The Pharma Innovation Journal 2022; 11(5): 1042-1051

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(5): 1042-1051

www.thepharmajournal.com
Received: 23-03-2022
Accepted: 31-04-2022

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Rhizosphere population dynamics and biocontrol potential of *Pseudomonas fluorescens* Pf1 against Wilt and collar rot pathogens in tomato

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Abstract
Pseudomonas fluorescens is a plant growth promoting rhizobacteria is proved to one of the most important and effective biocontrol agents against many plant diseases and a potential replacement for the fungicides in disease management. Ratherly used this antagonist by various ways through seed treatment, root dipping or soil application directly is not capable to uphold elevated population in soil/rhizosphere due to sharp competition from inhabitant microflora and therefore this application of *P. fluorescens* Pf1 talc formulation to the soil with and without farm yard manure was evaluated. Increase in population of 4.0 x 10 9 cfu g -1 of soil up to 20 days was observed when Pf1 applied along with FYM, while application without FYM resulted in decreased population from 10 days onwards. *P. fluorescens* Pf1 potentially inhibited the growth of the fungal mycelium in tomato soil borne pathogens like *Fusarium oxysporum* f.sp. *lycopersici* and *Sclerotium rolfsii*. Seed treatment and soil application of P. fluorescens Pf1 increased the germination and enhanced the seedling vigour also. Apart from PGPR activity Pf1 also induced accumulation of defense related enzymes *viz*., Phenylammonia lyase (PAL), Peroxidase (PO), Polyphenol oxidase (PPO) and the prolonged enzyme accumulation is well correlated with application of Pf1 with farm yard manure in tomato plants.

Keywords: Defense enzymes, Growth promotion, *Pseudomonas fluorescens*, Soil borne pathogens, Tomato

Introduction
In World level, the most important vegetable crop is Tomato (*Solanum lycopersicum* L.) and it's cultivated manly for its fleshy fruits. Day by day, the tomato cultivated area increasing due to it's highly demand, preference, more nutritious and more yield. It occupies the top most priority in their nutrient contribution to human diet supplemented with a more supply of essential amino acids, dietary fibers, minerals, organic acids and vitamins. In Tamil Nadu, the total production of 2,82,912 tonnes with grown in an area of 22,433 ha, with an average productivity of 12,611 kg/ha. In vegetables are affected by various diseases, among them *Fusarium* wilt is one of the most dangerous one and it's caused by *Fusarium oxysporum* f. sp. *lycopersici* Sacc. causes significant loss in fruit yield. The yield loss from 40 to 50 per cent was estimated due to *Fusarium* wilt disease (Kirankumar *et al*, 2008) [22]. It is the most damaging disease in the Southern and Northern states of India during excessively hot and dry seasons. In management of the tomato diseases, most of the farmers they widely used in chemicals. But continuous use chemicals cause more environmental pollution, health hazards and development of fungicidal resistance by the concerned pathogen leads to not effective by this chemical. Many authors are reported (Saravanakumar and Samiyappan, 2007) [32] by managing the pathogen in crop plants by biological approaches; one of the promising bio agents is *Pseudomonas fluorescen*s it acts as a growth promoting rhizobacteria (PGPR).
However, the delivery system for the biocontrol agents is an obstacle since; introduction of any antagonist directly in to the soil faces sensitive competition from occupant soil microorganisms, which has utilized by available substrates from the soil and firmly established and colonized in the soil as well as plant root surface. Normally antagonist populations not able to maintain highly in rhizosphere and soil, when the antagonist directly applied as seed treatment and soil application. Plants are utilized by own defense mechanism is present approach to move for managing the plant diseases are being experienced in all over the world. Plants have own induced systemic resistance which act as a latent defense mechanism against

all the pests, which was systemically induced by when the plants exposure of stress or infected by the plant pathogens. Several strains of *Pseudomonas* have been exposed to may active in plants by ISR against various plant diseases caused by fungi, bacteria and virus (Chen *et al*, 2000) [4].

How PGPR can stimulate plant growth has been assumed to give details about various mechanisms. Generally, these mechanisms are classified as direct or indirect by nature (Glick, 1995) [9]. Direct mechanisms draw out growth endorsement by production of various hormones likes giberellic acid, cytokinin, indole acetic acid and solubilization through phosphate (Idriss *et al*, 2002) [18]. They plant growth enhanced by repressing plant pathogens and harmful to microorganisms in the surrounding rhizosphere regions and other deleterious microorganisms. Induced systemic resistance (ISR) and suppression of harmful microorganisms are usually documented as potential a role in biological as a indirect mechanisms (Kloepper *et al*, 1992) [23]. Talc or powder-based carrier of plant growth promoting rhizobacteria maintain four months, it contains the more shelf life compare to others. The formulation is widely recommended as seed treatment, foliar and soil applications. Apart from this, enrichment of farm yard manure with *P. fluorescens* is also been recommended widely for soil application, since the multiplication and stimulation of activity of bacteria are high. Any biocontrol agents treated as seed get better germination of seed, seedling vigour, uniform crop growth and protects from soil and seed borne pathogens. With this supportive back ground information, the subsequent experiment were conducted to get the establishment of bacterial population in plant rhizosphere over the time period, impact on crop growth and antagonistic activity against major pathogens of tomato.

Materials and Methods

PKM 1 tomato seeds and bio agents *P. fluorescens*-Pf1strain obtained Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India be used in all the experiments.

Isolation and identification of the pathogen

The typical symptoms of wilt disease showing in tomato plants were collected from farmers' field. The pathogen was isolated from brown discoloured areas of split open infected root portion bits. The infected root bits were surface sterilized with sodium hypochlorite0.1 per cent for 30 second and subsequently rinsed in three changes of distilled water as sterile. Then, they were placed in Potato Dextrose Agar (PDA) medium containing sterilized petriplates and incubated for seven days at room temperature (25±2°C). Single hyphal tip method (Rangaswami, 2005) [31] was used to get the pure culture of *Fusarium oxysporum* f.sp. *lycopersici*. Similarly, collar rot infected plant samples were also collected, isolated, identified as *Sclerotium rolfsii* and pure culture developed hyphal tip method were maintained for further studies.

Enumeration of *P. fluorescens* Pf1 population dynamics in rhizosphere soil

Red soil: sand: cow dung manure @ 1:1:1 w/w/w consisted potting mixture was sterilized at 121 ˚C at 15 psi for 2 h in autoclave for two successive days. Talc formulation of *P. fluorescens* Pf1 with 2.5 X 10^8 per ml population load was mixed with the pot mixture @ 4g/250 g of soil. The sterilized pot mixture was filled with 12 cm diameter containing pots. The PKM 1 tomato seeds were treated with talc formulation of *P. fluorescens* Pf1 @ 10g/kg of seed were sown in the pots and watered periodically. Seedlings were pulled out at 5, 10, 15, 20, 25 and 30 days after sowing and the rhizosphere soil was collected and documented the viable bacterial cells as a number by using serial dilution method.

King's B medium was prepared and 15 ml of the sterilized medium was poured in sterile Petri plates. These plates were reserved for 48 hrs at room temperature. Petri dish outside bottom was equally marked in eight equal divisions. One replication of each dilution was used as four divisions, two dilutions were allowed for one plate. To get 10^{-1} dilution, one ml of serially diluted bacterial inoculums was prepared and transferred into sterile water in the quantity of 9 ml blank solution. In the same way, the dilutions were prepared and obtained upto 10^{-9} dilutions serially. Pipette out the 10 µl of this dilution and poured in to the respective divisions (quadrat) in the Petri plate. These plates were incubated at room temperature 28±1 °C for development of individual colonies without any disturbance and the drop plate method was used to count the individual colonies (Somasegaran and Hoben, 1994) [34]. Similar set of experiments were also carried out simultaneously without farm yard manure to assess the impact of FYM on population build up and plant growth.

In vitro screening of P. fluorescensPf1 against F. o. f. sp. lycopersici and S. rolfsii pathogens

Dual plate technique

F. o. f.sp. *lycopersici* and *S. rolfsii* culture discs in five days old (8mm) werepositioned at one end of 15 ml of solidified PDA medium containing sterilized Petri plate. The bacterial strain was streaked using flame sterilized inoculation needle 3 days after pathogen inoculation. In each treatment, maintained as three replications and incubated the inoculated plates were at room temperature (28±2°C). The measurement of mycelial inhibition zone (in mm) and mycelial growth of the pathogen were measured. The mycelial growth of the pathogen was measured as radial and the following formula was used by calculating the percent inhibition over control of the pathogen.

$$\text{Per cent inhibition over control} = \frac{C - T}{C} \times 100$$

Where, C - Mycelial growth of pathogen in control plate
T - Mycelial growth of pathogen in dual plate

Paper Disc method

Tomato diseases causing fungal pathogens like *F. oxysoporum* f. sp. *lycopersici* and
S. rolfsii mycelial discs of nine mm were positioned in the middle of the petridishes. Six mm filter paper discs (Sterile whatman No.40) were also placed in edges of the petriplates containing four sides. Prepared *P. fluorescens* Pf1 culture suspension were plunged in the filter paper discs at the quantity of twenty five micro litres. Occurrence of inhibition zone was observed in five days after incubation over the pathogen and nearer to bacterial zone. Instead of bacterial inoculum, sterile distilled water was maintained as control.

Plant growth promotion

A plant growth-promoting activity was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993) [19] of *P. fluorescens* Pf1 talc formulation. PKM

1 tomato seeds (Twenty five numbers) were treated with *P. fluorescens* talc formulation were placed over the pre- soaked germination paper. The one more germination paper strip by presoaked in nature by pressed gently with the placed by position of seeds. Seeds containing germination paper along with polythene sheet were rolled than incubated at growth chamber for 15 days. In each treatment contained three replications were maintained. The germination percentage of seeds was calculated and the individual seedlings shoot length and root length was also measured and this was compared with talc formulation of *P. fluorescens* treated seeds.
Abdul Baki and Anderson (1973) [1] described the formula of vigour index and calculated based on root, shoot length.

Vigour Index = (Mean root length + Mean shoot length) x Germination (%)

Talc based formulation for *P. fluorescens* Pf1 preparation
King's B broth was prepared and inoculated Pf1 at one loopful quantity was inoculated and placed at room temperature (28±1°C) for 72 hrs at 150 rpm in a rotary shaker. The preparation of Pf1talc-based formulation, incubation at 72 hrs after inoculation, the broth containing $9x10^8$ cfu/ml of formulation was used. As per the protocol was explained by Vidhyasekaran and Muthamilan (1995) [38], the following materials (bacterial suspension 400 ml, sterilized (105°C for 12 h) and purified talc powder 1 kg, neutralized pH (adjusted) calcium carbonate 15 g and 10 g carboxy methyl cellulose (CMC)) were mixed under sterile conditions. The mixed product, to get the moisture content of below 20 per cent for the mixed product was shade dried and packed by using polypropylene bag then sealed. The prepared talc based formulation of bacterial population count was adjusted by 2.5 to $3x10^8$ cfu/g of formulation, while for the application in time.

Assess the defense-related enzymes and compounds
Collection of samples
To study the assessment for the induction of defense related enzymes collected the individual treatment samples. The root samples were collected from initial days after sowing to 30th day of Pf 1 treated tomato plants.

Extraction of enzymes
Pf1 treated and control tomato plants root tissue samples were collected and extracted immediately with 0.1 M sodium citrate buffer (pH 5.0) with 2 ml at 4 °C. This extracted homogenate was centrifuged at 10,000 rpm for 20 minutes. The estimation of defense enzymes, prepared protein extracts from tomato root tissues was used. The extraction of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase enzymes by used 0.1 M Sodium phosphate buffer (pH 7.0).

Assay of phenylalanine ammonia-lyase (PAL)
Tomato root sample was weighed at one gram and homogenized with ice cold 3 ml of pH 7.0 Sodium borate buffer (0.1 M) and a mixture containing 2-mercaptoethanol at 1.4 mM and insoluble PVP at 50 mg. This homogenized extract was filtered by using cheese cloth and this filtrate was centrifuged at 4°C for 15 min at 20000g and the enzyme source by used as a cleared supernatant solution. The determination of this enzyme (PAL) activity was determined by the conversion rate of L-phenylalanine to trans-cinnamic acid at 290 nm. The reaction mixture sample containing the enzyme extract of 0.4 ml was incubated with pH 8.8 containing borate buffer (0.1M) at 0.5 ml and L-phenylalanine (12 mM) at 0.5 ml incubated for 30 min at 30°C. Synthesized amount of trans-cinnamic acid was calculated by using the extinction coefficient of 9630 $M^{-1}cm^{-1}$ (Dickerson *et al*, 1984) [7]. Enzyme activity of PAL was expressed in nmol trans-cinnamic acid min^{-1} mg^{-1} of plant tissue fresh weight basis.

Assay of Peroxidase (PO)
The peroxidase (PO) activity assay was conceded by the protocol developed by Hammerschmidt *et al*. (1982) [16]. Guaiacol @ 0.25 per cent (v/v) in pH 6.0 containing sodium phosphate buffer (0.01 M) and hydrogen peroxide (0.1 M) contained in a 2.5 ml of reaction mixture was prepared. To initiate the reaction, 0.1 ml of enzyme extract was added and immediately read by 470 mm for colorimetrically. The changes in absorbance also read at 470 nm of 0.1 to 0.2 absorbance units/min with the dilution of prepared crude enzyme. The Activity of PO was indicated as the boost in absorbance at 470 nm min^{-1} mg^{-1} of plant root tissue.

Assay of Polyphenoloxidase (PPO)
One gram of collected root sample was homogenized at 4°C with pH 6.5 containing sodium phosphate buffer (0.1 M) in 2ml. This homogenized solution was centrifuged at 4 °C for 15 min at 20,000g. The cleared supernatant solution served as enzyme source for the assay of PPO. The activity of polyphenoloxidase was determined as per the protocol developed by Mayer *et al.* (1965) [27]. The enzyme reaction mixture consisted of pH 6.5 containing sodium phosphate buffer (0.1 M) for 1.5 ml and the enzyme extract of 200 µl. To initiate the enzyme reaction 200 µl of catechol (0.01 M) was added and the activity was indicated as change in absorbance $min^{-1}mg^{-1}$ of plant root tissue.

Statistical analysis
The available data were statistically analyzed using the WASP version 2.0 developed by the ICAR- Central Coastal Agricultural Research Institute, Goa developed by Ashok Kumar Jangam and Pranjili Ninad Wadekar (Source :Gomez and Gomez, 1984) [11].

Results and Discussion
Tomato is one of the most important commercial vegetable crops and consumed diverse use for entire world. Over the past few decades agricultural production is likely more dependent on agrochemicals due to dependable method for plant protection with financial constancy of their operations. However, over use of agro chemicals like fungicides reason more than a few harmful effects *viz*., leads to health hazards, environmental pollution, toxicity and development of resistance against pathogens to the frequent use chemicals. It also reduces the beneficial microorganisms. Thus, it becomes necessary for finding alternative resources to decrease the agro chemicals (fungicides). In present days worldwide, they used biocontrol agents for the control of plant pathogens by direct and indirect ways and have a straight crash on financial help to farmers (Kirankumar *et al*, 2008) [22].
A population dynamics of *Pseudomonas fluorescens* Pf1 when applied in to soil along with FYM was analysed and the antagonistic activity was also tested for *in vitro* circumstances

against *Fusarium oxysporum* f.sp. *lycopersici* and *Sclerotium rolfsii*. Impact of seed germination, seedling emergence and their mechanism of inducing systemic resistance were also studied by the *P. fluorescens* Pf1 was applied as the soil and the results are presented here under. There are several microorganisms available for the control of soil borne diseases in different ways. Among them Plant Growth Promoting Rhizobacteria (PGPR), *P. fluorescens* take part in a most important function in promoting plant health with excellent antifungal activity through production of antimicrobial compounds etc. PGPR strains are an emerging area in plant protection to reducing the plants damaged by the pathogens or escape the plants; it's the recent development of biological techniques. Most of the PGPR strains were isolated from the various crops rhizosphere region, irrigation water, plants and soils in various countries by more than a few workers and experienced against number of diseases (Gupta *et al*, 2002) [13]. Many fluorescent pseudomonad strains can provoke pathogens either directly or in directly. In direct mechanism, through competition for nutrients in pathogens, antimicrobial compounds production or lytic enzymes secretion and stimulation plant host defenses by indirect mechanism. One of the most important promising defense tools for plant protection is induced systemic resistance (ISR) and its activated by PGPR strains when introduced in plants against pathogens. Chen *et al*, (2000) [4] reported that, this PGPR strains natural, eco-friendly safe and offered resistance against wide range of plant pathogens.

Isolation of the pathogen

The pathogen (*F. o*. f.sp. *lycopersici*) was isolated from the infected tomato plant root bits by half plate technique using potato dextrose agar (PDA) medium. After seven days the pathogen produced a dense, creamish white with pink coloured fluffy mycelial growth (*F. o*. f.sp. *lycopersici*) and pure white radiating mycelium producing brown sclerotia (*S. rolfsii*) on Petridish. The pure culture was maintained at 4^{o} C for refrigerator in PDA slants for further studies.

Mass multiplication of *P. fluorescens* Pf1

Talc based formulation of *P. fluorescens* Pf1 was prepared and packed when the moisture content reached up to 12 per cent. Enumeration of population density revealed that the product contained 2.0 x 10^{10}cfu/g of talc which is more than the quality standard recommended for commercial products. The product was free from common contaminants.

Enumeration of *P. fluorescens* Pf1 population density in the rhizosphere soils of tomato

Population density of the bacterial biocontrol agent was enumerated by using serial dilution technique. Initial population was almost same in both the treatments, while changes observed over the days after sowing. Population density was increasing with increase in DAS and the maximum of 4.0 x 10^{9} was observed on 20 DAS in soil applied with both FYM and Pf1 talc formulation. Thereafter, the density was decreased. Whereas, in the treatment applied with Pf1 alone, initial increase in population was noticed and started declining from 10th DAS and least population of 1.2 x 10^{6} was observed on 30th DAS in plant rhizosphere of Pf1 alone applied pots (Table 1; Fig. 1)

In plant rhizosphere root regions surrounded soils had influenced by improved more microbial activity than other regions. Normally the universal microbial community was more in healthy tomato plant rhizosphere soil than wilt affected soils. In the present research, healthy rhizosphere tomato soil contains more population of fungi, bacteria and actinomycetes than wilt affected rhizosphere soil. Similar type of result was observed by Kushaldas (2009) [25] who investigated microbial populations present in the healthy and *Fusarium* wilt infected soils and showed that diversity of microbial population maximum in healthy soils and *Fusarium* population maximum in infested soil. Zaidi and Singh (2004) [41] observed that the soil application of FYM amended *P. fluorescens* and *Trichoderma viride* to improve the plant growth and enriched the soils in Uttaranchal and Utter Pradesh states of India. Hence, the antagonist fairly frequently applied as seed as seed treatment, root as root dipping or soil application is not capable to uphold the more population in soil/rhizosphere.

In the present study, survival and adherence of *P. fluoresecens* (Pf1) talc formulations was tested at seed treatment and soil application in tomato. To standardize the dosage of talc formulations of Pf1, experiments were carried out with different inoculum levels *viz*.,5 g, 10 g and 15 g/kg of seed. It was found that maximum survival of bacterial at15 g of inoculum per kg of seeds applied pots and it was on a par with 10 g inoculum level /kg of seeds. For further study, the 10 g of inculum /kg of seeds taken. Kundu and Gaur, (1981) [24] observed that, adherence of more number of cells on the seed surface was observed inoculation of liquid formulation with rice gruel on seed, its due to sticky nature and more nutrient contents. In more adherence of *Bacillus* cells on seed surface was observed in seeds treated with sporulated *Bacillus* inoculums along with rice gruel treated seeds (Gomathy, 2003) [10]. Based on the above study, it was found that Pf1 Talc based formulation was better than liquid based formulation.

Soil application of Pf1 talc formulation was applied at initial population of 10^{8}cfu/g of soil which gradually increased to 10^{9}cfu/g of soil when applied with farm yard manure. In the present study, the population increased in up to 20 days after sowing and the population level slowly declined thereafter. These findings agreement with Shanthiyaa *et al.* (2013) [33], reported that the *C. globosum* applied as soil, it was showed that maximum population count 9.0 x 10^{4} (cfu/10g of soil) and *P. fluorescens* applied as soil showed the maximum population count 6.0 x 10^{6} (cfu/g of soil) in potato fields.

Screening of *P. fluorescens* Pf1 against *F. oxysporum* f. sp. *lycopersici* and *S. rolfsii* under *in vitro*

In vitro screening of *P. fluorescens* tested against *F. oxysporum* f. sp. *lycopersici* and *S. rolfsii* revealed that the biocontrol agent was highly effective against both the pathogens and recorded radial mycelial growth ranged from 41.6 to 42.0 mm and 43.20 to 44.40 mm in both

F. oxysporum f. sp. *lycopersici* and *S. rolfsii*, respectively. There was no difference in inhibitory activity among the methods used for evaluation (Table 2; Fig 2 and 3). Several workers (Van Loon and Van Strien, 1999; Haas and Defago, 2005) [36, 14] reported by the induction of resistance by fluorescent pseudomonads secreted secondary metabolites. The most important way of fluorescent pseudomonads as a biological control of plant pathogens by secretion of β-1,3-glucanase and chitinase as a lytic enzmes(Viswanathan and Samiyappan, 2001; Vivekananthan *et al*, 2004) [39, 40].

Manikandan *et al*. (2010) [26] reported that different age cultures of Pf1 liquid formulation of considerably abridged the mycelia growth of tomato wilt causing *Fusarium* pathogen under *in vitro*.

Evaluation of *P. fluorescens* Pf1 influence on plant growth promotion

PKM 1 tomato seeds were treated with *P. fluorescens* Pf as talc formulation and tested for plant growth promotion activity as a role. The observations are indicated in Table 3 and Fig 4. Seed treatment resulted in 100 per cent germination and maximum root length and shoot length of 8.46 mm and 13.80 mm, respectively with a high vigour index, while the control treatment recorded only 6.9 mm and 9.87 mm of shoot and root length, respectively.

Most of the rhizobacteria as a played a vital role for stimulate the plant growth under *in vitro* condition. Plant growth promoting activity of tomato and hot pepper plants were observed under the seeds were treated by fluorescent pseudomonads (Ramamoorthy *et al*., 2002) [30]. Kavimandan and Gaur, (1971) [21] reported that inducing of plant growth promoting substances like gibberellins and mineralizing phosphates by Pseudomonas spp. and not clearly understood by other mechanisms. *Pseudomonas* spp. has been very well known for its IAA producing ability, which is effectual for the help of plant growth activity (Dileep Kumar and Dube, 1992) [8]. In blueberry, soil application of *P. fluorescens* Pf5 treated plants showed increased leaf area, diameter of stem and more bush. This was reported by De Silva *et al.* (2000) [6]. Vivekananthan *et al.* (2004) [40] reported that chitin amended *P. fluorescens* strain FP7 in mango trees showed to enhance the panicle formation and induced more flowers. Similarly, the leaves have more nutrient content *viz*., N, P, K, Ca and Mg in PGPR treated mango trees than untreated trees. PGPR formulation significantly increased the plant growth promoting activity and vigour index in rice seedlings by Karthiba *et al.* (2009) [20] and Liquid formulation of Pf1 enhanced the same activity in tomato seedlings by roll towel method (Manikandan *et al*, 2010) [26].

Inductions of defense enzymes by biocontrol agents against Fusarium wilt in tomato under pot culture conditions

The induction of defense related enzyme activities *viz.*, Phenylalanine ammonia lyase (PAL), peroxidase and polyphenol oxidase were analyzed through biochemical analysis, the bio formulation applied tomato plants indicated as a induced systemic resistance mechanisms are presented here under.

Van Loon *et al*. (1998) [37] established a new defense mechanism by the plants protect themselves from any pests including insect and pathogen attack by the production of various bacterial metabolites and induction of systemic resistance by biocontrol agents against pests have been established as a direct antagonistic activity. Phenyl propanoid pathway and PR protein synthesis is the first step for induced resistance against diseases in several crops are connected with development of lignifications and also enhanced the activities of defense related enzymes (Hammerschmidt and Kuc, 1995) [15]. In our study, defense related enzyme molecules *viz*., phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenoloxidase (PPO) were establish to have been induced the plants applied with *P. fluorescens* Pf1and FYM as well as in Pf1 alone inoculated plants. The present result is in similar with earlier workers results and *P. fluorescens*strain WCS417r was exposed to activate an ISR response in a number of plant species like carnation, radish, tomato and *Arabidopsis* (Pieterse *et al*., 2001) [28].

Peroxidase (PO) activity

Peroxidase activity was measured from biocontrol agents and FYM tomato plants imposed roots, with the biocontrol agents treated alone and, healthy control. Generally the activity of peroxidase significantly increased due to application of *P. fluorescens* Pf1 when applied with or without FYM compared to healthy control. The maximum activity was noticed on 25thDAS in the treatment applied with FYM (1.58 Changes in absorbance min^{-1} g^{-1} of fresh tissue) and declined thereafter compared to Pf1 alone inoculated plants in which the maximum activity was noticed on 10th DAS (1.28 Changes in absorbance min^{-1} g^{-1} of fresh tissue) and started declining thereafter, while there is no change in peroxidase activity of control plants (Table 4).

Peroxidase is the most important enzyme for the lignin biosynthesis and fluorescent pseudomonads elicited the augmented activity of peroxidase enzyme in various crops such as rice, blackgram, groundnut, sugarcane, chillies and cucumber have been reported by various workers (Viswanathan and Samiyappan, 2001; Bharathi *et al*, 2004; Chen *et al*, 2000) [39,3,4]. Peroxidase enzyme has been concerned in a numeral of physiological functions that might contribute to various resistance actions with exudation of hydroxy cinnamyl alcohol into free radical intermediates, oxidation of phenol, cross linking of polysaccharide, cross linking of extensin monomers, lignification and are also connected with phenolic compounds deposited into the plant cell walls during incompatible interactions (Graham and Graham, 1991) [12]. This work supported by Vivekananthan *et al*. (2004) [40], the maximum induction of peroxidase activity was observed in chitin amended fluorescent pseudomonads strain FP7 bioformulation treated in mango trees against anthracnose causing pathogen. Combined application of EPB22 + Pf-1 strains treated banana and rice plants as applied individually and these strains activate high amount of peroxidase against virus and sheath blight pathogen (Harish, 2005, Radjacommare *et al.*, 2002) [17,29]. Additionally, Manikandan *et al*. (2010) [26] observed that combined application of root dipping + soil drench + foliar spray of both Pf1 liquid and talc formulations in tomato plants challenge inoculated with *A. solani* have induced six peroxidase isoenzymes.

Polyphenol oxidase (PPO) activity

The Polyphenol oxidase activity also increased in both the treatments significantly. The PPO activity was observed maximum in 20th DAS in Pf1 with FYM (2.98 changes in absorbance min^{-1} g^{-1}of fresh tissue) treated plants. Afterwards, this enzyme activity was declined. Similarly, the treatment with Pf1 alone recorded maximum activity changes in absorbance min^{-1} g^{-1}of fresh tissue at 1.65 on 10th DAS and declined further days after sowing. PPO activity in the healthy control plants remained unchanged (Table 5). Polyphenol oxidases (PPO) enzymes to catalyze the oxidation of mono phenolic and ortho diphenolic compounds by using molecular oxygen. PGPR strains were inoculated cucumber root tissues against *Pythium aphanidermatum* and it has induced high

PPO activity (Chen *et al*., 2000) [4]. Similar kind of results were observed in banana plants against virus and wilt pathogens, higher activity of PPO enzyme were observed in the plants treated with PGPR stains (Harish, 2005 and Akila *et al*, 2011) [17,2]. An enhanced initiation of PPO activity in *P. fluorescens* treated green gram was observed against the root rot pathogen *M. phaseolina* (Thilgavathi*et al*, 2007) [35]. Karthiba *et al*. (2009) [20] reported that maximum activity PPO was observed in rice plants treated PGPR consortium against sheath blight pathogen.

Phenylalanine ammonia lyase (PAL) activity

The increased Phenylalanine ammonia lyase activity was observed in both the treatments. The highest PAL activity was observed on 20th DAS in Pf1 with FYM (20.15 changes in absorbance min^{-1} $g^{-1}$of fresh tissue) treated plants. The activity of the enzyme was declined thereafter. Similarly, the treatment with Pf1 alone recorded maximum activity of 8.01 changes in absorbance min^{-1} $g^{-1}$of fresh tissue on 10th DAS and declined further days after sowing. PAL activity in the healthy control plants remained unchanged (Table 6).

During the plant pathogen interaction, a key and first important enzyme (Phenylalanine ammonia lyase (PAL)) produced during phenyl propanoid pathway and this enzyme role in bio synthesis of variety of defense chemicals (Bharathi *et al*, 2004) [3]. De Meyer *et al*. (1999) [5] reported that the colonization of *P. aeruginosa* 7 NSK 2 in bean rhizosphere region that activate PAL and accumulation of salicylic acid in higher levels in bean leaf tissues. Fluorescent pseudomonads treated plants showed maximum induction of PAL enzyme in various crops *viz*., tomato plants against wilt causing *Fusarium oxysporum* f.sp. *lycopersici* (Ramamoorthy *et al*, 2002) [30], cucumber against damping off causing *P. aphanidermatum* (Chen *et al*, 2000) [4], bean against blight causing *Botrytis cinerea* and mango against anthracnose causing *C. gloeosporioides* (Vivekananthan *et al*, 2004) [40]. Talc based formulation Pf1 was treated with green gram plants, which enhance activity of PAL enzyme against various pathogens (Thilagavathi *et al*., 2007) [35].

Table 1: Population of *P. fluorescens* Pf1 in rhizosphere soil

DAS*	*P. fluorescens* Pf1 (cfu#/g of soil)	
	With FYM	Without FYM
0	3.5×10^8	3.7×10^8
5	8.0×10^8	6.0×10^8
10	8.3×10^8	2.6×10^7
15	2.1×10^9	1.0×10^7
20	4.0×10^9	6.3×10^6
25	3.8×10^9	4.1×10^6
30	2.1×10^8	1.2×10^6

*Days after sowing

#cfu – Colony Forming Unit; Values are mean of three replications

Table 2: Effect of *P. fluorescens* Pf1 against *F. o.* f. sp. *lycopersic*i and *S. rolfsii* under *in vitro* condition

Treatments	Dual plate technique		Paper disc method	
	Radial mycelial growth* (mm)	Percent inhibition over control	Radial mycelial growth* (mm)	Percent inhibition over control
F. oxysporum f. sp. *lycopersici*	41.60	53.78	42.00	53.33
S. rolfsii	44.40	50.67	43.20	52.00
Control	90.00	-	90.00	-
CD (0.05)	8.59		8.52	

*Values are mean of seven replications

Table 3: Influence of *P. fluorescens* Pf1 on tomato seed germination and seedling growth on 15th DAS

Treatments	Germination* (%)	Shoot length* (cm)	Root length* (cm)	Vigour index
ST with Pf1 talc formulation	100	8.46	13.80	2226.0
Control	96	6.90	9.87	1610.0
CD (0.05)	2.82	0.22	0.25	39.71

*Values are mean of seven replications; ST Seed Treatment

Table 4: Induction of peroxidase (PO) activity in tomato plants treated with Pf 1

Treatments	Changes in absorbance min^{-1} g^{-1} of fresh tissue						
	0 DAS	5 DAS	10 DAS	15 DAS	20 DAS	25 DAS	30 DAS
ST+ SA of Pf 1 talc formulation along with FYM	1.26	1.29	1.36	1.40	1.49	1.58	1.39
ST+ SA of Pf 1 talc formulation alone	1.01	1.19	1.28	1.17	0.99	0.92	0.91
Control without FYM and Pf 1	0.50	0.51	0.53	0.53	0.54	0.54	0.52
CD (0.05)	0.03	0.03	0.05	0.03	0.03	0.04	0.03

Values are mean of seven replications; ST- Seed treatment (4g/kg); SA- Soil application (4g/250g of Soil); DAS- Days after sowing

Table 5: Influence of seed and soil application of Pf1 on induction of polyphenoloxidase (PPO) activity in tomato plants

Treatments	Changes inabsorbance min^{-1} g^{-1} of fresh tissue						
	0 DAS	5 DAS	10 DAS	15 DAS	20 DAS	25 DAS	30 DAS
ST+ SA of Pf 1 talc formulation along with FYM	1.26	1.39	1.61	1.93	2.98	2.31	2.10
ST+ SA of Pf 1 talc formulation alone	1.10	1.28	1.65	1.52	1.40	1.33	1.00
Control without FYM and Pf 1	0.89	0.90	0.90	0.91	0.91	0.91	0.89
CD (0.05)	0.12	0.13	0.19	0.19	0.19	0.21	0.17

Values are mean of seven replications; ST- Seed treatment (4g/kg); SA- Soil application (4g/250g of Soil); DAS- Days after sowing

Table 6: Induction of phenylalanine ammonia lyase (PAL) activity in tomato plants

Treatments	Changes inabsorbance min^{-1} g^{-1} of fresh tissue						
	0 DAS	5 DAS	10 DAS	15 DAS	20 DAS	25 DAS	30 DAS
ST + SA of Pf1 talc formulation along with FYM	8.43	9.00	12.00	14.60	20.15	18.75	18.71
ST + SA of Pf1 talc formulation alone	7.17	7.23	8.01	7.77	7.61	7.40	7.01
Control (without FYM and Pf1)	6.41	6.42	6.42	6.42	6.43	6.43	6.43
CD (0.05)	0.79	0.94	1.10	1.01	1.59	1.47	1.14

Values are mean of seven replications; ST- Seed treatment (4g/kg); SA- Soil application (4g/250g of Soil); DAS- Days after sowing

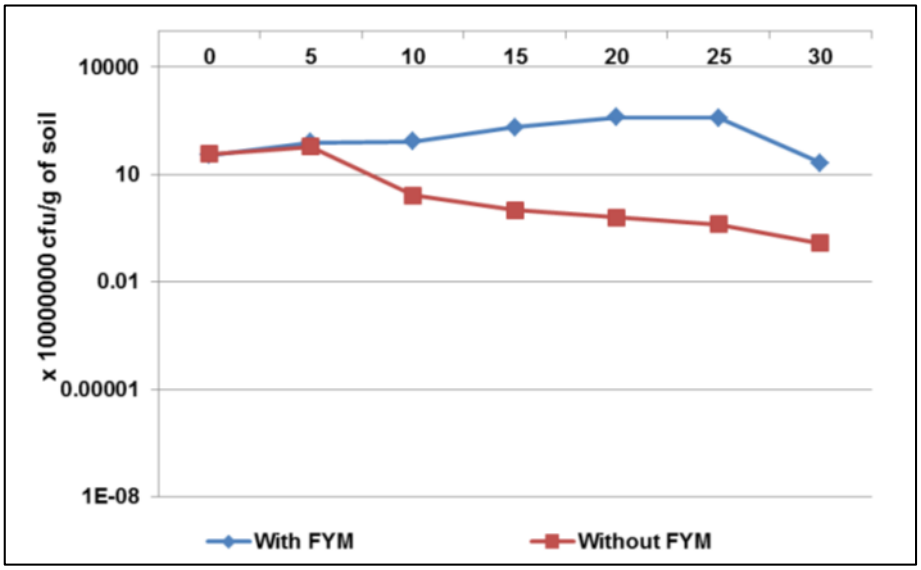
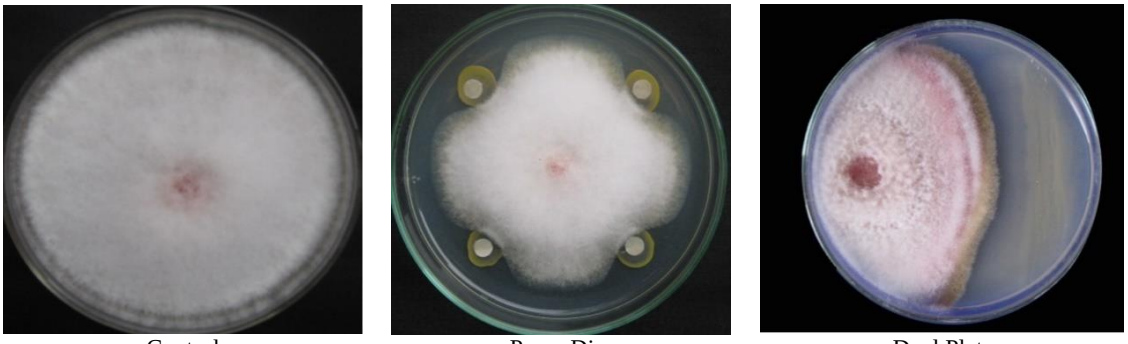
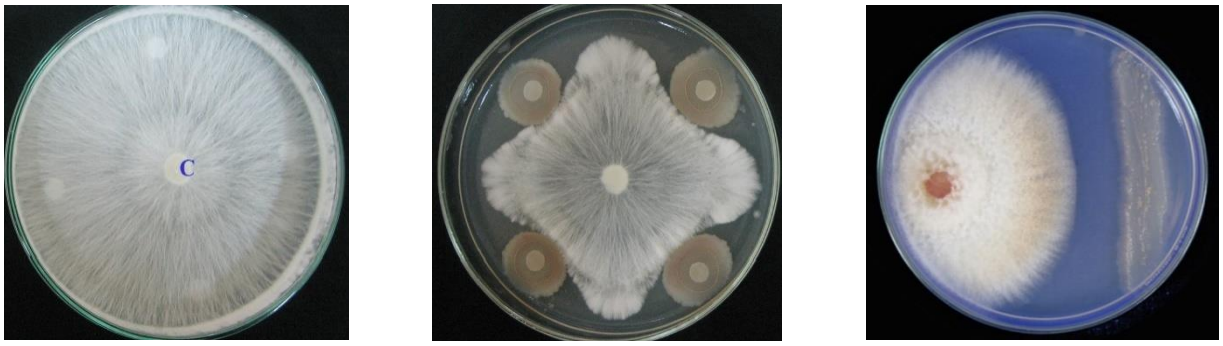


Fig 1: Population dynamics of *P. fluorescens* Pf1 applied to soil with and without FYM



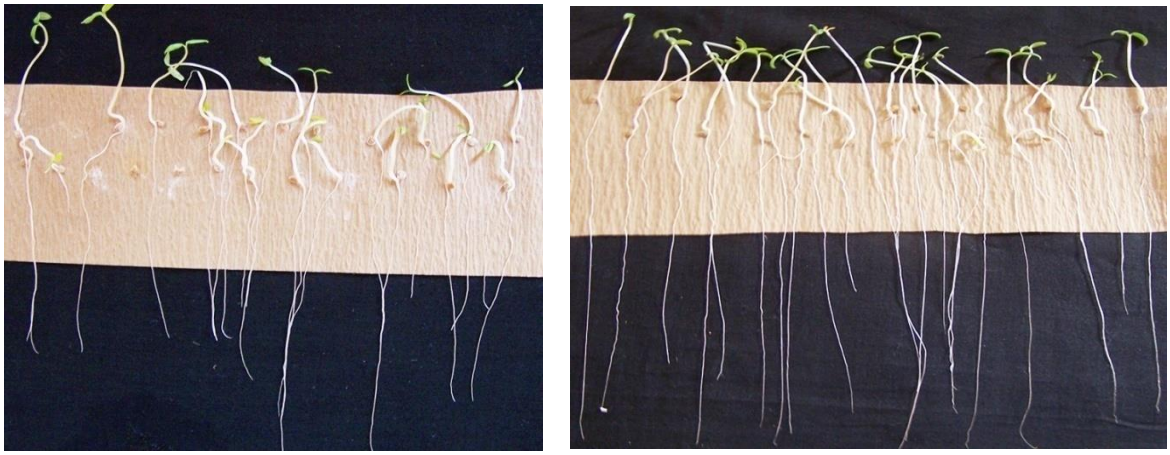
Control Paper Disc Dual Plate

Fig 2: Antagonistic activity of P. *fluorescens* Pf1 against F. *oxysporum* f.sp. *lycopersici* using dual plate technique and paper disc method



Control Paper disc Dual Plate

Fig 3: Antagonistic activity of *P. fluorescens* Pf1 against *Sclerotium rolfsii* using dual plate technique



Control *P. fluorescens* Pf 1 treated seeds

Fig 4: Effect of *P. fluorescens* Pf1 as seed treatment on tomato seed germination and seedling emergence on 15th DAS

Conclusion

The population dynamics of *Pseudomonas fluorescens* Pf1 when applied in to soil along with FYM was analyzed. The population density *P. fluorescens* Pf1 was increasedat 20 DAS and the maximum of 4.0 x 10^9 in both FYM and Pf1 talc formulation was applied as soil. For screening of *P. fluorescens* Pf1 against *F. oxysporum* f. sp. *lycopersici* and *S. rolfsii* revealed that the biocontrol agent was highly effective against both the pathogens under *in vitro*. In tomato seeds treated with *P. fluorescens* Pf1 resulted in per cent germination and maximum root and shoot length with a high vigour index. For induced systemic resistance, the maximum induction of defense related enzymes like phenylalanine ammonia lyase peroxidase and polyphenol oxidase activities were observed higher in 20th DAS in the treatment applied with FYM. Based on the above results, concluded that *P. fluorescens* Pf1 have more induction of plant growth harmones, antibiotic potential it's due to induction of defense related enzymes against tomato soil borne pathogens.

Acknowledgment

We are grateful to the Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, for the providing facility for carried out this research.

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