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ECO-FRIENDLY MANAGEMENT OF CHILLI ANTHRACNOSE

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Abstract

The present studies were undertaken to investigate the effect of Bio control agent *Pseudomonas fluorescens,* garlic bulb extract and mushroom spent compost tested against anthracnose disease. Thirty days old seedlings of the chilli anthracnose susceptible var. 'K 2' was transplanted at three per pot was used for this study. The seeds were treated @ 10 g/kg of seeds and were dried in shade over night before sowing. For soil application talc based formulation of *P. fluorescens,* were used @10kg/ha. Garlic bulb extract was used @ 15 percent concentration. Mancozeb 75 %WP was sprayed @ 0.25 per cent concentration was used as a comparison fungicide. The mushroom spent compost was incorporated in to the pots 5 tonnes/ ha five days before transplanting. The results revealed that among the various treatments, seed treatment with *P. fluorescens* @ 10 g/ kg, Foliar spray with *Allium sativum* @ 15% on 60 & 75 DAT and soil application with mushroom spent compost significantly reduced the chilli anthracnose disease severity, increased the plant height and increased the fruit yield. Also, there is a positive induction of host defense enzymes was observed due to the combined integrated treatment and challenge inoculation with the pathogen.

Key words : Chilli, Anthracnose, Biological control, Mushroom compost, defense enzymes.

Introduction

Chilli (*Capsicum annum* L.) is one of the most important constituent of the cuisines of tropical and subtropical countries and the fourth major crop cultivated globally. In India, chilli is cultivated in an area of 774.87 thousand ha with an annual production of 1492.14 thousand tones of dried chilli. Although production is high in India, the average productivity is less (1ton/ha), when compared to other important producers of chilli *viz*., China, Mexico, Taiwan where the productivity is three tonnes/ha. One of the major reason for this low productivity is diseases. (Sahitya *et al.,* 2014). Chilli crop is affected by several fungal, bacterial and viral diseases, of which chilli anthracnose causes considerable damage, inflicting severe quantitative and qualitative losses (Masoodi *et al.,* 2012). The estimated loss due to this disease ranged from 8 to 60 per cent in different parts of India (Kumar *et al.,* 2015). Several fungicides have been reported to be effective for management of fruit rot of chilli (Shovan *et al.,* 2008). However, indiscriminate use of chemicals led to the development of fungicidal resistance by the pathogen, environmental pollution, health hazards and high cost (Bajpai and Kang, 2010). Hence, a search for alternative methods of plant disease management is on increase.

Recently, the role of plant growth promoting rhizobacteria (PGPR) *viz*., *Pseudomonas fluorescen*s and *Bacillus subtilis* in biocontrol approaches for managing the pathogen in crop plants are well reported (Allu *et al.,* 2014). They are well adapted to rhizosphere and rhizoplane, Fluorescent pseudomonads exhibit diverse mechanisms of biocontrol which include antibiosis, competition for space and nutrients, HCN production, siderophore production and induced systemic resistance. Presently, there are number of commercial PGPR strains available in the market. However, the native isolates of certain biocontrol agents showed superiority over other isolates for the management of crop diseases (Dubey and Pandey, 2001). Moreover, there is an increased public

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demand for sustainable and chemical residue-free food production. In response to this, biofungicides, derived either from microbes or plants, emerged as promising alternative strategies. Neem (*Azadirachta indica*), garlic (*Allium sativum*), onion (*Allium cepa)* and few other plants inhibit chilli anthracnose (Ngullie *et al.,* 2010). One of the problems encountered with the use of biocontrol agents is that they may not perform equally well in a variety of environmental conditions. Hence, an integrated approach involving biocontrol agents, organic amendments and plant products would ensure the maximum suppression of chilli anthracnose and higher yield of chilli without any harmful effect on the ecosystem. Therefore, the present study was undertaken to investigate the effect of combined application of PGPR, organic amendments along with an effective plant extract for managing chilli anthracnose.

Materials and Methods

Efficacy of antagonists, plant extracts and organic amendments against fruit rot disease of chilli in pot culture

The pot culture study was conducted with nine treatments and three replications at Department of Plant Pathology, Annamalai University, and Annamalainagar. Five kilograms of topsoil collected from a chilli growing field was steam pasteurized and filled in 30 cm diameter earthen pots. Thirty day old seedlings of the chilli var. 'K 2' was transplanted at three per pot. The eco-friendly components *viz., P. fluorescens,* garlic bulb extract and mushroom spent compost and mancozeb were tested against anthracnose disease with following recommendation in a pot culture experiment. The talc based formulations of *P. fluorescens* were used @ 2×10^{-8} cfu g^{-1}. The seeds were treated @ 10 g/kg of seeds and were dried in shade over night before sowing. For soil application talc based formulation of *P. fluorescens,* were used @10kg/ha. Garlic bulb extract was used @ 15 percent concentration. Mancozeb 50%WP was sprayed @ 0.25 per cent concentration. The mushroom spent compost was incorporated in to the pots 5 tonnes/ ha five days before transplanting. The spore suspension (1×10^{6} ml^{-1}) of *C. capsici* was prepared from twenty days old culture grown on PDA slants using sterile distilled water was inoculated thoroughly over the plant canopy by pinpricking method on 90 days after transplanting. The inoculated plants were incubated in a humid chamber for 48h and subsequently moved to a greenhouse maintained at 22-28°C, 70-90% relative humidity, under a light intensity of 85 µmol m^{-1-} S^{-1}, 12h. photoperiod and subsequently transfer to pot culture yard. Below mentioned treatment schedule were designed on the basis of the above phenomena.

Treatment details

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

T_2 - Foliar spray with *P. fluorescens* @ 0.2% at 60 & 75 DAT

T_3 - T_1+ T_2

T_4 - SA with mushroom spent compost @ 5 t ha^{-1} + T_3

T_5 - Foliar spray with *A. sativum* @ 15% at 60 & 75 DAT

T_6 - T_1 + Foliar spray with *A. sativum* @ 15% at 60 & 75 DAT

T_7 - SA with mushroom spent compost @ 5 t ha^{-1} + T_6

T_8 - Mancozeb @ 0.25% as foliar spray at 60 &75 DAT

T_9 - Inoculated control

T_{10} – Control

Per cent Disease index

The fruit rot severity was assessed for the plants during their 100th, 125th and 150th day after transplanting. The intensity of fruit rot was calculated as per cent disease index (PDI) grade chart proposed by Reddy (1982) using the formula proposed by McKinney (1923).

Category value	Per cent fruit area diseased
0	0
1	1-5
2	6-10
3	11-25
4	26-50
5	51 and above

The per cent disease index (PDI) was calculated using McKinney (1923) infection index.

$$PDI = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruits observed}} \times \frac{100}{\text{Maximum category value}}$$

Induced Systemic Resistance

Sample Collection

A glasshouse experiment was laid out in completely randomized design using the chilli K2 variety to assess the induction of defense enzymes by IDM formulation against challenge inoculation of *C. capsici* with following treatments.

T_1 - Foliar spray with *P. fluorescens* at 60 DAT @ 0.2% at 60 DAT

T_2 - Foliar spray with Garlic @ 15 % at 60 DAT

T_3 - Foliar spray with Mancozeb 75% WP (0.25%) conc. @ 60 DAT

T_4 - Inoculated control

T_5 - Control

Ninety days after transplanting the plants were challenge inoculated with a conidial suspension of *C. capsici* with spore load of 1×10^6 ml^{-1}. The samples of the above treated plants were collected at different time interval (0, 1, 3, 5, 7, 9 and 11 days) after pathogen inoculation. Three replications were maintained in each treatment. Fresh plant samples were used for analysis.

Enzyme extraction

The plant tissues collected from plants were immediately homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1 M (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extract prepared from leaves were used for the estimation of peroxidase (PO), polyphenol oxidase (PPO) and L-phenylalanine ammonia-lyase (PAL).

Spectrophotometric assay

Peroxidase (PO)

Peroxidase activity was assayed spectrophotometrically (Hartee, 1955). 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 which was incubated at room temperature (28 ± 1°C). The change in absorbance at 420 hm was recorded at 30 sec. interval for 3 min and the boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} on fresh weight basis (Hammerschmidt *et al.,* 1982)

Polyphenol oxidase (PPO)

Polyphenoloxidase activity was determined as per the procedure given by Mayer *et al*. (1965). The reaction mixture consisted of 1.5 ml of 0.1M 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 changes in absorbance at 495 m min^{-1} g^{-1} fresh weight of tissue.

Phenylalanine ammonia-lyase (PAL)

The PAL assay was conducted as per the method described by Ross and Sederoff (1992). The assay mixture containing 100 µl of enzyme, 500 µl of 50 mM Tris HCl (pH 8.8) and 600 µl of 1mM L-phenylalanine was incubated for 60 min. The reaction was arrested by adding 2 N HCl. Later 1.5 ml of toluene was added, vortexed for 30 sec. centrifuged (1000 rpm, 5 min) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. Standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. The enzyme activity was expressed as hmoles of cinnamic acid min^{-1} g fresh $tissue^{-1}$.

β-1, 3-glucanase

The enzyme activity was calorimetrically assayed (Pan *et al.,* 1991). Crude enzyme extract of 62.5 µl was added to 62.5 µl of 4 per cent laminarin and incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid (DNS) and heated for 5 min on boiling water bath (DNS prepared by adding 300 ml of 4.5 per cent NaOH to 880 ml containing 8.8 g of DNS and 22.5 g potassium sodium tartarate). The resulting coloured solutions were diluted with distilled water, vortexed and the absorbance was read at 500 m. The crude extract preparation mixed with laminarin at zero time incubation served as blank. The enzyme activity was expressed as mg equivalents of glucose min^{-1} g fresh $weight^{-1}$.

Chitinase

The colorimetric assay of chitinase was carried out as per Boller and Mauch (1988). Reagents used were colloidal chitin, snail gut enzyme, dimethyl amino benzaldehyde (DMAB) and buffer (Annexure).

Assay procedure

The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg). After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 1000 rpm for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3 per cent (w/v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 min at 37°C and the absorbance was measured at 585 nm using *N*-acetylglucosamine (GlcNAc) as standard. The enzyme activity was expressed as nmoles GlcNAc equivalents min^{-1} g fresh $weight^{-1}$.

Total phenols

The content of the total phenols present in the fruits

was estimated following the procedure of Bray and Thorpe (1954). Fresh fruit samples of 0.5 g weight were blended with 10 ml of 80 per cent ethanol and boiled at 50ºC for 30 min. The extracts were filtered through cheese cloth and then with Whatman No. 41 filter paper and centrifuged. The volume was made up to 10 ml with ethanol. An aliquot of one ml was taken in a series of boiling tubes and made up to 3 ml with distilled water. To this, one ml of Folin ciocalteu reagent and two ml of 20 per cent sodium carbonate were added. The tubes were heated for one min in a boiling water bath and cooled in running water. The solution was diluted to 10 ml with distilled water and the intensity of the blue colour was measured at 660 nm in a spectrophotometer against a blank (a blank was maintained with three ml of distilled water instead of the extract and the colour was developed as described above) for which three replications were maintained. Catechol was used for preparing the standard graph from which the amount of phenol in the given sample was calculated. The content of the total phenols was expressed as catechol equivalents in mg 100 g fresh weight^{-1}.

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 per cent acrylamide concentration and stacking gel of 4 per cent acrylamide concentration were prepared. After electrophoresis, the gels were incubated in the solution containing 0.15 per cent benzidine in 6 per cent NH_4Cl for 30 min in dark. Then drops of 30 per cent 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)

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

Results and Discussion

Effect of *P. fluorescens*, *Allium sativum* and mushroom spent compost on chilli anthracnose incidence of chilli under pot culture experiment

The data presented in table 1 revealed that seed treatment with *P. fluorescens* @ 10 g/ kg and Foliar spray with *Allium sativum* @ 15% on 60 & 75 DAT and soil application with mushroom spent compost (T_7) significantly reduced the chilli anthracnose disease severity (13.46 PDI), increased the plant height (111.87 cm) and increased the fruit yield (388.35 g) (Plate 17). The test fungicide Mancozeb 75 WP (0.25%) as foliar spray at 60 and 75 DAT recorded 12.79 per cent anthracnose severity, which was on par with T_7 treatment.

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 *A. sativum* might have offered greater protection to the chilli crop against the attack of *C. capsici* causing anthracnose 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.

The antifungal activity of *A. sativum* may be due to the presence of sulphur compounds and allicin present in them (Sehajpal *et al.,* 2009). The mechanism of the action of sulfur compounds towards microorganisms is complex and has not yet been fully explained. It is generally recognised that the antimicrobial action of sulfur compounds depends on their hydrophilic or lipophilic character. 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. Akila *et al.,* (2011) positively correlated the treatment with combination of botanical and bacterial antagonist such as *P. fluorescens* and *B. subtilis* on the induction of defense enzymes against *Fusarium* wilt of banana. Zhang *et al.,* (1996) reported that compost improve the nutritional status of the host and induced systemic acquired resistance in cucumber to *Pythium* root rot and anthracnose.

Induction of Peroxidase (PO)

Among the various treatments, the treatment (T_2) involving foliar spray with garlic @ 15 % conc. on 60 DAT recorded highest peroxidase activity (15.07) on 7th day when compared with other treatments. This was followed by T_1 (14.19) and T_3 (13.07) treatments in the decreasing order of merit. The maximum peroxidase

Table 1: Effect of *P. fluorescens*, *A. sativum* and mushroom spent compost on chilli anthracnose severity of chilli under pot culture.

Tr. No.	Treatment	*Percent disease index	Percent reduction over control	*Plant height (cm)	*Fruit yield (g/plant)
T_1	Seed treatment with *P. fluorescens* @ 10g /kg	27.98[f]	21.74	100.76[e]	350.75[e]
T_2	Foliar spray with *P. fluorescens* @ 0.2% at 60 & 75 DAT	25.16[e]	29.46	103.30[d]	368.39[d]
T_3	$T_1 + T_2$	24.42[e]	31.53	102.99[d]	366.73[d]
T_4	SA with mushroom spent compost @ 5 t ha $^{-1}$ + T_3	20.13[d]	43.56	108.37[c]	375.23[c]
T_5	Foliar spray with *Allium sativum* @ 15% at 60 & 75 DAT	20.55[d]	42.38	108.58[c]	377.73[c]
T_6	T_1 + Foliar spray with *Allium sativum* @ 15% at 60 & 75 DAT	16.42[c]	53.96	111.60[b]	386.34[b]
T_7	SA with mushroom spent compost @ 5 t ha $^{-1}$ + T_6	13.46[b]	62.26	111.87[b]	388.35[b]
T_8	Mancozeb (0.25%) as foliar spray at 60 and 75 DAT	12.79[b]	64.14	114.11[a]	409.25[a]
T_9	Inoculated Control	35.67[g]	0.00	92.24[g]	283.86[g]
T_{10}	Healthy control	0.00[a]	100	99.48[e]	340.59[e]

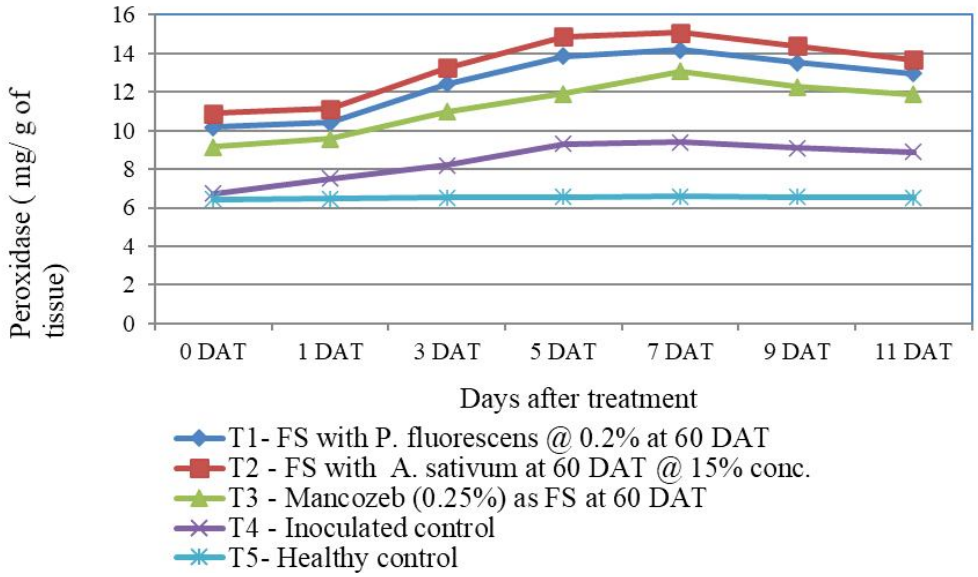
SA- Soil application.

DAT-Days after Transplanting.

*Values are mean of three replications.

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

Fig. 1. Induction in Peroxidase (PO) activity in *C. capsici* challenged chilli plants treated with *P. fluorescens* and *A. sativum* against under pot culture



activity was observed on the 7th 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).

Induction of polyphenoloxidase (PPO)

There was an increase in polyphenoloxidase (PPO) activity due to the treatment with *Allium sativum* and challenge inoculation with the pathogen. Among the treatments, foliar spray with garlic @ 15 % conc. on 60 DAT (T_2) recorded higher polyphenoloxidase (PPO) activity (1.41) when compared to other treatments. This was followed by T_1 (1.32) and T_3 (1.18). The maximum polyphenoloxidase activity was observed on the 7th day in all the treatments and thereafter a gradual decrease was observed.

Fig. 2. Induction in Polyphenoloxidase (PPO) activity in *C. capsici* challenged chilli plants treated with *P. fluorescens* and *A. sativum* against under pot culture

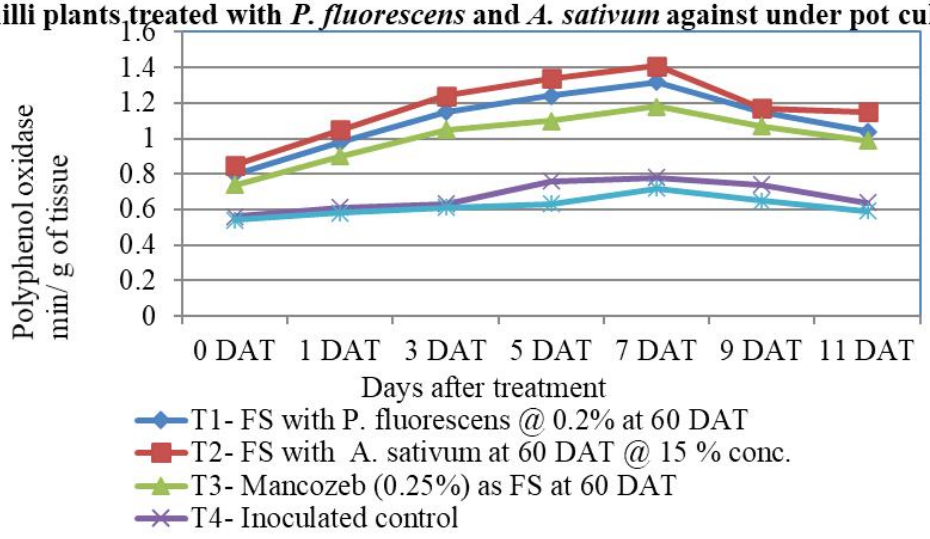
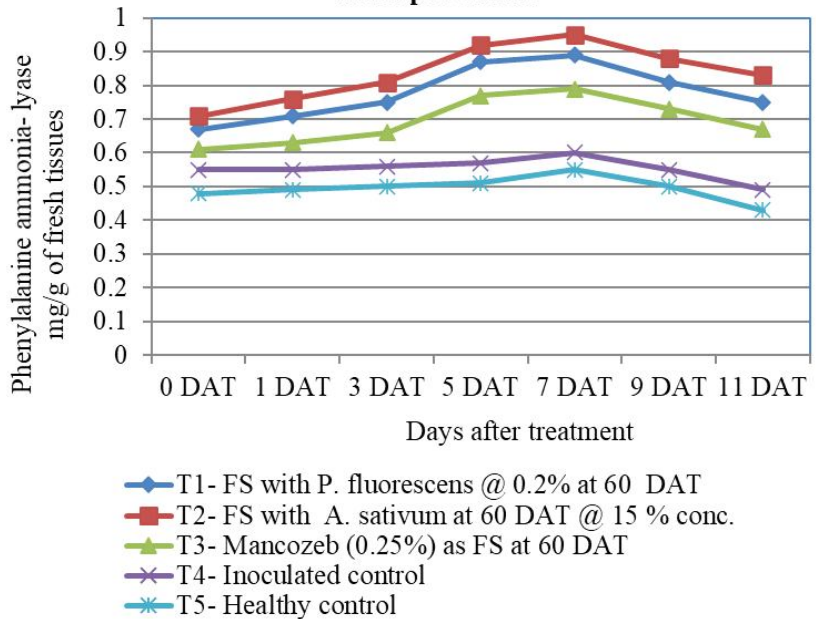


Fig. 3. Induction in Phenylalanine ammonia lyase (PAL) activity in *C. capsici* challenged chilli plants treated with *P. fluorescens* and *A. sativum* against under pot culture



Induction of phenylalanine ammonia lyase (PAL)

The results revealed that increased activity of phenylalanine ammonia lyase (PAL) was observed due to the treatment with *Allium sativum* and challenge inoculation with the pathogen. Among the treatments, foliar spray with *A. sativum* @ 15 % conc. on 60 DAT (T_2) recorded higher phenylalanine ammonia lyase activity (0.95) when compared to other treatments. This was followed by T_1 (0.89) and T_3 (0.79). The maximum phenylalanine ammonia lyase activity was observed on the 7th day in all the treatments and there after a gradual decrease was observed (Fig. 3). PAL is the key enzyme in inducing synthesis of salicylic acid (SA) which induces systemic resistance in many plants. Induction of PAL by Fluorescent *Pseudomonas* was reported in sugarcane against *C. falcatum* (Viswanathan and Samiappan, 1999).

Induction of Glucanase

Treatment with foliar spray of *Allium sativum* and challenge inoculation with the pathogen revealed increased activity of glucanase. Among the treatments, foliar spray with garlic @ 15 % conc. at 60 DAT (T_2) recorded higher glucanase activity (82.30) when compared to other treatments. This was followed by T_1 (77.17), and T_2 (68.80) treatment in the decreasing order of merit. The glucanase activity showed an increase up to the 7th day of observation in all the treatment and thereafter a gradual

Fig. 4. Induction in glucanase activity in chilli plants treated with *P. fluorescens* and *A. sativum* against *C. capsici* under pot culture

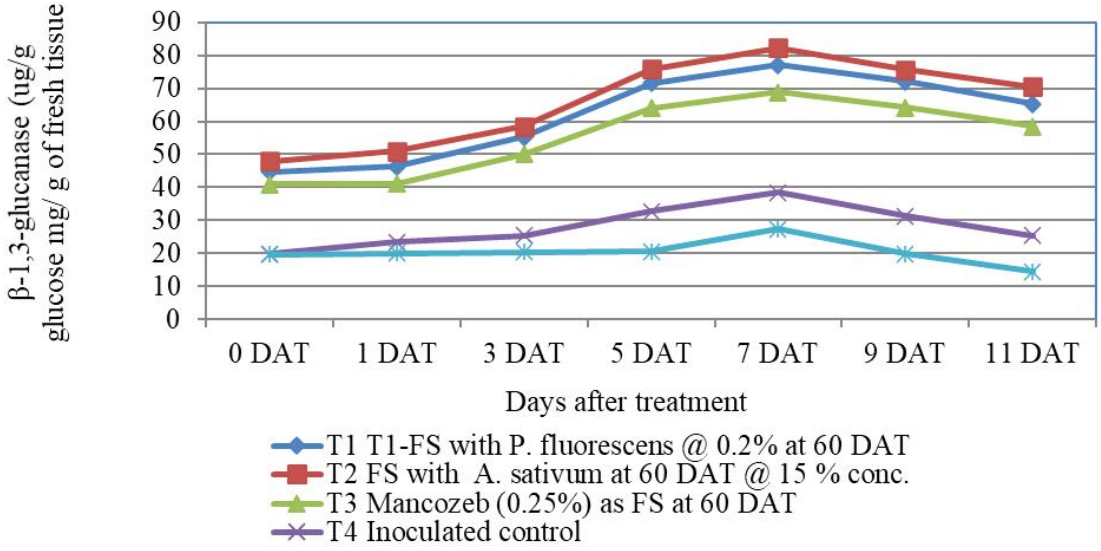
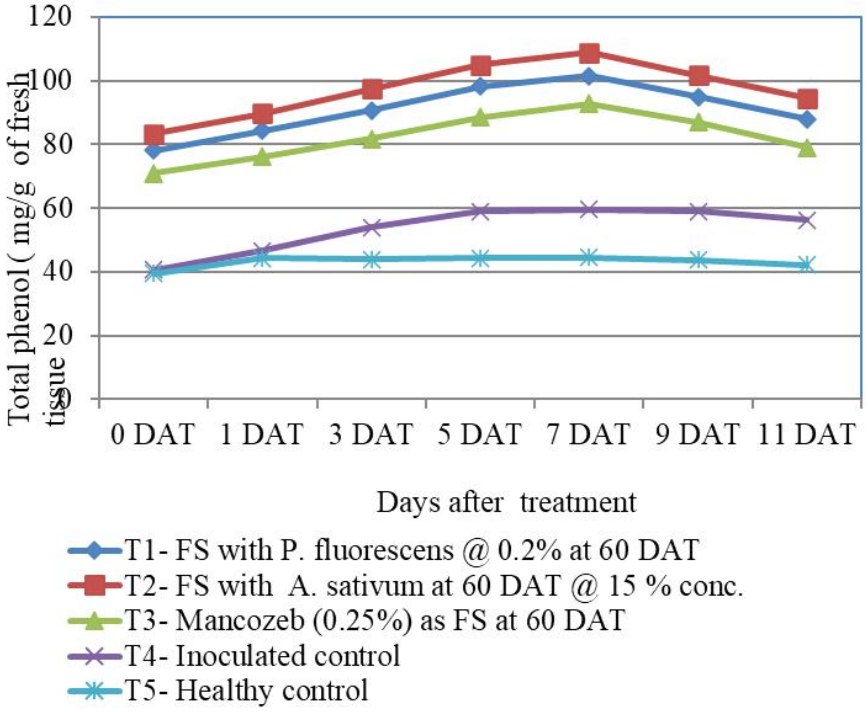


Fig. 5. Induction in Total phenol content activity in chilli plants treated with *P. fluorescens* and *A. sativum* against *C. capsici* under pot culture



decrease was observed (Fig. 4). In the present study, reduced disease severity might be due to increased activity of â-1, 3 glucanase in plants, foliar spray with *A. sativum* @ 15% at 60 DAT (T_2) and challenge inoculated with C. *capsici*. Normally fungal cells contain chitin and glucan as their cell wall constituents. The main mode of action of antagonistic activity of microbes are production of chitinase and â-1, 3 glucanase which act on cell walls of organisms which have chitin or glucan as their cell wall component and also through induced systemic resistance in plant system (Singh *et al.,* 1999).

Induction of total phenol

The results revealed that increased activity of phenol was observed due to the combined treatment with *P. fluorescens* and *Allium sativum* and challenge inoculation with the pathogen. Among the treatments foliar spray with garlic @ 15 % conc. at 60 DAT (T_2) recorded higher

phenol activity (108.87) when compared to other treatments. This was followed by T_1 (101.46), and T_3 (92.80) treatment in the decreasing order of merit. The maximum phenylalanine ammonia lyase activity was observed on the 7th day in all the treatments and thereafter a gradual decrease was observed (Fig. 5).

The results of the present study have clearly revealed that combination of *P. fluorescens* and mushroom spent compost and *A. 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 chilli and improved the consistency of biological control under varied climatic conditions.

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