Trichoderma asperelloides enhances local (LAR) and systemic acquired resistance (SAR) response under low nitrate nutrition in *Arabidopsis*

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Introduction

Results

Discussion

Material and Methods

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Summary

- Nitrogen (N) is essential for growth, development and defense but, how low N effects 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 LAR & SAR response when infected with avirulent and virulent *Pseudomonas syringae* DC3000. These responses were enhanced in the presence of *Trichoderma*. The mechanism of increased LAR and SAR mediated by *Trichoderma* involve increased N uptake and enhanced protein levels via modulation of nitrate transporter genes. *nrt2.1* mutant is compromised in LAR and SAR response suggesting a link between enhanced N transport and defense.

- Enhanced N uptake was mediated by *Trichoderma* elicited nitric oxide (NO). Low NO producing mutants *nia1,2*, *nsHb*$^{+}$ were unable to induce nitrate transporters and compromised defense in 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. SA deficient *NahG* and *npr1* mutants were compromised in LAR and SAR response in the presence of *Trichoderma*.

- The mechanism of enhanced plant defense under low N mediated by *Trichoderma* involve NO, ROS, SA production and induction of NRT and SAR marker genes.

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 the chlorophyll, nucleic acids and amino acids. N plays an important role in operation of primary and secondary metabolism and also involved in protection of plants against biotic and abiotic 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 the growth of plants 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 to support their survival under N deficiency (Li *et al.* 2017). These uptake systems are based on their affinity with nitrates (NO_3^-) mediated by the family of low and high affinity nitrate transporter proteins (LATs & HATs; Tsay *et al.* 2007).

N plays a very significant role in plant defense (Gupta *et al.* 2013; Mur *et al.* 2017). Hence, operation of efficient N transport system can help plants to defend against pathogens. These defense responses involve activation of innate immune response comprising of PTI (PAMP Triggered Immunity) and ETI (Effector-Triggered Immunity) (Alves *et al.* 2014; Jones and Dangl 2006) and requires N. The defense mechanism for 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 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 the 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 its concentration plays a 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^-. Therefore, N deficiency can also lead to reduced levels of NO. SA is known

to be induced by NO, hence, low N leads to low NO and reduced SA levels. Since NO production also requires NO_3^-, it may also play a role in systemic acquired resistance (SAR) which results in broad-spectrum disease resistance against secondary infections after primary infection (Cameron *et al.* 1994, 1999). SAR develops either as a consequence of HR where NO has its proven role (Delledonne *et al.* 1998), or in the course of a compatible interaction resulting in disease development (Gupta *et al.* 2014). SAR is dependent on SA or its derivatives (Park *et al.* 2007, Metraux *et al.* 1990) and PR gene expression (Ryals *et al.* 1996). Previously, it was shown that NO plays a role in nitrate uptake/assimilation during stress by modulating nitrate transporters (Frungillo *et al.* 2013). Thus, N plays multifaceted roles in plant defense.

Therefore, any mechanism that can increase N uptake may assist in plant defense under low NO_3^-. Many plant symbiotic microbes such as mycorrhiza, *Trichoderma*, PGPR 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 not known.

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 response (ISR). Previously, it was demonstrated that roots colonized by *Trichoderma* species can activate defense responses very rapidly and intensively via priming in *Arabidopsis* (Brotman *et al.* 2012). This occurs via modulation of transcription factors and key metabolites of plant defense (Shoresh *et al.* 2010). Upon pathogen attack, *Trichoderma* treated plants show accumulated transcript levels of *PAL* (Yedidia *et al.* 2003) and increased levels of defense-related plant enzymes (Shoresh *et al.* 2005).

If *Trichoderma* enhances N uptake, it can have positive effect on plant defense via LAR and SAR responses altogether. 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 *T203* enhances SAR response in Arabidopsis grown under low N via modulating nitrate transporters and this mechanism involves both NO and SA.

Results

Optimal and low nitrate effects LAR and SAR differently

Nitrogen plays an important role in plant defense, hence, we first tested the effect of low nitrate on LAR and SAR response. For this purpose, WT plants were grown under optimum (3 mM) and low (0.1 mM) NO_3^- conditions and infected with *P. syringae* DC3000 (*avrRpm1*) and observed local HR. As shown in Fig.**1a** (lower panel) within 24 hpi, the 3 mM NO_3^--fed plants showed HR (lesion formation) at the inoculation sites and it was enhanced till 48 hpi (Fig.**1a**) whereas 0.1 mM NO_3^- plants showed chlorotic lesions at the site of inoculation at 24 hpi and these spots were enhanced in 48 hpi. Electrolyte leakage (EL) (Fig.**1b**) was more rapid in 3 mM *Pst* infiltrated plants than 0.1 mM plants which is in accordance with HR response. A significant increase in CFU count was observed in 0.1 mM grown plants at 48 hpi in comparison to bacterial numbers in 3 mM grown plants (Fig. **1c**). We further found that, the *PR1* (a marker for HR) transcript levels have increased in 3 mM *Pst* treated plants (up to 25 fold till 48hpi), but in 0.1 mM WT plants, this gene was not at all induced at 48 hpi (Fig.**1d**). Taken together these results suggest that low NO_3^- grown plants are compromised with HR linked LAR response.

After observing LAR, we studied SAR (Fig. **S1b**; Cameron *et al.* 1994 and 1999). Three days post-secondary challenge with virulent *Pst* DC3000, EL, CFU and *PR1* gene expression were assessed. 0.1 mM *Pst*-challenged plants show more prominent necrotic and dark yellow lesions spread to both sides of the leaf (Fig. **2a**) confirming susceptible symptoms (yellow specks) whereas 3 mM grown plants showed reduced yellowing and necrotic symptoms. This clearly suggests that, 3 mM NO_3^- plants defended better during secondary challenge than 0.1 mM NO_3^- plants suggesting that optimal N is required for SAR development.

Trypan blue images of inoculated and uninoculated leaves are shown in Fig. **2b**. It was observed that, after 3 days post challenge, the cell death observed in *Pst* infiltrated tissues of 0.1 mM NO_3^- plants is profoundly less in comparison to 3 mM NO_3^- plants till 24 hpc. At 48 hpc, there was increased cell death in both 0.1 and 3

mM inoculated leaves but cell death phenotype is different. Interestingly, the uninoculated leaves of 0.1 and 3 mM WT plants, showed small and uniform spread of macrobursts throughout the leaf blades spread unanimously over the leaf, in all the time points, in exception that, 3 mM leaves displayed slightly larger macrobursts (Fig. **2b**). This suggests that during SAR establishment, the mobile signal perceived by the uninoculated leaves results in the occurrence of low frequency microscopic HRs (Cameron *et al.* 1999; Alvarez *et al.* 1998).

Then, we assessed EL (Fig. **2c**), we found leakage was significantly higher in 3 mM plants than 0.1 mM plants in both the cases (1° and 2°). But, much higher EL was observed during 2° challenge (Fig. **2c**-right panel). Mock inoculated plants showed moderate EL in both 0.1 mM and 3 mM plants (Fig. **S2a**). Higher bacterial growth was observed in 0.1 mM *Pst* treated plants as expected till 72 hpc, while there was a significant reduction in the bacterial numbers in 3 mM *Pst* inoculated plants (Fig. **2d**).

Low LAR under low nitrogen is reversed by *Trichoderma*

Next, we investigated whether *Trichoderma asperelloides* plays a role in improving LAR and SAR response under low NO_3^- availability. Interestingly, we found that under 0.1 mM NO_3^-, *Trichoderma* treated plants displayed healthy phenotype than untreated plants (absence of *Trichoderma*) (Fig. **S1a**), this enhanced growth was linked to increased leaf number, fresh weight and total chlorophyll content (Fig. **S2 b,c,d**). During LAR, *Trichoderma* grown 0.1 mM NO_3^- plants did not show any severe disease symptoms, rather they mimicked the HR phenotype of 3 mM plants and showed resistance (Fig. **3a**). This suggests that *Trichoderma* provides resistance under low N stress (Compare Fig. **3a** with Fig. **1a**). EL was significantly higher in both 3 mM and 0.1 mM *Trichoderma* treated plants (Fig. **3b**) in comparison to untreated plants (Compare Fig. **3b** with Fig. **1b**). As shown in Fig. **3c** there was a significant reduction in the bacterial count in 0.1 mM *Pst* plants at 24 hpi, suggesting that *Trichoderma* enhances LAR response under low N. The higher accumulation of *PR1* transcripts in both 0.1 mM (~9 folds at 48 hpi) and 3 mM (~28 folds at 48 hpi) in *Trichoderma* grown *Pst* treated plants (Compare Fig. **3d** with Fig. **1d**) again providing evidence that *Trichoderma* can enhance LAR under low N.

***Trichoderma* can induce SAR under low N**

During SAR assay secondary challenge there was complete reduction in yellowing in *T203* grown 0.1 mM plants in comparison to untreated plants (Compare Fig. **4a** with Fig. **2a**). In response to *T203*, 0.1 mM NO_3^- plants showed more Trypan blue spots during secondary challenge. Upon infection, cell death spots were more widely spread in these leaves (Compare Fig. **4b** with Fig. **2b**). EL was significantly higher (~4 fold) in both 0.1 and 3 mM *T203* treated plants during 2° challenge, in comparison to 1° inoculation at 24 and 48 hpc (Fig. **4c**). This suggests that *T203* treatment primes the defense responses in the uninoculated leaves immediately at 0 hpi and after 2° challenge. Bacterial count was in accordance with EL that, the *Pst* population decreased significantly in *T203* treated low N-fed plants, suggesting that *T203* imparts enhanced resistance to the NO_3^- stressed plants (compare Fig. **4d** with Fig **2d**).

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

Increased LAR and SAR response under low NO_3^- in the presence of *Trichoderma* is probably due to increased nutrient uptake facilitated by *Trichoderma* as these responses are absent in *Trichoderma* untreated plants. Increased nitrate transport is probably the reason for resistance hence, we checked the expression of both LATs (*CLC-A*, *NPF1.2*) and HATs (*NRT2.1*, *NRT2.2* and *NRT2.4*).

CLCa (the chloride channel family) is a tonoplast located antiporter channel system which drives nitrate accumulation in the vacuoles (Krapp *et al.* 2014). It was observed that, under low NO_3^-, C*LCa* is less inducible in *Pst* treated plants whereas it was significantly upregulated in *T203* grown 0.1 mM *Pst* treated plants*.* The uninoculated leaves of 0.1 mM *T203* grown WT plants showed slight induction of *CLCa* in comparision to non-*T203* grown plants (Compare Fig. **5a**,**b**). We further checked the expression of *NPF1.2;* which belongs to NRT1 (peptide transporter) family of nitrate transporters (Fig. **5c**) and 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 WT plants, *NPF1.2* levels were highly induced till 48 hpc, in comparison to 0.1 mM *Pst* inoculated leaves (Fig. **5c**). This might be the reason behind resistance of 3 mM plants and susceptibility of 0.1 mM plants after pathogen challenge. But, upon *T203* pre-treatment, *NPF1.2* transcript levels in *Pst*-inoculated 0.1 mM plants (Fig. **5d**), showed significant induction in comparison to same

treatment without *T203* (Compare Fig. **5c** & **5d**). This suggests that, *T203* may cause the regulation of *NPF1.2*.

NRT2 transporters (*NRT2.1*, *NRT2.2* and *NRT2.4*) are HATS and gets activated at low N concentrations (<1 mM). Previously, it was shown that *NRT2.1* is active only under N starving conditions (Dechorgnat *et al.* 2012). Under low NO_3^- conditions, *NRT2.1* was significantly induced in all treatments in *T203* grown *Pst* treated plants in comparison to untreated plants where there was already an induction of this gene at early time points (Fig. **6a,b**). The SAR establishment stage represented by uninoculated leaves showed dynamic and hiked up-regulation of *NRT2.1* transcripts (Fig. **6b**). This revealed that, *T203* colonization benefits the plant by facilitating the N supply even in low NO_3^- conditions thereby showing improved plant defense mediating SAR. A similar expression pattern was observed in *NRT2.2* expression levels 0.1 mM in uninoculated and inoculated plants (Fig. **6b**) and the expression was further accelerated several folds in the presence of *Trichoderma* (Fig. **6d**). This again revealed that faster N uptake by *T203* treated low NO_3^- plants can help to defend better. Consequently, we checked *NRT2.4* expression levels in both set of treatments. This gene showed early induction in 0.1 mM *Pst* treated in inoculated and uninoculated plants (Fig. **6e**) and the expression pattern was similar in response to *T203* treatment at early stages but a slight induction can be seen even at later time points (Fig. **6f)**.

Taken together, these results suggests that *Trichoderma* induces HATs 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. **6g**), it was not at all induced suggesting that *NRT2.1* plays an important role in increasing plant defense under low N mediated by *Trichoderma*. Then, we further found that in *nrt2.1* mutant, the expression of *NRT2.2* gene also suppressed in both control and *Trichoderma* treatment (Fig. **6h**) suggesting that *NRT2.1* is mainly responsible for increasing N uptake under low NO_3^- facilitated by *Trichoderma*. Then, we checked the protein levels in WT and *nrt2.1* mutant in the presence or absence of *Trichoderma* under low NO_3^-. In response to *Trichoderma*, protein levels have increased in WT, whereas in *nrt2.1* mutant protein levels were less than WT but upon

T203 treatment only slight increase was observed (Fig. **6i**). These result suggests that *T203* application can increase nitrate transport thus responsible for increased protein levels.

***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 NO could have a role in induction of nitrate transporter genes. Hence, we checked NO production in WT, *nia1,2* mutants and WT seedlings grown on NO scavenger cPTIO inoculated with *Trichoderma*. Control roots in 0.1 and 3 mM NO_3^- produced reduced levels of NO. Within 2 min of *T203* application, WT plants showed much increased levels of NO in both 0.1 and 3 mM nitrate (Fig. **7**) but the increase was slightly higher in 0.1 mM than 3 mM. But, in 10 minutes and 24 h of *T203* incubation, the WT plants showed extremely low fluorescence (Fig. **7**). This suggests *T203* greatly induces NO in low NO_3^- grown plants within short time. Thus, *Trichoderma* induced NO probably plays a role in priming and induction of HATs. Assessment of NO in *nia1,2* mutant revealed that NR is responsible for NO production. cPTIO grown plants showed reduced levels of NO.

Then, we further checked the importance of *Trichoderma* elicited early stages of NO in the induction of HATs, total protein levels and expression of *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 have increased in WT in response to *Trichoderma*, whereas in Hb$^+$, *nia1,2* and *nrt2.1* mutants these two HATs were not induced (Fig. **8a,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 induction of nitrate transporters mediated by *Trichoderma* elicited NO plays a role in increased N uptake under low NO_3^- (Fig. **8c**). Further, we found that *nia1,2* and Hb$^+$ were unable to induce *PR1* expression under these conditions (Fig. **8d**). Taken together, these results suggests that *Trichoderma* elicited NO plays a role in overall increase in defense response under low NO_3^-.

Further, we investigated the role of NO in LAR and SAR development in the presence of *Trichoderma* using NO mutants. Under low NO_3^-, WT plants performed better LAR response than Hb$^+$ and *nia1,2* mutant. Reduced bacterial growth and increased *PR1* gene expression was observed in WT than Hb$^+$ and *nia1,2* mutant

(Fig. **9a,b,c**). Similarly, SAR response was also compromised in than Hb+ and *nia1,2* like LAR, a similar reduced bacterial number and increased *PR1* gene expression was observed in WT in comparison to Hb^+ and *nia1,2* mutant (Fig. **9d,e,f**).

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

Both NO and ROS are involved in plant resistance response hence, we investigated the role of ROS. The distal un-inoculated leaves of 0.1 mM NO_3^- WT+T plants showed increased H_2O_2 production in comparison to 0.1 mM un-inoculated plants (Fig. **10a**). Also, the level of H_2O_2 was intense and distributed in 3 mM un-inoculated leaves of WT+T plants in comparison to untreated plants (Fig. **10a**). This is probably due to suppression of catalase activity by SA. This suggests that *T203* inoculation can enhance H_2O_2 levels in *Pst* uninoculated leaves during SAR, whereas, both the 3 mM *Pst* inoculated leaves from WT and WT+*T203* plants showed higher H_2O_2 production (Fig. **10a**). This clearly suggests that *T203* 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. **10b**). It was observed that, there was increased $O_2^{-\cdot}$ in inoculated leaves of both 0.1 and 3 mM fed WT and *T203* treated WT plants, but increase was much higher in *T203* treated WT plants than *Trichoderma* untreated plants. This suggests that there was a rapid oxidative burst (HR) which offers resistance to the plants in response to *T203* treatment. Taken together, the coordinated interplay of H_2O_2, $O_2^{-\cdot}$ and NO leads to the HR associated cell death that generously improvises the LAR and SAR responses in low N-stress plants.

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

Next, we checked the expression profiles of defense related genes (*PAL1, PR1, PR2* and *PR5*) during SAR. In both 3 mM and 0.1 mM *Pst* inoculated WT plants, *PAL1* transcripts were highly induced in all time points, but, the uninoculated leaves showed *PAL1* induction only till 6 hpc, but drastically declined at later time points (Fig. **11a-I**). *Trichoderma* grown WT plants under 3 mM and 0.1 mM, showed even more enhanced levels of *PAL1* transcripts (Fig. **11a-II**) in inoculated as well as uninoculated leaves till 48 hpc. Similar trend was observed in *PR1* (Fig. **11b**), *PR2* (Fig. **11c**) and *PR5* (Fig. **11d**) expression profiles, which got elevated after

Trichoderma application. Out of all the four defense genes examined, *PR1* gene (SA marker) displayed the extremely higher expression levels in *Trichoderma* grown plants suggesting that *T203* might induce SA levels to a greater extent during SAR.

***Trichoderma* enhances expression of SAR mediated regulatory genes**

Further, we checked whether *Trichoderma* can induce 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 both 0.1 and 3 mM inoculated and uninoculated WT plants (Fig. **12a-I**). But, in *T203* grown plants, a slight increased *DIR1* expression was observed in all time points (Fig. **12a-II**). Upon perception of SAR mobile signals, Non-Expresser of Pathogenesis-Related Genes1 (*NPR1*) activates defense in challenged plants (Cao *et al.* 1997). We found a similar trend in the *NPR1* expression profile like *DIR1* in WT plants (Fig. **12b-I**). Moreover, upon the *Trichoderma* treatment, the levels of *NPR1* gradually increased in both 0.1 and 3 mM inoculated and uninoculated challenged leaves (Fig. **12b-II**).

Next, we checked the expression of SAR DEFICIENT 1 (*SARD1*) a pathogen-induced transcription factor (Zhang *et al.* 2010) and a key regulator for Isochorismate Synthase 1 (*ICS1*) and SA synthesis (Wang *et al.* 2011). A remarkably stronger induction of *SARD1* expression levels in *T203* treated 0.1 mM uninoculated leaves at 6 hpc (~82 fold) revealed that *Trichoderma* is a potential inducer of SA biosynthesis (Fig. **12c-II)**. Another important regulatory gene, *TGA3*, is an *NPR1*-interacting protein (NIPs) and is a critical component in the SA signaling mechanism. This gene was induced in all treatments in response to *T203* (Fig. **12d**) suggesting that *Trichoderma* might act as unequivocal channel in SA signaling pathways.

nrt2.1 and npr1 mutants are compromised in LAR and SAR

Further, we checked LAR in *nrt2.1* and *npr1* in response to virulent *Pst*DC3000. The 0.1 mM N-fed *nrt2.1* and *npr1* mutants developed more severe symptoms than 0.1 mM WT plants (Fig. **13a**- 0.1 mM panel). Whereas the 3 mM

NO_3^--fed WT plants showed less disease symptoms as compared to 0.1 mM NO_3^- plants (Compare Fig. **1a** and Fig. **13a**- 3 mM panel). A continuous increase in CFU count was observed in 0.1 mM grown WT, *nrt2.1* and *npr1* at 24 and 48 hpi in comparison to bacterial numbers in 3 mM NO_3^- grown plants (Fig.**13b**). This suggests that optimum N concentration plays an important role in resistance response towards virulent *Pst* DC3000.

Similarly, we studied the SAR response in the WT, *nrt2.1* and *npr1* plants. 0.1 mM *Pst*-challenged WT plants show more prominent necrotic and discolored lesions spread to half of the leaf (Fig. **2a**) confirming susceptible symptoms (yellow specks). On the other hand, the mutant's *nrt2.1* and *npr1* showed even more severe disease symptoms (extensive chlorosis and necrosis) (Fig. **13c**) under low NO_3^- conditions. Results suggests that, 3 mM NO_3^- plants defended better during secondary challenge than 0.1 mM NO_3^- plants. Bacterial populations were also increased in *nrt2.1* and *npr1* mutants (Fig. **13d**) suggesting that *NRT2.1* and *NPR1* plays an important role in increasing defense mediated by *Trichoderma*.

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

Examination of SA levels revealed that *Trichoderma* presence accelerated total SA levels in 0.1 mM uninoculated distal leaves, in comparison to 3 mM uninoculated leaves. There was no significant increase in SA levels observed in inoculated leaves of 0.1 mM *Trichoderma* grown plants, while in 3 mM *Trichoderma* grown plants, a significant increase in SA levels were observed (Fig. **14a**). 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. **14b,c**). Surprisingly, *Trichoderma* grown *nahG* plants showed slightly enhanced *PR1* transcript levels (Fig. **14d)**.

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. Since N is also important for synthesis of various secondary metabolites, severe depletion of N can impact defense related pathways. Hence, plants may not be able to activate defense pathways for tolerance or resistance (Snoeijers *et al.* 2000). Plant take up N in the form of NH_4^+ or NO_3^- or 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 use NO_3^- as the preferable N source. Moreover, NO_3^- can help in better charge balance (Boudsocq *et al.* 2012). Nitrate nutrition can also enhance plant defence via increased generation of NO, polyamines and SA (Gupta *et al.* 2013; Fagard *et al.* 2014). Hence, we preferred to check the plant defense response under NO_3^- nutrition rather than under NH_4^+.

Plants which are grown under 0.1 mM NO_3^- showed reduced growth (Fig. **S1**) and morphological parameters (Fig. **S2**), suggesting that supplied 0.1 mM NO_3^- is not sufficient for the plants for growth and under these conditions plants become more susceptible. In our experiments, 0.1 mM NO_3^- grown plants were compromised in both LAR and SAR (Fig. **1,2**) response, suggesting that N is required for better defense. Plants grown on low N produced less SA (Fig. **14a**), further supporting that N is needed for SA biosynthesis. Since plants need N for growth and disease resistance, increasing N use efficiency can help them to defend better. Some group of *Trichoderma* help in nutrient absorption leading to increased growth and enhanced plant defense (Brotman *et al.* 2010). Previously, it was shown that the supplementation of *Trichoderma asperelloides* enhances plant growth (Brotman et *al.* 2012), and protects against abiotic and biotic stressors, moreover, it induces systemic resistance responses (Contreras-Cornejo *et al.* 2016; Brotman *et al.* 2012). *Trichoderma* induced increased growth attributed to auxin and ethylene (Garnica-Vergara *et al.* 2016) and induction of genes involved in carbon and N metabolism (Domínguez *et al.* 2016). However, there are hardly any reports on investigating the mechanism of improved plant growth and defense under N starvation mediated by

Trichoderma. In this 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 support in LAR & SAR response under low NO_3^- stress.

The low NO_3^- fed plants which were grown in the presence of *Trichoderma*, showed increased growth (Fig. **S2**) suggesting that *Trichoderma* can enhance growth under low NO_3^- (Fig. **S1a**). We found that this enhanced growth is partly due to increase N uptake which was evidenced by increased expression of *NRT2.1, NRT2.2, NRT 2.4* HATs (Fig. **6a,b,c,d,e,f**) and increased protein levels (Fig. **6i**). Since low NO_3^- fed plants becomes susceptible (Snoeijers *et al.* 2000), it is logical to assume that *Trichoderma* can increase N uptake and enhance resistance. Hence, in further experiments, we focused on LAR and SAR response under low and optimum NO_3^- in the presence or absence of *Trichoderma*. During local *Pst* infection, the plants display LAR response, and the systemic/distant uninoculated leaves induces SAR response. Both are important for plants to defend against pathogens.

During SAR, after primary inoculation, within 4–6 hours, in the inoculated site, where, localized cell death (HR) occurs, plant displays hyponastic response. 3 mM N-fed WT plants showed clear hyponasty after primary inoculation, whereas 0.1 mM did not display such response (Fig. **S1c).** In 3 mM WT plants, the defense response after secondary challenge was more rapid, robust and even long-lasting till 5 days post challenge (data not shown) whereas, 0.1 mM WT plants showed disease symptoms suggesting that NO_3^- concentration plays a role in LAR and SAR development (Fig. **2a**; Fig. **S1b**).

In our experiments, roots were treated with *Trichoderma* hence, we examined high and low affinity transporters to see whether *Trichoderma* modulates these transporters to enhance N uptake under low nitrate. *NRT2.1* is the main HAT, localized at the plasma membrane. Previously, it was shown that these transporters becomes active during N starvation and is severely inhibited when reduced nitrate sources such as glutamine or ammonium are provided (Dechorgnat *et al.* 2012). Similarly, *NRT2* also induced under low N (Dechorgnat *et al.* (2012). In response to *Trichoderma* treatment, a rapid re-programmed *NRT2.1, NRT2.2* transcripts in both 0.1 and 3 mM was observed during *Pst* inoculation. This feature is very important

since plants are NO_3^- starved. *NRT2.1* involvement was further evidenced by the fact that *nrt2.1* mutant produced less protein under low N and *Trichoderma* was unable to increase protein content in this mutant (Fig. **6f**). This mutant become very much susceptible to LAR and SAR (Fig. **13**).

We further unraveled the mechanism behind increased expression of LATs and HATs mediated by *Trichoderma*. One of the features of *Trichoderma* is the induction of short-term NO and ROS. These molecules play important role in induction of plant defense responses (Gupta *et al.* 2014). We suspected the role of NO in activating genes of these transporters. *Trichoderma* induced expression of *NRTs* are most likely mediated by short term increase in NO upon *Trichoderma* inoculation. The NR dependent NO elicited by *Trichoderma* probably responsible for the increased expression of HATs as our experiments revealed that in *nia1,2* and Hb^+, despite of growing in the presence of *Trichoderma* were unable to induce HATs they showed decline in protein levels (Fig. **8c**). *Trichoderma* triggers SA dependent SAR pathway (Pieterse *et al.* 2014). *Trichoderma* spp. induced ROS is involved in plant's resistance response against many biotic stressors (Asmawati *et al.* 2017). It also plays an important role in hypersensitive cell death together with NO (Durner and Klessig 1999, Dorey *et al.* 1999). Among ROS, H_2O_2 is the most stable form of ROIs that play an important role as signal transducer in the plant cell death process (Pieterse *et al.* 2014), and acts as key modulators of NO in triggering cell death. As shown in Fig. **10a**, it is observed that, *T203* treated low N fed plants displayed increased H_2O_2 levels, thus, enabling the SAR initiation and the establishment stage. Superoxides ($O_2^{-\cdot}$) are mainly produced via mitochondrial electron transport and *NADPH oxidase* chain during stress. Pathogens usually employ this mitochondrial disturbance as a strategy to suppress host immunity and thus, increased ROS may contribute some resistance to plants against pathogens (Torres *et al.* 2002, 2005). Earlier, it was shown that the extracellular elicitors isolated from *Trichoderma viridae* also induces the $O_2^{-\cdot}$ levels (Calderon *et al.* 1994). Here, we also found that *T203* grown, 0.1 mM NO_3^-, *Pst* inoculated plants show higher $O_2^{-\cdot}$ levels than untreated ones (Fig. **10b**), suggesting that *Trichoderma* 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. **11**) were also highly induced in the presence of *Trichoderma* in

low N-fed plants suggesting that *Trichoderma* mediated ROS, NO along with increased N 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 to be essential for local and systemic resistance (Delaney *et al.* 1994). *PAL1* levels were increased by *Trichoderma* is very intriguing. SAR response is associated with a specific set of SAR genes encoding pathogenesis related (PR) proteins (Pieterse *et al.* 1996). *PR* gene activates and accumulates during SAR (Fig. **11b,c,d**) which correlated with findings of Brotman *et al.* (2012) and Pieterse *et al.* (1996).

In our study, SAR regulatory genes *DIR1*, *NPR1* and *TGA3* are induced in the presence of *Trichoderma* under low N stress. The *npr1* mutant compromised in LAR and SAR in the presence of *Trichoderma* suggesting the role of this gene in enhancing defense. In *nahG* mutant, defense responses are slightly enhanced in the presence of *Trichoderma* despite of reduced SA levels, suggesting that apart from SA other factors such as increased NO, ROS probably responsible for defense in this mutant. Overall, this study proved that optimum N is required for LAR and SAR. *Trichoderma* can enhance N uptake via modulating N transporters via eliciting short term NO under low nitrate nutrition. The enhanced N uptake plays a role in enhancing SA levels and defense gene expression in local and distal levels to increase overall plant defense. These defense responses are not activated in *npr1, nahG, nrt2.1, Hb*$^{+}$ and *nia1,2* mutant providing strong evidence that *Trichoderma* mediated enhanced resistance under low nitrate involves synergistic roles of NO, ROS and SA.

Material and Methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia 0 (*Col0*; WT) was sown in plastic pots (10 cm diameter) containing soilrite: agropeat (1:1) mix (mix contains zero N) and stratified at 4°C in dark for 48 h. Then, the pots were kept in 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

solution and once with water. Then, 0.1 and 3 mM NO_3^- concentrations were given 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). 30-35 day old plants with fully developed rosette 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 poured with 10 ml of sterile water. The spores were evaluated up to $1\square10^9$ spores/ml. It was thoroughly mixed into the soilrite mix and distributed into the individual plastic pots.

Bacterial strain and growth conditions

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. The avirulent bacterial density was adjusted to 2×10^7 CFU ml^{-1} for primary inoculations and virulent *P. syringae* was used at 2×10^6 CFU ml^{-1} for challenge inoculations.

Bacterial infiltration in leaves

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. There were total four groups of plants; Group 1: 0.1 mM + $MgCl_2$ plants, Group 2: 3 mM + $MgCl_2$ plants, Group 3: 0.1 mM + *Pst* plants and Group 4: 3 mM + *Pst* plants. Each group consisted of 20-25 plants. SAR experiment initiated with primary inoculation (a*vrRpm1 Pst* DC3000) on one leaf per plant, and the secondary challenge inoculation with (virulent *P. syringae*) on 4 other (distant) leaves per plant, leaving 5-6 healthy un-inoculated leaves per plant (Cameron *et al*. 1999) after 2 days of primary inoculation. The Inoculated (I) and un-inoculated (U) leaves from each treatment were harvested (Fig. **S1**) for RNA extraction and other experiments post challenge inoculation. Leaves were analyzed for 0, 6, 24 and 48 hours post inoculation (hpi) or challenge (hpc) and the symptoms was determined periodically.

Electrolyte leakage

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

In planta bacterial number quantification assay

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

Expression profiling by qRT-PCR

The inoculated and uninoculated leaves were immediately frozen in liquid nitrogen and stored at -80°C. RNA extraction, cDNA synthesis and qPCR was performed according to Wany *et al.* (2017; 2018). The synthesized cDNAs were used as templates in qRT-PCRs using primers given in Table S1. Fold change in the target genes was normalized to *Arabidopsis* reference genes; ubiquitin, *18sRNA* (GQ380689) and *YSL8* gene (X69885.1). Fold expression relative to control treatment was determined by ΔΔCT values. Three biological experiments (with three independent replicates for each experiment) were performed for each treatment. The comparisons of the gene expression between the different treatments to control was performed by student's t-test. Differences between the treatments with $p<0.05$, $p<0.01$ and $p<0.001$ were considered significant.

Ex- vivo NO estimation

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

Determination of ROS levels

Production of hydrogen peroxide (H_2O_2) in inoculated and un-inoculated leaves was detected by Diaminobenzidine tetrahydrochloride (DAB) staining as per Daudi *et al*. (2012) with slight modifications. The superoxide levels were measured by *in vivo* staining with Nitroblue tetrazolium chloride (NBT, SA, USA) (Jambunathan 2010).

Histochemical detection of HR

Hypersensitive cell death in inoculated leaves was visualized by trypan 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).

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Author contribution

KJG supervised, designed the project and wrote the manuscript. AW designed, performed experiments and analysed whole data. PKP performed SA estimation.

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Figures

Fig 1

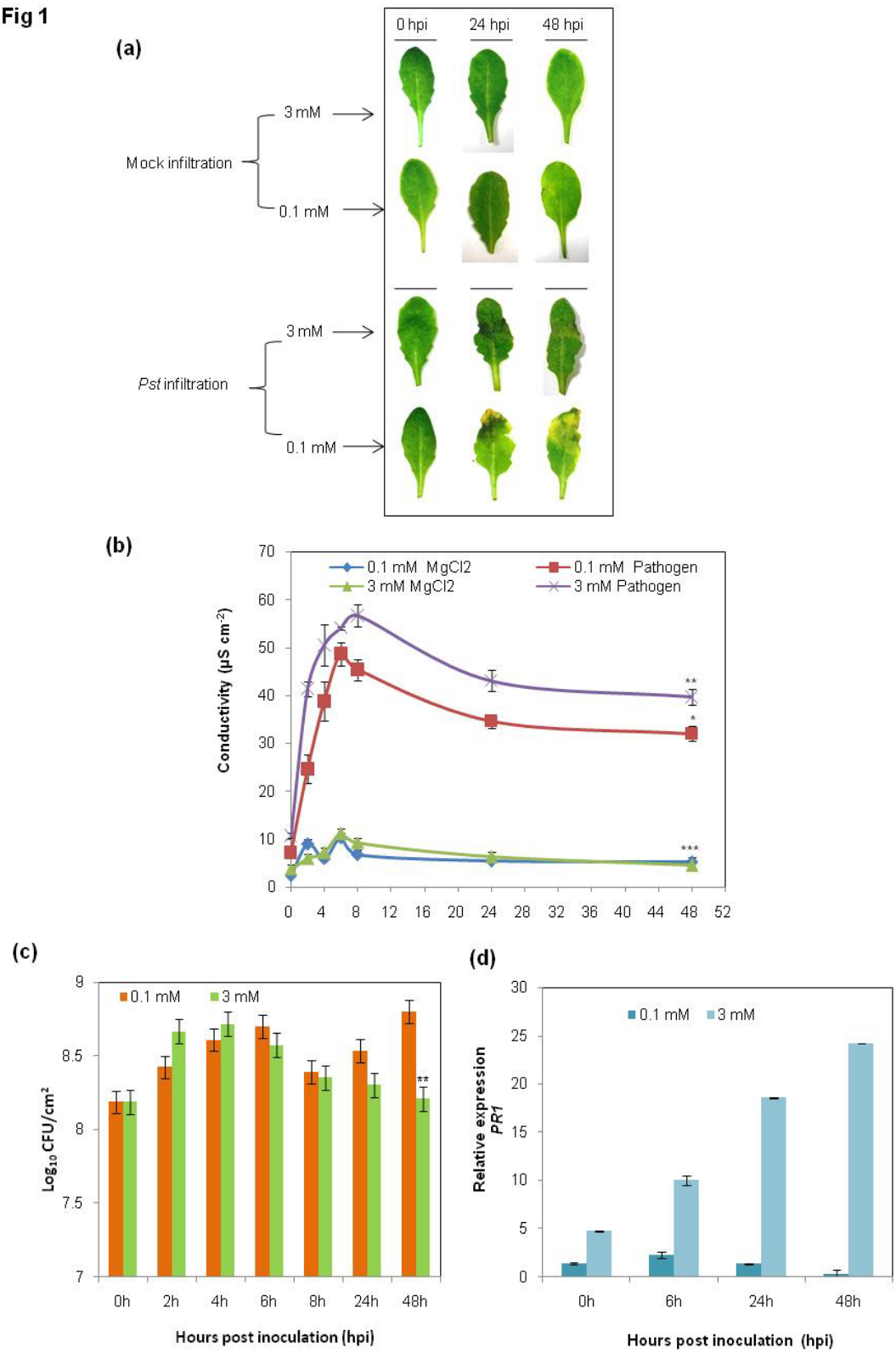


Fig. 1: **LAR response elicited by *Pst*DC3000/*avrRpm1* is compromised in low nitrate grown WT plants**

(a) *Pst*DC3000-*avrRpm1* mediated HR observed in 3mM and 0.1 mM NO_3^- grown WT plants at different time points. **(b)** Electrolyte leakage from leaf areas of 0.1 mM and 3 mM NO_3^- plants infiltrated with *Pst* and 10 mM $MgCl_2$. Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at $p< 0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3 mM $MgCl_2$ as control. **(c)** Bacterial number in log CFU in *Pst* infiltrated leaves of 0.1 mM and 3 mM NO_3^- plants immediately after infiltration (0 hpi). Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3 mM NO_3^- as control. **(d)** Relative *PR1* transcript levels from 0.1 and 3 mM *Pst* treated leaves at different time points. Data are mean values ± SE. Asterisks indicate statistical significance between 0.1 mM and 3 mM NO_3^- plants is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**). All the results obtained are representative of three independent experiments.

Fig. 2

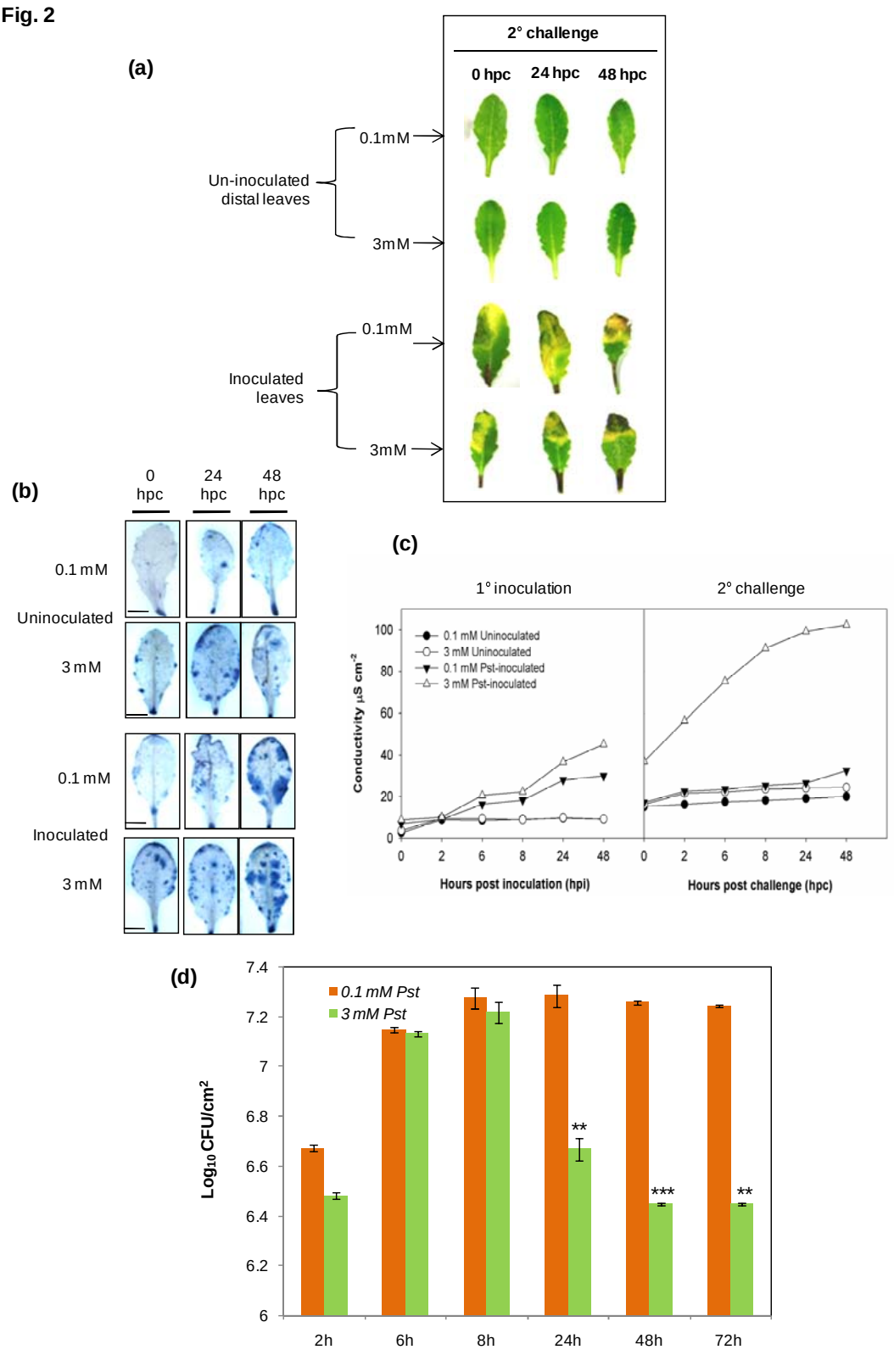

(a)
2° challenge
0 hpc
24 hpc
48 hpc
0.1mM
Un-inoculated distal leaves
3mM
0.1mM
Inoculated leaves
3mM
(b)
0 hpc
24 hpc
48 hpc
0.1 mM
Uninoculated
3 mM
0.1 mM
Inoculated
3 mM
(c)
1° inoculation
2° challenge
0.1 mM Uninoculated
3 mM Uninoculated
0.1 mM Pst-inoculated
3 mM Pst-inoculated
Conductivity µS cm-2
Hours post inoculation (hpi)
Hours post challenge (hpc)
(d)
0.1 mM Pst
3 mM Pst
Log10 CFU/cm2
2h
6h
8h
24h
48h
72h

Fig. 2: **Low nitrate grown WT plants show susceptible systemic responses during SAR**

(a) Lesion development post secondary challenge in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- plants. A characteristic lesion pattern in inoculated leaves of both 0.1 and 3 mM NO_3^- plants is noted **(b)** Histochemical staining for the detection of HR- mediated cell death post secondary challenge in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- plants. Scale bar-1 mm **(c)** Electrolyte leakage observed post primary (first panel) inoculation and secondary challenge (second panel) in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- plants. A significant difference between all treatments is analyzed by t-test at $p<0.05$ (*) with 3 mM WT $MgCl_2$ as control **(d)** Bacterial number in log CFU observed post secondary challenge in inoculated leaves of 0.1 mM and 3 mM NO_3^- plants. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*).

907 **Fig. 3**

908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928

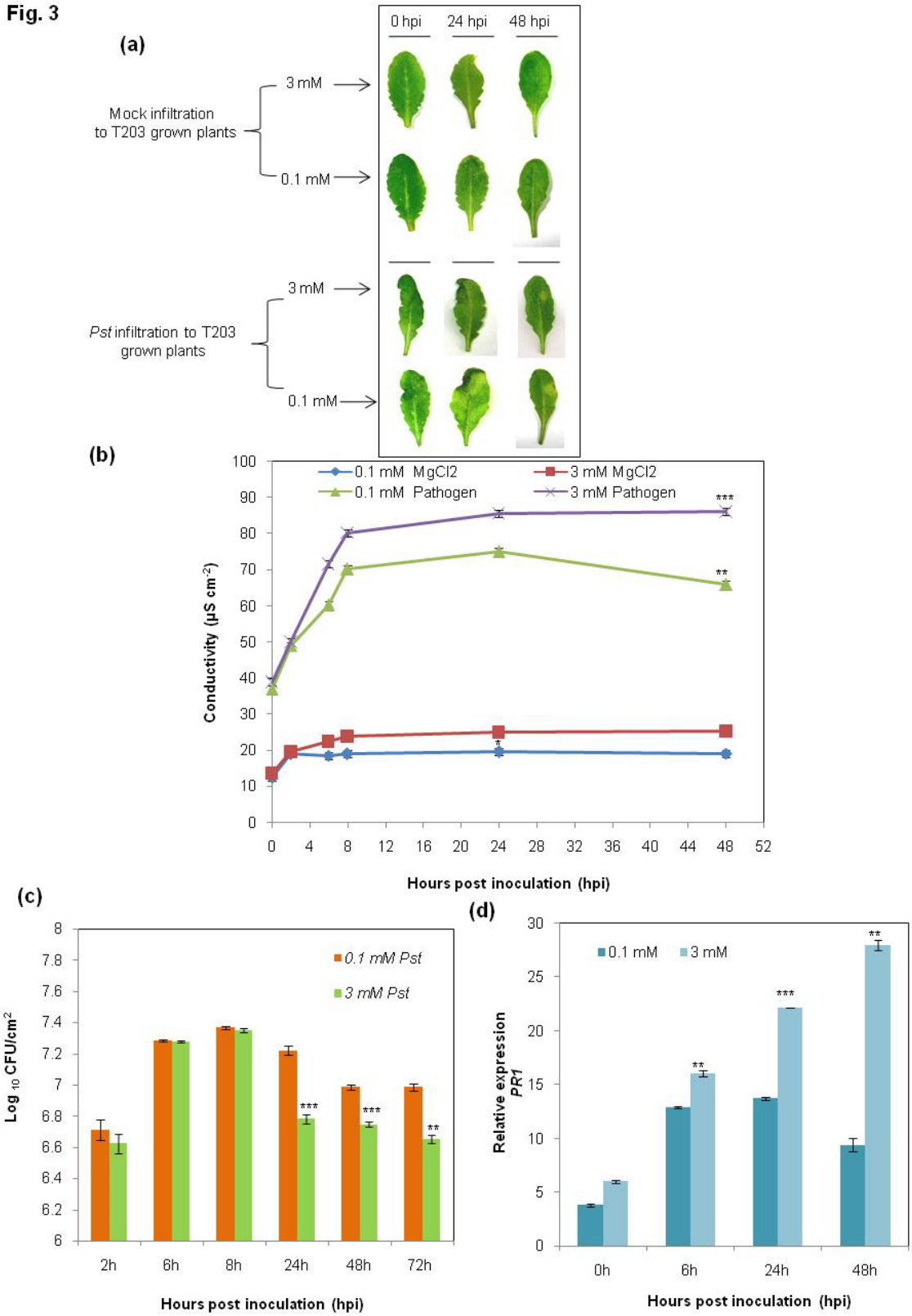


Fig. 3: ***Trichoderma* supplementation enhances LAR response in low nitrate grown WT plants**

(a) *Pst*DC3000-*avrRpm1* induced HR in 0.1 and 3 mM WT plants at different time points after *Trichoderma* (*T203*) supplementation. The images are representative of three independent experiments. First panel shows the mock infiltrated leaves i.e. control leaves treated with 10 mM $MgCl_2$ and second panel shows *Pst* infiltrated leaves of 0.1 and 3 mM NO_3^- concentrations **(b)** Electrolyte leakage from 0.1 mM and 3 mM WT + *T203* treated plants infiltrated with *Pst* and 10 mM $MgCl_2$. Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***) and $p<0.01$ (**) with 3 mM WT-$MgCl_2$ as control **(c)** Bacterial number in terms of log CFU in *Pst* infiltrated leaves of *T203* treated 0.1 mM and 3 mM NO_3^- WT plants. Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) **(d)** Relative *PR1* transcript levels from 0.1 and 3 mM *Pst* treated leaves of *T203* treated WT plants at different time points (hpi). Data are mean values ± SE. Asterisks indicate statistical significance between 0.1 mM and 3 mM plants is analyzed by t-test $p<0.001$ (***) and $p<0.01$ (**), by taking 3 mM plants as control.

Fig.4

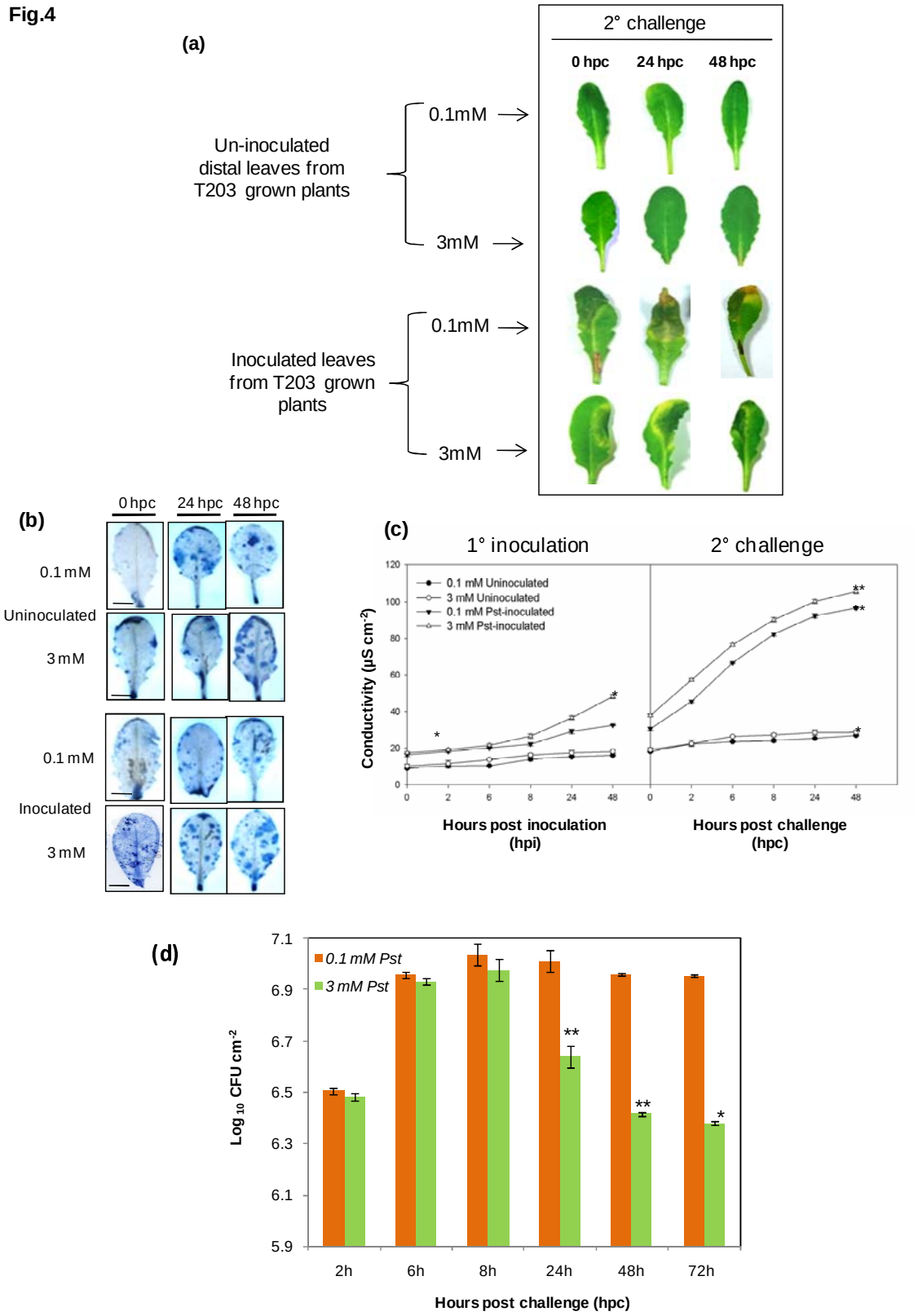

(a)
2° challenge
0 hpc
24 hpc
48 hpc
Un-inoculated distal leaves from T203 grown plants
0.1mM
3mM
Inoculated leaves from T203 grown plants
0.1mM
3mM
(b)
0 hpc
24 hpc
48 hpc
0.1 mM
Uninoculated
3 mM
0.1 mM
Inoculated
3 mM
(c)
1° inoculation
2° challenge
0.1 mM Uninoculated
3 mM Uninoculated
0.1 mM Pst-inoculated
3 mM Pst-inoculated
Conductivity (µS cm-2)
Hours post inoculation (hpi)
Hours post challenge (hpc)
(d)
0.1 mM Pst
3 mM Pst
Log10 CFU cm-2
2h
6h
8h
24h
48h
72h
Hours post challenge (hpc)

Fig. 4: ***Trichoderma* enhances SAR response in low nitrate plants**

(a) HR development post secondary challenge in inoculated and uninoculated leaves of 0.1 mM and 3 mM *T203* treated WT plants. **(b)** Histochemical staining for the detection of HR-mediated cell death post secondary challenge in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- , *T203* treated WT plants. Scale bar-1 mm **(c)** Electrolyte leakage observed post primary (first panel) inoculation and secondary challenge (second panel) in inoculated and uninoculated leaves of 0.1 mM and 3 mM *T203* treated WT plants. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3 mM WT $MgCl_2$ as control **(d)** Bacterial number in log CFU observed post secondary challenge in inoculated leaves of 0.1 mM and 3 mM, *T203* treated WT plants. A significant difference between 0.1 and 3 mM NO_3^- is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*).

Fig. 5

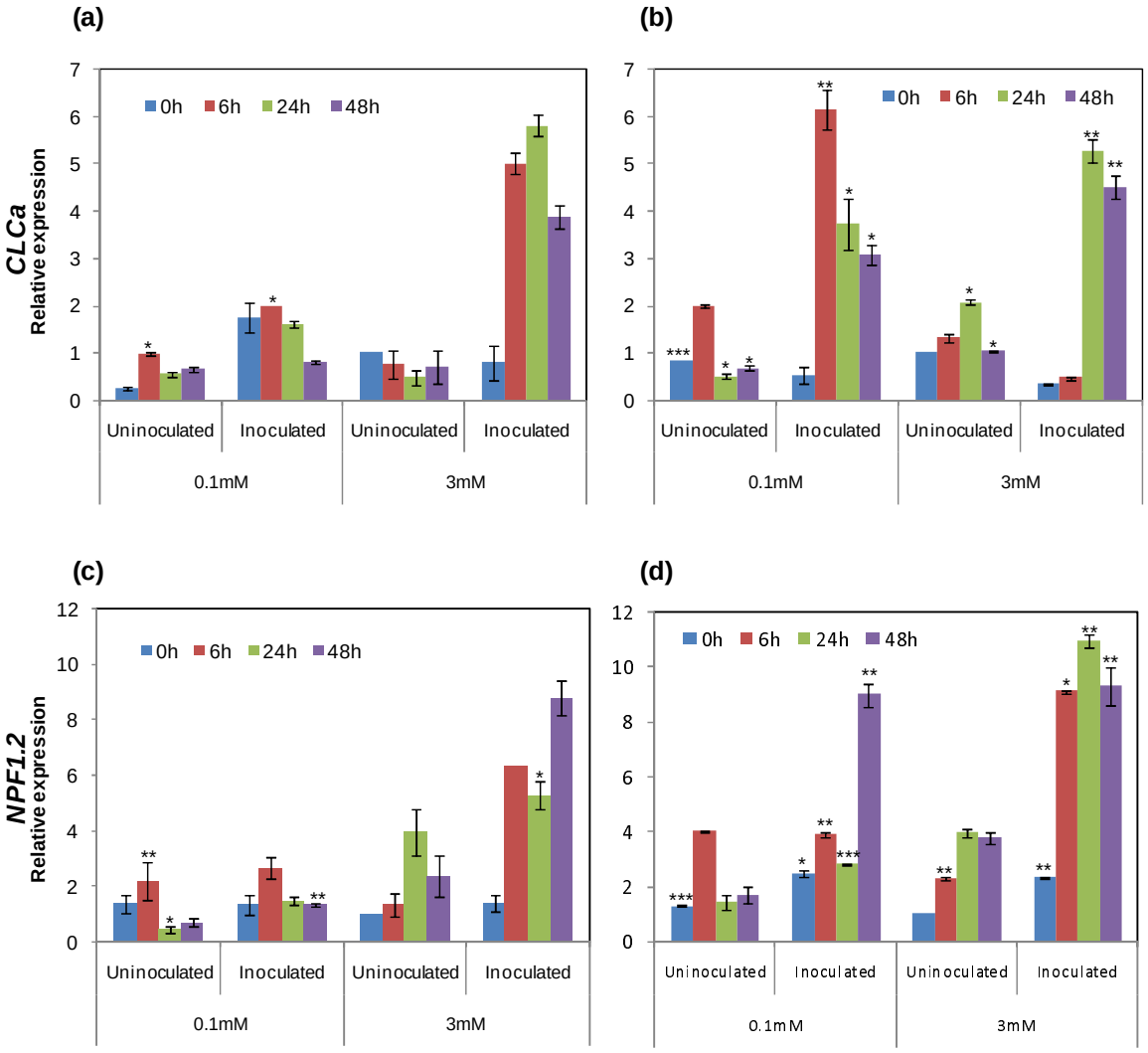
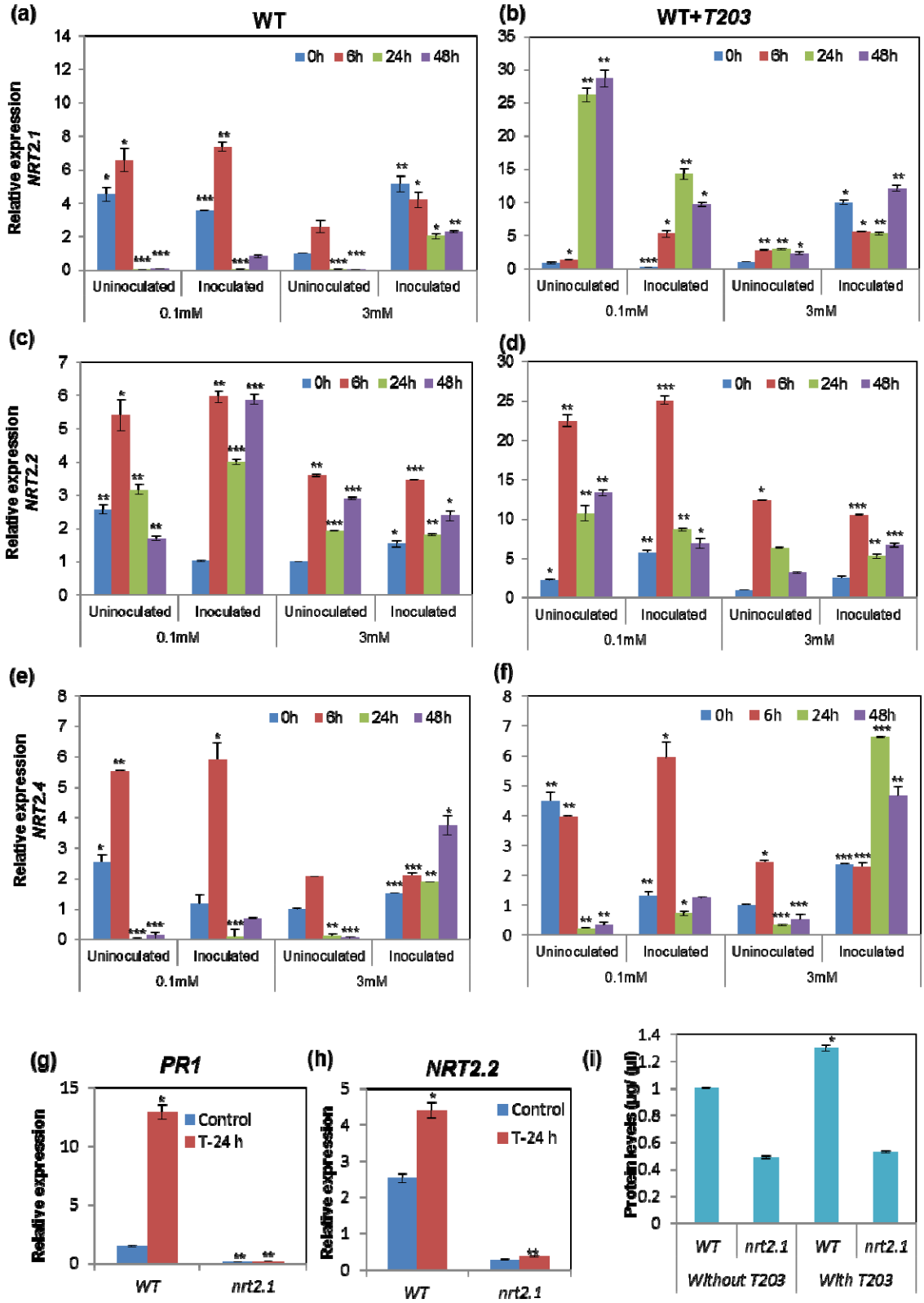


Fig. 5: **Expression profile of low affinity nitrate transporter genes (LATs) during SAR**

Relative expression of *CLCa* gene in **(a)** WT **(b)** WT+*T203* and *NPF1.2* gene in **(c)** WT **(d)** WT+*T203* grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and un-inoculated leaves. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3 mM un-inoculated leaves as control in each gene.

Fig 6


(a)
WT
(b)
WT+T203
0h 6h 24h 48h
Relative expression NRT2.1
Relative expression NRT2.2
Relative expression NRT2.4
Uninoculated
Inoculated
0.1mM
3mM
(c)
(d)
(e)
(f)
(g)
PR1
Control
T-24 h
Relative expression
WT
nrt2.1
(h)
NRT2.2
(i)
Protein levels (µg/ µl)
Without T203
With T203

1048

Fig. 6: 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 *NRT2.1* in **(a)** WT (I panel) and **(b)** WT+*T203* (II panel), *NRT2.2* in **(c)** WT (I panel) and **(d)** WT+*T203* (II panel) and *NRT2.4* in **(e)** WT (I panel) and **(f)** WT+*T203* (II panel) grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and un-inoculated leaves. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3 mM un-inoculated leaves as control in each gene **(g)** Relative *PR1* expression in roots of WT and *nrt2.1* plants grown under 0.1 mM NO_3^- concentration (with and without *T203* treatment; 24 h). A significant difference between with and without *T203* treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*) **(h)** Relative *NRT2.2* expression in roots of WT and *nrt2.1* plants grown under 0.1 mM NO_3^- concentration (with and without *T203* treatment; 24 h) **(i)** Protein levels in WT and *nrt2.1* plants grown under 0.1 mM NO_3^- concentration in the presence or absence of *T203*. A significant difference between with and without *T203* treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*).

Fig. 7

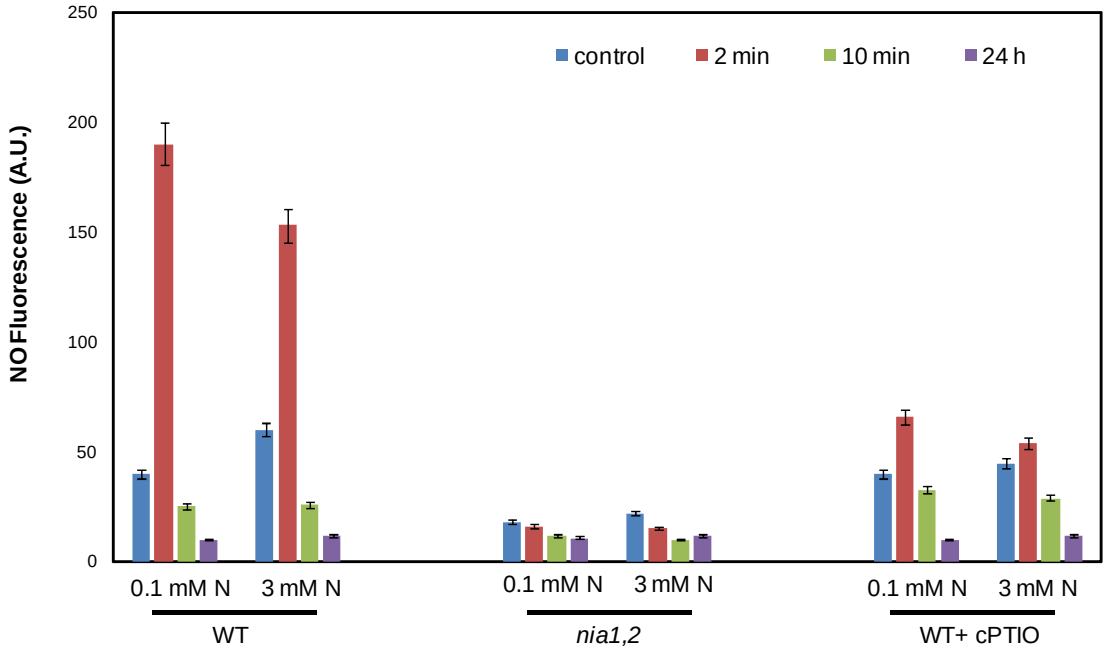


Fig. 7: Visualization of nitric oxide by diaminofluorescein (DAF) fluorescence Nitric oxide estimation by diaminofluorescein (DAF-FM) fluorescence under 0.1 and 3 mM NO_3^- concentrations in I. WT, II. *nia1,2* and III. cPTIO (100 µM) grown WT plants during different periods of *Trichoderma* inoculation. The experiment was performed three times independently with similar results.

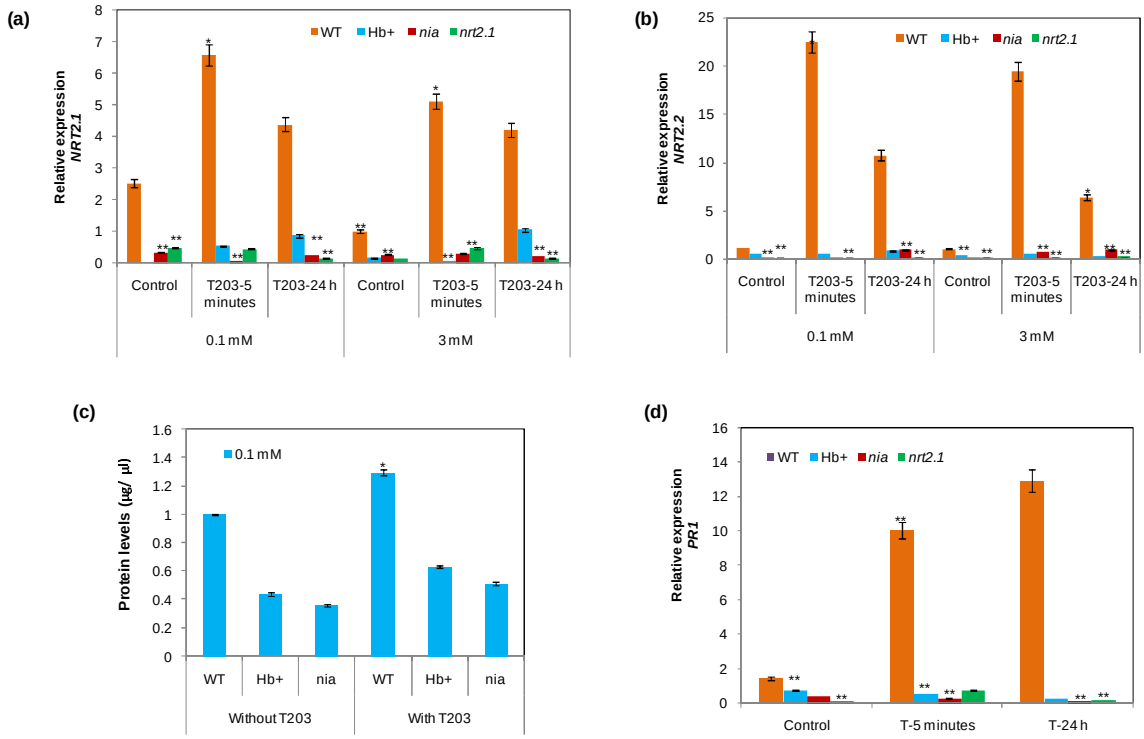


Fig. 8: Response of the genotypes during early stages of *Trichoderma* inoculation on *NRT2.1, 2.2* expression, protein levels and *PR1* expression

(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^- concentrations, with and without *T203* treatment, given for 5 minutes and 24 h to the plants. A significant difference between with and without *T203* treatment is analyzed by t-test at p<0.01 (**) and 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^-concentrations, with and without *T203* treatment, given for 5 minutes and 24 h to the plants. A significant difference between with and without *T203* treatment is analyzed by t-test at p<0.01 (**) and 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. *T203* treatment was given for 24 h to the plants. A significant difference between with and without *T203* treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*) **(d)** *PR1* gene expression in WT, Hb+, *nia1,2* and *nrt2.1* under 0.1 mM NO_3^- plants, with and without *Trichoderma*

treatment given for 5 minutes and 24 h to the plants. A significant difference between with and without *T203* treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*).

Fig. 9

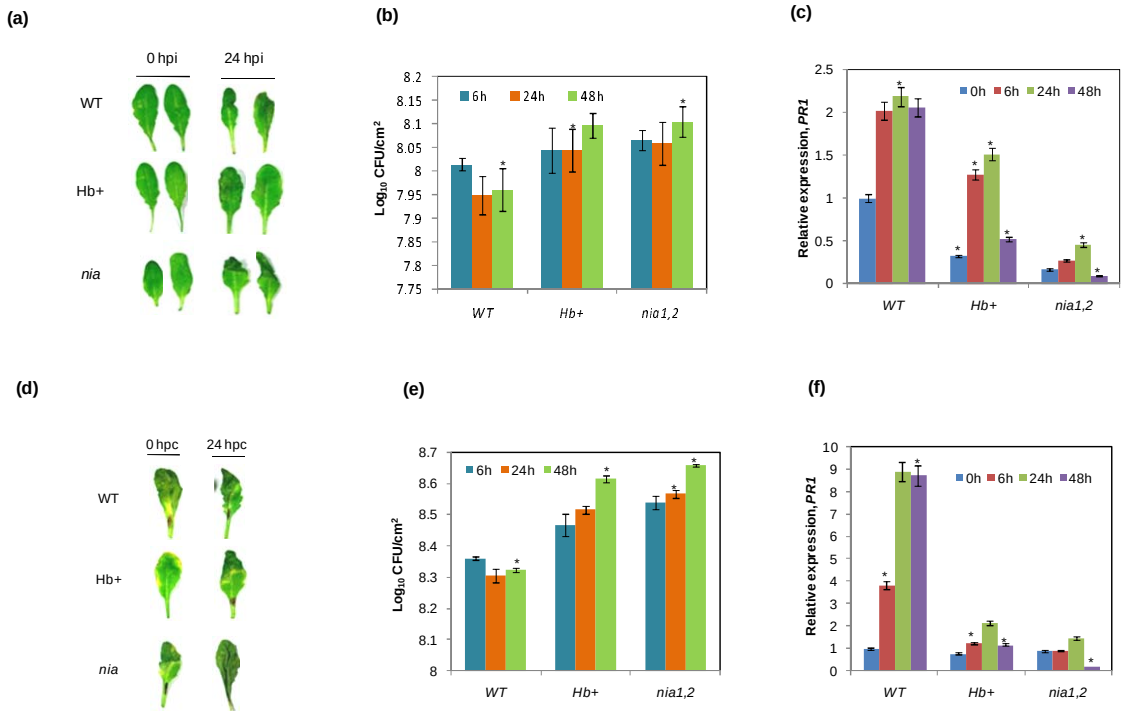


Fig. 9: Response of WT and NO mutants (Hb$^+$ and *nia1,2*) grown under 0.1 mM NO_3^- during LAR and SAR

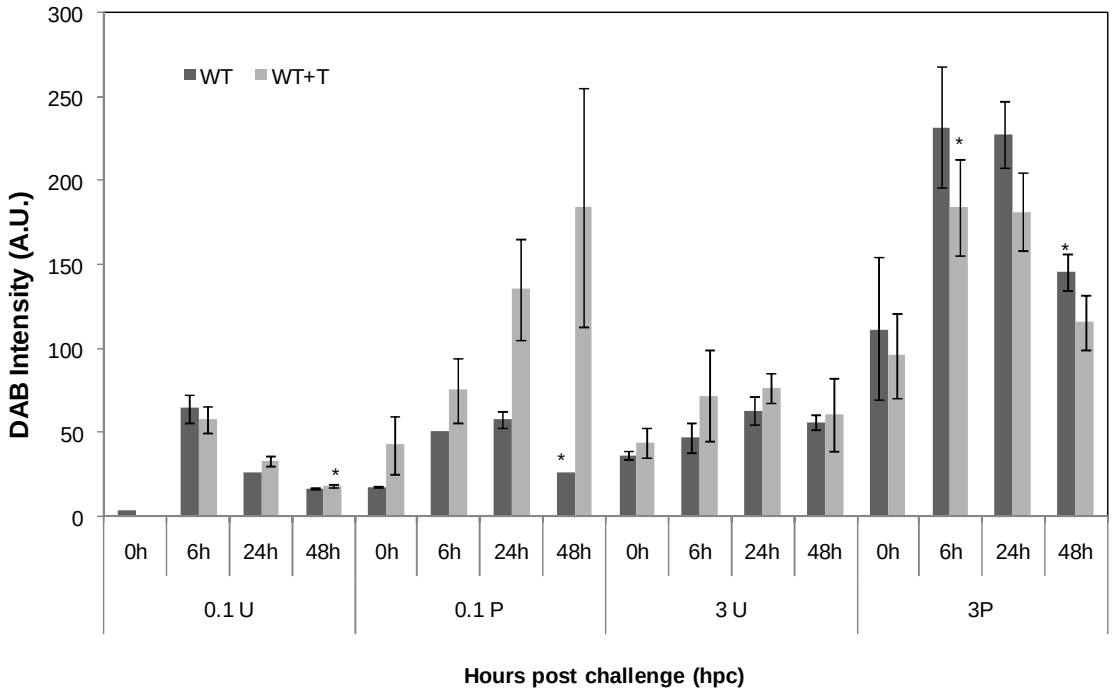
In LAR response **(a)** HR phenotype in inoculated leaves of WT, Hb$^+$ and *nia1,2* plants grown under 0.1 mM NO_3^- concentration at 0 and 24 hpi **(b)** *In planta* bacterial growth (log CFU) at 6, 24 and 48 hpi, asterisks indicate a significant difference (p<0.01; Student's t-test) taking WT as control **(c)** Relative *PR1* expression in infiltrated leaves during localized infiltration. Values are means (n=3) ± SE. Asterisks indicate a significant difference (p < 0.01; student's t-test) taking 0 hpi as control.

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 post secondary challenge **(e)** Bacterial population represented by log CFU at 6, 24 and 48 hpc, asterisks indicate a significant difference (p < 0.01; Student's t-test) taking 3 mM WT as control **(f)** Relative *PR1* gene expression in infiltrated leaves post secondary

challenge. Values are means (n=3) ± SE. Asterisks indicate a significant difference ($p < 0.01$; Student's t-test) taking 0 hpc as control.

Fig. 10

(a)



(b)

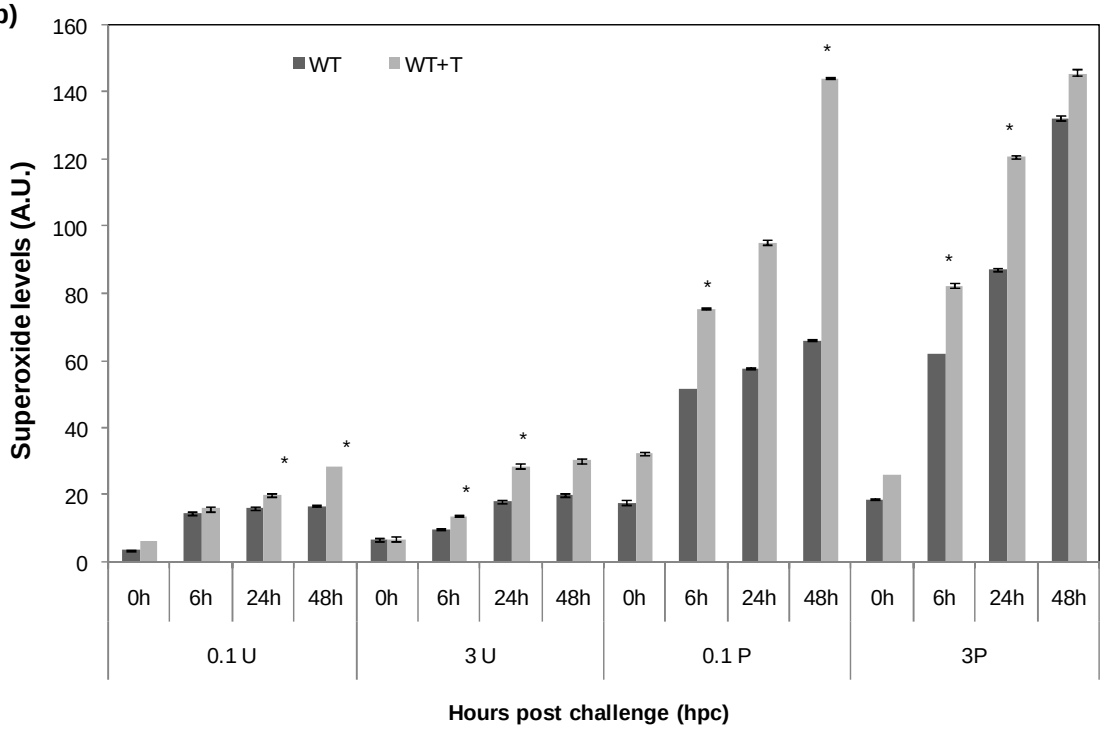


Fig. 10: Detection of ROS by measuring H_2O_2 and $O_2^{-\cdot}$ in 0.1 mM and 3 mM NO_3^- WT and *Trichoderma* grown WT plants during SAR response

(a) Quantification of DAB staining used to measure H_2O_2 levels in the inoculated leaves and uninoculated leaves after 3 days of 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.

(b) Quantification of NBT staining used to measure $O_2^{-\cdot}$ levels in the inoculated leaves and uninoculated 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.

Fig. 11

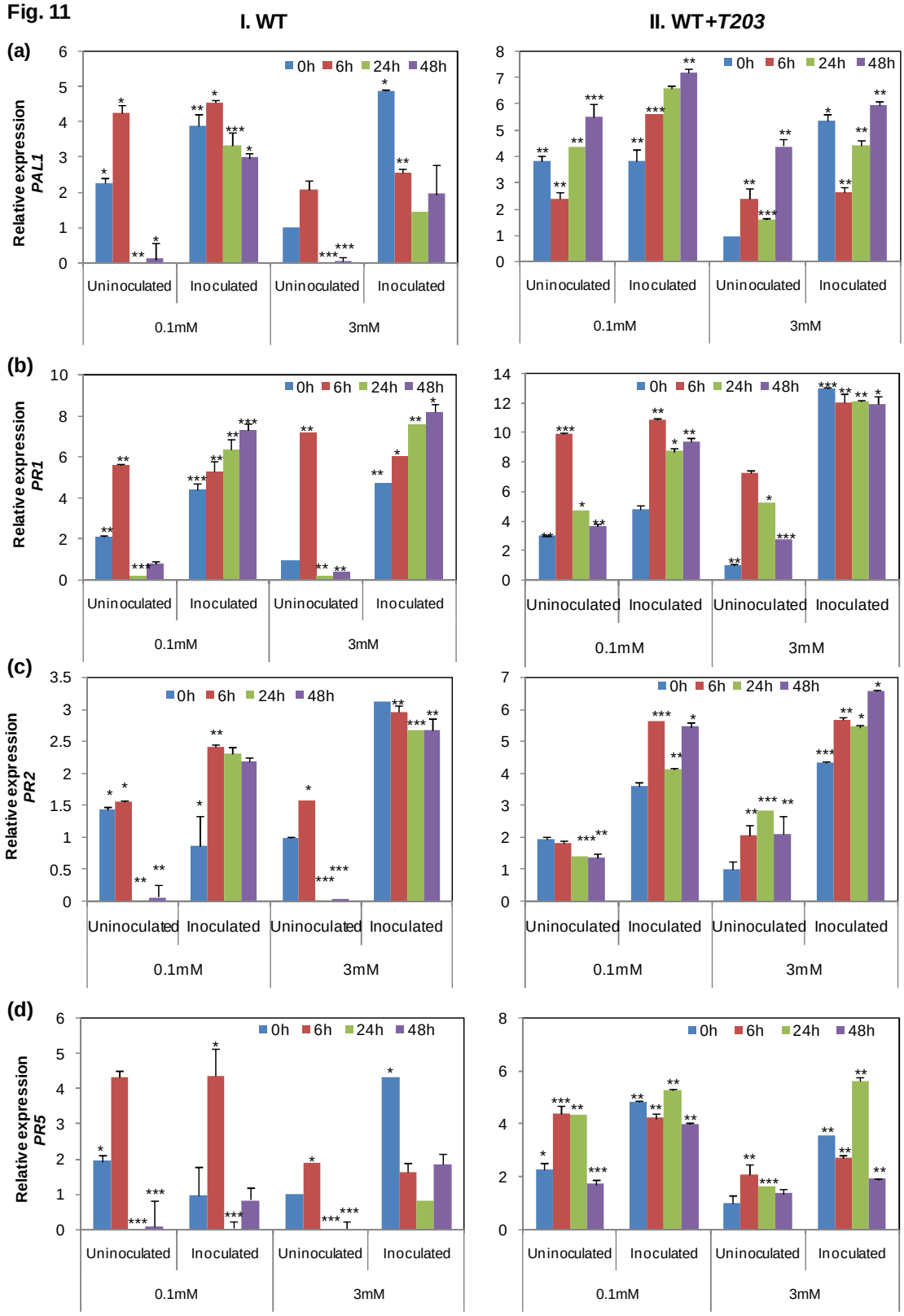

I. WT
II. WT+T203
(a)
Relative expression PAL1
(b)
Relative expression PR1
(c)
Relative expression PR2
(d)
Relative expression PR5
0h 6h 24h 48h
Uninoculated
Inoculated
0.1mM
3mM

Fig. 11: **Expression profiles of defense related genes during SAR**

Relative expression of defense related genes in WT (I panel) and WT+*T203* (II panel) grown under low (0.1 mM) and optimum (3 mM) NO_3^- concentration post secondary challenge of *Pst*DC3000 in both inoculated and uninoculated leaves **(a)** Relative expression of *PAL1* **(b)** Relative expression of *PR1* **(c)** Relative expression of *PR2* **(d)** Relative expression of *PR5*. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3 mM uninoculated leaves as control in each gene.

Fig. 12

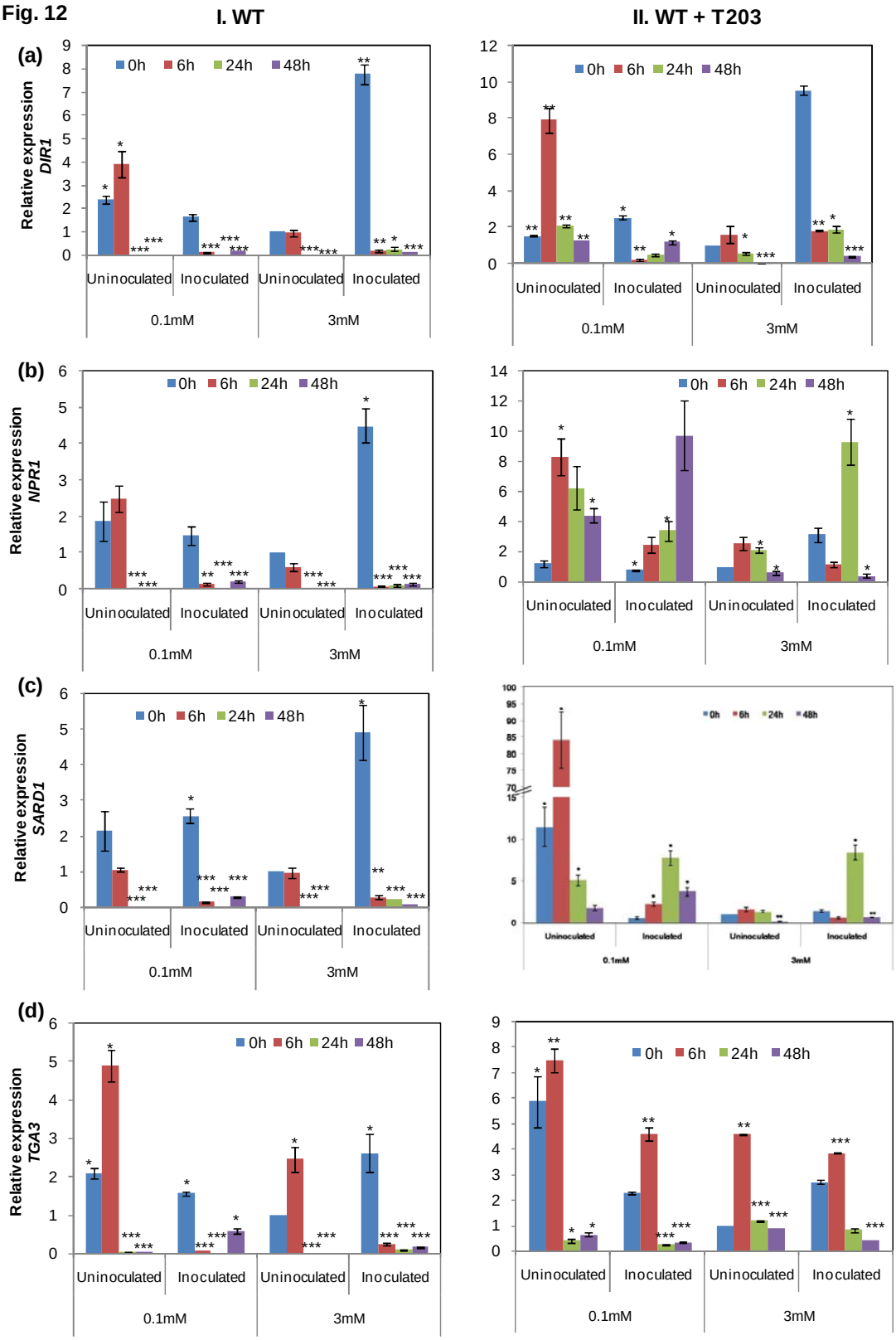

I. WT
II. WT + T203
(a)
(b)
(c)
(d)
Relative expression DIR1
Relative expression NPR1
Relative expression SARD1
Relative expression TGA3
0h 6h 24h 48h
Uninoculated
Inoculated
0.1mM
3mM

Fig. 12: **Expression profiles of regulatory SAR genes**

Relative expression of SAR regulatory gene in WT (I panel) and WT+*T203* (II panel) grown under low (0.1 mM) and optimum (3 mM) NO_3^- concentration post secondary challenge of *Pst*DC3000 in both inoculated and uninoculated leaves **(a)** Relative expression of *DIR1* **(b)** Relative expression of *NPR1* **(c)** Relative expression of *SARD1* **(d)** Relative expression of *TGA3*. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3mM uninoculated leaves as control in each gene.

Fig. 13

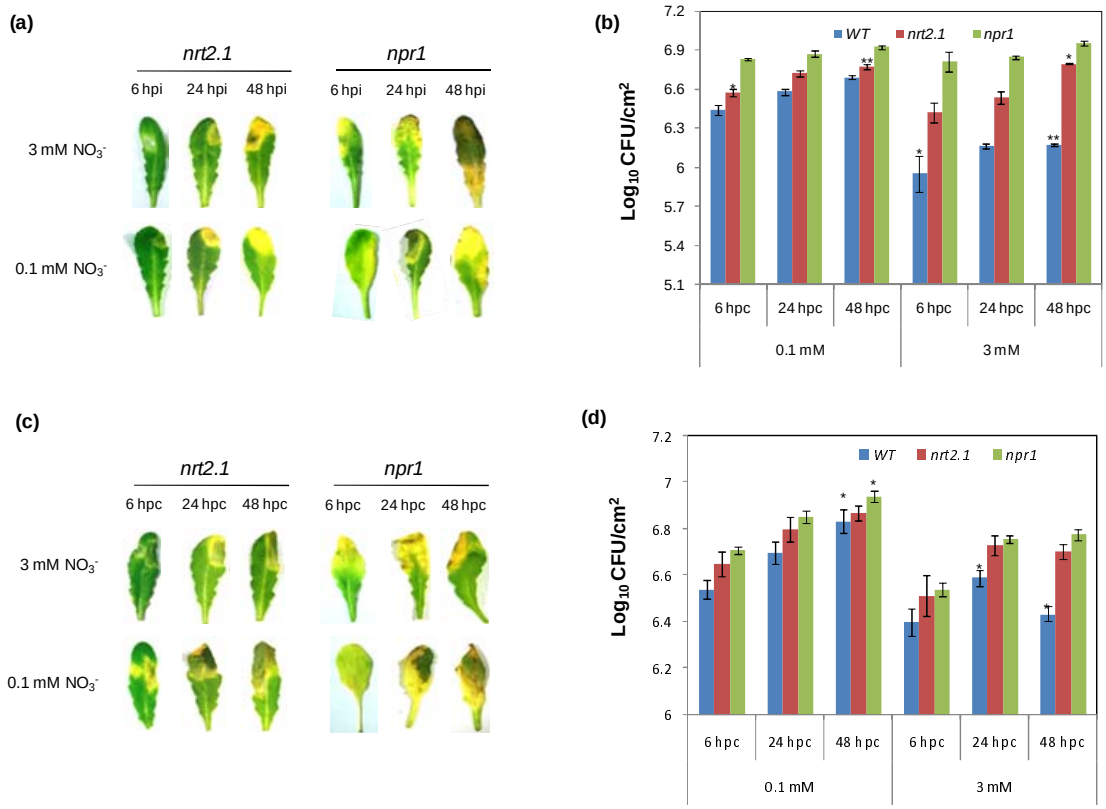


Fig. 13: **LAR and SAR response in *nrt2.1* and *npr1* mutants to virulent *Pst* DC3000**

(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. A significant difference between 0.1 and 3 mM nitrate is analyzed by t-test at p<0.01 (**) and p<0.05 (*).

Fig. 14

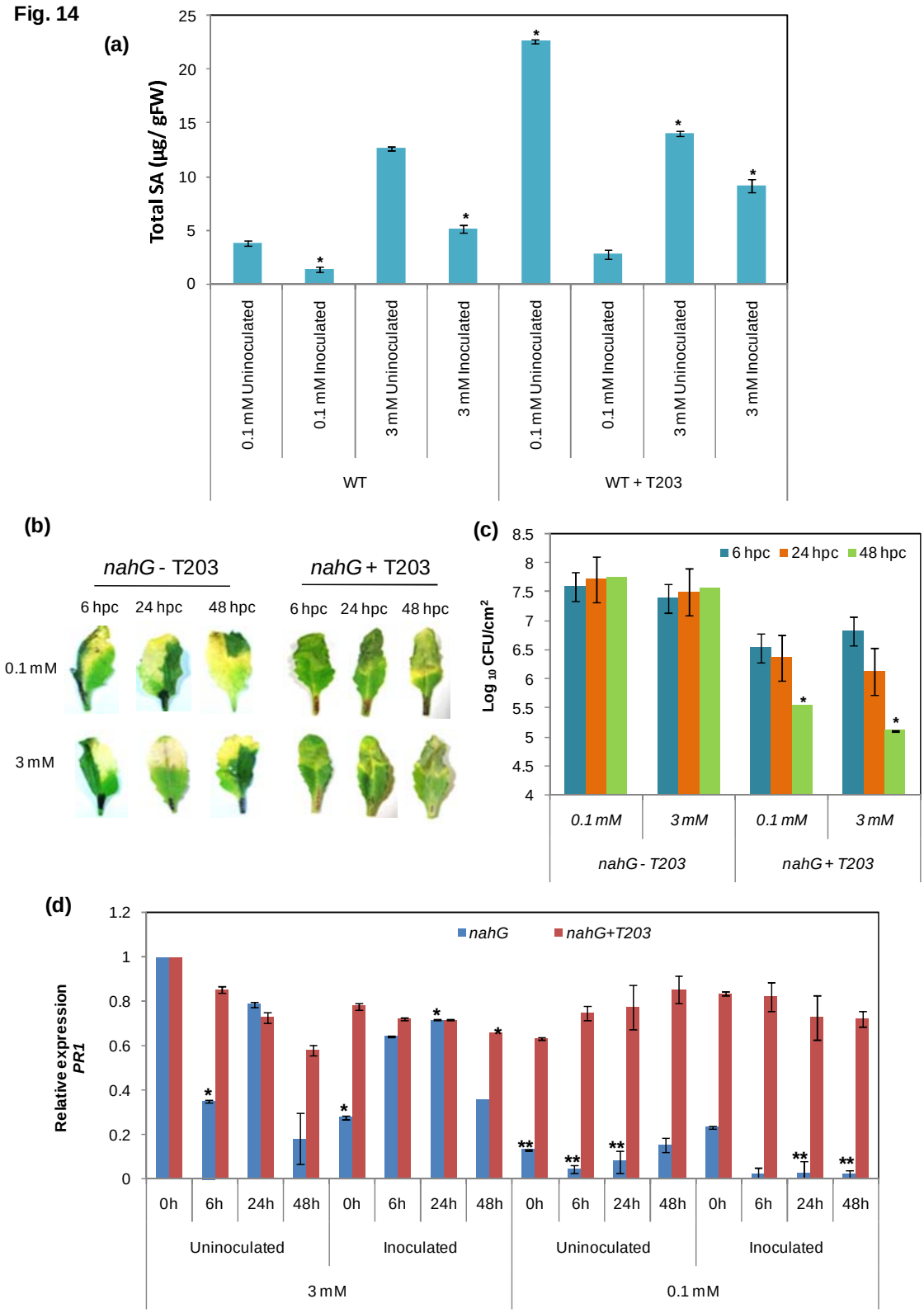


Fig. 14: **SA Accumulation during SAR, and response of *nahg* mutant in the presence and absence of *Trichoderma* during SAR**

(a) Total SA levels (µg g^{-1} fresh weight) were determined post challenge inoculation (24 hpc) in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- fed WT plants, with and without *Trichoderma*. Asterisks indicate mean values that are significantly different according to ($p<0.01$; Student's t-test) with 3 mM uninoculated leaves as control **(b)** HR phenotype in inoculated and un-inoculated distal leaves of *nahg* mutants grown under 0.1 and 3 mM NO_3^- 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 NO_3^- with and without *Trichoderma* treatment post secondary challenge. Values are means (n=3) ± SE. A significant difference between with and without *T203* treatment is analyzed by t-test at $p<0.01$ (**) and $p<0.05$ (*).

Fig. 15

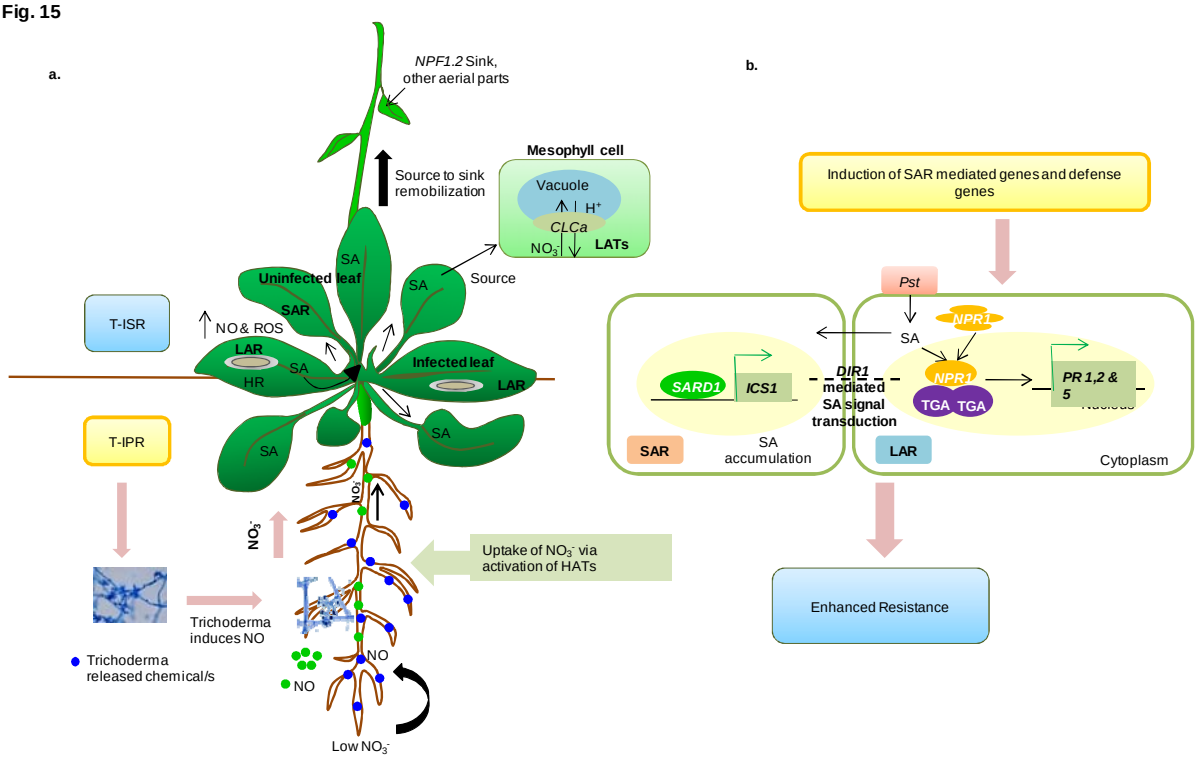


Fig. 15: A model depicting mechanism of *Trichoderma* induced systemic response (T-ISR) under low nitrate conditions during pathogen infection in Arabidopsis.

(a) After root colonization, *Trichoderma* secretes growth promoting elicitors (blue dots), and induces priming response (T-IPR) by eliciting short term nitric oxide (NO; green dots) which facilitate faster nitrate uptake by activating HATs (*NRT2.1, 2.2* and *2.4*) in the roots. The HATs in turn activates the vacuolar LATs (*CLCa and NPF1.2*) in the mesophyll cells allows source to sink re-mobilization of available nitrate from roots to other aerial parts. During primary inoculation (local pathogen attack; *avrRpm1*), NO and ROS signals both are produced during hypersensitive response (HR), along with salicylic acid (SA), but their basal levels are greatly enhanced due to *Trichoderma* treatment. Moreover, the *Trichoderma* induced short-term NO produced during priming and HR, plays a role in faster nitrate uptake by the HATs in low N stress plants. *Trichoderma* induced SA (produced during local infection) gets rapidly translocated to the other uninfected distal parts of the plant and pre-programs the stressed plant for subsequent pathogen attack.

(b) *Trichoderma* activates PR proteins and cause SA accumulation in locally infected 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.