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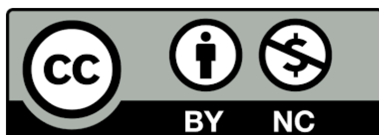

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Nitric oxide-induced modulation of physiological and molecular responses in tomato (*Solanum lycopersicum* L.) plants infected with *Xanthomonas perforans*

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Abstract: Bacterial spot, caused by *Xanthomonas perforans*, significantly threatens global tomato production. Traditional control methods have proven inadequate, necessitating innovative approaches. This study assessed nitric oxide (NO) pretreatment at 0.5, 1, and, 2 mM concentrations to induce abiotic resistance against *X. perforans* in greenhouse-grown tomatoes. Results indicated that NO pretreatment enhanced plant growth, biomass, and defense mechanisms under both normal and pathogen-stressed conditions. Specifically, NO regulated physiological processes such as lipid peroxidation, chlorophyll content, and fluorescence parameters. It also modulated key enzymes like NADPH oxidase and phenylalanine ammonia lyase (PAL), bolstering antioxidant defenses by scavenging reactive oxygen species, thereby reducing oxidative damage and improving stress tolerance. Additionally, NO induced sugar accumulation, increasing energy reserves during stress, and elevated amino acid content, facilitating defense protein synthesis. Notably, NO pretreatment significantly reduced disease severity by upregulating defense-related genes, including non-expressor of pathogenesis-related genes (NPR), PAL, and peroxidase (POD). Overall, NO pretreatment improved plant health, decreased pathogen proliferation, and increased biomass, highlighting its potential as an environmentally friendly strategy for managing bacterial diseases and activating specific resistance responses in tomato plants.

Keywords: Bacterial spot, abiotic resistance, disease management, systemic acquired resistance.

Introduction

Protecting crops from plant diseases is crucial to meet the global demand for food production. Pests and pathogens are widely recognized as major constraints in agricultural production systems, leading to significant yield losses (Suman et al., 2021). Among vegetable crops, tomatoes, being one of the most economically important, suffer the greatest damage due to their susceptibility to pests and diseases (Asiry et al., 2022; Haghpanah et al., 2023). The lack of pest- and disease-resistant tomato cultivars results in significant market losses (Akram et al., 2024). In 2022, the total global production of tomatoes reached 187 million tons. This production came from 5.5 million hectares of harvested land. Furthermore, tomato yields are expected to increase in the future (Asiry et al., 2022).

Various pests and diseases contribute to tomato crop losses, with tomato bacterial spot (BS) standing out as one of the most devastating diseases, causing yield reductions of over 50% during outbreaks (Osdaghi et al., 2021). This disease, caused by four *Xanthomonas* species (*X. euvesicatoria*, *X. gardneri*, *X. perforans*, *and X. vesicatoria*), has a global distribution, particularly in warm and humid regions (Osdaghi et al., 2021). BS Management has traditionally relied on antibiotics, copper (Cu)-based bactericides, and Cu-mancozeb. However, these methods raise concerns about negative environmental impacts (Lamichhane et al., 2018), the development of resistance genes (Strayer-Scherer et al., 2018), and potential hostile effects on human and animal health (Hristozov et al., 2018; Khan et al., 2023b). Consequently, it is imperative to develop sustainable plant conservation strategies that consider the intricate relationships between plants and biological threats (Haghpanah et al., 2024a).

Nitric oxide (NO) is a simple diatomic molecule consisting of one nitrogen atom double-bonded to one oxygen atom. As a signaling molecule and secondary messenger (Khan et al., 2023a; Aloui et al., 2024), NO counteracts reactive oxygen species (ROS)-induced toxicity and completely regulates numerous physiological processes under various stress conditions (Astier et al., 2018; Hussein, 2024). Earlier research highlighted its vital role in protecting plants against herbivores (Han et al., 2023), fungi (Khan et al., 2023b), viruses (Zhang et al., 2021a), and bacteria (Khan et al., 2023b). These compounds coordinate the plant defense system against bacterial pathogen infection by activating a series of defensive reactions that hinder pathogen growth and spread (Khan et al., 2023b). Furthermore, NO induces the defense-related genes and activates ROS production (Khan et al., 2023b). It also modulates the activity of plant defense proteins (Zhu et al., 2019) and induces the production of stress-related hormones, including Salicylic Acid (SA), Jasmonic Acid (JA), and Ethylene (ETH), which coordinate plant defense responses against bacterial pathogenic infections (Sami et al., 2018; Haghpanah and Namdari, 2024). Genes induced by NO have been shown to positively regulate systemic acquired resistance (SAR) in Arabidopsis leaves in response to the bacterial strain Pst DC3000 avrB. Inoculation of Arabidopsis with Pst DC3000 significantly reduced bacterial growth and ion leakage, leading to increased expression of pathogenesis-related (PR) genes PR1 and PR2 (Shahid et al., 2019). Furthermore, tomato plants treated with the NO donor sodium nitroprusside improved the expression of defense-related genes and decreased the severity of bacterial infection by inducing systemic resistance (Kolomiiets et al., 2021). Applying exogenous NO postharvest has been shown to reduce anthracnose disease caused by the fungus *Colletotrichum gloeosporioides* in custard apple (*Annona squamosa* L.) (Khaliq et al., 2021) and maintain apple fruit quality (Khaliq et al., 2021)and pepper (González-Gordo et al., 2023) during ripening. Increased NO production was observed in rice plants infected with rice black-streaked dwarf virus (RBSDV), and the defense system of these plants against RBSDV infection was enhanced using NO donors (Lu et al., 2020). NO also triggered defense responses in apple fruit against black spot disease caused by Alternaria alternata, reducing lesion diameter and accumulation of the Alternaria toxin (Han et al., 2023).

Despite evidence suggesting that NO can positively influence plant reactions to biotic and abiotic stresses, its role in managing tomato bacterial spot disease caused by *X. perforans* remained underexplored. This study hypothesizes that NO application can improve tomato resistance to

bacterial spot disease by enhancing growth and activating defense mechanisms. The objectives are: (1) to assess NO's effect on the growth of infected plants, (2) to examine its impact on key defense enzymes, and (3) to analyze its influence on defense gene expression. Understanding these effects may help develop new strategies to enhance disease resistance in tomatoes.

Materials and Methods

Sample preparation and treatment application

For this study, the tomato cultivar CH Falat, known for its susceptibility to *X. perforans*, was employed. Seeds were obtained from Falat Seed Company, disinfected, and sown in pots containing a soil mixture of perlite, coco pith, and peat moss in a 1:2:1 ratio. The plants were cultivated in a greenhouse under a photoperiod of 16 hours light and 8 hours darkness, with a temperature of 24°C and a relative humidity of 60%. At the three-true-leaf stage, 30-day-old plants were subjected to a 2×4 factorial experiment arranged in a completely randomized design (CRD) with three replications. Initially, the plants were treated with 0.5, 1 and 2 mmol sodium nitroprusside (SNP) as NO donor, followed by inoculation with the *X. perforans* pathogen 72 hourlater. The *X. perforans* strain, obtained from Plant Pathology Department at Sari Agricultural Sciences and Natural Resources University, was inoculated directly into the leaves at a concentration of 108 CFU/mL (Wang et al., 2017b). Experimental treatments labeling is presented in Supplementary Table S1.

Plant sampling and analysis

Plants samples were collected at 12, 36, 108, and 324 hour post incubation (hpi) with *X. perforans*. Plant tissues from three replicates (three plants per replicate) were instantly frozen in liquid nitrogen and stored at -80°C for subsequent genetic and biochemical analyses. To determine dry weight, samples were dried in an oven at 68°C for 48 hours.

Physiological parameters

Hydrogen peroxide (H_2O_2) content and lipid peroxidation measurement

The H_2O_2 content was measured using 0.1% trichloroacetic acid (TCA) (Alexieva et al., 2001). The absorbance of the samples was measured at 390 nm. Furthermore, lipid peroxidation content in membrane lipids was assessed by measuring the concentration of malondialdehyde (MDA) and other aldehydes generated by this process. Hence, fresh leaf tissue sample (0.2 g) was crushed in a porcelain mortar with 5 mL of 0.1% TCA. Then, 1 mL of the resulting supernatant after centrifugation was mixed with 4 mL of a 20% TCA solution containing 0.5% thiobarbituric acid (TBA). This mixture was heated in a water bath at 95°C for 30 minutes, cooled in ice, and centrifuged again at 10,000 g for 10 minutes. The solution absorbance was measured using a spectrophotometer at 532 and 600 nm, respectively (Valentovic et al., 2006). The concentration was measured using a standard curve plotted within the range of 100 25000 nmol H_2O_2 (Y = 0.4349X + 0.0771, R2 = 0.98).

Photosynthetic pigments and chlorophyll a fluorescence kinetics

Chlorophyll a and chlorophyll b contents were measured using the Lichtenthaler method (Valentovic et al., 2006) with 80% acetone as the solvent. Absorbance was measured at 460, 645, and 663 nm. The following formulas were used to calculate chlorophyll a, chlorophyll b, and carotenoids (Lichtenthaler 1987).

Chl*a* (μg. ml−1) = 16.72 A665.2 − 9.16 A652.4

Chl*b* (μg. ml−1) = 34.09 A652.4 −15.28 A665.2

Carotenoid (μg. ml−1) = (1000 A470 − 1.63 Chl*a* −104.96 Chl*b*)/221

Besides, chlorophyll fluorescence parameters were recorded from the last fully expanded leaf under dark-adapted conditions for 15 minutes and illuminated conditions using a PAM-2500 fluorometer (H. Walz, Effeltrich, Germany). Minimum fluorescence (Fo), with all reaction centers of photosystem II open, was determined by a low-intensity actinic light (1 μmol m^{-2} s^{-1}), and maximum fluorescence (Fm) was determined by a saturating pulse (8000 μmol m^{-2} s^{-1}) for one second on dark-adapted leaves. Subsequently, the variable fluorescence (Fv) and maximum efficiency of photosystem II (Fv/Fm) and the effective quantum yield of photosystem II photochemistry (Y(II)) were calculated using the parameters determined in dark-adapted and illuminated leaves. This calculation was based on (Fm - Fo)/Fm and (Fm -

Ft)/Fm´, respectively, as outlined by (Alexieva et al., 2001).

Antioxidant enzyme activity measurement

The enzyme extract content from tomato leaves was obtained by adding 1.8 mL of extraction buffer (50 mM Tris-HCl, pH 8, containing 1% Triton X-100 and 0.1% mercaptoethanol) to 200 mg of powdered leaf tissue (Bradford, 1976). The mixture was then centrifuged at 15,000 × g for 15 minutes at 4°C, and the resulting supernatant was collected for total protein quantification and enzyme activity analysis. Enzyme extracts were prepared for the analysis of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase, Superoxide Dismutase (SOD), and Cytosolic Peroxidase (POD) (Ghadirnezhad Shiade et al., 2023a). The assay mixture (1 mL) contained Tris buffer (pH 8.0, 50 mM), nitroblue tetrazolium (NBT), NADPH (0.1 mM), and 100 µL of the enzyme extract. Furthermore, SOD activity was measured according to (Beauchamp and Fridovich, 1971). Then, reaction mixture (5 mL) comprising potassium phosphate buffer (50 mM), methionine (0.013 mM), EDTA (0.1 µM), riboflavin (2 µM), and enzyme extract was subjected to the fairly intense light (300 Lmol m^{-2} s^{-1}) for 3 minute. One enzyme unit in this experiment was estimated to be equivalent to 50% of the NBT optical reduction at 560 nm compared to the control (Haghpanah et al., 2021). The peroxidase (POD) enzyme activity was determined following the method of (Tang and Newton, 2005). The reaction mixture contained 1 mL of potassium phosphate buffer (50 mM, pH 7.0), 50 µL of enzyme extract, 20 mM guaiacol, and 30 mM hydrogen peroxide. The reaction was initiated by the addition of hydrogen peroxide, and the absorbance increase at 470 nm was monitored for 2 minutes using a spectrophotometer. The POD activity was calculated based on an extinction coefficient of 26.6 mM^{-1} cm^{-1}. One unit of peroxidase activity was defined as the enzyme amount required to convert one micromole of substrate per minute. The extinction coefficient (0.0266 mM^{-1} cm^{-1}) and the formula $A = \varepsilon bc$ were applied to calculate enzyme activity.

Measurement of metabolite activity in the lignin biosynthesis pathway

Phenylalanine ammonia-lyase (PAL) measurement

PAL activity was assayed using (Hahlbrock and Grisebach, 1979). To initiate the enzyme reaction, 1 mL of extraction buffer, 10 mM phenylalanine, double-distilled water, and enzyme extract were combined and incubated at 37°C for 1 hour. The reaction was terminated by the addition of 6 M HCl, and the absorbance of the sample was determined at 290 nm. Enzyme activity was quantified as one unit, defined as 1 µmol/min/mg protein.

Cell wall peroxidase (CW-POD) activity was measured by (Patra and Mishra, 1979). CW-POD extraction was performed by homogenizing the tissue in 50 mM sodium phosphate buffer (pH 7.0, 1:5 w/v), followed by centrifugation at 10,000 × g for 15 min at 4°C. The pellet was resuspended in 1 M NaCl (in 50 mM phosphate buffer, pH 7.0) and incubated at 4°C for 1 hour with gentle shaking. After centrifugation, the supernatant was collected as the CW-POD extract.

Cell wall peroxidase activity activity (CW-POD) and total phenolic content (TPC) measurement

CW-POD activity was measured by determining of tetraguaiacol content produced in one minute in the presence of electrons generated from the decomposition of H_2O_2 by the enzyme. The analysis was conducted using a Shimadzu UV-1600 spectrophotometer (Shimadzu Corporation, Japan). The reaction mixture included 3 mL of 25 mM phosphate buffer (pH 6.8), guaiacol 20 mM, H_2O_2 40 mM, and 10 µL of enzyme extract. The extinction coefficient (ε) of tetraguaiacol at 470 nm is 6.26 L/(mmol·cm) (Patra and Mishra, 1979). Besides, TPC of the extracts was determined using the Folin-Ciocalteu method. 1.6 mL of phenolic extract was mixed with 0.4 mL of Folin-Ciocalteu reagent and 4 mL of sodium carbonate solution. The mixture was then incubated in the dark for 90 minutes at room temperature, followed by reading the absorbance at 760 nm. The TPC was calculated using a standard curve provided with gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh tissue (Singleton and Rossi, 1965).

Lignin content and amino acid content determination

To measure lignin content, the acetyl bromide method developed by Fukushima and Hatfield (2001)Fukushima and Hatfield (2001)Fukushima and Hatfield (2001)Fukushima and Hatfield

(2001)Fukushima and Hatfield (2001) was utilized. Accordingly, the sample's absorbance was measured at 280 nm using a spectrophotometer (Shimadzu Corporation, Japan). The lignin content was then calculated using the specific absorption coefficient of 20 g/L cm^2 at 280 nm. Moreover, the leaves' amino acid content (TAA) was determined using the Ninhydrin method with spectrophotometry at 570 nm (Cone et al., 1976).

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated using Trizol reagent (Riragene, Iran) following the manufacturer's protocol. To eliminate any contaminating DNA, the extracted RNA was subsequently treated with *DNase* I (Fermentas, Germany). purified RNA was used for cDNA synthesis using the RevertAid™ Reverse Transcriptase kit (Fermentas, Germany) according to the manufacturer's instructions. Specific primers for *Defensin*, *NPR1, PAL*, and *POD* genes were used to amplify PCR products (Supplementary Table S2) and the *Actin* gene was used as an internal reference. These primers were designed after aligning the sequences obtained from the NCBI gene bank (http://www.ncbi.nlm.nih.gov/) using BioEdit 7.0.9.0 and OligoExplorer V 1.4 software. The designed primers were confirmed for non-specific binding and dimer formation using Primer Blast on the whole genome. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). The Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used for qRT-PCR reactions. The BIORAD CFX96 Real-Time System was utilized for the analysis of gene expression data.

Statistical analysis

Analysis of variance and mean comparisons analyses were performed using Duncan's test through SPSS25 software (at the 5% probability level). Graphs were created using Excel version 2018 software.

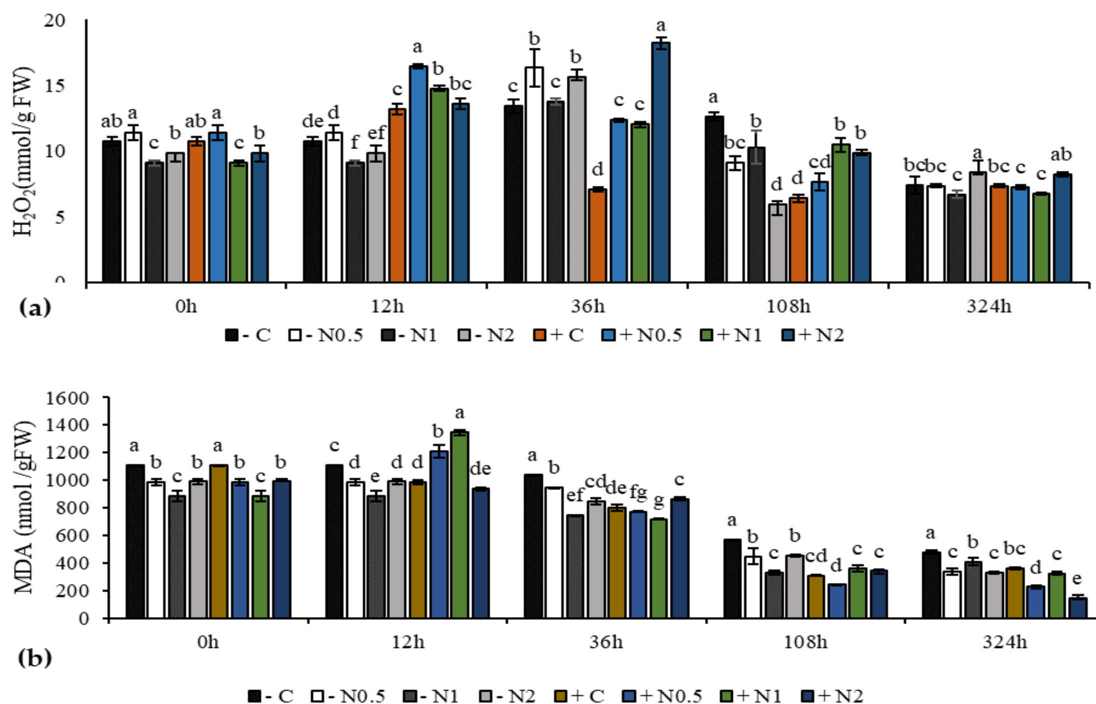

Figure 1. Effect of nitric oxide (NO) pre-treatment on H_2O_2 content (a) and lipid peroxidation (b) in tomato leaf samples treated and untreated with *Xanthomonas perforans* bacteria at time intervals of 0, 12, 36, 108, and 324 hours post inoculation (hpi). Data represent the mean of three replicates ± SE. Numbers with different letters have a significant difference ($P \leq 0.05$). (-C): treated with 5 mL of sterile distilled water; (-N0.5): pretreated with 0.5 mM SNP, (-N1): pretreated with 1mM SNP, (-N2): pretreated with 2 mM SNP; (+C): Control pretreated with *Xanthomonas perforans*, (+N0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+N1): pretreated with 1 mM SNP+ *X. perforans*, (+N2): pretreated with 2 mM SNP+ *X. perforans*

Results

NO boosts plant defense

X. perforans infection induced significant decline in leaf dry weight (LDW), with -C (infected without NO priming) plants exhibiting a 23.75% reduction at 108 hpi compared to 0 hpi, highlighting the detrimental impact of bacterial stress on biomass accumulation. However, NO pre-treatment effectively mitigated this loss, as LDW in +NO0.5, +NO1, and +NO2 plants increased by 19.9%, 15.1%, and 9.9%, respectively, relative to -C plants at 108 hpi. Notably, at 324 hpi, the +NO1 treatment displayed the most pronounced effect, enhancing LDW by 15.2%, suggesting a sustained protective role of NO (Table 1). Besides, the obtained images showed that inoculation with *X. perforans* bacteria induced typical hypersensitive response (HR) symptoms in tomato leaves within 24 hours, leading to the death of the inoculated leaf tissue. However, pre-treatment of tomato leaves with different NO concentrations prevented the development of *X. perforans*-induced HR. This effect was most pronounced at +NO0.5 treatment (Supplementary Figure 1). In contrast, +NO2 pre-treatment with resulted in general chlorosis of the inoculated leaf tissue. Diaminobenzidine (DAB) staining revealed a significant increase in POD activity upon pathogen inoculation, which was significantly reduced in +NO0.5 plants but increased again in +NO1 and +NO2 plants (Supplementary Figure 1).

Table 1. Effect of nitric oxide (NO; 0, 1, and 2 mM) on the leaf dry weight (LDW) changes at time intervals of 0, 12, 36, 108, and 324 hours (hpi) in tomato leaf samples treated and untreated with *Xanthomonas* perforans bacteria (+ and -).

	hpi (h)				
Treatment	**0**	**12**	**36**	**108**	**324**
+C	2.165 ± 0.07 b	2.165 ± 0.07 bc	2.465 ± 0.12 a	3.407 ± 0.04 ab	5.6 ± 0.03 cd
+NO0.5	2.51 ± 0.03 a	2.51 ± 0.03 a	2.325 ± 0.22 ab	3.253 ± 0.11 ab	6.337 ± 0.23 ab
+NO1	2.25 ± 0.09 b	2.25 ± 0.09 b	2.346 ± 0.16 ab	3.047± 0.14 bc	6.449 ± 0.24 a
+NO2	2.175 ± 0.02 b	2.175 ±0.02 bc	2.155 ± 0.01 ab	3.547± 0.17 a	6.291 ± 0.03 abc
-C	2.165 ± 0.07 b	2.03 ± 0.04 cd	2.408 ± 0.06 a	2.753 ± 0.03 c	5.684 ± 0.19 bcd
-NO0.5	2.51 ± 0.03 a	1.935 ± 0.04 d	1.979 ± 0.03 b	3.30 ± 0.23 ab	5.6 ± 0.3 cd
-NO1	2.25 ± 0.09 b	1.85 ± 0.01 d	2.206 ± 0.04 ab	3.167 ± 0.08 ab	5.46 ± 0.27 de
-NO2	2.175 ± 0.02 b	1.925 ± 0.12 d	2.108 ± 0.08 ab	3.027 ± 0.09 bc	4.853 ± 0.27 e

Data represent the mean of three replicates ± SE. Means with different letters have a significant difference ($P \geq 0.05$). hpi: hour post inoculation. (+C): Control pretreated with *X. perforans*, (+NO0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+NO1): pretreated with 1 mM SNP+ *X. perforans*, (+NO2): pretreated with 2 mM SNP+ *X. perforans*, (-C): treated with 5 mL of sterile distilled water; (-NO0.5): pretreated with 0.5 mM SNP, (-NO1): pretreated with 1mM SNP, (-NO2): pretreated with 2 mM SNP

H_2O_2 content and lipid peroxidation

The results showed that in control plants (without NO, -C), *X. perforans* inoculation initially caused a 22.9% increase in H_2O_2 content at 12 hpi, followed by a decrease of 47.2% and 49.1% at 36 and 108 hpi, respectively. However, after two weeks of bacterial infection, the H_2O_2 content did not differ significantly from that in control plants (Figure 1a). On the other hand, NO treatment in infected plants has led to a significant increase in H_2O_2 content in most treatments. For instance, 36 hours after disease, 0.5 mM, 1 mM, and 2 mM NO treatments increased H_2O_2 content by 74.3%, 69.5%, and 157%, respectively, compared to +C plants (Figure 1a). Conversely, *X. perforans* bacterial inoculation and NO priming alone caused a notable decrease in lipid peroxidation in most treatments compared to -C plants (Figure 1b). However, in infected plants at 12 and 36 hpi, NO priming resulted in a significant

increase in lipid peroxidation in most treatments compared to +C plants (Figure 1b).

Photosynthetic pigments and fluorescence kinetics

Regarding photosynthetic pigments, we observed a significant decrease in chlorophyll a content in +C plants at 12, 36, and 108 hpi with *X. perforans*. The reductions were 45.3%, 46.2%, and 48.1%, respectively. However, after two weeks of bacterial infection, the chlorophyll a content did not differ significantly from that of the control plants (Supplementary Figure 2a). Notably, NO treatment in infected plants resulted in a significant increase in chlorophyll a content in most treatments (Supplementary Figure 2a). The chlorophyll b content did not exhibit significant differences between +C and -C plants for most time points after stress. However, a decrease of 3.18% was observed after 108 hpi (Figure 2b). NO treatment in infected plants generally did not lead to a significant change in chlorophyll b content (Figure 2b). However, at 324 hpi, +NO0.5 and +NO2 pre-treatments improved chlorophyll b content in infected plants, while the 1 mM NO concentration reduced chlorophyll b content compared to +C at 108 and 324 hpi (Supplementary Figure 2b). Disease stress alone did not cause a significant change in the maximum quantum efficiency of photosystem II (Fv/Fm) in tomato plants (Figure2a).

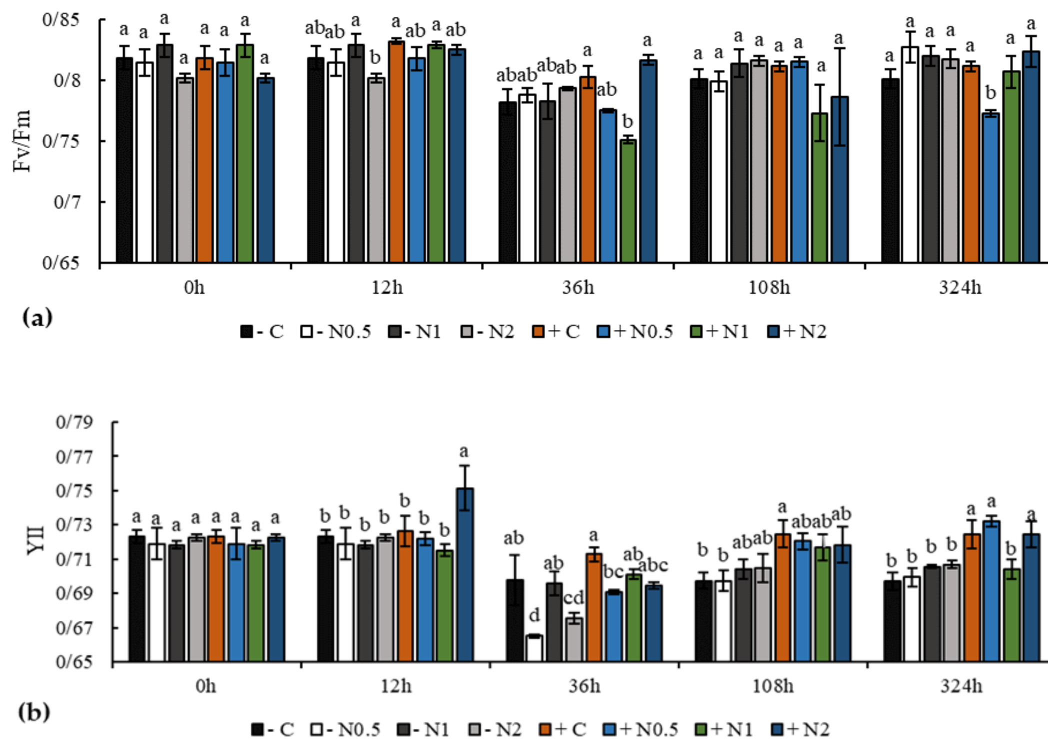

Figure 2. Effect of hydrogen sulfide (H2S; 0, 1, and 2 mM) on the time course changes of chlorophyll a fluorescence parameters, including (a) Fv/Fm, (b)Y(II) at 0, 12, 36, 108, and 324 hours post inoculation (hpi) at 36, 108, and 324 hpi in leaf samples of tomato plants treated and untreated with *Xanthomonas perforans* bacteria (+ and -). Data represent the mean of three replicates ± SE. (-C): treated with 5 mL of sterile distilled water; (-N0.5): pretreated with 0.5 mM SNP, (-N1): pretreated with 1mM SNP, (-N2): pretreated with 2 mM SNP; (+C): Control pretreated with *X. perforans*, (+N0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+N1): pretreated with 1 mM SNP+ *X. perforans*, (+N2): pretreated with 2 mM SNP+ *X. perforans*.

Furthermore, the interaction between bacteria and NO did not lead to a significant change in the maximum quantum efficiency of photosystem II (Fv/Fm) at most time points after the onset of

disease. However, at 36 and 324 hpi, concentrations of 1 and 0.5 mM of NO significantly decreased Fv/Fm by18.3% and 46.2%, respectively (Figure 2a). Moreover, inoculation with *X. perforans* resulted in a significant increase in the effective photochemical quantum yield of photosystem II (Y(II)) only at 324 hpi. Accordingly, NO treatment individually did not typically lead to a significant change. Howevere, at 36 hpi, +NO0.5 treatment caused a 7.4% decrease in Y(II), while +NO2 decreased this parameter by 2.3% (Figure 2b). Besides, the combination of bacteria and the inducer did not result in a significant change in the effective quantum efficiency of photosystem II photochemistry (Figure 2b). Whereas, it led to a 4.3% increase at a 2 mM NO treatment and 12 hpi and a significant decrease in Y(II) at 36 hpi and 324 hpi.

Activity of Antioxidant enzymes

After inoculation, the activity of NADPH oxidase in plants began to decrease. Compared to control plants, NADPH oxidase activity was reduced by 2.26% after 14 days of infection (Figure 3a).

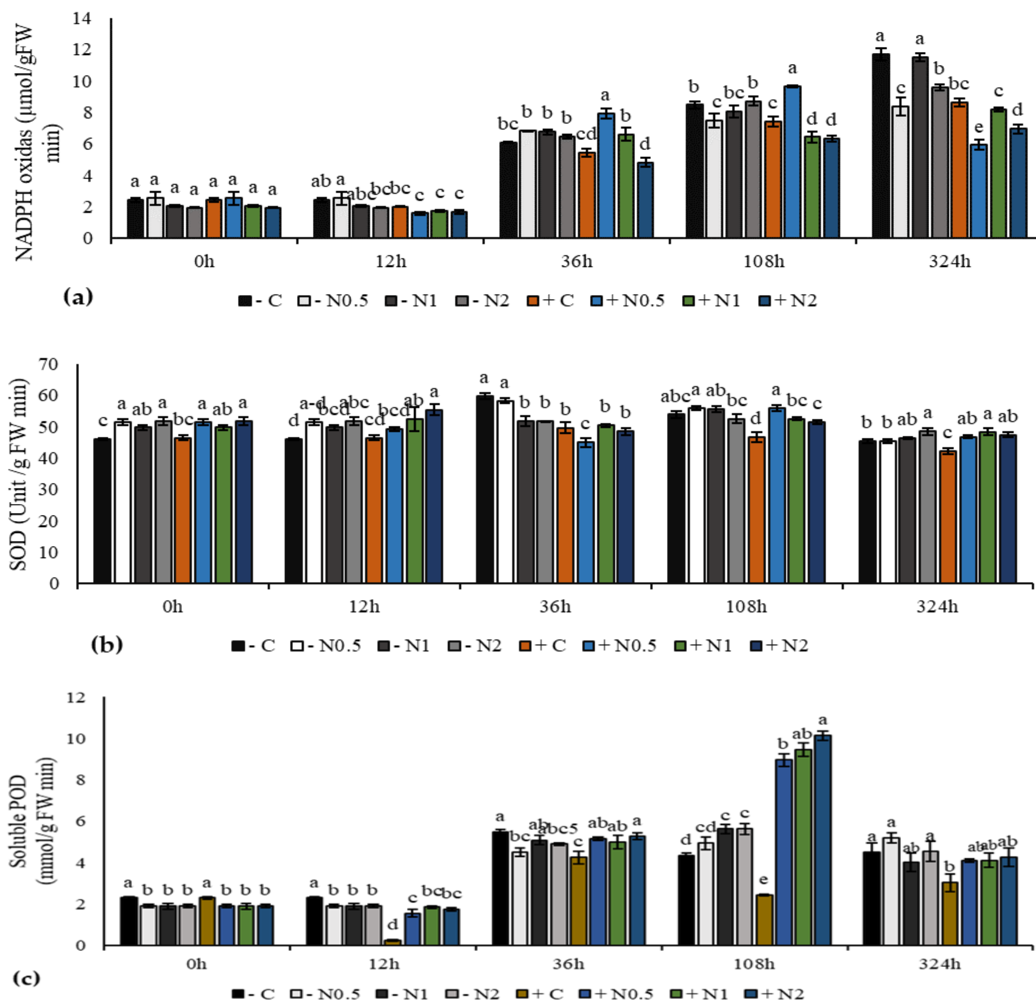

Figure 3. Time-course changes in the activity of (a) NADPH oxidase, (b)Super oxide dismutase (SOD), and (c) soluble Peroxidase (POD) at 0, 12, 36, 108, and 324hours post inoculation (hpi) in *Xanthomonas perforans*-treated and untreated tomato leaf samples. Data are means of three replicates ± SE. Numbers with different Latin letters have significant differences ($P \leq 0.05$). (-C): treated with 5 mL of sterile distilled water; (-N0.5): pretreated with 0.5 mM SNP, (-N1): pretreated with 1mM SNP, (-N2): pretreated with 2 mM SNP; (+C): Control pretreated with *X. perforans*, (+N0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+N1): pretreated with 1 mM SNP+ *X. perforans*, (+N2): pretreated with 2 mM SNP+ *X. perforans*

However, NO treatment in infected plants at 36 hpi led to a 30% and 46% increase in this enzyme activity at +NO0.5 and +NO1 treatments, respectively. At 108 hpi, +NO0.5 treatment resulted in a 30% increase in NADPH oxidase activity, while +NO1 and +NO2 treatments led to a 13% and 15% decrease, respectively. Additionally, 324 hours post-inoculation, at the same treatments (concentrations of 0.5 and 2 mM) resulted in a 3.31% and 4.19% decrease in NADPH oxidase activity, respectively (Figure 3a). The activity of the SOD enzyme did not exhibit a significant change in the early hours following bacterial infection. However, at 36, 108, and 324 hpi, SOD activity decreased by 17%, 4.13%, and 1.7%, respectively, compared to the uninfected control (Figure 3b). Treatment with 2 mM NO at 0 and 324 hpi increased SOD activity, while treatment at 36 hpi decreased it. SOD activity showed a significant increase in the *X. perforans* + NO interaction treatment at most sampling times (Figure 3b). Besides, POD acttivity decreased in *X. perforans* inoculated plants (Figure 3c). Besides, NO treatment individually led to a slight decrease in soluble POX in most cases, while it increased at 108 hpi. The interaction treatment of bacteria with the inducer significantly increased soluble POD activity in most cases, with the highest increase observed at 108 hpi (Figure 3c).

Lignin activity biosynthesis pathway enzymes

The results indicated that *X. perforans* inoculation did not lead to a significant change in PAL activity in plants without the NO inducer. However, when treated with NO, PAL activity decreased in most cases (Figure 4a). The combination of bacteria and the inducer did not consistently affect PAL activity. However, it led to a further decrease in PAL activity at all tested concentrations after 36 hpi. Moreover, +NO1 at 0 and 324 hpi caused a 4.20% and 3.18% reduction in PAL activity, respectively (Figure 4a). CW-POD activity did not show a significant change in *X. perforans* inoculated plants in the early hours of sampling, but it decreased by 3.14% and 5.17% at 108 and 324 hpi, respectively (Figure 4b). Besides, individual NO treatment did not cause a significant change in most treatments, while this value slightly decreased by the +NO1 treatment at 108 hpi and the +NO0.5 treatment at 324 hpi. While the interaction of bacteria with the inducer did not cause a significant change at 0 and 12 hpi, it significantly increased CW-POD activity at all NO concentrations used at 36, 108, and 324 hpi (Figure 4b). The highest increase in CW-POD was observed at 324 hpi. According to (Figure 4c), NO pre-treatment had mixed effects on TPC, with both increases and decreases observed depending on concentration and time point. The interaction between NO and bacterial infection was also evident, with some combinations leading to significant changes in TPC.

Lignin content did not change significantly with bacterial infection. At 324 hpi, pre-treatment with -NO0.5 resulted in a 17% decrease in lignin content in the plant. However, at 0 hpi, pre-treatment with -NO0.5 and -NO1 resulted in a 32.3% and 42% increase in lignin content compared to -C plants (Figure 4d). By 324 hpi, the interaction of 1 mM inducer and *X. perforans* bacteria led to a 30.8% decrease in lignin content. Besides, no significant differences was observed in the control conditions at other concentrations (Figure 4d).

Total amino acids (TAA) content

TAA content in the leaves increased by 39.1%, 66.3%, and 17.5% at 12, 36, and 324 hpi, respectively, compared to -C plants after bacterial infection. NO priming alone increased TAA content in most treatments. In *X. perforans*-inoculated plants, NO priming increased TAA content in most treatments but decreased at 36 hpi and did not cause a significant change at 12 and 108 hpi (Table 2)

Expression pattern of Defensin, NPR, PAL, and POD genes

According to the results, the expression level of the Defensin gene did not change significantly during X. perforans bacterial infection (Figure 5a). Treatment with -NO0.5 increased the expression level of this gene by 16.5 times 36 hpi. In addition, treatment with -NO1 resulted in a significant 31.8-fold increase in Defensin gene expression at 36 hpi. In the inducer-disease interaction treatments, treatments +NO1 increased the expression level of the Defensin gene by 16.5 and 2.8 times at 12 and 108 hpi, respectively. Besides, Figure 5b exhibited the expression levels of the NPR gene in response to NO inducer under X. perforans bacterial infection and non-infection treatments. Bacterial infection significantly reduced the expression level of the NPR gene.

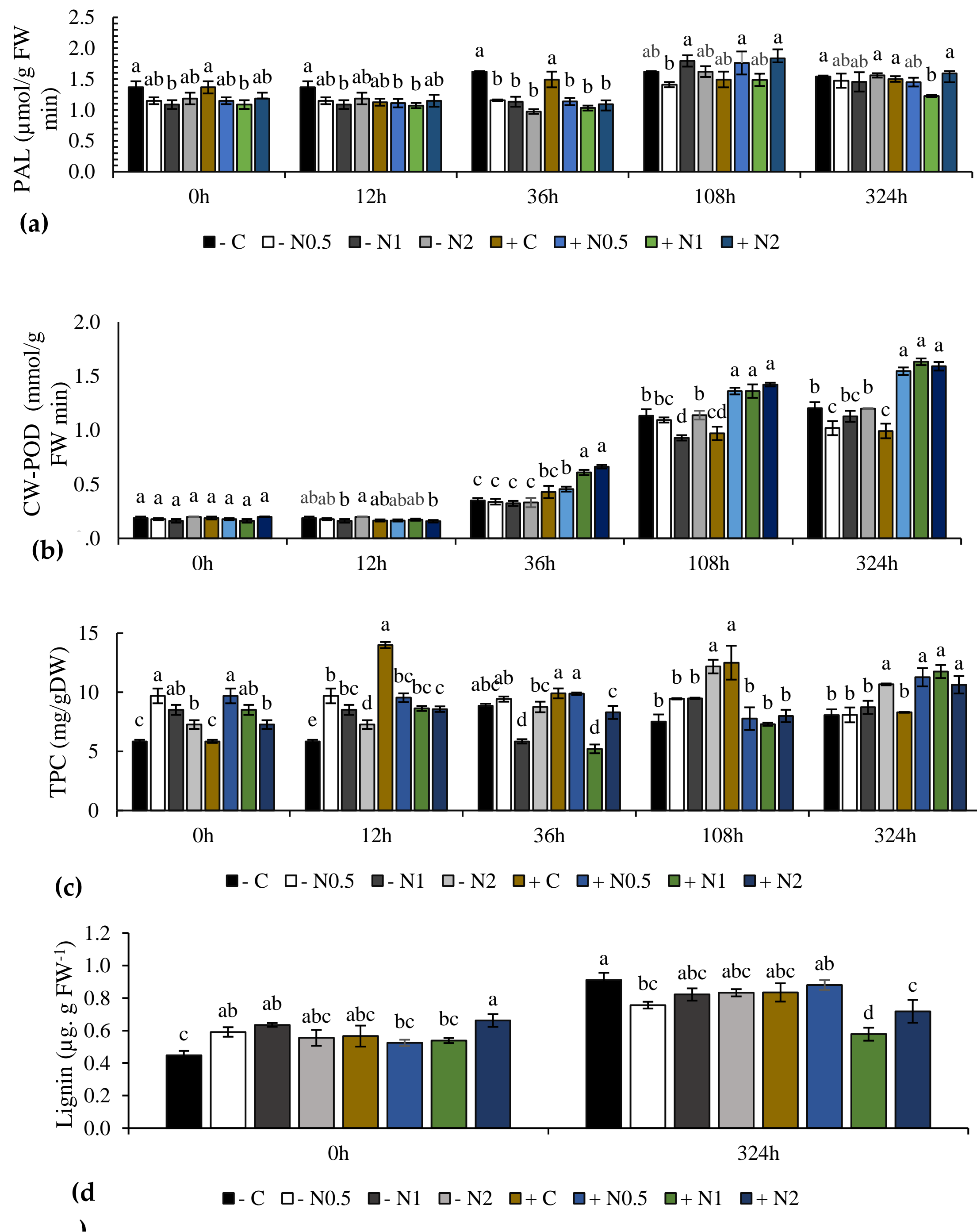

Figure 4. Time-course changes in (a)phenylalanine ammonia-lyase (PAL) enzyme, (b)cell wall peroxidase (CW-POD), (c) total phenol content (TPC), and (d)lignin at 0, 12, 36, 108, and 324 hours post inoculation (hpi) in *Xanthomonas perforans* treated and untreated tomato leaf samples. Data are means of three replicates ± SE. Numbers with different Latin letters have significant differences (P ≤ 0.05). (-C): treated with 5 mL of sterile distilled water; (-N0.5): pretreated with 0.5 mM SNP, (-N1): pretreated with 1mM SNP, (-N2): pretreated with 2 mM SNP; (+C): Control pretreated with *Xanthomonas perforans*, (+N0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+N1): pretreated with 1 mM SNP+ *X. perforans*, (+N2): pretreated with 2 mM SNP+ *X. perforans*

Table 2. Effect of nitric oxide (NO; 0, 1, and 2 mM) on the total amino acid (TAA) changes at time intervals of 0, 12, 36, 108, and 324 hours (hpi) in tomato leaf samples treated and untreated with *Xanthomonas perforans* bacteria (+ and -).

Treatment	hpi (h)				
	0	12	36	108	324
+C	15.00 ± 0.7 c	15.00 ± 0.7 c	17.00 ± 0.5 e	14.00 ± 0.6 b	10.00 ± 0.6 e
+NO0.5	20.06 ± 0.3 a	20.06 ± 0.3 a	20.17 ± 0.4 bc	13.23 ± 0.8 b	12.56 ± 0.3 c
+NO1	19.14 ± 0.9 ab	19.14 ± 0.9 ab	18.71 ± 0.9 d	14.02 ± 0.7 b	11.20 ± 0.8 de
+NO2	17.94 ± 0.2 b	17.94 ± 0.2 b	19.33 ± 0.6 cd	12.64 ± 0.5 b	11.90 ± 0.2 cd
-C	14.91 ± 0.7 c	20.75 ± 0.7 a	28.31 ± 0.6 a	13.23 ± 0.7 a	11.29 ± 0.8 cd
-NO0.5	20.06 ± 0.3 a	20.21 ± 0.3 a	19.56 ± 0.7 cd	16.98 ± 0.5 a	15.06 ± 0.3 b
-NO1	19.14 ± 0. 9 ab	20.39 ± 0.9 a	21.21 ± 0.89 b	16.29 ± 0.9 a	15.35 ± 0.6 a
-NO2	17.94 ± 0. 2 b	19.91 ± 0.2 b	20.21 ± 0.5 bc	17.48 ± 0.1 a	17.08 ± 0.2 b

Data represent the mean of three replicates ± SE. Means with different letters have a significant difference ($P \geq 0.05$). hpi: hour post inoculation (+C): Control pretreated with *Xanthomonas perforans*, (+NO0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+NO1): pretreated with 1 mM SNP+ *X. perforans*, (+NO2): pretreated with 2 mM SNP+ *X. perforans*, (-C): treated with 5 mL of sterile distilled water; (-NO0.5): pretreated with 0.5 mM SNP, (-NO1): pretreated with 1mM SNP, (-NO2): pretreated with 2 mM SNP.

Application of +NO1 and +NO2 inducer significantly increased the expression level of NPR in plant leaves in the absence of disease stress by 3.1 and 5.1 times at 108 hpi sampling times, respectively. At 12 hpi, the increase was observed only at 2 mM, and at 36 hpi, an increase was seen at all concentrations used. The expression level of this gene was observed to increase (Figure 5b). Treatment with *X. perforans* had no significant effect on the expression of *PAL* and *POD* genes (Figs. 5c, d). However, after 12 hpi, the NO inducer significantly increased the expression levels of genes at all concentrations studied in the absence of disease stress. Specifically, the expression level of *POD* gene increased by 18.5 times, with 2 mM NO treatments (Figs. 5 c). Furthermore, the disease-inducer interaction treatment significantly enhabced the expression levels of *PAL* gene at 12 hpi in 0.5 mM NO concentration. Treatment with +NO0.5 increased the expression levels of *PAL* by 2.5 times (Figs. 5c, d).

Discussion

This study examined the effectiveness of nitric oxide (NO) pretreatment in enhancing resistance to tomato bacterial spot disease caused by *X. perforans*. It assessed biochemical and physiological changes in tomatoes treated with NO at different concentrations. The results revealed that *X. perforans* treatment caused typical bacterial spot disease symptoms, including chlorosis, necrosis, and hypersensitive response (HR) lesions. These symptoms appeared as water-soaked, dark, circular to irregular spots surrounded by chlorotic halos. These findings are consistent with those of previous reports on tomatoes(Potnis et al., 2015) and peppers (González-Gordo et al., 2023). Enlargement of these lesions decreases the amount of photosynthetic leaf tissue because of an increased ratio of necrotic tissue, which promotes chlorophyll degradation(Potnis et al., 2015). Necrotic tissue and gradual leaf yellowing were observed in inoculated plants, probably due to reduced concentrations of chlorophyll a and b. Chlorophylls absorb light energy and are essential for initiating photosynthetic events (Ghadirnezhad Shiade et al., 2023b).

Lipid peroxidation typically increases under both biotic and abiotic stress (Haghpanah et al., 2024b) , leading to elevated levels of lipid radicals and the subsequent production of MDA (Ghadirnezhad et al., 2020). However, the findings of the present study contrast with previous research on plant-pathogen interactions, as a reduction in MDA production was observed in plants under disease stress (Haghpanah et al., 2024b).

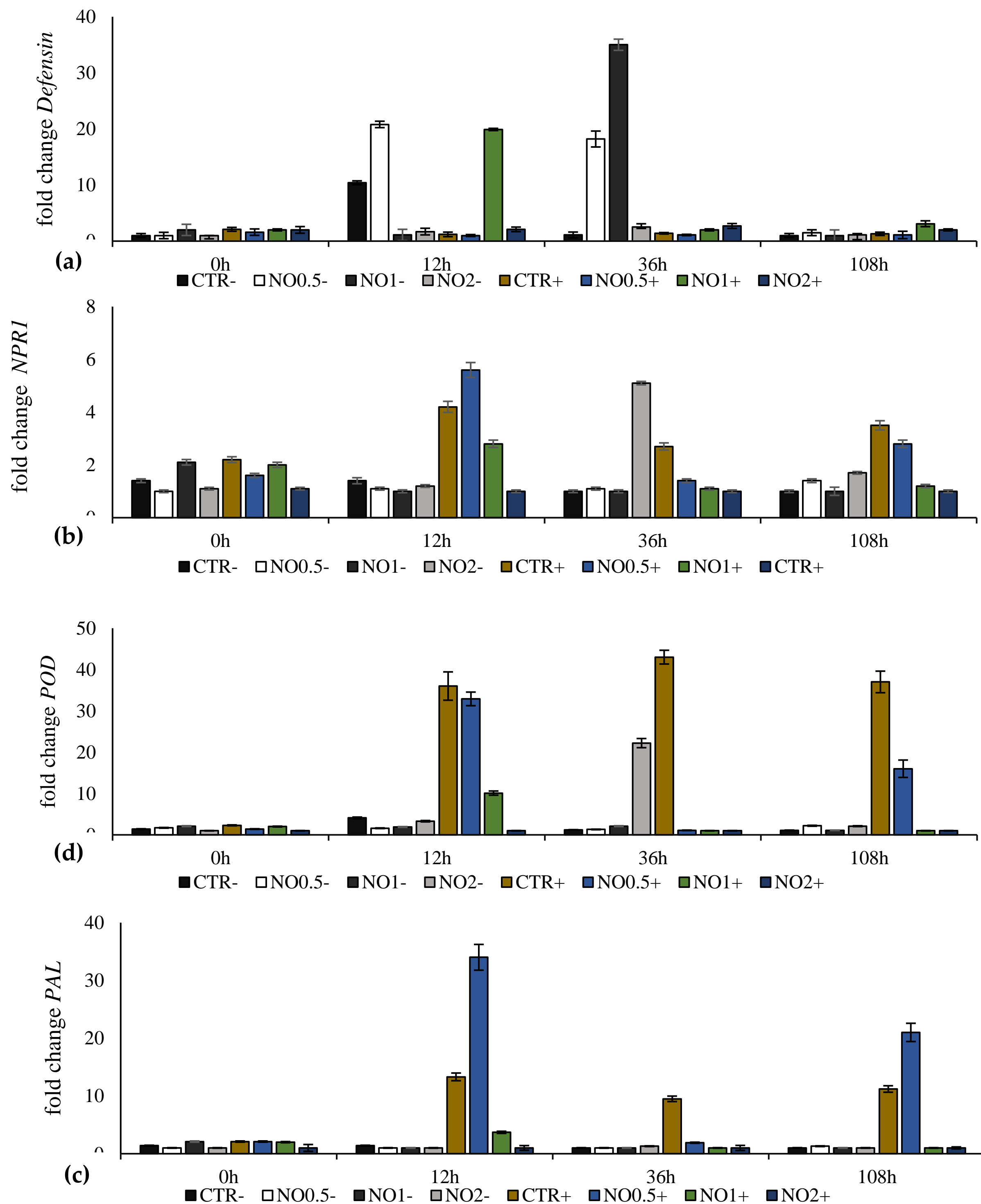

Figure 5. Effect of nitrite oxide (NO) (0, 1, and 2 mM) on the time course of relative gene expression of (a) *Defensin*, (b) *nonexpresser of pathogenesis-related genes 1 NPR* , (c) *phenylalanine ammonia-lyase (PAL)* , and (d) *cytosolic peroxidase (POD)* at 0, 12, 36, and 108 hours post inoculation (hpi) in *Xanthomonas perforans* treated and untreated tomato leaf samples (+ and -). Data are means of three replicates ± SE. (-C): treated with 5 mL of sterile distilled water; (-N0.5): pretreated with 0.5 mM SNP, (-N1): pretreated with 1mM SNP, (-N2): pretreated with 2 mM SNP; (+C): Control pretreated with *X. perforans*, (+N0.5): pretreated with 0.5 mM sodium nitroprusside (SNP)+ *X. perforans*, (+N1): pretreated with 1 mM SNP+ *X. perforans*, (+N2): pretreated with 2 mM SNP+ *X. perforans.*

It has been proposed that an increase in endogenous NO may directly scavenge superoxide (O_2^-) in addition to enhancing catalase (CAT) activity (Khan et al., 2023a). Furthermore, the addition of an NO scavenger negates the beneficial effects of H_2O_2 treatment, supporting the hypothesis that NO plays a critical role in reducing ROS and MDA through its inhibitory properties, while also acting as a signaling molecule to enhance antioxidant enzyme activity (Santana et al., 2017).

The initial plants response to pathogen attack is the ROS generation at the infection site, resulting in oxidative damage to nucleic acids, proteins, lipids, and photosynthetic pigments (Potnis et al., 2015). Our study revealed that inoculation with *X. perforans* significantly decreased chlorophyll a and b, as well as carotenoids in tomato plants during the early stages, indicating damage to the photosynthetic system. Similar findings have been reported under fusarium wilt in tomato (Kabashnikova et al., 2020). Furthermore, plant-pathogen interactions often result in changes in the expression levels of photosynthesis-related genes (He et al., 2022). Previous research has demonstrated that various photosynthesis-related genes, such as chlorophyll a/b binding proteins and photosystem reaction center proteins, were significantly downregulated in susceptible poplar leaves nine days after inoculation with Melampsora medusa (He et al., 2022). This reduction in chlorophyll content may also be explained by detoxification of pathogen-derived compounds. However, in our study, these reductions were compensated for at 324 hpi and showed no significant difference compared to the control plants. Additionally, infected plants exhibited an increase in carotenoid levels at 324 hpi. These results are consistent with those of (Wang et al., 2017a), who demonstrated that *X. perforans* differentially regulates the expression of genes involved in photosynthesis and oxidative phosphorylation pathways in susceptible and relatively resistant tomato plants. Previous studies have shown that exogenous NO enhances chlorophyll biosynthesis in plants by promoting iron uptake, translocation, and internal accessibility(Dong et al., 2016). Photosynthesis is a vital process that is vulnerable to a wide range of stresses. NO supplementation has demonstrated a remarkable ability to integrate various molecules, resulting in a significant impact on plant photosynthesis and stomatal regulation in a unique manner. Its mode of action is mainly through post-translational modifications such as nitration and S-nitrosylation (Sami et al., 2018).

As for fluorescence parameters, Fv/Fm is a widely used indicator of plant stress, as it reflects the quantum efficiency of PSII (Sami et al., 2018). In non-stressed plants, this parameter typically reaches values close to 0.8, and lower values may indicate photoinhibition and damage to PSII (Sami et al., 2018). Contrary to the common decrease in the photosynthetic parameters Fv/Fm and YII, in the present study, Fv/Fm, stomatal conductance, and maximum photosynthetic rate were not significantly altered in plants subjected to disease stress. However, *X. perforans* inoculation led to an increase in the effective quantum yield of photosystem II photochemistry, carotenoids, and the maximum photosynthetic rate. These results are consistent with the findings of Du et al. (2015)Du et al. (2015)Du et al. (2015)Du et al. (2015)Du et al. (2015) on tomato plants, who demonstrated that the X. perforans T3 strain has the opposite effect on photosynthesis between resistant and susceptible tomato cultivars. Accordingly, the pathogen appears to neutralize the photosynthetic downregulation process to secure nutrients for survival. Therefore, the observed improvement in photosynthetic parameters in this study suggests a positive effect on the photosynthetic apparatus and enhanced protection of photosystem II as a result of *X. perforans* inoculation. In susceptible plants, cells increase the production of carbohydrates and energy sources to support *X. perforans* bacteria during the early stages of infection. In contrast, relatively resistant tomato plants downregulate the expression of genes involved in photosynthesis and oxidative phosphorylation pathways (Wang et al., 2017a). This suggests that cells in relatively resistant plants respond to *X. perforans* invasion by limiting biomass accumulation and energy resources, thereby suppressing the growth and proliferation of the invading bacteria.

As previously mentioned, NO is an important signaling molecule in plants that regulates the transcription of various physiological processes, including growth and development, response to

abiotic and biotic stress, and photosynthesis(Hager et al., 2016). Chromatin immunoprecipitation sequencing and RNA-seq analysis have revealed that NO also plays a role in metabolic changes during plant growth and development under stressful conditions (González-Gordo et al., 2023). One of the serious injuries caused by stress in plants is the overproduction of ROS, such as H2O2 and superoxide anions, leading to oxidative damage to cellular components, ultimately resulting in autophagy and programmed cell death (Suman et al., 2021). Our results revealed that *X. perforans* treatment increased the accumulation of toxic H_2O_2 compounds at 12 hpi. However, NO effectively increased antioxidant enzyme activity, thereby reducing ROS levels and protecting biological membranes. Upon pathogen infection, NO can inhibit enzyme activity through S-nitrosylation of NADPH Cys890 oxidoreductase enzyme, thereby reducing ROS production (Khan et al., 2023a). NO plays a pivotal role in plant-pathogen interactions by inducing a hypersensitive response, triggering the expression of defense-related genes, and contributing to the establishment of systemic acquired resistance. An increase in NO is accompanied by enhanced ROS activity, and the combined action of NO and H_2O_2 leads to the death of hypersensitive cells. In this dual system, these two molecules could not independently induce cell death. In Arabidopsis thaliana overexpressing chloroplastic ascorbate peroxidase (APX), which reduces H_2O_2 content, NO treatment causes fewer cell damage symptoms than the wild type (Suman et al., 2021).

Regarding antioxidant enzyme activity, NADPH oxidase content decreased after inoculation, but NO treatment significantly increased its activity, with varying effects at later time points depending on NO concentration. The mitigating effect of NO can be attributed to its role as a signaling molecule that regulates the plant's antioxidant defense mechanisms. By enhancing the activity of antioxidant enzymes, such as NADPH oxidase, NO helps to neutralize ROS, thereby reducing oxidative stress and promoting cellular homeostasis (Khan et al., 2023a). Similarly, SOD and POD activity decreased after infection but increased by NO treatment at certain time points. At lower concentrations, NO interreacts with organic molecules and oxygen species, triggering NO-mediated cellular signaling, thereby eliciting antioxidant stress responses. However, at high concentrations, NO increases nitroxide reactions, causing severe cellular damage (Santana et al., 2017). This model effectively explains the early observed interaction between phytopathogens and host plants, which is associated with the plant immune oxidative burst. Moreover, unchecked amplification of ROS/NO signaling may lead to nitrosative stress and, ultimately, irreversible cellular damage (Santana et al., 2017). In summary, ROS and NO are predominantly found in the form of free radicals that are active in plant cells under stress conditions. The regulatory mechanism involves ROS acting as an intermediate for NO formation, while NO counteracts excessive ROS accumulation through the antioxidant system, thereby maintaining the cellular REDOX balance.

In the present study, NO increased the accumulation of total phenols and cell wall POX at 324 hpi, while it decreased PAL activity and lignin at high concentrations. These findings are consistent with previous research on melatonin-treated such as litchi (Zhang et al., 2021b), kiwi (Yang et al., 2021), and peach (Li et al., 2017) treated with NO. The phenylpropanoid metabolic pathway, with phenylalanine as the primary substrate, has been widely studied and has been proven to play a significant role in plant disease resistance (Noorbakhsh and Taheri, 2016). Activation of the phenylpropanoid metabolic pathway is catalyzed by PAL, leading to the production of phenols, flavonoids, and lignin, which are common antimicrobial compounds (Ge et al., 2018). In this study, *X. perforans* inoculation did not cause a significant change in PAL activity or gene expression levels but led to a marked increase in total phenol content. Previous studies have shown that increased PAL activity and gene expression in tomato plants results in higher levels of phenylpropanoid metabolites, and consequently, enhanced disease resistance (Zhang et al., 2021a). Moreover, the accumulation of higher levels of phenols and their precursors has been observed in diseased plant tissues. In line with this, the assessment of the structural responses of three tomato lines to *X. perforans* revealed that the cell wall defense response is the major difference

between susceptible and resistant tomato lines (Wang et al., 2017a). Smilarly, Ren et al. (2020)Ren et al. (2020)Ren et al. (2020)Ren et al. (2020)Ren et al. (2020) in a study on improving mango fruit resistance to anthracnose with NO, demonstrated that SNP as a NO donor enhanced the accumulation of total phenols and lignin.

In the present study, the TAA content of the leaves increased with bacterial infection. In *X. perforans*-inoculated plants, amino acid content was enhanced by NO priming at 0 and 324 hpi but decreased at hpi 36. Beyond their fundamental role as protein building blocks, amino acids actively participate in plant growth and the regulation of responses to environmental stressors. Furthermore, amino acids serve as precursors for a diverse array of primary and secondary metabolites (Shiade et al., 2024). In rice (*Oryza sativa* L.) seedlings exposed to arsenic (As), a notable decline in amino acid content was observed compared to the control group, except for Histidine (His), Arginine (Arg), and Methionine (Met).

Notably, the supplementation of NO donors led to a reversal of this trend, resulting in an improvement of amino acid content compared to the arsenic-treated seedlings alone. These findings suggest that NO-supplemented rice seedlings exhibit enhanced tolerance to As toxicity by modulating their amino acid profiles. This adaptive response not only mitigates the adverse effects of arsenic but also leads to an increase in both essential and non-essential amino acids, thereby enhancing the nutritional quality of the rice seedlings (Praveen et al., 2020). NO has been shown to modulate the activities of mitochondrial aconitate decarboxylase and oxidase, thereby assisting plants in shifting from primary metabolism to amino acid biosynthesis (Gupta et al., 2012). These results suggested that the reduced tissue damage observed in this study may be associated with increased amino acid levels.

In the present study, disease inoculation did not result in significant changes in the transcriptional expression of any of the genes analyzed. However, treatment with NO led to a significant upregulation in the expression levels of all the studied genes. The significant upregulation of gene expression observed following NO treatment suggests that NO plays a key role in reprogramming plant defense mechanisms at the transcriptional level. This includes the activation of genes related to defense, the production of antimicrobial secondary metabolites, and the hypersensitive response (Martínez-Medina et al., 2019). Specifically, the upregulation of *PAL* by NO pretreatment aligns with previous findings where NO donors, such as sodium nitroprusside, stimulated *PAL* activity in sweet potato roots (Wang et al., 2017b) This demonstrates NO's ability to enhance plant resistance by activating key defense-related genes. Our results revealed that bacterial inoculation significantly decreased the expression level of the *NPR* gene at 12 and 108 hpi, indicating the susceptibility of tomato plants to this pathogen. In contrast, NO elicitor application significantly increased the expression of *NPR* in plant leaves during disease stress. In tomato fruits, exogenous ferulic acid treatment has been shown to enhance resistance to Botrytis cinerea by regulating the NO signaling pathway (Shu et al., 2021). It is now well accepted that there is a link between plant defence and NO and salicylic acid (SA) receptor genes (*NPR1*, *NPR3*, and *NPR4*) (Belt et al., 2017). *NPR* proteins have extensive functions and regulate other transcription factors (TFs). In Arabidopsis, the SA-dependent defense response is generated by NO, where the application of an NO donor increases *NPR1* expression and SA content, leading to the activation of *PR* genes and resistance to Pseudomonas. In addition, NO causes a rapid change in glutathione (GSH) levels, which play an important role in activating *NPR1* (Kovacs et al., 2015).

Moreover, in the present study, the effect of NO treatment was concentration-dependent, with 1 mM NO leading to an earlier increase in *NPR1* expression under pathogen stress conditions. *NPR1* and *TGA1* are key regulators of systemically acquired resistance in plants through redox control. Reduced *NPR1* monomers interact with decreased *TGA1*, which targets the sequence 1 (as-1) element activation of the defense proteins promoter, leading to increased DNA-TGA1 binding and PR gene expression activation. In addition, NO promoted the nuclear import of *NPR1*. Consequently, NO is a redox regulator of the *NPR1*/*TGA1* system and underscores the importance of NO in plant defense responses (Wawrzyńska and Sirko, 2024).

Furthermore, a recent study found that NO upregulates certain *POD* genes in pepper (*Capsicum annuum* L.) during fruit ripening. This study identified 75 *POD* genes, of which NO treatment significantly upregulated two of these genes during the ripening process (González-Gordo et al., 2023).

Conclusion

Our findings demonstrate that nitric oxide (NO) plays a multifaceted role in enhancing plant growth, biomass, and resistance against *X. perforans* under stressful conditions. NO contributes to plant resilience through several key mechanisms. It regulates gene expression related to defense responses, including the upregulation of genes involved in systemic acquired resistance and secondary metabolite production. Additionally, NO modulates both enzymatic and non-enzymatic antioxidant activities, which helps in scavenging ROS and reducing oxidative stress.

By influencing the activity of critical enzymes such as NADPH oxidase and phenylalanine ammonia lyase (PAL), NO enhances the plant's ability to manage stress and resist pathogen attacks. Furthermore, NO affects carbohydrate metabolism and the production of antimicrobial compounds, which collectively bolster the plant's defense system. These findings highlight the potential of NO as a valuable, environmentally friendly tool for improving plant growth, stress tolerance, and disease resistance, offering a promising strategy for sustainable agriculture and crop protection. Future research should focus on optimizing NO application methods, concentrations, and timing for various crops, and conducting long-term field trials to assess its practical benefits and sustainability. Additionally, exploring the molecular mechanisms of NO's effects, its interactions with other stress management strategies, and its impact on soil health and economic feasibility will be crucial for advancing its use in sustainable agriculture.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_722457.html.

Supplementary Table 1. Experimental treatments labeling.

Supplementary Table 2. Specifications of the used primers used in the study.

Supplementary Figure 1. Effect of Nitric oxide (NO) pre-treatment on tomato leaf symptoms after inoculation with *X. perforans* bacteria.

Supplementary Figure 2. Effect of nitric oxide (NO) pre-treatment on (a) chlorophyll a, chlorophyll b (b) in tomato leaf samples treated and untreated with *X. perforans* bacteria at time intervals of 0, 12, 36, 108, and 324 hours post inoculation (hpi).

Author Contributions

Conceptualization: M.J., H.P., A.D.; Methodology: M.J., H.P., A.D., P.M., V.B.; Data collection: M.J.; Formal analysis: M.J.; Writing—original draft preparation: M.J., H.P., A.D.; Writing—review and editing: M.J., H.P., A.D., P.M., V.B.; Supervision: H.P., A.D., P.M., V.B.; Funding acquisition: H.P., A.D.; All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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نقش تعدیل‌کنندگی اکسید نیتریک در خصوصیات فیزیولوژیکی و مولکولی گیاه گوجه‌فرنگی (Solanum lycopersicum L) آلوده شده با *Xanthomonas perforans*

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مقاله پژوهشی

چکیده: لکه برگی باکتریایی، که توسط Xanthomonas perforans ایجاد می‌شود، تهدیدی جدی برای تولید گوجه‌فرنگی در سطح جهانی به شمار می‌آید. روش‌های سنتی کنترل این بیماری نتوانسته‌اند به‌طور مؤثر عمل کنند، و ضرورت به‌کارگیری رویکردهای نوآورانه بیشتر مورد نیاز است. در این مطالعه، اثر پیش‌تیمار با نیتریک اکسید (NO) در غلظت‌های ۰.۵، ۱ و ۲ میلی‌مولار برای القای مقاومت غیرزیستی در برابر *X. perforans* در گوجه‌فرنگی‌های کشت‌شده در گلخانه ارزیابی شد. نتایج نشان داد که پیش‌تیمار با NO باعث بهبود رشد گیاه، بیوماس و مکانیسم‌های دفاعی در شرایط عادی و همچنین در شرایط تنش ناشی از پاتوژن شد. به‌طور خاص، NO فرآیندهای فیزیولوژیکی نظیر پراکسیداسیون لیپیدها، محتوای کلروفیل و پارامترهای فلورسانس را تنظیم نمود. همچنین این ترکیب باعث تعدیل آنزیم‌های کلیدی مانند NADPH اکسیداز و فنیل‌آلانین آمونیالیاز (PAL) شد و از طریق مقابله با گونه‌های فعال اکسیژن، پاسخ‌های دفاعی آنتی‌اکسیدانی را تقویت کرده و آسیب‌های اکسیداتیو را کاهش داد، که به نوبه خود باعث افزایش تحمل به تنش گردید. علاوه بر این، NO موجب تجمع قندها شده و ذخایر انرژی را در شرایط تنش تقویت کرد، همچنین با افزایش محتوای آمینو اسید، به تسهیل سنتز پروتئین‌های دفاعی کمک نمود. به‌ویژه، پیش‌تیمار با NO موجب کاهش چشمگیر شدت بیماری از طریق افزایش بیان ژن‌های دفاعی، از جمله ژن‌های غیر بیان‌کننده مرتبط با بیماری‌زا (NPR)، فنیل‌آلانین آمونیا لیاز (PAL) و پراکسیداز (POD) شد. به‌طور کلی، پیش‌تیمار با NO به بهبود سلامت گیاه، کاهش تکثیر پاتوژن‌ها و افزایش بیوماس منجر شد، که این امر قابلیت بالای این روش را به‌عنوان یک استراتژی پایدار و دوستدار محیط‌زیست برای مدیریت بیماری‌های باکتریایی و فعال‌سازی پاسخ‌های اختصاصی مقاومت در گیاهان گوجه‌فرنگی نشان می‌دهد.

کلمات کلیدی: لکه باکتریایی، مقاومت غیرزیستی، مدیریت بیماری، مقاومت اکتسابی سیستمیک.

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