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EFFICACY OF NATIVE ISOLATES OF *PSEUDOMONAS FLUORESCENS* AND PLANT EXTRACTS FOR THE MANAGEMENT OF EARLY BLIGHT IN TOMATO

Sutha Raja Kumar R.*, Magesh M., Darwin Christdhas Henry L., Jaiganesh V. and Udhaya Kumar R.

Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar -608 002 (Tamil Nadu), India.

Abstract

Early blight disease of tomato caused by *Alternaria solani* is one of the most common and damaging disease occurring nearly every season, wherever tomatoes are grown and is a threat to tomato cultivation. The present studies were undertaken to investigate the effect of native isolates bacterial bio control agent and plant extracts against Early blight of tomato. In pot culture and field experiments, application of Bacterial bio protectant *P. fluorescens* as seed treatment @ (10ml /kg of seed) plus a prophylactic spray with the extracts of *Eucalyptus globules* @ 15 per cent concentration as foliar spray on the 45th and 60th DAT (Days After Transplanting) was found to be on par with the comparison fungicide Mancozeb 75% WP in reducing early blight disease incidence and recording a significant increase in the fruit yield when compared to control. Also, the same treatment recorded higher induction of defense enzymes *viz.,* PO, PPO and PAL when compared to control and comparison fungicide.

Key words : Early blight, Tomato, Bio control agent, *Pseudomonas fluorescens.*

Introduction

India is the fourth largest producer of tomato globally, contributing around 11.9 MT/ year. However, its average per hectare production is lesser (19.6 MT) when compared to the world average (28.2 MT) (Anonymous, 2019). The yield of tomato is restricted to a great extent due to various diseases and insect pests associated with tomato cultivation. Amongst these, tomato is highly susceptible to early blight, late blight and *Fusarium* wilt (Panthee and Chen, 2010). In India, the yield loss due to early blight disease was estimated to be 10 to 80 per cent (Abada *et al.,* 2008). Most important methods of managing *Alternaria* leaf blight include cultural methods and development of the host plant resistance with the application of fungicides. Cultivation of resistant varieties is the ultimate control of this disease. However, farmers in pursuance of high yield are inclined to cultivate some varieties which may be less resistant to disease. Also unplanned and wide use of fungicides often leads to serious environmental problems besides affecting the health of users and consumers (Deepti and Nidhi 2015). Hence, a search for alternative methods of plant disease management is on increase.

PGPR are the major root colonizers, belong to different genera and most reported strains are from species of *Pseudomonas, Bacillus* and *Serratia*. The strains of PGPR are known to survive both in rhizosphere and phyllosphere (Krishnamurthy and Gnanamanickam, 1998). Several *Pseudomonas* strains have been shown to activate ISR in plants against many fungal, bacterial and viral diseases (Chen *et al.,* 2000). Recent investigations on mechanisms of biological control by plant growth promoting fluorescent pseudomonas 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 (M'Piga *et al.,*

Author for correspondence : E-mail: subipatho@gmail.com

1997) and production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Nagarajkumar *et al.,* 2004). Plant peroxidases and polyphenol oxidases are involved in plant defense mechanism especially during pathogen attack or exposed to harsh environmental conditions. In response to this, biofungicides, derived either from microbes or plants, has emerged as promising alternative strategy. Neem (*Azadirachta indica*), garlic (*Allium sativum*), onion (*Allium cepa)* and few other plants inhibit early blight in potato and tomato (Mate *et al.,* 2005). Therefore the present study was aimed to assess the efficacy of native isolates *P. fluorescens* and plant extracts against *Alternaria* leaf blight of tomato.

Materials and Methods

Effect *P. fluorescens* and *Allium sativum* on the management of early blight of tomato under pot culture

Preparation of inoculums

The isolates of *P. fluorescens* was grown in King's B broth under constant shaking at 150 rpm for 48 h. at room temperature (28±2°C). The culture at its stationary phase of growth was centrifuged at 6000 rpm for 10 minutes and bacterial cells were re-suspended in 10 mM phosphate buffer (pH 7.0). The concentration was adjusted to 9 x 10^8cfu ml^{-1}. Then 2 per cent carboxy methyl cellulose was mixed with the bacterial suspension as a sticking agent and used as bacterial inoculums (Thomson, 1996).

Seed treatment with antagonist

Seeds of tomato (PKM1) were surface sterilized with two per cent sodium hypochlorite for 30 seconds rinsed in sterile dist. water and dried overnight. One gram of seed was soaked for 2 h. in ten ml of antagonist inoculums taken in a Petri dish and added with 100mg of carboxy methyl cellulose (CMC).

Preparation of aqueous extract

The freshly collected plant leaf materials were separately washed with tap water, then with alcohol and finally with repeated changes of sterile distilled water. They were separately ground in sterile dist. water at the rate of one ml/g of the leaf tissue in a sterilized pestle and mortar. The extract was stained through two layer of muslin cloth subsequently filtered through Whatman No: 1 filter paper and finally passed through Seitz filter to eliminate bacterial contamination. This formed the standard plant extract solution (100%) (Shekhawat and Prasad, 1971). This extract was further diluted to the desired concentration by adding requisite quantities of sterile dist. water. All the extracts were used at 100% concentration for screening antifungal activity. The plant species showed effectiveness in the preliminary screening alone were further diluted to different concentration (5, 10, 15 & 20 %) and tested against *A. solani*.

The early blight susceptible variety PKM1 grown in earthen pots was used for the study. *Allium sativum* leaf extract @ 15 % conc. was sprayed two days after inoculation of the pathogen and second spray was given at fortnightly interval. The crop was maintained in poly house with frequent spraying of water to provide adequate moisture and relative humidity to enable successful infection by the pathogen. The experiment were conducted in a randomized block design with three replications for each treatment and a suitable control. The fungicide Mancozeb 75% WP (0.25%) was used for comparison and the standard agronomic practices recommended by the State Agricultural Department were followed. The observations on PDI was assessed

Foliar spray

Liquid based bioformulations were mixed with the water @ 2 ml lit^{-1} and used as foliar spray at 30^{th} and 45^{th} day after transplanting.

Chemical spray

Mancozeb @ 0.2% was used for seed treatment and foliar spray were given on 30^{th} and 45^{th} day after transplanting, both in pot culture as well as field experiments. Mancozeb served as chemical check.

Enzyme extraction

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

Assay of peroxidase (PO)

Assay of PO (EC 1.11.1.7) activity was carried out as per the procedure described by Hammerschmidt *et al*. (1982). The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1ml) was added to initiate the reaction, which was followed colorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme was used as blank. Activity

was expressed as the increase in absorbance at 470 nm min^{-1} mg^{-1} of plant tissue.

Assay of polyphenoloxidase (PPO)

One gram of sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 20,000 rpm for 15 min at 4°C. The supernatant served as enzyme source and polyphenoloxidase activity was determined as per the procedure given by Mayer *et al*. (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 ml of the enzyme extract. To start the reaction, 200 ml of 0.01 M catechol was added and the activity was expressed as change in absorbance $min^{-1}mg^{-1}$ of plant tissue.

Assay of phenylalanine ammonia-lyase (PAL)

One gram of plant sample was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0, containing 1.4 mM of 2-mercaptoethanol and 50 mg of insoluble polyvinyl pyrrolidone (PVP). The resulting extract was filtered through cheese cloth and the filtrate was centrifuged at 20,000 rpm for 15 min at 4°C and the supernatant was used as the enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH8.8 and 0.5 ml of 12 mM L -phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 $M^{-1}cm^{-1}$ (Dickerson *et al.,* 1984). Enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid min^{-1} mg^{-1} of plant tissue.

Activity gel electrophoresis

Peroxidase (PO)

To study the expression pattern of different isoforms of peroxidases in different treatments, activity gel electrophoresis was carried out. For native anionic polyacrylamide gel electrophoresis, resolving gel of 8% and stacking gel of 4% were prepared. After electrophoresis, the gels were incubated in a solution containing 0.15% benzidine in 6 per cent NH_4Cl for 30 min in dark. Then drops of 30% H_2O_2 were added with constant shaking till the bands appear. After staining, the gel was washed with distilled water and photographed (Sindhu *et al.,* 1984).

Polyphenol oxidase (PPO)

Enzyme was extracted by homogenizing one g of tissue in 0.01 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 20,000 rpm for 15 min. at 4°C and the supernatant was used as enzyme source. After native electrophoresis, the gel was equilibrated for 30 min in 0.1% *p*-phenylene diamine in 0.1M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete bands (Jayaraman *et al.,* 1987).

Results and Discussion

Effect of *P. fluorescens* plus *Allium sativum* on early blight incidence of tomato under pot culture experiment

The data presented in table 1 revealed that the seed treatment with *P. fluorescens* @ 10ml /kg of seed plus foliar spray with *Allium sativum* @ 15% at 30 and 45 DAT (T_7) managed the early blight disease incidence (20.14 PDI), increased the plant height (85.55 cm) and increased the fruit yield (313.52 g). The test fungicide Mancozeb 75 WP (0.25%) as foliar spray at 30 and 45 DAT recorded 19.60 per cent early blight incidence, which was on par with T_7 treatment.

Induction of Peroxidase (PO) activity in tomato plants treated with *P. fluorescens* and *Allium sativum* against *A. solani* under pot culture experiment

Among the various treatments, the combination treatment (T_7) involving, seed treatment with *P. fluorescens* @ 10ml /kg of seed plus foliar spray with *Allium sativum* @ 15% at 30 and 45 DAT recorded higher peroxidase activity (3.469) on 9th day when compared with other treatments. The treatment T_3 (seed treatment with *P. fluorescens* @ 10ml /kg of seed plus foliar spray with *P. fluorescens* @ 0.2% at 30 & 45 DAT) also showed statistically similar results in inducing the peroxidase activity (3.281). This was followed by T_6 (2.958), T_5 (1.256), T_2 (1.149) and T8 (1.091) treatments in the decreasing order of merit. The maximum peroxidase activity was observed on the 9th day in all the treatments and thereafter a gradual decrease was observed (Fig. 1).

The increased PO activity has been correlated with resistance and these enzymes are involved in the polymerization of proteins and lignin or precursor into plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration of cell walls (Bradley *et al.,* 1992). Peroxidases have been implicated in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, cross linking of extension monomers, oxidation of hydroxyl–cinnamyl alcohols into free radical intermediates and wound healing (Vidhyasekaran *et al.,* 1997).

Table 1: Effect of *P. fluorescens* and *Allium sativum* on early blight incidence of tomato under Pot culture condition.

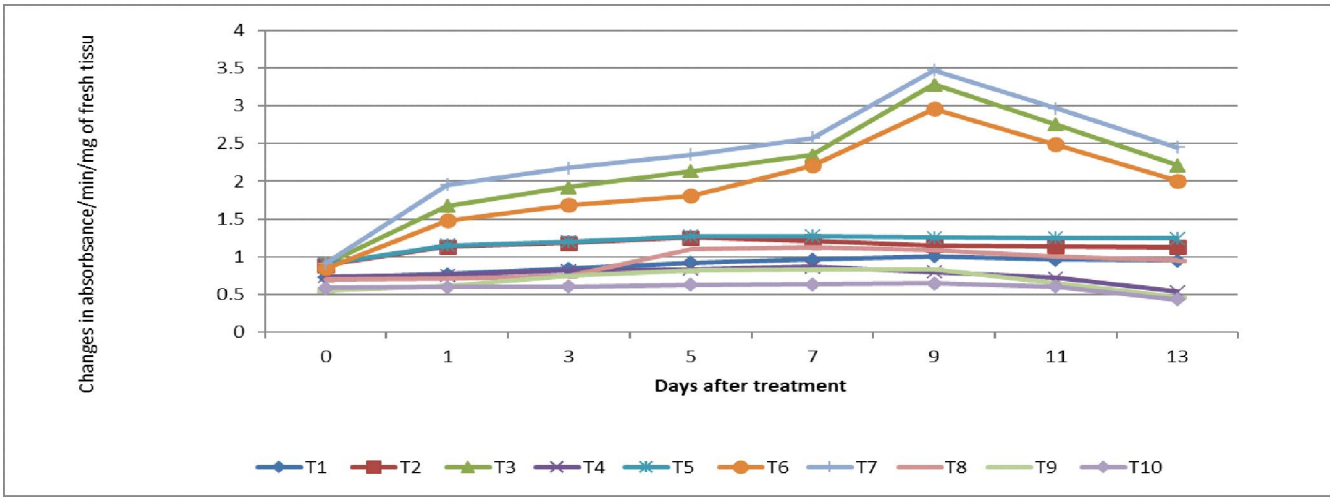
Treatment	Percent disease index	Percent reduction over control	Plant height (cm)	Fruit yield (g/plant)
T_1 - Seed treatment with *P. fluorescens* @ 10ml /kg	35.85^{e}	34.77^{g}	70.50^{e}	196.35^{e}
T_2 - Foliar spray with *P. fluorescens* @ 0.2% at 30 & 45 DAT	32.72^{d}	40.46^{f}	75.73^{d}	197.31^{e}
T_3 - T_1 + T_2	23.78^{b}	56.73^{c}	83.60^{b}	259.14^{b}
T_4 - Foliar spray with *Allium sativum* @ 15% at 30 DAT	37.14^{f}	32.42^{h}	69.50^{e}	195.21^{e}
T_5 - Foliar spray with *Allium sativum* @ 15% at 30 & 45DAT	26.86^{c}	51.12^{e}	76.42^{d}	199.46^{e}
T_6 - T1 + T_4	24.62^{b}	55.20^{d}	79.62^{c}	245.11^{c}
T_7 - T_1 + T_5	20.14^{a}	62.68^{b}	85.55^{a}	313.52^{a}
T_8 - Mancozeb (0.25%) as foliar spray at 30 & 45 DAT	19.60^{a}	64.33^{a}	74.85^{d}	210.78^{d}
T_9 - Inoculated control	54.96^{g}	-	59.20^{f}	165.76^{f}

Values in the column followed by same letters not differ significantly by DMRT (p=0.05).

Table 2: Effect *P. fluorescens* and *Allium sativum* on early blight incidence of tomato under field condition.

Treatments	Percent disease index	Percent reduction over control	Fruit yield (t/ha)
T_1 - Seed treatment with *P.fluorescens* @ 10ml /kg	17.02$_{f}$	40.77	52.42$_{g}$
T_2 - Foliar spray with *P.fluorescens* @ 0.2% at 30 & 45 DAT	16.86$_{f}$	41.33	52.65$_{f}$
T_3 - T_1 + T_2	12.61$_{c}$	56.12	55.09$_{c}$
T_4 - Foliar spray with *Allium sativum* @ 15% at 30DAT	17.14$_{f}$	40.36	51.92$_{h}$
T_5 - Foliar spray with *Allium sativum* @ 15% at 30 & 45 DAT	15.00$_{e}$	47.80	53.89$_{e}$
T_6 - T1 + T_4	13.52$_{d}$	52.95	54.00$_{d}$
T_7 - T_1 + T_5	10.17$_{b}$	62.87	59.31$_{a}$
T_8 - Mancozeb (0.25%) as foliar spray at 30& 45 DAT	10.00$_{a}$	64.61	55.60$_{b}$
T_9 - Inoculated control	28.74$_{g}$	.....	47.00$_{i}$

Values in the column followed by same letters not differ significantly by DMRT (p=0.05).



T_1 - Seed treatment with *P. fluorescens* @ 10ml /kg

T_2 - Foliar spray with *P. fluorescens* @ 0.2% at 30 & 45 DAT

T_3 - T_1 + T_2

T_4 - Foliar spray with *Allium sativum* @ 15% at 30 DAT

T_5 - Foliar spray with *Allium sativum* @ 15% at 30 & 45 DAT

T_6 - T1 + T_4

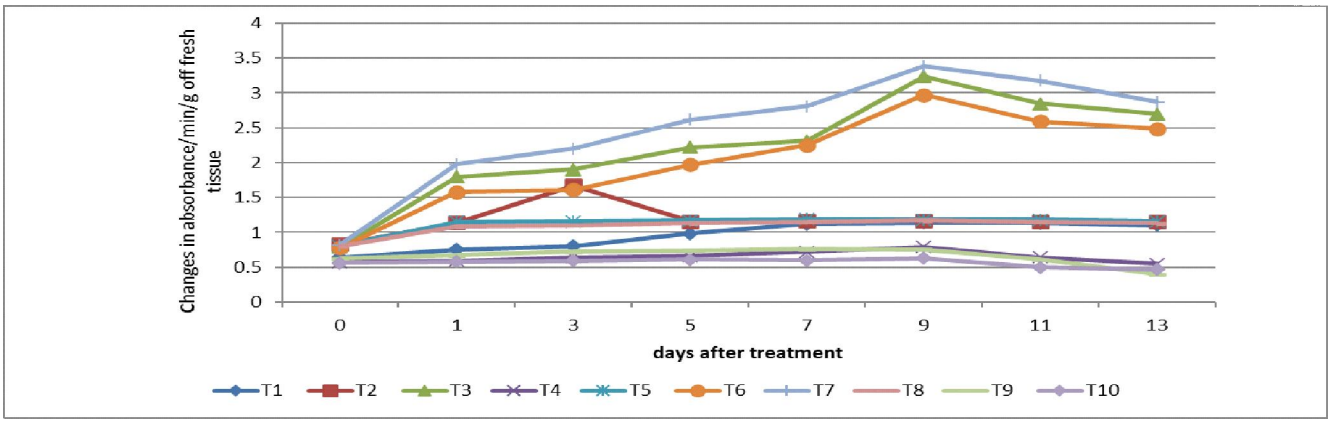
T_7 - T_1 + T_5

T_8 - Mancozeb (0.25%) as foliar spray at 30 & 45 DAT

T_9 - Inoculated control

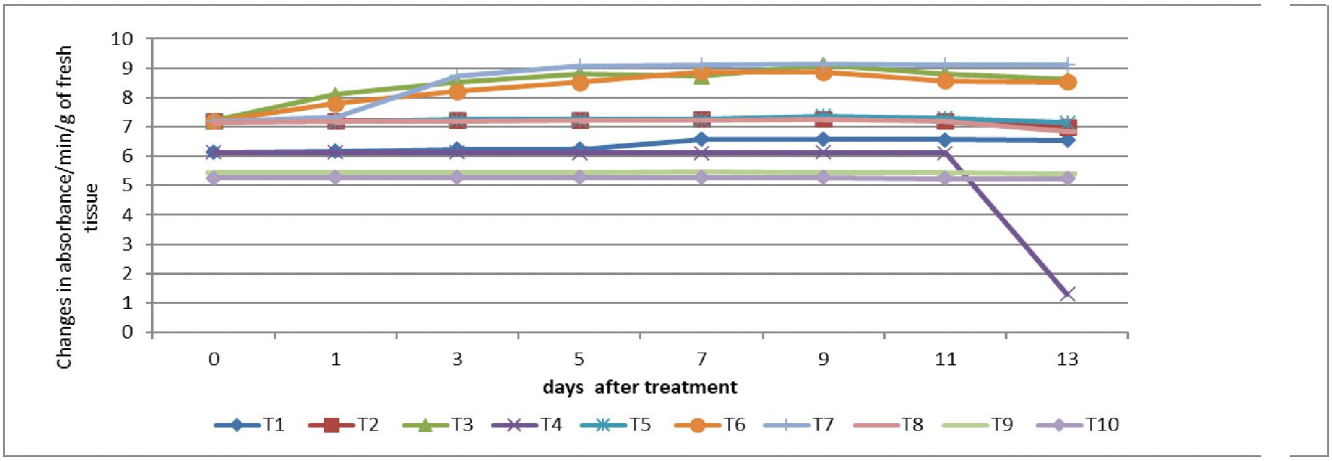
T_{10} - Healthy control

Fig. 1: Induction of Peroxidase (PO) activity in tomato plants treated with *P. fluorescens* and *Allium sativum* against *A. solani* under pot culture experiment.



T_1 - Seed treatment with *P. fluorescens* @ 10ml /kg
T_2 - Foliar spray with *P. fluorescens* @ 0.2% at 30 & 45 DAT
T_3 - T_1 + T_2
T_4 - Foliar spray with *Allium sativum* @ 15% at 30 DAT
T_5 - Foliar spray with *Allium sativum* @ 15% at 30 & 45 DAT
T_6 - T1 + T_4
T_7 - T_1 + T_5
T_8 - Mancozeb (0.25%) as foliar spray at 30 & 45 DAT
T_9 - Inoculated control
T_{10} - Healthy control

Fig. 2: Induction of polyphenoloxidase (PPO) activity in tomato plants treated with *P. fluroescens* and *Allium sativum* against *A. solani* under pot culture experiment.



T_1 - Seed treatment with *P. fluorescens* @ 10ml /kg
T_2 - Foliar spray with *P. fluorescens* @ 0.2% at 30 & 45 DAT
T_3 - T_1 + T_2
T_4 - Foliar spray with *Allium sativum* @ 15% at 30 DAT
T_5 - Foliar spray with *Allium sativum* @ 15% at 30 & 45 DAT
T_6 - T1 + T_4
T_7 - T_1 + T_5
T_8 - Mancozeb (0.25%) as foliar spray at 30 & 45 DAT
T_9 - Inoculated control
T_{10} - Healthy control

Fig. 3: Induction of phenylalanine ammonia lyase (PAL) activity in tomato plants treated with *P. fluorescens* and *Allium sativum* against *A. solani* under pot culture experiment.

Induction of polyphenoloxidase (PPO) activity in tomato plants treated with *P. fluroescens* and *Allium sativum* against *A. solani* under pot culture experiment

There was an increase in polyphenoloxidase (PPO) activity due to the treatment with *P. fluorescens* and *Allium sativum* and challenge inoculation with the pathogen. Among the treatments, seed treatment with *P. fluorescens* @ 10ml /kg of seed plus foliar spray with *Allium sativum* @ 15% at 30 and 45 DAT (T_7) recorded higher polyphenoloxidase (PPO) activity (3.38) when compared to other treatments. The treatment T_3 (seed treatment with *P. fluorescens* @ 10ml /kg of seed plus foliar spray with *P. fluorescens* @ 0.2% at 30 and 45 DAT) also showed statistically similar results in inducing the polyphenoloxidase (PPO) (3.24). This was followed by T_6 (2.25) and T_5 (1.18) treatments. The maximum polyphenoloxidase activity was observed on the 9th day in all the treatments and thereafter a gradual decrease was observed (Fig. 2).

Induction of phenylalanine ammonia lyase (PAL) activity in tomato plants treated with *P. fluorescens* and *Allium sativum* against *A. solani* under pot culture experiment

The results revealed that increased activity of phenylalanine ammonia lyase (PAL) was observed due to the combined treatment with *P. fluorescens* and *Allium sativum* and challenge inoculation with the pathogen. Among the treatments, seed treatment with *P. fluorescens* @ 10ml /kg of seeds plus foliar spray with *Allium sativum* @ 15 per cent at 30 and 45 DAT (T_7) recorded higher phenylalanine ammonia lyase activity (9.13) when compared to other treatments. The treatments T_3 (seed treatment with *P. fluorescens* @ 10ml/kg of seed plus foliar spray with *P. fluorescens* @ 0.2% at 30 and 45 DAT) also showed statistically similar results in inducing the phenylalanine ammonia lyase (9.12). This was followed by T_6 (8.85), T_5 (7.37) T_2 (7.24) and T8 (7.0) treatments in the decreasing order of merit. The maximum phenylalanine ammonia lyase activity was observed on the 9th day in all the treatments and there after a gradual decrease was observed (Fig. 3). The maximum accumulation of PAL upto 9 days constituted for enhancing the resistance in tomato plants against early blight disease. PAL is the key enzyme in inducing synthesis of salicylic acid (SA) which induces systemic resistance in many plants. (Li *et al.,* 1993).

Effect *P. fluorescens* and *Allium sativum* on early blight incidence of tomato under field condition

Experiment carried out under field conditions (table 2) revealed that the treatment T_7 with *P. fluorescens* as seed treatment (10ml /kg of seed) plus *Allium sativum* foliar spray (15 per cent at 30 and 45DAT) reduced the early blight disease incidence (10.67 per cent), with maximum per cent disease reduction (62.87%) and fruit yield (59.31 t/ha) which was on par with Mancozeb 75% WP (0.25%) which recorded (64.61%) per cent reduction of early blight incidence over control. The control treatment recorded the maximum disease incidence (28.74%) and the least fruit yield (47.00 t/ha). The result indicated that different plant colonization pattern and different mechanism of disease suppression elicited by the combination of *P. fluorescens* and the extract of *Allium sativum* might have offered greater protection to the tomato crop against the attack of *A. solani* causing early blight disease. *Pseudomonas* spp. have been shown to produce wide array of antibiotics which includes DAPG, HCN, Kanosamine, phenazine, pyoluteorin and pyrrolnitrin as well as several other uncharacterized moieties (Whipps, 1997). O'Dowling and O'Gara, (1994) listed as many as 38 secondary metabolites produced by *Pseudomonas* spp. involved in the suppression of various diseases. Production of such antibiotics and metabolites could have contributed to the suppression of early blight incidence observed in the study. Besides the ISR triggered by *P. fluorescens* could have also contributed to the disease suppression. (Meera *et al.,* 2013).

The antifungal activity of *Allium sativum* may be due to the presence of sulphur compounds and allicin present in them (Anil Sehajpal *et al.,* 2009) Govindappa *et al.,* (2011) reported that induction of resistance in rice with *A. vasica* was evident from increase accumulation of PR proteins and other related compounds and garlic extract against *Fusarium* wilt of tomato. The results of the present study have clearly revealed that combination of *P. fluorescens* along with *Allium sativum* extract would have exerted a synergism and also different mechanisms of disease control which certainly enhanced greater disease suppression and increased fruit yield of tomato and improved the consistency of biological control under varied climatic conditions.

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