CHAPTER 4

Induction of pathogenesis-related (*PR*) genes expression in tomato against *Fusarium* wilt disease by *Streptomyces*

4.1 Introduction

Plants defense themselves are demonstrated as natural resistance to pathogens based on the combined effects of natural barriers and inducible mechanisms that activated upon pathogen attack through a wide variety (Dixon, 1986; Keen, 1990; Ryals *et al*., 1992; Ryals *et al*., 1994). However, two different types of induced resistance have been extensively studied: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR, induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of the phytohormone salicylate (salicylic acid), and associated with the accumulation of pathogenesis-related (*PR*) proteins, whereas rhizobacteria-mediated ISR, induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of the phytohormones ethylene and jasmonate (jasmonic acid), independent of salicylate, and is not associated with the accumulation of *PR* proteins (or transcripts). However, both responses are also effective against a broad spectrum of virulent plant pathogens (Ward *et al*., 1991; Uknes *et al*., 1992; Pieterse *et al*., 1996, 1998; Knoester *et al*., 1999; Maleck *et al*., 2000; Schenk *et al*., 2000; van Wees *et al*., 2000; McGovern, 2015).

The better understanding of plant signalling pathways has led o the discovery of natural and synthetic compounds called “elicitors” that induce similar defense responses in plants as induced by the pathogen infection (Hammond-Kosack and Jones, 2000; Gómez-Vásquez *et al*., 2004). Following elicitor perception, the activation of signal transduction pathways generally lead to act as signals that stimulate the synthesis of natural products, phytoalexins and especially pathogenesis-related (*PR*) proteins

(Benhamou and Theriault, 1992; Ebel and Cosio, 1994; van Loon and van Strien, 1999), one of the most important and effective plant defense mechanisms against various pathogens. The *PR* proteins are encoded from defense-related genes which subsequently prevent various pathogen invasions (Bowles, 1990). van Loon *et al.* (2006) defined the term "pathogenesis-related proteins" is microbe-induced proteins and their homologouses to the extent that enzymes, which are generally presented constitutively and only increased during severely infection. Production of *PR* proteins in the uninfected parts of plants can prevent the affected plants from further infection (Ryals *et al*., 1996; Delaney, 1997).

According to Conrath *et al.* (2002) who suggested that the non-pathogenic bacteria prime the plant for accelerated and enhanced response to a second stress stimulus, such as a pathogen, and *Streptomyces* species can also induce systemic and localized resistance to plant pathogens and improve plant growth and metabolism (Conn *et al*. 2008; Lehr *et al*. 2008). Besides, other reports were represented *Streptomyces* spp. elicited a systemic defense response as biotic elicitors to protect many plant diseases, such as common scab of potato tuber (Beausejour *et al*., 2003), soil-borne pathogens *Erwinia carotovora* and *F. oxysporum* in flowering plant *Arabidopsis thaliana* (Conn *et al*., 2008), oak powdery mildew (Kurth *et al*., 2014) and seed-borne *Sclerotinia sclerotiorum* (Srivastava *et al*., 2015). Perhaps, the effective strain, *Streptomyces* NSP3, might be showed antifungal activity to control pathogenic fungi and based on this aim, the examining expression of plant defense related genes mRNA in response to *Streptomyces* NSP3 and challenged inoculation of *F. oxysporum* f. sp. *lycopersici* to tomato plants were demonstrated.

The objectives of this chapter were as follows:-

1. To evaluate the efficiency of *Streptomyces* NSP3 for plant defense response by induce immunity in tomato plant
2. To induction of accumulation of *PR* proteins by *Streptomyces* NSP3 against challenge inoculation with *F. oxysporum* f. sp. *lycopersici*

4.2 Materials and methods

Fol inoculum

The pathogenic *F. oxysporum* f. sp. *lycopersici* (*Fol*) isolate *Fol*CK_117, the most virulent isolate to tomato seedlings cv. 'Bonny Best', was tested in this experiment. The strain was grown on PDA at RT for 7 days. The microconidia of *Fol* was then prepared as conidial suspension by flooded *Fol* colony with 10 ml of sterile distilled water (Singleton *et al*., 1992). Mycelia were dislodged by scraping the surface of *Fol* colony with a sterile microscope glass slide. The mycelia suspension was then filtered through a sterile cheese cloth. The concentration of conidia in suspension was determined on a heamacytometer and adjusted to 1×10^7 conidia/ml.

Streptomyces preparation

Streptomyces strain NSP3 was selected to test in this experiment. The strains were grown on GYM at RT for 20 days. The NSP3 was then prepared as culture suspension by flooded *Streptomyces* plate with 10 ml of sterile distilled water. Colonies were dislodged by scraping the surface of *Streptomyces* colonies with a sterile microscope glass slide. The concentration of *Streptomyces* in suspension was determined by colony counting on GYM plates.

The colony counting was conducted according to serial dilution. One ml of *Streptomyces* suspension (original solution) was drawn with a pipetted and transferred to another tube containing 9 ml of sterile distilled water to produce 10 ml of the dilute solution, and then mixed with a vortex mixer. One ml of the dilute solution was moved to another tube and repeated as many times as necessary to achieve the desired solution using the technique described previously. The concentration of NSP3 in suspension was counted using spread plate technique by separately plating 100 µl volumes of the dilute suspension on GYM plates, and incubated at RT for 3 days. The grown colonies were counted, and then transformed to the colony-forming units (cfu)/ml. Only plates (or replicate plates from the same dilution) with 30 – 300 colonies were counted.

Formula of calculation the colony-forming units (cfu)/ml

$$\text{cfu/ml} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of culture plate (ml)}}$$

Plant materials

Seeds of tomato (*Lycopersicon esculentum* Mill.) were surface-sterilized with 1% sodium hypochlorite (10% Clorox) for 1 minute, then rinsed three times in sterile distilled water and dried under a sterile air stream. Sterilized seeds were separately soaked for 12 h, then air dried overnight before sowed in 72-cell plastic seeding tray (28 × 54 cm) containing Peat Moss growing media (Klasmann®) mixed with coconut coir dust (1:1 ratio) and maintained in greenhouse at 30 ± 2°C with 12 h photoperiod. The experiments were performed with the uniform 30-day-old tomato plants with four expanded leaves. The tomato cv. 'Bonny Best' (susceptible to *Fusarium* wilt) was used for study. The cultivar was kindly provided by Hortigenetics Research (S.E. Asia) Limited.

Experimental design for induction of defense mechanisms (modified from Ramamoorthy *et al.*, 2002; Aimé *et al.*, 2008; Goel and Paul, 2014)

Seed treatment (ST)

Ten gram of surface-sterilized tomato seeds were soaked in 10 ml of *Streptomyces* suspension for 12 h, and then air dried overnight. Treated-seeds were sown and maintained in greenhouse according to the method described previously. Seeds soaked in sterile distilled water were served as control.

Soil application (SA)

Ten ml of *Streptomyces* suspension, containing 9×10^8 cfu/ml, was drenched into each pot at 5 days before *Fol* inoculation. Sterile distilled water was served as control.

Fol inoculation (Fol-inoc)

Five days after soil application, each seedling was challenge inoculated with 10 ml of *Fol* containing 1×10^7 conidia/ml as soil-drench. Seedlings without prior treatment of *Fol* were served as control.

Wounding root

Tomato roots were wounded by cutting through actually rhizosphere soil at one time. Non-wounding roots were served as control.

Experimental treatments

The experiment was applied as followed treatments, including (T_1) ST + *Fol*-inoc, (T_2) SA + *Fol*-inoc, (T_3) combination of ST + SA + *Fol*-inoc, (C_1) ST, (C_2) SA, (C_3) combination of ST + SA, (C_4) *Fol*-inoc, (C_5) wounding root and (C_6) healthy seedlings (Table 4.1). Three replications were arranged in RCB, with a plant per replicate. The plants were maintained in the greenhouse at 30 ± 2°C with 12 h photoperiod until sampling for total mRNA extraction.

Table 4.1 Experimental treatment design for induction of defense mechanisms (Modified from Aimé *et al.*, 2008; Goel and Paul, 2014)

Treatments	Seed treatment	Soil application	*Fol*-inoculation	Wounding root
T_1	✓	-	✓	-
T_2	-	✓	✓	-
T_3	✓	✓	✓	-
C_1	✓	-	-	-
C_2	-	✓	-	-
C_3	✓	✓	-	-
C_4	-	-	✓	-
C_5	-	-	-	✓
C_6	-	-	-	-

✓ = present, - = absence

Sample collection from tomato leaves

The 3rd leaf blade (fully expanded) of treated-tomato plant was carefully cut without causing damage to tissue at different time intervals; 0, 3, 6, 12 and 24 h (a plant at time) after *Fol* inoculation, and then immediately stored in deep freezer (-80°C) until mRNA extraction.

RNA isolation from tomato leaves

RNA extraction: Total RNA was extracted from 0.5 g of tomato leaf samples. Plant tissues were ground in liquid nitrogen, and followed by 1.0 ml of TRIzol® Reagent (Invitrogen™, USA) according to the manufacturer's instructions. Each homogenization samples were transferred into a microcentrifuge tube (Eppendorf®) before incubated at RT for 5 min. Two hundred microlitres of chloroform was added and mixed vigorously on vortex for 15 sec before incubated at RT for 3 min. Samples were centrifuged at 12,000 × g for 20 min at 4°C, then the mixture were separated for 3 phases. The upper colorless aqueous phase, approximately 400 - 500 µl, was transferred to a new tube, RNA remains exclusively in this phase.

RNA precipitation: An equal volume of 100% isopropanol was added to aqueous solution and incubated for 10 min at RT, then centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was discarded and RNA pellet were remained in this phase.

RNA washing: One microlitres of 75% ethanol was added and mixed with a vortex mixer. The tube was then centrifuged at 7500 × g for 5 min at 4°C and discarded the supernatant. The RNA pellets were air-dried for 5 min to remove remained ethanol.

RNA resuspension: The RNA pellets were resuspended in 50 µl of diethyl pyrocarbonate (DEPC)-treated water by passing the solution up and down several times through a pipette tip and incubated in heat block set at 55–60°C for 10 min. Yield of RNA was determined and measured at 260 nm and 280 nm absorbance to determine concentration (the formula; A260 × dilution × 40 = µg RNA/ml) by NanoDrop™ Spectrophotometer.

Complementary DNA (cDNA) synthesis

Double- stranded cDNA was then synthesized from 1 ng of total RNA using a ReverTra Ace® qPCR RT Master Mix (TOYOBO, Japan) according to the manufacturer's instructions.

Quantitative real time-PCR (qRT-PCR) analysis

qRT-PCR assays were performed in a C1000™ Thermal Cycler with a CFX96™ Real-Time System (Bio-Rad, USA). The following primer sets were used and shown in Table 4.1.

qRT-PCR reactions were carried out with 100 ng of cDNA, 400 µM of each primer, 10 µl of 2x SYBR green master mix (SensiFAST™ SYBR® No-Rox Kit, Bioline™, UK) and nuclease-free water in a final volume of 20 µl. In negative control cDNA were replaced by nuclease-free water. The program used for real-time qPCR reaction was 95ºC for 2 min, 50 cycles of denaturation for 5 sec at 95ºC, annealing for 30 sec at 58ºC and extension for 20 sec at 72ºC; at the end of which the fluorescence was measured. The *Actin* (a housekeeping gene) was used as an internal standard to normalize each cDNA sample (Livak and Schmittgen, 2001) and calculated the relative expression values using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Beaubois *et al*., 2007). A complete 2-factor factorial design is an experimental design.

Statistical analysis

Data were computed analysis of variance (ANOVA). Treatments mean were compared using Fisher's Least Significant Difference (LSD) at $P = 0.05$.

Table 4.2 Primers designed from gene sequences (GenBank) and used in real-time qRT-PCR to amplify the genes encoding the selected *PR* proteins in tomato (Modified from Aimé *et al.*, 2008; Goel and Paul, 2014)

Primer		Nucleotide sequence (5´–3´)	Amplicon size (pb)	Accession number (GenBank)	Target gene
Actin	F	AGGCACACAGGTGTTATGGT	177	U60480	*Actin*
	R	AGCAACTCGAAGCTCATTGT			
*Le*PR1a	F	TCTTGTGAGGCCCAAAATTC	246	AJ011520	*PR-1a*
	R	ATAGTCTGGCCTCTCGGACA			
*Le*Chi3	F	TGCAGGAACATTCACTGGAG	248	Z15141	acidic chitinase
	R	TAACGTTGTGGCATGATGGT			
*Le*Chi9	F	GAAATTGCTGCTTTCCTTGC	235	Z15140	basic chitinase
	R	CTCCAATGGCTCTTCCACAT			
CEVI-1	F	GCAACAAGCCCAAAGTACCG	219	Y19023	peroxidase
	R	GAAACAACGCCAGGACACAC			

4.3 Results

Treatment effects on the expression of defense related genes in tomato 0, 3, 6, 12 and 24 h post-*Fol* inoculation (hpi) were determined and validated using the *Actin* gene as the internal control. Tomato seedlings responded to the *Streptomyces* NSP3 treatments. The levels of transcripts encoding the *PR* proteins observed, *PR-1a*, *Chi3*, *Chi9* and *CEVI-1* genes, they increased to the maximum following treatment with *Streptomyces* NSP3 after challenge-inoculation with *Fol* (T_1 – T_3) within 24 hpi. These *PR* proteins were also provided by the wounding treatment (C_5), but they expressed lower level. In contrast, the accumulation of these genes was always lower in other controls (C_1 – C_4), comparable to healthy seedlings (C_6). Statistical analysis showed significant differences in the relationship between the treatments and time-sampling in observed *PR* proteins (Figure 4.1).

PR-1a

The maximum accumulation of *PR-1a* was detected at 3 hpi in combination of seed treatment and soil application before challenge inoculation with *Fol* (T_3) (73.1 fold) before found to be stable at 6 -12 hpi (average 51.8 fold), and then decreased to average 34.9 fold until 24 hpi. Soil application before challenge inoculation with *Fol* (T_2) showed a maximum relative *PR-1a* expression of 14.2 fold (at 3 hpi), then found to be stable until 24 hpi (average 12.5 fold). Seed treatment before challenge inoculation with *Fol* (T_1) showed a stable *PR-1a* expression from 3 – 6 hpi (average 10.2 fold), before increased to maximum at 12 hpi (13.4 fold) and slightly decreased at 24 hpi (12.5 fold). Although the accumulation of *PR-1a* in treatment of wounding roots (C_5) was increased, the expression was reaching a maximum only 4.9 fold at 24 hpi. However, significantly lower *PR-1a* expression levels were found in four different control treatments in plants inoculated with *Streptomyces* NSP3 or *Fol* alone (C_1 – C_4), comparable to healthy seedlings (C_6) (Table 4.2; Figure 4.1a).

Chi3

The maximum accumulation of *Chi3* was also detected in the combination of seed treatment and soil application before challenge inoculation with *Fol* (T_3); the relative *PR-1a* expression was found to be relatively constant at 3 – 6 hpi (average 19.3 fold), before increased to 41.9 fold (12 hpi) and reached a maximum at 24 hpi (56.1 fold). Soil application before challenge inoculation with *Fol* (T_2) showed a stable of *Chi3* expression during 3 – 12 hpi (average 12.3 fold), then increased to a maximum 19.0 fold at 24 hpi. The expression of *Chi3* gene in seed treatment before challenge inoculation with *Fol* (T_1) was found to be stable until 6 h after treatment (average 8.2 fold), then increased to 16.0 fold at 12 hpi before increasing to be a maximum at 24 hpi (18.6 fold). Although the accumulation of *Chi3* in treatment of wounding roots (C_5) was increased, the expression was reaching a maximum only 5.2 fold at 24 hpi. However, significantly lower *Chi3* expression levels were found in four different control treatments in plants inoculated with *Streptomyces* NSP3 or *Fol* alone (C_1 – C_4), comparable to healthy seedlings (C_6) (Table 4.3; Figure 4.1b).

Chi9

The maximum accumulation of *Chi9* was detected in the combination of seed treatment and soil application before challenge inoculation with *Fol* (T_3); the *Chi9* gene expression was successively increased at 3 and 6 hpi (23.7 and 31.3 fold, respectively) before reaching a maximum at 12 hpi (50.7 fold), the decreased slightly at 24 hpi (42.8 fold). The accumulation of *Chi9* in soil application before challenge inoculation with *Fol* (T_2) showed a stable expression from 3 – 6 hpi (average 14.9 fold), before increasing to a maximum until 24 hpi (average 18.9 fold). The *Chi9* gene expression of seed treatment before challenge inoculation with *Fol* (T_1) was found to be relatively constant at 3 – 6 hpi (average 10.3 fold), then reaching a maximum at 12 hpi (13.29 fold) before decreased slightly at 24 hpi (12.0 fold). In the same way, the accumulation of *Chi9* in treatment of wounding roots (C_5) was increased to maximum only 4.7 fold at 24 hpi. However, significantly lower *Chi*9 expression levels were found in four different control treatments in plants inoculated with *Streptomyces* NSP3 or *Fol* alone (C_1 – C_4), comparable to healthy seedlings (C_6) (Table 4.4; Figure 4.1c).

CEVI-1

The maximum accumulation of *CEVI-1* was also detected in the combination of seed treatment and soil application before challenge inoculation with *Fol* (T_3), which found to be stable at 3 – 6 hpi (average 25.6 fold), then increased to maximum (43.3 fold) at 12 hpi before decreasing thereafter (37.1 fold at 24 hpi). The *CEVI-1* gene expression in soil application before challenge inoculation with *Fol* (T_2) showed 16.7 fold upregulation at 3 hpi, then relatively unchanging *CEVI-1* gene expression until 12 hpi (average 18.9 fold), then increased to a the maximum of 26.0 fold at 24 hpi. Seed treatment before challenge inoculation with *Fol* (T_1) showed *CEVI-1* gene expression stable until 6 hpi (average 14.9 fold), then gene expression increased to a maximum at 12 hpi (27.7 fold) before decreasing to 20.5 fold at 24 hpi. There were similarly to other *PR* gene expression in treatment of wounding roots (C_5); although the accumulation of *CEVI-1* in was increased, the expression was reaching a maximum only 6.8 fold at 24 hpi. However, significantly lower *CEVI-1* expression levels were found in four different control treatments in plants inoculated with *Streptomyces* NSP3 or *Fol* alone (C_1 – C_4), comparable to healthy seedlings (C_6) (Table 4.3; Figure 4.1d).

Table 4.3 qRT-PCR analysis of *PR-1a* gene expression in tomato leaves in response to the application of *Streptomyces* NSP3 with or without challenge inoculation with *Fusarium oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. Relative expression levels of transcripts were assessed 0, 3, 6, 12 and 24 h post-*Fol* inoculation

Treatments[1/]	Relative expression of *PR-1a* genes (normalized to *Actin*)[2/]									
	0 h		3 h		6 h		12 h		24 h	
T_1	1.25	Aa[3/]	10.29	Ba	10.19	Ba	13.36	Ba	12.49	Ba
T_2	1.26	Aa	14.15	Ba	12.92	Ba	12.00	Ba	12.55	Ba
T_3	1.17	Ad	73.10	Aa	51.11	Ab	52.51	Ab	34.91	Ac
C_1	1.52	Aa	1.21	Ba	0.55	Ba	0.49	Ba	1.08	Ba
C_2	1.76	Aa	0.49	Ba	0.33	Ba	0.46	Ba	0.47	Ba
C_3	2.40	Aa	0.59	Ba	0.39	Ba	0.81	Ba	1.37	Ba
C_4	1.46	Aa	0.65	Ba	0.48	Ba	0.39	Ba	0.95	Ba
C_5	1.45	Aa	3.65	Ba	2.00	Ba	3.85	Ba	4.90	Ba
C_6	1.00	Aa	1.00	Ba	1.00	Ba	1.00	Ba	1.00	Ba
A (treatment)			***		$LSD_{0.05}$ = 6.81					
B (time-sampling)			**		$LSD_{0.05}$ = 5.08					
A*B			***		$LSD_{0.05}$ = 15.23					
CV (%)			119.89							

[1/] Experimental treatments: (T_1) seed treatment + *Fol*-inoculation, (T_2) soil appliction + *Fol*-inoculation, (T_3) combine of seed treatment + soil appliction + *Fol*-inoculation, (C_1) seed treatment, (C_2) soil appliction, (C_3) combine of seed treatment + soil appliction, (C_4) *Fol*-inoculation, (C_5) wounding root and (C_6) healthy seedling. Control plants were treated with sterile distilled water.

[2/] The mean of three replications were analyzed by 2-factor ANOVA (1 plant/rep).

[3/] The differences between treatments in the column and time-sampling in the row of each pair are indicated by upper and lower case letters, respectively. Significant treatment effects were determined by LSD at $P \leq 0.05$ (treatment × time-sampling interaction).

*** significantly different at $P \leq 0.001$

Table 4.4 qRT-PCR analysis of *Chi3* gene expression in tomato leaves in response to the application of *Streptomyces* NSP3 with or without challenge inoculation with *Fusarium oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. Relative expression levels of transcripts were assessed 0, 3, 6, 12 and 24 h post-*Fol* inoculation

Treatments[1/]	Relative expression of *Chi3* genes (normalized to *Actin*)[2/]					
	0 h	3 h	6 h	12 h	24 h	
T_1	1.06 Ab	9.48 BCb	6.94 Bb	15.95 Bab	18.55 Ba	
T_2	1.06 Ab	10.00 Ba	13.90 Aba	13.05 Ba	19.04 Ba	
T_3	1.40 Ad	20.99 Ac	17.57 Ac	41.93 Ab	56.13 Aa	
C_1	1.11 Aa	0.62 Ca	0.40 Ba	0.62 Ca	1.11 Ca	
C_2	1.39 Aa	0.30 Ca	0.31 Ba	1.59 Ca	1.00 Ca	
C_3	2.18 Aa	0.30 Ca	0.41 Ba	0.56 Ca	1.02 Ca	
C_4	1.10 Aa	0.50 Ca	0.29 Ba	0.62 Ca	1.06 Ca	
C_5	1.19 Aa	2.30 BCa	1.70 Ba	4.20 Ca	5.18 Ca	
C_6	1.00 Aa	1.00 Ca	1.00 Ba	1.00 Ca	1.00 Ca	
A (treatment)		***	$LSD_{0.05}$ = 3.92			
B (time-sampling)		***	$LSD_{0.05}$ = 2.92			
A*B		***	$LSD_{0.05}$ = 8.76			
CV (%)		85.84				

[1/] Experimental treatments: (T_1) seed treatment + *Fol*-inoculation, (T_2) soil appliction + *Fol*-inoculation, (T_3) combine of seed treatment + soil appliction + *Fol*-inoculation, (C_1) seed treatment, (C_2) soil appliction, (C_3) combine of seed treatment + soil appliction, (C_4) *Fol*-inoculation, (C_5) wounding root and (C_6) healthy seedling. Control plants were treated with sterile distilled water.

[2/] The mean of three replications were analyzed by 2-factor ANOVA (1 plant/rep).

[3/] The differences between treatments in the column and time-sampling in the row of each pair are indicated by upper and lower case letters, respectively. Significant treatment effects were determined by LSD at $P \leq 0.05$ (treatment × time-sampling interaction).

** significantly different at $P \leq 0.01$, *** significantly different at $P \leq 0.001$

Table 4.5 qRT-PCR analysis of *Chi9* gene expression in tomato leaves in response to the application of *Streptomyces* NSP3 with or without challenge inoculation with *Fusarium oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. Relative expression levels of transcripts were assessed 0, 3, 6, 12 and 24 h post-*Fol* inoculation

Treatments[1/]	Relative expression of *Chi9* genes (normalized to *Actin*)[2/]									
	0 h		3 h		6 h		12 h		24 h	
T_1	1.07	Aa	10.05	Ba	10.58	BCa	13.29	BCa	11.96	BCa
T_2	0.75	Ab	14.20	ABa	15.56	Ba	19.00	Ba	18.81	Ba
T_3	0.73	Ac	23.65	ABb	31.33	Ab	50.74	Aa	42.84	Aab
C_1	0.79	Aa	0.60	Ba	0.38	Ca	0.38	Ca	1.09	Ca
C_2	0.83	Aa	0.33	Ba	0.25	Ca	1.01	Ca	0.69	Ca
C_3	1.27	Aa	0.78	Ba	0.43	Ca	0.83	Ca	0.99	Ca
C_4	0.70	Aa	0.67	Ba	0.37	Ca	0.71	Ca	1.01	Ca
C_5	1.05	Aa	3.12	Ba	2.04	Ca	3.83	Ca	4.66	Ca
C_6	1.00	Aa	1.00	Ba	1.00	Ca	1.00	Ca	1.00	Ca
A (treatment)			***		$LSD_{0.05}$ = 5.83					
B (time-sampling)			***		$LSD_{0.05}$ = 4.34					
A*B			***		$LSD_{0.05}$ = 13.03					
CV (%)			121.13							

[1/] Experimental treatments: (T_1) seed treatment + *Fol*-inoculation, (T_2) soil appliction + *Fol*-inoculation, (T_3) combine of seed treatment + soil appliction + *Fol*-inoculation, (C_1) seed treatment, (C_2) soil appliction, (C_3) combine of seed treatment + soil appliction, (C_4) *Fol*-inoculation, (C_5) wounding root and (C_6) healthy seedling. Control plants were treated with sterile distilled water.

[2/] The mean of three replications were analyzed by 2-factor ANOVA (1 plant/rep).

[3/] The differences between treatments in the column and time-sampling in the row of each pair are indicated by upper and lower case letters, respectively. Significant treatment effects were determined by LSD at $P \leq 0.05$ (treatment × time-sampling interaction).

ns = non significant, *** significantly different at $P \leq 0.001$

Table 4.6 qRT-PCR analysis of *CEVI-1* gene expression in tomato leaves in response to the application of *Streptomyces* NSP3 with or without challenge inoculation with *Fusarium oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. Relative expression levels of transcripts were assessed 0, 3, 6, 12 and 24 h post-*Fol* inoculation

Treatments[1/]	Relative expression of *CEVI-1* genes (normalized to *Actin*)[2/]					
	0 h	3 h	6 h	12 h	24 h	
T_1	0.62 Ac	14.28 ABb	15.60 Aab	27.65 Ba	20.49	Bab
T_2	0.85 Ab	16.65 Aa	18.88 Aa	18.98 Ba	25.99	ABa
T_3	0.74 Ac	25.81 Ab	25.42 Ab	43.34 Aa	37.08	Aab
C_1	1.10 Aa	0.85 Ba	0.96 Ba	0.43 Ca	0.83	Ca
C_2	0.88 Aa	0.45 Ba	0.28 Ba	1.42 Ca	0.90	Ca
C_3	1.35 Aa	1.13 Ba	0.57 Ba	1.21 Ca	0.95	Ca
C_4	0.63 Aa	0.77 Ba	0.31 Ba	0.44 Ca	0.64	Ca
C_5	0.82 Aa	3.31 Ba	2.51 Ba	3.87 Ca	6.79	Ca
C_6	1.00 Aa	1.00 Ba	1.00 Ba	1.00 Ca	1.00	Ca
A (treatment)		***	$LSD_{0.05}$ = 5.64			
B (time-sampling)		***	$LSD_{0.05}$ = 4.20			
A*B		***	$LSD_{0.05}$ = 12.61			
CV (%)		105.72				

[1/] Experimental treatments: (T_1) seed treatment + *Fol*-inoculation, (T_2) soil appliction + *Fol*-inoculation, (T_3) combine of seed treatment + soil appliction + *Fol*-inoculation, (C_1) seed treatment, (C_2) soil appliction, (C_3) combine of seed treatment + soil appliction, (C_4) *Fol*-inoculation, (C_5) wounding root and (C_6) healthy seedling. Control plants were treated with sterile distilled water.

[2/] The mean of three replications were analyzed by 2-factor ANOVA (1 plant/rep).

[3/] The differences between treatments in the column and time-sampling in the row of each pair are indicated by upper and lower case letters, respectively. Significant treatment effects were determined by LSD at $P \leq 0.05$ (treatment × time-sampling interaction).

ns = non significant, * significantly different at $P \leq 0.05$, *** significantly different at $P \leq 0.001$

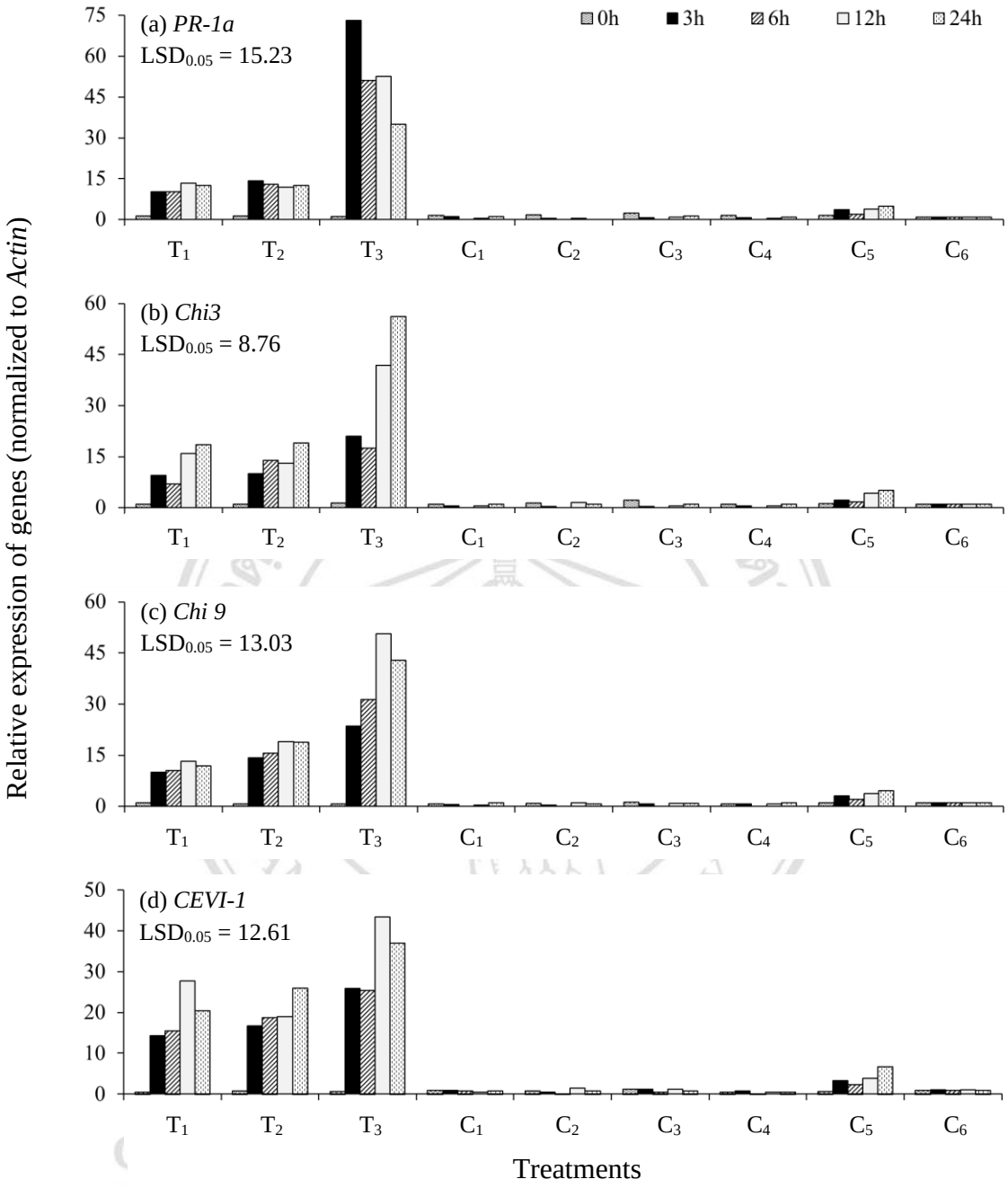

Figure 4.1 Real-time qPCR analysis of *PR* gene expression in tomato leaves in response to application of *Streptomyces* NSP3 with or without challenge inoculation with *Fusarium oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. Control plants were treated with sterile distilled water. Bars represent relative expression levels of transcripts assessed 0, 3, 6, 12 and 24 h post-*Fol* inoculation. The mean of three replications were analysed by 2-factor ANOVA. Significant treatment effects were determined by LSD at $P \leq 0.05$ (treatment × time-sampling interaction), while ns = non significantly different. Experimental treatments included (T_1) ST + *Fol*-inoc, (T_2) SA + *Fol*-inoc, (T_3) combine of ST + SA + *Fol*-inoc, (C_1) ST, (C_2) SA, (C_3) combine of ST + SA, (C_4) *Fol*-inoc and (C_5) wounding root and (C_6) non-inoculated seedlings

4.4 Discussion

Plants have endogenous defense mechanisms or latent defensive systems that are induced upon response to attack by insects and pathogens, were activated. Induced resistance in plants refers to a state of heightened defensive capacity created by a prior stimulus. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy (Hammerschmidt and Kuc, 1995; Ramamoorthy *et al*., 2002; McGovern, 2015). Systemic acquired resistance (SAR) is induced systemically throughout the plant in response to a pathogen infected plant (Ryals *et al*., 1996; Sticher *et al.*, 1997; Durrant and Dong, 2004), conferring a broad-spectrum of pathogen resistance against (Ryals *et al*., 1996; Sticher *et al*., 1997; Durrant and Dong, 2004). The development of SAR is associated with the induction of *PR* proteins (White, 1979), which are mostly of low molecular weight, preferentially extracted at low pH, resistant to proteolysis, and localized predominantly in the intercellular spaces of leaves (Taheri and Tarighi, 2012). The hypothesis was that the non-pathogenic rhizobacteria have been shown to enhance disease resistance by stimulating the systemic defense pathways (Hammerschmidt 1999). Many reports have demonstrated the efficiencies of *Streptomyces* spp. in controlling plant diseases caused by pathogenic fungi (Abd-Allah, 1995; Yuan and Crawford, 1995; Hardy and Sivasithamparam, 1995; El-Shanshoury *et al*., 1996; Nemec *et al*., 1996; Singh *et al*., 1999; Abd-Allah, 2001; Getha1 and Vikineswary, 2002; Sabaratnam and Traquair, 2002; Anitha and Rabeeth 2009; De Oliveira *et al*., 2010; Baharlouei1 *et al*., 2010; Kekuda *et al*., 2013). However, little is known about the ability of *Streptomyces* to trigger SAR in tomato against *Fol.*

The aim of the present study was to investigate the accumulation of transcript encoding some induced *PR* proteins in tomato plants response towards *Streptomyces* NSP3 challenge inoculated with or without *F. oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. In this study, four *PR* proteins, including *PR-1a, Chi3, Chi9* and *CEVI-1*, were investigated the upregulation by real-time RT-PCR. The *PR-1* genes have been frequently used as marker genes for SAR in many plant species as previously described by Mitsuhara *et al.* (2008). This study exhibited the strongly increase of

PR-1a expression immediately after *Fol*-inoculation. This result was in accordance with those already published by Conn *et al*. (2008) who reported that inoculation *Arabidopsis thaliana* seeds with *Streptomyces* sp. strain EN27 and EN28 challenged inoculation with *F. oxysporum* resulted in activation of the SAR pathway 4 day after pathogen inoculation via induction of the *PR*-1 gene, which increased expression 850 and 47.4 fold respectively, compared with the uninoculated control. Related to further reports of Berrocal-Lobo and Molina (2004) demonstrated the induction of the *PR-1* gene in *Arabidopsis thaliana* transcript 4 days after infection with *F. oxysporum*, lead to activation of the SAR pathways. However, the function of *PR-1* gene is still unclear (van Loon *et al.*, 2006) as deccribed by Silvar *et al*. (2008) showed that *PR-1* and sesquiterpene cyclase genes were up-regulated in infected stems of the pepper markedly by 24 h post-inoculation especially in resistant cultivars and suggested that the precise biological role of the *PR-1* proteins remains unknown, perhaps they appear to be important role in restricting pathogen colonization in resistant cultivar and related to biosynthesis of defense-related sesquiterpene phytoalexins.

Additionally, the *PR-3* (*Chi3*) and *PR*-4 (*Chi9*) genes are comprised of chitinases, which well-known that are constitutively expressed at low levels in plants, but are dramatically induced when plants respond to infection by fungal, bacterial, or viral pathogens (Leubner-Metzger and Meins, 1999; Neuhaus, 1999; van Loon, 1999). Chitinases have the potential to hydrolyse chitin, which is a major component of fungal cell walls. Chitin and glucan oligomers released during degradation of fungal cell walls act as elicitors that elicit various defence mechanisms in the plants (Frindlender *et al*., 1993). In this study, the expression level of *Chi3* was highest at 3 h post-inoculation, while *Chi9* was investigated during 6 – 12 h. It was previously shown by Taheri and Tarighi (2012) that observed resistance in tomato plants against *Rhizoctonia solani* between in Sunny 6066 (cv. resistant) and Rio Grande (cv. susceptible). The results revealed the highest elevated levels of chitinase gene (*LOC544149*) expression in both cultivar at 24 h post-inoculation. However, higher level of *LOC544149* gene expression and earlier upregulation were observed in Sunny compared to Rio Grande cultivar at 12 h post-inoculation. Likewise, Sridevi *et al*. (2008) reported on the role of chitinase in resistance of rice plants to *R. solani*. Similarly in tomato, investigations of Chen *et al*. (2009) revealed the involvement of chitinase in defense of the plants against *Botrytis*

cinerea. Also, Cachinero *et al*. (2002) demonstrated that inoculation of chickpea with protective strains of *F. oxysporum* resulted in an increased accumulation of chitinase, *β*-1,3-glucanases and peroxidase activities in roots correlated with plant resistance to *Fusarium wilt*. Similarly, Ito *et al*. (2005) showed an enhanced expression of acidic chitinase gene (*Chi3*) in tomato plants inoculated with strains belonging to *formae speciales* non-pathogenic on tomato. The result indicated that *PR-3* and *PR*-4 encoding chitinase gene may play an important role in host plant defense.

The *PR-9* or peroxidases (*CEVI-1*) are key enzymes in the cell wall building process, and it has been suggested that extracellular or wall-bound peroxidases would enhance resistance in various plant species against phytopathogens by the construction of a cell wall barrier that may hamper pathogen ingress and spread in plant cells. They often increase in response to stress and one of the principal roles of peroxidase appears to be cellular protection from oxidative reactions imposed by various stresses (Siegel, 1993; Taheri and Tarighi, 2012). Plant peroxidase produces antimicrobial phenolic compounds in the chemical defense systems against plant pathogens (Kobayashi *et al*. 1994). In this study, *Chi9* mRNA expression was increased to maximum after 3 h post-inoculation. Resembly, Taheri and Tarighi (2012) investigated the expression level of peroxidase gene (*CEVI-1*) the time point of upregulation in tomato plants against *Rhizoctonia solani* between in Sunny 6066 (cv. resistant) and Rio Grande (cv. susceptible). The results showed that peroxidase gene increased at 12 hpi and reached its maximum at 48 hpi in the Rio Grande plants. However, an increase in peroxidase gene expression at 6 hpi and peaking at 18 hpi was observed in cv. Sunny. Additionally, Xue *et al*. (1998) reported that resistance of bean plants, against the root rot pathogen *Rhizoctonia solani* and the anthracnose pathogen *Colletotrichum lindemuthianum*, were elicited after inoculated with nonpathogenic *Rhizoctonia* species by released peroxidases, *β*-1,3-glucanases and chitinases at 48 h after challenged inoculation. Recently, Boominathan and Sivakumaar (2013) found that *Bacillus megaterium* strain AUM72, a plant growth promoting rhizobacteria (PGPR), had ability to control rhizome rot in turmeric (*Curcuma longa* L) by increasing activities of peroxidase, *β*-1,3-glucanase, chitinase, phenylalanine ammonia lyase and polyphenol oxidase.

In the present experiment revealed that the treatments of *Streptomyces* NSP3 challenge inoculated with *F. oxysporum* f. sp. *lycopersici Fol*CK_117 was greatly exhibited high levels of studied *PR* proteins either seed treatment or soil application. Moreover, the combination of these two methods elicited the highest of interested *PR* genes expression. Induction resistance to *Fol* mediated by *Streptomyces* NSP3 occurred primarily by the SAR pathway. The studied *PR* genes in the plant challenged inoculate with *Fol*CK_117 and NSP3 were responsed at the higher level than plants challenged inoculate with NSP3 alone or *Fol*-inoculation plants. Besides wounding is reported to triggers defense mechanisms in plant defense system, this study showed that the maximum accumulation reaching at 24 hours after tomato plants were wounded. Furthermore, no upreguration of *PR* proteins were found in healthy plants. Related to Ramamoorthy *et al*. (2002) the protective strain *Pseudomonas fluorescens* isolate Pf1 was found to protect tomato plants by exhibited *PR* proteins and phenolics, including PAL, peroxidase, PPO, chitinase and TLP. The accumulation was higher responded after challenged inoculate with the pathogen *Fol*. Results of this study indicated that the selected *Streptomyces* NSP3 severed as excellent trigger in defense mechanism against *Fusarium* wilt disease in tomato plants as described by Suwan (2012) that the selected NF-*Streptomyces* NSP-167 (*Streptomyces* NSP3 in this study) might be activated the plant defense genes in the absence of a pathogen inoculation, suggesting that are detected as "minor" pathogens which do not trigger a full resistance response on their own, because they do not show pathogenic determinants, and this may result in more effective priming of the defense response against *Colletotrichum gloeosporioides* isolate TPCMCg60 causing chili anthracnose. In the result of this chapter indicated that whole interested *PR* proteins may play an important role in host plant defense. These results suggest that induction of *PR* proteins involved in SAR pathway might have contributed to restriction of invasion of *F. oxysporum* f. sp. *lycopersici* in tomato plants.

4.5 Conclusions

Applications of *Streptomyces* NSP3, including seed treatment, soil application and combination of these two methods were investigated for the activation of four plant defenses related genes. The average accumulation *PR-1a* was found the highest level,

followed by *Chi3* encoding acidic chitinase, *Chi9* encoding basic chitinase and *CEVI-1* encoding peroxidase. The results implied that these *PR* proteins appeared earlier and accumulated to higher levels (within 24 h) when plants were treated with *Streptomyces* NSP3 and challenged inoculated with *F. oxysporum* f. sp. *lycopersici Fol*CK_117 and compared to non-treated plants or those treated with *Streptomyces* NSP3 alone, or the pathogen *Fol* alone. Combination of seed treatment and soil application is more effective for accumulation of these *PR* proteins than either method alone.