INDUCTION OF SYSTEMIC RESISTANCE IN TOMATO BY FRUIT EXTRACTS OF *AZADIRACHTA INDICA*

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Abstract:

Systemic acquired resistance (SAR) is a well-known phenomenon in plant kingdom. Biotic induction of systemic resistance is an environment-friendly method of plant protection. The present study focussed on deciphering the molecular mechanism of SAR induction in tomato by aqueous neem fruit extract against *Pseudomonas syringae* pv. *tomato*. The alterations in the activities of Peroxidase (POX), Polyphenol Oxidase (PPO) and Lipoxygenase (LOX) and their acidic isozymes were monitored. Quantitative Real-Time PCR (qRT-PCR) of *CEVI1*, *PPOB* and *TomLoxD* genes was performed to study the change in the mRNA levels of Peroxidase, Polyphenol Oxidase and Lipoxygenase respectively. The results demonstrate effectivity of neem extract in inducing systemic resistance in tomato. The activity of these defense enzymes was increased and additional isozymes of POX, PPO and LOX were expressed after neem treatment. The qRT-PCR results demonstrate the inhibitory effects of pathogen on the neem-elicited resistance.

Key words: neem, SAR, POX, PPO, LOX, qRT-PCR.

INTRODUCTION

Tomato (*Lycopersicum esculentum*), an important globally cultivated vegetable is extensively attacked by *Pseudomonas syringae* pv. *tomato* (Barone *et al*., 2008). Chemical

methods to control the pathogen and reduction in the incidence of bacterial speck caused by it are environmentally unacceptable (Luna *et al*., 2012). Therefore, a biological control method such as induction of Systemic Acquired Resistance (SAR) in crop plants has emerged showing great potency in plant protection against a large array of pathogens (Vallad and Goodman, 2004). SAR is a phenomenon in which prior application of biological or chemical inducers activates the defense system of the plant against subsequent attack of bacterial, fungal or viral pathogen (Percival, 2001). Aqueous extracts of *Artemisia camphorata* (camphor) when sprayed prior to pathogen inoculation could induce systemic resistance in wheat against *Bipolaris sorokiniana* (Franzener *et al*., 2003). Application of ginger mass to soil near base of lettuce plants reduced disease incidence due to enhanced host resistance (Rodrigues *et al*., 2007).

Plant defense responses primed by SAR are followed by the enhanced expression of several defense related proteins (Pathogenesis-related 'PR' proteins) which provide broad-spectrum resistance against a large number of pathogens (Durrant and Dong, 2004). Peroxidase (POX), Polyphenol Oxidase (PPO) and Lipoxygenase (LOX) are few such defense proteins which have wide implications in protection of host plants from the invading pathogens and are known to be induced during pathogen attack or by application of elicitors (Porta and Rocha-Sosa, 2002; Wang *et al*., 2005; Bhuvaneshwari and Paul, 2012).

Peroxidases (POXs) are haem-containing glycoproteins which oxidize a wide variety of compounds in the presence of hydrogen peroxide (H_2O_2) (Hiraga *et al*., 2001). Gaspar *et al.* (1991) reported that peroxidases are involved in auxin and ethylene metabolism, redox reactions in plasma membranes, cell wall modifications (lignification and suberinization) as well as in developmental and defense processes. POXs are involved in the production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\circ -}$), hydroxyl radical (OH^-) and H_2O_2 as one of the earliest cellular responses following successful pathogen recognition. The production of ROS has also been related to hypersensitive response and induction of SAR in the host plant (El-Khallal *et al*., 2007; Liu *et al*., 2010).

Polyphenol oxidases (PPOs) (EC 1.14.18.1 or EC 1.10.3.2) are ubiquitously present enzymes which catalyze the O_2-dependent oxidation of mono and *o*-diphenols to *o*-diquinones, whose secondary reactions are believed to be responsible for the oxidative browning which accompanies plant senescence, wounding, and responses to pathogens (Thipyapong *et al.*, 2004). The defensive roles of PPO against disease and insect pests have been clearly established (Newman *et al*., 2011). Induction of POX and PPO by neem extract has been demonstrated by Bhuvaneshwari *et al*. (2012).

LOXs are a class of non-heme, iron-containing, monomeric proteins which catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) containing *cis*,*cis*-1, 4 pentadiene moiety such as linoleic acid and linolenic acid (Hu *et al*., 2011) to convert them into hydroxyperoxides which in turn is responsible for signaling and defense response in plants (Vardar and Unal, 2011). LOXs are known to be elicited in response to wounding or pathogen interaction, water and drought stresses (Yang *et al*., 2011) and following inoculation with pathogens or treatment with elicitors (Peever and Higgins, 1989).

In the present study, efforts have been made to analyse the inductive effects of a biotic elicitor [aqueous fruit extracts of *Azadirachta indica* (neem)] on the activity and expression of POX, PPO and LOX which are instrumental in imparting resistance to tomato, and the interactive effects of the pathogen it.

MATERIALS AND METHODS

Raising of plants

Tomato seeds (Roopsi variety, Century seeds) were raised in sterile culture room maintained at 25 ± 1°C with a relative humidity of 70% and a photoperiod of 12 h of light and dark. Trays were watered daily with sterilised distilled water and once a week with Hoagland's solution.

Neem fruit extract (elicitor) preparation

Fully mature but green neem fruits (fruit coat green but seed hard) were washed twice with sterilized Type I water and dried under aseptic condition. 20 % (w/v) aqueous extract of the fruits was prepared in sterilized Type I water by macerating in a pre-chilled pestle and mortar. The obtained extract was filtered through four folds of Muslin cloth and the filtrate was centrifuged at 8000xg at 4°C for 30 min. The supernatant obtained was filtered through 0.45 μm membrane filter and used as aqueous neem fruit extract.

Preparation of pathogen inoculum

Pseudomonas syringae pv. *tomato* was isolated from naturally-infected tomato fruits collected from the fields on King's B agar medium. It was sub-cultured on King's B broth and after 24 h its concentration was adjusted to 10^8 cfu/ml. This was subsequently used for inoculating tomato plants.

Treatment of plants

8 weeks old plants were used for the study. The plants were divided into six groups of 50 plants each. The third nodal leaf from the base of each plant was treated with elicitor. The six groups of treatments were as follows:

(1) Group 1: Sprayed with autoclaved Type I sterile water (control).
(2) Group 2: Pathogen inoculated 24 h prior to elicitor application.
(3) Group 3: Pathogen inoculated 24 h after elicitor application.
(4) Group 4: Simultaneous application of pathogen and elicitor.
(5) Group 5: Inoculated with pathogen only.
(6) Group 6: Treated with elicitor only.

Samples were collected from treated third node and distal untreated nodes at 0, 24, 48, 72, 96 h and 2 weeks post treatment

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Disease severity

Disease severity was evaluated by inoculating the newly emerged leaves with the pathogen and visually observing bacterial speck lesions on them after 2 days of inoculation. Disease severity was scored using a disease index with a range of 0 to 3 (0 signifies a healthy-looking plant; 1 signifies 2 to 5 specks together or spread over each leaf; 2 signifies 6 to 10 specks; and 3 signifies more than 10 specks).

Enzyme extraction

300 mg of leaf sample was homogenized in 1.2 mL of ice-cold sodium phosphate buffer (0.1 M, pH 9.0) containing 0.001 % Triton X-100, 10 mM β-mercaptoethanol, 10 % (w/w) PVP , 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 1 mM EDTA at 4°C. The homogenate was centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant thus obtained was used as an enzyme extract for POX, PPO and LOX estimation. Five replicates were taken for each sample.

Peroxidase activity assay

POX activity assay was carried out by making necessary modifications in the method earlier described by Neto *et al*. (2006). The reaction mixture consisted of 0.245 mL of sodium-phosphate buffer (1M, pH 7.0), 0.25mL of Guaiacol (0.1 M), 0.05 mL Hydrogen Peroxide (H_2O_2), 0.05 mL of crude enzyme extract and 1.655 mL of Type I water. The reaction mixture was incubated at 25±1°C for 5 min and reaction was terminated by addition of 0.5 mL 10% v/v Sulphuric acid. Absorbance was recorded at 470 nm using UV-VIS spectrophotometer (Shimadzu, 1650). Reaction mixture without enzyme extract served as blank. The molar extinction coefficient taken for the calculation of enzyme activity for POX was, $\varepsilon = 26.6\ mM^{-1}cm^{-1}$. Enzyme activity was expressed as mM $min^{-1}g^{-1}$ fresh weight.

Polyphenol Oxidase activity assay

PPO activity assay was performed as per the method described earlier by Bhuvaneshwari and Paul (2012). Enzyme activity was expressed as units g^{-1} min^{-1} fresh weight. One unit of enzyme activity was defined as the amount of enzyme required for a change in absorbance of

0.001 per minute. The number of active units in a sample was calculated from the total proteins present in that respective sample.

$$No. of\ active\ enzyme\ units\ = \frac{A\ \times V}{v\ \times t \times\ w}$$

Where, A = Absorbance of reaction mixture at 420 nm
V = Volume of the reaction mixture
v = volume of crude enzyme added to the reaction mixture
t = time of incubation of reaction in minutes
w = weight of sample crushed per unit volume of the buffer

Lipoxygenase activity assay

LOX activity assay was carried out by the modifications in the method earlier described by Fortunato *et al*. (2004). The modified reaction mixture consisted of 1.955 mL of sodium phosphate buffer (0.1M, pH 7.0), 0.025 mL of Linoleic Acid (5 mM), 0.02 mL of crude enzyme extract. The reaction mixture was incubated at 25±1°C for 2 min and terminated by addition of 0.5 mL 5% v/v Sulphuric acid. Absorbance was recorded at 234 nm using UV-VIS spectrophotometer (Shimadzu, 1650). The molar extinction coefficient for LOX used was, ε=25 $mM^{-1}cm^{-1}$. Reaction mixture without enzyme extract served as blank. Enzyme activity was expressed in mM min^{-1} g^{-1}fresh weight.

Native-Basic PAGE and in-gel-activity-staining

The isozyme profiles of cytoplasmic acidic POX, PPO and LOX were analysed by native basic PAGE (Laemmli, 1970), without SDS. Electrolyte for electrode reservoirs was Tris-glycine (pH 8.3). Bromophenol blue (0.01%) was used as tracking dye. For each sample 75 µg proteins were loaded onto the native basic polyacrylamide gel for isoform analysis. The native gel consisted of 10% resolving gel and 4% stacking gel. Electrophoresis was carried out at 70mA/gel for 3 hours at 4°C. After electrophoresis, the gels were stained for iso-POX by incubating in 0.1 M Sodium-phosphate buffer (pH 7.0) containing 10 mM Guaiacol and 0.75% H_2O_2 (Neto *et al*.,

2006). Acidic PPO isoforms were visualized by the modified method of Anand *et al*. (2007), by equilibrating the gel in 0.1% p-phenylene diamine followed by addition of 50 mM catechol in 0.1M Sodium-phosphate buffer (pH 7.0). LOX isoforms were stained by incubating the gel in 50 mM Potassium phosphate buffer (pH 6.0) containing 0.1% linoleic acid and 0.02% o-dianisidine (Wang and Yang, 2005). The stained isoforms were distinguished by calculating the relative distance (Rf value) (Bhuvaneshwari and Paul, 2012) of each isozyme band from each zymogram using the following equation: Rf value = Distance migrated by the isoenzyme band from the start of the resolving gel/Distance migrated by tracking dye from the start of the resolving gel.

Statistical analysis of the data

The data were statistically analyzed for analysis of variance (ANOVA) using the general linear model procedure and the least squares means test of the statistical software SAS (version 9.2 developed by SAS institute Inc., Cary, NC, USA). Multiple pairwise comparison tests using least-square means were performed for post-hoc comparisons after two way with treatment and time as the two factor with replications. The corrections used for multiple comparisons were Tukey's honest significantly differences test (HSD) procedure. Data for disease incidence and severity were statistically analyzed by SPSS software for windows version 16 (SPSS Inc., Chicago, Illinois, USA) using univariate general linear model procedures and one-way ANOVA respectively followed by post-hoc comparisons using Tukey's HSD.

Relative gene expression levels of the defense enzymes

Relative gene expression quantification of peroxidase *CEVI-1*, lipoxygenase *TomLoxD* and polyphenol oxidase *PPOB* genes was performed by real time polymerase chain reaction (qRT-PCR) using 18S gene as endogenous control. The qRT-PCR was outsourced to Xcelris Genomics Pvt. Ltd., Ahmedabad, India. As per the company policy, the protocols were not shared with us.

Primer Sequences

The primer sequences for the three target tomato genes were obtained from www.ncbi.nlm.nih.gov by performing BLAST of the mRNA of the corresponding gene.

Gene Name	Direction	Sequence 5'-3'	Primer Length	Amplicon length
Peroxidase *CEVI1* gene	F primer	GCAACAAGCCCAAAGTACCG	**20**	**219**
	R primer	GAAACAACGCCAGGACACAC	**20**	
Polyphenol Oxidase *PPOB* gene	F primer	AATTCCTCCCGAAAGCCAGG	**20**	**375**
	R primer	TTTGGTACCAGAGTCACCGC	**20**	
Lipoxygenase *TomloxD* gene	F primer	GCAGATCGCTAAAGCACACG	**20**	**123**
	R primer	GCGCTTAACTGCCTATGTGC	**20**	

Results

The disease severity observations indicate that neem fruit extract could effectively induce resistance in tomato against *Pseudomonas syringae* pv. *tomato*. Neem application either singly or in combination with the pathogen could significantly ($p \leq 0.05$) reduce disease symptoms in the leaves emerging after 2 weeks of treatments in the host plants. When the plants were treated with neem extract alone, prior to or after pathogen inoculation, the severity of disease was reduced by ~ 50 %. The plants which were treated with pathogen and elicitor simultaneously had 30 % reduction in disease symptoms. The pathogen only treated plants had highest disease severity (Figure 1).

Significant ($P \leq 0.05$) increase in POX activity was observed at 24 h in the entire 3rd node treated and distal untreated leaves except control and pathogen only inoculated plants (Figure 2), which continued upto 96 h of sampling. Two POX isoforms (Rf = 0.01 and 0.32) were constitutively expressed in all the samples. Induction of one additional acidic POX isoforms (Rf

= 0.34) was observed at 48 h in all the treatments except control, 3rd node leaves inoculated with pathogen prior to neem application and distal untreated leaves in plants which were inoculated with pathogen after neem application or simultaneously with it (Figure 3).

Significant ($P \leq 0.05$) increase in active PPO units was observed at 24 h in the 3rd and distal node leaves of neem alone treated plants which continued upto 96 h. Significant ($P \leq 0.05$) increase in PPO active units was noted after 48 h in distal leaves of plants treated with neem followed by pathogen inoculation. The 3rd node samples of plants treated with pathogen and neem simultaneously showed significant ($P \leq 0.05$) rise in active PPO units at 72 h. The new leaves emerging after 2 weeks of neem treatments had significantly ($P \leq 0.05$) higher active PPO units in all the samples except control and pathogen only inoculated plants (Figure 4). Five PPO isoforms (Rf = 0.24, 0.32, 0.38, 0.40 and 0.42) were constitutively expressed in all the samples including control. A PPO isoform (Rf = 0.02) was induced in all the samples except control and distal untreated leaves of neem alone treated plants. Another PPO isoform (Rf = 0.44) was induced in all the samples except control and plants inoculated with pathogen prior to neem treatment (Figure 5).

Significant ($P \leq 0.05$) increase in LOX activity was observed in both the 3rd node and distal leaves of plants inoculated with pathogen inoculation prior to or after neem treatment and distal leaves of plants inoculated simultaneously with pathogen and neem extract at 24 h. The 3rd node leaves treated with neem alone or when followed by pathogen inoculation also had significantly ($P \leq 0.05$) higher LOX activity after 24 h of treatment. In the distal leaves of plants inoculated with pathogen prior to, after or simultaneous treatments significantly ($P \leq 0.05$) higher LOX activity was noted at 96 h. Significant ($P \leq 0.05$) increase in LOX activity was observed in the new emerging leaves of all treated plants except control, pathogen only and neem only treated plants after 2 weeks (Figure 6). A single LOX isoform (Rf = 0.28) was constitutively expressed in all the samples including control. An additional LOX isoform (Rf = 0.31) was observed in the 3rd node leaves of plants treated with neem alone and in the distal leaves of plants treated with neem after pathogen inoculation or in conjunction with it (Figure 7).

The qRTPCR of the peroxidase, lipoxygenase and polyphenol oxidase genes demonstrated mixed response of the genes' expression after treatment with neem fruit extract. The Polyphenol Oxiadse gene *PPOB* was significantly ($P \leq 0.05$) upregulated in the 3rd nodal leaves when the neem fruit extract was sprayed either before or after pathogen inoculation. However, the other samples didn't show any significant ($P \leq 0.05$) change in the expression level of *PPOB*. The Lipoxygenase gene *TomLoxD* didn't show significant variation in expression in any treatment except in the distal untreated leaves of the plants treated with neem extract prior to pathogen inoculation, where it was observed to be significantly ($P \leq 0.05$) downregulated. Similar significant ($P \leq 0.05$) downregulation of Peroxidase gene *CEVI1* was observed in both the 3rd nodal treated and distal untreated samples of pathogen inoculation followed by neem extract treatment (Figure 8).

DISCUSSION

The implications of bio-elicitors in enhancing the resistance of host plants against pathogens have been studied for long now. However, the molecular mechanism of the induction of resistance in these plants is largely unknown.

In the present study, neem fruit extract was significantly ($P \leq 0.05$) effective in inducing SAR in tomato against *Pseudomonas syringae* pv. *tomato* and reducing the incidence of bacterial speck in tomato up to half as compared to control.

Extracts from different parts of neem have been used as biocontrol agents for protection of plants from several pathogens. Aqueous extract of leaves of neem provided control of leaf stripe pathogen on barley (*Drechslera graminea*) indirectly by inducing plant defense reactions (Paul and Sharma, 2002; Bhuvaneshwari *et al*., 2012). Neem seed powder significantly reduced the disease severity of *Fusarium* and root-knot in both greenhouse and field in tomato plants (Agbenin *et al*., 2004). The incidence of crinkle virus disease in Urd bean could be effectively reduced by neem extract application (Binyamin *et al*., 2011). Neem seed extract has been recommended for controlling leaf spot disease in eggplant (Nwogbaga and Utobo, 2012).

Neem extract was observed to significantly reduce the early blight and leaf spot disease in tomato (Pattnaik *et al*., 2012).

The application of neem extract on a single leaf of the plant could enhance the activity of POX, PPO and LOX in the host plants both locally as well as systemically. Also, it was effectively able to induce novel POX, PPO and LOX isozymes in them. Elevated levels of activity of these defense enzymes and enhanced expression of additional acidic isoforms in tomato plants after neem treatment bespeak its potent role in assuring the getting ready of plants for any possible encounter with the pathogens in near future and successfully defending the host from any such circumstances. The appearance of additional isoforms after neem treatment suggest that either the already expressed but inactive POX, PPO and LOX isoforms were activated or new ones were expressed as a result of neem elicited reactions.

Enhanced peroxidase activity has been associated with induced systemic resistance of cucumber to *Colletotrichum lagenarium* (Hammerschmidt *et al.,* 1982). It has been suggested that increase in activity of a specific anionic isoform of POX in some resistant inbred lines of maize, due to virus inoculation, could be related to a defense mechanism against this virus (De Souza *et al*., 2003). *Ep5C* (corresponding to *CEVI16*) gene expression was induced in tomato leaves upon inoculation with *Pseudomonas syringae* pv. *tomato* in a fashion similar to that observed for the induced expression of the classical PR genes (Coego *et al*., 2005). Increase in peroxidase activity in Faba bean resulted in lower disease incidence (Hassan *et al.,* 2007). The results obtained by Aboshosha *et al.* (2008) revealed the validity of peroxidase activity and its isozymes' pattern as genetic markers for resistance and susceptibility in sunflower to *M. phaseolina.*

Li and Steffens (2002) reported that PPO over-expressing tomato plants could hinder the ingress of the bacterial pathogen *Pseudomonas syringae* pv. *tomato.* Mohammadi and Kazemi (2002) suggested that induced resistance in falat wheat heads could be attributed to over-expression of PPO. Nakkeeran *et al*. (2006) reported that application of *Bacillus subtilis* strain BSCBE4 and *Pseudomonas chlororaphis* strain PA23 mediated induction of PPO, which was

effective in controlling damping-off of hot pepper. Induced systemic resistance (ISR) in cucumber against *P. cubensis* and *Erysiphe cichoracearum* was mediated by expression of additional POX and PPO isoforms (Anand *et al*., 2007). Elevated PPO activity was observed while using chemical elicitors for induction of resistance against leaf blight in onion (Abo-Elyousr *et al.*, 2008). The transcript levels of PPO genes were altered and its activity increased after application of commercial extract from the brown seaweed *Ascophyllum nodosum* during the control of fungal diseases in cucumber (Jayaraman *et al*., 2011). Higher number of PPO isoforms were observed in the resistant pearl millets cultivars following application of different inducers (Lavanya *et al*., 2012). The induction of resistance in tomato plants against *Pseudomonas syringae* pv. *tomato* has been correlated to the increased activity of POX and PPO and increase in number of their isoforms (Bhuvaneshwari and Paul, 2012).

Accumulation of LOX mRNA in tomato upon interaction with *Pseudomonas syringae* has been previously reported (Koch *et al*., 1992). Sailaja *et al*. (1997) reported the involvement of LOX both in growth and development as well as in plant-pathogen interactions, particularly induced disease resistance. Fortunato *et al*. (2004) reported the possible induction of LOX by the application of cell culture or elicitor in tomato. Devi *et al*. (2006) reported that the induction of LOX isenzymes were responsible for enhanced resistance of pigeon pea seedlings against *Fusarium udum*. Akram *et al*. (2008) demonstrated the induction of SAR preceded by enhanced levels of LOX activity in tomato by application of non-pathogenic bacteria in tomato. Salt stress (Delaplace *et al*., 2009) and herbivore-response in passion fruit (Jardim *et al*., 2010) induced expression of enzymes involved in lipoxygenase pathway were local and systemic induction of LOX. Yang *et al*. (2011) demonstrated that the wound-induced JA was regulated by LOX at both transcriptional and enzymic levels. Its activation was necessary in wound-mediated defense response and could enhance the tolerance of pea seedlings to wounding.

PPOB gene was significantly upregulated at the site of treatment in plants inoculated with pathogen prior to after neem treatment. It seems that when the pathogen inoculation preceded neem treatment, either the elicitor was able to overcome the inhibitory effects of

pathogen or the pathogen was unable to significantly inhibit *PPOB* gene expression. Similarly, when neem treatment preceded pathogen inoculation, the pathogen could not suppress its elicitation effects.

TomLoxD gene was downregulated in distal node leaves of plants inoculated with pathogen after neem treatment. The effector molecules from the pathogen probably suppressed the neem-induced defense response in this gene. The *CEVI1* POX gene was downregulated in both the 3rd and distal node leaves of plants inoculated with pathogen prior to neem treatment. The pathogen seems to interfere and eventually suppress the inductive effect of neem, thereby suppressing the expression of *CEVI1*gene. The neem treatment could not overcome the inhibitory effects of the pathogen. Moreover, it appears that the pathogen interferes with the elicitation abilities of the neem fruit extract because significantly lower expression of RNA was observed in the 3rd node leaves of plants simultaneously treated with pathogen and neem extract. This could be due to possible modulation of the host genomic machinery by the effector molecules secreted by the pathogen into the host cytoplasm. This appears to be in agreement to the findings of Rico and Preston (2008), who reported that such effector proteins can potentially inactivate plant surveillance mechanisms and signaling pathways, thus allowing the survival of the pathogen on the leaf surface.

The findings of this study outline the events occurring in the tomato cytoplasm during successful induction of systemic resistance. However, the successful induction of defense response is a result of complex molecular interactions between the host machinery, pathogen effector molecules and the biotic elicitor. Pathogen hinders the inducing effects of the elicitor thereby probably reducing its efficiency. Therefore, further research is required to target the pathogen establishment on the surface of the plants and reduce its interactions and subsequent release of its effector molecules into the host cytoplasm. The implications of aqueous neem fruit extract as a potent biocide could be a potent method of protection of tomato plants from the bacterial speck disease.

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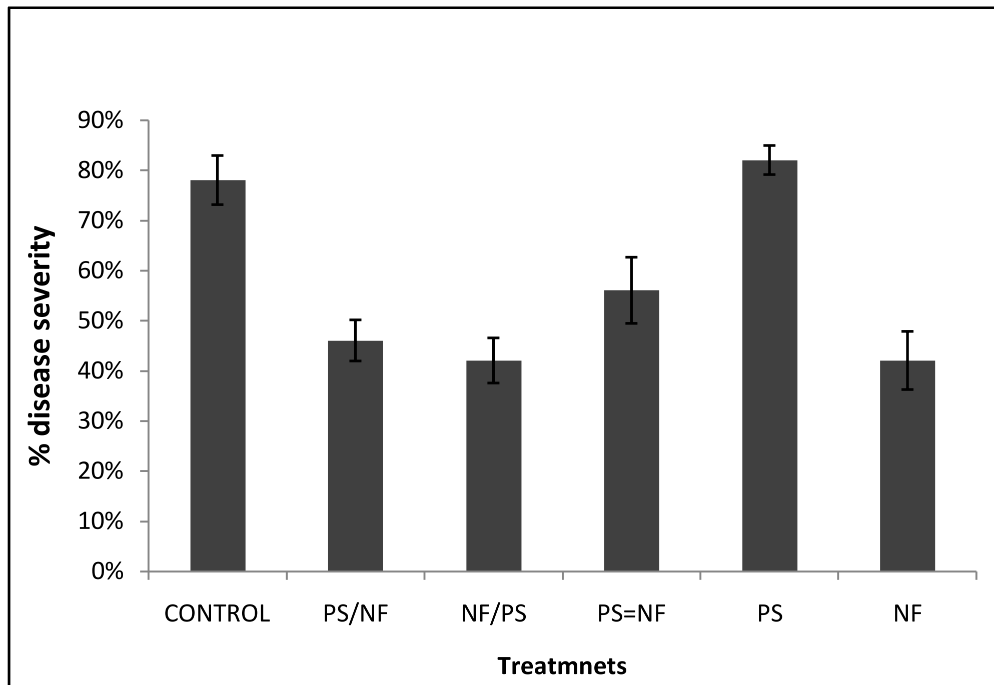

Figure 1. % disease severity in treated tomato plants.

The different treatments are as follows : Control – control samples, PS/NF - pathogen inoculation prior to elicitor treatment, NF/PS - elicitor treatment prior to pathogen inoculation, PS=NF - pathogen and elicitor simultaneous treatment, PS - pathogen inoculation only, NF - elicitor only treatment.

(The vertical bars at the top represent the standard error).

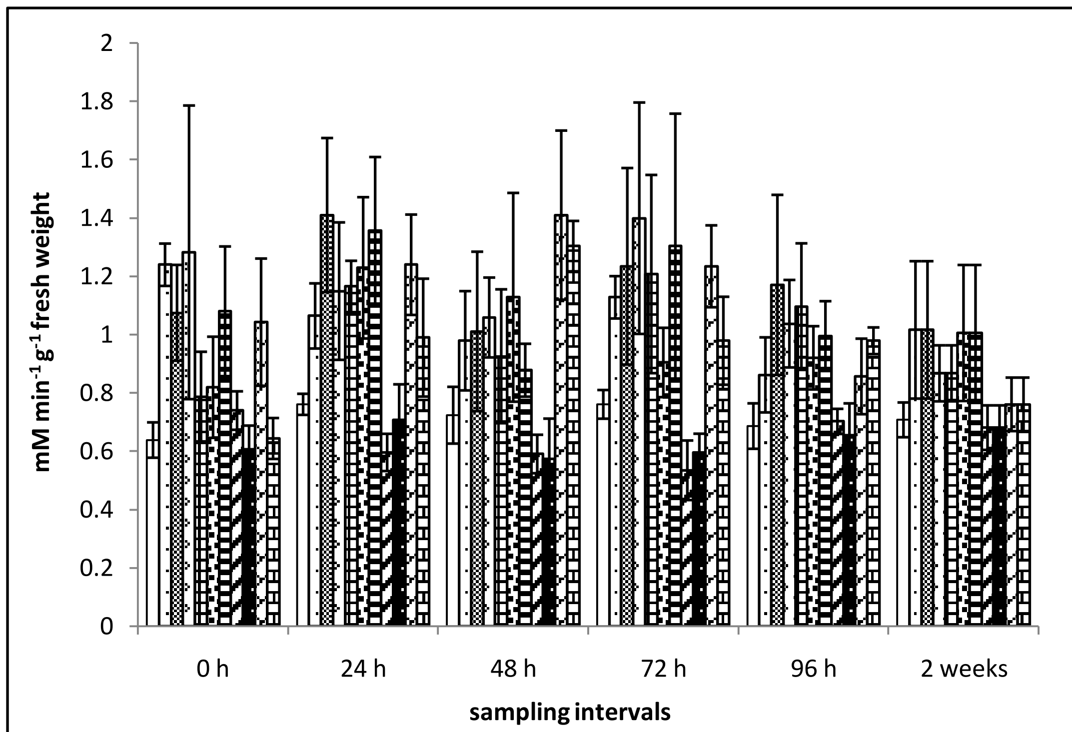

Figure 2. POX activity in treated plants.

□ control ◘ 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment ▩ distal untreated leaf of plants inoculated with pathogen prior to elicitor treatment ◘ 3rd node leaf of plant inoculated with pathogen after elicitor treatment ⊞ distal untreated leaf of plant inoculated with pathogen after elicitor treatment ◘ 3rd node leaf of plant treated simultaneously with pathogen and elicitor ◘ distal untreated leaf of plants treated simultaneously with pathogen and elicitor ◘ 3rd node leaf of plant inoculated with pathogen only ■ distal untreated leaf of plant inoculated with pathogen only ⊠ 3rd node leaf of plant treated with elicitor only ⊟ distal untreated leaf of plant treated with elicitor only.

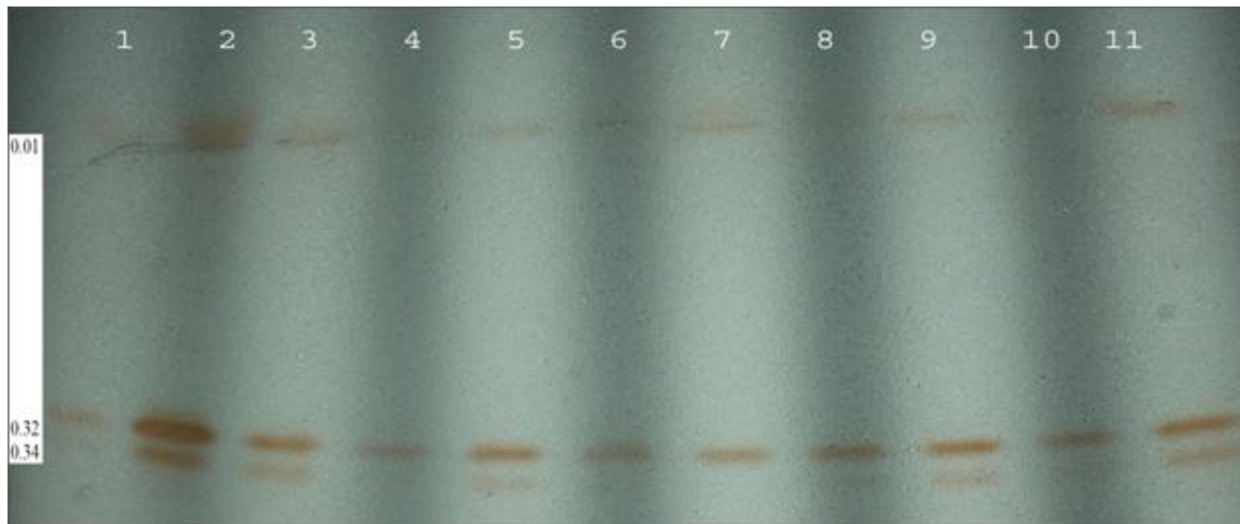

Figure 3. POX zymogram of treated plants.

Lane 1= 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment, lane 2= distal untreated leaf of plant inoculated with pathogen prior to elicitor treatment, lane 3= 3rd node leaf of plant inoculated with pathogen after elicitor treatment, lane 4= distal untreated leaf of plant inoculated with pathogen after elicitor treatment, lane 5= 3rd node leaf of plant treated simultaneously with pathogen and elicitor, lane 6= distal untreated leaf of plant treated simultaneously with pathogen and elicitor, lane 7= control, lane 8= 3rd node leaf of plant inoculated with pathogen only, lane 9= distal untreated leaf of plant inoculated with pathogen only, lane 10= 3rd node leaf of plant treated with elicitor only, lane 11= distal untreated leaf of plant treated with elicitor only.

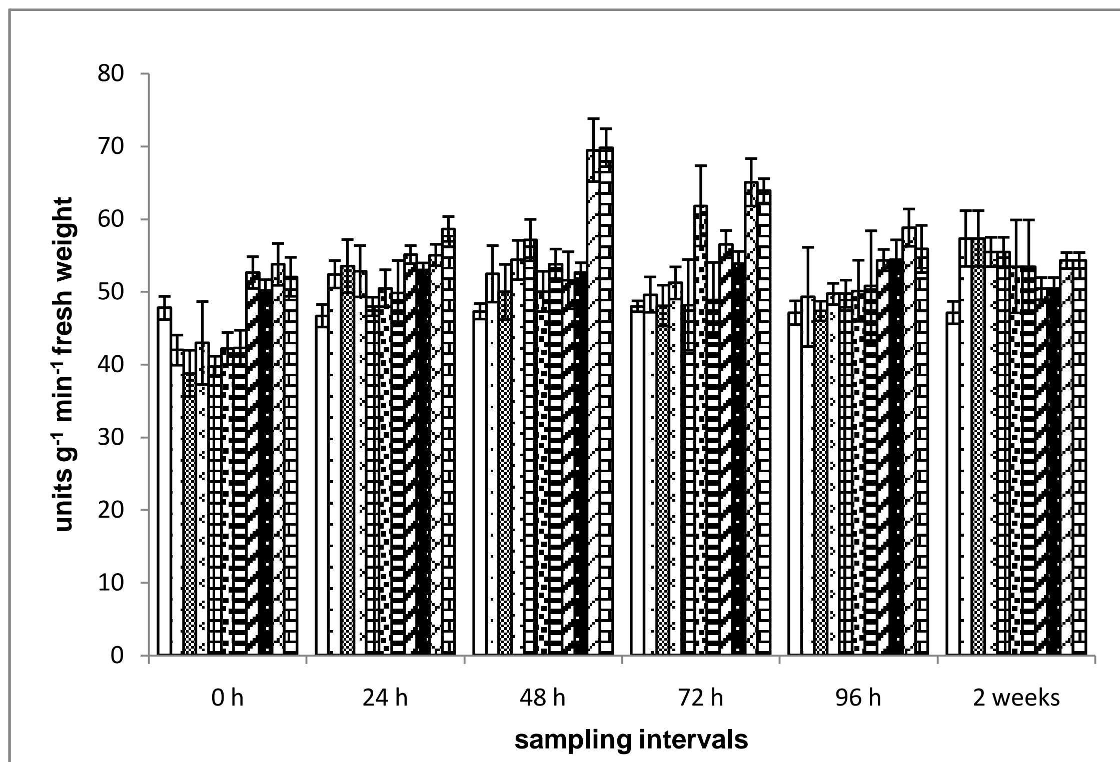

Figure 4. PPO activity in treated plants.

□ control ⊡ 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment ▩ distal untreated leaf of plants inoculated with pathogen prior to elicitor treatment ⊡ 3rd node leaf of plant inoculated with pathogen after elicitor treatment ⊞ distal untreated leaf of plant inoculated with pathogen after elicitor treatment ⊡ 3rd node leaf of plant treated simultaneously with pathogen and elicitor ⊟ distal untreated leaf of plants treated simultaneously with pathogen and elicitor ◪ 3rd node leaf of plant inoculated with pathogen only ■ distal untreated leaf of plant inoculated with pathogen only ⊠ 3rd node leaf of plant treated with elicitor only ⊟ distal untreated leaf of plant treated with elicitor only.

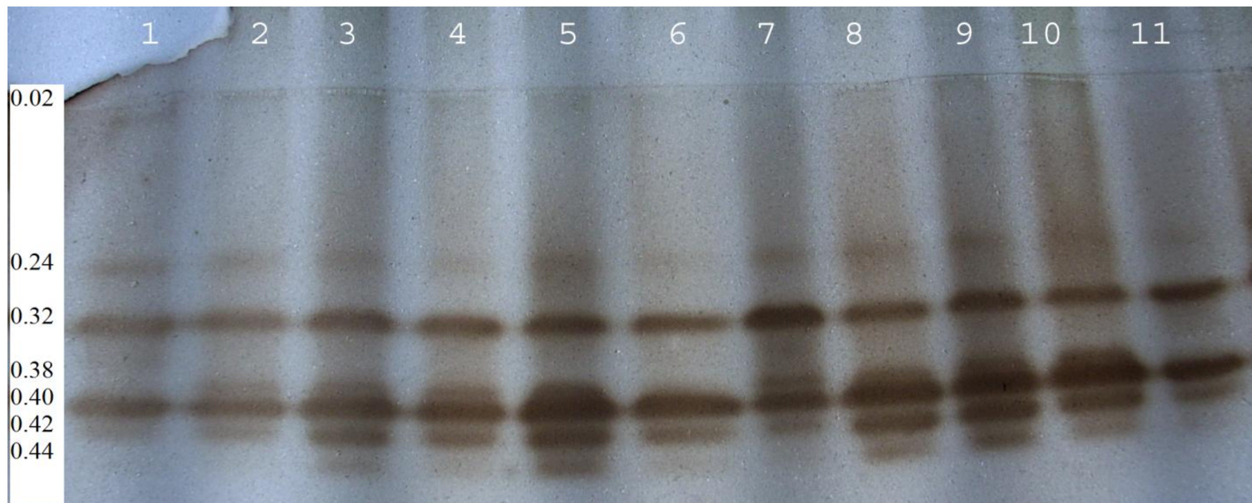

Figure 5. PPO zymogram of treated plants.

Lane 1= 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment, lane 2= distal untreated leaf of plant inoculated with pathogen prior to elicitor treatment, lane 3= 3rd node leaf of plant inoculated with pathogen after elicitor treatment, lane 4= distal untreated leaf of plant inoculated with pathogen after elicitor treatment, lane 5= 3rd node leaf of plant treated simultaneously with pathogen and elicitor, lane 6= distal untreated leaf of plant treated simultaneously with pathogen and elicitor, lane 7= control, lane 8= 3rd node leaf of plant inoculated with pathogen only, lane 9= distal untreated leaf of plant inoculated with pathogen only, lane 10= 3rd node leaf of plant treated with elicitor only, lane 11= distal untreated leaf of plant treated with elicitor only.

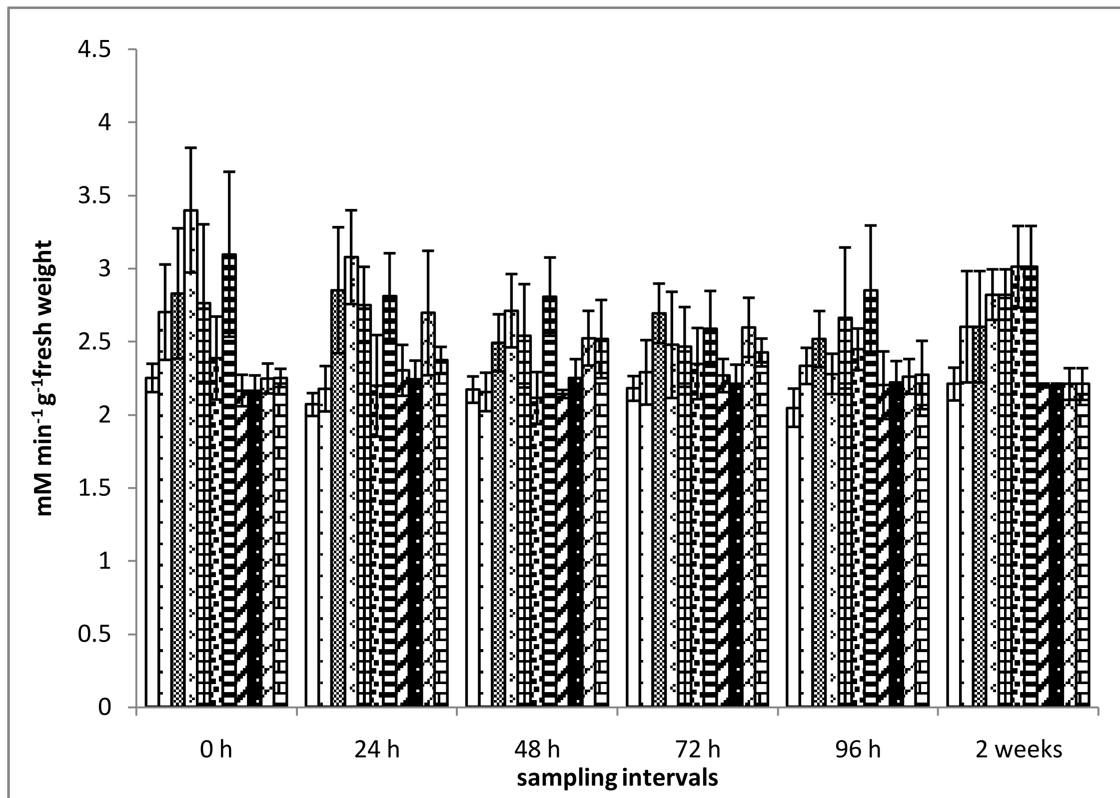

Figure 6. LOX activity in treated tomato plants.

□ control □ 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment ▩ distal untreated leaf of plants inoculated with pathogen prior to elicitor treatment ⊡ 3rd node leaf of plant inoculated with pathogen after elicitor treatment ⊞ distal untreated leaf of plant inoculated with pathogen after elicitor treatment ⊡ 3rd node leaf of plant treated simultaneously with pathogen and elicitor ⊟ distal untreated leaf of plants treated simultaneously with pathogen and elicitor ◪ 3rd node leaf of plant inoculated with pathogen only ■ distal untreated leaf of plant inoculated with pathogen only ⊠ 3rd node leaf of plant treated with elicitor only ⊟ distal untreated leaf of plant treated with elicitor only.

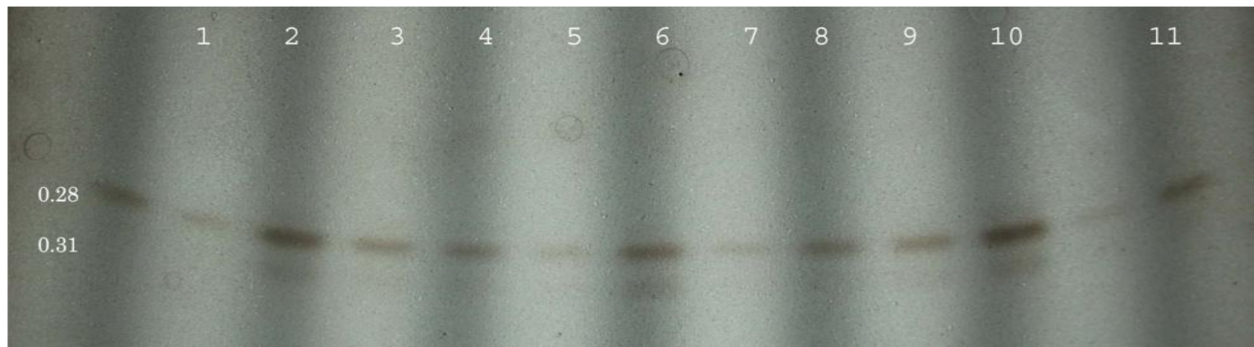

Figure 7. LOX zymogram of treated plants.

Lane 1= 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment, lane 2= distal untreated leaf of plant inoculated with pathogen prior to elicitor treatment, lane 3= 3rd node leaf of plant inoculated with pathogen after elicitor treatment, lane 4= distal untreated leaf of plant inoculated with pathogen after elicitor treatment, lane 5= 3rd node leaf of plant treated simultaneously with pathogen and elicitor, lane 6= distal untreated leaf of plant treated simultaneously with pathogen and elicitor, lane 7= control, lane 8= 3rd node leaf of plant inoculated with pathogen only, lane 9= distal untreated leaf of plant inoculated with pathogen only, lane 10= 3rd node leaf of plant treated with elicitor only, lane 11= distal untreated leaf of plant treated with elicitor only.

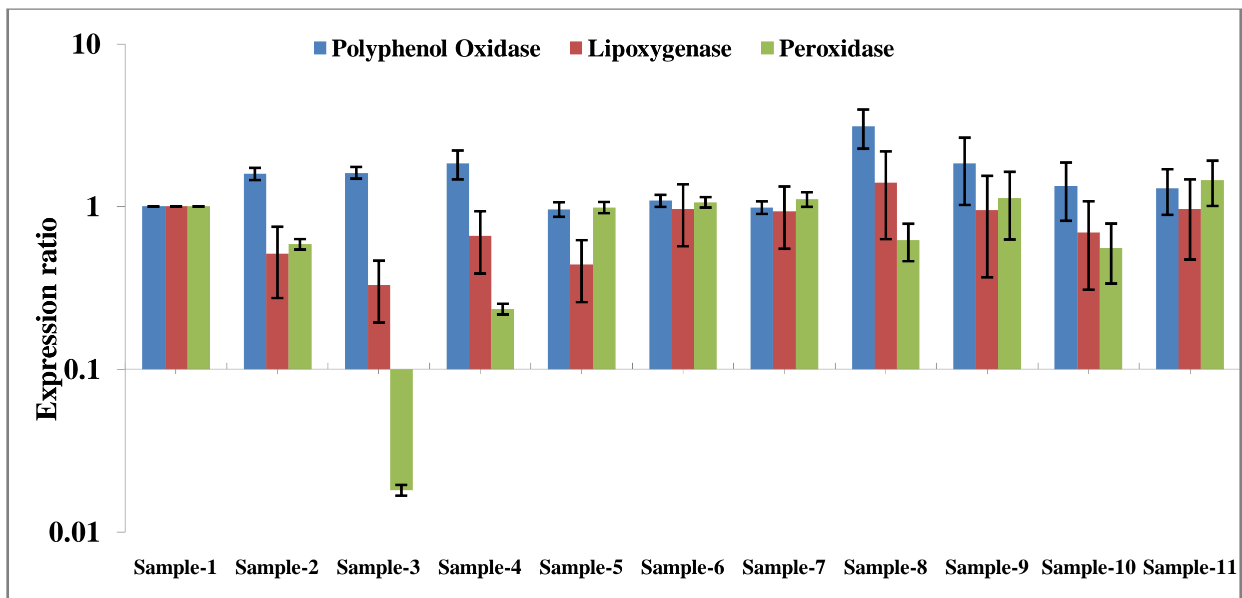

Figure 8. Expression ratios of peroxidase, lipoxygenase and polyphenol oxidase genes in treated tomato plants.

Sample 1= control, Sample 2= 3rd node leaf of plant inoculated with pathogen prior to elicitor treatment, Sample 3= distal untreated leaf of plant inoculated with pathogen prior to elicitor treatment, Sample 4= 3rd node leaf of plant inoculated with pathogen after elicitor treatment, Sample 5= distal untreated leaf of plant inoculated with pathogen after elicitor treatment, Sample 6= 3rd node leaf of plant treated simultaneously with pathogen and elicitor, Sample 7= distal untreated leaf of plant treated simultaneously with pathogen and elicitor, Sample 8= 3rd node leaf of plant inoculated with pathogen only, Sample 9= distal untreated leaf of plant inoculated with pathogen only, Sample 10= 3rd node leaf of plant treated with elicitor only, Sample 11= distal untreated leaf of plant treated with elicitor only.