

Full Length Research Paper

Enhancement of the expression of defense genes in tomato against *Ralstonia solanacearum* by N-octanoyl-L-homoserine lactone

Shiva Kumar Jayanna and Sharanaiah Umesha*

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore-570006, Karnataka, India.

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Many Gram-negative bacteria use N-acyl-homoserine lactone (AHLs) as quorum sensing (QS) signalling molecules to monitor their population density and to regulate gene expression in a density dependent manner. Recently, it has been shown that AHLs are detected by the plants and they trigger plant defense responses. In this study, N-octanoyl-L-homoserine lactone (C8-HSL) has been used as resistant inducer against bacterial wilt disease of tomato caused by *Ralstonia solanacearum*. The present investigation focused on the role of defense related enzymes (phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and lipoxygenase) in imparting resistance in tomato against *R. solanacearum*. Activities of these defense enzymes, increased in C8-HSL treated tomato plants, which were challenged with *R. solanacearum*. The transcripts accumulation studies for these enzymes were carried out using semi-quantitative reverse transcription PCR, with maximum mRNA accumulation in resistant cultivar upon treatment with C8-HSL. Quantitative real time-PCR (qRT-PCR) confirmed the maximum induction of all these four genes in C8-HSL treated plants. However, the expression of defense genes was higher in C8-HSL treated resistant cultivar than that of susceptible cultivar. Therefore, the results support the view that C8-HSL molecule enhances disease protection against *R. solanacearum* infection in tomato through the activation of defense genes.

Key words: N-octanoyl-L-homoserine lactone, *Ralstonia solanacearum*, tomato, defense genes, quantitative real time-polymerase chain reaction (PCR).

INTRODUCTION

Many bacteria use small signalling molecule to communicate with each other and to co-ordinate their growth activities, this process is commonly referred to as quorum sensing (QS). The most common signalling

molecules in Gram negative bacteria are N-acyl-L-homoserine-lactones (AHLs). The AHLs are composed of a conserved homoserine lactone moiety and a fatty acid side chain that can vary in length (4 to 18 carbon atoms)

*Corresponding author. E-mail: su@appbot.uni-mysore.ac.in or umeshgroup@yahoo.co.in. Tel: +91 821 2419884. Fax: +91 821 2419884.

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(Decho et al., 2011). To date, approximately 50 types of AHL signalling molecules are found in bacteria (Jin et al., 2012). Recent reports indicate that bacteria commonly associated with plants are capable of producing a variety of AHLs (Cha et al., 1998). However, only little is known about the molecular ways of plants reacting with these bacterial QS signals. Bacteria like *Xanthomonas oryzae* pv. *oryzae*, *Ralstonia solanacearum*, *Pseudomonas syringae* and *Dickeya didantii* cause disease on plants. In recent years, evidence has accumulated that AHL molecules are able to function as priming agents. AHL molecules induce resistance against a broad spectrum of plant pathogens in different plant species (Schikora et al., 2016). Schenk and Schikora (2015) showed that AHL primed plants, upon a challenge with pathogens accumulate callose and phenolic compounds. Similar to AHL molecules produced by bacteria, commercially available pure AHL molecules also induce priming. The transport of AHLs within plants has been studied initially in Barley and *Arabidopsis* (Gotz et al., 2007; von Rad et al., 2008) by using radioactive labelled AHLs. Mathesius et al. (2003) observed that the legume plant, *Medicago truncatula* are able to respond to nanomolar concentrations of synthetic and purified AHLs and these compounds elicit major changes in protein expression. These changes suggest that bacterial QS signalling molecules might regulate the functions of these proteins, which include roles in defense responses of host plants, primary metabolism, plant hormonal response, transcriptional gene regulation, protein processing and activities of the cytoskeleton (Mathesius et al., 2003). Schuhegger et al. (2006) showed that treatment of roots with synthetic N-hexanoyl-L-homoserine lactone (AHLs (C6-HSL)) enhanced the expression of salicylic acid and ethylene dependent defense genes in tomato against the fungal leaf pathogen, *Alternaria alternata*. A transcriptomic approach by von Rad et al. (2008) in *Arabidopsis* showed gene expression changes for several hundred genes in shoots and roots in response to 10 μ M concentration of C6-HSL. However, if plant can detect low concentration of AHLs, they might be able to respond before pathogen concentration build up (Teplitski et al., 2010). The long chain AHL, oxo-C14-HSL, activates resistance towards different obligate bio-trophic pathogens such as *Golovinomyces orontii* and *Blumeria graminis* in *Arabidopsis* and barley, respectively (Schenk et al., 2014). All these studies demonstrate that AHLs can induce resistance in plant by activating the defense mechanism.

Tomato (*Solanum lycopersicum* Mill.) is one of the important vegetable grown and consumed worldwide. Tomato is prone to a number of bacterial diseases among which bacterial wilt caused by *R. solanacearum* (Smith) is a very destructive harmful disease resulting in complete loss of the crop (Vanitha and Umesha, 2008; Prakasha et al., 2016). Control of bacterial wilt has been difficult due to the high variability of the pathogen, high

ability to survive in diverse environments and its extremely wide host range. Using chemicals to control plant diseases is hazardous to the environment and living beings, so using biological control can overcome this problem. Schuhegger et al. (2006) results suggest that AHL molecule play an important role in the biocontrol activity of *Serratia liquefaciens* and other rhizobacteria in tomato, act as mediators of communication between prokaryotes and eukaryotes. AHLs may therefore be considered as potential candidates for a new group of general elicitors of plant defense as they induce expression of typical defense related proteins resulting in increased resistance against pathogen (Venturi and Fuqua, 2013).

Plants possess a range of active defense responses that contribute to resistance against a variety of pathogens. They respond to bacterial pathogen attack by activating various defense responses that are associated with the accumulation of several factors like defense related enzymes and inhibitors that serve to prevent pathogen infection. The interaction between the pathogen and host plant induces some changes in cell metabolism, primarily in the enzyme activities, including that of phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), lipoxygenase (LOX), superoxide dismutase and β -1,3-glucanase (Kavitha and Umesha, 2008). These enzymes play a crucial role with respect to the degree of host resistance, by increasing anti microbial activity, bio synthetic processes related to wall development such as phenol, lignification, polymerization of hydroxyproline-rich glycoproteins, regulation of cell wall elongation and wound healing (Belkhadir et al., 2004).

The aim of this study was to investigate whether C8-HSL induce resistance in tomato and also its efficacy in controlling bacterial wilt disease through differential expression of defense genes (PAL, POX, PPO and LOX). The changes in the gene expression patterns were also studied using specific primers for these enzymes. Defense genes were assessed to determine possible relationships between the activation of these enzymes and the protection of plants following treatment with C8-HSL and its mRNA accumulation was measured by quantitative real time-PCR upon challenge inoculation with the pathogen.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of tomato cultivar, resistant (R) (cv. Golden) and susceptible (S) (cv. Rasi) to bacterial wilt were procured from private seed agencies in Mysore, Karnataka, India. From earlier studies in our laboratory, these two cultivars of tomato were selected on the basis of their response to bacterial wilt disease caused by *R. solanacearum* inoculation (Vanitha and Umesha, 2008). All seed samples were surface sterilized with 3% (v/v) sodium hypochlorite solution for 5 min and washed with distilled water three times.

Table 1. List of primers.

Gene product	Forward primer (5'-3')	Reverse primer (5'-3')
Phenylalanine ammonia lyase	GTGACTAACCCACGTCCAAAG	CAAAGCGCCACGAGATAG
Peroxidase	GTTGCCTTGGTTGCTAGAG	GACGTCTGGAGACTGGAA
Polyphenol oxidase	ACTACGGAGGTGCCATAC	CGGCTAATCGCCAGATTG
Lipoxygenase	GGACATGGCGACAAGAAA	GTAGGGCGATTAGGGAGATA
18S rRNA	GTGCATGGCCGTTCTTAGTTG	CAGGCTGAGGTCTCGTTCGT

Tomato plants were raised in plastic trays filled with mixture of sterilized soil, sand and farm yard manure (2:1:1). Tray were maintained in the green house conditions and watered as and when required.

Treatment with C8-HSL and challenge with *R. solanacearum*

Four-week-old plants grown in sterilized soil were treated with 10 μ M C8-HSL (Cayman, USA) (Schuhegger et al., 2006). A100 x stock in dimethyl sulfoxide (DMSO) was diluted in 5 ml sterile distilled water and pipetted on the soil to avoid contact with shoots and leaves. Control plants received equal amounts of DMSO in water.

The wilt causing *R. solanacearum* (strain: RS-lpxC-DOB-2) inoculum was prepared by growing bacteria on 2,3,5-triphenyltetrazolium chloride (TZC) agar medium for 48 h at 30°C (Kumar et al., 2016). The bacterial cells were collected in sterile distilled water and pelleted by centrifugation at 12,000 rpm for 10 min. The pellet was resuspended in distilled water and bacterial concentration was adjusted to 1×10^8 cfu/ml at absorbance 610 nm using UV-visible spectrophotometer (Hitachi U-2000, Japan) according to Ran et al. (2005). 15 ml of bacterial suspension was poured onto the soil near the roots of tomato plants.

The four-week-old tomato plants inoculated and uninoculated were harvested at 0, 3, 6, 9, up to 72 h post inoculation (hpi) and stored at -80°C for subsequent analysis.

Enzyme studies

Phenylalanine ammonia lyase (PAL) activity was performed according to Lisker et al. (1983). One gram of tomato seedling, fresh mass was homogenized to fine paste in a pre-chilled mortar with 25 mM Tris-HCl buffer (pH 8.8). The homogenate was centrifuged at 10,000 rpm for 12 min at 4°C and the supernatant was directly used as enzyme source. The enzyme activity was determined by measuring the production of trans-cinnamic acid from L-phenylalanine using spectrophotometer (Hitachi U-2000, Japan). The reaction mixture contained 1 ml enzyme extract, 0.5 ml 50 mM L-phenylalanine and 0.4 ml 25 mM Tris-HCl buffer (pH 8.8). After incubation for 2 h at 40°C, the activity was stopped by the addition of 60 μ l 5 M HCl and the absorbance was read at 290 nm against the same volume of reaction mixture without L-phenylalanine which served as blank.

Peroxidase (POX) assay was carried out as described by Hammerschmidt et al. (1982). One gram of fresh mass of plants was homogenized in 1 ml of 10 mM phosphate buffer (pH 6.0) and centrifuged at 10,000 rpm for 12 min at 4°C and the supernatant served as enzyme source. The reaction mixture consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.0) containing 10 mM H₂O₂. Addition of 0.1 ml of crude enzyme extract initiated the reaction and absorbance at 470 nm was measured for 1 min.

Polyphenol oxidase (PPO) activity was determined according to Mayer et al. (1966). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 ml of the enzyme extract. The reaction was started with the addition of 0.2 ml of 10 mM catechol. The increase in absorbance was measured at 420 nm for 1 min.

Lipoxygenase (LOX) activity was estimated according to Borthakur et al. (1987). The activity was determined spectrophotometrically by monitoring the appearance of conjugated diene hydroperoxide, absorbing at 234 nm. The reaction mixture contained 2.7 mL of 0.2 M sodium phosphate buffer (pH 6.5), 0.3 ml of 10 mM linoleic acid in Tween 20 and 0.05 ml of the enzyme extract. Protein contents of the extracts were determined according to standard procedure of Bradford (1976) using BSA (Sigma, USA) as standard.

Analysis of defense genes by semi-quantitative reverse transcription PCR

The total RNA from four-week-old plants of resistant and susceptible tomato cultivars based on enzyme assays (PAL, POX, PPO and LOX) were extracted for analysis. RNA isolation was done using RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction.

The complementary DNA was synthesised using 2 μ g of RNA and first strand cDNA synthesis kit (Thermo Scientific, India). The reverse transcribed RNA was used as PCR template with gene specific primers for all the four genes (PAL, POX, PPO and LOX). 18S rRNA gene primer specific to tomato was used as a constitutive control in all gene expression studies (Chandrashekar and Umesha, 2014). All the primer sequences were reconfirmed by BLAST analysis. The primers used are shown in Table 1. Semiquantitative RT-PCR assay conditions were, initial 3 min denaturation at 94°C, followed by 94°C for 30 s, 60°C for 40 s, and 72°C for 1 min and a final extension for 10 min at 72°C. The number of cycles was 35.

Analysis of defense gene expression by quantitative real time PCR

Each qPCR reaction (20 μ l) consisted of 1 x SYBR Green (Thermo Scientific, India) PCR master mix, 3 pmol of each primer and 20 ng each of cDNA by using StepOnePlus™ Real Time PCR machine (Applied Biosystems, USA). qPCR steps were: denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, 60 s at 60°C. For calculating the fold change in expression of genes in plants, the transcripts in both the control and treated were normalized to 18S rRNA and the difference in the 18S rRNA normalized cycle threshold value ($\Delta\Delta$ CT) was used to obtain fold change (Livak and Schmittgen, 2001), with standard error being calculated from three replicated derived from each independent experiment.

Statistical analysis

All enzyme assay experiments were carried out in triplicates. Further, the experimental results were subjected to Duncan's multiple range tests at a significance level of $P < 0.05$. All statistical tests were performed using SPSS software.

RESULTS

Enzyme studies

Plants have endogenous defence mechanisms that can be induced in response to attack by pathogens. Inducing the plants own defense mechanisms by prior application of AHL (biological inducer) is thought to be a novel plant protection strategy. The temporal changes of all four enzyme activities of 10 μM C8-HSL primed followed by challenge inoculated with the pathogen along with their respective uninoculated controls were assayed. The temporal pattern studies of enzymes were undertaken to estimate the PAL, POX, PPO and LOX highest activities at regular intervals from 0 to 72 h.

In the resistant tomato cultivar, the temporal pattern of PAL enzyme revealed the maximum activity at 9 hpi (140 units) (Figure 1a), while in susceptible tomato cultivar, PAL enzyme revealed the maximum activity at 18 hpi (85 units) (Figure 1b). In the resistant tomato cultivar, the temporal pattern of POX enzyme revealed the maximum activity at 6 hpi (65 units) (Figure 1c), while in susceptible tomato cultivar POX enzyme revealed the maximum activity at 15 hpi (28 units) (Figure 1d). In the resistant tomato cultivar, the temporal pattern of PPO enzyme revealed the maximum activity at 12 hpi (72 units) (Figure 1e), while in susceptible tomato cultivar, PPO enzyme revealed the maximum activity at 24 hpi (35 units) (Figure 1f). In the resistant tomato cultivar, the temporal pattern of LOX enzyme revealed the maximum activity at 6 hpi (40 units) (Figure 1g), while in susceptible tomato cultivar, LOX enzyme revealed the maximum activity at 12 hpi (23 units) (Figure 1h). Therefore, gene expression studies were concentrated on only that particular time interval.

Semi-quantitative reverse transcription PCR

The gene expression pattern was altered when the plants were treated with C8-HSL and challenged with *R. solanacearum* in both resistant and susceptible tomato cultivars. The PAL, POX, PPO and LOX gene expressions was higher in resistant cultivar when the plants were treated with C8-HSL. The respective controls also expressed the accumulation of gene expression but they were not significant. The housekeeping gene, 18S rRNA expression was found to be unaltered in both resistant and susceptible cultivar in all the treatments (Figure 2).

Gene expression analysis by quantitative real-time PCR

Both resistant and susceptible tomato cultivars were raised and the four-week-old plants were treated with C8-HSL and inoculated with *R. solanacearum* (concentration of 1×10^8 cfu/ml). The gene expression studies were carried out based on the temporal pattern studies of enzymes. Based on the temporal activity of PAL (Figure 1a and b) and followed by semi quantitative RT-PCR (Figure 2); the authors selected 9 and 18 hpi for resistant and susceptible tomato cultivars, respectively. Based on the temporal activity of POX (Figure 1c and d) and followed by semi quantitative RT-PCR (Figure 2); 6 and 15 hpi were selected for resistant and susceptible tomato cultivars, respectively. Based on the temporal activity of PPO (Figure 1e and f) and followed by semi quantitative RT-PCR (Figure 2); 12 and 24 hpi were selected for resistant and susceptible tomato cultivars, respectively. Based on the temporal activity of LOX (Figure 1g and h) and followed by semi quantitative RT-PCR (Figure 2); 6 and 12 hpi were selected for resistant and susceptible tomato cultivars, respectively. For qRT-PCR analysis, the authors have selected the time interval which showed highest activity in both temporal as well RT-PCR analysis for PAL, POX, PPO and LOX gene and the total RNA was isolated and converted into cDNA (Thermo Scientific, India) as per manufacturer's instructions.

In the resistant tomato cultivar, the relative gene expression of PAL was up-regulated to 20 fold upon 10 μM C8-HSL treatment as compared to the control. Whereas pathogen inoculated resistant tomato cultivar up-regulates PAL activity to 16 fold which was significantly increased to 30 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3a). In the case of susceptible tomato cultivar, the relative gene expression of PAL was up-regulated to 12 fold upon 10 μM C8-HSL treatment as compared to the control. Whereas pathogen inoculated susceptible tomato cultivar down-regulates PAL activity to 4 fold which was significantly increased to 20 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3b).

In the resistant tomato cultivar, the relative gene expression of POX was up-regulated to 10 fold upon 10 μM C8-HSL treatment as compared to the control. Whereas, pathogen inoculated resistant tomato cultivar up-regulates POX activity to 5 fold which was significantly increased to 20 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3c). In the case of susceptible tomato cultivar, the relative gene expression of POX was up-regulated to 7 fold upon 10 μM C8-HSL treatment as compared to the control. Whereas pathogen inoculated susceptible tomato cultivar down-regulates POX activity to 2 fold which was significantly increased to 12 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3d).

In the resistant tomato cultivar, the relative gene

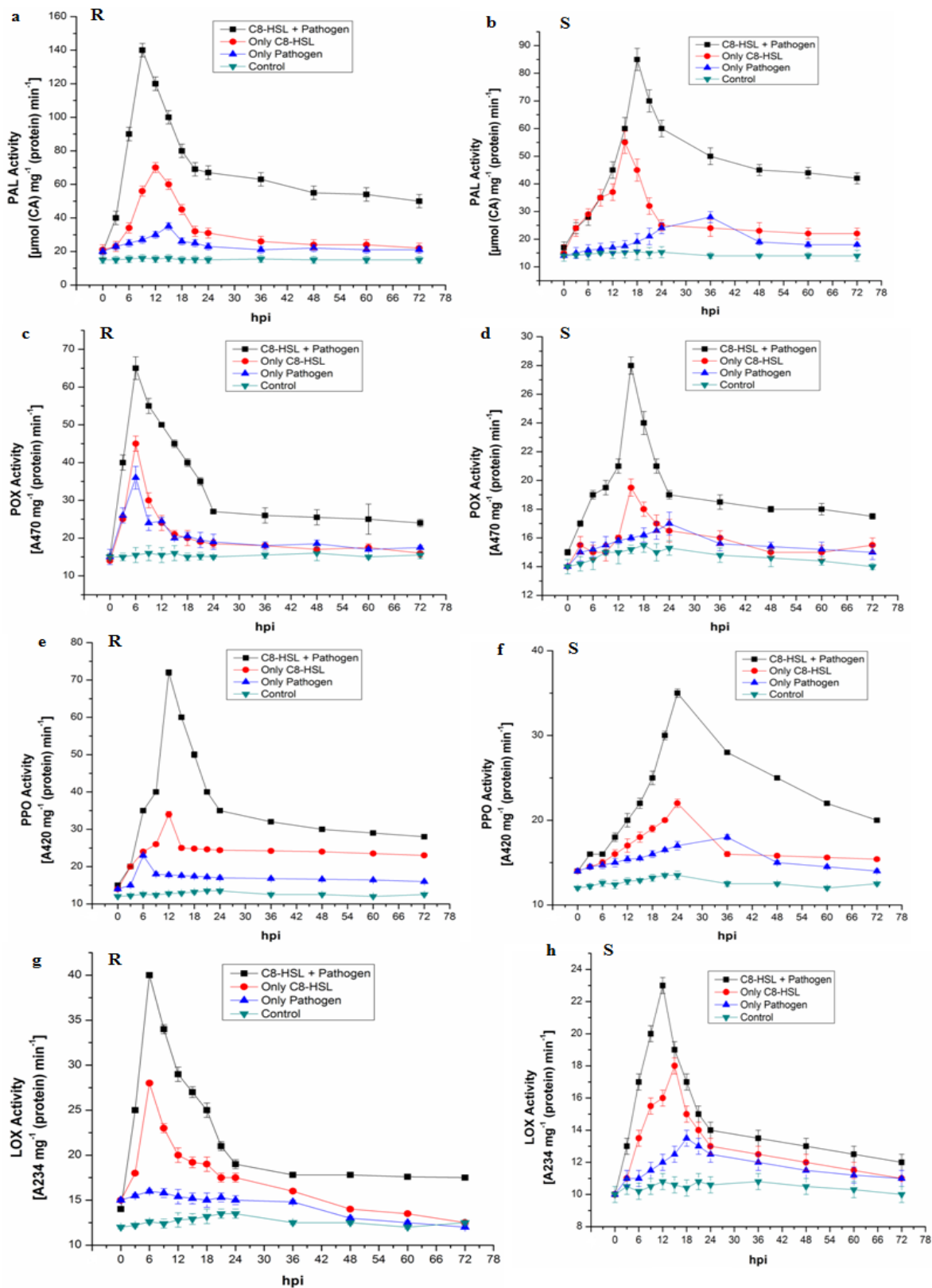


Figure 1. Temporal pattern study of PAL, POX, PPO and LOX activity in resistant (R) and susceptible (S) tomato cultivars. Four-week-old plants were treated with C8-HSL, followed by challenged with pathogen. Both treated and control plants were harvested at different hours after pathogen inoculation, and subjected to enzyme estimation. The data are expressed as the average of three independent experiments with three replicates each. Bars indicate standard errors.

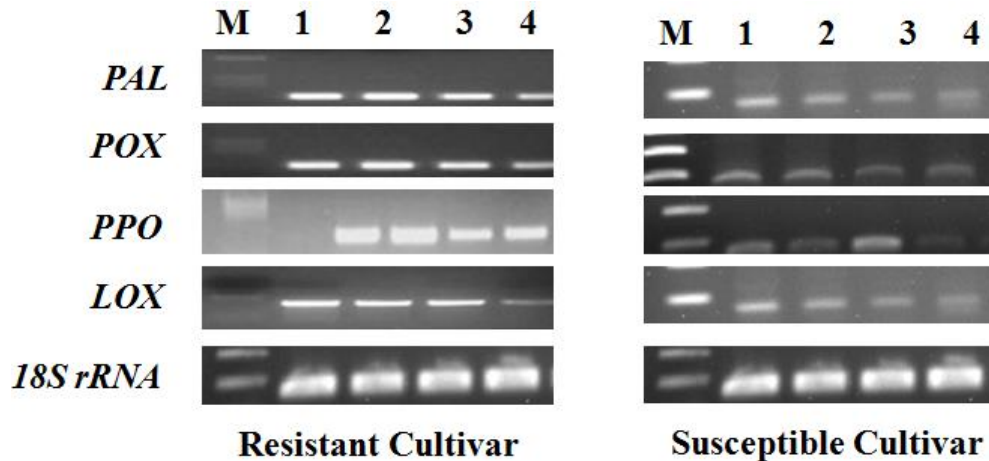


Figure 2. The PAL, POX, PPO and LOX gene expression was assessed by semi-quantitative PCR. After the C8-HSL (10 μ M) treatment, the PCR product was assayed by electrophoresis (2% agarose gels) stained with ethidium bromide. Lane M: DNA ladder; Lane 1: pathogen + C8-HSL; Lane 2: C8-HSL; Lane 3: pathogen only; Lane 4: control.

expression of PPO was up-regulated to 12 fold upon 10 μ M C8-HSL treatment as compared to the control. Whereas, pathogen inoculated resistant tomato cultivar up-regulates PPO activity to 7 fold which was significantly increased to 25 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3e). In the case of susceptible tomato cultivar, the relative gene expression of PPO was up-regulated to 9 fold upon 10 μ M C8-HSL treatment as compared to the control. Whereas, pathogen inoculated susceptible tomato cultivar down-regulates PPO activity to 1 fold which was significantly increased to 15 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3f).

In the resistant tomato cultivar, the relative gene expression of LOX was up-regulated to 5 fold upon 10 μ M C8-HSL treatment as compared to the control. Whereas pathogen inoculated resistant tomato cultivar up-regulates LOX activity to 4.5 fold which was significantly increased to 12 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3g). In the case of susceptible tomato cultivar, the relative gene expression of LOX was up-regulated to 4 fold upon 10 μ M C8-HSL treatment as compared to the control. Whereas pathogen inoculated susceptible tomato cultivar down-regulates LOX activity to 1.5 fold which was significantly increased to 9 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3h).

DISCUSSION

Resistance in plants is a highly regulated phenomenon depending on several signalling pathways, each activated by a set of different biotic and abiotic stimuli (Schuhegger et al., 2006). Recently, it has become evident that plants

can sense and respond appropriately to bacterial AHLs. It is reasonable that cross-kingdom signalling exists between plants and bacteria because plants and bacteria co-habited the earth for millions of years during which they might have evolved complex signalling networks consisting of different signalling molecules (Jin et al., 2012). In addition, plants seem to be able to detect various AHLs at quite low concentration (Mthesius et al., 2003). Schuhegger et al. (2006) in tomato reported that C6-HSL were able to induce resistance to the fungal leaf pathogen, *Alternaria alternata*. Mathesuis et al. (2003) found that over 150 proteins of approximately 2000 resolved protein spots were significantly altered in their accumulation in *M. truncatula* roots after the treatment with low concentration of 3OC12-HSL and 3OC16-HSL. In addition, von Rad et al. (2008) showed that the contact of *Arabidopsis* roots with C6-HSL resulted in distinct transcriptional change in the roots. Miao et al. (2012) found significant changes in protein accumulation for approximately 6.5% proteins of the total resolved proteins on 2-DE gels after the interaction of *Arabidopsis* roots with 3OC8-HSL, indicating that the responses of plants to AHLs are quite extensive. However, it is becoming increasingly evident that AHLs plays a positive role in activation of defense gene expression and pathogen defense. These data suggested that AHL play an important role in plant bacterial communication and a possible role in pathogen defense, and the authors decided to analyze the effect of C8-HSL on tomato plant along with *R. solanacearum* challenge inoculation.

Early and elevated levels of expressions of various defense enzymes are important features of plant resistance to pathogens. This is the first report where the role of defense related enzymes such as PAL, POX, PPO and LOX during the C8-HSL mediated elicitation of

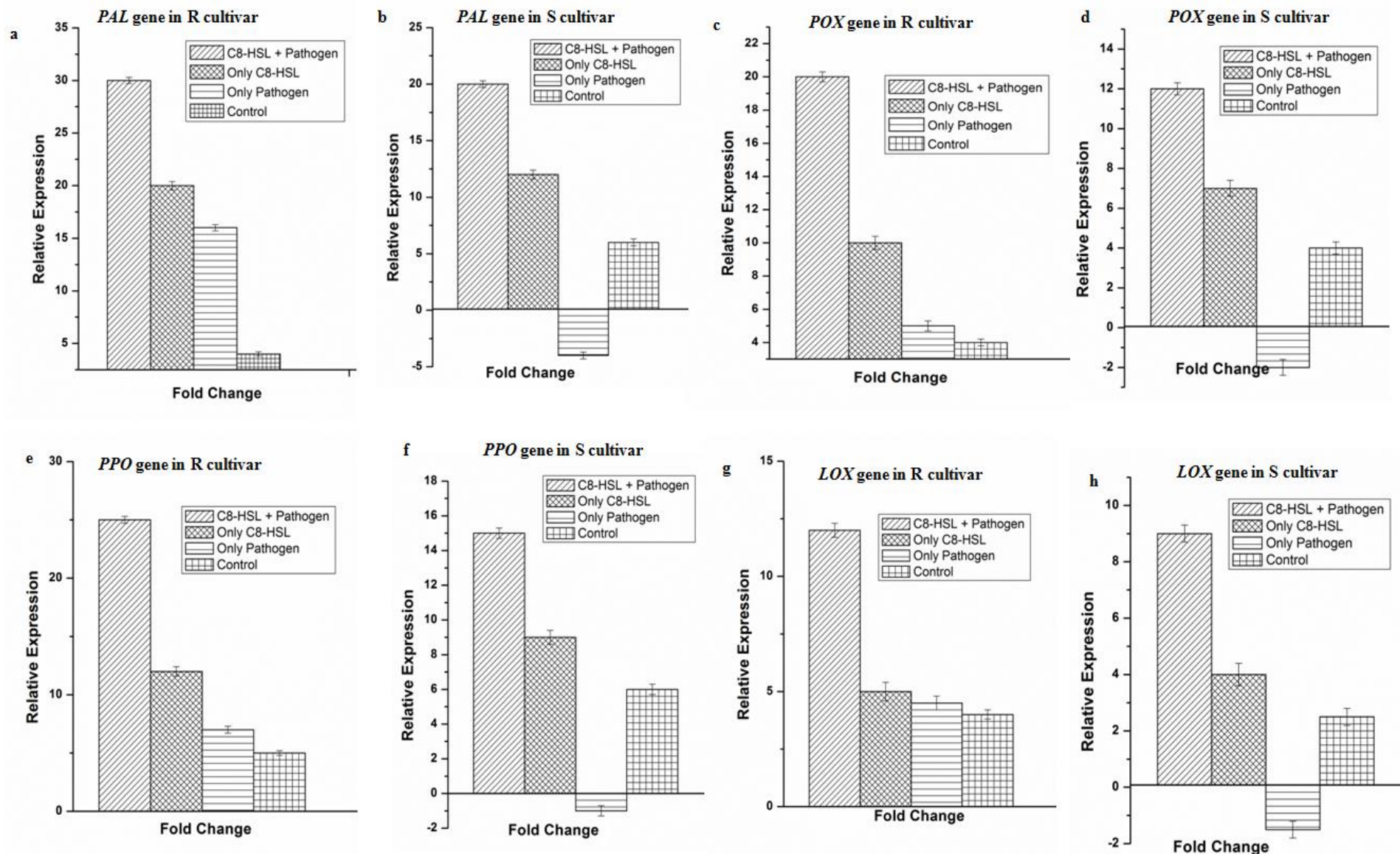


Figure 3. Relative expression levels of PAL, POX, PPO and LOX genes in four-week-old plants of both R and S tomato cultivars upon C8-HSL (10 μ M) treatment and challenged with the pathogen. Total RNA (2 μ g) was used to synthesis the cDNA of which 20 ng of individual cDNA was used to check the fold change of the PAL, POX, PPO and LOX genes which were carried out in three replicates. The gene expression levels were measured by qRT-PCR and normalized to the constitutive 18S rRNA gene. Each bar represents the mean of three independent experiments with standard error. R: resistant (cv. Golden); S: susceptible (cv. Rasi).

resistance in tomato against *R. solanacearum* was studied. Expression of these defense related enzymes (PAL, POX, PPO and LOX) are known to play a major role in determining the host resistance against various phytopathogens. These enzymes are either directly or indirectly involved in hypersensitive reaction (HR) development (Rusterucci et al., 1999), biosynthesis of cell wall strengthening material (lignin and suberin) and anti-microbial compounds (phytoalexins, furanocoumarin, quinines and pterocarpan) (Daayf et al., 1997), as also signalling molecules (salicylic acid and jasmonic acid) (Creelman and Mullet, 1997; Hammerschmidt, 1999).

Early induction of PAL is more important because it is the first key regulatory enzyme in the phenyl propanoid pathway leading to the production of phytoalexins and phenolic substances (Wang et al., 2004). In this study, maximum PAL activity was 9 h after inoculation (hpi). PAL activity increased in C8-HSL treated tomato plants challenged with the *R. solanacearum*, while tomato plants inoculated with the *R. solanacearum* alone had lower PAL activity. The role of PAL in imparting resistance to tomato against bacterial canker disease has been discussed by Umesha (2006). In contrast with the results from the present study, the PAL activity in roots of pepper plants from a resistant cultivar was high than for a susceptible cultivar after inoculation with *Phytophthora capsici* (Zhang et al., 2013). Iqbal et al. (2005) showed that during the infection of *F. solani* f. sp. *glycines* on the roots of soybean plants from a resistant cultivar, the PAL enzyme was up-regulated, and this was not observed in the susceptible cultivar.

POX is a key enzyme in the biosynthesis of lignin, in addition to its antimicrobial activity (Torres et al., 2006). Increased activity of cell wall bound peroxidases has been elicited in different plants due to pathogen infection. In this study, POX activity up-regulated after inoculation and reached its maximum at 6 hpi. Similar to PAL activity, tomato plants inoculated with the *R. solanacearum* alone recorded lower POX activity than C8-HSL treated plants. Leite et al. (2014) reported that POX activity was higher in the plants of a resistance genotype of common bean in response to *Sclerotinia sclerotiorum* infection than for a susceptible genotype.

PPO catalyses the oxidation of phenolic compounds to highly toxic quinines which play an important role in plant disease resistance. In this study, the activity of PPO reached maximum at 12 hpi in tomato plants. The PPO activity in plants treated with C8-HSL alone did not reach the level of activity seen in the plants treated with C8-HSL and inoculated with the *R. solanacearum*. PPO also plays a critical role in tomato's disease resistance to *Pseudomonas syringae* pv. tomato (Thipyapong et al., 2004).

The lipoxygenase enzyme initiates a metabolic route leading to the synthesis of various antimicrobial compounds involved in plant defense. In this study, the LOX activity was maximum at 6 hpi in plants treated with

C8-HSL and challenged with *R. solanacearum*. Similar to the above mentioned enzymes, LOX activity were lower in other treatments. However, high LOX activity may constitute in plants resistance to pathogens but with an addition increase upon infection (Devi et al., 2000).

The plants treated with C8-HSL followed by pathogen inoculation accumulated increased amounts of defense enzymes (PAL, POX, PPO and LOX) when compared with untreated control. Similar results were reported when the activities of PAL, POX, PPO, LOX were increased in tomato plants pre-treated with *Pseudomonas fluorescens* and challenged with *R. solanacearum* (Vanitha and Umesha, 2011). The RT-PCR studies were carried out to investigate the gene expression pattern of defense related enzymes (PAL, POX, PPO and LOX). The genes were compared with the internal control being 18S rRNA. The 18S rRNA was expressed in both cultivars. The expression of the defense genes was higher in resistant cultivar.

qRT-PCR was performed to evaluate the mRNA accumulation of differentially expressed defense genes in both resistant cultivar and susceptible cultivar. In the resistant tomato cultivar, the relative gene expression of defense genes (PAL, POX, PPO and LOX) was up-regulated upon C8-HSL treatment as compared to the control, and significantly increased upon *R. solanacearum* inoculation. Whereas, in susceptible cultivar, the defense genes (PAL, POX, PPO and LOX) were down-regulated upon *R. solanacearum* inoculation as compared to the control, and interestingly up-regulated upon C8-HSL treatment. Thus, the results show that C8-HSL can induce significant defense gene (PAL, POX, PPO and LOX) in both the tomato cultivar (Figure 3). Our findings were in accordance with Lata et al. (2010) who have showed the relationship of PEG-induced dehydration stress in tolerant and sensitive millet, plants where transcripts showed a differential expression pattern in both cultivars at different time points of stress treatment as analyzed by qRT-PCR. Song et al. (2011) results showed the treatment with abscisic acid (ABA) on tomato against *Alternaria solani*, effectively reduced disease severity in tomato plants, as enzyme activities were maintained at higher levels in ABA pre-treated and *A. solani* challenged tomato plants. Tomato defense genes were rapidly and significantly up-regulated by ABA treatment which is well correlated with the present study.

In conclusion, the results of the present study confirm that C8-HSL trigger the defense mechanism in tomato plants by activating the defense enzymes and protect itself from the pathogen attack. Thus, this investigation shows that C8-HSL can be considered as potential candidates for elicitors for tomato plants against wilt disease, as they induce expression of typical defense related enzymes resulting in increased resistance against *R. solanacearum*. To the best of the authors' knowledge, this is the first report on the interaction between the C8-

HSL – tomato plants and its defense enzymes against *R. solanacearum* under greenhouse conditions.

Conflict of interest

The authors have not declared any conflict of interest.

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