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Trichoderma asperellum T42 induces local defense against *Xanthomonas oryzae* pv. *oryzae* under nitrate and ammonium nutrients in tobacco†

Bansh Narayan Singh,[ab] Padmanabh Dwivedi,*[b] Birinchi Kumar Sarma[c] and Harikesh Bahadur Singh[c]

Trichoderma has been explored and found to play a vital role in the defense mechanism of plants. However, its effects on host disease management in the presence of N nutrients remains elusive. The present study aimed to assess the latent effects of *Trichoderma asperellum* T42 on oxidative burst-mediated defense mechanisms against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in tobacco plants fed 10 mM NO_3^- and 3 mM NH_4^+ nutrients. The nitrate-fed tobacco plants displayed an increased HR when *Xoo* infected, which was enhanced in the *Trichoderma*-treated plants. This mechanism was enhanced by the involvement of *Trichoderma*, which elicited NO production and enhanced the expression pattern of NO-modulating genes (*NR*, *NOA* and *ARC*). The real-time NO fluorescence intensity was alleviated in the NH_4^+-fed tobacco plants compared to that fed NO_3^- nutrient, suggesting the significant role of *Trichoderma*-elicited NO. The nitrite content and NR activity demonstration further confirmed that nitrate metabolism led to NO generation. The production of ROS (H_2O_2) in the plant leaves well-corroborated that the disease resistance was mediated through the oxidative burst mechanism. Nitrate application resulted in greater ROS production compared to NH_4^+ nutrient after *Xoo* infection at 12 h post-infection (hpi). Additionally, the mechanism of enhanced plant defense under NO_3^- and NH_4^+ nutrients mediated by *Trichoderma* involved NO, ROS production and induction of *PR1a MEK3* and antioxidant enzyme transcription level. Moreover, the use of sodium nitroprusside (100 μM) with *Xoo* suspension in the leaves matched the disease resistance mediated *via* NO burst. Altogether, this study provides novel insights into the fundamental mechanism behind the role of *Trichoderma* in the activation of plant defense against non-host pathogens under N nutrients.

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1. Introduction

Nitrogen (N) is an essential component for plant metabolism. It is the main component of chlorophyll, nucleic acid, and amino acid biosynthesis. Nitrogen plays a crucial role in plant development and disease management.[1–6] The primary issue of N scarcity in soil is due to its slow mineralization, leaching, and de-nitrification process. Accordingly, a lack of N availability to plants results in several physiological and morphological responses.[7] Thus, to minimize N deficiency, plants have a unique mechanism through which they overcome the problem of low N. Specifically, different transporters residing on the surface of the roots of plants can enhance the nitrogen uptake efficiency. These transporters have different affinities and subclasses for nitrate (NO_3^-) and ammonium (NH_4^+) uptake.[5] N is applied in agricultural soil in the form of nitrate (NO_3^-), ammonium (NH_4^+) or combined. These forms of N activate several defense mechanisms in plant systems against pathogens.[1,2] These defense mechanisms are associated with the involvement of PAMP or ETI (effector-triggered immunity), which reprograms different nuclear genes,[8,9] and thus N is required for enhancing plant immunity.[6] The PAMP defense response involving the rapid development of plant immunity in response to pathogen infections is referred to as the hypersensitive response (HR), which seems to appear after avirulent pathogen and plant interactions.[10] A characteristic of this local HR is the rapid generation of reactive oxygen species (O_2^- and H_2O_2) and nitric oxide (NO). N is an essential component that facilitates the HR response against avirulent pathogens.[1] However, N is not only involved in increasing the plant nutrient status, but its content affects disease defense. NO is a signaling molecule that was

[a]*Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi 221005, India*

[b]*Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221005, India. E-mail: pdwivedi25@rediffmail.com*

[c]*Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221005, India*

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observed to be rapidly generated during plant pathogen-interactions and considered one of the important components in defense activators.[11] The involvement of NO in the activation of defense signaling cascades has been well studied. The reactions of NO with cysteine-rich thiol groups and nitration of tyrosine-rich groups with different proteins are referred as *S*-nitrosylation, indicating the regulatory results mediated by NO.[12,13] The modification of the SA marker protein (NPR1) and ROS generating complex (RBOH) in *Arabidopsis* during defense responses are considered good examples of the role of *S*-nitrosylation.[14–16] In plants, RBOH production leads to localized ROS bursts to regulate plant development and immune responses under stress conditions. Induced plant immune responses are associated with the interaction of elicitors with the plant. Different response molecules and physiological changes occur during the interaction of elicitor molecules and plants.[17,18] The interaction of *Trichoderma* as an elicitor with plants stimulates various defense molecules such as NO, reactive oxygen species, SA, and genes related to defense. Moreover, *Trichoderma* may induce multiple secondary metabolites, auxin production and phenolic compounds, which may enhance the defense mechanisms in plants.[19–21]

Therefore, systems that can enhance the affinity for N uptake and promote disease resistance in plants are necessary. Several plant symbiotic microbes are well known as plant growth promoters, which facilitate nutrient availability to plants. However, the minimal use of these microbes in plant defense occurs in the presence of nitrate and ammonium or both supplements. Several species of *Trichoderma* have been demonstrated to play a key role in disease management against biotic stresses. They promote plant defense *via* various processes such as mycoparasitism, antibiosis, and activation of the basal defense mechanism. Colonization of *Trichoderma* within apoplastic cells led to an enhanced defense response in *Arabidopsis*[6,22] and pea[20] rapidly. Moreover, the colonization of *Trichoderma* with plant roots activated different defense-related enzyme activities and regulation of PR pathogenesis gene.[6,12] Perhaps, the activation of these defense responses is due to the modulation of metabolites and defense genes involved in the resistance mechanism.[20,23]

If *Trichoderma* initiates an enhanced N uptake efficiency[6] and NO accumulation,[12] it may be implicated in plant defense *via* HR-mediated cell death. *Trichoderma asperellum* T42 as a biological control agent and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were used in the present experiment. In the previous studies, the T42 strain was used as the most effective microbe in nitrogen utilization and root development in tobacco.[4] Herein, we aimed to assess the effects of two forms of N (NO_3^- and NH_4^+) nutrients on the defense response in tobacco plants *via* HR-mediated cell death against the non-host bacterial pathogen *Xoo* induced by the symbiotic rhizospheric fungus *Trichoderma asperellum* T42. We demonstrated that *Trichoderma asperellum* T42 promotes the oxidative burst response in tobacco grown under nitrate and ammonium nutrient media *via* the involvement of both NO and ROS.

2. Experimental setup

2.1 The growth of *Trichoderma asperellum* T42 and *Xanthomonas oryzae* pv. *oryzae* and inoculation preparation

T. asperellum (T42 strain; Gene Bank Accession: JN128894) was routinely cultured on potato dextrose agar (PDA) plates at 28 ± 2 °C for 6–7 days until sporulation. Before treatment with the T42 strain, the spores of the fungus were washed with 0.80% NaCl solution and the spore suspension was diluted to 1×10^6 CFU mL^{-1}. The *Xanthomonas oryzae* pv. *oryzae* strain was purchased from the Centre for Cellular & Molecular Biology (Hyderabad, India), and used as a non-host pathogen (incompatible) in tobacco plants. Before infiltration in the tobacco leaves, *Xoo* bacteria were grown for four days in peptone sugar agar (PSA) media. After incubation, the bacterial cell density was diluted to 1×10^7 CFU mL^{-1} to ensure each experiment contained an approximately accurate number of cells during infiltration.

2.2 Plant material and experimental setup conditions

Six-week-old tobacco plants (*Nicotiana tabacum* cv. Xanthi) were used in the experiment. Before two-week-old tobacco plantlets were transferred to Perspex tubes, sterilized tobacco seeds were primed with a spore suspension of T42 strain according to the previously described method,[4,24] and allowed to germinate without nitrogen supplements at 25 °C in the culture lab. After 2 weeks, the tobacco plants were transferred to Perspex tubes (capacity 1.8 L, 0.5 m high, and 15 cm inner diameter) containing a hydroponic solution for four weeks and provided continuous growth conditions (D/N, 16/8 h; temperature, 22 °C/20 °C; relative humidity, 70%; artificial light of photosynthetic photon flux density (PPDF), 350–400 μmol m^{-2} s^{-1}). The hydroponic culture solution for nitrate nutrient (pH 6.3) consisted of 10 mM KNO_3, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 μM NaFe-EDTA, 0.5 mM K_2HPO_4, and 1 mM KH_2PO_4, while for ammonium nutrition (pH 6.0–6.2), 3 mM NH_4Cl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 μM NaFe-EDTA, 0.5 mM K_2HPO_4, 1 mM KH_2PO_4 (ref. 4 and 25) and trace elements.[26] The nutrient solutions were supplemented to the growing plants and routinely replaced every two days. Distilled water used in place of the nitrogen supplement was treated as the control.

2.3 Infiltration of chemicals and bacterial strain in tobacco leaves

To study the modulation of endogenous NO in the HR response, 100 μM SNP (sodium nitroprusside) and 200 μM cPTIO (carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; Alexis Biochemicals) were infiltrated with *Xoo* suspension. Stock solutions of SNP and cPTIO were prepared in distilled water (Alexis Biochemicals). The suspension culture containing *Xoo* inoculum was prepared in 10 mM $MgCl_2$. There were four treatments: 10 mM $MgCl_2$ (control), *Xoo* suspension, *Xoo* suspension + 100 μM SNP and *Xoo* suspension + 100 μM SNP + 200 μM cPTIO. Each treatment was infiltrated in a total of five groups of tobacco plants, T0: distilled water plants, T1: T42 strain inoculated plants, T2: NO_3^--fed plants, T3: NO_3^--fed +

T42 strain inoculated plants, T4: NH_4^+-fed plants, and T5: NH_4^+-fed + T42 strain inoculated plants. Infiltration without *Xoo* cells and any chemical treatments was considered as the control (only 10 mM $MgCl_2$). All the treatments were infiltrated on the abaxial side of the tobacco leaves.

In addition, we studied the effect of SNP on the growth of *Xoo* under *in vitro* conditions. Different concentrations of SNP were freshly prepared and added to PSA media at 4 °C. Then, the spore suspension (0.05 OD_{600}) of *Xoo* was spread on the PSA media and the growth of the *Xoo* colony was examined after 4–5 days (ESI Fig. 1†).

The lesion-induced leaves from each group of plants from each treatment were harvested. Twenty regions per leaf and 6 leaves per plant were selected from each experiment. The bacterial number in the leaves from each treatment during HR induction was counted according to the protocols mentioned in a previous study.[27]

2.4 Cell death assay

Cell death assay in the leaves of the different treatment groups of plants was estimated using Evan's blue solution with slight modification.[28] The membrane damage or destabilized membrane is a hallmark of biotic stress. Evan's blue, an azo dye, is used to determine the cell viability of plant cells. Evan's blue quickly penetrates the ruptured membrane and stains cell. Accordingly, plant cells subjected to biotic stress exhibited a greater accumulation of Evan's blue dye compared to the control. In contrast, the healthy plant cells maintained their membrane integrity and did not allow blue protoplasmic staining, which could be easily quantified spectrophotometrically or visualized without the use of a camera.

2.5 Assay for histochemical visualization and quantification of H_2O_2 content

The histochemical staining of H_2O_2 was performed as previously described[29] with a few modifications. In the case of H_2O_2, HR lesions of leaves were dipped in 3,3′-diaminobenzidine (DAB; 1 mg ml^{-1}, pH 3) and incubated for 6–8 h in the dark at 25 °C. Dechlorophylization was performed by transferring the HR samples to a bleaching solution of ethanol : acetic acid- : glycerol (3 : 1 : 1, v/v) and boiling on a water bath for 10–15 min at 90 °C. The leaves were rinsed briefly in distilled water and mounted in lactic acid : phenol : water (1 : 1 : 1, v/v), then observed under a microscope. Estimation of the H_2O_2 content from the different treatment groups performed according to the previously described method.[30]

2.6 Chlorophyll content determination

Leaf discs from HR-induced regions were collected, and the leaf tissues homogenized in 80% acetone. The total chlorophyll content was monitored spectrophotometrically at 645 and 663 nm in the inoculated and uninoculated leaves, respectively, and chlorophyll content was calculated as described in Arnon.[31]

2.7 Antioxidant enzyme assays

One gram of HR lesion sample was homogenized with a chilled mortar and pestle at 4 °C in 4 mL 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% PVP. The homogenate was centrifuged at 13 000*g* for 20 min at 4 °C and the supernatant was used for the subsequent enzyme activity assays. Total protein content was determined by the method described previously.[32]

Superoxide dismutase (SOD; EC 1.15.1.1) activity was estimated by the method proposed previously.[33] Samples without enzyme extract were taken as the controls. The absorbance was recorded at 560 nm and one unit of enzyme was taken as the quantity of enzyme that reduced the absorbance reading of the sample to 50% in comparison to the tubes lacking enzyme. Catalase activity (CAT: EC 1.11.1.6) was measured using the previously described method.[34] Ascorbate peroxidase (APX: EC 1.11.1.11), which acts as an H_2O_2 scavenger, was measured using the previously described method.[35]

2.8 Assay for nitrite content

Nitrite level was measured according to the previously described method with some modifications.[25] HR-induced leaf samples (1 g) were crushed in liquid N_2 and 1200 μL (per sample) reaction mixture containing 500 μL sulphanilamide (1%), 500 μL *N*-(1)-(naphthyl) ethylenediamine-dihydrochloride (0.02%), and 200 μL zinc acetate (0.5 M) immediately added, and incubated at 25 °C for 3–4 h. Subsequently, the reaction mixture was centrifuged at 16 000*g* for 5 min. The absorbance of the chromophoric azo product was measured at 540 nm.

2.9 Assay of nitrate reductase activity

NR activity in the HR lesion samples was determined through the previously described method.[36] The intensity of the pink color was measured with a spectrophotometer (SpectraMax Me2, Molecular Devices, USA) at 540 nm.

2.10 Spectrofluorometry-based detection of NO production

For fluorometric NO determination, the fluorophore 4,5-diaminofluorescein-FM diacetate (DAF-FM DA) (Alexis Biochemicals, Gruenberg, Germany) was used. DAF-FM DA (10 μM) probing dye (stock prepared in 100 mM HEPES-KOH, pH 7.5) was preloaded in the leaves before infiltration. The leaves were kept in the dark at 25 °C for 30 min. Then, the different treatment plant groups were re-infiltrated in the same region where DAF-FM DA was preloaded. $MgCl_2$ (10 mM) was used as a control. Then, 100 mg leaves was excised from the HR-induced leaves with the help of a cork borer (size 0.79 cm^2) and immediately crushed in liq. N_2. Then, 700 μL of pre-chilled 100 mM HEPES-KOH (pH 7.5) buffer was added and centrifuged (16 000*g*, 15 min, at 4 °C). The reaction product (supernatant) was used for the measurement of NO production at 488 nm excitation and 500–515 nm emission wavelengths (2 nm bandwidth) with spectrofluorimetry (Spectra-Max 2, Molecular Devices, USA). Relative fluorescence was expressed as arbitrary units (AU) with some modifications.[25]

2.11 *In vivo* measurement of NO emission

The HR-induced leaves were incubated in 10 μM DAF-FM DA probe (4-amino-5-methylamino-2′,7′-difluorofluorescein-diacetate) in 100 mM HEPES-KOH buffer (pH 7.5) and placed in cavity glass slides and incubated for 5 min in the dark at 25 °C.[37] The procedure for DAF-FM DA loading and infiltration was similar to that described above. Then, the leaf samples were rinsed three times for 5 min with fresh 10 mM HEPES-KOH (pH 7.5) buffer and transferred onto a slide and visualized (Nikon Eclipse 90i fluorescence microscope, Nikon Instruments Inc. America, excitation at 488 nm, emission at 500–515 nm).

2.12 RNA extraction, cDNA preparation and qRT-PCR

Total RNA was harvested from the HR-induced leaf samples from each group of plants after post-infiltration at 6, 12, and 24 h according to the method described previously.[20] cDNA preparation and qPCR were performed according to the method by Singh *et al.*[4] The synthesized cDNA used as a template for the analysis of targeted genes is listed in ESI Table 1.† qRT-PCR was carried out in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Munchen, Germany) using SYBR Green chemistry (Affymetrix SYBR® Green Supermix Kit). The transcript level of mRNA was normalized and determined with the level of housekeeping gene β-tubulin for tobacco. The data obtained by real-time PCR of the different treatment groups was normalized with the $2^{-\Delta\Delta C_T}$ value method.[38] Three biological replicates were used for each group of plants for each treatment.

2.13 Statistical analysis

The data obtained from the different experiments is shown as mean ± standard error (SE) and subjected to analysis of variance (ANOVA). The treatment mean values were compared by Duncan's multiple range test at the $p < 0.05$ significance level and analyzed by SPSS ver. 16 (SPSS Inc., Chicago, IL). ClustVis, an online web tool, was used for heat map development.

3. Result and discussion

Crop production is challenging for farmers in the 21st century.[39] The continuous increment of microbes in the ecosystem increases pressure on agricultural production. Nitrogen availability in different forms directly affects the growth and development of plants.[7] Since N is an essential component of macronutrients, it plays a major role in the defense mechanisms of plants.[1] Ammonium ions are readily available to plants in comparison to nitrate ions, but an excess amount cause toxic effects to plant.[1,6] However, plants prefer NO_3^- as the N source for growth and disease resistance.[1,5] In addition, nitrate availability in the soil can help charge balance.[40] However, due to leaching, plants are unable to uptake sufficient amounts of nitrate. Several investigations have highlighted the role of nitrate in different physiological aspects, for example, root differentiation in tobacco[4] and local defense response against *Pseudomonas syringae*,[6] but there has been much less work done concerning HR-mediated disease resistance. Currently, the continuous increase in disease incidence and nutrient deficiency make plants more prone to pathogen invasion, especially since the R-gene-mediated HR-linked defense is often ephemeral.[41] Therefore, alternative strategies including microbial recruitment by plant roots may help solve this problem. Accordingly, it is necessary to characterize this type of pathological approach, which may compromise or increase HR-mediated disease resistance. In the present investigation, we present the effects of NO_3^- and NH_4^+ nutrition on the defense response with or without *Trichoderma asperellum* T42 in tobacco plants.

3.1 Nitrogen nutrients and *Trichoderma* recruitment affect defense response *via* HR-mediated cell death

Plant resistance is a complex process and exploring the signal mechanisms is the primary goal to understand host responses against invading pathogens. Oxidative burst in the plasma membrane is a primary and rapid event occurring in the defense mechanism of plants.[28] Evidence has supported that the growth of incompatible or compatible pathogens is restricted by early host cell death during the HR reaction.[42] The HR is frequently triggered by the rapid generation of reactive oxygen species (O_2^-, H_2O_2, OH^-, *etc.*), particularly superoxide, which is a very unstable free radical and rapidly mutated into H_2O_2 and molecular oxygen. In this study, we demonstrate the HR in the infiltrated region after challenged with *Xoo* (Fig. 1). According to the comparison in Fig. 1A (T0, T2 and T4 plants) 12 hpi, the NO_3^- (T2)-fed plants showed a higher level of HR compared to NH_4^+ (T4). In contrast, no HR was observed at the sites of infiltration in the plants grown without nutrient (T0). Also, the induction of cell death was found to be more significant in the NO_3^- grown plants at 12 hpi in comparison to the control (T0) and NH_4^+-fed (T4) plants (compare Fig. 1C and D, respectively). We further found that maximum cell death increased in the NO_3^- grown plants (Fig. 1C). These results suggest that N is essential for the defense response, and the NO_3^- nutrient is better than NH_4^+ for the activation of the local defense response against non-host pathogens. Further, we studied the effect of *Trichoderma asperellum* T42 in the activation of the local defense response. We found that the HR was greater in the plants grown in *Trichoderma* plus NO_3^- (T3) than *Trichoderma* plus NH_4^+ (T5). While, in the case of the T42 grown plants (T1), the HR was greater than the control plants (T0) (Fig. 1A). Thus, these results further support that plants require N for growth and disease development and *Trichoderma asperellum* T42 inoculation can help in increasing the N utilization efficiency.[4] Similarly, the inoculation of some groups with *Trichoderma* promoted their nutrient absorption capacity, which elevated the local defense mechanism.[22] It was evidenced that *Trichoderma asperelloides* inoculation elevated the local defense mechanisms in plants against both biotic and abiotic stresses.[43]

Furthermore, the CFU count and cell death observations suggest that the NH_4^+-grown plants displayed compromised disease resistance both in the plants inoculated with and without *Trichoderma* (Fig. 1B and C, respectively). After evaluating the effects of N nutrients and *Trichoderma* in the local

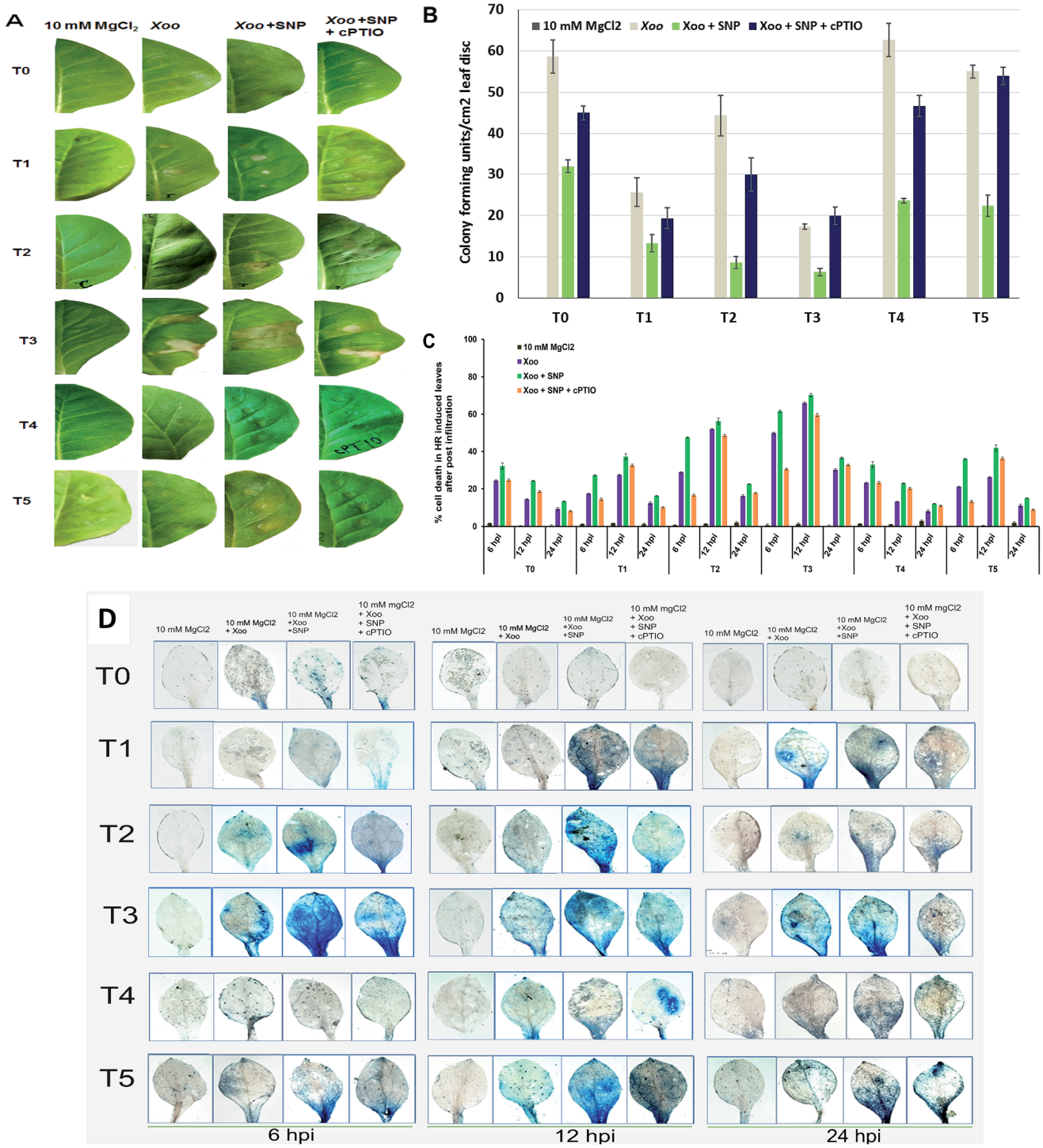

Fig. 1 Response of *Trichoderma* in the induction of the local defense response in plants grown under different forms of N nutrient. HR phenotypes were observed in the tobacco leaves at 24 hpi after infiltration with the NO donor SNP at 100 μM and NO scavenger, 200 μM cPTIO, with *Xoo* suspension culture in tobacco leaves (A), and number of colony-forming units per leaf disc (1 cm^2) observed in the HR-induced leaves of tobacco at 6–24 hpi (B). Percentage of dead cells observed after elicitation in tobacco leaves at 6–24 hpi (C). Evan's blue staining used to measure cell death in the inoculated leaves and uninoculated leaves post challenge inoculation at 6, 24 and 48 hpi (D). Blue colour shows the level of intensity of cell death. Plants were grown in different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition, and T5 = T42 + NH_4^+ nutrition. The lesion was monitored 24 h after elicitor infiltration, and no HR response was monitored in 10 mM $MgCl_2$. Results are the mean of twelve independent experiments ± SE.

defense response against *Xoo*, we further assessed the impact of the NO donor (SNP) in HR induction. Co-infiltration of SNP with *Xoo* increased the intensities of the HR compared to without NO donor in all the treated plants (Fig. 1). The application of cPTIO confirmed the importance of the NO donor in the induction of HR. These findings indicate that *Trichoderma asperellum* T42

inoculation sustained the HR against bacterial pathogen *Xoo* interaction, and an especially greater response was found in NO_3^- nutrient. However, there are scarce reports available on demonstrating the role of *Trichoderma asperellum* T42 in the activation of the local defense mechanism in plants grown under different forms of N nutrients. Hence, in this experiment, we studied the comparative impact of *Trichoderma* on enhancing the local defense response against *Xoo* stress under NO_3^- and NH_4^+ nutrients.

We also observed cell death efficiency through histochemical staining using the Evan's blue method (Fig. 1D). The cell death efficiency was observed to be higher in the NO_3^--grown plants compared to NH_4^+ at 12 hpi. Meanwhile, T42 inoculation in the plants further enhanced the cell death efficiency and the maximum efficiency was observed in NO_3^- nutrient after challenged with *Xoo* pathogen. A reduction in cell death was observed in the case of the plants grown without any treatments (Fig. 1D, upper panel). This efficiency was further increased through SNP application, suggesting that NO_3^- nutrient has a greater effect in the local defense response (HR induction), where even inoculation with *Trichoderma* promoted HR induction. Previous reports have studied the induced local defense response with *Trichoderma virens* inoculation in tomato leaves after exposure to *Rhizoctonia solani*.[12,44] Here, the *Trichoderma asperellum* T42-treated tobacco plants activated local defense mechanisms, which influenced *Xoo* invasion. Our results suggest that the suppression of local disease symptoms in the *Trichoderma asperellum* T42 plus NO_3^- plants may be correlated with the activation of the plant defense response through other signaling networks, as demonstrated by Wany *et al.* (2018) in *Arabidopsis* plants protected by *Trichoderma* strains against *Pseudomonas syringae*.[6]

3.2 H_2O_2 production and *RBOH* transcript accumulation helps HR induction in *Xoo* challenged plants

ROS generation is a continuous process, but in the presence of elicitors/or incompatible pathogens, it drastically increases. ROS generation is a result of constant metabolism processes and may involve NADPH oxidase/RBOH activities.[45] Furthermore, ROS are by-products generated from different part of plant organs such as chloroplast, mitochondria, and peroxisome; however, ROS production participates in HR-mediated cell death.[1] Evidence has highlighted that *Trichoderma* spp. induces ROS against several biotic stresses[46] and together with NO, plays an important role in HR-mediated cell death.[47] H_2O_2 is the most critical ROS molecule, which acts as a signal transducer in HR induction.[48] Herein, we investigated whether H_2O_2 production participating in cell death is linked to disease resistance, which is regulated by plant homologs membrane-associated *RBOH*s in plants against pathogens. We found that the induction of H_2O_2 production was significantly greater after *Xoo* challenged, and particularly greater in the NO_3^--fed plant leaves (T2) compared to the NH_4^+-fed plants (T4) (Fig. 2A), similarly to H_2O_2 production in *Arabidopsis*.[42] However, it was low without any treatment (T0), even after *Xoo* challenged. H_2O_2 production was found to be maximum in the plant leaves grown under *Trichoderma* plus NO_3^- treatment (3.72 μmol g^{-1} FW) at 6 hpi among the treatments (Fig. 2A). DAB staining also followed a similar pattern of observations (Fig. 2B; H_2O_2 staining images at 6 and 24 hpi not shown). A similar view was also reported in maize, where H_2O_2 production was generated in the early stage of infection of a fungal hypha of *Colletotrichum graminicola*.[20,49] Moreover, H_2O_2 production was also observed in *Arabidopsis* after colonization with *T. virens*[43] and in pea by *Trichoderma asperellum*.[20] The higher accumulation of H_2O_2 production in the *Trichoderma*-inoculated plants grown in N nutrients provides evidence that *Trichoderma* can promote H_2O_2 production under N nutrients during *Xoo* infection. A previous study demonstrated that the T42 strain increased the nitrogen use efficiency in tobacco plants.[4] However, H_2O_2 production was reduced after co-infiltration of 100 μM SNP with *Xoo*, and the lowest production was observed in the control (T0) plant leaves (0.079 μmol g^{-1} FW). Interestingly, cPTIO reversed the effect of SNP in all the treatment groups.

*RBOH*s are one of the primary sources for ROS generation upon pathogen elicitation, which participate in defense responses mediated through cell death in tobacco[50,51] and pea leaves.[52] Here, we also found that the higher accumulation of *NtRBOH* transcripts was activated at a late stage of infection, *i.e.*, 24 hpi in the T1, T2, and T4 treatment groups, except for T3 and T5, where *Trichoderma* association promoted earlier transcript accumulation at 12 hpi (comparison in Fig. 9). A reduction in *NtRBOH* transcript expression was observed in the NH_4^+ (T4) fed plants (0.8 fold) compared to that in NO_3^- (T2) nutrient (5.3 folds) after *Xoo* challenged, indicating the first cue that the NO_3^- form of N nutrient promotes the RBOH level during incompatible *Xoo* pathogen interaction. These results are consistent with the H_2O_2 production and staining results (compare Fig. 2 with Fig. 9), where ROS production was demonstrated to be higher in the NO_3^--fed leaves as compared to NH_4^+. The use of SNP further elevated the *NtRBOH* transcripts level, suggesting that RBOH is involved in plant cell death.

3.3 *Trichoderma* and N nutrients overcome chlorophyll degradation

Besides, the activation of RBOH-dependent ROS generation leads to continuous oxidative reaction in plants systems, causing membrane instability, and thus chlorophyll degradation is the first visual sign of oxidative damage. It was evidenced that nitrogen metabolism plays important in role in preventing chlorophyll degradation in potato leaves infected with *Phytophthora infestans*.[53]

Considering the putative biological roles of N fertilizers and *Trichoderma* in disease management, a study was conducted to determine if N fertilizers and *Trichoderma* have any visual effects in tobacco leaves infected by bacterial *Xoo* pathogen using seven-week-old plant leaves. We found that the chlorophyll content was significantly degraded/reduced after *Xoo* infiltrated the tobacco leaves compared to in the absence of pathogen infection at 6–24 hpi (Fig. 3). It was observed that at 6 hpi, the chlorophyll content in the infiltrated tobacco leaves was reduced, and maximum reduction was observed in the control

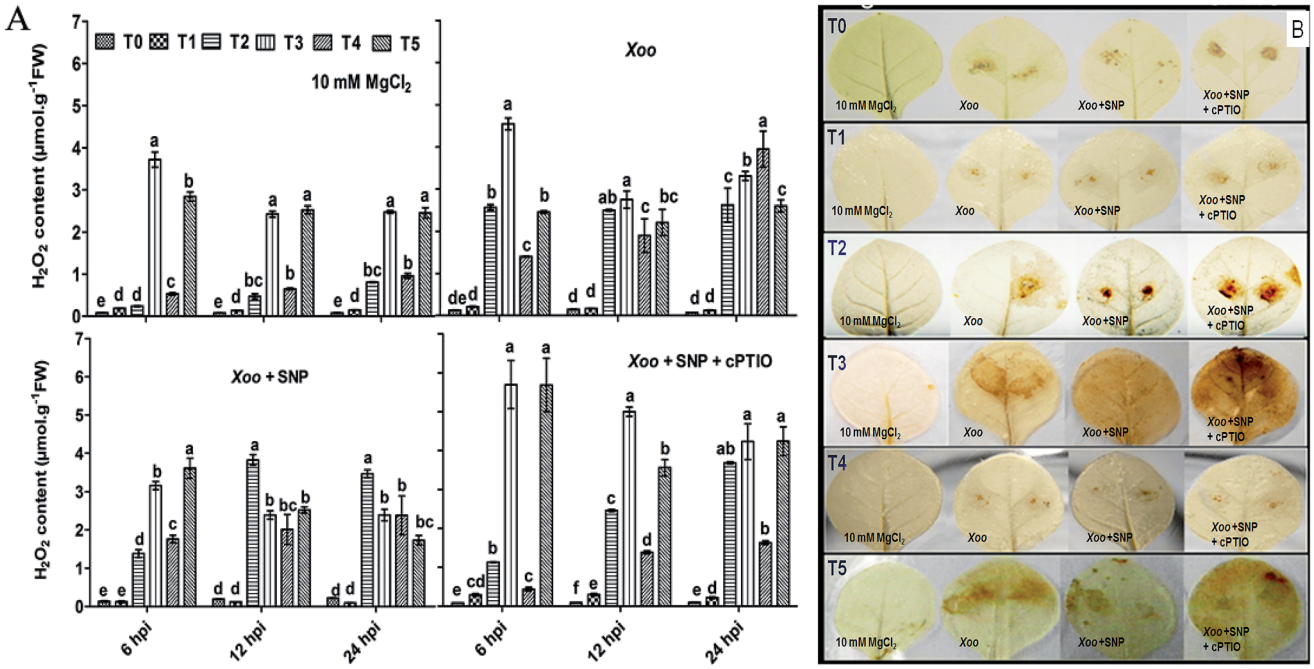

Fig. 2 Detection of ROS production in HR-induced tobacco leaves. Reactive oxygen species (H_2O_2) generation in HR-induced tobacco leaves modulated by *Xoo*, as analyzed spectrophotometrically at 6 to 24 hpi (Fig. 2A). Histochemical staining of H_2O_2 production with DAB dye in tobacco leaves at 12 hpi (Fig. 2B). H_2O_2 accumulation in tobacco leaves grown in different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_{4+} nutrition, and T5 = T42 + NH_4^+ nutrition at 6, 12 and 24 hpi. Suspension cultures of *Xoo* infiltrated with 100 μM SNP and 200 μM cPTIO. Suspension culture of *Xoo* was prepared in 10 mM $MgCl_2$ as a control. Error bars represent SEs from the mean of three measurements. Different superscript letters indicate significant differences from other treatments ($p \leq 0.05$; Duncan's multiple range test) (Fig. 2A). Brown color represents H_2O_2 deposition. The color intensity represents higher ROS (H_2O_2) generation during plant–pathogen interactions (Fig. 2B).

plants (T0) (0.03 mg g^{-1} FW) where *Xoo* was challenged, while the lowest reduction was observed in the *Trichoderma* plus NO_3^- fed plants (T3) (0.35 mg g^{-1} FW). No significant difference was found in the NO_3^- and NH_4^+ fed plant leaves during 6–24 hpi, indicating that *Trichoderma* prevented chlorophyll degradation only in the plants grown in NO_3^- nutrient. Considering that NO donors play diverse roles in biological systems and help in chlorophyll development, 100 μM SNP was infiltrated with *Xoo*, and it was observed that the loss of chlorophyll degradation was reverted with time (Fig. 3).

3.4 Induction of antioxidant enzymes activities and gene expression-promoted defense in *Trichoderma*-treated tobacco plants grown under N nutrients

Overall, these studies demonstrate that the *Trichoderma asperellum* T42 strain is efficient in promoting plant immunity against *Xoo*. The T42-induced local defense response may be essential in *Xoo* resistance in tobacco. The T42 strain treatment of tobacco plants stimulated ROS molecules (H_2O_2), which may activate other defense-related pathways, leading to an increase antioxidant enzymatic activity. ROS are naturally occurring signal molecules involved in plant defense.[11] They are rapidly generated at the site of infection caused by biotrophic or non-biotrophic pathogens in plants.[42] Previously, it was reported that several antioxidant enzymatic activities increased during *Ralstonia solani*-induced disease in sunflower.[54] These antioxidant properties were further elevated when sunflower seeds were treated with *Trichoderma*. Moreover, SOD, CAT, and APX elevation during oxidative stress is actively involved in plant cell wall strengthening and preventing cellular damage.[44,55] Several pieces of evidence have been demonstrated that plant growth-promoting microbes induce the generation of antioxidant enzymes such as SOD and POx during plant host–pathogen interactions.[56,57]

The present experiment revealed the key role of the *Trichoderma asperellum* T42 strain in triggering antioxidant enzymatic pathways, including SOD, CAT and APx. This is similar to previous reports, where the antioxidant potential increased in mustard and tomato after *Trichoderma* inoculation.[58,59] Herein, we observed that compared to the untreated plants, NO_3^--fed (T2) tobacco showed greater SOD activity (13.9 EU g^{-1} FW) than the NH_4^+-fed plants (T4; 11.8 EU g^{-1} FW) at 6–24 hpi after *Xoo* challenged (Fig. 4A). Meanwhile, *Trichoderma* inoculation significantly increased SOD activity, similar to tomato plants,[60] and this effect was comparatively greater in the NO_3^--fed (T3) plants (67.1 EU g^{-1} FW) than the NH_4^+-fed (T5) plants (42.3 EU g^{-1} FW) 6–24 hpi. No significant difference in the untreated (T0) and *Trichoderma* (T1) treatment groups were noticed during the infection period (Fig. 4A). Moreover, enhanced SOD activity was observed in the presence of SNP in the NO_3^- and *Trichoderma* plus NO_3^- fed plants compared to the other treatments,

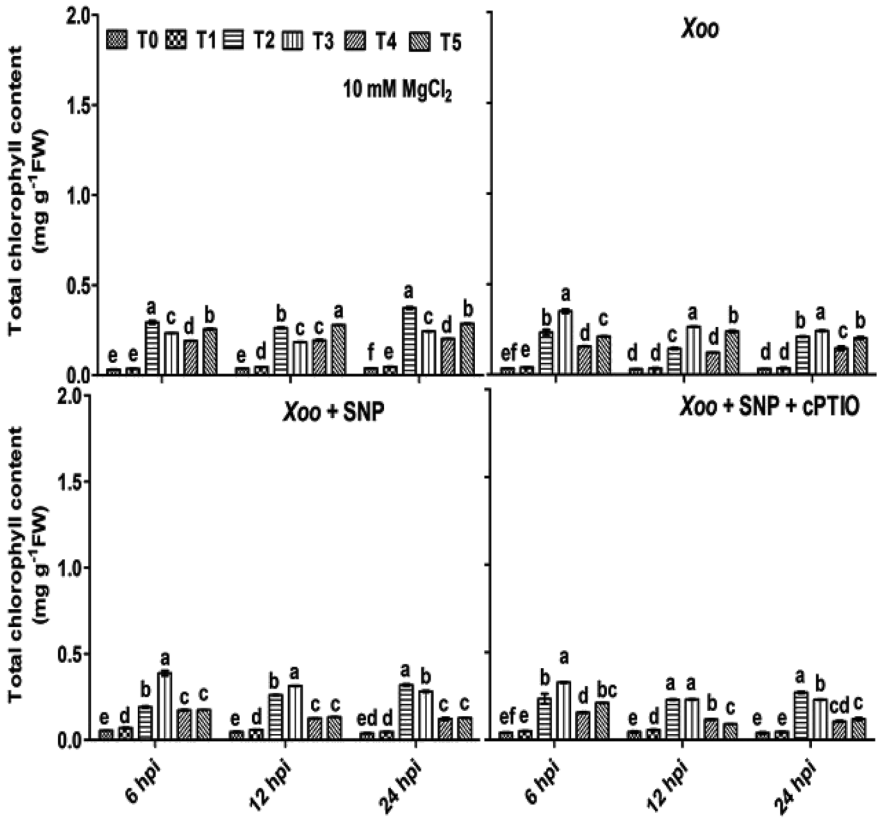

Fig. 3 Effect of *Trichoderma* and forms of N nutrient on total chlorophyll content (TCC) in tobacco leaves during *Xoo* challenged. TCC measured in tobacco leaves grown in different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition, and T5 = T42 + NH_4^+ nutrition after 6, 12 and 24 hpi. Suspension culture of *Xoo* was infiltrated with 100 μM SNP and 200 μM cPTIO. Suspension culture of *Xoo* was prepared in 10 mM $MgCl_2$ as a control. Error bars represent SEs from mean of three measurements. Different superscript letters indicate data are significantly different from the other treatments ($p \leq 0.05$; Duncan's multiple range test).

indicating that NO may be an essential factor for the induction of a local defense response against *Xoo* in tobacco.

Then, we further checked the role of T42 strain-elicited transcript accumulation of the *SOD* gene in the T2 and T4 treatment groups. The expression level of SOD level increased 0.2- and 0.15-fold in the T2 and T4 treated plants, respectively and no significant difference was recorded in the T0 and T1 groups (Fig. 9). *Trichoderma* inoculation elevated the transcript accumulation of the *SOD* gene similarly to SOD enzyme activity and maximum folding occurred in *Trichoderma* plus NO_3^- nutrient (T3; 0.23 folds) followed by T42 plus NH_4^+ (T5; 0.21 folds) 6–24 hpi (compare Fig. 4A with Fig. 9). Thus, these findings suggest that *Trichoderma* inoculation enhanced the SOD activity in the presence of NO_3^- compared to without any treatment (control). Co-infiltration of 100 μM SNP with *Xoo* followed the same pattern as SOD activity. Interestingly, after SNP use, the SOD expression level was greater in the T2 plants compared to the T4 plants (Fig. 4A), while the *Trichoderma*-treated plants showed a greater SOD expression level compared to the T0 plants (Fig. 4A). The effects of SNP were further abolished using 200 μM cPTIO.

During the local defense response assay, *Trichoderma* and N nutrients treatments also affected the CAT activity in the plant host and *Xoo* interactions. Herein, CAT activity was significantly increased in T2 compared to that in the T4-treated plants 6–24 hpi (Fig. 4B). A contrasting report was earlier demonstrated where the maximum CAT activity was observed in NH_4^+-fed plants in comparison to NO_3^-.[61] The CAT activity was enhanced more in the *Trichoderma* treatment group (T1) compared to the untreated plants (T0). Consistent with our results, Zhang *et al.* (2016) found that the overexpression of *Tachi* (a chitinase gene form *Trichoderma asperellum*) in Glycine max abolished disease infection caused by *Sclerotinia sclerotiorum* by enhancing CAT activity.[62] A similar result was also reported in tomato plants, where *Trichoderma* inoculation enhanced CAT activity under water-deficit conditions.[60] In the present study, the effect of *Trichoderma* treatment was evaluated in N nutrient conditions. The CAT activity was found to be greater in the *Trichoderma* plus NO_3^- fed (T3) plants compared to the *Trichoderma* plus NH_4^+ fed (T5) after *Xoo* challenged. Transcriptomic profiling of the *CAT* gene followed a similar pattern as CAT activity, and the expression level increased in tobacco after challenged with *Xoo* (compare Fig. 4B with Fig. 9). The transcript level of the *CAT* gene in the NO_3^--fed plants was greater than that in the NH_4^+-fed plants compared to the untreated plants (0.25 fold). Moreover, *Trichoderma* inoculation elevated the transcript accumulation of the *CAT* gene and it was found to be significantly higher in the T3 group (8.9 folds) among the treatment groups

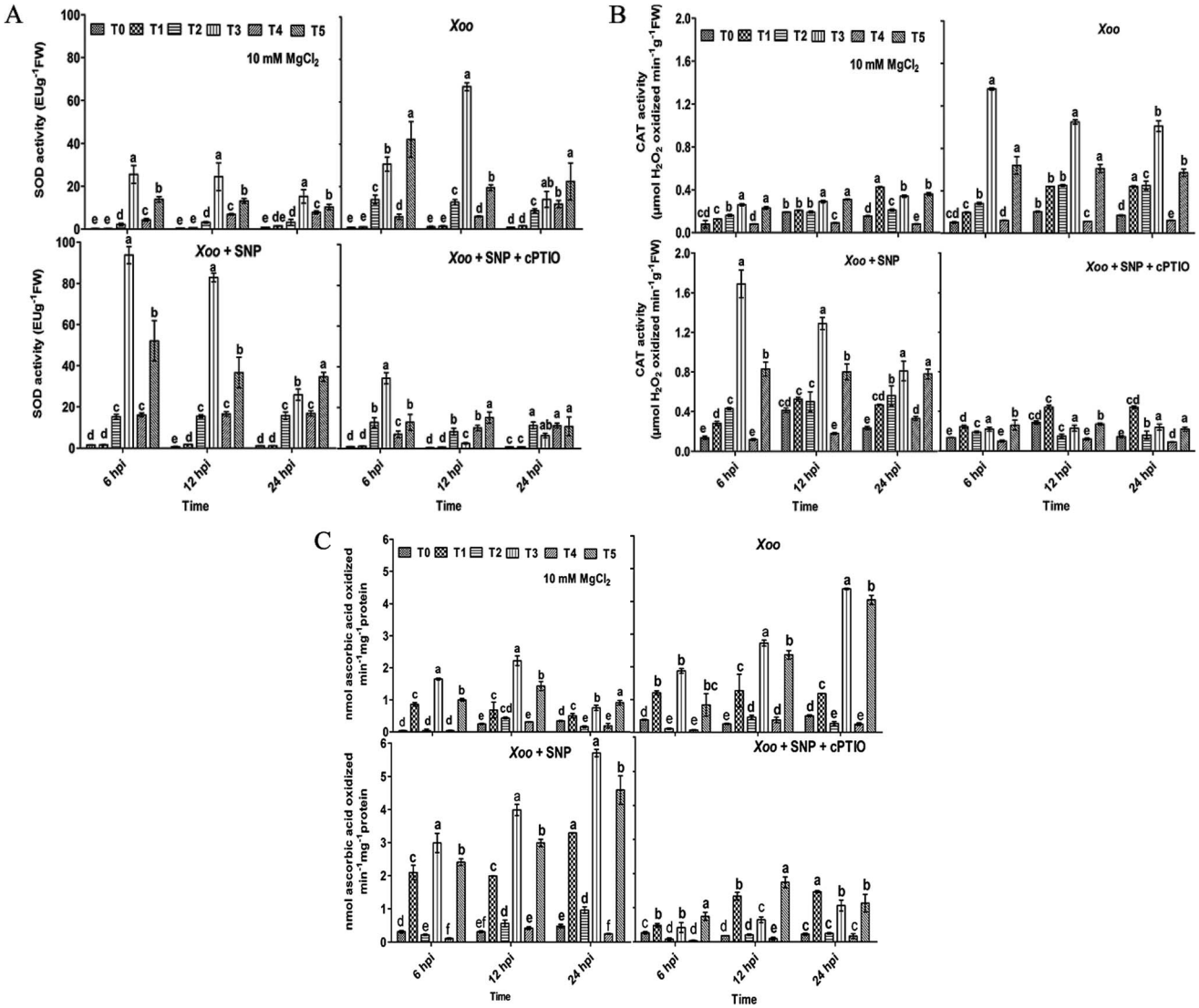

Fig. 4 Superoxide dismutase (A), catalase (B) and ascorbate peroxidase (C) activity in induced tobacco leaves modulated in the presence of *Xoo* were analyzed through spectrophotometrically. The enzyme activity was measured in tobacco leaves grown in different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition, and T5 = T42 + NH_4^+ nutrition 6, 12 and 24 hpi. Suspension culture of *Xoo* infiltrated with 100 μM SNP and 200 μM cPTIO. Suspension culture of *Xoo* (cells) was prepared in 10 mM $MgCl_2$ as a control. Error bars represent SE from mean of three measurements. Different superscript letters indicate data significantly different from the other treatments ($p \leq 0.05$; Duncan's multiple range test).

6–24 hpi. However, the CAT expression level increased 3.7-fold in the *Trichoderma* inoculated plant grown under *N*-deprived conditions (T1; in the presence of *Trichoderma*) compared to T0 treatment. The higher accumulation of *CAT* transcripts and activity in the *Trichoderma*-grown NO_3^- fed plants in comparison to the other treatments in the presence of NO donor (SNP) again provides evidence that *Trichoderma* can enhance the local defense response under nitrogen (compare Fig. 4B with Fig. 9).

Consequently, in case of APx activity, *Trichoderma* inoculation enhanced the APx activity in comparison to the untreated plants, similarly to that in an earlier report in tomato plants under water-deficit conditions.[60] This effect was observed to be remarkably higher in the T3 plants (0.35 nmol) followed by T5 (0.13 nmol) compared to the untreated plants (T0) after *Xoo* challenged (Fig. 4C). No significant difference in the T2 and T4 treated plants were noticed after *Xoo* infection, suggesting that *Trichoderma* can enhance APx activity in the presence of NO_3^- nutrient. Further, the transcript level of *APx* gene after *Xoo* challenged was observed. The maximum APx transcript level after *Xoo* challenged in the plant leaves was observed to be 0.38, 0.87, 4.5, 4.6, 1.2 and 3.8 fold in the T0, T1, T2, T3, T4, and T5 treatment groups, respectively, 6–24 hpi (Fig. 9). These findings indicate that the maximum APx expression level increased in the *Trichoderma* plus NO_3^- fed plants (T3). The application of SNP significantly affected APx activity and transcript accumulation in all the treatment groups, which was especially higher in the T3 treatment group compared to the control (T0) (compare Fig. 4C with Fig. 9).

Consistent with the above results, *Trichoderma* treatment increased the SOD, CAT, and APx activity in tobacco plants after *Xoo* challenged. As observed earlier, *Trichoderma* inoculation resulted in greater antioxidant activity in cucumber roots[63] and in tomato[60] under different biotic and abiotic stresses. The increased activity of SOD, CAT and APx may result from the additional protection of cells through the involvement of NO in the plant cell defense mechanism.[56,64] During colonization, the overproduction of antioxidant enzymes in nitrate nutrient occurs as a result of the use of SNP, which was demonstrated previously in *Cassia tora* roots[65] and leaves of tall fescue[66] under different stresses as a mechanism to protect host plants.

3.5 NO-mediated defense is augmented by NO_3^- nutrition and *Trichoderma asperellum* T42 against *Xoo*

Here, we further checked the mechanism behind the elevated local defense response mediated through *Trichoderma*. It has been demonstrated that the key features of *Trichoderma* is the induction of reactive oxygen species and NO-dependent defense. The induction of ROS plays a key role in defense responses.[67] Recently, short-term NO production was induced by elicitation upon *Trichoderma*,[6,68] most probably mediated by an increase in the expression of nitrate transporters. A mutation studied with the *nia 1,2* tobacco mutant revealed that NO production is mediated through nitrate reductase in plants. Several arguments suggested that nitrate reductase is a key enzyme for NO generation in plants through NR-dependent pathways.[69] However, the mechanism of NO generation in plants is still a debate. The main source of NO in plants is nitrate reductase or nitric oxide associated genes, which was clearly identified in plants.[68,70,71] In contrast, nitric oxide synthase (NOS) is a key source for NO generation in animals. Nitrate is reduced to nitrite by nitrate reductase, and thus an increased level of nitrite in the system is sign that NO conversion occurred.[72] The study by Modolo *et al.* (2005) demonstrated the production of NO from nitrite in *Arabidopsis* as a result of nitrate reductase activity, which showed disease resistance against *Pseudomonas syringae*.[73] Also, NO production was detected in other plants upon subjected to nitrite treatment.[74]

In our experiments, to check whether the nitrite content and nitrate reductase activity can help in NO production and defense, the plants were treated with *Trichoderma*. We studied the nitrite content and nitrate reductase activity in all the treated plants. It was found that in the absence of *Xoo* infiltration, the nitrite content was 0.58, 0.53, 19.95, 21.42, 1.32 and 16.61 μmol g^{-1} h^{-1} FW in the T0, T1, T2, T3, T4, and T5 plants, respectively (Fig. 5). These findings indicate that the nitrite content was higher in the NO_3^--fed plants (T2) in comparison to the NH_4^+-fed plants (T4). However, *Trichoderma* inoculation increased the nitrite content, especially in the plants grown in NO_3^- nutrient. No significant difference in the untreated plants (T0) and *Trichoderma*-treated plants (T1, alone) was observed. Meanwhile, a similar pattern as the nitrite content was also recorded with NR activity in all the treated plant leaves except in the T2 and T3 treatment groups, where no significance difference was observed (Fig. 6). To study the effect of *Xoo* infection, nitrite content and NR activity were further analyzed in the *Trichoderma* and N nutrient-treated tobacco plants (Fig. 5 and 6, respectively). The nitrite content significantly increased more in the NO_3^- grown plants (T2) than NH_4^+ (T4) 6–24 hpi. No significant difference in the T0 and T1 groups was noticed. However, in the presence of *Trichoderma*, the nitrite content increased in the NO_3^- fed (T3) and NH_4^+ fed (T5) nutrient groups during *Xoo* infection (Fig. 5), suggesting that *Trichoderma* has potential to assimilate nitrate.[6] Meanwhile, the NR activity followed the same pattern as nitrite content. The maximum NR activity was noticed in *Trichoderma* plus NO_3^- treated plants (T3) (Fig. 6). No significant difference was observed in the T0 and T1 plants, providing evidence of the role *Trichoderma* in nitrate acquisition. Co-infiltration of SNP with *Xoo* significantly increased both nitrite content and NR activity in the tobacco leaves grown in all the treatment groups 6–24 hpi, indicating that NO may play a role in the mechanism for promoting nitrate acquisition from roots, but this mechanism is still unclear.

To confirm NO production by tobacco after infiltration with *Xoo*, spectrofluorimetry-based detection of NO intensity and microscopy experiments with DAF-FM dye were performed.[12] Previously, NO was identified as a regulator of physiological response in animals, but now, it is a crucial component for plant immune response.[1,47] The interaction of NO with H_2O_2 leads to the activation of HR-mediated cell death during non-host–pathogen interactions.[42,75,76] NO played a crucial role in the activation of the defense mechanism during the interaction between plants and the bacterial pathogen *Pseudomonas syringae*,[77,78] but the contribution of NO against necrotrophic and biotrophic fungi has also been demonstrated.[79–82]

Our findings demonstrate that NO production was induced in the tobacco leaves after infiltration with *Xoo*. The real-time spectrofluorimetry-based detection of NO intensity showed NO production continuously increased in the tobacco leaves (Fig. 7). The maximum NO fluorescence intensity was observed at 90 min in the T2-treated plants after *Xoo* challenged, while in the T4-treated plants, at 180 min. Further, *Trichoderma* inoculation elevated the NO production in the plants (Fig. 7). Significant differences in NO intensity were detected in the T3 and T5 plants after they were infected with *Xoo*, suggesting that *Trichoderma* inoculation enhanced the NO production. However, in the absence of *Trichoderma* inoculation, the relative NO fluorescence intensity was lower in the T0-treated plants (Fig. 7). Moreover, the results of the DAF-FM T intensities in the HR leaves closely match the fluorescence signal observed in the fluorescence microscopy study with all the treatments except the infiltration of 10 mM $MgCl_2$ in the T0 tobacco leaves (control) (Fig. 8). The microscopic analysis showed that in the NO_3^- and *Trichoderma* plus NO_3^- grown plants, stronger fluorescence signals of NO were detected compared to the control (T0) and T1 plants (Fig. 8). In all the treated plants, NO accumulation was detected in the tobacco leaves at a different time intervals after *Xoo* challenged. The intensities of NO production in the tobacco leaves were enhanced by co-infiltration of SNP together with *Xoo* in all the treatment groups. However, the effect of SNP was confirmed using an NO scavenger. The

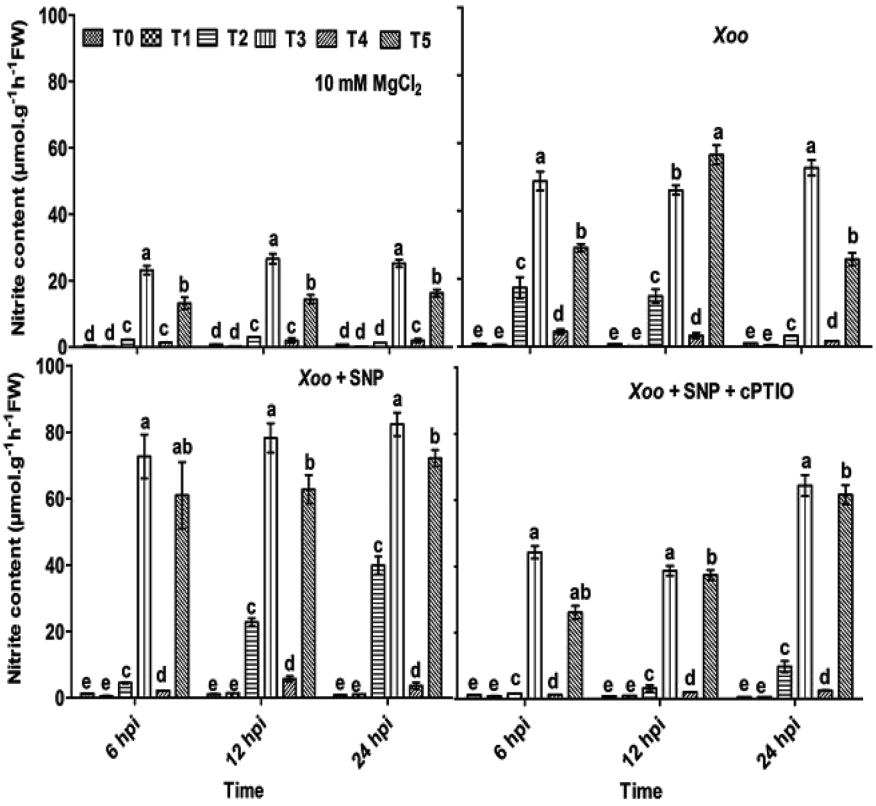

Fig. 5 Effect of *Trichoderma* on level of nitrite content in the tobacco plants grown in N nutrients during HR response. Nitrite content was measured in tobacco leaves grown in different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition, and T5 = T42 + NH_4^+ nutrition 6, 12 and 24 hpi. Suspension culture of *Xoo* infiltrated with 100 µM SNP and 200 µM cPTIO. Suspension culture of *Xoo* was prepared in 10 mM $MgCl_2$ as a control. Error bars represent SE from mean of three measurements. Different superscript letters indicate data significantly different from the other treatments ($p \leq 0.05$; Duncan's multiple range test).

reduction in the fluorimetry-based NO signal and microscopy DAF-FM T intensity was associated with the cPTIO application (Fig. 8).

Consistent with this, we demonstrated that NO generation showed comparatively higher disease resistance in the NO_3^- nutrient group as compared to NH_4^+ due to the possibility of utilizing nitrate as a substrate.[25] In our experimental approach, NO generation in the presence of *Trichoderma asperellum* T42 was activated, which may be an essential component of signaling of the defense response induced in the presence of nitrate. The combination with other signaling molecules, including H_2O_2 formation during pathogen interaction, may mutually influence their activities. NO accumulation was enhanced in the presence of nitrate nutrient in the *Trichoderma*-inoculated tobacco plants. Thus, the question arises, how is NO_3^- nutrition involved in the cell death mediated response? One of the likely pathways was explained by Romero-Puertas *et al.*[52] Several well-known pathways have been identified for the production of NO such as L-arginine-, polyamine- or NAD(P)H-linked reduction of NO_3^- by cytosolic NR.[74] Our assessment of NO production from the *nia30* mutant plant demonstrated that the NO_3^- nutrition was the major source of NO production,[4] similarly to that in the tobacco plant.[1] Considering this, we observed that significant NO was generated in the NO_3^--fed tobacco leaves after elicitation with *Xoo*, whereas a lower intensity was observed in the NH_4^+-fed tobacco plants. Previously, Shoresh and Harman *et al.*[23] demonstrated that *Trichoderma* inoculation induced NO generation. Our observation showed that NO generation activated by *Trichoderma asperellum* T42 inoculation may influence the *Xoo* infection greater in nitrate nutrient; however, the tobacco plants were compromised to infection against *Xoo* in ammonium nutrient. Consistent with the presence of *Xoo*-induced NO emission in tobacco leaves, NO donor (SNP) had a more significant effect on *Xoo*-induced cell death. The NO_3^--fed plant leaves showed greater induced HR compared to the NH_4^+-fed tobacco leaves. Notably, the inoculation of tobacco plants by *T. asperellum* resulted in the ability to counter HR in the NO_3^--fed leaves in infiltrated with *Xoo* pathogen. This result suggests that priming with *T. asperellum* equipped the plants to better respond to pathogen infection,[23,83] especially more in NO_3^- nutrition.

3.6 *T. asperellum* T42 recruitment favors activation of NO-modulating, *PR1a* and *MEK3* genes in N nutrition, suppressing incompatible pathogen *Xoo*

The nitrite content and NR activity levels in the tobacco leaves increased in the presence of *Xoo* infection in the different treatment groups, which are indicative of the fact that NO

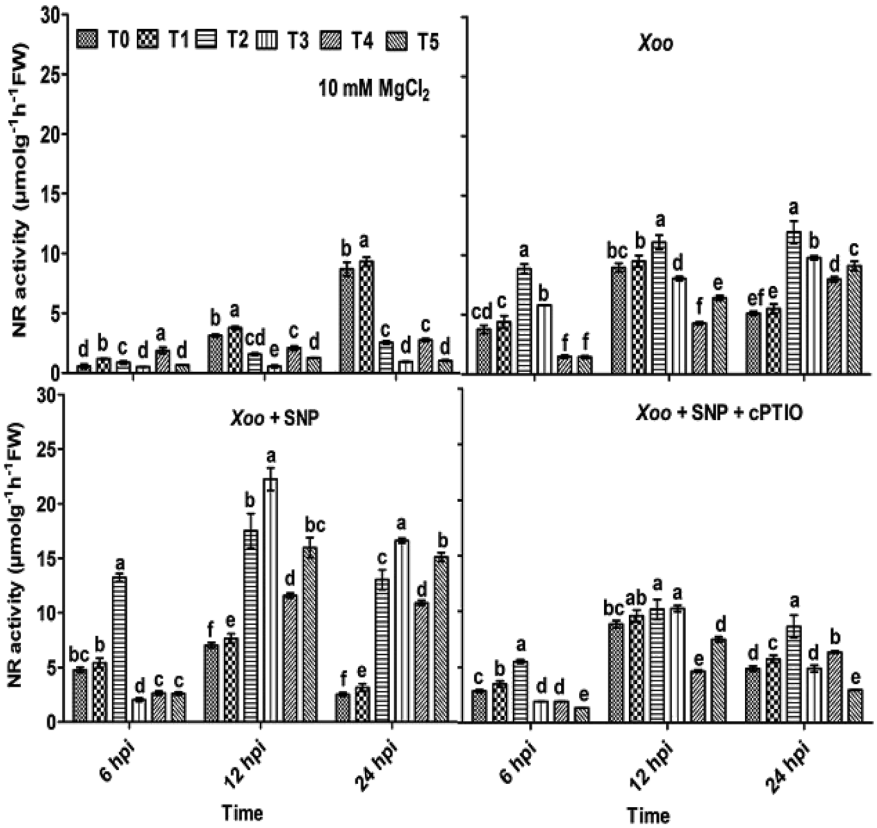

Fig. 6 Effect of *Trichoderma* inoculation in induction of nitrate reductase (NR) activity in tobacco leaves during the local defense response. NR activity was measured in tobacco leaves grown in different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition, and T5 = T42 + NH_4^+ nutrition 6, 12 and 24 hpi. Suspension culture of *Xoo* infiltrated with 100 μM SNP and 200 μM cPTIO. Suspension culture of *Xoo* prepared in 10 mM $MgCl_2$ served as a control. Different superscript letters indicate data significantly different from the other treatments ($p \leq 0.05$; Duncan's multiple range test).

production depends on nitrite content and NR activity. A previous study reported that *NR* expression was highly induced by N nutrient in plants.[5] In the present study, the expression level of *NR* and *NOA* was found to increase in comparison to unchallenged *Xoo* in all the treatments (Fig. 9). The maximum expression level of *NR* and *NOA* increased at 12 hpi in all the tobacco-treated plants after *Xoo* challenged except T0 and T2, where the maximum *NR* expression level was observed at 24 hpi. *NR* expression was induced at an early stage, *i.e.* at 6 hpi in the T1-treated plants. Nitrate nutrient had a significant effect and induced greater *NR* and *NOA* expression in the NO_3^- grown plants (T2) compared to the NH_4^+ grown plants (T4). Down-regulation of *NR* expression in the NH_4^+ (T4) tobacco leaves was noticed. Interestingly, *Trichoderma* inoculation in the tobacco plants significantly up-regulated the *NR* and *NOA* expression in the plants grown in NO_3^- nutrient (T3 plants) compared to the NH_4^+-fed tobacco leaves (T5 plants). Co-infiltration of SNP with *Xoo* further induced a higher expression level by several folds in the NO-modulating genes in all the treatments except the T5 treatment, where SNP did not affect the expression of the NO-modulating gene. These results were further confirmed by the use of an NO scavenger with *Xoo* and SNP. Meanwhile, the transcript accumulation of *ARC* increased in all the treatment groups after *Xoo* infiltration except T0 (Fig. 9), where *ARC* was down-regulated. The maximum *ARC* transcript accumulation occurred in the *Trichoderma* plus NO_3^- grown plants (T3) (3.3-fold) followed by the NH_4^+ grown plants (T4) (2-fold) 6–24 hpi. The application of SNP with *Xoo* negatively affected the ARC expression level. Thus, these findings suggest that *Trichoderma* inoculation may enhance *ARC* accumulation, particularly in the presence of NO_3^- nutrient.

To further characterize the N nutritional effects and *T. asperellum* T42 recruitment on defense, we focused on the MAP kinase gene response in the defense mechanism against *Xoo* in tobacco leaves. It was also remarkable that in our assessments of the defense gene, the expression of NO_3^- effects seemed to be confirmed by MEK3-regulated oxidative burst. According to Asai *et al.* (2008), the expression of *MEK2-SIPK* is crucial for the induction of *RBOH* in *N. benthamiana*, which is elicited by INF1, causing oxidative burst.[79] In our experiment, the transcript accumulation of *MEK3* was correlated with the regulation of *RBOH* expression in response to *Xoo* infection in the tobacco leaves (Fig. 9). We found that the transcript accumulation of *MEK3* increased more in T0 (0.4-fold) compared to T1 (0.14 folds) 6–24 hpi. No significant difference in *MEK3* expression level in the T2 and T4 grown plants was observed after *Xoo* challenged (Fig. 9). On the other hand, upon T42 recruitment, *MEK3* expression was found earlier (6 hpi) in T42 plus NO_3^-

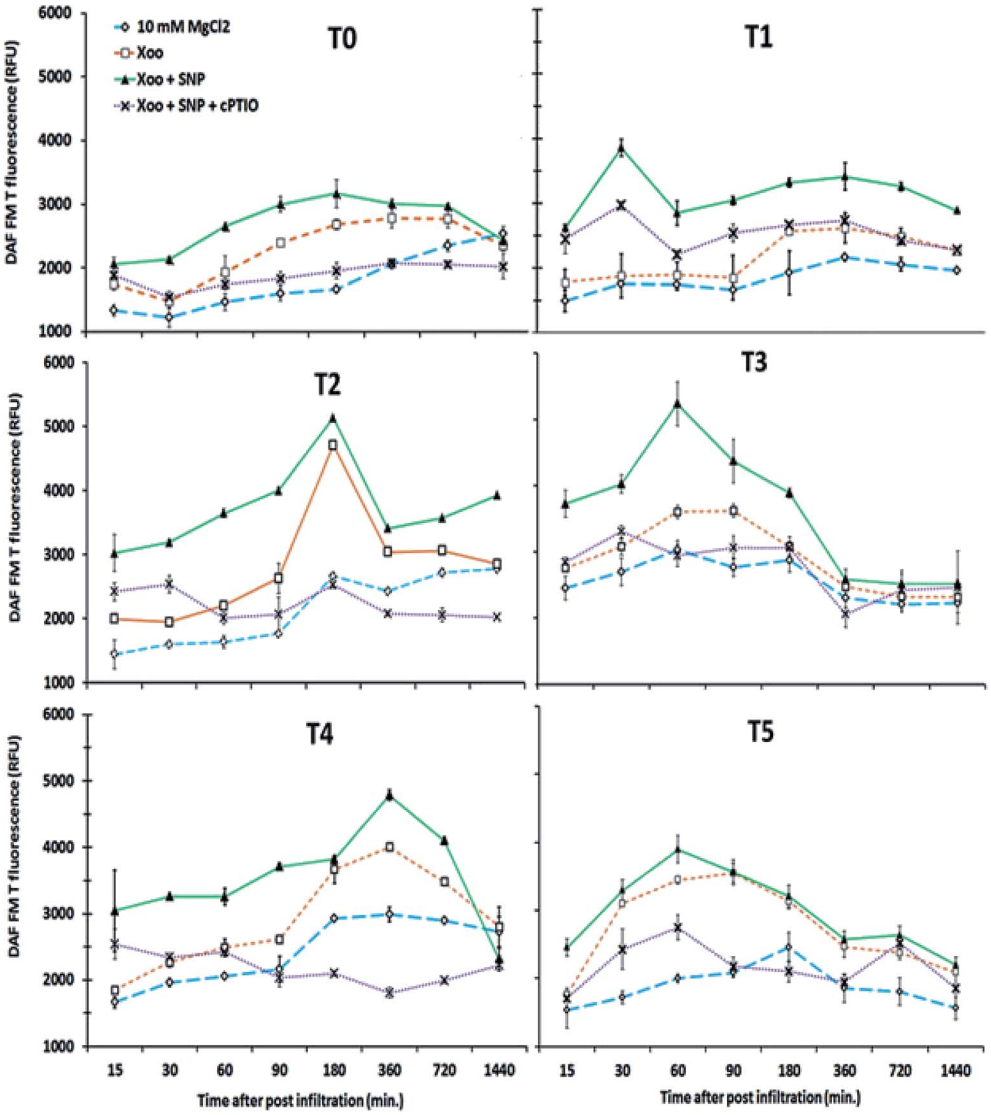

Fig. 7 *Xoo*-induced diaminofluorescein-FM T fluorescence intensity in tobacco leaves. Plants grown under different treatments: T0 = dist. H_2O, T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition, and T5 = T42 + NH_4^+ nutrition up to 24 hpi. After preloading cells with DAF-FM (as described in the Experimental setup section), *Xoo* suspension culture singly, *Xoo* + 100 μM SNP, and *Xoo* + 100 μM SNP + 200 μM cPTIO were infiltrated. Cell suspensions for *Xoo* were prepared in 10 mM $MgCl_2$ and infiltration of $MgCl_2$ served as a control. At the indicated times, 0.75 cm^2 discs were removed from the leaves for fluorescence observation. Note that the reaction product of DAF and NO, DAF-FM T, is stable for a long time. Therefore, a continuous increase in fluorescence indicates a constant rate of NO formation. Each value represents the mean ± SD of eight independent experiments.

compared to T42 plus NH_4^+ treatment indicating that *Trichoderma* priming involves activation of *MEK3*/*RBOH* and, promotes cell death during infection. However, the transcript accumulation of *MEK3* increased several folds in the T5-grown plants (7.1-fold) than the T3-grown plants (3.8-fold) at a later stage. Co-infiltration of SNP with *Xoo* in tobacco leaves up-regulated the MEK3 expression in all the treated plants except in the T5 plants, where a negative effect of SNP on MEK3 was observed. The lack of complete information about the correlation of NO and MEK3 in NO_3^- or NH_4^+ feeding modifying HR in the presence of *T. asperellum* T42 requires further characterization to determine how *Trichoderma* recruitment changes HR against infection of non-host–pathogens through NO_3^- or NH_4^+.

NO is known to trigger the salicylic acid marker gene,[47] and it has the ability to induce oxidative burst to augment cell death during HR.[80] Thus, the induction of *PR1a* gene activation in response to *Xoo* infection in the NO_3^--fed tobacco leaves is most likely linked to induced NO production (Fig. 9), which was further investigated. The expression level of *PR1a* increased 0.3-fold under nutrient-deprived (control; T0) conditions at 6 hpi (Fig. 9). The *PR1a* expression was high in the NO_3^--fed plants compared to NH_4^+ nutrient, suggesting that the plants fed in NO_3^- nutrient showed more *PR*-mediated disease resistance compared to NH_4^+ nutrient against incompatible *Xoo*, similarly to the findings of Gupta *et al.*,[1] where the form of N nutrition affected cell death linked to resistance in response to elicitation of HR against *Pseudomonas syringe* in tobacco leaves. However, switching of

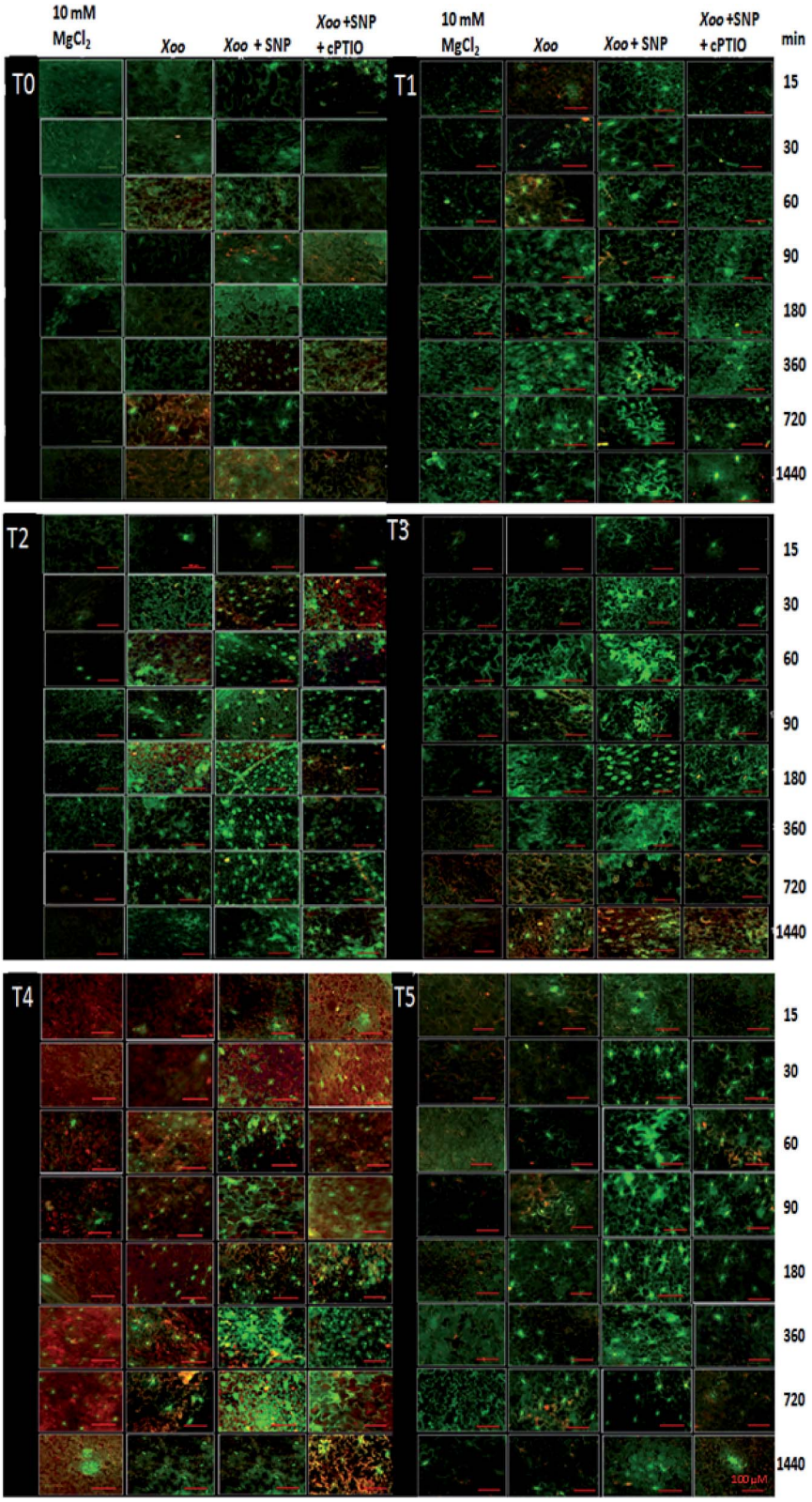

Fig. 8 Detection of NO accumulation using diaminofluorescein-FM fluorescence in tobacco leaves after *Xoo* infiltration. *Xoo*-induced DAF-FM T fluorescence under different treatments: dist. H_2O (T0), T42 treated (T1), nitrate (T2), nitrate + T42 strain (T3), ammonium (T4) and ammonium + T42 strain (T5) grown tobacco leaves. After preloading cells with DAF-FM (as described in the Experimental setup section), *Xoo* suspension culture singly, *Xoo* + 100 μM SNP, and *Xoo* + 100 μM SNP + 200 μM cPTIO were infiltrated. Cell suspensions for *Xoo* were prepared in 10 mM $MgCl_2$ and infiltration of $MgCl_2$ served as a control. At the indicated times, 0.75 cm^2 discs were removed from the leaves for fluorescence observation. Note that the reaction product of DAF and NO, DAF-FM T, is stable for a long time. Therefore, a continuous increase in fluorescence indicates a constant rate of NO formation. Bars represent = 100 μM.

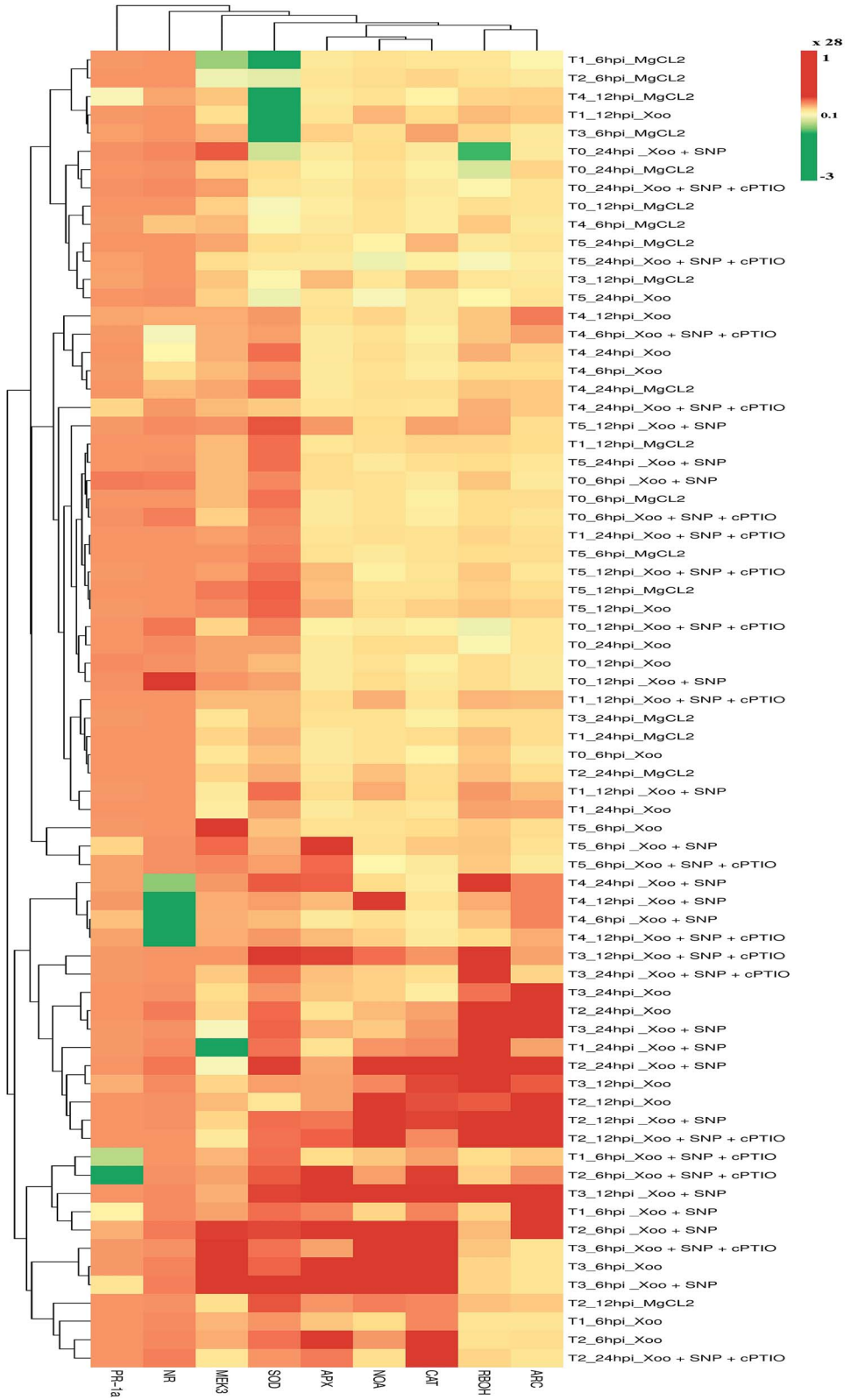

Fig. 9 Real-time qPCR expression profiling of NO synthesis, MAK, SAR and ROS induced genes in HR-induced tobacco leaves. The expression patterns of *NR*, *NOA RBOH*, *MAK3*, *CAT*, *APxI*, *SOD*, *PR1a* and *ARC* indicated their important roles in the *Xoo*-induced HR in different treatments: T0 = dist. H_2O,T1 = T42 treatment, T2 = NO_3^- nutrition, T3 = T42 + NO_3^- nutrition, T4 = NH_4^+ nutrition and T5 = T42 + NH_4^+ nutrition at 6, 12 and 24 hpi. Application of 100 μM SNP and 200 μM cPTIO with *Xoo* suspension was infiltrated in the leaves of the tobacco plants with each treatment. Suspension culture was prepared in 10 mM $MgCl_2$ as a control. The heatmap was generated by a folding pattern of the real-time PCR data presented as $\Delta\Delta^{C_T}$. Tobacco ubiquitin gene was used as an internal control. Red and green colors show up- and down-regulation of genes, respectively; however, yellow color was used for the transcript expression level for control plants.

PR1a expression by *T. asperellum* T42 inoculum in the N nutrient-fed tobacco plants depends on the mode of infection mechanism. Its expression level was down-regulated in the T1, T3, T4, and T5 grown plants (Fig. 9). The induction of *PR1a* expression in the presence of NO donor (SNP) linked with disease resistance mediated through NO and *PR1a* was demonstrated in tobacco leaves, similarly to another observation.[25]

4. Conclusion

In conclusion, in the present experiment, we found that *Trichoderma asperellum* T42, saprophytic filamentous fungi, could significantly promote the plant defense response against *Xanthomonas oryzae* pv. *oryzae* by modulating reactive oxygen species and nitric oxide molecules in tobacco plants. *Trichoderma*-induced disease resistance to *Xanthomonas oryzae* pv. *oryzae* was demonstrated to induce NO *via* the NR and NOA pathways, and accumulation of H_2O_2 by an increase in antioxidant enzyme activity. Moreover, the application of nitrate as a nitrogen source was promoted by *Trichoderma* inoculation, which helped the plants maintain NO and H_2O_2 generation during the hypersensitive response against *Xanthomonas* infection. Our study provides new insight into the mechanistic approach of *Trichoderma*, which reprograms disease resistance against non-host–pathogen *Xanthomonas oryzae via* NO and H_2O_2 production in response to nitrogen nutrition. Further, these findings will open new gateways for researchers to study how and which mechanisms are associated with *Trichoderma* for increasing N acquisition. It may be possible that certain novel signaling molecules are volatile compounds; however, additional experiments are essential to determine this.

Abbreviations

DAF-FM	Diacetate 4,5-diaminofluorescein-FM
HR	Hypersensitive response
MAPK	Mitogen-activated protein kinase
NR	Nitrate reductase
NO	Nitric oxide
$ONOO^-$	Peroxynitrite ion
PCD	Programmed cell death
PR-1	Pathogenesis-related protein
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase
SNP	Sodium nitroprusside
cPTIO	μM cPTIO (carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide)

Author contributions

Contributor BNS performed laboratory experiments, data collection, and statistical analysis. PD designed the research, revised the manuscript for publication and supervised the work. Contributed reagents/materials/analysis tools/equipment facilities: PD, BKS, HBS.

Conflicts of interest

The authors declare that research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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