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Title of retracted article: Induction of Systemic Resistance in Tomato against Ralstonia solanacearum by Pseudomonas fluorescens

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The paper does not meet the standards of "American Journal of Plant Sciences".

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Induction of Systemic Resistance in Tomato against *Ralstonia solanacearum* by *Pseudomonas fluorescens*

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Abstract

The biological control agent *Pseudomonas fluorescens* was used against the bacterial wilt causing *Ralstonia solanacearum*. The present investigation focuses on the role of defense related enzymes in imparting resistance in tomato against *R. solanacearum.* A total of ten rhizobacterial isolates were screened against *R. solanacearum*, of which three isolates (Pf3, Pf5 & Pf8) showed a maximum inhibition against the pathogen and were further identified as *P. fluorescens* by 16S rRNA analysis. Seedling treatment with *P. fluorescens* isolates significantly enhanced the quality of seed germination and seedling vigor. The three *P. fluorescens* strains were further tested for their ability to induce the production of defense-related enzymes in plants. Involvement of defense related enzymes in bacterial wilt pathogenesis was studied in susceptible tomato cultivar (Arka Meghali). Root dip inoculation was performed with bacterial suspensions of *R. solanacearum* and *P. fluorescens* (1×10^8 cfu/ml) on ten days old seedlings and harvested at different time intervals (0, 3, 6, 9, 12, 15, etc. up to 72 h) and assayed for the defense related enzyme activity. The seedling treatment of *P. fluorescens* isolates induced a significant increase in the activities of peroxidase (POX), Polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), and *β*-1, 3-glucanase in treated tomato plants and the same trend of increase in enzyme activity was observed in *P. fluorescens* treated tomato seedlings challenged with *R. solanacearum*. The activities of the enzymes PAL, POX, PPO and *β*-1, 3-glucanase reached maximum at 24, 18, 24 and 24 h after inoculation respectively. Higher accumulation of phenolics was noticed in plants pre-treated with *P. fluorescens* and challenge inoculated with *R. solanacearum*. Native PAGE analysis of both Peroxidase (POX) and Polyphenol oxidase (PPO) was carried out for the time course of enzyme activities and the isoforms of POX and PPO were examined.

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Keywords

***Pseudomonas fluorescens*, *Ralstonia solanacearum*, Induced Systemic Resistance, Tomato, Peroxidase, Phenylalanine Ammonia Lyase, Polyphenol Oxidase, *β*-1, 3-Glucanase**

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is contemplated to be one of the most important plant diseases in tropical agriculture [1] [2]. It has a large host range of more than 200 species in 50 families [3]. These globally dispersed and heterogeneous strains cause bacterial wilt diseases, which have major socio economic impacts [4]. Several hundred species of tropical, subtropical and warm temperature plants are susceptible to one or more races of *R. solanacearum* and affect a wide range of economically important crops such as tomato, potato, eggplant, chilli and non *Solanaceous* crops such as banana and groundnut in India resulting in massive losses [5]. Bacterial wilt is said to be causing 15% to 55% crop losses around the world. In India, a study showed 10% to 100% incidence of bacterial wilt during the summer [6]. Infested soil and surface water, including irrigation water, are the primary sources of inoculum. The pathogen infects roots of susceptible plants, usually through wounds [7]. Colonization by the bacterium within the xylem prevents water movement into upper portion of the plant tissue [8]. The symptoms start as leaf drooping followed by wilting of whole plant initially and slowly results in a permanent wilt leading to total plant collapse. The roots and lower portion of the stem have a browning of their vascular system. The invaded roots may rot due to infection from secondary bacteria [9].

Chemical control of plant diseases is usually expensive and may have a negative impact on the environment and on public health. Biological control makes management of plant diseases less dependent on the use of high risk chemicals and is environmentally friendly. Fluorescent Pseudomonads are amongst the most effective biological control agents against soil borne plant pathogens. Several isolates of *P. fluorescens*, *P. putida*, and *P. aureofaciens* suppress the soil borne pathogens through rhizosphere colonization, antibiosis and iron chelation by siderophore production. Certain fluorescent pseudomonads are also found to promote plant growth by production of plant growth promoting substances and thus are called Plant Growth Promoting Rhizobacteria (PGPR). PGPR are known to induce resistance against fungal, bacterial and viral diseases. In addition to plant growth promotion and direct antimicrobial activity, activation of defense genes by PGPR application is a novel strategy in plant protection. PGPR systemically activates the plant's latent defense mechanism against pathogens called Induced Systemic Resistance (ISR) [10]. This mechanism operates through the activation of multiple defense compounds at sites distant from the point of pathogen attack. Recent studies on mechanisms of biological control by PGPR reveals 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, Peroxidase (POX), Phenylalanine ammonia-lyase (PAL), Polyphenol oxidase and (PPO) [11].

Induction of Systemic Resistance against various disease causing pathogens in crops such as banana, bean, rice, and cucumber by *Pseudomonas* and *Bacillus* strains have been reported widely [12]. The Induction of systemic resistance by *Pseudomonas* strains were demonstrated in bean, carnation, rice, and cucumber [13]. The strains of *Pseudomonas* spp. were found to induce resistance against different pathogens in cucumber against *C. orbiculare* under field conditions [14] and radish [15]. However, a better understanding of the microbial interactions that result in increased plant growth will significantly upsurge the success rate of field applications. *Pseudomonas* spp. is widespread in agricultural soils and has many traits that make them sound contenders as PGPR. The most effective strains of *Pseudomonads* are gram negative, motile, rod shaped bacteria and have various phyto beneficial traits which include production of hydrogen cyanide, siderophores, protease, antimicrobials and phosphate solubilizing enzymes [16]. Initial studies of PGPR focused primarily on fluorescent *Pseudomonads*, but it is now known that PGPR include a diverse assemblage of bacteria representing a broad spectrum of genera. PGPR strains are aggressive colonies of the rhizosphere environment and can persist for the duration of the growing season [17]. PGPR have the ability to promote the growth of plants following inoculation onto seeds or subterranean plant parts by secreting plant hormones and are established to protect the roots of certain crop plants. Nevertheless, fluorescent Pseudomonads have emerged as the largest and potentially most promising

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group of plant growth promoting rhizobacteria involved in the biocontrol of plant diseases.

The potential of P. *fluorescens* in providing disease resistance and plant growth promotion has been proved in a variety of crops and pathogen interaction, as in sheath blight, sheath rot, bacterial blight of cotton [18], bacterial leaf blight of rice [19], wilt disease of tomato [20], *Botrytis cinerea* in Strawberry [21] and Pythium disease of tomato and hot pepper [22].

The objective of the present study deals with the induction of defense enzymes such as phenylalanine ammonialyase (PAL), peroxidases (POX), polyphenol oxidase (PPO), total phenolics and 1,3-*β* glucanase by *P. fluorescens* against challenge inoculation with *R. solanacearum*.

2. Materials and Methods

2.1. Isolation and Identification of *R. solanacearum*

Affected tomato plants showing typical symptoms of wilt were collected from different agro climatic zones of Karnataka. The isolates were subjected to identification and confirmation based on the morphological, physiological, cultural, biochemical and pathogenicity studies [23] [24].

2.2. Isolation and Identification of *Pseudomonas fluorescens*

Fluorescent *Pseudomonads* were isolated from rhizosphere soil of tomato fields from Karnataka, India. Isolation of fluorescent *pseudomonads* was carried out by serial dilution technique using King's B medium [25]. The colonies were examined for morphological characteristics such as shape, size, structure and pigmentation. Presence of fluorescence in UV light was used to select putative *P. fluorescens* colonies. The individual colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in refrigerator at 4˚C for further use [26].

The identification of the selected strains was further confirmed by molecular methods based on 16 s rRNA sequencing for *R. solanacearum* and *P. fluorescens*. NCBI BLAST search was performed and the top hit sequences were multiple aligned and phylogenetic tree was constructed using CLUSTAL X2 2.1 (Windows version) software by Neighbor Joining (NJ) analysis with 1000 bootstrap replications based on the algorithm [27]. The sequences were deposited to NCBI database.

2.3. Effect of *P. fluorescens* on Tomato Seed Germination and Seedling Vigor Index

The effect of *P. fluorescens* on seed germination and vigor of seedlings along with *R. solanacearum* was evaluated under laboratory conditions. Wilt susceptible tomato cultivar (Arka Meghali) was procured from Indian Institute of Horticultural Research (IIHR) Bangalore, India.

Preparation of Bacterial Inoculums

Pseudomonas fluorescens (Pf3, Pf5, and Pf8) were cultured on King's B agar medium [25]. *P. fluorescens* was multiplied in nutrient broth for 24 h and bacterial cells were collected by centrifugation and population was adjusted to 1×10^8 colony forming units (CFU).

Inoculum of *R. solanacearum* was prepared by growing it on TZC agar medium, for 48 h at 30˚C. Colonies were multiplied in sucrose peptone broth [28]. 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 suspensions were spectrophotometrically adjusted to O.D 600 nm = 0.1 (approximately 10^8 CFU·ml^{-1}) [29].

The germination tests for fresh *R. solanacearum* and *P. fluorescens* suspensions were carried out according to the paper towel method [30]. One hundred seeds were placed at equidistant on the germination paper presoaked in distilled water and covered with another presoaked paper towel and wrapped with polythene to prevent drying of towels. The rolled towels were incubated for ten days at 24˚C ± 2˚C. After incubation, paper towels were unrolled and germinated seeds were counted and represented in percentage. Seeds treated with distilled water in a similar method served as negative controls. The vigor index was calculated by using the formula VI = (mean root length + mean shoot length) × Germination percentage [31]. To evaluate vigor, the length of the root and shoot of an individual seedling was measured. The experiment was conducted with four replicates of hundred seeds each and the entire experiment was repeated thrice.

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2.4. Induction of Defense Mechanisms and Experimental Design

2.4.1. Preparations of Crude Enzyme Extracts

Tomato seeds of Arka Meghali were treated with the *P. fluorescens* suspension for 12 h and then were germinated on moist blotter discs placed in Petri dishes, at 25 seeds per plate following standard procedure [30]. The plates were incubated at 28˚C ± 2˚C for 8 days until cotyledons were completely opened. The roots of 10 days old seedlings were dip-inoculated by pouring *R. solanacearum* suspension into the Petri dishes. A set of three controls were maintained, *i.e*., only *P. fluorescens* treated tomato seeds, water and *R. solanacearum* treated seeds. The inoculated and uninoculated seedlings were harvested at different time intervals: 0, 3, 6, 9, 12, 15, 18, 21, 24 up to 72 after pathogen inoculation and stored at −80˚C for subsequent analysis. Distilled water inoculated samples served as control.

One gram of tomato seedlings were macerated to a fine paste in a prechilled mortar with 25 mM Tris HC1 buffer (pH 8.8) (w/v; 1:1). The homogenate was centrifuged at 10,000 rpm for 30 min at 4˚C and the supernatant was used directly for PAL enzyme assay. One gram of tomato seedlings were homogenized in 10 mM phosphate buffer (pH 6.0) in a prechilled mortar and pestle on ice (w/v; 1:1). The homogenate was centrifuged at 12,000 rpm for 20 min at 4˚C and the supernatant served as enzyme source for POX and PPO. Protein contents of the extracts were determined according to standard procedure of Bradford [32] using Bovine serum albumin (BSA) as the standard.

2.4.2. Determination of Phenylalanine Ammonialyase (PAL) Activity

The enzyme activity was determined by spectrophotometric measurement of the production of trans cinnamic acid from L-phenylalanine. The reaction mixture contained 1ml enzyme extract, 0.5 ml substrate, 50 mM L-phenylalanine and 0.4 ml 25 mM Tris HC1 buffer (pH 8.8). After incubation for 2 h at 40˚C the activity was stopped by the addition of 0.06 ml of 5 N HC1; the absorbance was read at 290 nm against the same volume of reaction mixture without L-phenylalanine that served as blank. The enzyme activity was expressed as mol of trans cinnamic acid mg^{-1} protein h^{-1} [33]. Experiments were conducted in three replicates and were repeated three times.

2.4.3. Determination of Peroxidase (POX)

The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 per cent H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2˚C). The changes in absorbance at 420 nm were recorded at 30 s interval for 3 min. The enzyme activity was expressed as changes in the absorbance $min^{-1}mg^{-1}$ protein [34]. All the experiments were repeated thrice.

2.4.4. Determination of Polyphenol Oxidase (PPO)

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* [35]. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm. The activity was expressed as change in absorbance $min^{-1}\cdot mg^{-1}$. All the experiments were conducted in four replicates and repeated three times.

2.4.5. Determination of *β*-1, 3-Glucanase

Activity of *β*-1, 3-glucanase was assayed by the Laminarin dinitrosalicylic acid method [36]. Tomato seedlings (1 g) were extracted with 2 mL of 0.05 M sodium acetate buffer (pH 5.0) and centrifuged at 16,000 rpm for 15 min at 4˚C. The supernatant was used for the enzyme assay. The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40˚C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic acid and heating for 5 min on boiling water, vortexed and its absorbance was measured at 500 nm. The enzyme activity was expressed as µg glucose released $min^{-1}\cdot mg^{-1}$ protein.

2.4.6. Determination of Phenol

Tomato seedlings (1 g) were homogenized in 10 mL of 80% methanol and agitated for 15 min at 70˚C [37]. One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin Ciocalteu reagent (1 N) and the solution was kept at 25˚C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol

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mg^{-1} Protein.

2.5. Native PAGE Analysis of POX and PPO Enzymes

The isoform profiles of POX and PPO were examined by discontinuous Native polyacrylamide gel electrophoresis (Native PAGE) with slight modifications [38]. Both treated and control tomato seedlings were collected at 18 and 24 h for POX and PPO enzymes, respectively. The protein extracts were prepared by homogenizing 1 g of seedlings in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 18,000 rpm for 15 min at 4˚C. Samples (POX and PPO) were loaded onto 8% (w/v) polyacrylamide gels in a vertical minigel electrophoresis. The electrode buffer was Trisbase (3.0 g Tris base, 7.2 g glycine and 1000 ml distilled water). Electrophoresis was performed at a constant voltage of 50 V initially for 1 h and of 100 V to complete electrophoresis.

2.5.1. Activity Staining for POX

After electrophoresis, POX isoforms were visualized by soaking the gels in staining solution containing 100 mg benzidine dissolved in 1 ml of absolute alcohol and made up to 40 ml using distilled water. Clear solution was obtained by adding 500 ml of glacial acetic acid to the above mixture and undissolved particles of benzidine were removed by filtering the solution through cotton. H_2O_2 (250 ml) was added to the filtered solution at the end and gels were incubated in the solution until bands appeared [39].

2.5.2. Activity Staining for PPO

The activity staining for the isoforms of PPO was performed by incubating the gels in 50 mM Tris buffer (pH 6.8) containing 500 mg catechol and 300 mg of L-3, 4-dihydroxyphenylalanine (L-DOPA) on a rotary shaker. After 10min of incubation, dark bands indicative of PPO isozymes appeared in the gel. Bands were revealed after 20 min incubation at room temperature.

3. Results

3.1. Isolation and Characterization *Pseudomonas fluorescens*

Ten isolates of fluorescent pseudomonads were isolated from the rhizosphere soil of different fields and named them as Pf3, Pf2, Pf3, Pf4, Pf5, Pf6, Pf7, Pf8, Pf9, and Pf10. All the isolates were found effective against *R. solanacearum*. Hence, these isolates were further subjected to morphological and biochemical characterization and plant growth promotion activity of tomato plants. All 10 strains of *P. fluorescens* tested showed antagonistic effects against highly virulent *R. solanacearum*; with inhibition zone radii ranging from 3 to 29 mm. *P. fluorescens* strain 5 was the most potent in inhibiting all test pathogen strains, followed by Pf3 and Pf8 and hence were selected for further ISR studies [24].

The amplified PCR products were sequenced and a phylogenetic tree was constructed by the blast analysis and multiple sequence alignment data (**Figure 1**). The sequences were deposited in NCBI GenBank with Accession No. Pf3: KF679344, Pf5: KF679345, and Pf8: KF679346.

3.2. Effect of *P. fluorescens* on Tomato Seed Germination and Seedling Vigor Index

There was an improvement in seedling vigor upon *P. fluorescens* seed treatment whereas seed germination of tomato seeds upon *R. solanacearum* inoculation showed reduction. The *P. fluorescens* treatment enhanced the vigor index when compared to control. The highest germination was recorded in *P. fluorescens* treated seeds as tabulated in (**Table 1**).

Under laboratory conditions, all the isolates showed significantly higher mean root length, higher mean shoot length, and vigor index with respect to control (**Figure 2**). Pure cultures of Pf3, Pf5, Pf8 (1×10^8 cfu/ml) increased the seedling vigour of tomato seeds by 1308, 1255 and 1230 and showed an improved seed germination which increased by 48%, 47% and 45% respectively upon challenge inoculation with *R. solanacearum*. In comparison to the control, maximum germination was recorded in Pf3, Pf5, and Pf8 seeds treated with *P. fluorescens* (92%, 91% and 99%) (**Table 1**).

3.3. Induction of Systemic Resistance by *P. fluorescens*

The activities of the enzymes were estimated and detected in *P. fluorescens* isolates treated seedlings, which

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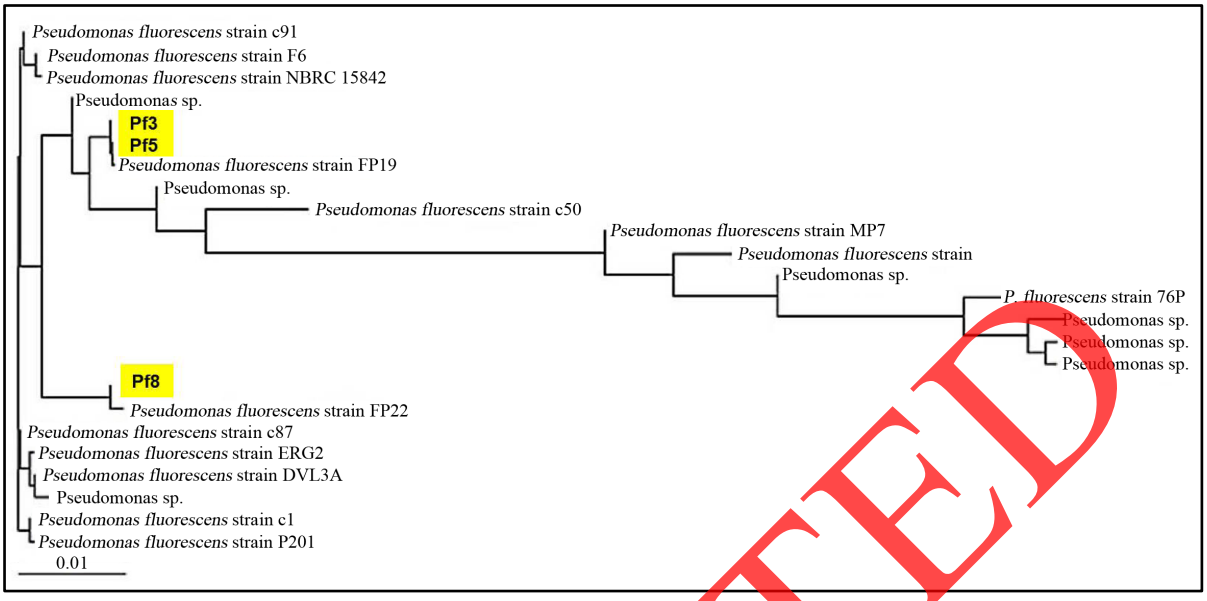

Figure 1. Phylogenetic relationships of *P. fluorescens* (Pf3, Pf5 and Pf8) isolates inferred by neighbor-Joining (NJ) bootstrap tree analysis of 16s rRNA sequences. Sequences used for this comparison were obtained from GenBank.

Table 1. Effect of seed treatment with *R. solanacearum* and *P. fluorescens* strains on seed germination and seedling vigour of tomato under laboratory conditions.

Treatments	Germination (%)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (gm.)	Dry Weight (gm.)	Vigor Index
Control	91.33 ± 3.46^{d}	3.78 ± 0.25^{c}	5.66 ± 0.33^{b}	0.94 ± 0.011^{b}	0.22 ± 0.005^{b}	863.11 ± 4.61^{b}
RS1	34.0 ± 1.52^{a}	2.95 ± 0.043^{b}	3.32 ± 0.025^{a}	0.40 ± 0.033^{a}	0.12 ± 0.003^{a}	213.41 ± 5.77^{a}
RS2	35.0 ± 1.15^{a}	2.59^{ab} ± 0.011^{ab}	3.16 ± 0.057^{a}	0.37 ± 0.011^{a}	0.13 ± 0.006^{a}	201.57 ± 6.35^{a}
RS3	32.66 ± 1.2^{a}	2.75 ± 0.057^{ab}	3.26 ± 0.025^{a}	0.37 ± 0.05^{a}	0.12 ± 0.002^{a}	196.64 ± 3.46^{a}
RS4	35.33 ± 1.15^{a}	2.65 ± 0.011^{ab}	3.11 ± 0.033^{a}	0.40 ± 0.057^{a}	0.12 ± 0.001^{a}	203.67 ± 5.77^{a}
RS5	34.33 ± 0.88^{a}	2.40 ± 0.011^{a}	3.17^{a} ± 0.057^{a}	0.34 ± 0.033^{a}	0.12 ± 0.003^{a}	191.59 ± 6.57^{a}
RS6	35.66 ± 1.12^{a}	2.57 ± 0.033^{ab}	3.17 ± 0.012^{a}	0.35 ± 0.066^{a}	0.12 ± 0.003^{a}	204.86 ± 5.19^{a}
RS7	34.0 ± 1.86^{a}	2.50 ± 0.028^{a}	3.19 ± 0.045^{a}	0.45 ± 0.025^{a}	0.12 ± 0.005^{a}	193.60 ± 4.61^{a}
RS8	35.0 ± 1.7^{a}	2.85 ± 0.057^{ab}	3.18 ± 0.066^{a}	0.40 ± 0.011^{a}	0.12 ± 0.006^{a}	211.35 ± 3.46^{a}
RS9	34.66 ± 1.12^{a}	2.68 ± 0.011^{ab}	3.15 ± 0.033^{a}	0.36 ± 0.021^{a}	0.12 ± 0.005^{a}	202.41 ± 4.61^{a}
RS10	35.33 ± 1.57^{a}	2.67 ± 0.033^{ab}	3.24 ± 0.057^{a}	0.36 ± 0.011^{a}	0.12 ± 0.003^{a}	209.11 ± 6.92^{a}
Pf3	92.0 ± 3.43^{d}	5.76 ± 0.15^{e}	8.45^{e} ± 0.15	1.19 ± 0.033^{c}	0.37 ± 0.006^{d}	1308.20 ± 28.92^{e}
Pf5	91.33 ± 1.57^{d}	5.62 ± 0.17^{e}	8.23 ± 0.25^{de}	1.21 ± 0.057^{c}	0.29 ± 0.002^{c}	1255.84 ± 22.57^{e}
Pf8	89.66 ± 1.52^{cd}	5.58 ± 0.28^{e}	8.40 ± 0.66^{de}	2.22 ± 0.066^{c}	0.26 ± 0.005^{bc}	1230.80 ± 20.27^{e}
Pf3 + RS	85.66 ± 1.15^{bcd}	5.25 ± 0.15^{de}	7.80 ± 0.33^{cd}	1.15 ± 0.021^{c}	0.27 ± 0.006^{bc}	1127.16 ± 14.43^{d}
Pf5 + RS	83.33 ± 1.52^{bc}	5.103 ± 0.57^{de}	7.51 ± 0.15^{c}	1.13 ± 0.011^{c}	0.26 ± 0.003^{bc}	1067.81 ± 17.89^{cd}
Pf8 + RS	82.0 ± 1.2^{b}	5.04 ± 0.15^{de}	7.64 ± 0.57^{c}	1.10 ± 0.005^{c}	0.23 ± 0.005^{bc}	1040.03 ± 16.16^{c}

Means ± SE (standard error) followed by the same letter do not differ significantly according to Duncan's multiple range test at $P = 0.05$. Scheffe post hoc test Means sharing different alphabetical (a, b, c, d, e) superscripts in a column significantly different ($P \leq 0.05$). RS: *Ralstonia solanacearum*, Pf: *Pseudomonas fluorescens.*

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were challenge inoculated with *R. solanacearum*. Maximum PAL activity was observed 24 h after inoculation (hpi). PAL activity increased in *P. fluorescens* pretreated seedlings challenged with the pathogen while seedlings inoculated with the pathogen alone had lower PAL activity. PAL activity in seedlings treated with only *P. fluorescens* remained almost unchanged throughout the experiment but was slightly higher compared to control (**Figure 3**).

In our study, activity of POX increased after inoculation and reached its maximum at 18 hpi. Tomato seedlings inoculated with the pathogen alone recorded lower POX activity than treated seedlings. The seedlings treated with *P. fluorescens* alone demonstrated higher activity than the untreated control seedlings. The activity of POX reached the highest level in all the treatments on 18 h after challenge inoculation and then slowly decreased as compared to control. The highest activity of POX was observed with *P. fluorescens* isolates challenge inoculated with *R. solanacearum* (**Figure 4**).

Treatment with *P. fluorescens* on tomato seedlings exhibited ISR associated with enhanced PPO activities. At 24 hpi, the activity of PPO was maximal in seedlings treated with *P. fluorescens* and challenge inoculated with *R. solanacearum*. Seedlings treated with *R. solanacearum* alone also showed increased PPO activity but the increase was moderately less. The PPO activity in seedlings treated with *P. fluorescens* alone and control never reached to the level of activity observed in the seedlings treated with *P. fluorescens* and challenge inoculated with *R. solanacearum* (**Figure 5**).

A significant increase in *β*-1, 3-glucanase activity was also observed in tomato seedlings treated with *P. fluorescens* and *R. solanacearum*. The *β*-1, 3-glucanase activity increased after challenge inoculation, reached the

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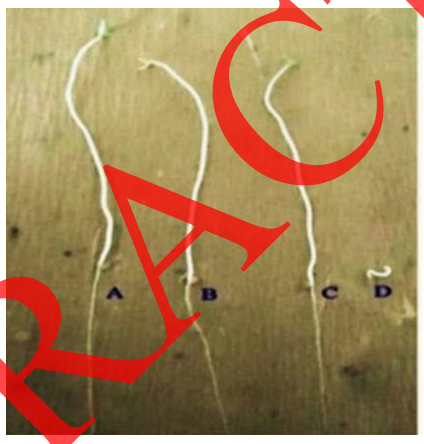

Figure 2. Effect of *P. fluorescens* on tomato seed germination and seedling vigor index. Seed germination of tomato seeds A and B: *P. fluorescens* treatment, C: Control and D: Pathogen treatments seeds.

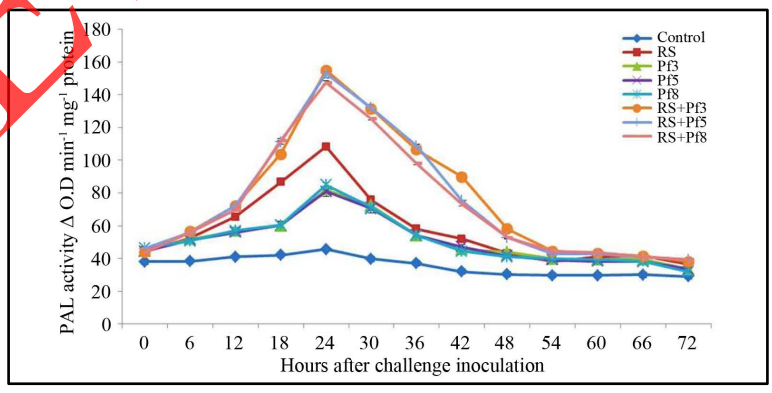

Figure 3. The effect of treatment of *P. fluorescens* isolates on the activity of phenylalanine ammonia lyase (PAL) in tomato seedlings. Values are the mean of three replications and bars represent ± SE. C—Control, Pf—*P. fluorescens* and RS—*R. solanacearum*.

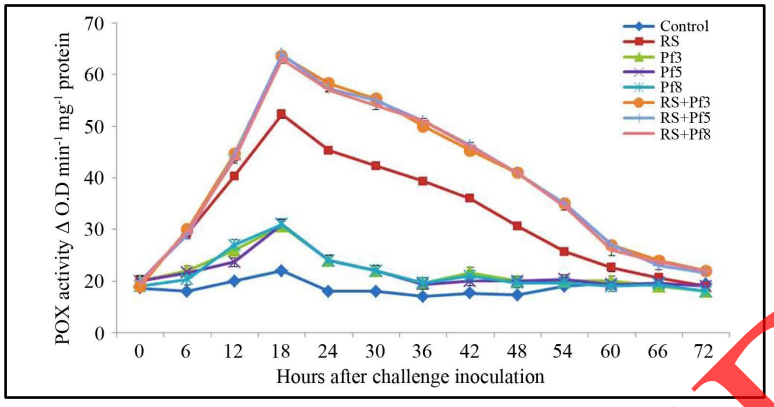

Figure 4. The effect of treatment of *P. fluorescens* isolates on the activity of Peroxidase (POX) in tomato seedlings. Values are the mean of three replications and bars represent ± SE. C—Control, Pf—*P. fluorescens* and RS—*R. solanacearum*.

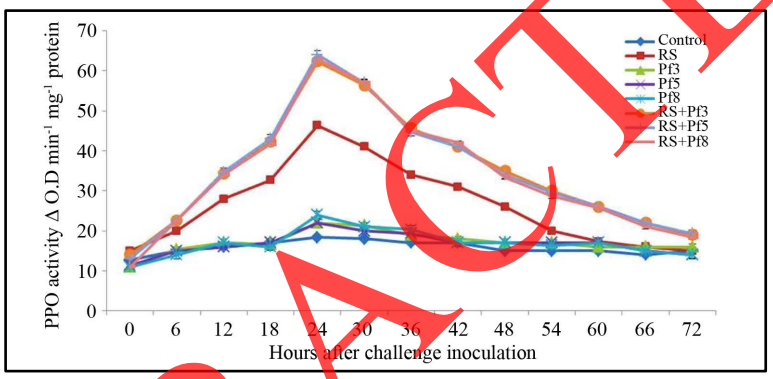

Figure 5. The effect of treatment of *P. fluorescens* isolates on the activity of Polyphenol oxidase (PPO) in tomato seedlings. Values are the mean of three replications and bars represent ± SE. C—Control, Pf—*P. fluorescens* and RS—*R. solanacearum*.

highest level at 24 h and declined thereafter. Application of *P. fluorescens* resulted in increase of *β*-1, 3-glucanase activity compared to the control (**Figure 6**).

Treatment of tomato seedlings with *P. fluorescens* resulted in a high phenol accumulation in plant extracts. Seedlings treatment of *P. fluorescens*, resulted in the maximum accumulation of phenol (440 $\mu g \cdot g^{-1}$ catechol) when compared to the control (120 $\mu g \cdot g^{-1}$ catechol). The phenol increased after challenge inoculation with *R. solanacearum* and reached the maximum level on 24 h (**Figure 7**).

3.4. Native PAGE Analysis of Peroxidase (POX) and Poly Phenol Oxidase (PPO)

The protein samples of seedlings of tomato were analyzed for expression of POX. A total of four samples (C—Control, T1—*R. solanacearum*, T2—*P. fluorescens* and T3—*P. fluorescens* + RS) of different treatments were expressed and the band intensities varied between control and inoculated seedlings. Native PAGE analysis revealed that five POX isoforms designated as POX1, POX2, POX3, POX4 and POX5. The expression of POX3 and POX4 were more prominent in T3 treatments. The intensities of the bands observed in T1 and T2 treatments were lower as compared to T3. Protein extracts from control exhibited only 2 isozymes when compared to other treatments (**Figure 8**).

The protein samples of treated and untreated tomato seedlings were analyzed for expression of PPO isoforms. A difference in number and intensity of isoforms was observed between T1, T2, T3 and control seedlings. Totally four isoforms of PPO, PPO1, PPO2, PPO3 and PPO4 were expressed in seedlings raised from *P. fluorescens* treated seedlings and challenge inoculated with *R. solanacearum* (T3), compared to control (untreated) seedlings.

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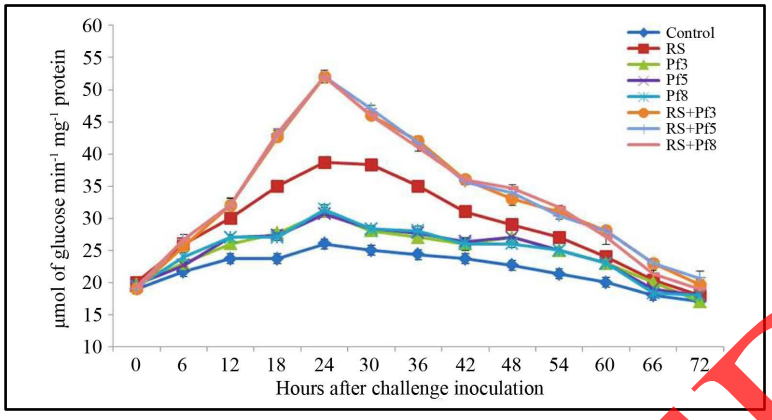

Figure 6. The effect of treatment of *P. fluorescens* isolates on the activity of *β*-1, 3 glucanase in tomato seedlings. Values are the mean of three replications and bars represent ± SE. C—Control, Pf—*P. fluorescens* and RS–*R. solanacearum*.

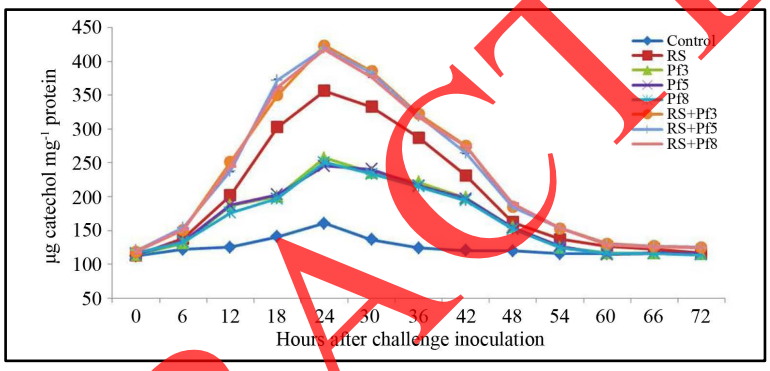

Figure 7. The effect of treatment of *P. fluorescens* isolates on the activity of total phenolics in tomato seedlings. Values are the mean of three replications and bars represent ± SE. C—Control, Pf—*P. fluorescens* and RS—*R. solanacearum*.

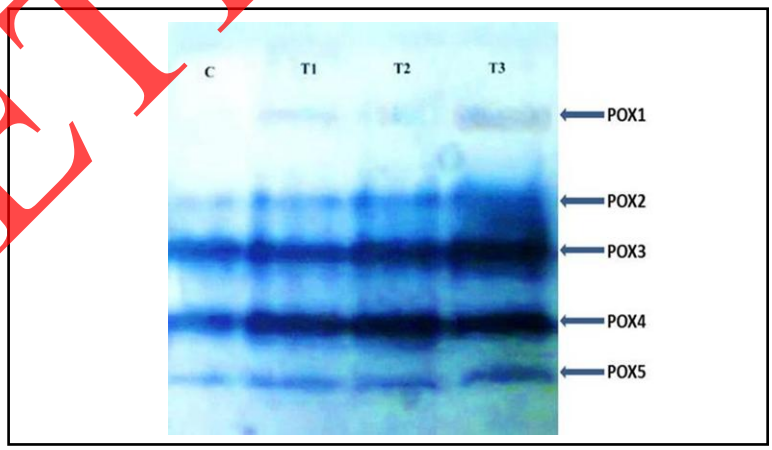

Figure 8. Native PAGE analyses for peroxidase (POX) isoforms induced by *Pseudomonas fluorescens* in tomato seedlings challenged with or without the pathogen *Ralstonia solanacearum*. C—Untreated seeds (Control), T1—Challenge inoculated with *R. solanacearum*, T2—Bacterized with *P. fluorescens* seedlings and T3—Bacterized *P. fluorescens* with seedlings and challenge inoculated with *R. solanacearum*.

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The isoforms PPO3 and PPO4 exhibited higher activity in T3 treatment when compared to control. The four PPO isoforms expressed lower intensity bands in the control lane than the other bacterized treatments (T1, T2 and T3) (**Figure 9**).

4. Discussion

The isolates used in this investigation were isolated from tomato rhizospheres as they are well adapted to utilize exudates from their original host plants. The success of plant growth promotion by the rhizobacteria mainly depends on their timely establishment and persistence throughout the growing season at sites where the pathogen may become active. Many of the fluorescent pseudomonads, mainly *P. fluorescens*, have been isolated from suppressive soil for the management of soil borne diseases. Systemic resistance was enhanced in response to *R. solanacearum* challenge in tomato due to high accumulation of defense enzymes [23].

The present work was an effort to analyze the defense related enzyme activity in bacterial wilt pathogenesis of tomato. Infection by pathogens is one of the major stress stimuli that plants often encounter. In response to the infection, the host induces a cascade of pathogen inducible enzymes, which are implemented in defense against phytopathogens. Early and elevated levels of expressions of various defense enzymes are an important feature of plant resistance to pathogens. Plants have their own enzymatic resources including PAL, POX and PPO during host pathogen interactions. Many studies have suggested that PAL, POX and PPO activities increased plant growth challenged with pathogens [40]. Induced systemic resistance by PGPR fortifies plant cell wall strength and alters host physiology and metabolic responses, leading to an improved production of plant defense chemicals upon pathogen challenge and/or abiotic stress factors [41]. The induced protection by selected strains of PGPR is often associated with the onset of defense mechanisms by expression of various defense related enzymes such as β-1-3-glucanase, Chitinase and accumulation of phenols [19] and has been shown to promote plant growth [13] [42]. Investigation of that watermelon plants pretreated with bio agents exhibited higher activity of PAL, POX, PPO, β-1-3-glucanase as well as accumulation of phenol upon challenge inoculation with the pathogen [43].

Peroxidases are used primarily for the synthesis of secondary metabolites and are known to be induced by various types of stresses including pathogen infection [44]. Peroxidases have been implicated in a number of physiological functions that may contribute to resistance phenol oxidation, lignification and in the deposition of phenolic material into plant cell walls during resistant interaction [45].

Both PAL and POX play important roles in biosynthesis of phenolics, phytoalexins and lignin, the three key factors responsible for disease resistance [46]. Phenylalanine ammonialyase catalyzes the conversion of phenylalanine to trans cinnamic acid, a key intermediate in the synthesis of salicylic acid. Enhanced PAL and POX activity was reported in tomato infected by *Fusarium oxysporum* [20]. Also our findings support that quick and

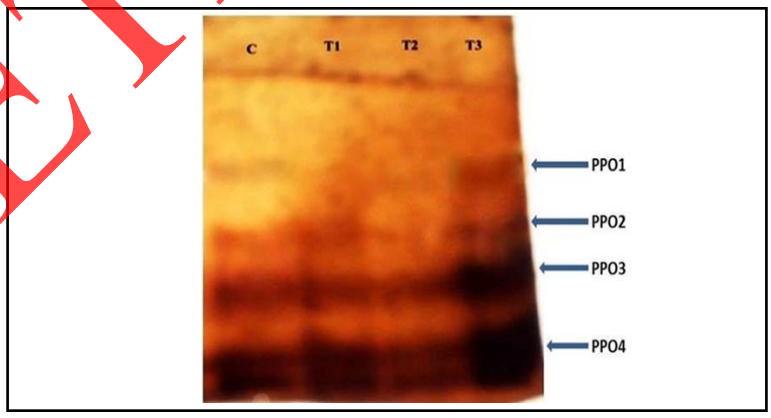

Figure 9. Native PAGE analyses for Poly phenol oxidase (PPO) isoforms induced by *Pseudomonas fluorescens* in tomato seedlings challenged with or without the pathogen *Ralstonia solanacearum*. C—Untreated seeds (Control), T1—Challenge inoculated with *R. solanacearum*, T2—Bacterized with *P. fluorescens* seedlings and T3—Bacterized *P. fluorescens* with seedlings and challenge inoculated with *R. solanacearum*.

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high induction of PAL and POX was observed in *P. fluorescens* pretreated tomato seedlings, which were inoculated with *R. solanacearum* [23].

In the present study, increased activity of PAL and POX was recorded in tomato seedlings grown from seeds treated with *P. fluorescens* (Pf3, Pf5, and Pf8) after challenge inoculation with the pathogen. In our study, a significant increase in PAL activity till the 24hpi was observed in the *P. fluorescens* treated seedlings challenge inoculated with *R. solanacearum* indicating the induction of resistance in host plants. The control seedlings with or without pathogen infection reported the lowest PAL activity without much variation. However, the seedlings inoculated with the *P. fluorescens* isolates alone also exhibited high PAL activity in comparison with the control. Peroxidase catalyzes the last step in the biosynthesis of lignin and other oxidative phenols.

Seed treatment with *P. fluorescens* induced the defense relate activities of POX. Our study reports a significant increase in POX activity at 18 hpi, observed in the *P. fluorescens* treated seedlings challenge inoculated with *R. solanacearum* indicating the induction of resistance in tomato plants. The control seedlings reported the lowest POX activity with or without pathogen infection with no variation. However, the seedlings inoculated with the *P. fluorescens* isolates alone also exhibited high POX activity in comparison with the control.

Polyphenol oxidase, a copper containing enzyme, oxidizes phenolics to highly toxic quinines and is involved in the terminal oxidation of diseased plant tissue and is attributed for its role in disease resistance. Various rhizobacteria and *P. aphanidermatum* induced the PPO activity in cucumber root tissues [47]. Accumulation of PPO was increased in *P. fluorescens* treated tomato [48].

This report also finds the high accumulation of phenols in plant extracts treated with *P. fluorescens* isolates when compared to the control, the maximum level being attained at 24 hpi. The activity of PPO was highest in seedlings pretreated with *P. fluorescens* and later challenge inoculated with *R. solanacearum*. *Pseudomonas fluorescens* induced resistance against *R. solanacearum* in tomato seedlings is associated with the enhanced expression of genes for defense related enzymes. Pretreatment of tomato plants with *P. fluorescens* triggered the increased PAL, POX, PPO and GLU activities in response to attack by *R. solanacearum*.

5. Conclusion

The present study proves the induction of systemic resistance by *P. fluorescens* against *R. solanacearum* in tomato seedlings for defense related enzymes. The application of biocontrol agents as seed treatments could prove to be a beneficial component of integrated pest management. These *P. fluorescens* isolates, apart from their action against bacterial wilt pathogen, are good growth promoters, and are able to induce systemic resistance in tomato plants, which is an added advantage for practical agricultural system. It is evident that rhizobacteria could possibly serve as ecofriendly and sustainable alternatives to the hazardous chemicals used for growth promotion and management of plant diseases.

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