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Systemic induction of defense-related genes in tomato by *Pseudomonas fluorescens* suppresses bacterial wilt disease incited by *Ralstonia solanacearum*

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Abstract
Bacterial wilt incited by *Ralstonia solanacearum* Yabucchi *et al.* is one of the most devastating disease of solanaceous crops. In this study, the bacterial wilt suppressing activity of *Pseudomonas fluorescens* Migula. was examined in tomato plants during plant-PGPR-pathogen interaction. The RT-PCR analysis revealed that, the transcription level of chitinase (133.06%) and β-1,3 glucanse (133.06%) in tomato plants treated with *Ralstonia solanacearum* + *Pseudomonas fluorescens* and *Pseudomonas fluorescens* increased with increasing time intervals (96 hpi). Unexpectedly, the defensin gene alone downregulated at 48th hpi and interestingly the transcription fold (126.67%) increased again at 96 hpi. Comparatively, the transcription levels of all the three defense genes upregulated sequentially with the progressive time interval during tripartite interaction than in mock control and *Ralstonia solanacearum* inoculated plants. Collectively, the results suggest that the simultaneous upregulation of all the three defense-related genes triggers the systemic resistance in tomato plants against the bacterial wilt.

Keywords: Bacterial wilt, tomato, *Pseudomonas fluorescens*, tripartite interaction, RT-PCR, ISR genes, systemic resistance

Introduction
Tomato (*Lycopersicum esculentum* Mill.) is one of the most important versatile vegetable due to its special nutritive value and wide usage in Indian culinary tradition. Tomato is an important source of vitamin A and C, minerals, some dietary fiber, a minute protein and copious water (Pest Control manual, PCM 1983) [29]. Tomato tops the list of canned vegetables worldwide. India is the second largest producer of tomato contributing 11 per cent of world`s production with area coverage of 797000 hectare, producing of 18399000 tonnes per year and the productivity of 26.0 tonnes per hectare (India stat, 2018).

Diseases are the foremost obstacle in tomato farming. Among tomato diseases, the bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabucchi *et al.* (1996) is the most devastating disease of tomato causing yield losses up to 90 per cent (Mallikarjun *et al*., 2008) [24]. Rapid and complete wilting of well-established plants causing lethal effect is the typical symptom of bacterial wilt. During warm and wet climatic environments the plants exposes the symptom of sudden drooping of leaves, without yellowing, often accompanied by rotting of the stem. Even though, the stem gets rotted, the roots of the infected plant remains healthy and well developed. Based on the pathogenicity trait of pathogen on different plant host, the RS strains in India were categorized under Race-1 (Chandrashekara *et al.*, 2012) [3]. It is evident that in India Race 1 and Biovar 3 of the pathogen *R. solanacearum* infects the tomato plants and the strains in India are highly virulent in nature.

Since *R. solanacearum* is a soil-borne pathogen and host resistance is limited, bacterial wilt is difficult to control (Saddler, 2005) [30]. Plant growth promoting rhizobacteria (PGPR) are the diverse group of microorganisms that play a major role in the biocontrol of plant pathogens and growth promoting activities. The concept of biological control of plant diseases has evidenced that some plant growth promoting rhizobacteria (PGPR) showed bio inhibition over some of the plant parasitic pathogens. Among PGPRs, fluorescent pseudomonads have been reported to be successful in effective management against a broad spectrum of plant pathogens (Loon *et al.*, 1998) [21].

Ferrnandez *et al.* (1989) indicated in their studies that the biological control of bacterial wilt disease could be accomplished using antagonistic bacteria.

Interactions of PGPR with pathogens, plants and the rhizosphere play a major role in inducing different defense strategies against the plant pathogens. Indirectly, the PGPR effectively check the deleterious effects of one or more plant pathogenic organism by producing siderophores that limit the available iron to the pathogen, producing antibiotics against pathogen, antibiosis and inducing systemic resistance in plant. PGPR also make cell wall structural modifications and biochemical/physiological changes of host plant leading to the enhanced synthesis of proteins and chemicals involved in plant defense mechanisms. Recent investigations on mechanisms of biological control by PGPR revealed that several strains protect the plants from pathogen attack by strengthening the epidermal and cortical cell walls with deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics and by activating defense genes encoding chitinase, POX, PPO and PAL (M'Piga *et al.,* 1997, Chen *et al.,* 2000) [22, 4].

Pseudomonas spp. suppress the disease development not only directly through the synthesis of metabolites with anti-microbial activity, but also indirectly through the inducing defense response mechanisms. These indirect mechanisms are linked to the synthesis of ISR and SAR mechanism (Pieterse *et al.,* 2014) [28] that are regulated by phytohormones such as SA, ABA, JA, ethylene (García *et al.,* 2013; Pieterse *et al.,* 2012) [11, 27], as well as CLPs (Vleesschauwer and Hofte, 2009) [34]. The ability of PGPR to synthesize ABA, especially under stressful conditions and to influence its level in plants was found in many strains of bacteria including the genera *Bacillus*, *Azospirillum*, *Pseudomonas*, *Brevibacterium* and *Lysinibacillus* (Dodd *et al.,* 2010; Kudoyarova *et al.,* 2014) [9, 20]. The ISR caused by endophytic bacteria is preserved in plants for a long time and effectively works against pathogens under natural conditions (Maksimov *et al.,* 2011) [23]. It manifests in early cascade and rapid accumulation of ROS, including H_2O_2, after the onset of infection, and corresponds with the up-regulation of redox-sensitive transcription factors and PR genes (Maksimov *et al.,* 2011) [23]. Thus, *Pseudomonas putida* LSW17S induced a rapid accumulation of transcription PR genes and production of H_2O_2 in tomato plants infected by *P. syringae* pv. tomato DC3000, which inhibited pathogen development (Ahn *et al.,* 2011) [1].

The current research was mainly focused to diminish the bacterial wilt of tomato an emerging disease in Tamil Nadu using PGPR (*Pseudomonas fluorescens* (PF)). Considering the lack of information on tripartite interaction between Tomato-Pathogen-PGPR, the project was aimed to find the transcription level of major defense genes expressed during tripartite interaction that induce systemic resistance against *R. solanacaerum.* To our knowledge, this is the first demonstration in India analyzing the level of ISR gene (chitinase, β-1, 3 glucanse & defensin) expression in tomato plants during tripartite interaction with *P. fluorescens* against *R. solanacearum*.

Materials and Methods

Plant material and microorganisms

Truthfully labelled healthy untreated tomato hybrid (Shivam) seeds were obtained from Rasi HyVeg Pvt. Ltd. The seeds were surface sterilized with 0.5 per cent sodium hypochlorite (NaOCl) solution and washed thrice with sterile distilled water to remove traces of NaOCl. The surface sterilized seeds were sown in sterile pro-trays containing sterilized well decomposed coir pith. The pro-trays were kept in germination chamber conditioned with controlled environment *viz.,* 25 °C temperature and 85 per cent relative humidity under 14 h light (70 µmol m^{-2} s^{-1}) and 10 h dark condition per day. The seedlings were watered with sterile fresh water and fertilized with Hoagland`s nutrient solution at three days interval. The seedlings were allowed to grow for 24 days under controlled condition without interference of any biotic or abiotic factors.

The *Ralstonia solanacearum* isolate KK2 was isolated from the wilted tomato plants collected from farmer's field at Kinathukadavu (10.8225° N, 77.0161° E), India. The authenticated *Pseudomonas fluorescens* culture TNAU PF1 (Accession No. ITCC BE0005) strain was obtained from Department of Plant Pathology, TNAU, Coimbatore was used as bio-control agent against the bacterial wilt pathogen.

Effect on defense gene expression in tomato plants treated with *Pseudomonas fluorescens* (TNAU PF1) and *Ralstonia solanacearum* (KK2)

Seedling production

The following four treatments (Table 1) were adopted to analyze the transcription level defense genes during tripartite interaction between tomato, *Pseudomonas fluorescens* and *Ralstonia solanacearum*.

Table 1: Treatments adopted for the three way interaction study

Treatment number		Treatment Particulars
Treatment 1(T1)	:	Control (Sterile distilled water)
Treatment 2 (T2)	:	*Ralstonia solanacearum* (KK2) + *Pseudomonas fluorescens* (TNAU PF1)
Treatment 3 (T3)	:	*Ralstonia solanacearum* (KK2)
Treatment 4 (T4)	:	*Pseudomonas fluorescens* (TNAU PF1)

At 25 DAS, the each seedlings transplanted to the pro-tray with cavity size of 8 cm^3 containing sterile soil mixture at 3:1:1 (Red soil: Sand: FYM) ratio. To analyze the defense gene expression the seedlings were grown in under continuous fluorescent light (70 µmol m^{-2} s^{-1}) and fertilized with Hoagland`s nutrient solution at three days interval. The seedlings were drenched with 30 ml of their respective 24h old bacterial suspension as per each treatment. The experiment was arranged in a completely randomized block design (CRD) and labelled. Five replications were maintained for each treatment. The samples from all the treatments were harvested at discrete time intervals at 0, 24, 48, 72 and 96 hours post inoculation (hpi). The harvested plants were washed under running tap water to remove soil particles and evaluated for plant vigour index.

The relative expression of defense genes (Chitinase, β-1,3 Glucanse & Defensin) induced by *P. fluorescens* in tomato plants against *R. solanacearum* was quantified using RT-PCR (Real time-Polymerase chain reaction) technique.

RNA extraction and cDNA synthesis

The tomato seedlings were withdrawn from pro-tray on 0^{th} h, 24^{th} h, 48^{th} h, 72^{nd} and 96^{th} h post inoculation. Total RNA from the treated plant samples was extracted using Trizol (Takara IsoPlus) (Chomczynski and Sacchi, 1987) [5] and was checked on 1.2 per cent agarose gel. The RNA extracted from the plant samples were subjected to DNase treatment to remove the residual DNA (Biolabs New England).

cDNA synthesis and polymerase chain reaction

From the DNA-free RNA samples, a quantity of 12 µl (about 2 µg) was used for cDNA synthesis. With the above RNA, 1 µl of oligo dT primer (100 pmol/ µl) was added, incubated at 65 ºC for 5 mins PCR thermocycler and immediately placed on ice. To the above content, 4 µl of 5x reverse transcriptase buffer; 2 µl of 10mM dNTP mixture and 1 µl of Revert Aid H minus Reverse transcriptase was added and mixed on ice and followed the incubation in PCR thermocycler (Eppendorf Mastercycler, Nexus Gradient, Germany) at 42 ºC for 60 mins; 50 ºC for 15 mins; 70 ºC for 15 min. After the cDNA synthesis, the samples were stored at -20 ºC for further use.

Relative quantification of defense gene analysis by RT-PCR

The transcription level defense genes were quantified using Roche Light Cycler® 96 using SYBR green (Takkara) as detection system along with the reference housekeeping gene (Actin). The defense gene primer sequences (Table 2) synthesized from Eurofins Genomics India Pvt. Ltd.

Table 2: Primers used in the study

Defense Gene	Primer name	Primer sequence	References
Chitinase	Achi-F	GCACTGTCTTGTCTCTTTTTC	Mitsuro *et al.*, 2013 [25]
	Achi-R	ATGGTTTATTATCCTGTTCTG	
β-1,3 Glucanase	Bgl-F	ATTGTTGGGTTTTTGAGGGAT	Mitsuro *et al.*, 2013 [25]
	Bgl-R	TTTAGGTTGTATTTTGGCTGC	
Defensin	Def-F	CAATGTAACTTAAAGTGCCTAATTATG	Hafez *et al.*, 2013 [12]
	Def-R	CTTATCAGATCTCAATGGAGAAATC	
Actin	Act-F	GGGGAGGTAGTGACAATAAATAACAA	Mitsuro *et al.*, 2013 [25]
	Act-R	GACTGTGAAACTGCGAATGGC	

The cDNA samples were diluted 5 times with nuclease free milliQ water. For each mix, 5 µl of SYBR green qPCR mixture; 3 µl of primer mix (Respective primers at 10 pmol/ µl Act-F and Act-R for housekeeping gene); 2 µl of diluted cDNA samples were added and the fluorescens was assessed in RT-PCR thermocycler following the programmed recommended by the manufacturer SYBR green (Takkara, Japan). The thermocycler programme includes (95 ºC for 30 sec), 45cycles of PCR (Includes denaturation 95 ºC for 5 sec, amplification at 60 ºC for 30 sec) and melting curve (Includes denaturation 95 ºC for 5 sec, amplification at 60 ºC for 60 sec). Changes in transcription level of the defense genes in each sample were calculated by relative quantification (ΔΔCt) method using threshold cycle (Ct) values of target and reference genes. For all real-time RT-PCR analyses, three biological replicates and two technical replicates were used. The size and intensity of amplified fragments were confirmed by gel electrophoresis. The relative quantification of defense gene of tomato was compared for 0, 24, 48, 72 and 96 hpi among each treatment.

Statistical analysis

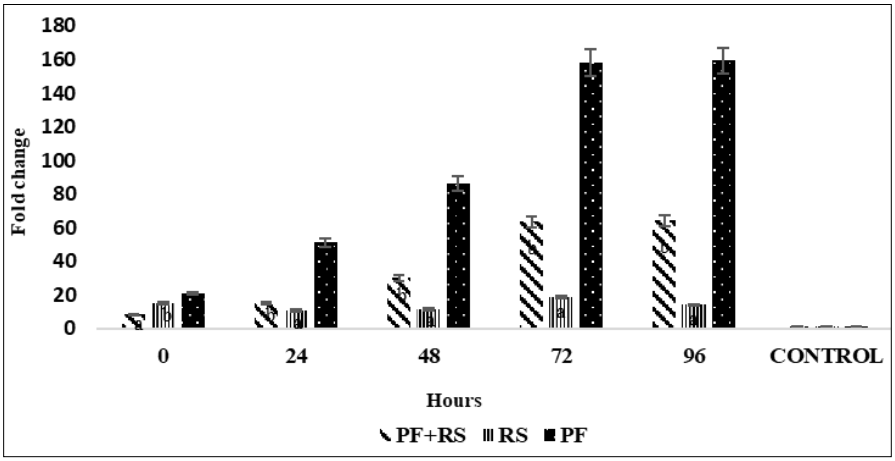
Lab experiments were carried out under Completely Randomized Block Design (CRD). One-way analysis of variance (ANOVA) was performed to identify the significant difference between the treatments and the means were separated by Duncan`s multiple range tests (DMRT).

Results

Transcript analysis of defense genes in tomato during tripartite interaction with *Pesudomonas fluorescens* (TNAU PF1) and *Ralstonia solanacearum* (KK2)

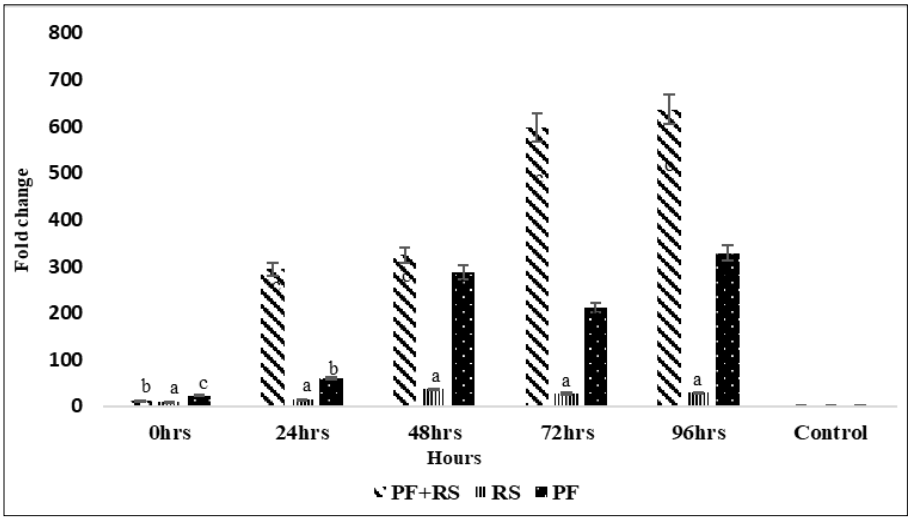
The relative amounts of mRNA detected from target genes (Chitinase, β-1,3 Glucanse & Defensin)**,** present in samples collected 0, 24, 48, 72 and 96 h post inoculation of treatments T2 and T4 were compared with plants infected with *Ralstonia solanacearum* and the mock healthy control to determine whether *Pesudomonas fluorescens* (TNAU PF1) application might control the bacterial wilt and induce resistance by bourgeoning the transcription of defense related genes. The relative transcription level of defense genes Chitinase, β-1,3 Glucanse and Defensin were normalized with Actin (reference gene) at different time intervals.

Among the four treatments, T4 up-regulated the chitinase gene in tomato leaves as compared to un-inoculated control (129.464% & 133.06% at 72 & 96 hpi, respectively), while the T3 had less influence on it. Moreover, T2 also upregulated the level of Chitinase gene up to 54.22 per cent at 96 hpi (Fig. 1). The early response of β-1,3 glucanase as influenced by PGPR / pathogen inoculation in tomato presented in fig. 2. Compared to 0^{th} hour after treatment, the PGPR or pathogen inoculation significantly altered the level of transcription of β-1,3 glucanase in tomato seedlings. Among the four treatments, T2 up-regulated the quantity of β-1,3 glucanse significantly than T3 in tomato leaves harvested at 48 hpi. The sequentially upregulating β-1,3 glucanase gene abruptly downregulated to 75.44 per cent at 72^{nd} hpi in T4 plants. Interestingly, at 96 hpi the β-1,3 glucanase increased over 116.98 per cent in T4 plants. Subsequently, the T4 up-regulated the β-1,3 glucanase (328.55% on 96 hpi) in tomato plants than in T3 plants. Accordingly, the highest level of defensin was observed in T4 plants. Where, the treatment T4 upregulated the relative transcription of defensin to 126.67 per cent at 96^{th} hour from 0^{th} hour post inoculation. Followed by, the plants in T2 expressed with 92.09 per cent at 96 hours post inoculation (Fig. 3). Similar to β-1,3 glucanase, the transcription level of defensin tends to decrease at 48^{th} hpi and reminisced to the increasing phase of defensin at progressive time intervals in T4.



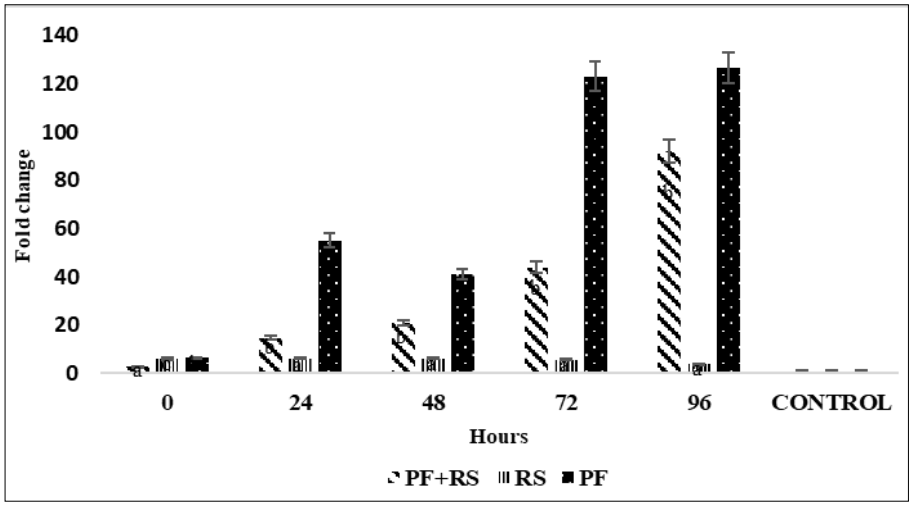
Quantification of gene expression transcript for acidic Chitinase in four treatments at five different intervals. Different letters indicate significant differences (Coincidence level of 95%) among treatments. Error bars represent one standard deviation from the mean (n=3).

Fig 1: Expression of Chitinase gene in tomato plants during PGPR-Pathogen interaction



Quantification of gene expression transcript for β-1,3 Glucanse in four treatments at five different intervals. Different letters indicate significant differences (Coincidence level of 95%) among treatments. Error bars represent one standard deviation from the mean (n=3).

Fig 2: Expression of β-1,3 Glucanse gene in tomato plants during PGPR-Pathogen interaction



Quantification of gene expression transcript for Defensin in four treatments at five different intervals. Different letters indicate significant differences (Coincidence level of 95%) among treatments. Error bars represent one standard deviation from the mean (n=3).

Fig 3: Expression of Defensin gene gene in tomato plants during PGPR-Pathogen interaction

Discussion

In the present investigation, the RT-PCR results revealed that at initial stage of infection, *Ralstonia solanacearum* primarily triggered the defense genes in tomato plants and receded at later stages. All the above results depicts that, the *Pseudomonas fluorescens* strain TNAU PF1 triggers the consecutive expression of all the three defense-related genes in tomato plants challenge inoculated with *Ralstonia solanacearum*. The results found that the transcription level of defense genes is directly proportional to the population level of microorganism at progressive time intervals.

In recent years, Plant growth-promoting rhizobacteria (PGPR) were well known for their beneficial effects in growth enhancement and potentiality in reducing the pathogen population, thereby suppressing diseases by colonizing the rhizosphere of the host plant (Kloepper *et al.,* 1988; Van *et al.,* 1991; Wei *et al.,* 1991) [18, 35, 35]. Among the PGPR, the *Pseudomonas fluorescens* can efficiently check the colony multiplication of *R. solanacearum* and the development of wilt symptoms in tomato plants. *Pseudomonas fluorescens* generally produces several antibiotic compounds, including antimicrobial substances that include bacterial toxins, antibiotics *viz.*, 2,4-diacetyl phloroglucinol, pyrollnitrin, HCN production, phenazine-1-carboxylic acid, degrading enzymes, bacteriocins and a signal molecule in the bacterial quorum-sensing system (Girija and Manoj, 2005) [10].

Accordingly, the multiplication of *R. solanacearum* colonies in *P. fluorescens* treated soil mixture was suppressed in pro-tray experiment due to the presence of anti-microbial extracellular and intracellular compounds produced by *P. fluorescens*. In our study, the plants inoculated with *Ralstonia solanacearum* (T3) started to wilt at fifth DAI (Day after inoculation) and the typical symptoms were observed and confirmed with ooze test. Our study found that it is not only the antimicrobial compounds from *P. fluorescens* inhibits the development of wilt symptoms in the tomato plant, the transcription level of defense-related gene (Chitinase, β-1,3 glucanase and defensin) induced by *P. fluorescens* plays a significant role in inducing systemic resistance in treated plants than the untreated and challenge inoculated plants. The level of relative expression of defense genes varied among each other in respective to the different time interval after treatment. Hafez *et al.* (2013) [12], reported that the increase in time of incubation after treatment with bio-agents increased the level of Chitinase (PR protein) gene expression (2.76 fold) at 24 h post inoculation in tomato plants. Similarly, the highest expression level of defensin was observed in the plants treated with *F. oxysporum* + *B. subtilis* (0.69 fold) at 24 h post inoculation. Our results were in correlation with Mitsuro *et al.* (2012) findings on the analysis of defense gene expression in tomato plants treated with culture filtrate (CF) of *B. thuringiensis* and challenge inoculated with *Ralstonia solanacearum*. The findings concluded that the tomato plants treated with culture filtrate (CF) of *B. thuringiensis* with challenge inoculation with *Ralstonia solanacearum* reduced the symptom development by one third than the pathogen inoculated plants.

Moreover, our results clearly indicated the up-regulation of defense-related genes such as, acidic chitinase and β-1,3 glucanase and defensin in the symptom less treated plants than in wilted plants. Chitinases, which are clustered in type three family of pathogen-related protein (PR-3) are the fundamental enzymes involved in plant-microbe interactions. The novel findings of our study identified that the combined expression of chitinases gene along with other tested plant-defense proteins *viz.,* β-1,3 glucanase and defensin at same time interval enhances the systemic resistance in tomato plants against the bacterial wilt disease of tomato.

The former studies on bacterial bio-agents articulates that the cellular compounds of bacterial antagonist inhibit the pathogen colonization and initiate the innate immune system of the plant system against specific or broad range of pathogen. ISR elicited by PGPR is mainly mediated by Jasmonic acid/Ethylene dependent signaling pathways. The extracellular compounds produced during the plant pathogen interaction mimics as elicitors in activation of signaling pathways to enhance the plant defense system (Bargabus *et al.,* 2002; Jetiyanon *et al.,* 2003; Kloepper *et al.,* 2004, Murphy *et al.,* 2003; Zhou *et al.,* 2008) [2, 14, 17, 26, 37].

The comparative variation among the transcription level all the three defense-related genes at shorter and longer hour post-inoculation (HPI) time intervals indicates the different levels of resistance level against bacterial wilt of tomato. The consecutive upregulation of defense gene at longer inoculation periods might be related to the quorum sensing effects by increasing the antagonistic population over the rhizosphere region of the host. Zhang *et al.* (2004) [36] reported that the definite critical bacterial population density is required to produce antibiotics and /or signal molecules that induces the systemic protection in the host plant. For further confirmation, influence of bacterial population of PGPR need to be investigated with host-pathogen interaction.

During the synthesis of ISR (Induced Systemic Resistance) induced by endophytic bacteria, generation of ROS in plants can play a critical role in the formation of the priming effect. The ISR priming phenomenon in the host genome influenced by bacterial bio-agents produce hypersensitivity reaction to foreign substances. The ISR is characterized by faster and stronger activation of cellular mechanisms of plant protection under pathogens or insect invasion and can last for a rather long time. This will lead to an increase of plant resistance. It is suggested that such priming in response to bacterial infection is associated with a change in the status of DNA methylation in the plant genome (Da *et al.,* 2012) [7].

With the above results on induced systemic resistance (ISR) during tripartite interaction between tomato, *P. fluorescens* and *R. solanacearum*, it was observed that the resistance level in tomato plants by combined application of PGPR and pathogen was higher than the individual application of *P. fluorescens* and *R. solanacearum*. Simultaneous inoculation of tomato plants with PGPR and pathogen can induce defense responses. PGPR induced systemic resistance was associated with an enhanced capacity for the rapid activation of cellular defense responses, which were induced only after the contact of challenging pathogen (Ku´c, 1987; Conrath *et al.,* 2002) [19, 6]. A phenomenon termed priming of defense responses has been defined in rhizobacterium-mediated induced systemic resistance (Conrath *et al.,* 2002) [6].

Conclusion

Collectively, up-regulation of defense-related genes chitinase, β-1,3 glucanase and defensin by *P. fluorescens* strain TNAU PF1 against *R. solanacearum* strain KK2 in tomato plants indicates that PGPR may cause a sequence of physiological, molecular and biochemical changes which lead to systemic resistance against pathogens and stimulating the plant canopy. Our results indicate that, the PGPR strain TNAU PF1 is an effective bio control agent that enhances the inherent defense

system against the bacterial wilt pathogen *Ralstonia solanacearum* (Race-1 & Phyllotype-1) by increasing the transcription fold of the defense genes expressed in tomato during tripartite interaction. Identifying the defense genes/PR-proteins and their expression level at different time interval during tripartite interaction for the specific crop against a specific disease may provide an appreciated outcome to the researchers on subsequent development of resistant cultivars.

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