contribution

KJG supervised, designed the project and wrote the manuscript with help from ARF. AW designed, performed experiments and analyzed whole data. PKP performed experiments.

One-sentence summary: *Trichoderma* enhances local and systemic acquired resistance under low nitrate nutrition

Abstract

Nitrogen (N) is essential for growth, development and defense but, how low N affects defense and the role of *Trichoderma* in enhancing defense under low nitrate is not known. Low nitrate fed Arabidopsis plants displayed reduced growth and compromised local and systemic acquired resistance responses when infected with both avirulent and virulent *Pseudomonas syringae* DC3000. These responses were enhanced in the presence of *Trichoderma*. The mechanism of increased local and systemic acquired resistance mediated by *Trichoderma* involved increased N uptake and enhanced protein levels via modulation of nitrate transporter genes. The *nrt2.1* mutant is compromised in local and systemic acquired resistance responses suggesting a link between enhanced N transport and defense. Enhanced N uptake was mediated by *Trichoderma* elicited nitric oxide (NO). Low NO producing *nia1,2* mutant and *nsHb*$^{+}$ over expressing lines were unable to induce nitrate transporters and thereby compromised defense in the presence of *Trichoderma* under low N suggesting a signaling role of *Trichoderma* elicited NO. *Trichoderma* also induced SA and defense gene expression under low N. The SA deficient *NahG* transgenic line and the *npr1* mutant were also compromised in *Trichoderma*-mediated local and systemic acquired resistance responses. Collectively our results indicated that the mechanism of enhanced plant defense under low N mediated by *Trichoderma* involves NO, ROS, SA production as well as the induction of NRT and marker genes for systemic acquired resistance.

Key words: Nitric oxide, nitrate transporters, *Pseudomonas syringae,* SAR, *Trichoderma*

Introduction

Nitrogen (N) is essential for growth and development of plants. It is a crucial component in chlorophyll, nucleic acids and amino acids as well as a large number of specialized metabolites. N, furthermore, plays an important role in the regulation of primary and secondary metabolism and also in protection of plants against biotic and abiotic stresses since it triggers defense response against stresses (O'Brien *et al.* 2016; Mur *et al.* 2017). N deficiency occurs in soil due to slow mineralization, lack of sufficient organic matter, leaching due to heavy rainfalls and increased activities of denitrifying bacteria. Low N can retard plant growth and cause severe physiological and morphological defects (Walker *et al.* 2001; Landrein *et al*. 2018). To cope with this, plants have evolved N uptake systems that support their survival under N deficiency (Li *et al.* 2017). These uptake systems are based on their affinity with transport of nitrates (NO_3^-), being mediated by the low and high affinity nitrate transporter protein families (LATs & HATs; Tsay *et al.* 2007).

N plays a very significant role in plant defense against both virulent and avirulent pathogens (Dietrich *et al.* 2004; Gupta *et al.* 2013; Mur *et al.* 2017). Hence, operation of an efficient N transport system can help plants to obtain sufficient nitrogen to defend themselves against bacterial pathogens. These defense responses involve activation of innate immune response comprising of pathogen-triggered immunity (PTI and effector-triggered immunity (ETI) (Alves *et al.* 2014; Jones and Dangl 2006) and require N. One of the defense mechanisms against invading pathogen importantly includes rapid programmed cell death known as the hypersensitive response (HR), which develops during incompatible plant-pathogen (*R-avr*) interactions (Delledonne *et al.* 1998). An early characteristic of HR is the rapid generation of superoxide ($O_2^{-\cdot}$), nitric oxide (NO) and the accumulation of H_2O_2 (Lamb and Dixon, 1997). Moreover, SA produced during HR, plays an important role in plant defense (Mur *et al.* 2000, 2008; Gupta *et al.* 2013). The host mobilizes salicylic acid (SA) for plant defense (Oliva and Quibod 2017, Mur *et al.* 2017) and this pathway itself requires N for synthesis of various intermediates leading to SA production. Thus, N not only improves the nutritional status of the plant, but, also it also plays a more direct role in defense. Moreover, the form of N nutrition can greatly influence the HR-mediated resistance in plants (Gupta *et al.* 2013).

Nitrate (NO_3^-) nutrition greatly influences HR via the production of NO which is a regulatory signal in plant defense (Delledonne *et al.* 1998). NO production depends on NO_3^- or L-Arginine (Planchet *et al*. 2005, Astier *et al.* 2018) As such, N deficiency can also lead to reduced levels of NO. Moreover, SA is known to be induced by NO, hence, low NO_3^- leads both to low NO and reduced SA levels. Since NO production also requires NO_3^-, it may also play a role in local acquired resistance (LAR) where resistance is developed at the site of infection and also in systemic acquired resistance (SAR) which results in broad-spectrum disease resistance against secondary infections following the primary infection (Cameron *et al.* 1994, 1999). SAR develops either as a consequence of HR where NO has a proven role (Delledonne *et al.* 1998). SAR is dependent on SA or its derivatives (Park *et al.* 2007, Metraux *et al.* 1990) as well as pathogen responsive (*PR*) gene expression (Ryals *et al.* 1996). It was previously shown that NO plays a role in nitrate uptake/assimilation during stress by modulation of the expression of nitrate transporters (Frungillo *et al.* 2013). Thus, N plays likely plays multifaceted roles in plant defense. Indeed, whilst it has been shown that a very high levels of N can increase susceptibility to pathogen infection (Fagard *et al.* 2014) a low level of N can also increase susceptibility (Dietrich *et al.* 2004). For this reason we set out to test whether increasing N uptake can assist in plant defense under low NO_3^-. Many plant symbiotic microbes such as mycorrhiza, *Trichoderma* and plant growth promoting rhizobia are known to increase nutrient uptake. However, the operation of plant defense under low N and the effect of these microbes in increasing plant defense under N deficiency is currently unknown.

Several species of *Trichoderma* play an important role in plant growth promotion and resistance against various biotic and abiotic stresses. They confer resistance to plants via various mechanisms such as mycoparasitism, activation of basal and induced systemic resistance (ISR) responses (Brotman *et al.* 2012). Upon pathogen attack, *Trichoderma* treated plants show both elevated elevated phenylalanine ammonia-lyase (*PAL*) transcript levels (Yedidia *et al.* 2003) and increased levels of defense-related plant enzymes (Shoresh *et al.* 2005).

If *Trichoderma* enhances N uptake, it could be anticipated to have positive effects on plant defense via both LAR and SAR responses. So far is it not known whether *Trichoderma* can increase plant defense under low N. Therefore, in the

present study, we have assessed, the systemic defense response of low N fed *Arabidopsis thaliana* plants to the phytopathogen; *Pseudomonas syringae* p.v. tomato DC3000 induced by the beneficial fungus *Trichoderma asperelloides* (*T203*) under low and optimum nitrate concentrations. Here, we describe that *Trichoderma* enhances the SAR response in Arabidopsis grown under low N by enhancing NO_3^- uptake via enhancing nitrate transporter expression with this mechanism involving both NO and SA.

Results

Low nitrogen compromises LAR response and *Trichoderma* enhances LAR under low N condition

Nitrogen plays an important role in plant defense, hence, we first tested the effect of low nitrate on the LAR response. For this purpose, WT plants were grown under high (3 mM) and low (0.1 mM) NO_3^- conditions and infected with avirulent *P. syringae* DC3000 (*avrRpm1*) and the local HR response was observed (Fig. **1a**, Fig. **S3b**). As shown in Fig.**1a-WT panel**, the 3 mM NO_3^--fed *Pst* infiltrated plants showed HR at the inoculation sites at 24 and 48 hours post infection (hpi) whereas plants grown in the presence of 0.1 mM NO_3^- plants displayed chlorotic lesions at the site of inoculation at 24 and 48 hpi. We next studied the effect of *Trichoderma* on the LAR response Surprisingly, 0.1 mM NO_3^- grown WT plants in the presence of *Trichoderma* did not show any severe disease symptoms, rather they mimicked the HR phenotype of 3 mM NO_3^- grown plants and displayed resistance (Fig. **1a-WT+T panel**). Interestingly, we also found that under 0.1 mM NO_3^-, *Trichoderma* treated plants displayed an enhanced growth phenotype compared to plants in the absence of *Trichoderma* (Fig. **S1a**), this enhanced growth was linked to an increased leaf number, leaf fresh weight and total chlorophyll content (Fig. **S2 b, c, d**). These results suggest that *Trichoderma* provides resistance under low N stress. Furthermore, HR was evidenced in electrolyte leakage (EL) assays where higher and more rapid leakage was recorded in the presence of *Trichoderma* irrespective of the concentration of nitrate supplied (Fig.**1b**) with these results being in close accordance with the observed HR response.

A significant increase in the colony forming unit (CFU) count during LAR was observed in 0.1 mM WT plants supplied with 0.1 mM nitrate was observed in

157 comparison to the bacterial numbers of the same plants grown in the presence of
158 *Trichoderma* (Fig. **1c**). We further found that, *PR1* (a marker for HR) transcript levels
159 increased in *Pst* treated plants grown in the presence of either 0.1 mM (~9 fold at 48
160 hpi) or 3 mM (~28 folds at 48 hpi) NO_3^- in the presence of *Trichoderma* grown in
161 comparison to WT plants grown on 0.1 mM NO_3^- and in the absence of
162 *Trichoderma*, where expression of this gene remained unaltered at 48 hpi (Fig.**1d**).
163 Collectively these data thus suggest that *Trichoderma asperelloides* plays a role in
164 improving the LAR response under low NO_3^- availability.

165 **_Trichoderma_ can induce the SAR response under low N**

166 Having characterized LAR responses, we next studied responses SAR by
167 infecting the secondary leaves with virulent *Pst* DC3000 (Fig. **S1b**; Cameron *et al.*
168 1994 and 1999). Three days post-secondary challenge with virulent *Pst* DC3000, EL,
169 CFU and *PR1* gene expression were assessed (Fig. S3c). During this SAR assay
170 *Pst*-challenged WT plants grown on 0.1 mM NO_3 showed more prominent necrotic
171 and dark yellow lesions that spread to both sides of the leaf than *Pst*-challenged WT
172 plants grown on 3 mM NO_3. This suggests that, the plants grown on 3 mM NO_3^- had
173 stronger defenses during secondary challenge than the plants grown on 0.1 mM
174 NO_3^- suggesting that sufficient N is required for SAR development. In the presence
175 of *Trichoderma* there was significant reduction of yellowing in the 0.1 mM NO_3^- plants
176 in comparison to plants in which *Trichoderma* was absent (Compare Fig. **2a-WT+T**
177 **panel** with **WT panel**).

178 Trypan blue images of inoculated and distal leaves are shown in Fig. **2b** and
179 **Fig. S5b**. In response to *Trichoderma*, plants grown on 0.1 mM NO_3^- showed more
180 trypan blue spots during secondary challenge. Upon infection, cell death spots were
181 more widely spread in these leaves (compare Fig. **2b WT+T panel** with **WT panel**).
182 At 48 hpc, there was an increased cell death in inoculated WT leaves grown both on
183 0.1 and 3 mM NO_3^- but the cell death phenotype differs. The distal leaves plants in
184 the presence of *Trichoderma*, showed a uniform spread of microbursts throughout
185 the leaf blades, at all the time points but with the plants grown on 3 mM NO_3^-
186 displaying slightly larger microbursts than those grown on 0.1 mM NO_3^- (Fig. **2b**).
187 The presence of these microbursts suggests that during SAR establishment the

188 mobile signal perceived by the distal leaves results in the occurrence of low
189 frequency microscopic HRs (Cameron *et al.* 1999; Alvarez *et al.* 1998).

190 We next investigated electrolyte leakage during SAR. Mock inoculated plants
191 showed moderate EL in plants grown on either 0.1 mM or 3 mM NO_3^- (Fig. **S2a**). EL
192 was, however, significantly higher (~4 fold) in both 0.1 and 3 mM NO_3^- grown plants
193 treated with *Trichoderma* during secondary challenge, in comparison to 0.1 mM
194 nitrate grown plants in the absence of *Trichoderma* at 24 and 48 hpc (Fig. **2c**).This
195 suggests that *Trichoderma* treatment primes the defense responses in the leaves.
196 EL data of distal leaves during SAR is also shown in Fig. **S5a**.

197 Bacterial count in terms of Log CFU (Fig. **2d**) revealed that, the *Pst* population
198 significantly decreased in *Trichoderma* treated low N-fed plants, suggesting that
199 *Trichoderma* imparts enhanced systemic resistance to the NO_3^- stressed plants.

200 ***Trichoderma* activates nitrate transporters, facilitates N uptake and promotes**
201 **SAR in low N-fed plants**

202 Increased LAR and SAR responses under low NO_3^- in the presence of
203 *Trichoderma* are probably due to an increased nutrient uptake as these responses
204 were all absent in the absence of *Trichoderma*. We, therefore, checked the NO_3^-
205 levels in the leaves of 0.1 mM and 3 mM NO_3^- grown WT plants in the presence and
206 absence of *Trichoderma* and represented it as percent nitrate uptake up to Day 15
207 in leaves (Fig. 3a). The experimental design and protocol for measuring NO_3^- levels
208 and uptake in leaves is demonstrated in Fig. **S4a, b** and **d**. It was observed that %
209 nitrate uptake was significantly increased in the presence of *Trichoderma* in
210 comparison to in the absence of *Trichoderma* enhancing the nitrate uptake of 0.1
211 mM NO_3^- fed WT plants by 17%. The basal levels of NO_3^- present in the soilrite:
212 agropeat mixture is also shown in Fig **S4c**. This increased NO_3^- transport is probably
213 the reason for resistance hence, we next checked the expression of both LATs
214 chloride channel *CLC-A)*, *NRT1/ PTR* family 1.2 (*NPF1.2*) and high affinity nitrate
215 transporters (HATs; *NRT2.1*, *NRT2.2* and *NRT2.4*).

216 *CLCA* is a tonoplast located antiporter channel system which drives NO_3^-
217 accumulation in the vacuoles (Krapp *et al.* 2014). It was observed that, under low
218 NO_3^-, C*LCA* is less inducible in *Pst* treated plants whereas it was significantly
219 upregulated in 0.1 mM NO_3^- grown, *Pst* treated plants in the presence of

Trichoderma (Fig. 3b). We further checked the expression of *NPF1.2;* (Fig. **3c**) which is involved in the transfer of xylem-borne nitrate to the phloem in the petiole (Krapp *et al.* 2014). It was found that, in *Pst* treated 3 mM NO_3^- grown WT plants, *NPF1.2* levels were highly induced till 48 hpc, in comparison to 0.1 mM *Pst* inoculated leaves (Fig. **3c**). This might be the reason behind the resistance of 3 mM plants and susceptibility of 0.1 mM plants following pathogen challenge. However, upon *Trichoderma* pre-treatment, *NPF1.2* transcript levels in *Pst*-inoculated 0.1 mM NO_3^- grown plants, showed significant induction in comparison to same treatment in the absence of *Trichoderma*. This suggests that, *Trichoderma* may cause the regulation of *NPF1.2*.

NRT2 transporters (*NRT2.1*, *NRT2.2* and *NRT2.4*) are HATS which become activated at low N concentrations (<1 mM). Previously, it was shown that *NRT2.1* is active only under conditions of N starvation (Dechorgnat *et al.* 2012). Under low NO_3^- conditions, *NRT2.1* was significantly induced in all treatments in the presence of *Trichoderma* in *Pst*- treated plants in comparison to plant in the absence of *Trichoderma* where there was already an induction of this gene at early time points (Fig. **4a**). The SAR establishment stage represented by non-inoculated leaves (distal) showed dynamic and elevated up-regulation of *NRT2.1* transcripts (Fig. **4a**). This revealed that, *Trichoderma* colonization benefits the plant by facilitating the N supply even under low NO_3^- conditions thereby improving plant defense via the mediation of SAR. *NRT2.2* expression levels have increased several folds in 0.1 mM distal and inoculated leaves of *Trichoderma* grown plants (Fig. **4b & 6b)**). This again suggests that faster N uptake by *Trichoderma* treated plants grown on low NO_3^- can aid in *Pst* defense. We next checked *NRT2.4* expression levels across the treatments. This gene showed early induction (6 h) in 0.1 mM *Pst* treated in inoculated and distal leaves (Fig. **4c**) and the early induction was similar in *Trichoderma* treatment.

Taken together, these data suggest that *Trichoderma* induces HATs (*NRT 2.1 and NRT 2.2*) to facilitate N uptake under low NO_3^- conditions. To confirm the role of HATs in increasing plant defense via N uptake, we checked the *PR1* expression in WT and *nrt2.1* mutants. The expression of *PR1* gene in *Trichoderma* inoculated plants increased 12-13 folds in 24 h but in the case of *nrt2.1* mutant (Fig. **4d**), it was not at all induced suggesting that *NRT2.1* plays an important role in increasing plant

defense under low N mediated by *Trichoderma*. We subsequently found that the expression of the *NRT2.2* gene was also suppressed in the *nrt2.1* mutant, both in and the presence and absence of *Trichoderma* (Fig. **4e**) suggesting that *NRT2.1* is mainly responsible for increasing N uptake under low NO_3^- facilitated by *Trichoderma*. We next checked the protein levels in WT and the *nrt2.1* mutant in the presence or absence of *Trichoderma* under low NO_3^- nutrition. In response to *Trichoderma*, protein levels were increased in the WT. In the *nrt2.1* mutant protein levels were lower than in WT and upon *Trichoderma* treatment only a slight increase in their levels was observed (Fig. **4f**). These results suggest that *Trichoderma* application can increase NO_3^- transport.

Trichoderma **elicits NO production during early stages of inoculation which is required for induction of HATs and *PR* gene expression**

Previously, it was shown that *Trichoderma* elicits NO at early stages (Gupta *et al.* 2014). This elicitation could subsequently play a role in the induction of NO_3^- transporter genes. Hence, we checked NO production in WT, *nia1,2* mutants and WT seedlings inoculated with *Trichoderma* and grown on the NO scavenger cPTIO. Control roots in 0.1 and 3 mM NO_3^- produced reduced levels of NO. Within 2 min of *Trichoderma* application, WT plants displayed highly increased levels of NO in both 0.1 mM and 3 mM NO_3^- (Fig. **5**) but the increase was slightly higher in 0.1 mM than 3 mM. That said, after 10 minutes and 24 h of *Trichoderma* incubation, the WT plants showed extremely low fluorescence (Fig. **5**). This suggests *Trichoderma* greatly induces NO in low NO_3^- grown plants within a short time period. Thus, *Trichoderma* induced NO production likely plays a role in priming and induction of HATs. Assessment of NO in the *nia1,2* mutant revealed that nitrate reductase (NR) is responsible for the *Trichoderma*-induced NO production. Whilst plants grown in the presence of cPTIO displayed reduced levels of NO. We further checked the importance of *Trichoderma*-elicited NO production in the induction of HATs (*NRT 2.1 & 2.2*), total protein levels and expression of the *PR1* gene in low NO producing non-symbiotic hemoglobin over-expressing line (*nsHb*$^+$), *nia1,2* and *nrt2.1* mutants. Expression of *NRT2.1* and *2.2* levels increased in WT in response to *Trichoderma*, whereas in Hb$^+$ lines, *nia1,2* and *nrt2.1* mutants these two HATs were not induced (Fig. **6a,b**) suggesting that *Trichoderma* elicited NO is responsible for induction of HATs. Further, reduced protein levels were observed in *nia1,2* and Hb$^+$ plants

suggesting that the induction of NO_3^- transporters mediated by *Trichoderma* elicited NO plays a role in increased N uptake under low NO_3^- (Fig. **6c**). Furthermore, we found that *nia1,2* and Hb^+ were unable to induce *PR1* expression under these conditions (Fig. **6d**). Taken together, these results suggest that *Trichoderma* elicited NO plays a role in overall increase in defense response under low NO_3^-.

We next investigated the role of NO in LAR and SAR development in the presence of *Trichoderma* using NO mutants. Under low NO_3^-, WT plants displayed a stronger LAR response than either the Hb^+ lines or the *nia1,2* mutant. Furthermore, reduced bacterial growth and increased *PR1* gene expression was observed in WT compared to either the Hb^+ lines or the *nia1,2* mutant (Fig. **7 a,b,c**). Similarly, the SAR response was also more compromised in the WT than in the Hb+ lines and the *nia1,2* mutant ((Fig. **7 d,e,f**). As for LAR, a similarly reduced bacterial number and increased *PR1* gene expression was observed in WT in comparison to the Hb^+ lines and the *nia1,2* mutant

ROS is a component of the *Trichoderma*-induced increased resistance via SAR

Both NO and ROS are involved in the plant resistance response hence we next investigated the role of ROS. The distal leaves of 0.1 mM NO_3^- grown WT plants in the presence of *Trichoderma* showed increased H_2O_2 production in comparison to 0.1 mM NO_3^- grown distal leaves (Fig. **8a**). Also, the level of H_2O_2 was more in 3 mM NO_3^- grown distal leaves of WT plants in the presence of *Trichoderma* in comparison to untreated plants (Fig. **8a**). This is probably due to the suppression of catalase activity by SA. This suggests that *Trichoderma* inoculation can enhance H_2O_2 levels in distal leaves during SAR, whereas, both the *Pst* inoculated leaves from WT grown under 3 mM NO_3^- and WT plants in the presence of *Trichoderma* displayed higher H_2O_2 production (Fig. **8a**). This clearly suggests that *Trichoderma* plays a role in inducing H_2O_2 in low NO_3^- fed WT plants during SAR.

$O_2^{-\cdot}$ is a key player in cell death (Fig. **8b**). It was observed that, there was increased $O_2^{-\cdot}$ in inoculated leaves of both 0.1 and 3 mM NO_3^- fed WT and *Trichoderma* treated WT plants, but the increase was much higher in the presence than in the absence of *Trichoderma*. This suggests that there was a rapid oxidative burst (HR) which offers resistance to the plants in response to *Trichoderma* treatment. Taken together, the coordinated interplay of H_2O_2, $O_2^{-\cdot}$ and NO leads to

the HR associated cell death that greatly improves the LAR and SAR responses of low N-stress plants.

Trichoderma induces defense genes during SAR in low N-fed plants

We next checked the expression levels of defense related genes (*PAL1, PR1, PR2* and *PR5*) during SAR. In both 0.1 mM and 3 mM NO_3^- grown, *Pst* inoculated WT plants, *PAL1* transcripts were highly induced in all time points, but, the distal leaves showed *PAL1* induction only until 6 hpc, and this dramatically declined at later time points (Fig. **9a**). In the presence of *Trichoderma* WT plants grown under 0.1 mM and 3 mM NO_3^- regimes, showed even greater enhancements in the levels of *PAL1* transcripts) in inoculated as well as distal leaves until 48 hpc. A similar trend was observed in the *PR1* (Fig.**9b**), *PR2* (Fig. **9c**) and *PR5* (Fig. **9d**) expression profiles, which displayed elevation following *Trichoderma* application. Of the four defense genes examined, the *PR1* gene displayed the highest expression levels in plants grown in the presence of *Trichoderma* suggesting that *Trichoderma* might induce SA levels to a greater extent during SAR.

Trichoderma enhances expression of SAR-mediated regulatory genes

Having established the link between *Trichoderma* and SAR we next checked whether *Trichoderma* can induce the SAR response via induction of regulatory genes such as *DIR1, NPR1, SARD1 and TGA3*. The lipid transfer protein, DEFECTIVE IN INDUCED RESISTANCE1 (*DIR1*) is a key mobile component of SAR response (Maldonaldo *et al.* 2002) involved in long-distance translocation from local to distant leaves (Carella *et al.* 2015, Champigny *et al.* 2013). It was found that, *DIR1* induction took place only in the initial time points in inoculated and distal leaves of both 0.1 and 3 mM NO_3^- grown plants (Fig. **10a**). However, in the presence of *Trichoderma*, a slightly increased *DIR1* expression was observed at all time points (Fig. **10a**). Upon perception of SAR mobile signals, *Non-Expresser of Pathogenesis-Related gene1* (*NPR1*) activates defense in challenged plants (Cao *et al.* 1997). We found a similar trend in the *NPR1* expression profile as we did for *DIR1* (Fig. **10b**). Moreover, upon *Trichoderma* treatment, the levels of *NPR1* inoculated and distal challenged leaves gradually increased in both 0.1 and 3 mM NO_3^- grown (Fig. **10b**). Following this we checked the expression of *SAR DEFICIENT 1* (*SARD1*) a pathogen-induced transcription factor (Zhang *et al.* 2010) and a key regulator of

352 *Isochorismate Synthase 1* (*ICS1*) and SA synthesis (Wang *et al.* 2011). A
353 remarkably stronger induction of *SARD1* expression levels in *Trichoderma* treated
354 distal leaves of 0.1 mM NO_3^- grown plants at 6 hpc (~82 fold) revealed that is a
355 potential inducer of SA biosynthesis (Fig. **10c)**. Another important regulatory gene,
356 *TGA3*, is an *NPR1*-interacting protein (NIP) as well as a critical component in the SA
357 signaling mechanism. This gene was induced in all treatments in response to
358 *Trichoderma* (Fig. **10d**) highlighting the important *Trichoderma*-mediated promotion
359 of SA signaling pathways.

360 ***nrt2.1 and npr1 mutants are compromised in LAR and SAR responses***

361 Having clearly demonstrate the link between N nutrition and defense we next
362 decided to characterize LAR in *nrt2.1* and *npr1* mutants in response to virulent
363 *Pst*DC3000. The 0.1 mM NO_3^- fed *nrt2.1* and *npr1* mutants developed more severe
364 symptoms than 0.1 mM NO_3^--fed WT plants (Fig. **11a** 0.1 mM panel). An increased
365 CFU count was observed in 0.1 mM NO_3^- fed WT and *nrt2.1* and *npr1* mutants at 24
366 and 48 hpi in comparison to bacterial numbers in 3 mM NO_3^- grown plants in which
367 overall bacterial count of WT was less but a significant increase in CFU was
368 observed in *nrt2.1* and *npr1* mutants (Fig.**11b**). This suggests that optimum NO_3^-
369 concentration plays an important role in resistance response towards virulent *Pst*
370 DC3000.

371 Similarly, we studied the SAR response in the WT, *nrt2.1* and *npr1* plants. 0.1
372 mM NO_3^- grown *Pst*-challenged WT plants show more prominent necrotic and
373 discolored lesions spread to half of the leaf (Fig. **2a**) confirming susceptible
374 symptoms (yellow specks). On the other hand, the *nrt2.1* and *npr1* mutants showed
375 even more severe disease symptoms – namely extensive chlorosis and necrosis
376 (Fig. **11c**) under low NO_3^- growth conditions. Our results suggest that, 3 mM NO_3^-
377 grown plants proved more resilient to secondary challenge than 0.1 mM NO_3^- grown
378 plants. Bacterial populations were also increased in the *nrt2.1* and *npr1* mutants (Fig.
379 **11d**) suggesting that *NRT2.1* and *NPR1* plays an important role in increasing
380 defense mediated by *Trichoderma*.

381 **Salicylic acid pathway is a part of enhanced plant resistance mediated by**
382 ***Trichoderma* under low N**

Examination of SA levels revealed that *Trichoderma* presence accelerated total SA levels in 0.1 mM distal leaves, in comparison to 3 mM distal leaves. There was significant increase in SA levels observed in inoculated and distal leaves of *Trichoderma* grown plants, in comparison to SA levels in WT plants (Fig 12a). We sought to further confirm role of SA in *Trichoderma* increased SAR, hence *nahG* plants were challenged in the presence or absence of *Trichoderma*. An intense chlorotic lesion was evident in both 0.1 and 3 mM grown *nahG* plants in response to challenge inoculation while *Trichoderma* grown *nahG* plants when challenged they defended much better evidenced by decreased chlorotic lesions and reduced bacterial numbers (Fig. **12 b,c**). Surprisingly, *Trichoderma* grown *nahG* plants showed slightly enhanced *PR1* transcript levels (Fig. **12d)**.

Discussion

Nitrogen availability and supply can severely impact growth and development of plants (Walker *et al.* 2001; Landrein *et al.* 2018). N deficiency can cause chlorosis, which can impact photosynthesis and overall energy demand for growth and defense with investment in plant resistance-related compounds being drastically constrained under limiting nitrogen supply (Dietrich *et al.* 2004). This suggest a possibility that increased N uptake under limited supply might boost defense. Previously it was shown that N levels have been shown to affect the synthesis of constitutive defences based on secondary metabolites such as alkaloids (Stout *et al.*, 1998) and (poly) phenolics under varying N regimes (Johnson *et al.*, 1987). Since N is also important for synthesis of various secondary metabolites, severe depletion of N can also impact defense related pathways. Hence, plants may not be able to activate adequate defense pathways for tolerance or resistance (Snoeijers *et al.* 2000). Ward *et al.* (2010) have previously shown that plant reconfigure their metabolism in response to N supply. Plants take up N in the form of NH_4^+ or NO_3^- or a combination of both. Different N forms differentially effect various free radicals such as NO and ROS (Wany *et al.* 2018; Gupta *et al.* 2013). Ammonium uptake and assimilation is less costly to the plants in comparison to NO_3^- but excess of NH_4^+ can cause toxic effects to the plants (Boudsocq *et al.* 2012; Liu *et al.* 2017), hence, many plants preferentially use NO_3^- as N source. NO_3^- nutrition can also enhance plant defence via increased generation of NO, polyamines and SA (Gupta *et al.* 2013; Fagard *et al.* 2014). Under NH_4^+ nutrition plants compromise in resistance due to

reduced NO, increased sugars, aminoacids and enhancement of γ-aminobutyric acid (GABA) levels which may be source of nutrition for pathogens (Gupta *et al.* 2013) Hence, here we chose to check the plant defense response under NO_3^- nutrition rather than under NH_4^+.

Plants grown on 0.1 mM NO_3^- showed reduced growth (Fig. **S3**) and morphological parameters (Fig. **S3**), suggesting that the supplied 0.1 mM NO_3^- is insufficient for optimal growth of plants and consequently would likely reduce the available resources which could be allocated to defense. In our experiments, 0.1 mM NO_3^- grown plants were compromised in both LAR and SAR (Fig. **1,2**) responses, suggesting that NO_3^- is required for better defense. Plants grown on low NO_3^- produced less SA (Fig. **12a**), further supporting the notion that NO_3^- is needed for SA biosynthesis. Since plants need N for growth and disease resistance, increasing their N use efficiency may be an effective manner to improve their resistance. Certain types of *Trichoderma* aid in nutrient absorption leading to increased growth and enhanced plant defense (Brotman *et al.* 2010). It was previously demonstrated that supplementation of plants with *Trichoderma asperelloides* enhances plant growth (Brotman et *al.* 2012), and protects against abiotic and biotic stressors- moreover, It was, moreover, demonstrated to induce systemic resistance responses (Contreras-Cornejo *et al.* 2016; Brotman *et al.* 2012). *Trichoderma* induced increased growth has been varyingly attributed to auxin and ethylene (Garnica-Vergara *et al.* 2016) and the induction of genes involved in carbon and N metabolism (Domínguez *et al.* 2016). However, there are hardly any reports concerning the mechanism(s) underlying *Trichoderma*-mediated plant growth and defense improvement under N starvation. In the current study, we unraveled the mechanism of *Trichoderma*-induced plant growth and defense under low N. Hence, in this current work, we studied the impact of *Trichoderma* on enhancing N uptake and supporting both LAR and SAR responses under low NO_3^- stress.

The enhanced growth of plants grown in the presence of *Trichoderma* is due to an increased NO_3^- uptake (Fig. 3a) which was also evidenced by increased expression of *NRT2.1, NRT2.2* (Fig. **4a,b,c,d,e**) and increased total cellular protein levels (Fig. **4f**). *NRT2.1* is the main HAT, localized at the plasma membrane. Previously, it was shown that these transporters become active during N starvation and are severely inhibited when reduced nitrate sources such as glutamine or

449 ammonium are provided (Dechorgnat *et al.* 2012). Similarly, *NRT2* is also induced
450 under low N (Dechorgnat *et al.* 2012). In response to *Trichoderma* treatment, a rapid
451 induction of *NRT2.1, NRT2.2* transcripts in both plants grown with 0.1 and 3 mM
452 NO_3^- was observed during *Pst* inoculation. *NRT2.1* involvement was further
453 evidenced by the fact that the *nrt2.1* mutant produced less protein under low N and
454 even the addition of *Trichoderma* was unable to increase protein content in this
455 mutant (Fig. **4f**). Furthermore, this mutant become highly susceptible to LAR and
456 SAR under low N (Fig. **11**). It thus appears reasonable to assume that *Trichoderma*
457 can increase N uptake and by this means enhance resistance

458 Hence, in further experiments, we focused on the LAR and SAR responses under
459 low and optimum NO_3^- in the presence or absence of *Trichoderma*. During local *Pst*
460 infection, the plants display a LAR response, and the systemic/distal leaves induce a
461 SAR response. Both are critically important for plant defense against pathogens.

462 In 3 mM NO_3^--fed WT plants, the defense response after the secondary
463 challenge was also more rapid, robust and even longer-lasting till 5 days post
464 challenge (data not shown) whereas, 0.1 mM NO_3^- fed WT plants showed disease
465 symptoms suggesting that NO_3^- concentration plays a key role in the development of
466 both LAR and SAR (Fig. 1a, **2a** and **S1b**).

467 One of the features of *Trichoderma* is the induction of short-term spikes
468 of NO This molecule play an important role in induction of plant defense responses
469 (Gupta *et al.* 2014). We suspected the role of NO in activating genes of these
470 transporters. Indeed, *Trichoderma* induced expression of *NRTs* are most likely
471 mediated by short term increase in NO upon *Trichoderma* inoculation. NR-
472 dependent NO elicited by *Trichoderma* is probably responsible for the increased
473 expression of HATs since our experiments revealed that the Hb^+ lines and the
474 *nia1,2* mutant, were unable to induce HATs and showed decline in protein levels
475 even in the presence of *Trichoderma* (Fig. **6c**). *Trichoderma* trigger the SA-
476 dependent SAR pathway (Pieterse *et al.* 2014), they induced ROS involved in the
477 plant's resistance response against many biotic stressors (Asmawati *et al.* 2017).
478 They additionally plays an important role in hypersensitive cell death together with
479 NO (Durner and Klessig 1999, Dorey *et al.* 1999). Among ROS, H_2O_2 is the most
480 stable form which plays an important role as a signal transducer in the plant cell

death process (Pieterse *et al.* 2014), and acts as a key modulator of NO in triggering cell death. As shown in Fig. **8a** *Trichoderma* treated low N fed plants displayed increased H_2O_2 levels, thus, enabling the initiation and the establishment of SAR . Superoxides ($O_2^{-\cdot}$) are mainly produced via mitochondrial electron transport and NADPH oxidases during stress plays a role in plant defense (Torres *et al.* 2002, 2005). Earlier, it was shown that the extracellular elicitors isolated from *Trichoderma viridae* also induces $O_2^{-\cdot}$ levels (Calderon *et al.* 1994). Here, we also found that in the presence of *Trichoderma* , 0.1 mM NO_3^- grown *Pst* inoculated plants show higher $O_2^{-\cdot}$ levels than in the absence of *Trichoderma* (Fig.**8b**), suggesting that it can enhance $O_2^{-\cdot}$ production during infection which can aid in defense. Consequently, the expression of defense marker genes such as *PR1*, *PR2*, *PR5* and *PAL1* (Fig. **9**) were also highly induced in the presence of *Trichoderma* in low NO_3^--fed plants suggesting that *Trichoderma*-mediated ROS andNO along with increased N are probably responsible for higher induction of these genes. Martinez-Medina *et al.* (2013), reported several *Trichoderma* strains are known to induce systemic responses by acting as a "short circuit" in plant defense signaling. *PAL1* is an important marker gene in SA mediated defense (Kim and Hwang 2014) and accumulates in cells undergoing HR (Dorey *et al.* 1997) and is thought to be essential for local and systemic resistance (Delaney *et al.* 1994). The fact that *PAL1* levels were increased by *Trichoderma* is intriguing. The SAR response is associated with a specific set of SAR genes encoding pathogenesis related (PR) proteins (Pieterse *et al.* 1996). In our study these *PR* genes are activated and consequently accumulate during SAR (Fig.**9b,c,d**) in keeping with the earlier findings of Brotman *et al.* (2012) and Pieterse *et al.* (1996).

In our study, the SAR regulatory genes *DIR1*, *NPR1* and *TGA3* are induced in the presence of *Trichoderma* under low N stress. The *npr1* mutant is compromised in LAR and SAR in the presence of *Trichoderma* suggesting a role for this gene in enhancing defense. In the *nahG* transgenic line, defense responses are slightly enhanced in the presence of *Trichoderma* in spite of reduced SA levels, suggesting that apart from SA other factors such as increased NO and ROS are probably responsible for defense in this mutant. Overall, our study demonstrated that optimum N is required for both LAR and SAR. *Trichoderma* can enhance N uptake via modulating N transporters and via eliciting short term NO under low NO_3^- nutrition.

The enhanced N uptake plays a role in enhancing SA levels and defense gene expression in local and distal leaves to increase overall plant defense (Fig. **13**). These defense responses are neither activated in *npr1, , nrt2.1,* or *nia1,2* mutants; nor in the *nahG* and Hb^{+} transgenic lines providing corroborative evidence that *Trichoderma* mediated enhanced resistance under low NO_3^- involves synergistic roles of NO, ROS and SA. Further work is needed on how this mechanism works in key crop plants. Understanding the role of *Trichoderma* in improving nitrogen use efficiency (NUE) in response to different pathogens which have diverse strategies can help in improving plant resistance to pathogens and improving plant productivity when limited N available. N is important for plant defense as well as growth hence using beneficial *Trichoderma* can be a great tool to confer multifaceted benefits to the plants and improve crop resilience under reduced N availability

Material and Methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia 0 (*Col0*; WT) were sown in plastic pots (6.5 X 7.5 cm) containing autoclaved soilrite: agropeat (1:1) mix and stratified at 4°C in the dark for 48 h. The pots were then kept in a growth room under short day conditions (8h-light, 16h-dark), 22/18°C (day/night) temperatures, relative humidity of 60% and 180-200 µE m^{-2} s^{-1} light intensity. Initially, plants were bottom irrigated for a week, once with half strength Hoagland's solution and once with water. Then, 0.1 and 3 mM NO_3^- concentrations were provided to the growing plants weekly. The NO_3^- nutrient solution in Hoagland's media contained either 0.1 mM or 3 mM KNO_3, according to modified Hoagland's nutrient solution (Hoagland and Arnon, 1950). 28-30 day-old plants with fully developed rosettes were used for the experiments. The seeds of *nrt2.1* (SALK_035429C) and *nia1,2* (N6936) were procured from ABRC.

Trichoderma supplementation

T. asperelloides (*T203* strain) was grown on potato dextrose agar (PDA; Himedia) plates for 15 days under low light conditions until sporulation. Conidia were harvested by gently scraping the petridish and pouring 10 ml of sterile water over the surface and collecting the resultant suspension. Spores were evaluated up to $1 \square 10^9$

spores/ml. It was thoroughly mixed into the soilrite mix and distributed into the individual plastic pots. WT plants in *Trichoderma* supplemented pots were also grown similarly as described above.

Pathogen preparation and infiltration

Pseudomonas syringae pv. tomato DC3000 (*Pst*DC3000; *avrRpm1,* avirulent) were grown in King's B (KB) medium containing 50 µg ml^{-1} rifampicin. Primary and secondary culture was prepared according to standard protocols (Liu *et al.* 2015). The avirulent bacterial density was adjusted to 2×10^7 CFU ml^{-1} for LAR assay and primary inoculations and virulent *P. syringae* was used at 2×10^6 CFU ml^{-1} for challenge inoculations. Mock infiltration (control) was performed with 10 mM $MgCl_2$. *Pst* inoculations were made by syringe infiltration on the abaxial side of the leaves manually (Liu et al. 2015).

LAR Assay

The procedure of the LAR assay is described schematically in Fig. **S3 a&b**. 30-day-old plants were used for this experimental set up and were transferred to an infection room prior to pathogen infiltration. Two sets of experiments were performed simultaneously, the first set were mock infiltrated (10 mM $MgCl_2$) plants and the second set were the pathogen infiltrated (avirulent *Pst*DC3000; *avr*Rpm1) plants. The pathogen inoculated leaves were harvested at 0, 6, 24 and 48 hours post inoculation for RNA extraction and other assays. This assay was repeated more than three times in order to ensure the reproducibility of the results.

SAR Assay

The procedure of the SAR assay is described schematically in Fig. **S3c**. The SAR assay was performed according to the protocol described in Cameron *et al.* (1999) in a specific infection room with appropriate growth conditions. Primary inoculation was performed with avirulent *Pst*DC3000 (a*vrRpm1*) on one leaf per plant (marked with pink sticker; Fig. **S1b**) and the entire experimental set up was left for two days. Thereafter, a secondary (challenge) inoculation was performed with virulent *P. syringae* on four outer leaves (marked with black marker pen) other than primary inoculated leaf (Fig. **S1b**). Further, there were 5-6 non-inoculated distal leaves left per plant. Hereafter, the inoculated leaves are described as *Pst* or

inoculated leaves and non-inoculated leaves are described as distal throughout the text. The inoculated and distal leaves from each treatment were harvested (Fig. **S3**) for RNA extraction and other experiments post challenge inoculation. This assay was repeated more than three times in order to obtain reproducible results.

Electrolyte leakage

Leaf discs (5 mm diameter) were taken and electrolyte leakage was monitored exactly as described in (Gupta *et al*. 2013).

***In planta* bacterial number quantification assay**

The bacterial number in leaves from *Pst* treated plants during LAR and SAR responses were assessed was calculated as per Gupta *et al.* (2016).

Determination of nitrate levels and nitrate uptake assays

Nitrate levels in leaves were determined using the protocol described in Hachiya and Okamoto (2017). The schematic representation of the experimental design and nitrate uptake assay in leaves is shown in Fig. **S4a and b**. Nitrate levels were also determined in 1 g each of S: A mixture, 0.1 mM N fed S: A mixture and 3 mM N fed S: A mixture (with and without *Trichoderma*; Fig. **S4c**). The procedure for nitrate determination in S: A mix and leaves is mentioned below.

Nitrate determination in S: A mix

For determining the basal nitrate levels, the individual pots were filled with S: A mixture (with and without *Trichoderma*) and 0.1 mM and 3 mM nitrate was supplied, allowed to soak for 2 hours and excess was drained off by pressing. The detailed protocol is described in Fig. **S4a and b** (Supplementary Information). Apparent nitrate was calculated from a standard curve of nitrate concentration (Fig. **S4d**) using the absorbance values at 410 nm.

Nitrate determination in leaves

Nitrate uptake was measured in leaves of 0.1 mM and 3 mM nitrate grown WT in the presence and absence of *Trichoderma* plants till 15 days and is represented as percent (%) nitrate uptake up to Day 15 (Fig. **S4b, Fig. 3a**). For each nitrate treatment, six pots containing healthy rosettes (22 d old) were filled with autoclaved S: A mixture (with and without *Trichoderma*). The detailed protocol is described in Supplementary Information of Fig. **S4b**.

615

616 **Expression profiling by qRT-PCR**

617 Inoculated and non-inoculated leaves were immediately frozen in liquid
618 nitrogen and stored at -80°C. RNA extraction, cDNA synthesis and qPCR was
619 performed according to Wany *et al.* (2017; 2018). The synthesized cDNAs were
620 used as templates in qRT-PCRs using the primers listed in Table S1. Fold change in
621 the expression of the target genes was normalized to the *Arabidopsis* reference
622 genes; ubiquitin, *18sRNA* (GQ380689) and *YSL8* (X69885.1). Fold expression
623 relative to control treatment was determined by ΔΔCT values. Three biological
624 experiments (with three independent replicates for each experiment) were performed
625 for each treatment.

626

627 **NO estimation**

628 For this experiment, NO was measured from the following five different
629 combinations in roots; 1. WT; 2. WT + *T203* (*Trichoderma*); 3. *nia1,2* double mutants
630 +*T203*; 4. WT + cPTIO (carboxy-PTIO potassium salt); 5. WT + cPTIO + *T203*. WT
631 and *nia1,2* plants were grown for one week in plates containing 0.1 mM and 3 mM
632 NO_3^- concentrations. A spore suspension of *Trichoderma* was poured over these 7d
633 old plants and incubated for 2 minutes, 10 minutes and 24 hours, respectively. Then,
634 the roots were incubated in 10 µM DAF-FM DA (4-amino-5-methylamino- 2′,7′-
635 difluorofluorescein diacetate) in 100 mM HEPES buffer (pH 7.2) placed in a 1.5 ml
636 tube, incubated for 15 minutes in the dark and photographed using a fluorescence
637 microscope (Nikon*80i*, Japan) set at 495 nm excitation and 515 nm emission
638 wavelengths.

639

640 **Determination of ROS levels**

641 Production of hydrogen peroxide (H_2O_2) in inoculated and distal leaves was
642 detected by Diaminobenzidine tetrahydrochloride (DAB) staining as per Daudi *et al*.
643 (2012) Superoxide levels were measured by *in vivo* staining with Nitroblue
644 tetrazolium chloride (NBT, SA, USA) (Jambunathan, 2010).

645

646 **Histochemical detection of HR**

647 Hypersensitive cell death in inoculated leaves was visualized by the trypan
648 blue staining method according to Fernández-Bautista *et al.* (2002).

SA levels

The SA levels were measured by HPLC according to the protocol described in Singh *et al.* (2013).

Acknowledgements

We thank Dr. Yariv Brotman for providing the *T203* strain. Seeds of *nahG* and *npr1* mutants were provided by Prof. Ashis Nandi, JNU. Seeds of *nsHb*$^{+}$ *over-expressing transgenic lines* was provided by Dr. Kim Hebelstrup. This research was funded by SERB, DST (NPDF to AW), UGC (SRF to PKP) and SERB-ECR and DBT-IYBA award to KJG. CIF of NIPGR is greatly acknowledged.

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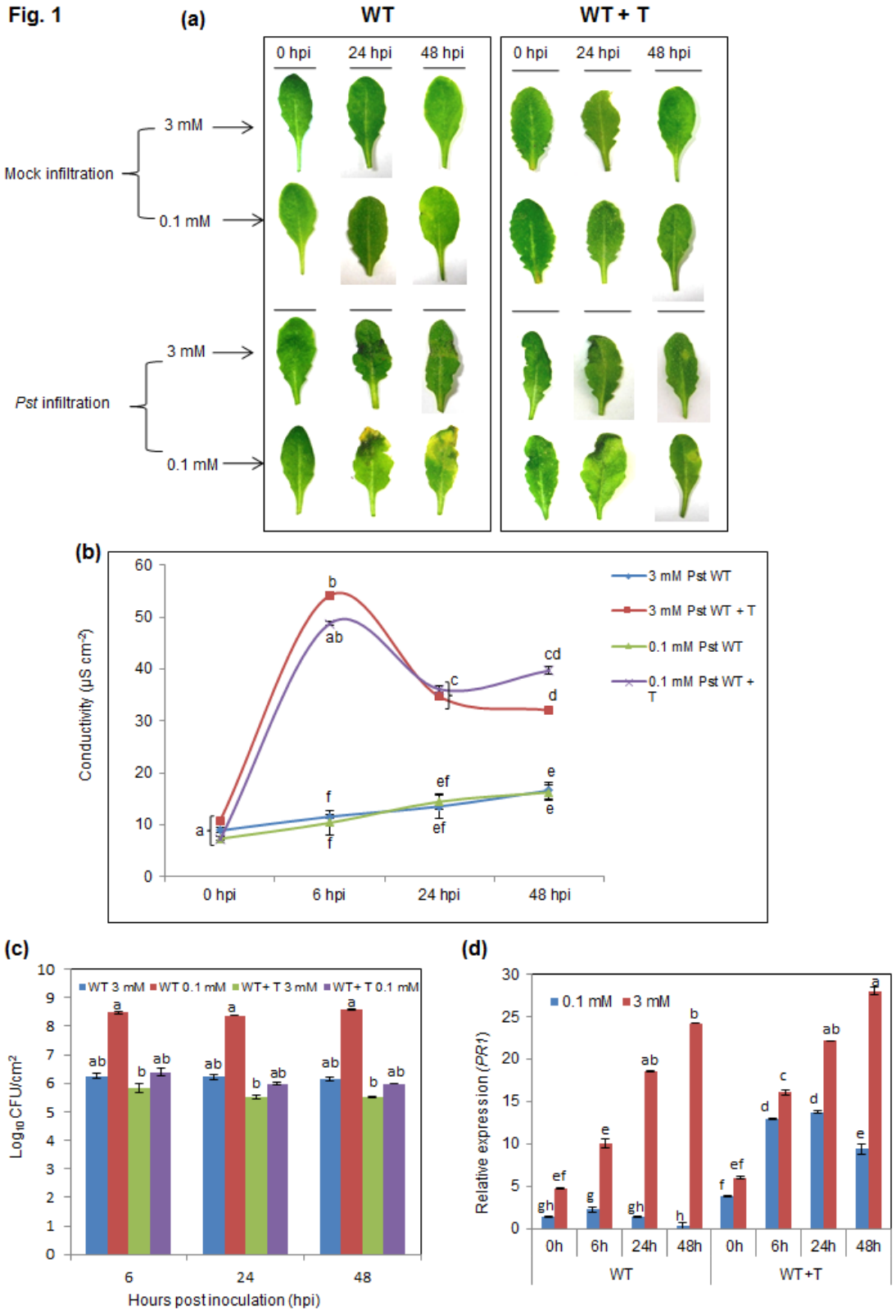

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865 **Fig 1**: ***Trichoderma*** **supplementation enhances LAR response elicited by**

866 ***Pst*****DC3000/avrRpm1 in low nitrate grown WT plants**

LAR response shown by WT and WT+T grown plants fed with 0.1 mM and 3 mM nitrate nutrition subjected to *Pst*DC3000/avrRpm1 infection. Two sets of plants viz., control WT plants for *Pst* treatment were grown in autoclaved un-inoculated soilrite: agropeat mixture and treated WT plants were grown in pots with soilrite: agropeat with *Trichoderma* spore suspension and denoted as WT+T plants.

(a) *Pst*DC3000-avrRpm1 mediated HR phenotype observed in 3 mM and 0.1 mM NO_3^- grown WT and WT+ T plants at different time points. First panel shows the mock infiltrated leaves i.e., control leaves treated with 10 mM $MgCl_2$ and second panel shows *Pst* infiltrated leaves. Image is representative of 4 independent replicates.

(b) Electrolyte leakage from infiltrated leaf areas with *Pst* in 0.1 mM and 3 mM NO_3^- grown WT and WT + T plants at different time points. Line graph represents the average of five biological replicates ± SEM.

(c) Bacterial number in log CFU in *Pst* infiltrated leaves of WT and WT+T plants at different time points. Column graph represents the average of three biological replicates ± SEM.

(d) Relative *PR1* transcript levels from WT and WT+T plants at different time points. Data are average mean values ± SE with n=3.

In all the experiments, statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 2

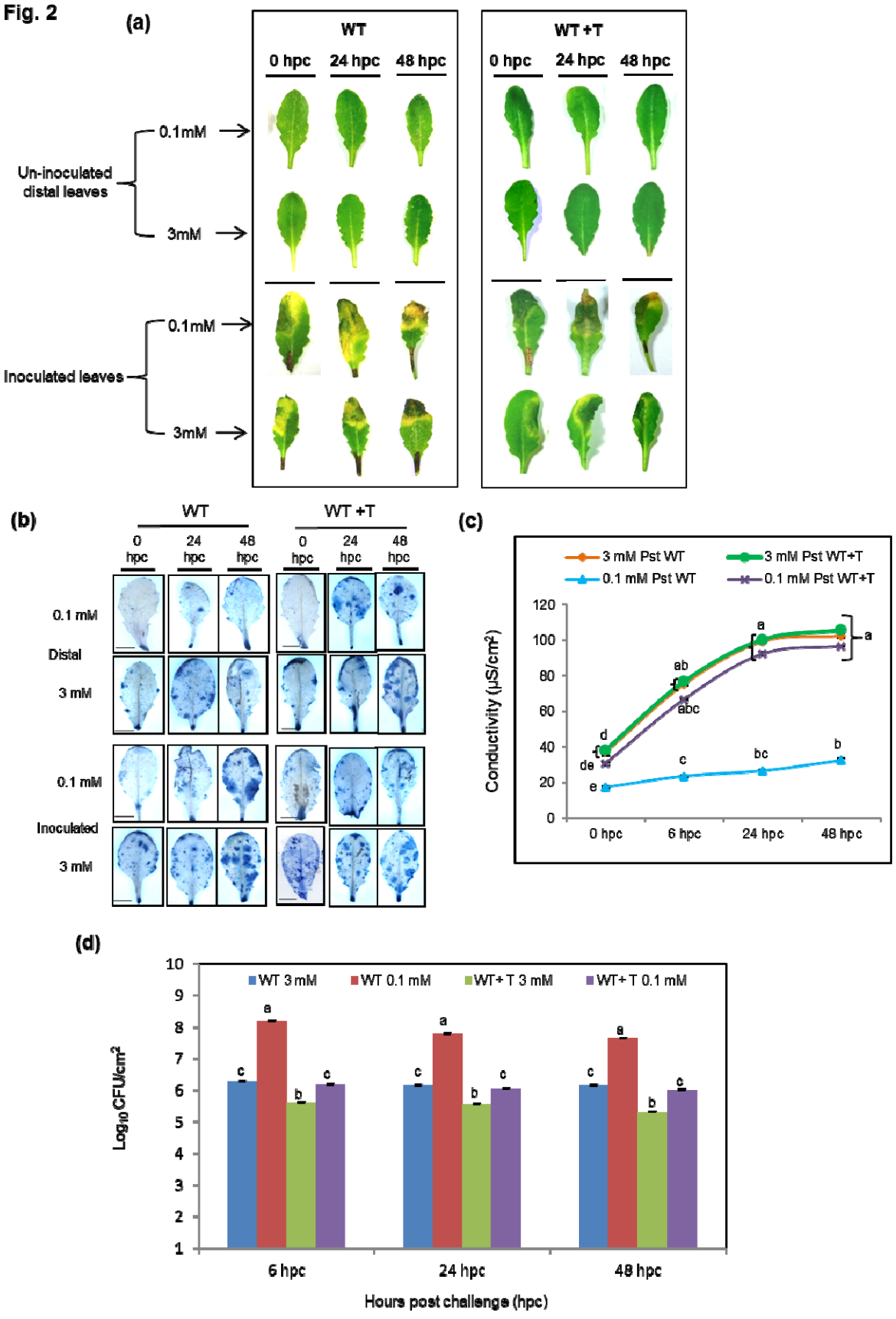

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Fig. 2. *Trichoderma* enhances SAR response in low nitrate grown WT plants

SAR response shown by WT and WT+T plants grown in 0.1 mM and 3 mM nitrate nutrition were initially subjected to primary inoculation on one leaf per plant with *Pst*DC3000/avrRpm1, left for two days followed by secondary challenge inoculation with virulent *P. syringae* on 4 other (distal) leaves per plant, leaving 5-6 healthy un-inoculated leaves per plant (Cameron et al. 1999). These plants are left for 3 days and then the Inoculated (I) and un-inoculated/distal (U) leaves were assayed for HR phenotype, Trypan blue imaging, EL pattern and bacterial count experiments. The uninoculated leaves from now is mentioned as distal leaves.

(a) HR phenotype post-secondary challenge in inoculated and uninoculated leaves of WT and WT+T plants at different time points in 0.1 mM and 3 mM NO_3^-.

(b) Histochemical staining using Trypan Blue for the detection of HR-mediated cell death post-secondary challenge in inoculated leaves of WT and WT+T plants at different time points in 0.1 mM and 3 mM NO_3^-. Images were observed under the 0.5 X objective of AZ100 Stereo Microscope. Scale bar-1 mm.

(c) Electrolyte leakage observed post-secondary challenge in inoculated and uninoculated leaves of WT and WT+T plants at different time points in 0.1 mM and 3 mM NO_3^-.

(d) Bacterial number in log CFU post-secondary challenge in inoculated leaves of WT and WT+T plants at different time points in 0.1 mM and 3 mM NO_3^-. Data are average mean values ± SE with n=3. In all the experiments, statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 3

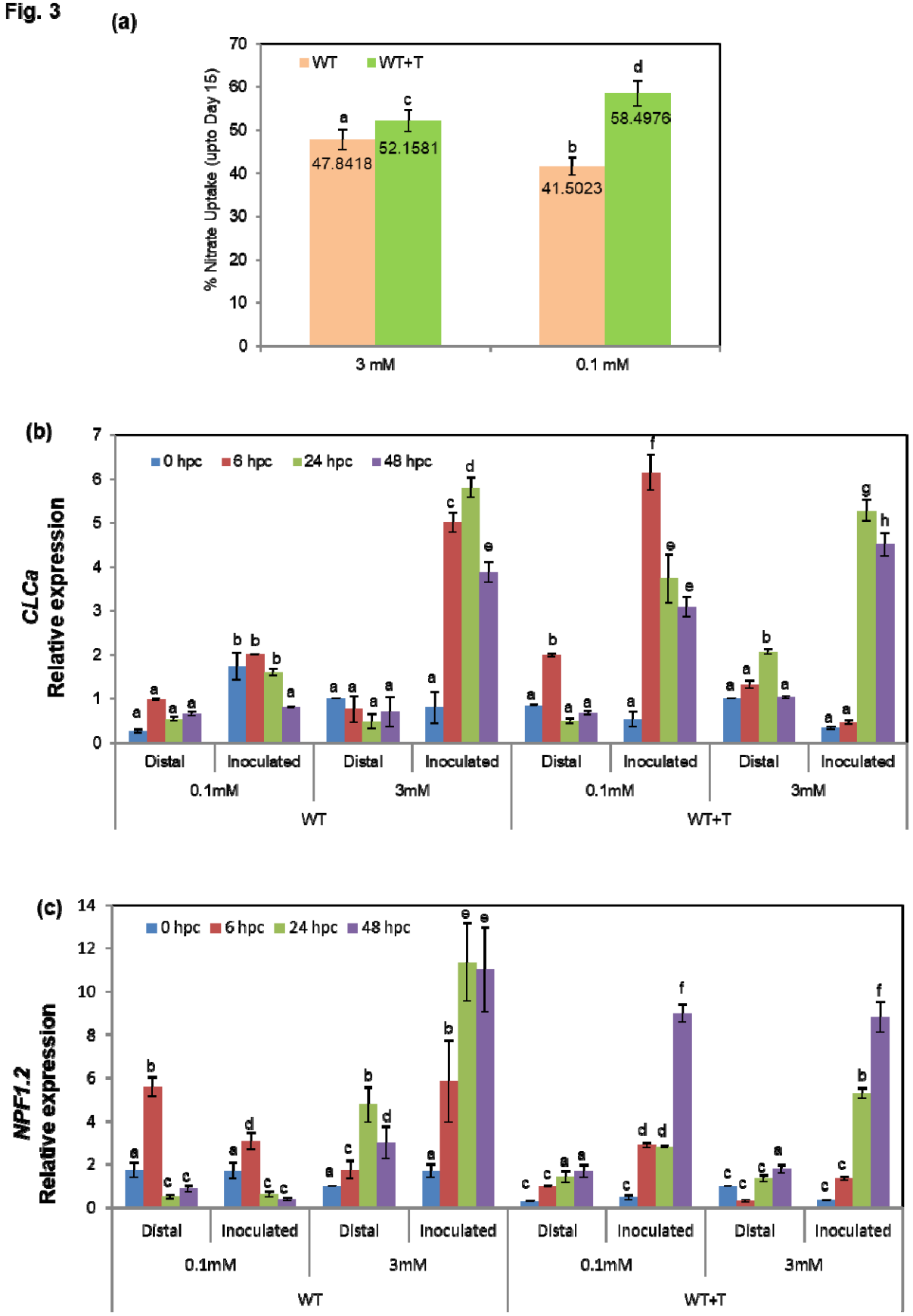

Fig. 3: **Nitrate uptake and expression analysis of low affinity nitrate transporters**

(a) Column graph represents the % nitrate levels utilized by the plants till 15th day from the initial day in 0.1 mM and 3 mM WT and WT+T leaves. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Expression profile of low affinity nitrate transporter genes (LATs) during SAR. Relative expression of **(b)** *CLCA* gene in WT and WT+T plants and **(c)** *NPF1.2* gene in WT and WT+T plants grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and distal leaves. For all the target genes, fold expression values are means (n=3) ± SE. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 4

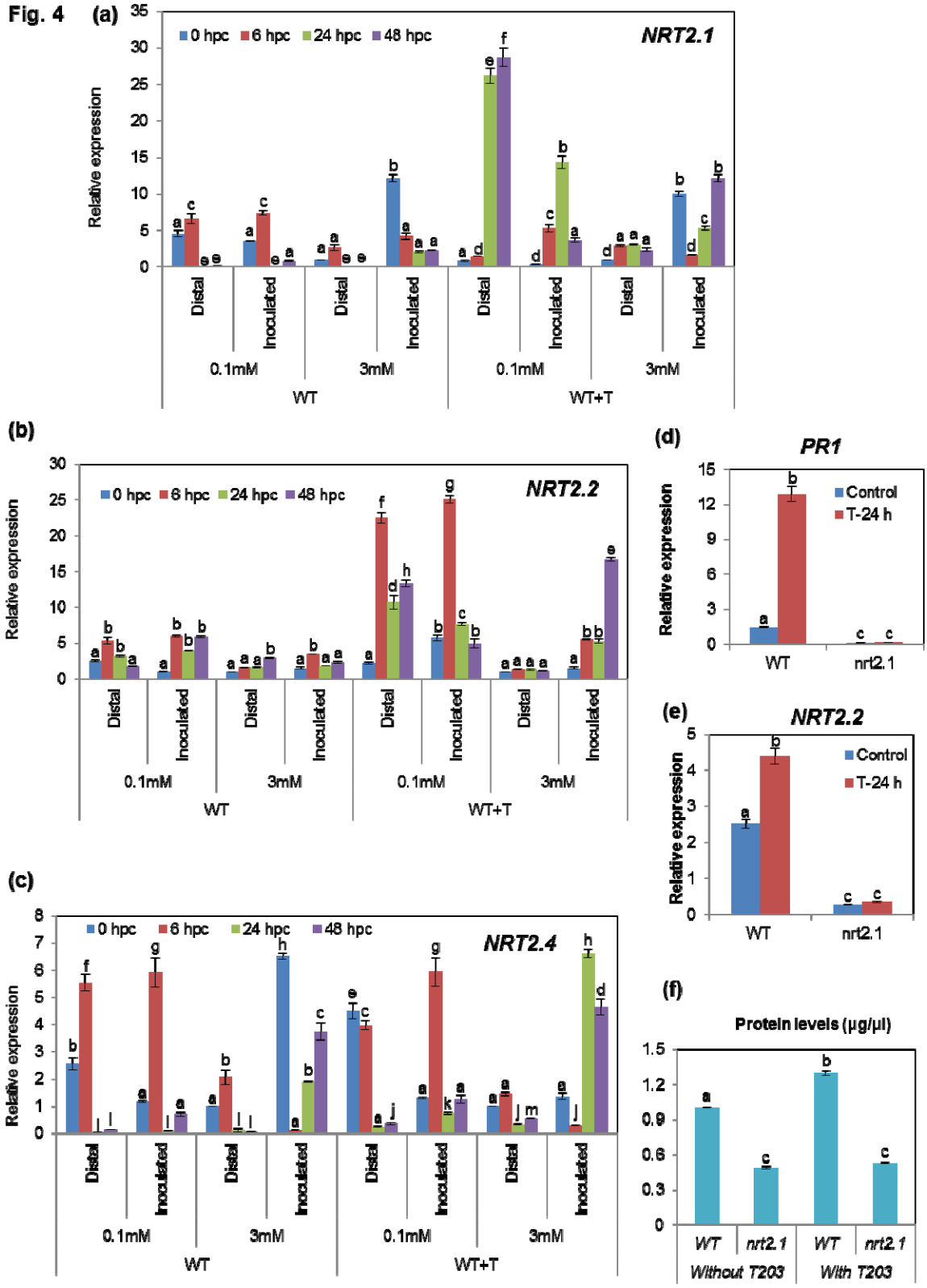

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Fig. 4: Expression profile of high affinity nitrate transporter genes (HATs) during SAR and response of genotypes (WT and *nrt2.1*) on priming effect of 24 h *Trichoderma* pre-treatment w.r.t *PR1* and *NRT2.2* expression on low nitrate fed WT plants

Relative expression of (a) *NRT2.1*, (b) *NRT2.2*, (c) *NRT2.4* in WT and WT+T plants grown under 0.1 mM and 3 mM NO_3^- concentration post-secondary challenge in both inoculated and distal leaves. For all the target genes, fold expression values are means (n=3) ± SE. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

(d) Relative *PR1* expression in roots of WT and *nrt2.1* plants grown under 0.1 mM NO_3^-concentration (with and without *Trichoderma* treatment; 24 h). Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at $p < 0.05$.

(e) Relative *NRT2.2* expression in roots of WT and *nrt2.1* plants grown under 0.1 mM NO_3^-concentration (with and without *Trichoderma* treatment; 24h). Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at $p < 0.05$.

(f) Protein levels in WT and *nrt2.1* plants grown under 0.1 mM NO_3^- concentration in the presence or absence of *Trichoderma*. Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 5

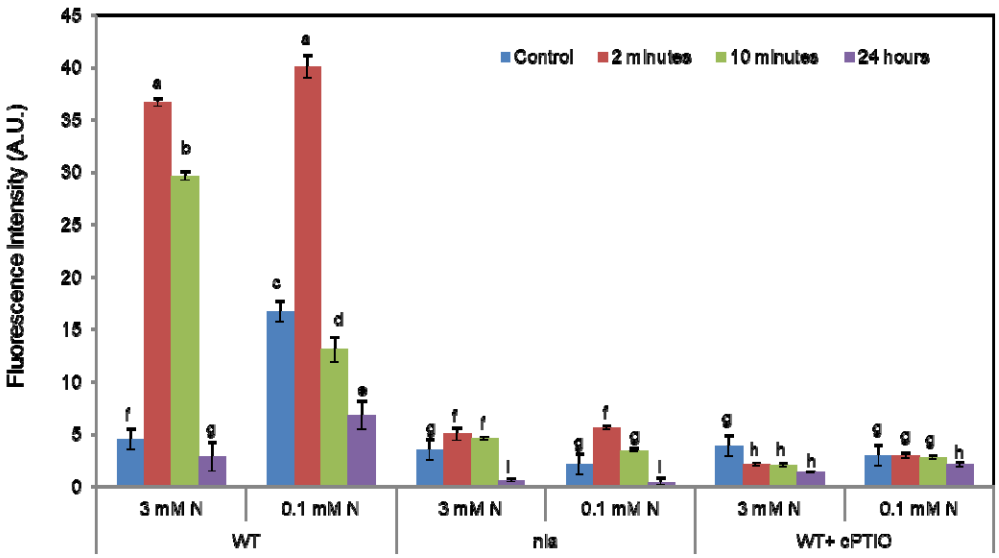
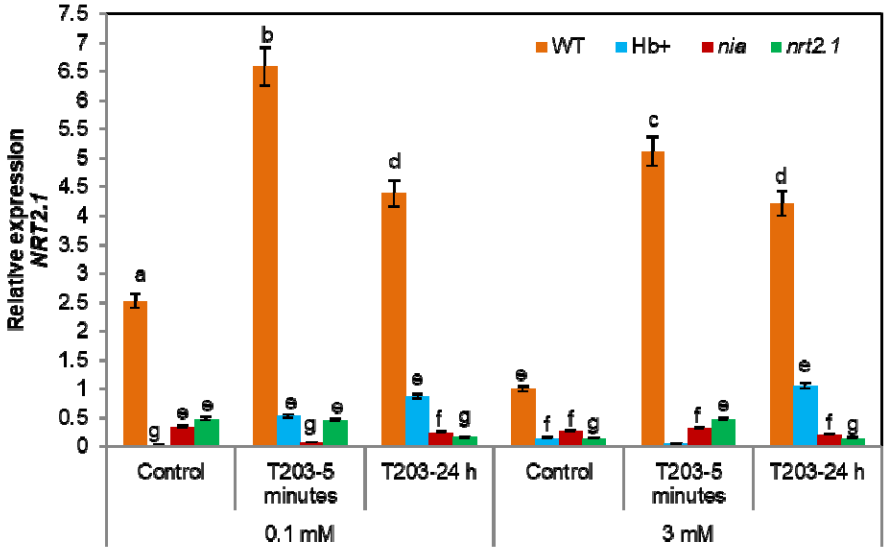


Fig. 5: Visualization of nitric oxide by diaminofluorescein (DAF) fluorescence Nitric oxide estimation by diaminofluorescein (DAF-FM) fluorescence under 0.1 and 3 mM NO_3^- concentration in WT, *nia1,2* and cPTIO (100 µM) grown WT plants during different periods of *Trichoderma* inoculation. The experiment was performed three times independently with similar results. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at p < 0.05.

Fig. 6 (a)



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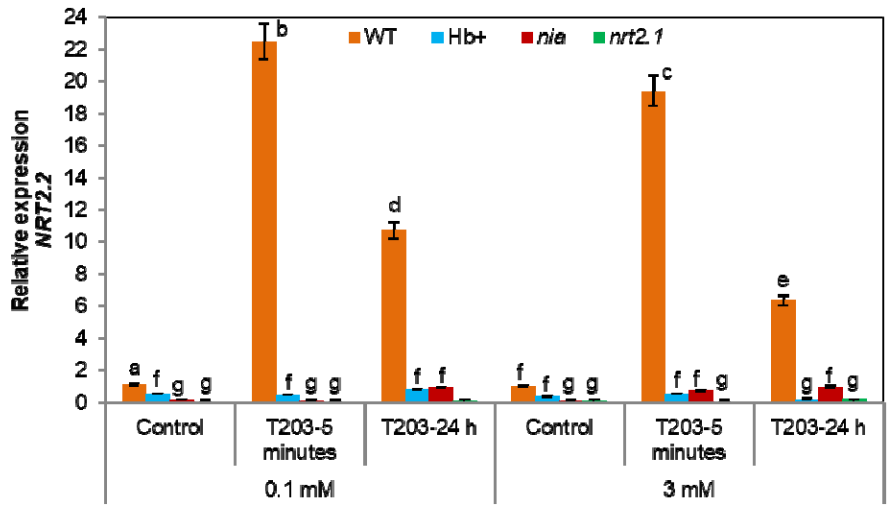

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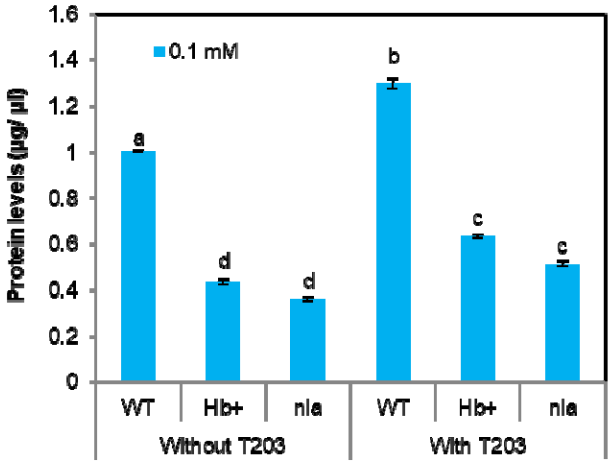

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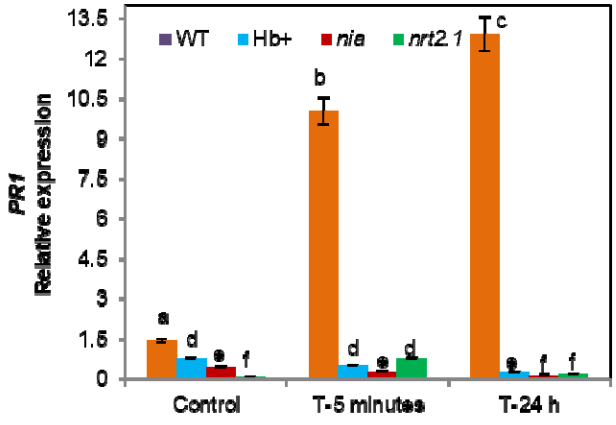

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Fig. 6: Response of the genotypes during early stages of *Trichoderma* inoculation on *NRT2.1*, *NRT2.2* expression, protein levels and *PR1* expression

(a) Relative *NRT2.1* expression in roots of WT, Hb^+, *nia1,2* and *nrt2.1* plants grown under 0.1 mM and 3 mM NO_3^- concentration, with and without *Trichoderma* treatment, given for 5 minutes and 24 h to the plants. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

(b) Relative *NRT2.2* expression in roots of WT, Hb^+, *nia1,2* and *nrt2.1* plants grown under 0.1 mM and 3 mM NO_3^- concentration, with and without *Trichoderma* treatment, given for 5 minutes and 24 h to the plants. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

(c) Protein levels measured in WT, Hb^+ and *nia1,2* seedlings grown under 0.1 mM NO_3^- for 15 days in vertical plates using Bradford's assay. *Trichoderma* treatment was given for 24 h to the plants. Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at $p < 0.05$.

(d) *PR1* gene expression in WT, Hb^+, *nia1,2* and *nrt2.1* plants under 0.1 mM NO_3^- plants, with and without *Trichoderma* treatment, given for 5 minutes and 24 h to the plants. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 7

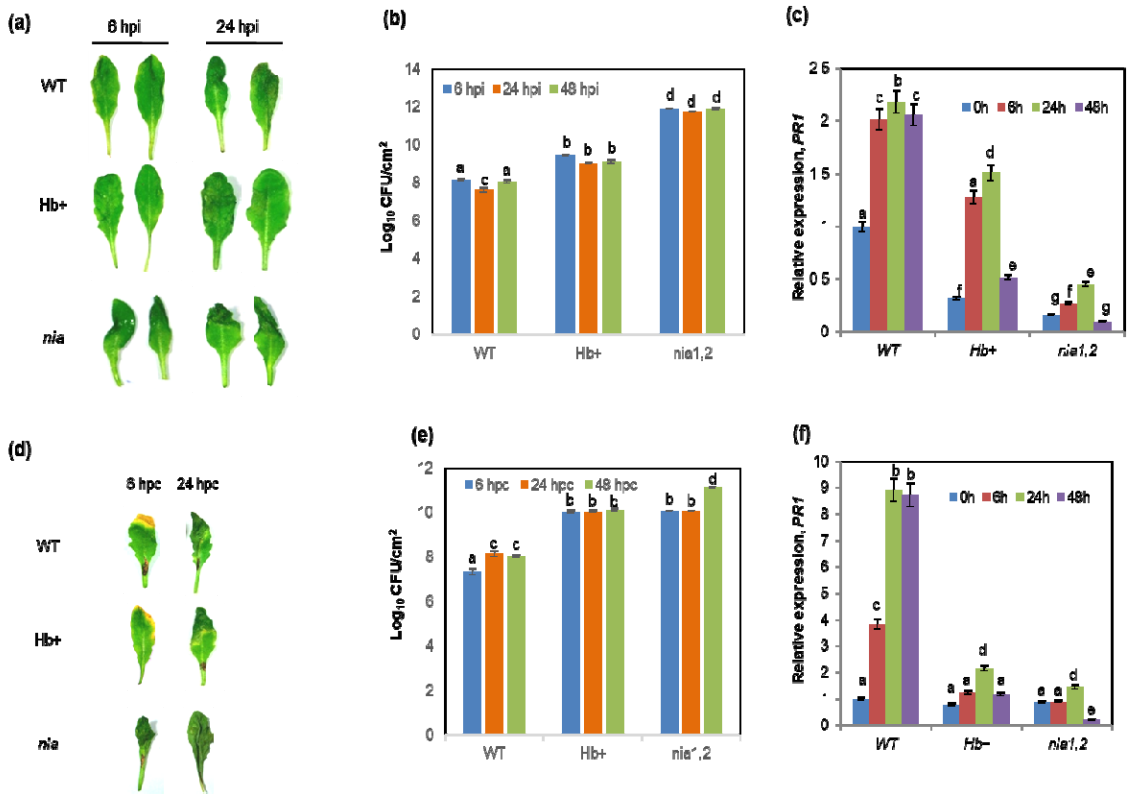


Fig. 7: Response of WT and NO mutants (Hb^+ lines and *nia1,2*) grown under 0.1 mM NO_3^- during LAR and SAR

In LAR response (a) HR phenotype in inoculated leaves of WT, Hb^+ and *nia1,2* plants grown under 0.1 mM NO_3^- concentration at 0 and 24 hpi .

(b) In planta bacterial growth (log CFU) at 6, 24 and 48 hpi. Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at p < 0.05.

(c) Relative *PR1* expression in infiltrated leaves during localized infiltration. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at p < 0.05.

In SAR response (d) HR phenotype in inoculated and uninoculated leaves of WT, Hb^+ and *nia1,2* plants grown under 0.1 mM NO_3^- concentration at 0 and 24 hours post secondary challenge

(e) Bacterial population represented by log CFU at 6, 24 and 48 hpc. Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at $p < 0.05$.

(f) Relative *PR1* gene expression in infiltrated leaves post secondary challenge. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 8

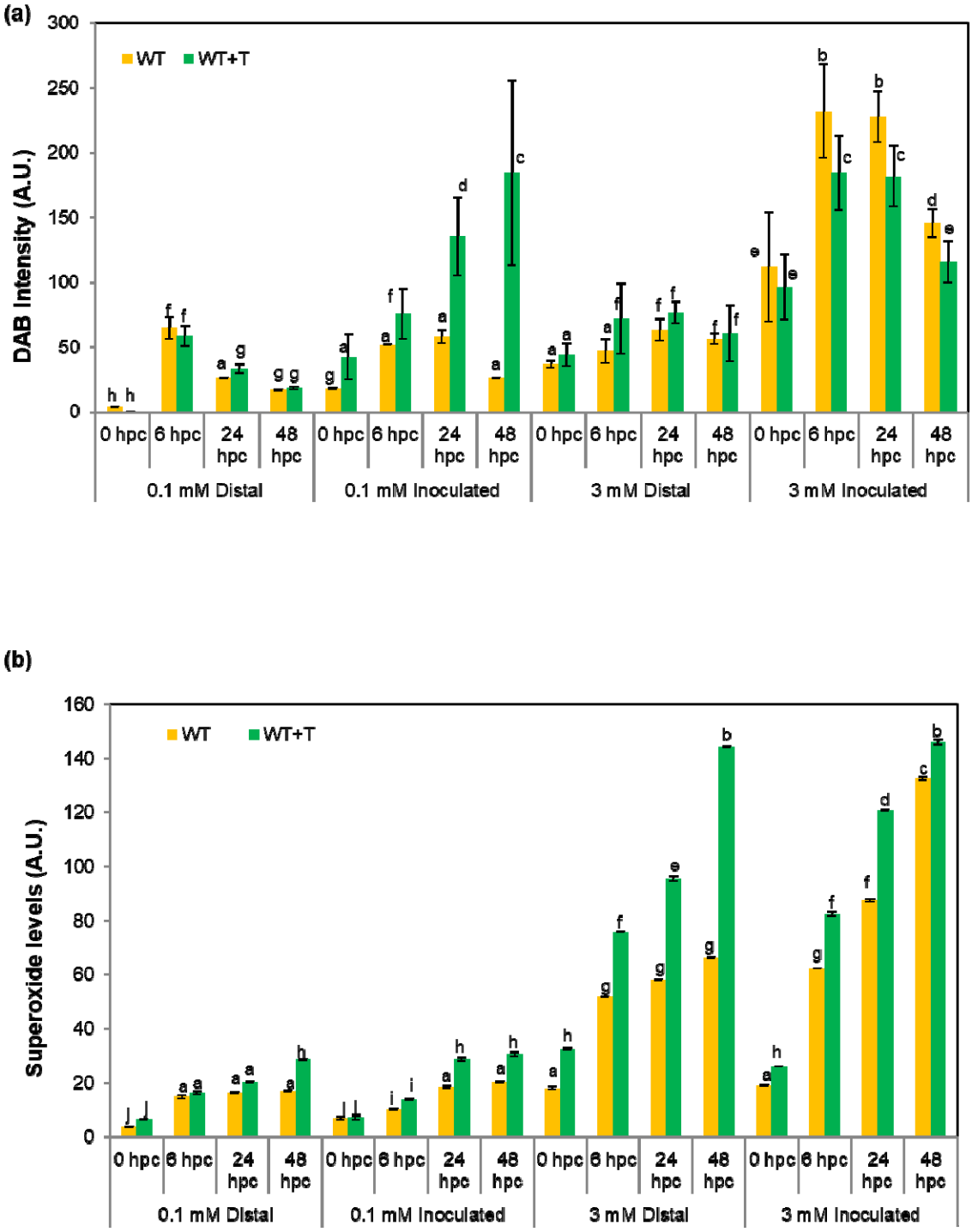


Fig. 8: Detection of ROS by measuring H_2O_2 and $O_2^{-\cdot}$ in 0.1 mM and 3 mM NO_3^- WT and *Trichoderma* grown WT plants during SAR response

(a) Quantification of DAB staining used to measure H_2O_2 levels in the inoculated leaves and distal leaves post challenge inoculation at 0, 6, 24 and 48 hpc is shown as intensity per stained area from ten leaves per treatment measured using ImageJ in arbitrary units with the mean values ± SE. Column graph represents the DAB intensity calculated as stained leaf area compared with the total surface of leaf analyzed in terms of % stained area upon the % control area by ImageJ (ver 3.2). The control leaves were completely decolorized using bleaching agent and then the area was measured using ImageJ and % was calculated with total area and the result obtained was termed % control area. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

(b) Quantification of NBT staining used to measure $O_2^{\cdot-}$ levels in the inoculated leaves and distal leaves post challenge inoculation at 0, 6, 24 and 48 hpc shown as intensity per stained area from ten leaves per treatment measured using ImageJ in arbitrary units with the mean values ± SE. Similar method was used to measure % stained area for NBT stained images w.r.t control leaves. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 9

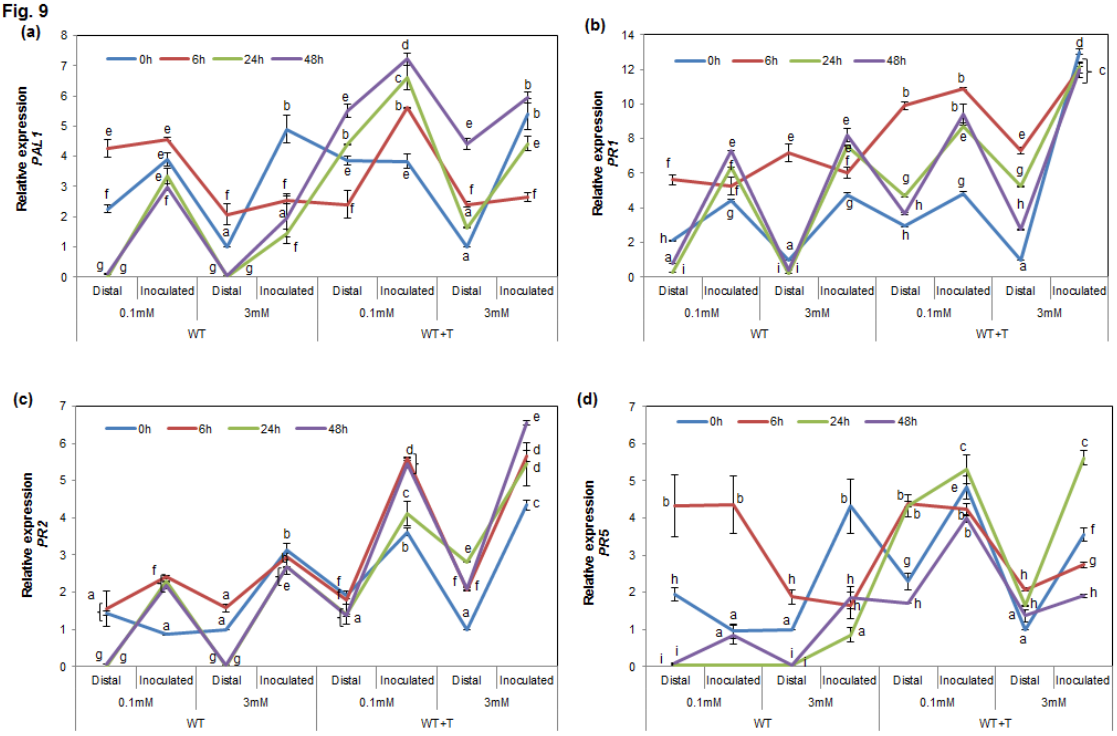


Fig. 9: Expression profiles of defense related genes during SAR

Relative expression of defense related genes in WT and WT+T grown under low (0.1 mM) and optimum (3 mM) NO_3^- concentration post-secondary challenge of *Pst*DC3000 in both inoculated and distal leaves (a) Relative expression of *PAL1* (b) *PR1* (c) *PR2* (d) *PR5*. For all the target genes, fold expression values are means (n=3) ± SE. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

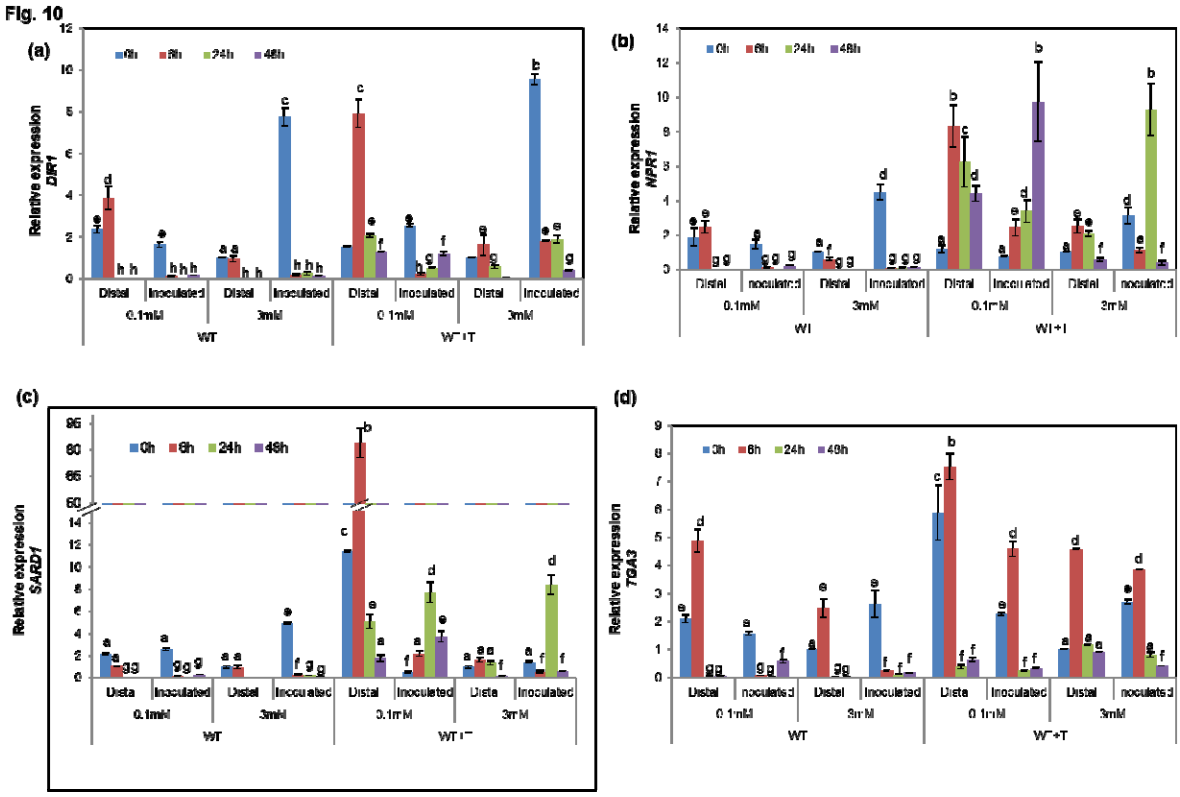


Fig. 10: Expression profiles of regulatory SAR genes

Relative expression of SAR regulatory gene in WT and WT+T grown under low (0.1 mM) and optimum (3 mM) NO_3^- concentration post-secondary challenge of *Pst*DC3000 in both inoculated and distal leaves. Relative expression of (a) *DIR1* (b) *NPR1* (c) *SARD1* (d) *TGA3*. For all the target genes, fold expression values are means (n=3) ± SE. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at $p < 0.05$.

Fig. 11

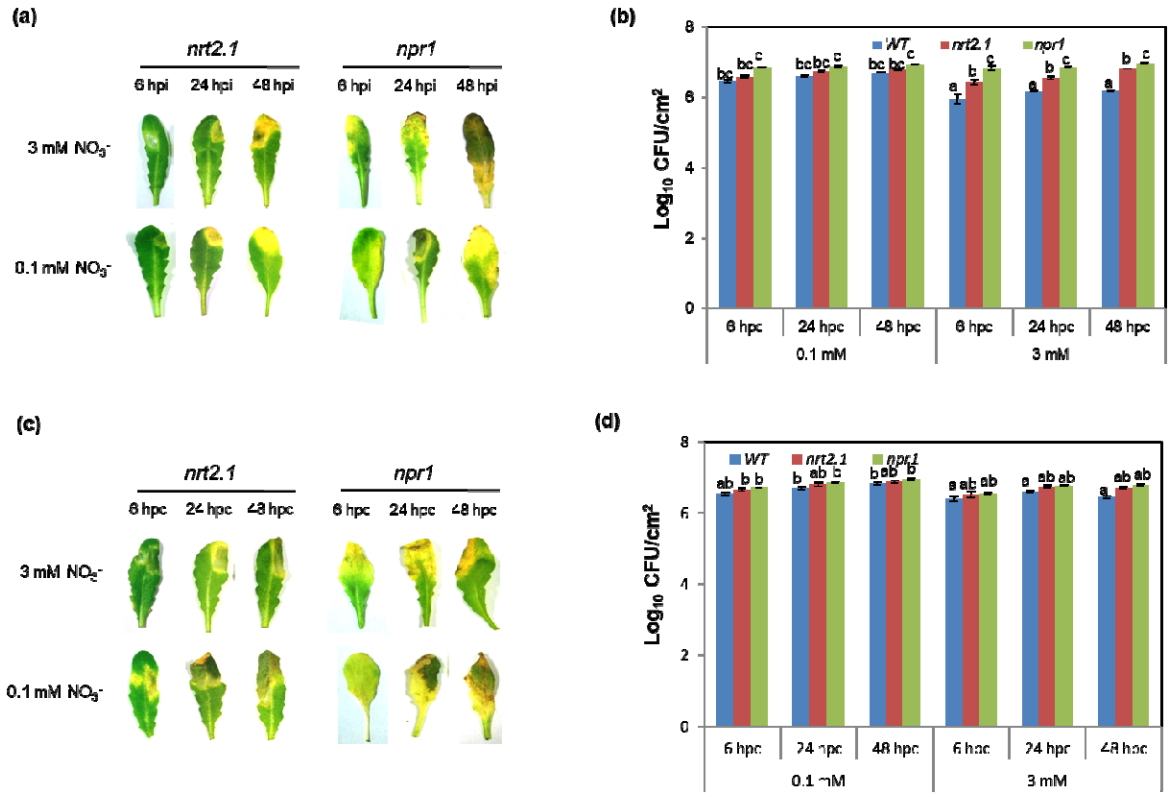


Fig. 11: LAR and SAR response in *nrt2.1* and *npr1* mutants to virulent *Pst* DC3000

(a) LAR disease symptoms to virulent Pst DC3000 in *nrt2.1* and *npr1* mutant in 0.1 and 3 mM nitrate (b) Bacterial growth in WT, *nrt2.1* and *npr1* mutant (c) Phenotype during SAR response in *nrt2.1* and *npr1* mutant in 0.1 and 3 mM nitrate (d) Bacterial growth in WT, *nrt2.1* and *npr1* mutant during SAR response.

Fig. 12

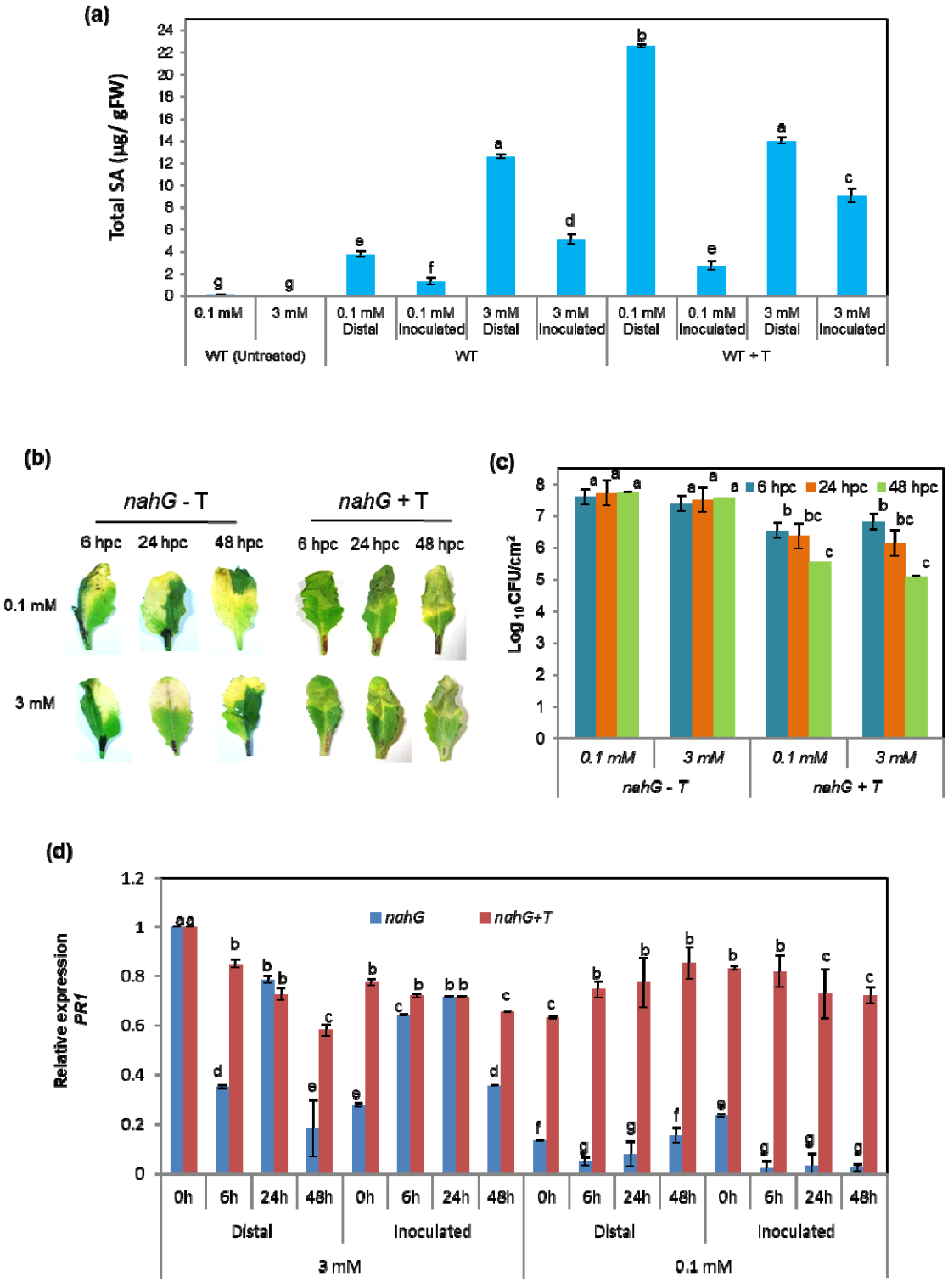
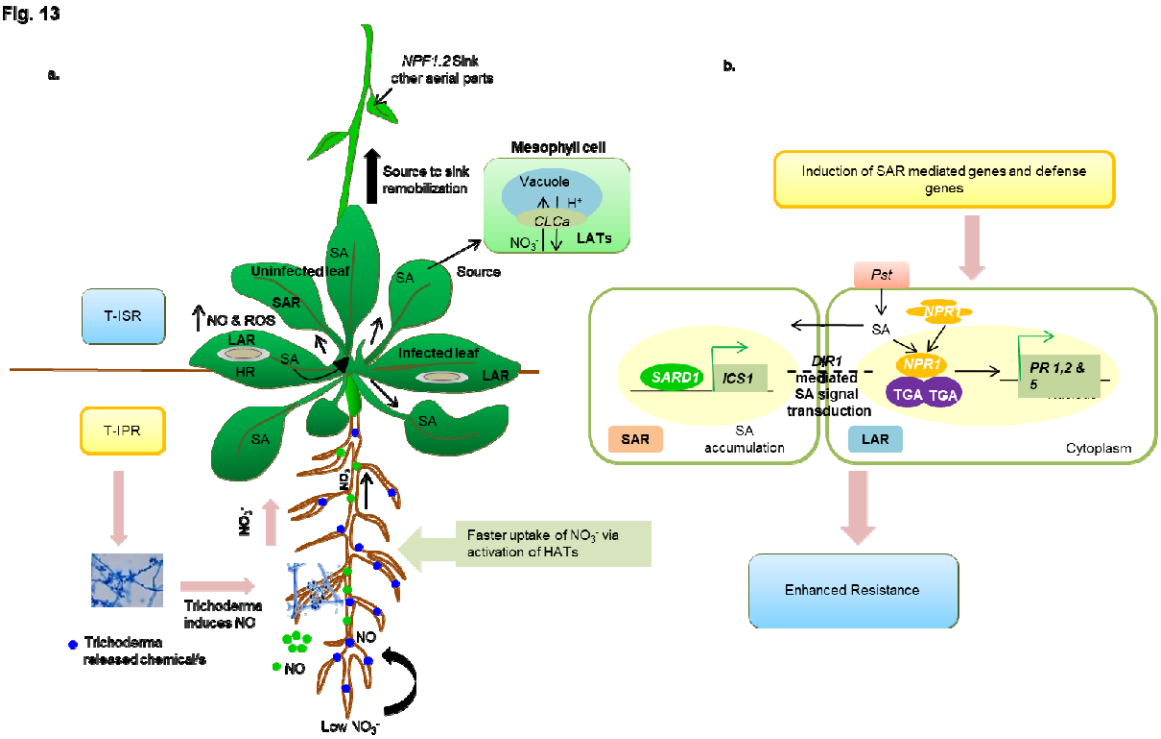


Fig. 12: SA Accumulation during SAR, and response of *nahg* in the presence and absence of *Trichoderma* during SAR

(a) Total SA levels (µg g^{-1} fresh weight) were determined in untreated WT leaves (without *Trichoderma* and *Pst* treatment) and in inoculated and distal leaves of 0.1 mM and 3 mM nitrate- fed WT plants post challenge inoculation (24 hpc) with and without *Trichoderma*. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at p < 0.05.

(b) HR phenotype in inoculated and un-inoculated distal leaves of *nahg* mutants grown under 0.1 and 3 mM nitrate concentration post-secondary challenge at 6, 24 and 48 hpc (c) Bacterial number represented by log CFU at 6, 24 and 48 hpc from the inoculated leaves post secondary challenge

(d) *PR1* gene expression in inoculated and uninoculated leaves of *nahG* mutants grown under 0.1 and 3 mM nitrate, with and without *Trichoderma* treatment post-secondary challenge. Data are average mean values ± SE with n=3. Statistical significance was tested by two-way ANOVA followed by Tukey's All-Pairwise Comparisons post-hoc test. The different letters above each column represent significance difference between means at p < 0.05.



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1178 **Fig. 13: A model depicting mechanism of *Trichoderma* induced systemic**
1179 **response (T-ISR) under low nitrate conditions during pathogen infection in**
1180 **Arabidopsis**

1181 (a) After root colonization, *Trichoderma* secretes growth promoting elicitors (blue
1182 dots), and induces priming response (T-IPR) by eliciting short term nitric oxide (NO;
1183 green dots) which facilitate faster nitrate uptake by activating HATs (*NRT2.1*, *2.2* and
1184 *2.4*) in the roots. The HATs in turn activates the vacuolar LATs (*CLCa* and *NPF1.2*)
1185 in the mesophyll cells allows source to sink re-mobilization of available nitrate from
1186 roots to other aerial parts. During primary inoculation (local pathogen attack;
1187 *avr*Rpm1), NO and ROS signals both are produced during hypersensitive response
1188 (HR), along with salicylic acid (SA), but their basal levels are greatly enhanced due
1189 to *Trichoderma* treatment. Moreover, the *Trichoderma* induced short term NO
1190 produced during priming and HR, plays a role in faster nitrate uptake by the HATs in
1191 low N stress plants. *Trichoderma* induced SA (produced during local infection) gets
1192 rapidly translocated to the other uninfected distal parts of the plant and pre-programs
1193 the stressed plant for subsequent pathogen attack.

1194 (b) *Trichoderma* activates PR proteins and cause SA accumulation in locally infected
1195 leaves during T-ISR. These signals are transported to the other part of the plant by

activating a set of regulatory genes (*DIR1*, *NPR1*, *SARD1*, *TGA3*) involved in SAR response. The SA signal transduction mediated by *DIR1 NPR1 and TGA3* was evidenced by their induced expression in systemic leaves in the presence of *Trichoderma*. Moreover, induced *SARD1* expression activates the SA biosynthetic genes (*ICS1*) in the systemic leaves. All these genes are involved in translocating these signals from locally infected leaf to the uninfected parts of the plant. Consequently, *Trichoderma* helps in the accumulation of PR proteins and SA in the uninfected leaves, thus allowing the low nitrate stress plants to show enhanced resistance.

Supplementary/Supporting Information

Figure Legends of Supplementary files:

Fig. S1: Phenotype of plants grown in different nitrate nutrition pre- and post-challenge inoculation **(a)** The growth pattern and phenotype of WT and *Trichoderma* treated WT plants in 3 mM and 0.1 mM NO_3^- nutrition; **(b)** Effect of post-secondary challenge on plant phenotype, 1°- primary inoculation, 2°-secondary challenge and D-Distal leaves; **(c)** Hyponastic response (petiole elongation) shown by 0.1 mM and 3 mM NO_3^- plants after primary inoculation.

Fig. S2: Electrolyte leakage of mock plants and morphological growth parameters during SAR **(a)** Electrolyte leakage from mock infiltrated plants in SAR; Morphological growth parameters observed in WT and treated WT plants; **(b)** Leaf number; **(c)** Biomass measured as fresh weight (FW); **(d)** Total chlorophyll content in *Pst* treated and untreated plants.

Fig. S3: Experimental design of LAR and SAR assay

(a) Description of plant samples taken for study and description of plants in absolute control, control and treatment. The sample size for each pot described here is n= 20-25 and plant's age was 30 days old.

(b) In LAR assay, there were two sets of experiments performed at the same time. Set 1 comprised of mock infiltrated plants, most of the healthy leaves of the plants are syringe infiltrated by 10 mM $MgCl_2$. Set 2 comprised of pathogen infiltrated plants, most of the healthy leaves were syringe infiltrated by avirulent *Pst*DC3000. The plants were left to acclimatize for 1 hour and then sample collection was performed at 0, 6, 24 and 48 hours post inoculation (hpi). Lowermost bar also shows

the age of the plant during the experiment. All the infections were performed in infection room under standard conditions.

(c) In SAR assay, there were two sets of experiments performed at the same time. Set 1 comprised of plants initially infiltrated with primary inoculation of avirulent (*Pst*DC3000;*avr*Rpm1) and then secondary or challenge inoculation with virulent *Pst*DC3000. Set 2 comprised of mock infiltrated plants with both primary and secondary inoculation with 10 mM $MgCl_2$. The incubation time for each inoculation is described in the figure. The time of primary and secondary inoculation was same (i.e. 10 am) and time selected for sampling is after the completion of incubation of challenge inoculation. The samples were collected after completing 3 days of challenge inoculation are denoted as 0 (10 am), 6 (4 pm), 24 (10 am next day) and 48 (10 am next to next day) hours post challenge (hpc).

Fig. S4 Experimental design and methodology of nitrate determination.

(a) Schematic representation of nitrate determination experimental design is shown here. First, the nitrate levels were determined in absolute control and soilrite: agropeat mix (with and without *Trichoderma*) according to Hachiya and Okamoto, 2017. 1 g of these S: A mixes were taken in 50 ml centrifuge tubes and immediately added pre-heated 10 ml of ultrapure water. Heat will denature nitrate reductase present in S: A mix and will be unable to utilize available nitrate in the substrate. Gently vortexed the tubes and kept at 100°C water bath and shaken every 5 minutes. After incubation, allowed the samples to cool down and settle down completely. Decanted the supernatant in another fresh tube. Centrifuged at maximum speed for 20 minutes at RT. Gently removed the clear supernatant and measured nitrate according to assay described in Hachiya and Okamoto (2017).

(b) Nitrate uptake assay was evaluated in rosette leaves of WT and WT+T plants (with and without *Trichoderma*) in a day-wise set up and percent nitrate uptake up to Day 15 is calculated. The trays containing pots were bottom irrigated with 0.1 mM and 3 mM nitrate nutrient Hoagland's solution, allowed the pots to soak the entire nutrient solution for 2 hours and excess nutrient solution was drained off by pressing the S: A mixture. The leaves were sampled from Day 0 till Day 15 (even days) for nitrate uptake assay in the same set of plants (one leaf from each pot each day of sampling). During this period, nutrient was not supplied to the plants, they were only watered to ensure no drying of the S: A mixture and cause no drought to plants. A separate set of 6 pots of WT rosettes (absolute control) was also kept for calibrating

the basal levels of nitrate present in leaves without flooding with 0.1 or 3 mM nitrate solution. The nitrate levels (leaves) obtained from this absolute control were subtracted from the nitrate treated pots. Each day-wise nitrate levels were calculated by subtracting the values of Day 2, 4, 6, 8, 10, 12 and 15 from Day 0 nitrate levels and subsequently % nitrate uptake was evaluated, shown in Fig. 3(a).

(c) Nitrate levels were also determined in 1 g of soilrite: agropeat mixture (blue bar), 0.1 mM fed S: A mix (light orange bar), 3 mM fed S: A mix (light green bar), 0.1 mM fed S: A mix with *Trichoderma* (light purple bar) and 3 mM fed S: A mix with *Trichoderma* (yellow bar). Data are average mean values ± SE with n=3. Statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparison test. The different letters above each column represent significance difference between means at $p < 0.05$.

(d) Standard curve of potassium nitrate used to calculate apparent nitrate concentration. Nitrate standard series of 2, 4, 6, 8, 10 and 12 mM were prepared and assay was performed according to Hachiya and Okamoto, (2017). A straight line curve generated was used to determine the nitrate concentration (mM) using the formula = Abs/0.055, where 0.055 is the value according to straight line curve.

Fig S5: **(a)** Electrolyte leakage in distal leaves in WT and WT+T leaves during SAR **(b)** % cell death quantified in Trypan blue images of both inoculated (Pst) and uninoculated (distal) leaves in WT and WT+T leaves during SAR using ImageJ. Results are presented as % necrotized leaf area compared with the total surface of leaf analyzed by ImageJ (ver 3.2) and represent means ± SE of 6 leaves per treatment.

Table S1: List of primers

S.No	*Gene*	Accession ID	Orientation	Sequence
1	*Chloride channel A (AtCLC-A)*	AT5G40890	Forward	GCTTCACTCATGGCTGGTTC
			Reverse	CATCCACGGCTCTGGATTTG
2	*Nitrate transporter (AtNPF1.2)*	AT1G52190	Forward	CGGTTTAGGAATGGCTGTGG
			Reverse	GGACCATACGACCAGCTACA
3	*High affinity nitrate transporter (AtNRT2.1)*	AT1G08090	Forward	TCTTCTCCTTCGCCAAACCT
			Reverse	CTCCCATCACGAGCCTAGAG
4	*High affinity nitrate transporter (AtNRT2.2)*	AT1G08100	Forward	AGCGGGGATTATAGCAGCAT
			Reverse	CACACAAAAGAGACCACCGG
5	*High affinity nitrate transporter (AtNRT2.4)*	AT5G60770	Forward	GTTCATGATCGGGTTCTGCC
			Reverse	TGAAGGACGTGGACCCTAAC
6	*Pathogenesis related-1 (AtPR1)*	AT2G14610	Forward	AGGCACGAGGAGCGGTAGG
			Reverse	CATGTTCACGGCGGAGACG
7	*β-1,3-Glucanase (AtPR2)*	AT3G57260	Forward	TGGTGTCAGATTCCGGTACA
			Reverse	TCATCCCTGAACCTTCCTTG
8	*Pathogenesis related-gene 5 (AtPR5)*	AT1G75040	Forward	CGTACAGGCTGCAACTTTGA
			Reverse	TGAATTCAGCCAGAGTGACG
9	*Phenyl Ammonia Lyase1 (AtPAL1)*	AT2G37040	Forward	GGCAGTGCTACCGAAAGAAG
			Reverse	TCTCCGGTCAAAAGCTCTGT
10	*SAR Deficient 1 (AtSARD1)*	AT1G73805	Forward	GCGATGACTGAAGCGATTGT
			Reverse	CAGTGTTGATGTGGCGAGAG
11	Non-expresser of PR genes	At1g64280	Forward	GAATCCGTCTTTGACTCGCC

	(AtNPR1)		Reverse	GCGGTGTTGTTGGAGTCTTT
12	*TGA1A-related gene 3 (AtTGA3)*	At1g22070	Forward	CGCCCATCCGAGCTTTTAAA
			Reverse	ATGCTTTCGACCAAACCCTG
13	*Defective in Induced Resistance 1 (AtDIR1)*	At5g48485	Forward	CATGAGCCAGGATGAGTTGA
			Reverse	ACTGTTTGGGGAGAGCAGAA

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