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Nitric oxide: a signaling molecule which activates cell wall-associated defense of tomato against *Rhizoctonia solani*

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Abstract We investigated the role of sodium nitroprusside (SNP), as a NO donor, in activating cell wall-related defense responses of tomato against *R. solani.* Based on previous knowledge on the cross talk of NO with various pathways and its effect on cell wall components, experiments were carried out to investigate the function of cell wall-related defense responses, octadecanoid and phenylpropanoid pathways (which are associated with cell wall modification) not only in SNP-activated defense but also in basal resistance of tomato to the pathogen. In detached leaves and intact plants, we observed a considerable decrease in disease progress on both partially resistant (CH Falat) and susceptible (Mobil) tomato cultivars treated with SNP. The SNP treatment regulated malondialdehyde (MDA), H_2O_2 and O_2^-levels in plant cells, whereas SNP primed callose depsition, phenolics and lignification, as defense responses related to cell wall modification in the inoculated tomato plants. Priming in the activity and expression of lipoxygenase (*LOX*) and phenylalanine ammonia-lyase (*PAL*) as key markers of octadecanoid and phenylpropanoid pathways, respectively, was observed in SNP-treated inoculated plants. Co-application of LOX or PAL inhibitors and SNP completely suppressed SNP-activated defense responses. Using DEA-NONOate, as an independent NO releaser, supported the data obtained by SNP on disease progress and the role of PAL and LOX in NO-related protection of tomato against the pathogen. These findings highlight the role of phenylpropanoid and octadecanoid pathways and involvement of cell wall modifications in NO-associated induction of defense as novel mechanisms of SNP-IR in tomato–*R. solani* pathosystem.

Keywords Basal resistance · Cell wall · Induced resistance · Nitric oxide · *Rhizoctonia solani* · *Solanum lycopersicum*

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Introduction

Plants suffer from biotic stresses caused by various pathogens. So, they have the capability of developing defense strategies during interactions with phytopathogens. In addition to several resistance sterategies, the plant cell wall is a first defense line for protecting plants against pathogens. The cell wall is an active border for interaction of various pathogens with the host plant cells which supplies carbohydrates for pathogen growth. It is known as a physical barrier that is suppresses pathogen development in plant tissues, and an integrity sensory system capable of activating defense-related signaling pathways in plant cells (Nafisi et al. 2014). Plants have developed pre-invasive structural defenses, including a cuticular layer, and cell wall modifications that serve to block the progress of an intruder pathogen (Pastor et al. 2013; Van Kan 2006). Chemical composition of the modified cell wall and deposition speed of new

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substances are important for a successful defense response (Huckelhoven 2007).

The cell wall is actively reinforced via deposition of cell wall appositions, known as papillae, at sites of interaction with pathogens (Voigt 2014). The major constituent of papillae is the β-(1,3)-glucan polymer callose as a cell wall polysaccharide. However, several other compounds are associated with papillae formation including reactive oxygen species (ROS), phenolics, cell wall proteins and polysaccharides (Bestwick et al. 1997; Hematy et al. 2009; Paris et al. 2007). By slowing pathogen invasion in the attacked tissue, papillae formation can gain time for induction of additional defense responses that may require gene upregulation, which leads to changes in metabolic and hormonal profiles (Boller and Felix 2009; Garcia-Andrade et al. 2011).

Nitric oxide or nitrogen monoxide (NO) is a gaseous free radical, which plays regulatory function in plant growth, development and defense responses against biotic and abiotic stresses (Beligni and Lamattina 2002). Since intercellular and intracellular NO accumulation in plants usually occurs in response to biotic (Floryszak-Wieczorek et al. 2012) and abiotic stresses (Gill et al. 2013), its major role is involvement in signaling and regulating plant defense. Chemical characteristics of NO, including its small size, short half-life, absence of charge and high diffusivity would serve an ideal signaling molecule in plant defense (Wang et al. 2005). Recently, involvement of NO in defense responses during plant-pathogen interactions has been well documented (Floryszak-Wieczorek et al. 2012; Kim et al. 2013). Especially, NO may be implicated in some of resistance responses mediated by ROS, such as defense gene activation, hypersensitive cell death, production of phytoalexins (Delledonne et al. 1998; Durner et al. 1998), flavonoids (Ganjewala et al. 2008) and cell wall modifications via callose deposition (Paris et al. 2007). Both NO and ROS are versatile molecules that mediate a variety of cytomolecular responses in plants. It has been suggested that ROS alone are not always sufficient to mediate a strong disease resistance response in plants, and their combination with NO can act synergistically to activate a stronger response (Wang et al. 2005). However, accumulation of ROS can disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Møller et al. 2007).

Previous evidence indicated that a low concentration of the NO donor SNP enhanced callose deposition in tomato roots (Correa-Aragunde et al. 2008). Furthermore, a recent study demonstrated the role of octadecanoid pathway in the activation of phenylpropanoid metabolism leading to cell wall modification as a major mechanism of plant defense against the necrotrophic fungus *Rhizoctonia solani* (Taheri and Tarighi 2010). Jasmonic acid (JA) and its methyl ester methyl jasmonate (MeJA), known as jasmonates, are the key products of the octadecanoid pathway. Investigations revealed that NO is involved in JA mediated plant defense strategies, including the inhibition of wound-induced hydrogen peroxide (H_2O_2) production in tomato leaves (Orozco-Cardenas and Ryan 2002). Also, Hung and Kao (2004) reported an inhibitory effect of NO on the MeJA-induced H_2O_2 burst in rice plants. However, the effect of NO on production and accumulation of other ROS, such as superoxide anion (O_2^-) and hydroxyl radical (OH^-) is not known, so far.

Phenolics are among the main compounds produced via phenylpropanoid pathway during cell wall modification process and are effective antioxidants in plants (Qingming et al. 2010; Boubakri et al. 2013). Increased levels of phenolics may provide an adequate substrate for oxidative reactions catalyzed by peroxidase, which results in lignification and makes the plant cells unfavorable for further pathogen progress (Pauwels et al. 2008; Taheri and Tarighi 2011). Phenolics may act as soluble antimicrobial compounds or they may cross-link with callose and proteins into cell walls, so inhibiting penetration and absorption of nutrients by pathogens (Hukkanen et al. 2007). However, there is still insufficient knowledge on the cross talk of NO with phenolics, lignin, callose, ROS, octadecanoid and phenylpropanoid pathways as major components of the complex network involved in cell wall-associated resistance mechanisms.

In the present work, we examined the effect of NO on defense responses of tomato against *R. solani* by treating the intact plants and detached leaf discs with SNP. The ability of SNP treatment to modulate H_2O_2 and O_2^- levels, callose deposition, phenolics production, and lignification, as defense responses related to cell wall modification, was analysed. Involvement of the octadecanoid pathway in NO-activated defense responses was investigated by determining lipoxigenase (LOX) activity and transcript accumulation of *LOX*B isoform, as a key marker of this pathway, in SNP-treated plants compared to controls. In addition, the role of phenylpropanoid pathway in SNP-induced resistance

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(SNP-IR) was investigated by determining phenylalanine ammonia lyase (PAL) activity and its gene expression levels. Also, the PAL and LOX inhibitors were used to analyse the function of phenylpropanoid and octadecanoid signaling pathways on SNP-IR and basal resistance in this pathosystem. Additionally, the effect of NO on malondialdehyde (MDA) content, as an indicator of membrane lipid peroxidation was investigated in our pathosystem.

Materials and methods

Tomato genotypes and growth conditions

The tomato genotypes CH Falat and Mobil, as partially resistant and susceptible cultivars to *R. solani*, respectively (Nikraftar et al. 2013), were used in this study. The seeds were surface sterilized with 1 % sodium hypochlorite for 1 min, rinsed 3 times with sterile distilled water and directly sown in the 15 cm-diameter plastic pots. The pots were filled with a commercial potting soil which had been autoclaved at 121 °C for 60 min and the plants were grown in greenhouse (30± 4 °C; 16/8 h light/dark photoperiod). Four-week-old plants were used for inoculation.

Pathogen inoculation and disease assessment

R. solani isolate KZ17, belonging to anastomosis group (AG) 3 with high level of virulence on tomato (Nikraftar et al. 2013), was used to investigate the role of NO in activating plant defense responses. The fungal isolate was cultured on potato dextrose agar (PDA) and incubated at 28 °C for 7 days. Following complete growth in the Petri dish, culture was kept at 4 °C for short-term storage and restored monthly by mycelium passage into a fresh PDA medium. Inoculation with *R. solani* was carried out using colonized wheat grains at the stem base of four-week-old tomato plants as previously described (Nikraftar et al. 2013). Disease severity was estimated at 7 days post inoculation (dpi) by measuring the lesion length.

In the bioassay with detached leaf discs, the discs were punched out from the tertiary leaves of 4-week-old tomato plants using a 2-cm diameter cork borer. Each leaf disc was placed on a glass slide inside a Petri dish containing a moist filter paper. The pathogen inoculation on tomato leaf discs and disease evaluation were done as previously described (Nikraftar et al. 2013). Intensity of disease symptoms was graded into five classes based on the leaf area infected and disease index (DI) was calculated as described by Taheri and Tarighi (2010).

Treatment with sodium nitroprusside (SNP), DEA-NONOate and inhibitors

Four-week-old tomato plants were treated with 100 μM SNP (Małolepsza and Rózalska 2005) by foliar spray and exposed to light. The specific PAL and LOX inhibitors were used in detached leaf disc bioassay to determine the contribution of phenylpropanoid and octadecanoid pathways in basal resistance and SNP-IR in tomato-*R. solani* interaction. Inhibitor treatments consisted of the PAL inhibitor α-aminooxy-β-phenylpropionic acid (AOPP; Darmstadt) and 5, 8, 11,14-eicosatetraynoic acid (ETYA; Sigma-Aldrich) as a LOX inhibitor . The leaf discs were pre-treated for 2 h with 100 μM AOPP or 20 μM ETYA prior to SNP treatment (24 h). Then, the treated leaf discs were used for inoculation with *R. solani* in a detached leaf disc bioassay. For SNP treatment, which was dissolved in water, sterile distilled water was used as control. In the cases of AOPP and ETYA, which were dissolved in methanol prior to dissolving in water, equivalent volume of methanol was added to control treatment to ensure that it did not interfere with the experiment. Another set of experiments on both intact tomato plants and leaf discs were carried out to compare the effect of SNP with that of an alternative NO donor, diethylamine(DEA)-NONOate (Kovacik et al. 2014), at 100 μM concentration.

Detection of hydrogen peroxide, superoxide and nitric oxide

Formation of H_2O_2 at various time points after inoculation was assayed via the histochemical detection method using a color reaction with 3,3′-diaminobenzidine (DAB), as described by Thordal-Christensen et al. (1997). Polymerization of the DAB molecule at the site of H_2O_2 accumulation and peroxidase activity results in a reddish brown polymer which could be visualized via microscopy. After DAB staining, the leaves were decolorized in ethanol: glycerol (9:1) at 70 °C for 3–4 h (Taheri et al. 2014). After cooling, the leaves were

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mounted in 50 % (*v*/*v*) glycerol and investigated under microscope (Olympus BX41).

In order to detect O_2^- accumulation in the leaves, nitro blue tetrazolium (NBT) staining was performed according to Dong et al. (2009). By NBT reaction with O_2^-, a dark blue foramazan compound could be produced. Removing chlorophyll of the leaves and microscopic analysis were carried out as mentioned above. Finally, both DAB and NBT staining intensities were quantified using Image J (http://rsb.info.nih.gov/ij/index.html) software.

Endogenous NO levels were measured to investigate the effect of PAL and LOX inhibitors on NO accumulation. The NO content was quantified by Griess reagent in plant homogenates prepared using sodium acetate buffer (pH 3.6) as described by Vitecek et al. (2008).

Callose deposition assay

A common plant defense response is deposition of callose, which is involved in cell wall thickening. Callose deposition was investigated by aniline blue staining as described by Nguyen et al. (2010). The stained leaf discs were mounted with 60 % glycerol on glass slides and observed from the adaxial surface of the disc by epifluorescence microscopy (Olympus BX51; USA) using ultraviolet light. Callose intensity was quantified by determining the number of pixels per million pixels using image J software.

Extraction and analysis of phenolic contents

For analysis of total soluble phenolics, 1.0 g of fresh leaves were weighed and extracted by homogenization in 80:20 (*v*/*v*) methanol:water (De Ascensao and Dubery 2003) and centrifugation at 12,000*g* for 10 min . The obtained supernatant was collected and the pellet was homogenized in 80:20 (*v*/*v*) methanol:water and centrifuged again as mentioned above. The two supernatants were combined and used for quantification of total soluble phenolics. The pellet was used for determining concentration of NaOH-hydrolysable cell wall-bound insoluble phenolics according to the method described by Hukkanen et al. (2007). Total content of both soluble phenolic and cell wall-bound phenolic extracts was determined with the Folin-Ciocalteu reagent according to the method of Li et al. (2007). Gallic acid (0–500 mg/l) was used for the standard calibration curve. The results were expressed as gallic acid equivalent (GAE)/g fresh weight (FW).

Quantitative thioglycolic acid assay

Lignification in the leaves of 4-week-old tomato plants with or without SNP treatment at various time points after the pathogen inoculation was quantitatively measured. Lignin was quantified using thioglycolic-acid (TGA) assay in which the lignin bounds to TGA to form thioglycolic acid lignin (TGAL) derivatives which can be extracted from tissue using NaOH and measured spectrophotometrically. Each sample, consisting of 0.5 g of fresh plant tissue sampled at different time points after inoculation, was ground in liquid nitrogen. TGAL derivatives were purified as described by Suzuki et al. (2009). The purified TGAL derivatives were dissolved in 1 M NaOH, and the absorbance was measured at 280 nm using spectrophotometer. The values were calculated based on the lignin curve and expressed as μg of soluble lignin per mg of dry weight.

Estimation of lipid peroxidation

A quantitative index of lipid peroxidation, malondialdehyde (MDA) content, was measured in tomato leaves by the thiobarbituric acid (TBA) reaction as described by Hodges et al. (1999) . Briefly, 1.0 g (FW) of leaf tissue was homogenized in 20 ml 96 % ethanol:water (80:20; *v*/*v*), followed by centrifugation at 3000×*g* for 10 min. Two 0.5 ml aliquotes of the alcoholic extract were taken, one was mixed with 0.5 ml (i)+TBA solution containing 20 % trichloroacetic acid, 0.01 % butylated hydroxytoluene (BHT) and 0.65 % TBA, and the other was mixed with (ii)−TBA solution that had the same composition as solution (i) but without TBA. The mixture was heated at 95 °C for 25 min, cooled and then centrifuged at 4000×*g* for 10 min. Absorbance was measured at 440, 532 and 600 nm . The MDA equivalent was derived from the absorbance according to Hodges et al. (1999).

Enzyme extraction and activity analysis

Activity of PAL was evaluated according to the method of Ramamoorthy et al. (2002) and Dickerson et al. (1984) by slight modifications. Leaf samples (0.5 g) were homogenized in 3 mL of ice cold 0.1 M sodium borate buffer (pH 7.0) containing 1.4 mM of 2-mercaptoethanol and

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0.1 g of insoluble polyvenylpyrrolidone. The extract was centrifuged at 16,000×*g* for 25 min and supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Sample containing 0.4 mL of enzyme extract was incubated with 0.5 mL of 0.1 M borate buffer (pH 8.8) and 0.5 mL of 12 m M L-phenylalanine in the same buffer for 30 min at 30 °C. The reaction was stopped using 0.1 mL HCl 6 N. The amount of trans-cinnamic acid formed was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1} (Dickerson et al. 1984). Enzyme activity was presented as nmol trans-cinnamic acid min^{-1} mg^{-1}protein (Ramamoorthy et al. 2002).

The LOX enzyme was assayed by the method of Rodriguez-Saona et al. (1995), which is based on the increase in absorbance at 234 nm (ΔA234) of the conjugated dienes formed when linoleic acid (used as substrate) was oxidized in the presence of LOX. Absorbance readings were carried out every 30 s for 10 min at room temperature (25 °C) using a spectrophotometer (Biochrom WPA Biowave II, UK). The rate of product formation (V) was calculated by the equation $V=\Delta A234/e\,I\Delta t$. A value of 25.000 M^{-1} $cm^{-1}$was used for the molar extinction coefficient (e) of linoleic acid (Axelrod et al. 1981); the path length (I) was one cm and the reaction time (Δt) was 10 min. LOX activity was expressed as μmol product formed min^{-1} mg^{-1} protein.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from tomato leaves by sodium citrate method (Oñate-Sánchez and Vicente-Carbajosa 2008). After treatment with RNase-free DNase (TURBO DNA free kit, Ambion, USA) to remove contaminating DNA, the RNA was quantified spectrophotometrically. Complementary DNA (cDNA) was synthesised from 100 ng of total RNA using oligo $(dT)_{18}$ primer and SuperScript Reverse Transcriptase (Invitrogen, Germany) and used for PCR amplification. The gene specific primers of *PAL* (accession number: XM004253470.2; listed by Peng et al. 2005), *LOX*B (accession number: XM010325709.1; Song et al. 2010), and *Actin* (accession number: U60480; Carmel- Goren et al. 2003) as an internal control, were used for amplification of corresponding cDNA in different samples. The PCR program used was 3 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing (for *LOX*B: 30 s at 57 °C; *PAL*: 60 s at 55 °C; and for *Actin*: 30 s at 56 °C) and extension at 72 °C (for *LOX*B: 30 s; *PAL*: 60 s; and for *Actin*: 45 s) extention 7 min at 72 °C. Intensities of the obtained bands were quantified using Image J software.

Statistical analysis

All experiments included three independent repetitions carried out with four replications in each repetition. After investigating the variance homogeneity and normality of the obtained data, statistical analyses were performed via non-parametric method using the Kruskall-Wallis multiple comparison tests completed by Mann–Whitney analysis at $P=0.05$ in the SPSS (version 21.0) software.

Results

Application of SNP reduced the disease caused by *R. solani* on tomato leaves via involvement of both octadecanoid and phenylpropanoid pathways.

Based on previous knowledge on the cross talk of NO with various signaling pathways, the experiments were carried out to explore the possibility of using the NO donor SNP for protection of tomato against *R. solani* and investigate the involvement of octadecanoid and phenylpropanoid pathways in this process. In detached leaf disc bioassays, we observed considerable decrease in disease progress and significant reduction of disease index(DI) caused by the pathogen (Fig. 1a) on both partially resistant (CH Falat) and susceptible (Mobil) cultivars treated with 100 μM SNP. Similarly, foliar application of SNP at the same concentration on the intact plants led to significant decrease in the crown and root rot disease caused by *R. solani* on both tomato cultivars in the greenhouse conditions (Fig. 1b). SNP is known to break down and release cyanide and NO. So, to investigate the possibility of attributing the results obtained using SPN to NO accumulation, we conducted subsequent experiments with application of another NO donor, DEA-NONOate. The results obtained using DEA-NONOate were similar to those of the SNP treatment without significant differences, at the used concentrations of NO donors, on both leaf discs (Fig. 1a) and intact plants (Fig. 1b). Application of 100 μM SNP or DEA-NONOate in vitro had no direct effect on vegetative growth of the pathogen.

To determine the role of octadecanoid and phenylpropanoid pathways in SNP-IR to *R. solani* in tomato, detached leaf disc assay was used for investigating

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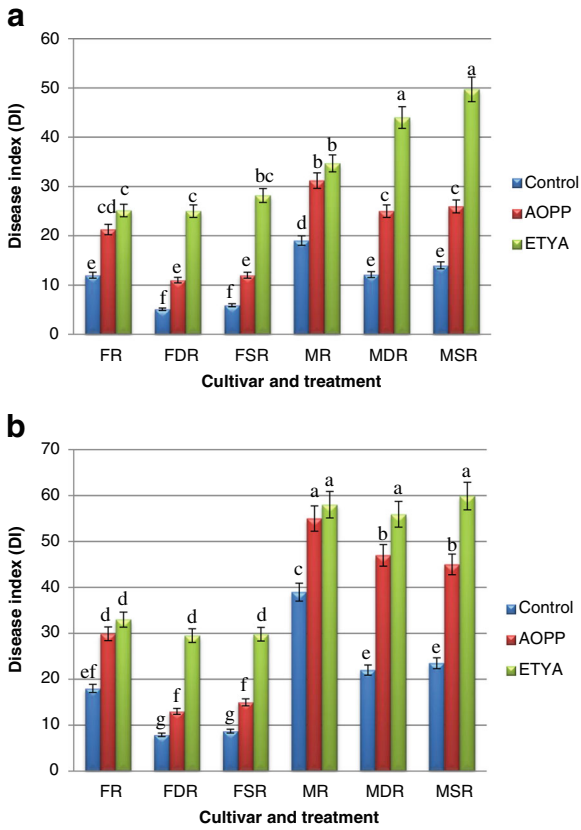

Fig. 1 Disease index on the leaf discs (**a**) and intact tomato plants (**b**) of cultivars CH Falat and Mobil pretreated with sodium nitroprusside (SNP) or DEA-NONOate and then treated with the PAL inhibitor (AOPP) or the LOX inhibitor (ETYA). Disease was evaluated at 5 days post inoculation with *Rhizoctonia solani*. For each cultivar, at least 18 leaf discs from six plants were infected. Statistical data analysis was carried out using SPSS (Version 21.0) software. Different letters indicate significant differences according to Kruskal-Wallis followed by the Mann–Whitney comparison test ($P = 0.05$). Abbreviations in all figures: FR, Falat inoculated by *R. solani*; FDR, Falat treated with DEA-NONOate and inoculated by *R. solani*; FSR, Falat treated with SNP and inoculated by *R. solani*; MR, Mobil inoculated by *R. solani*; MDR, Mobil treated with DEA-NONOate and inoculated by *R. solani*; MSR, Mobil treated with SNP and inoculated by *R. solani*; + SNP, treated with SNP; −SNP, without SNP treatment

effect of the LOX inhibitor ETYA and the PAL inhibitor AOPP, respectively. The in vitro investigations revealed no direct effect of both inhibitors on pathogen growth (data not shown). The ETYA and AOPP were used alone or together with SNP in detached leaf disc bioassay. Both inhibitor treatments had strong effect on basal resistance of tomato leaves to *R. solani* and significantly enhanced susceptibility to the disease in both cultivars. The ETYA treatment led to slightly higher DI compared to AOPP in each cultivars. Co-application of SNP and ETYA or AOPP significantly increased the DI compared to plants only treated with SNP. No significant difference between the DI of CH Falat leaves treated with AOPP plus SNP compared to water treated control revealed that AOPP completely suppressed SNP-IR in this cultivar. Significantly higher DI on the CH Falat leaves treated with ETYA plus SNP compared to control revealed that ETYA completely inhibited SNP-induced defense responses in this cultivar. Similarly, higher disease severity on the Mobil leaves treated with ETYA or AOPP plus SNP compared to control revealed that both inhibitors completely suppressed SNP-IR. Higher DI of the CH Falat and Mobil leaves treated with ETYA compared to controls showed higher importance of ETYA compared to AOPP in suppressing basal resistance and SNP-IR in our pathosystem (Fig. 1a). In all experiments, there was no significant difference between the DI of control leaf discs treated with water and the discs treated with water containing equal amount of methanol as was used in the AOPP and ETYA solution for dissolving it in water (Control+M). So, Control+M treatment did not affect the investigated processes and was not included in the Fig. 1.

Investigation of endogenous NO contents revealed no effect of AOPP and/or ETYA treatments at the used concentrations on levels of this small gaseous molecule in both tomato cultivars tested. However, significantly higher NO accumulation was observed in the partially resistant CH Falat compared to the susceptible Mobil cultivar (Fig. 2).

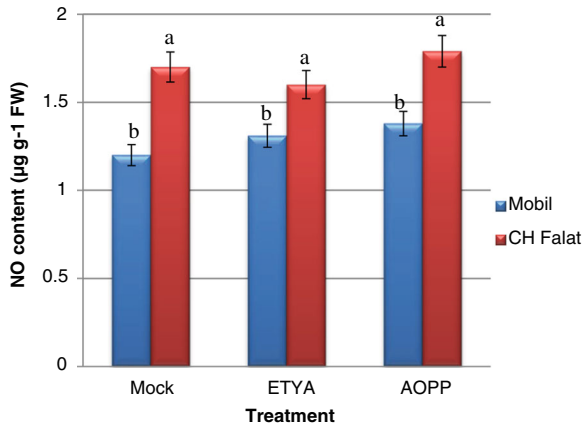

Fig. 2 Effect of AOPP and ETYA on NO content of tomato leaf discs. The leaf discs of cultivars CH Falat and Mobil were treated with 100 µM AOPP or 20 µM ETYA for 2 h and then were used for NO measurement. Equivalent volume of methanol, used for dissolving the inhibitors, was added to water in mock treatment to ensure that it did not interfere with the experiment

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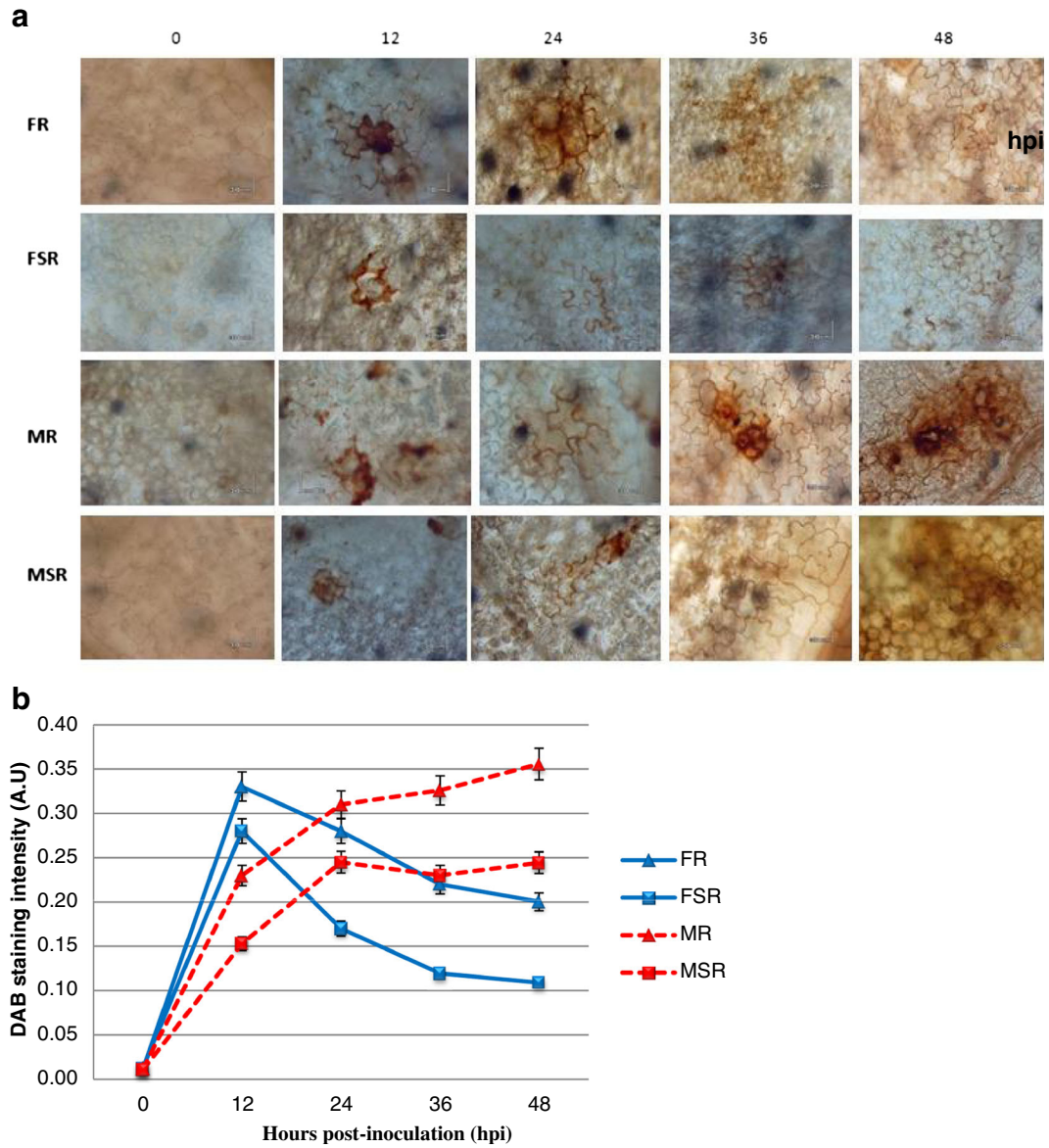

Fig. 3 Histochemical detection of H_2O_2 in the leaves of resistant (CH Falat) and susceptible (Mobil) tomato cultivars pretreated with sodium nitroprusside (SNP) at various time points after inoculation with *Rhizoctonia solani*. The levels of H_2O_2 were detected by 3,30-diaminobenzidine (DAB) staining (**a**) and the staining intensities were quantified using MATLAB software (**b**). Different *letters* indicate significant differences according to Kruskal-Wallis followed by the Mann–Whitney comparison test ($P = 0.05$)

Effect of NO on the levels of ROS in plant cells

Histochemical analyses revealed the presence of $H_2O_2$and O_2^- in the leaves of both tomato cultivars treated with SNP at various time points after inoculation with *R. solani* (Figs. 3 and 4). Accumulation of H_2O_2 at the site of inoculation was higher in CH Falat compared to Mobil cultivar at 12 hpi (Figs. 3a and b). Whereas, in SNP treated samples of both cultivars, considerable decrease was observed in H_2O_2 accumulation at various time points (Fig. 3b). Similar to H_2O_2 burst, priming O_2^- production was observed in the inoculated CH Falat compared to Mobil leaves. At each time point

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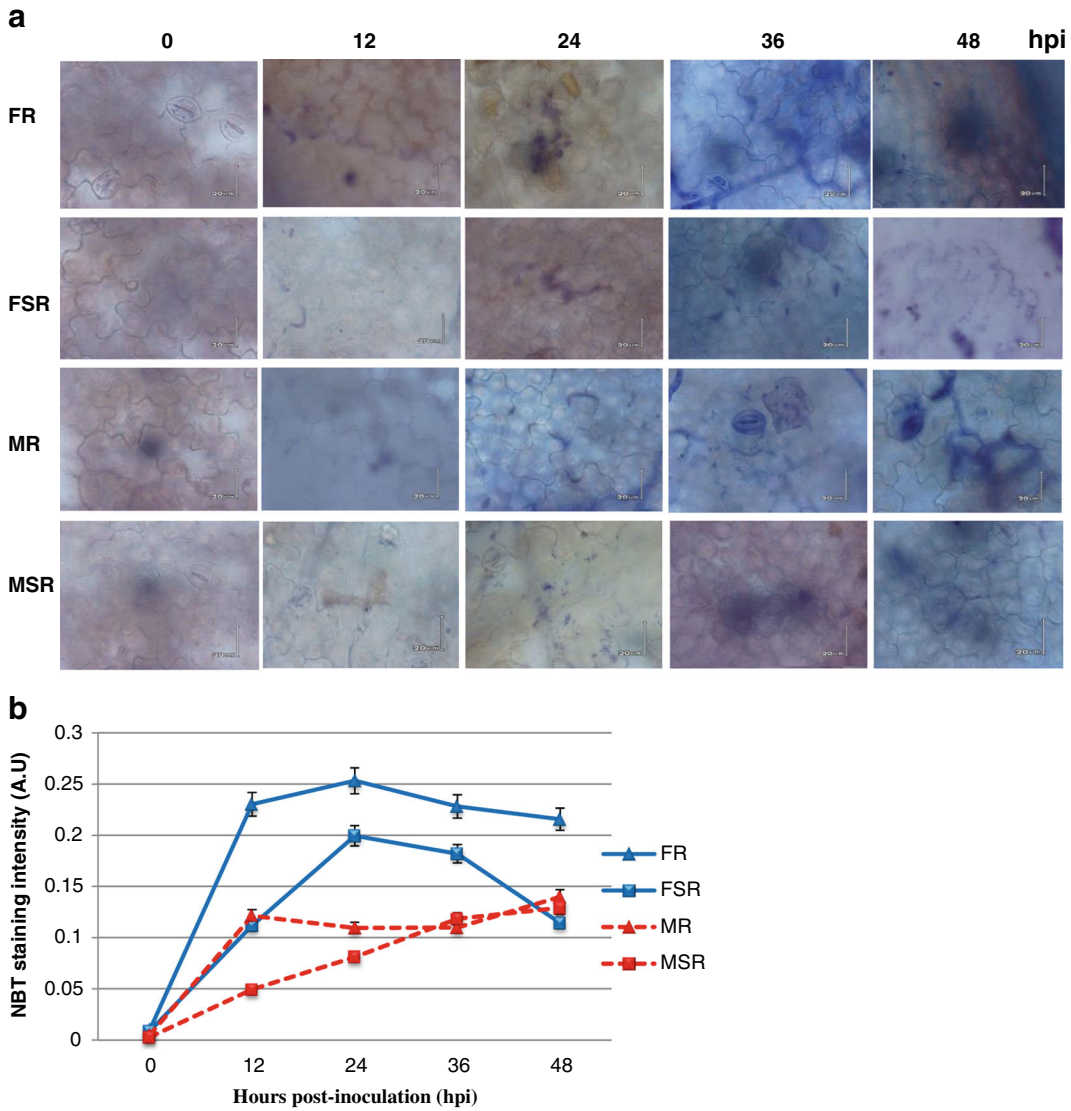

Fig. 4 Histochemical detection of O_2^- in the leaves of resistant (CH Falat) and susceptible (Mobil) tomato cultivars pretreated with sodium nitroprusside (SNP) at various time points after inoculation with *Rhizoctonia solani*. Thelevels of O_2^- wered etected by nitro blue tetrazolium (NBT) staining (**a**) and the staining intensities were quantified using MATLAB software (**b**). Different letters indicate significant differences according to Kruskal-Wallis followed by the Mann–Whitney comparison test ($P = 0.05$)

investigated, O2- production in the SNP-treated CH Falat leaves was considerably lower than that of controls without SNP treatment. In both SNP-treated and non treated inoculated CH Falat samples, the O_2^- level reached to its maximum at 24 hpi and decreased afterwards (Fig. 4a and b). In Mobil cultivar without SNP treatment, higher O_2^- levels were detected compared to SNP-treated Mobil leaves except at 36 hpi (Fig. 4b).

Callose deposition

We analyzed callose deposition, a common response by plants to fungal attack, in the form of at site of penetration. Callose deposition in the epidermal cells of tomato leaves with or without SNP treatment at various time points after inoculation by *R. solani* was investigated. At 12 hpi, higher level of callose deposition was observed in the leaves of partially resistant CH Falat compared to

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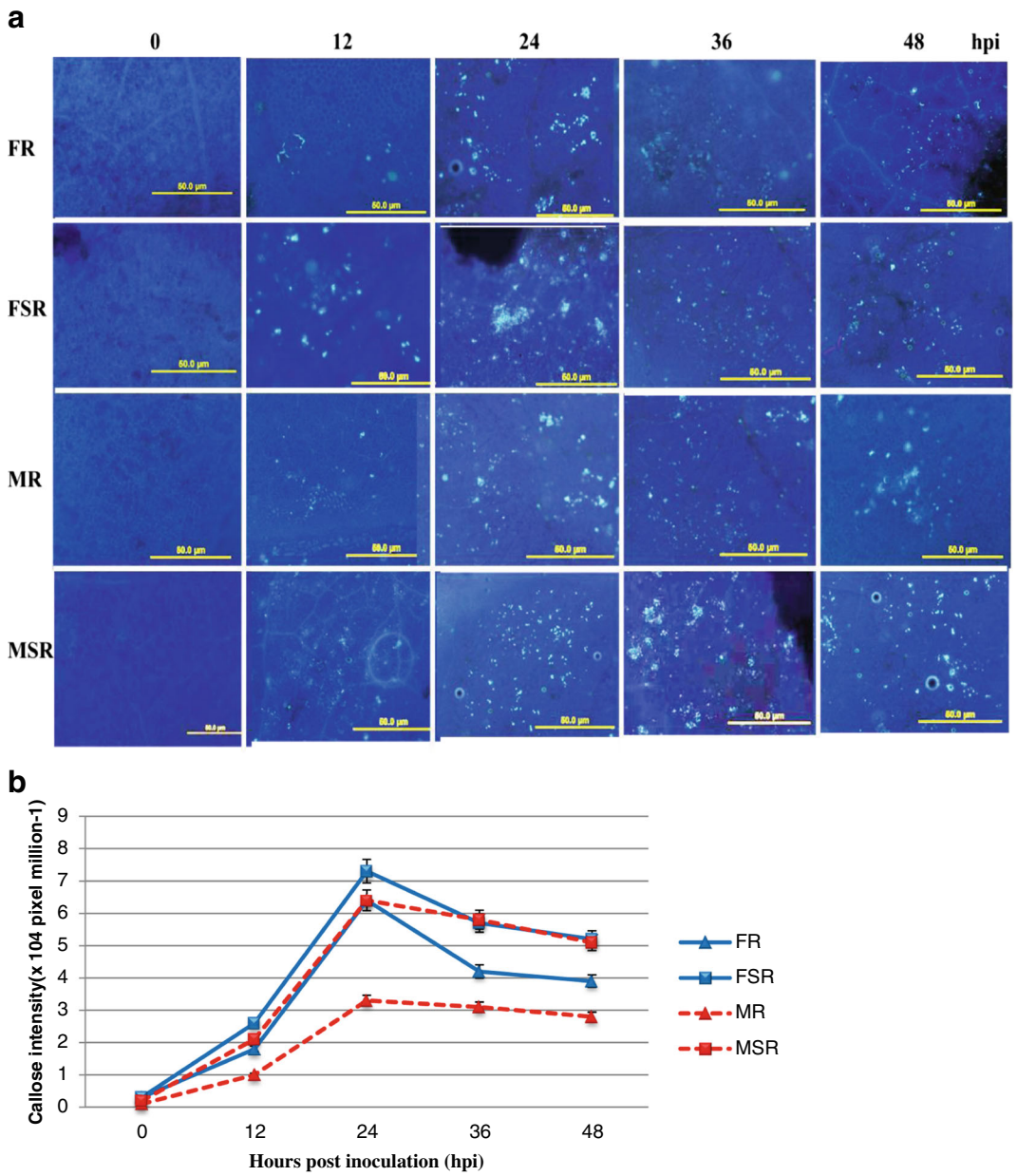

Fig. 5 Callose-staining imaging in the leaf discs of two tomato cultivars pretreated with sodium nitroprusside (SNP; **a**) and callose intensity (**b**) was quantified by determining the number of pixels per million pixels using image J software. Different letters indicate significant differences according to Kruskal-Wallis followed by the Mann–Whitney comparison test ($P = 0.05$)

susceptible Mobil cultivar (Fig. 5a and b). Callose formation in SNP-treated CH Falat was higher than that of untreated leaves of this cultivar at different time points. Similar case was observed for Mobil leaves. At 24 hpi, callose deposition reached to the maximum level in SNP-treated CH Falat and decreased afterward for all samples (Fig. 5b).

Determination of soluble and cell wall-bound phenolic compounds

In the inoculated CH Falat cultivar treated with SNP, the levels of soluble total phenolics were higher than non-treated tissues of the same cultivar and Mobil samples at all time points tested. Production of soluble phenolics

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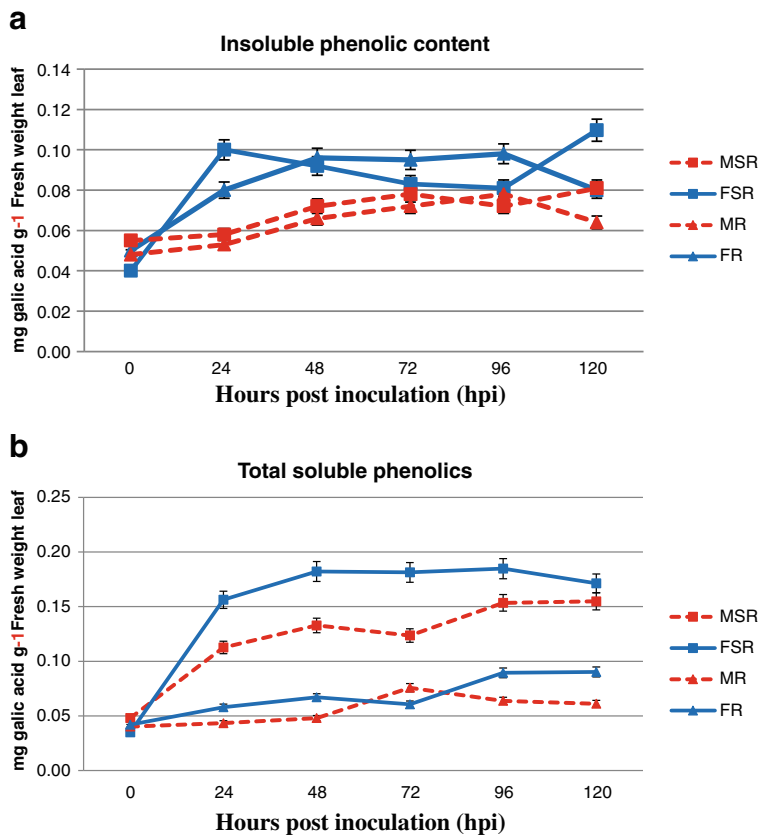

Fig. 6 Total soluble phenolics (**a**) and insoluble phenolic content (**b**) of sodium nitroprussuide. (SNP) treated or untreated leaves of susceptible (Mobil) and partially resistant (CH Falat) tomato cultivars at various time points after inoculation with *Rhizoctonia solani*. Data are means (± standard error) of three replicates of a representative experiment. Each replicate consisted of one sample pooled from six individual plants. The experiment was repeated three times with similar results

reached its maximum at 96 hpi in the SNP-treated CH Falat which was considerably higher than that of Mobil plants at this time point (Fig. 6a).

Compared to soluble phenolics, production of NaOH hydrolysable (insoluble) phenolics were lower. However, priming insoluble cell wall-bound phenolics was observed in SNP-treated inoculated CH Falat plants. Faster and higher accumulation of these phenolics was detected in the FSR samples at 24 hpi as the first peak (Fig. 6b). Afterward, a decreasing rate of cell wall-bound phenolics was observed in SNP-treated CH Falat (FSR) samples until 96 hpi followed by the second peak at 120 hpi. In SNP-treated Mobil leaves, insoluble phenolics increased until 72 hpi and then slightly decreased at 96 hpi with an increase at 120 hpi. Untreated plants in both cultivars showed increasing levels of insoluble phenolics until 96 hpi which followed by a decrease at 120 hpi.

Correlation between SNP treatment and lignification

Thioglycolic acid (TGA) extractable cell wall complexes were measured in order to quantitatively investigate the effect of SNP treatment on lignification in tomato-*R. solani* interaction. The TGA assay revealed that lignin accumulation in both cultivars treated with SNP were considerably higher than that of untreated samples at most of the time points investigated (Fig. 7a). Increasing levels of lignification were observed in all different treatments (FR, FSR, MR, and MSR) by time and it reached to the maximum at 120 hpi for all samples tested. At each time point, highest level of lignin was quantified in FSR samples (Fig. 7a).

Analysis of lipid peroxidation

Investigating lipid peroxidation, as the main mechanism involved in oxidative damage to cell membranes and toxicity process that leads to cell death, showed the levels of thiobarbituric acid reactive substances (TBARS) in tomato cultivars with or without SNP treatment at various time points after *R. solani* inoculation . In SNP-treated CH Falat cultivar, a decreasing trend was observed until 24hpi and then increased till 96hpi.

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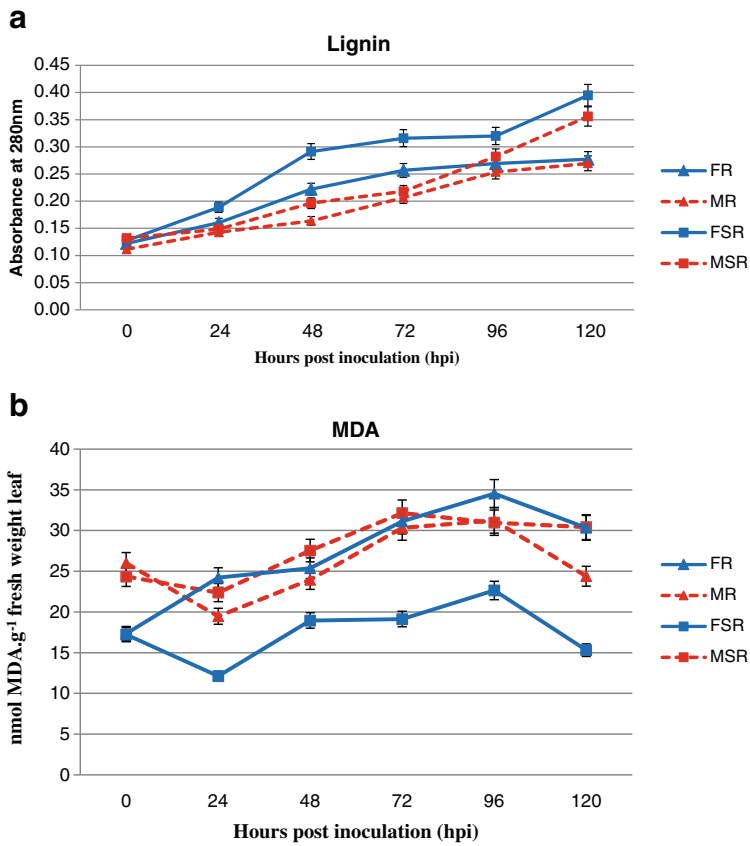

Fig. 7 Lignin (**a**) and malondialdehyde (MDA) content (**b**) in sodium nitroprussuide (SNP) treated or untreated leaves of susceptible (Mobil) and partially resistant (CH Falat) tomato cultivars at various time points after inoculation with *Rhizoctonia solani*. Data are means (± standard error) of three replicates of a representative experiment. Each replicate consisted of one sample pooled from six individual plants. The experiment was repeated three times with similar results

Whereas, MDA increased till 96 hpi in the same cultivar without SNP treatment and then decreased. In Mobil with or without SNP treatment, decreasing MDA content was measured till 24 hpi, followed by an increasing slope until 72 hpi for MSR and 96 hpi for MR samples and then decreased (Fig. 7b). In overall, SNP treatment of the partially resistant CH Falat cultivar leads to lower amount of MDA formation compared to all other samples tested.

Involvement of phenylpropanoid and octadecanoid pathways in basal resistance and SNP-IR in tomato to *R. solani*

To investigate molecular defense mechanisms involved in basal resistance and SNP-IR, the activity and expression of *PAL* and *LOX* were investigated as main markers of phenylpropanoid and octadecanoid pathways, respectively (Figs. 8 and 9). Comparative analysis revealed higher level of the PAL activity in the inoculated CH Falat compared to Mobil cultivar at various time points (Fig. 8a). Application of SNP on the partially resistant CH Falat cultivar increased the PAL activity which reached to maximum level at 48 hpi (Fig. 8a). But, SNP treatment of the susceptible Mobil cultivar slightly decreased the PAL activity at various time points after inoculation.

Priming the LOX activity was observed in CH Falat compared to Mobil plants (Fig. 8a). Maximum activity of this enzyme in CH Falat was observed at 48 hpi, which was in a considerably higher level and earlier time point compared to that of Mobil at 96 hpi (Fig. 8b). In both cultivars, SNP treatment resulted in induced LOX activity at various time points (Fig. 8b).

Both *PAL* and *LOX* genes are known to be involved in basal resistance and responsive to infection by necrotrophic pathogens (Glazebrook 2005; Rance et al. 1998; Taheri and Tarighi 2010). These findings prompted us to investigate whether basal resistance and SNP-related defense responses are associated with these pathways in tomato. The RT-PCR analysis revealed presence of significant differences in *PAL*

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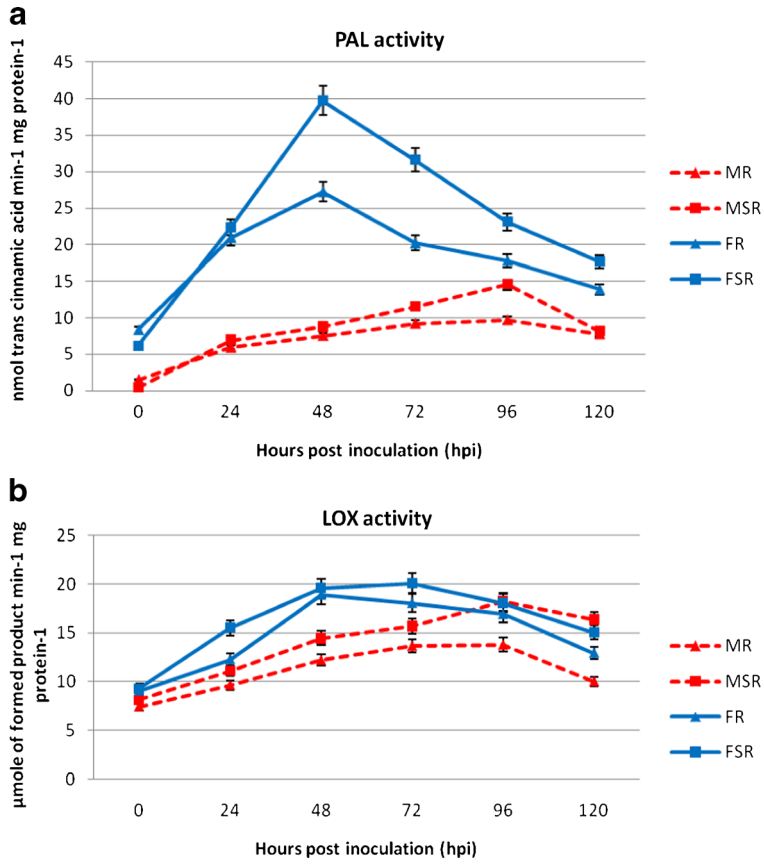

Fig. 8 The activity of Phenylalanine ammonia lyase (PAL; A) and Lipoxygenase (LOX; B) enzymes in sodium nitroprussuide (SNP) treated or untreated leaves of susceptible (Mobil) and partially resistant (CH Falat) tomato cultivars at various time points after inoculation with *Rhizoctonia solani*. Data are means (± standard error) of three replicates of a representative experiment. The experiment was repeated three times with similar results

expression between SNP-treated and untreated plants at most of the time points investigated. Exogenous application of SNP elevated *PAL* expression in both cultivars, which peaked at 48 hpi in CH Falat and later (at 96 hpi) in Mobil. However, in untreated plants, the *PAL* transcripts showed lower levels of accumulation at different time points (Fig. 9a, b). Similarly, the *LOXB* expression was significantly higher in SNP-treated plants of both cultivars compared to untreated controls at most of the time points (Fig. 9a, c). Interestingly, in the partially resistant cultivar, priming transcript accumulation of both *PAL* and *LOXB* genes was observed compared to the susceptible cultivar.

Discussion

Plant cell wall is a dynamic physical complex of polysaccharides and glycoproteins which is a barrier against pathogens and its reinforcement leads to limitation of pathogen progress in the host plant tissue (Underwood 2012). Cell wall modification can affect plant development and responses to pathogens by two modes of response: changes in mechanistic properties and initiation of signaling pathways (Pogorelko et al. 2013). Some plants may alter the cell wall biosynthetic pathways leading to better defense by reinforcing cell walls via accumulation of callose, lignin, phenolics, and glycoproteins (Bellincampi et al. 2014; Mandal et al. 2013).

Involvement of NO in cell wall-related defense responses of two tomato genotypes, with different basal defense against *R. solani*, was investigated in the present study. This experimental setup revealed involvement of the same defense mechanisms in both genotypes tested. We demonstrated that NO plays a crucial role in the initiation of fast defense responses of tomato leaves to the necrotrophic pathogen *R. solani*. Nitric oxide can react with O_2^- to form the peroxinitrite anion ($ONOO^-$), which is resulted in decreased level of ROS (Asai and Yoshioka 2009). So, decreased levels of O_2^- and H_2O_2 observed in tomato-*R. solani* interaction in the present study could be explained by this phenomenon.

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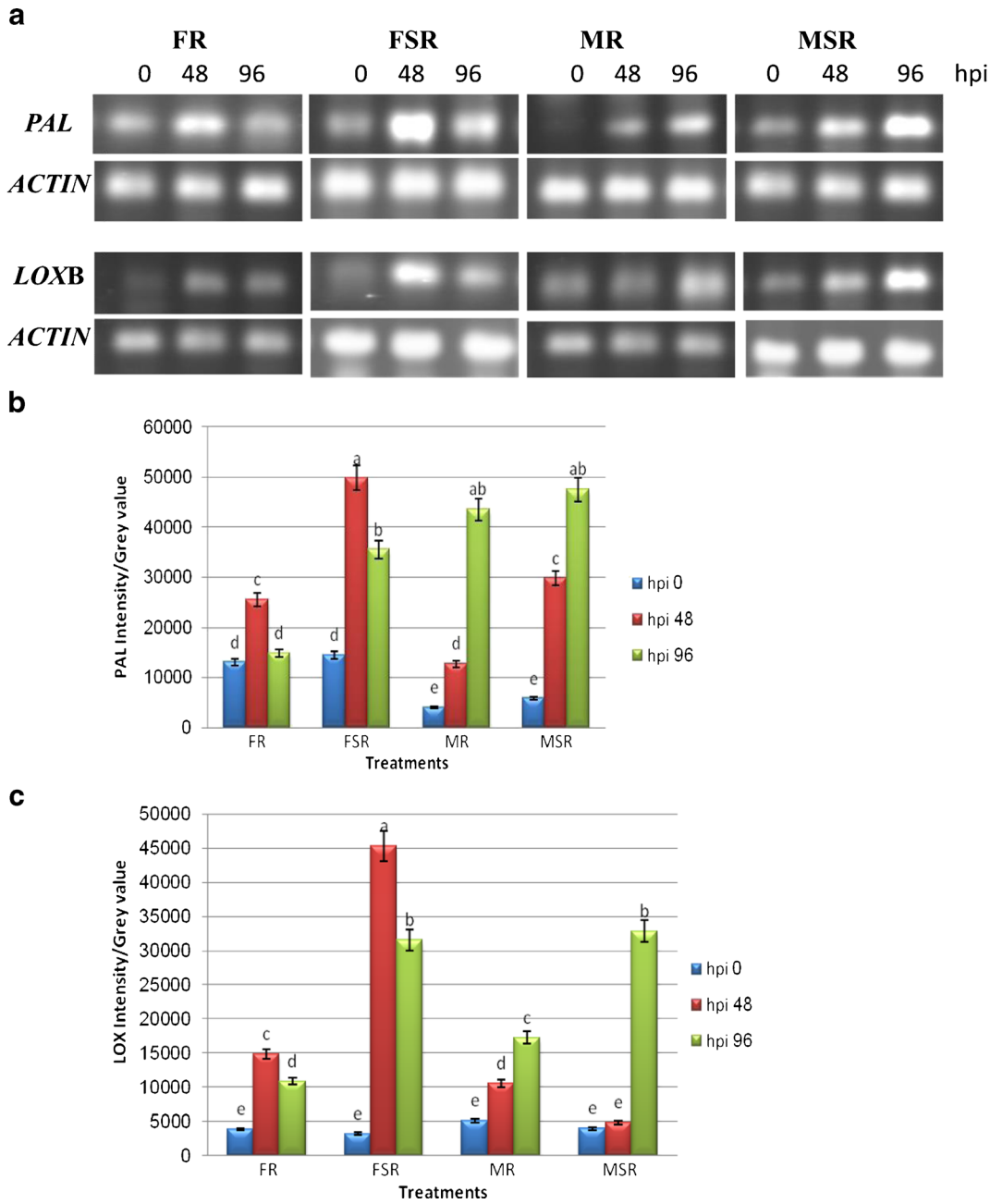

Fig. 9 Effect of sodium nitroprusside (SNP) on *PAL* and *LOX* transcript accumulation in *Rhizoctonia solani* infected tomato cultivars. The 4-week-old tomato plants were sprayed with SNP or mock (0.05 % Tween 20) until runoff and inoculated with *R. solani* at 1 dpt. At each of the indicated time points after inoculation, fully expanded third leaves from six plants were collected, pooled and subjected to RNA isolation for using in RT-PCR (**a**). Intensities of the obtained bands (**b**, **c**) were quantified using Image J software. Statistical data analysis was carried out using SPSS 21.0 for Windows. Different letters indicate significant differences according to Kruskal–Wallis followed by the Mann–Whitney comparison test ($P = 0.05$)

Similarly, NO inhibited wound-inducible H_2O_2 generation in tomato (Orozco-Cardenas and Ryan 1999) andthe same effects were observed in tomato –*Botrytis cinerea* interaction (Yang et al. 2011). Recent findings confirmed that NO promotes the wound-healing response of plant tissue (Arasimowicz et al. 2008), and

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plants attacked by an avirulent pathogen leading to cell wall reinforcement (Grob et al. 2013). Also, Talukdar (2013) showed enhanced resistance in common bean by exogenous NO in arsenic stress through detoxifying ROS and significant increase of peroxidase activity.

The level of H_2O_2 at the earliest stage of penetration and various time points has been shown to be decisive for the outcome of several tomato-pathogen interactions (Asselbergh et al. 2007; Nikraftar et al. 2013). Also, H_2O_2 could be involved in cell wall reinforcement by increasing protein cross-linking and enhancing phenolics production (Taheri and Tarighi 2011; Asselbergh et al. 2007).

This research demonstrated that NO triggered the priming state of tomato defense, since the SNP-treated plants revealed increased capability to express cytomolecular defense responses after *R. solani* inoculation. Application of SNP led to increased resistance of tomato cultivars to the pathogen which was associated with priming PAL and LOX activity and expression, lignin formation, callose deposition and phenolics accumulation. Similar to these findings, Durner et al. (1998) reported that the first enzyme of phenylpropanoid biosynthesis pathway, PAL, is induced after infiltration of tobacco leaves and cells with NO donors. Furthermore, involvement of NO in oxidative burst regulation and PAL activation induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures has been shown by Wang and associates (2006) which is in agreement with our data. We found higher LOX activity in SNP-treated plants which was similar to the findings of Wang and Wu in *Taxus* cells (2005).

The *PAL* gene was upregulated by SNP treatment, suggesting an important role of NO in activating phenylpropanoid pathway in tomato-*R. solani* interaction. Priming *PAL* expression and the activity of corresponding enzyme in the partially resistant compared to the susceptible plants indicated the involvement of PAL in tomato basal resistance to *R. solani*. Increases were observed in the PAL activity and its transcript accumulation in both cultivars with or without SNP treatment at various time points after infection. This finding is in accordance with previous reports about enhanced PAL activity after *R. solani* inoculation as a mechanism involved in basal resistance (Singh et al. 2012) and biologically induced resistance (Patil et al. 2011) in tomato.

For more in depth investigating the role of PAL and phenylpropanoid pathway in resistance against phytopathogens, several PAL inhibitors have been used to suppress *PAL* expression and activity (Moerschbacher et al. 1990; Carver et al. 1994). The PAL inhibitor AOPP reduced basal resistance and strongly suppressed SNP-IR in both cultivars, whereas, it did not affect the endogenous NO levels in tomato leaf discs. In a similar investigation, using AOPP led to increased susceptibility of barley lines to powdery mildew caused by *Erysiphe graminis* (Carver et al. 1994). Also, treatment of resistant wheat plants with PAL inhibitors decreased the frequency of lignified host cells and concomitantly led to increased fungal growth (Moerschbacher et al. 1990). So, these studies support the relationship between formation of lignin and host plant resistance to phytopathogens as we demonstrated in tomato-*R. solani* pathosystem.

In tomato, five isoforms of lipoxygenases (LOXA, LOXB, LOXC, LOXD, and LOXE) have been reported so far (Chen et al. 2004). However, the role of each isoform in basal resistance and NO-associated defense responses against pathogens is unclear. To further determine the function of LOX and the octadecanoid pathway in tomato basal resistance to *R. solani*, we used ETYA as a potent inhibitor of *LOX*B expression and LOX activity, which has previously been used in several pathosystems (Hamiduzzaman et al. 2005; Taheri and Tarighi 2010). In this research, the LOX inhibitor ETYA reduced basal resistance of tomato to the pathogen and completely inhibited SNP-activated defense responses in both partially resistant and susceptible cultivars. ETYA treatment had no effect on endogenous NO levels in tomato leaf discs. Consequently, the LOX and octadecanoid signaling pathway plays an important role in basal resistance and NO-related defense responses in this pathosystem. These findings are in accordance with the effect of ETYA on increasing plant susceptibility and suppressing riboflavin-induced resistance in rice-*R. solani* interaction (Taheri and Tarighi 2010). Application of the PAL and LOX inhibitors led to reduction of BABA-IR, suggesting that callose deposition is a defense mechanism associated with phenylpropanoid and octadecanoid pathways (Hamiduzzaman et al. 2005). Using DEA-NONOate, as an independent NO donor, supported the data obtained by SNP on disease progress and the role of PAL and LOX in NO-related protection of tomato against *R. solani*. Detection of higher endogenous NO levels in the partially resistant CH Falat compared to the susceptible Mobil cultivar supported the role of this defense-related signaling molecule in resistance of tomato plants to *R. solani*.

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Our investigations revealed higher levels of callose deposition in the partially resistant CH Falat plants compared to the susceptible Mobil cultivar. Also, SNP treatment enhanced callose formation in both cultivars tested, which was correlated with SNP-activated defense. Likewise, Paris et al. (2007) found that application of SNP as a NO donor resulted in activation of callose deposition and wound healing-related defense genes (e.g. *PAL*), in damaged areas of potato leaflets. Also, Arasimowicz et al. (2008) demonstrated that using SNP led to increased callose and lignin formation in pelargonium leaves. Therefore, NO might be involved in callose deposition as a cell wall-related defense mechanism in various plants against pathogens.

Deposition of callose at the site of infection in early stage of penetration was observed in this study. Importance of timely elevated early callose deposition at the site of penetration to slow or even stop pathogen invasion in powdery mildew-Arabidopsis interaction has been shown by Ellinger et al. (2013)). Interaction of NO and H_2O_2 in initial tomato defense against *Colletotrichum coccodes* via cell wall modification at sites of appressoria formation was noted by Wang and Higgins (2006) and an increased numbers of penetration sites with cross-linked proteins and callose was observed when NO was increased via SNP application, which is in agreement with our data. According to Prats et al. (2005), NO might be a potent messenger in cell wall–associated defense responses which support the results presented here.

Soluble and cell wall-bound phenolics accumulate in plant tissues challenged by fungal pathogens. Lignins are complex cell wall phenolics which result from oxidative polymerization of monolignols (Gabaldón et al. 2004). This study revealed involvement of lignifications in basal resistance and SNP-activated defense responses in our pathosystem. Deposition of lignin has been shown to be involved in cotton resistance to *Verticillium dahliae* and in defense of *Camelina sativa* to *Sclerotinia sclerotiorum* (Xu et al. 2011; Eynck et al. 2012). Similarly, a possible regulatory effect of NO on xylem cell wall lignification of *Zinnia elegans* has been proposed by Ferrer and Ros Barceló (1999).

The MDA contents are used as an index of membrane peroxidation, which indicates the degree of injury to membranes due to ROS accumulation. The NO may act as an antioxidant by scavenging ROS to protect plant cells from oxidative damage (Grob et al. 2013). Exogenous SNP treatment decreased MDA, H_2O_2 and O_2^- contents in the plants exposed to salt stress conditions (Wu et al. 2011; Shi et al. 2007), which is similar to our findings in tomato-*R. solani* interaction.

The present study demonstrated NO activated tomato defense mechanisms against *R. solani* via priming octadecanoid and phenylpropanoid pathways, which resulted in cell wall-related defense responses. We observed earlier and stronger cell wall modifications including callose deposition, phenolics accumulation and lignification at early time points after infection by the necrotrophic fungus *R. solani* in the SNP-treated plants compared to controls. These defense mechanisms were involved not only in SNP-activated defense, but also in basal resistance in the tomato-*R. solani* pathosystem. Also, an increasing trend of phenolics accumulation, *PAL* and *LOX* expression and activity in time course of 0 to 48 hpi suggested that NO had a positive effect on both the phenylpropanoid and octadecanoid pathways. The major role of NO in resistance to *R. solani* can be interpreted in its regulatory function on defense gene expression, ROS levels and cell wall modification. However, further experiments are necessary to more fully investigate the role of NO as a signal inducer and defense activator in the context of plant-pathogen interactions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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