Transcriptional and translational regulation of defense enzymes induced by neem fruit extract in tomato

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Transcriptional and translational regulation of defense enzymes induced by neem fruit extract in tomato

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Fruit extract of neem (Azadirachta indica A. Juss.) induces defense response through enhanced activities of phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), polyphenol oxidase (PPO), peroxidase (POX) along with isoenzymes of PPO and POX in tomato. The increase in PAL, POX activity and induction of specific isoenzymes of acidic PPO, POX and basic POX by neem fruit extract were completely inhibited due to pre-treatment with actinomycin D or cycloheximide. Their activation was found to be time course-dependent. Synergistic enhancement of TAL activity by neem extract was observed when plants were pre-treated with cycloheximide, whereas similar observations were made for PPO activity by neem extract when plants were pre-treated with either actinomycin D or cycloheximide. These results suggest that the enhanced activities of defense enzymes and altered isoenzyme patterns of PPO and POX by neem extract was regulated at both transcriptional and translational levels which may contribute to the induction of defense responses in tomato.

Keywords: actinomycin D; cycloheximide; peroxidase; phenylalanine ammonia-lyase; polyphenol oxidase; tyrosine ammonia-lyase

1. Introduction

The development of novel plant activators (inducers and elicitors) to activate host defense mechanisms with the induction of a wide range of defense-related genes (pathogenesis-related genes) is of high interest for organic agriculture. Increased activity of polyphenol oxidase (PPO; EC 1.10.3.1 or EC 1.14.18.1), peroxidase (POX; EC 1.11.1.7), and phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) has been reported in plants treated with various biotic and abiotic inducers of resistance (Raghvendra et al. 2007). Reported increases in defense enzyme activity could be the result of de novo synthesis or an activation of latent enzymes. Experiments with inhibitors of nucleic acid (actinomycin D) and protein synthesis (cycloheximide) have demonstrated as evidence of de novo synthesis of defense enzymes. Actinomycin D is considered to be inhibitory through a specific blocking of DNA-dependent RNA synthesis (Goldberg et al. 1962). Cycloheximide (Actidione) has found extensive use as an inhibitor of enzyme synthesis in plant cells by blocking nuclear gene expression but organelle translation is unaffected. However, in some of these
experiments, the amount of inhibition was variable and in other cases slight stimulatory effects were also observed. Individual isoperoxidases (iso-POX) in bean discs were reported to exhibit different sensitivities to some of these inhibitors (Novacky and Wheeler 1971).

Only limited studies have shown that the plant extracts can reduce the disease progression in plants through induction of acquired resistance. One of the problems in formalising a general hypothesis for induction of defense-related enzymes and phytoalexin from data obtained with abiotic elicitors is that not all plant species respond uniformly to the same chemicals (Varns et al. 1971). Therefore, there is a need to study the regulation of induction by elicitors on enzymes and isoenzyme profiles. The regulating effects of neem extracts on defense-related gene expression is not yet understood. Our earlier studies have shown that neem fruit extract induces systemic acquired resistance in tomato plants (*Lycopersicum esculentum*) against *Pseudomonas syringae* pv. tomato (Bhuvaneshwari et al. 2009). In this study, an effort was made to understand the nature of gene expression and its regulation leading to induction of defense response in tomato by application of fruit extract of neem (*Azadirachta indica* A. Juss.). Transcriptional and translational inhibitors were utilised along with neem extract to understand the nature of induction by neem extract on activities of defense enzymes leading to defense responses.

2. Materials and methods

2.1. Sowing of seeds

Seeds of S 22 variety of tomato were sown in soilrite in plastic trays (35 cm × 25 cm × 6 cm; L × W × H). Plants were raised in sterile culture room at 25 ± 1°C with a relative humidity (RH) of 70% and 12 h of light and dark photoperiod adjustment. Trays were watered daily with sterile distilled water and once a week with 100% Hoagland’s nutrient solution. Seven-week-old seedlings were used for the study.

2.2. Preparation of neem fruit extract

About 5 g of fully mature but green neem fruits (fruit coat green but seed hard) were washed twice with sterilised distilled water and dried under aseptic condition. Seeds were macerated in 20 ml of sterilised Type I water in a pre-chilled sterilised pestle and mortar. The extract thus obtained was filtered through four folds of Muslin cloth then centrifuged at 9500 rpm at 4°C for 30 min. The supernatant obtained was filtered through 0.45 μm membrane filter and used as neem fruit extract.

2.3. Treatment of plants

Experiments were conducted in a completely randomised fashion. Six groups of plants having three replicates each were treated as following. Each replicate had 52 tomato plants.

(1) First group of plants were sprayed with sterile Type I water and used as control plants.
Second group of plants were pre-treated with transcriptional inhibitor (actinomycin D, 0.05 mg/ml) followed by spraying with sterile Type I water and designated as transcriptional inhibitor control plants.

Third group of plants were pre-treated with translational inhibitor (cycloheximide, 0.5 mg/ml of ethanol) followed by spraying with sterile Type I water and designated as translational inhibitor control plants.

Fourth group of plants were sprayed with neem fruit extract only and designated as neem fruit extract treated plants.

Fifth group of plants were pre-treated with transcriptional inhibitor (actinomycin D, 0.05 mg/ml) followed by spraying with neem fruit extract and designated as transcriptional inhibitor neem treated plants.

Sixth group of plants were pre-treated with translational inhibitor (cycloheximide, 0.5 mg/ml of ethanol) followed by spraying with neem fruit extract and designated as translational inhibitor neem treated plants.

Spraying was done using automiser to produce fine spray droplets. All the above operations were carried out under aseptic conditions. For all analysis, leaves of third node from the base were sampled from both control and treated plants at 0 h, 24 h, 48 h, 72 h and 96 h after treatment. Immediately after sampling, the leaves were frozen in liquid nitrogen and stored at −80°C and used subsequently for estimating the activities of PAL, tyrosine ammonia-lyase (TAL), PPO, POX and isoforms of PPO, POX.

2.4. Preparation of extract and estimation of PAL and TAL

Frozen leaf tissue (300 mg) was homogenised in 1.2 ml of ice cold 0.05 M borate buffer (pH 7.0) containing 1 mM Phenyl methyl sulfonyl fluoride (PMSF), 0.001% Triton X-100, 1 mM EDTA and 10% (w/w) polyvinyl pyrrolidone (PVP) at 4°C. The homogenate was centrifuged at 12,800 rpm for 20 min at 4°C. The supernatant was used as enzyme extract for estimating PAL and TAL activity. Two replicates were taken for each sample.

The reaction mixture for PAL containing 0.05 ml of enzyme extract, 0.95 ml of 0.05 M borate buffer (pH 7.0), 0.11 ml of 100 mM L-phenylalanine was incubated in a water bath at 40°C for 30 min and terminated by adding 2% w/v of trichloroacetic acid. For estimation of TAL activity, L-phenylalanine was replaced with L-tyrosine. Absorbance was recorded on a UV-VIS spectrophotometer (Shimadzu, 1650) at 275 nm and 310 nm for PAL and TAL activity, respectively. Reaction mixture without enzyme extract served as blank. The PAL and TAL activity was calculated using their respective molar extinction coefficient $\varepsilon = 15.56 \times 10^{-3}$ μM$^{-1}$ cm$^{-1}$ and $\varepsilon = 9.554 \times 10^{-3}$ μM$^{-1}$ cm$^{-1}$. Enzyme activity was expressed as μM g$^{-1}$ fresh weight min$^{-1}$. Two replicates were taken for each replicate sample.

2.5. Preparation of extract and estimation of PPO and POX

Frozen leaf tissue (300 mg) was homogenised at 4°C in 1.2 ml of ice cold 0.1 M sodium phosphate buffer (pH 9.0 for PPO/pH 6.5 for POX) containing 1 mM PMSF, 0.001% Triton X-100, 1 mM EDTA and 10% (w/w) PVP. The homogenate was centrifuged at 10,250 rpm for 20 min at 4°C. The supernatant was used as
enzyme extract for estimating activity and detecting isoenzyme pattern of PPO and POX. Two replicates were taken for each sample.

The reaction mixture for PPO consisted of 0.5 ml of phosphate buffer (1 M, pH 9.0), 1.25 ml of catechol solution (0.2 M), 0.01 ml of enzyme extract and 0.2 ml of sterilised type I water. The reaction mixture for POX consisted of 0.245 ml of phosphate buffer (1 M, pH 6.5), 0.25 ml of pyrogallol (0.1 M), 0.05 ml of 100 mM hydrogen peroxide, 0.01 ml of enzyme extract and 1.445 ml of type I water. Both the reaction mixtures were incubated at 25°C for 5 min and terminated by addition of 0.5 ml of 10% (v/v) sulphuric acid. Absorbance was recorded at 420 nm using UV-VIS spectrophotometer (Shimadzu, 1650). Reaction mixture without enzyme extract served as blank. Enzyme activity of PPO was expressed as units g⁻¹ fresh weight min⁻¹. Enzyme activity of POX was calculated using the molar extinction coefficient ε = 2.47 × 10⁻³ μM⁻¹ cm⁻¹ and expressed as μM g⁻¹ fresh weight min⁻¹. Two replicates of each replicate enzyme extract were taken for each replicate sample.

### 2.6. Native-BASIC PAGE analysis

The isozyme profile of acidic POX and PPO was examined by native basic polyacrylamide gel electrophoresis (Laemmli 1970) without SDS. For acidic proteins, separating and stacking gels were made of 10% polyacrylamide at pH 8.8% and 5% polyacrylamide at pH 6.8, respectively. Electrolyte for electrode reservoirs was Tris-glycine, pH 8.3. Bromophenol blue (0.01%) was used as tracking dye. For each sample, 75 μg of crude extract for POX isoforms and 50 μg of crude extract for PPO isoforms were loaded onto the native basic polyacrylamide gel. Electrophoresis was carried out for 6 h at 30 mA/gel and 4°C. After electrophoresis, native basic gels were stained for iso-POX by incubating in 0.1 M sodium phosphate buffer pH 6.5 containing 50 mM pyrogallol and 0.75% H₂O₂. Acidic PPO isoforms were visualised by the modified method of Jayaraman et al. (1987) by equilibrating the gel in 0.1% p-phenylene diamine followed by addition of 50 mM catechol in 0.1 M sodium phosphate buffer pH 9.0.

### 2.7. Native-acidic PAGE analysis

Acidic native-PAGE was performed by the modified method of Reisfeld et al. (1962). For basic proteins (pI > 7), 10% resolving gel prepared in a 0.385 M acetic acid-KOH solution, pH 4.3 and 5% stacking gel prepared in 0.0625 M acetic acid-KOH solution, pH 6.8. The electrophoresis was carried out with acidic running buffer (0.14 M acetic acid and 0.35 M β-alanine, pH 4.3) and a reverse polarity electrode, using methyl green as the tracking dye. For each sample, 75 μg of crude extract was loaded to the gel. Electrophoresis was performed at 4°C, 20 mA/gel for 30 min, followed by 40 mA/gel for 3 h. Native acidic gels were stained for iso-POX by incubating in 0.1 M sodium phosphate buffer pH 9.0 containing 0.1% p-phenylene diamine, 50 mM catechol and followed by addition of 0.75% H₂O₂ for 30 min. Isoenzyme pattern in gel was visualised on a transilluminator in white fluorescent light. The relative distance (Rf value) of each isozyme band was calculated from each zymogram using the following equation: (Rf value = Distance migrated by the isoenzyme band from the origin/Distance migrated by tracking dye from the origin).

Difference in isoenzyme banding pattern was identified according to the number of bands, their Rf values and their level of expression.
2.8. Data analysis
Each sample of extract was measured twice in each replicate, and three replications were performed per analysis. The data were statistically analysed by 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). Multiple pairwise-comparison tests using least-square means were performed for post-hoc comparisons after two-way ANOVA with treatment and time as two factor with replications. The corrections used for multiple comparisons were Tukey’s honest significantly differences (HSD) test, Dunnett and Bonferroni procedure.

3. Results and discussions
3.1. Activity of PAL
PAL activity significantly increased after 96 h in neem-treated plants \((P<0.001)\). However, on pre-treatment with inhibitors, induction of PAL activity by neem fruit extract was inhibited by both actinomycin D (transcriptional inhibitor) and cycloheximide (translational inhibitor) (Figure 1). This implies that the induction of PAL activity by neem fruit extract was due to de novo biosynthesis of this enzyme being regulated at both transcriptional and translational level.

Similarly, Matsushita and Uritani (1975) reported the increase of PAL activity in response to wounding in sweet potato was inhibited by both cycloheximide and actinomycin D. Chen et al. (2006) also reported the induction of PAL expression by salicylic acid in harvested grape berries could be blocked by pre-treatment with cycloheximide and actinomycin D.

3.2. Activity of TAL
TAL activity significantly increased after 48 h \((P<0.001)\) of neem treatment and reached maximum with three-fold enhancement after 96 h \((P<0.001)\). TAL activity

Figure 1. Effect of actinomycin D and cycloheximide on induction of PAL activity by neem fruit extract in tomato plants. Vertical bars indicate standard deviations.

- Control plants,
- Transcriptional inhibitor control plants,
- Translational inhibitor control plants,
- Neem treated plants,
- Transcriptional inhibitor neem treated plants,
- Translational inhibitor neem treated plants.

3.2. Activity of TAL
TAL activity significantly increased after 48 h \((P<0.001)\) of neem treatment and reached maximum with three-fold enhancement after 96 h \((P<0.001)\). TAL activity
significantly increased after 48 h of treatment with actinomycin D, cycloheximide and in neem extract preceded by actinomycin D or cycloheximide \((P < 0.001)\). Actinomycin D and cycloheximide had no inhibitory effect on the TAL activity. Both the inhibitors induced TAL activity similar to that observed in plants treated with neem fruit extract alone. There was no synergistic enhancement of TAL activity when neem was applied along with actinomycin D. Four-fold synergistic enhancement of TAL activity was observed after 96 h when neem treatment preceded with cycloheximide \((P < 0.001)\) (Figure 2). Induction of TAL activity by neem treatment was not regulated at transcriptional and translational level.

Our results suggest the existence of different inactivating systems between PAL and TAL. To explain the apparent paradox of the induction of TAL synthesis with protein synthesis inhibitor, the super induction mechanism similar to the previous studies implies that either expressed protein inhibitor masks the activity of the already existing enzyme or the concentration of the repressor is reduced by a partial inhibition of protein synthesis (low concentrations of inhibitor) resulting in derepression of the gene. (Attridge and Smith 1973; Tao and Khan 1975). Cycloheximide and actinomycin D elicit defense-related enzymes and phytoalexin production at low concentrations but higher concentrations are inhibitory (Hadwiger and Schwochau 1971).

**3.3. Activity of PPO**

PPO activity significantly increased after 48 h \((P < 0.01)\) in neem-treated plants, reached maximum with six-fold induction after 96 h \((P < 0.001)\). PPO activity was high after 48 h of treatment with actinomycin D \((P < 0.05)\) and cycloheximide \((P < 0.001)\). Three-fold synergistic enhancement of PPO activity was observed after 24 h of treatment with neem preceded with either actinomycin D \((P < 0.001)\) or cycloheximide \((P < 0.001)\) (Figure 3). Inspite of inhibition of acidic iso-PPO (Rf 0.24 and 0.34) after 24 h of neem extract treatment preceded with actinomycin D or cycloheximide (Figure 5), the total activity of PPO was higher (Figure 3). Even the
increased total activity of PPO was maintained till 48 h of the neem treatment preceded with cycloheximide (Figure 3). This particular aspect of PPO seems to be very interesting and is probably due to the increased activities of alternate iso-PPO. Lee (1971) also observed increase in indoleacetic acid oxidase activity despite the inhibition of its specific isoenzymes by actinomycin D and cycloheximide along with low concentration of cytokinin in tobacco callus culture. Although cycloheximide is extremely inhibitory to protein synthesis in vivo in higher plants, the reported insensitivity of some plant ribosomes suggests that it may not invariably act at the ribosomal level (Ellis and MacDonald 1970). Similarly actinomycin D and cycloheximide showed no inhibitory effect on the PPO activity in controls as well as in gibberellic acid-treated half-seeds and caused a two- to three-fold stimulation of enzyme activity similar to that observed in endosperm treated with gibberellic acid alone. However, there was no additive or synergistic enhancement of PPO activity when gibberellic acid was tested in combination with cycloheximide or actinomycin D (Taneja and Sachar 1974b). Synergistic enhancement of PPO activity in neem treatment preceded with actinomycin D or cycloheximide may be due to previous existence of inactive pool of this enzyme, and the maintenance of this pool in an inactive state could depend on RNA and protein synthesis. This is an unusual and interesting phenomenon, similar to the “superinduction” explained in earlier reports (Taneja and Sachar 1974b; Tao and Khan 1975).

3.4. Activity of POX

POX activity significantly increases after 24 h of neem treatment ($P < 0.001$). The induction of POX activity by neem extract was completely inhibited by both actinomycin D (transcriptional inhibitor) and cycloheximide (translational inhibitor) after 24 h ($P < 0.001$) (Figure 4). This implies that the induction of POX expression by neem fruit extract was regulated at transcriptional and translational level.
Similar to our result, induction of POX activity by ethylene in root disks of sweet potato (*Ipomoea batatas* (L.) Lam.) was inhibited by actinomycin D and cycloheximide, indicating *de novo* protein synthesis (Gahagan et al. 1968). The increase of POX activity in response to wounding of sweet potato was inhibited by cycloheximide but was not inhibited by actinomycin D, which repressed RNA synthesis 60% to 70% (Matsushita and Uritani 1975). Sweet potato slices treated with inhibitors of protein synthesis also inhibited the increment in POX activity (Kanazawa et al. 1965). Wounding of tobacco induces POX activity and cycloheximide treatment prevented this induction (Lagrimini and Rothstein 1987).

3.5. *Acidic isopolyphenol oxidases (iso-PPO)*

Control plants had seven constitutively expressed acidic iso-PPO (Rf 0.24, 0.34, 0.41, 0.44, 0.48, 0.52 and 0.58). Neem fruit extract treated leaves had increased expression of acidic iso-PPO (Rf 0.24 and 0.34) after 24 h of treatment. When neem treatment preceded with either actinomycin D or cycloheximide, the expression of acidic iso-PPO (Rf 0.24 and 0.34) was completely inhibited within 24 h of treatment (Figure 5). The inhibition of acidic iso-PPO observed in neem treated plants by actinomycin D may be regarded as an indication of requirement for DNA-dependent RNA synthesis for the enhanced activity of these isoenzymes. Therefore, it can be postulated that the induction of acidic iso-PPO (Rf 0.24 and 0.34) activity by neem fruit extract treated plants was due to *de novo* expression of these isoenzymes and their transcriptional and translational regulation.

Cycloheximide inhibited the appearance of isoforms of PPO in excised coleoptiles and roots of 4–5-day-old wheat seedlings, whereas actinomycin D did not inhibit implying that protein synthesis for formation of new isoforms (Taneja and Sachar 1974a).
3.6. Acidic iso-POX

Control plants had three constitutive acidic iso-POX with Rf 0.29, 0.43, 0.47. Four new acidic iso-POX (Rf 0.12, 0.21, 0.37, 0.50) were expressed after 24 h of treatment with neem fruit extract. Neem treatment preceded by actinomycin D inhibited all acidic iso-POX except with Rf 0.43 after 24 h. Neem treatment preceded with cycloheximide suppressed all the expression of acidic iso-POX except with Rf 0.29, 0.43, 0.47 after 24 h (Figure 6). The inhibition of acidic iso-POX in neem-treated plants by actinomycin D probably indicates the requirement of DNA-dependent RNA synthesis for expression of these isoenzymes. Therefore, it implies that probably the induction of acidic iso-POX activity by neem fruit extract could be due to transcriptional and translational regulation of biosynthesis.

Increase in PPO, POX and iso-POX due to coronatine treatment in potato tuber has been markedly inhibited by cycloheximide or actinomycin D (Mino et al. 1980). The rapid increase in POX and iso-POX activity in sweet potato slices results from de novo synthesis (Shannon et al. 1971).

Figure 5. Changes in acidic iso-PPO profile after 24 h of neem fruit extract treatment preceded with transcriptional and translational inhibitors. Lane 1: Control plants; Lane 2: transcriptional inhibitor control plants; Lane 3: translational inhibitor control plants; Lane 4: neem fruit extract treated plants; Lane 5: transcriptional inhibitor neem treated plants; Lane 6: translational inhibitor neem treated plants.

Figure 6. Changes in acidic iso-POX profile after 24 h of neem fruit extract treatment preceded with transcriptional and translational inhibitors. Lane 1: Control plants; Lane 2: transcriptional inhibitor control plants; Lane 3: translational inhibitor control plants; Lane 4: neem fruit extract treated plants; Lane 5: transcriptional inhibitor neem treated plants; Lane 6: translational inhibitor neem treated plants.
3.7. Basic iso-POX

Control plants had two constitutive basic iso-POX (Rf 0.25, 0.55). The expression of basic iso-POX (Rf 0.25) progressively increased after 24 h in plants treated with neem fruit extract only. But when neem extract treatment preceded with transcriptional inhibitor (actinomycin D) or translational inhibitor (cycloheximide), the expression of basic iso-POX (Rf 0.25) was completely inhibited after 24 h (Figure 7). It implies that probably transcriptional and translational regulation controls the expression of basic iso-POX (Rf 0.25) induced by neem treatment.

Wounding the tobacco plant triggered the expression of several basic iso-POX in the leaf and both basic and acidic iso-POX in pith tissue and the induction was inhibited by cycloheximide (Lagrimini and Rothstein 1987). Vance and his coworkers showed that cycloheximide inhibited the resistance of canarygrass to Helminthosporium avenae, also blocked lignin biosynthesis and activity of enzymes involved in lignin formation, including POX (Vance and Sherwood 1976; Vance et al. 1976). Change in PAL activity and isozyme patterns of PPO and POX was observed in cowpea treated with salicylic acid, leading to enhance resistance against Rhizoctonia solani (Chandra et al. 2007).

4. Conclusions

It was concluded from our study that the induction of PAL, POX, specific iso-PPO and iso-POX activity by neem extract is regulated at transcriptional and translational level which may contribute to the induction of defense responses in tomato. Inhibitory effect observed on expression of specific isoenzymes of PPO and POX could probably be due to selective inhibitory effect of actinomycin D and cycloheximide on expression of genes for these isoenzymes. The paradoxical stimulation of TAL and PPO due to the neem treatment proceeded with actinomycin D or cycloheximide suggested the activation of inactive forms. Superinduction of PPO activity by actinomycin D was possibly due to (a) inhibition of synthesis of the mRNA coding for specific repressor proteins which inhibit this enzyme and (b) differential stability of mRNAs in presence of actinomycin D, and competition among mRNAs for factors limiting translation, thus favouring synthesis of this enzyme. Superinduction of TAL, PPO activity by cycloheximide may be because of

[Figure 7. Changes in basic iso-POX profile after 24 h of neem fruit extract treatment preceded with transcriptional and translational inhibitors. Lane 1: Control plants; Lane 2: transcriptional inhibitor control plants; Lane 3: translational inhibitor control plants; Lane 4: neem fruit extract treated plants; Lane 5: transcriptional inhibitor neem treated plants; Lane 6: translational inhibitor neem treated plants.]
(a) inhibition of synthesis of proteolytic enzymes which leads to continued synthesis of these enzymes in the presence of cycloheximide or (b) suppressed translation and expression of repressor proteins which are involved in regulation of gene expression of TAL, PPO. Absence of such repressors removes inhibitory control thereby leading to continued synthesis of these enzymes.

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References


