European Journal of Biotechnology and Bioscience
ISSN: 2321-9122; Impact Factor: RJIF 5.44
www.biosciencejournals.com
Volume 3; Issue 9; September 2015; Page No. 56-65

Induction of systemic resistance by certain biocontrol agents against fusarium wilt complex disease of tomato

T Sivakumar, K Sanjeevkumar, P Balabaskar
Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, Tamil Nadu, India

Abstract
Tomato (*Lycopersicon esculentum* Mill) is one of the important vegetable crops in the world. Among the pathogens affecting tomato, *Fusarium oxysporum* f. sp. *lycopersici* is considered as the major pathogen. *F. oxysporum* f. sp. *lycopersici* along with *M. incognita*, causes more damage to tomato than either of the pathogen alone. Considering the illeffects of chemical fungicides an attempt was made to exploit the ISR induced by antagonists for the management of wilt complex of tomato. The reults revealed that the plants treated with combination of Pf + Bc + Pl and challenge inoculation with *F.oxysporum f. sp. lycopersici* and Pf + Bc + Pl and challenge inoculation with Fol + nematode showed induction of earlier and increased levels of defense enzymes viz., PO, PPO, PAL and Phenol content as well as PR Proteins viz., chitinase and β-1, 3 glucanase in both leaves and root tissues.

Keywords: *Lycopersicon esculentum,* fusarium wilt

Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the important vegetable crops in the world. It occupies a pride of place in view of its high nutritive value coupled with multivarious uses and it tops the list of processed vegetables. Tomato is a good source of vitamin-A, vitamin-C and various minerals. It is used directly as a raw vegetable in sandwiches, salads etc., and also processed into several food items like paste, puree, syrup, juice, ketchup, sauce, drinks, whole peeled tomato etc.

Tomato is cultivated throughout the world in an area of 3.54 million ha with estimated annual production of 95.13 million tones. In India tomato is grown in 4.57 lakh ha and producing 74.27 lakh tones with a productivity of 16.3 tonnes/ha (Dharamveer *et al.* 2005) [11].

In Tamil nadu, tomato is cultivated in about 21.055 ha in the districts of Theni, Madurai, Coimbatore, Dharmapuri and Erode (Anon, 1997) [1].

Tomato crop grown throughout the year is susceptible to several diseases and more than 200 pathogens affect the crop resulting in 75-95 per cent yield loss (Lukyanenko, 1991) [20]. Among these, *Fusarium oxysporum* f. sp. *lycopersici* considered as the major pathogen often results in 10-50 per cent crop losses around the world. The isolates of *F. oxysporum* f. sp. *lycopersici* differed in their potentiality to induce wilt incidence in tomato (Padmodaya and Reddy, 1996) [27]. Besides fungal diseases, a number of plant parasitic nematodes affect tomato. Sasser (1979) [33] reported that the major nematode damaging tomato is the root-knot nematode Yield loss due to this nematode range from 15 to 71 per cent in tomato (Jain *et al.* 1994) [16].

Bora (1977) [5] reported about the association of *F. oxysporum* f. sp. *lycopersici* with *M. incognita*, which causes more damage to tomato than either of the pathogen alone. *Meloidogyne-Fusarium* wilt complex disease are import and widely distributed causing substantial yield losses on many important vegetable crops (Mai and Abawi, 1987) [21].

Thus, it is imperative that the management *Fusarium-Meloidogyne* wilt complex disease of tomato is of atmost importance for economic productivity of the crop. In modern agriculture, large quantities of several volatile and non-volatile pesticides are being used inevitably to control soil borne disease and nematodes. In India, pesticides are not indigenously manufactured and involve exorbitant cost. Besides, indiscriminate and improper use of these chemicals lead to environmental pollution, groundwater contamination, health hazards due to pesticide residues in the harvested produce, toxicity to non-target organisms and adverse effects on beneficial microflora and fauna of the soil. Hence, there is a need to develop an eco-friendly, economical and alternative method for effective management of *Fusarium-Meloidogyne* wilt complex disease in tomato.

Biological control of soil borne plant pathogens by addition of antagonistic micro-organisms to the soil is a potential non-chemical means (Harman, 1991) [15] and is known to be a cheap and effective method for the management of soil and seed-borne diseases (Cook and Baker, 1983) [8]. In addition, the native isolates of certain bio control agents showed superiority over other isolates for the management of soil borne diseases (Dubey and Patel, 2001) [12]. Hence, the possibility of exploiting the native antagonistic organism was thought off for the management of tomato *Fusarium-Melodogyne* wilt complex disease.

Application of a single antagonist often results in inconsistent management of the wilt complex disease. One of the strategies to overcome this problem is to combine the disease suppressive activity of two or more beneficial antagonists in a bio-control preparation. Such combinations would have potential for more extensive colonization of the rhizosphere, more consistent expression of beneficial traits under a broad range of soil conditions and antagonism to a large number of pathogens (Meyer and Roberts, 2002) [9].

Until today, no single method is found to be very effective and economical for the effective management of *fusarium* root-knot nematode wilt complex disease of tomato. Hence, an integrated approach would always ensure maximum suppression of the disease and higher yield without any deleterious effect on the ecosystem. In this context, it was thought that application of organic amendments could serve as the nutrient base for the antagonist and as well for the

crop. Similarly, application of micronutrients like manganese sulphate, zinc sulphate and ferrous sulphate can improve the vigour of the crop, which may useful for the indirect suppression of the disease. With this background, the present investigation was conducted with the induction of defense related enzymes in tomato plants pre-treated with biocontrol agents and challenge inoculated with pathogen

Materials and Methods

Preparation of bacterial inoculum

The bacterial isolates were grown on KB and NA broth with constant shaking at 150 rpm for 48 h. at room temperature 28±2 ºC. The bacterial cells were re-suspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu/ml (OD 593 = 0.3) and used as bacterial inoculum (Thompson, 1996) [36].

Seed Treatment with Antagonist (*Pseudomonas* sp. and *Bacillus* sp.)

Seeds of tomato (cv. PKM-1) were surface sterilized with two per cent sodium hypochlorite for 30 sec., rinsed in sterile dist. water and dried overnight. One gram of surface sterilized seeds were soaked in 10 ml of antagonistic suspension (containing $3x10^8$ cfu ml^{-1}) for 2 h and air dried overnight in a sterile Petri dish. To this, 100 mg of Carboxy methyl cellulose (CMC) was added as an adhesive material.

Induction of Defense Related Enzymes and Proteins as Influenced by Antagonists and Challenge Inoculation of *F. oxysporum* f. sp. *lycopersici*

Collection of plant samples

The tomato seeds pre-treated with different bioformulations were sown in earthen pots. At fifteen DAS, the pots were challenge inoculated with the pathogen and also treated with the inoculum suspension containing adequate cfu of the biocontrol agents. The leaves and roots samples were collected starting from zero to seven days after challenge inoculation of the pathogen and used for analysis.

Treatments

T_1: *P. fluorescens* + FOL
T_2: *B. cereus* + FOL
T_3: T_1 + T_3+ FOL
T_4: FOL inoculated
T_5: Healthy control

Enzyme Extraction

One g. of leaves and root sample were homogenized with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4 ºC. The homogenate was centrifuged for 20 min. at 10,000 rpm. The supernatant was used as crude enzyme extract for assaying chitinase activity. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL).

Peroxidase (PO)

Peroxidase activity was assayed as per the procedure described by Hammerschmidt *et al*. (1982) [14]. 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 N hydrogen peroxide. Enzyme extract (0.1 ml) was added to initiate the reaction, which was followed colorimetrically at 470 nm. Crude enzyme preparation were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme preparation served as blank. Activity was expressed as changes in absorbance at 470 nm min^{-1} g^{-1} of fresh tissue.

Polyphenol oxidase (PPO)

Polyphenol oxidase 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 µl of the enzyme extract. To start the reaction 0.01 M catechol was added and the activity was expressed as changes in absorbance at 470 nm min^{-1} g^{-1} of fresh tissue.

Phenylalanine Ammonia Lyase (PAL)

The PAL activity was assayed as per the method described by Ross and Sederoff (1992). The assay mixture containing 100 µl of enzyme of 50 mM Tris Hcl (pH 8.8) and 600 µl of 1 mm L-phenyalanine was incubated for 60 min. and 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 nmoles of cinnamic acid min^{-1} g^{-1} of fresh tissue.

β-1, 3 glucanase

The activity of β-1, 3 glucanase was assayed by the laminarin dinitrosalicylic acid method (Pan *et al*. 1991). The reaction mixture consisted of 62.5 µL of 4 per cent laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40 ºC for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic acid and heated for 5 min in boiling water, vortexed and the absorbance was measured at 500 nm. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissue.

Chitinase

The colorimetric assay of chitinase was carried out as per Boller and Mauch (1998) [4]. Reagents used were colloidal chitin, snail gut enzyme, dimethyl amino benzaldehyde (DMAB) and buffer (Annexure-1).

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 37ºC the reaction was stopped by centrifugation at 1000 rpm for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (W/V) snail gut enzyme for 1 h. After 1 h. the reaction mixture was brought pH 8.90 by the addition of 70 µl of 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 37ºC and immediately thereafter the absorbance was measured at 585 nm. N-acetylglucosamine (GlcNac) was used as a standard. The enzyme activity was expressed as nmoles Glc Nac equivalents Min^{-1} g^{-1} of fresh tissue.

Phenol Content

Phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One g. of leaves and root

fresh tissue was homogenized in 10 ml of 80 per cent methanol and agitated for 15 min. at 70ºC. One ml of the methanol extract was added to 5 ml of distilled water and 250 µl of Folin Cicocalteau reagent (1N) and the solution was kept at 25 ºC. After three min. one ml of saturated solution of Na_2 Co_3 and one ml of dist. water was added and the reaction mixture was incubated for 1 h. at 25 ºC. The absorption of the developed blue colour was measured using a spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reagent with a phenol solution (C_6 H_5 OH) and expressed as catechol equivalents g^{-1} of fresh tissue.

Induction of Defense Related Enzymes and Proteins as Influenced by Antagonists and Challenge Inoculation of *F. oxysporum* f. sp. *lycopersici* and Nematodes

A separate experiment was conducted with the same set of treatments as mentioned in the previous chapter. In this experiment, at fifteen DAS, the pots were treated with the inoculum suspension containing adequate cfu of the biocontrol agents and challenge inoculated with the pathogen and nematode. The samples of both root and leaves of tomato plants collected at different intervals were analysed for PR-proteins such as chitinase, β- 1, 3 glucanase and defense related enzymes viz., PO, PPO, PAL and total phenol content as per the methods described earlier.

Treatment details

T_1: Pf + FOL + Nem
T_2: Bc + FOL + Nem
T_3: Pl + FOL + Nem
T_4: T_1 + T_2 + T_3
T_5: FOL + Nem
T^6: Control

Results and Discussion

Studies on the Induction of Systemic Resistance as Induced by Antagonists and Challenge Inoculation of Pathogens

Peroxidase (Po) Activity - *Fusarium* Alone

Peroxidase was estimated from leaves and roots of tomato plants treated with bio control agents individually and in combination. Enzyme activity in the combination of treatments was found to be increased significantly when compared to individual antagonistic treatments. Variation in the induction of Po activity in leaves and roots are given in fig. 1 and fig.2, respectively. Po activity started increasing from the second day after application and reached its peak on the 5th day and then started declining both in the leaves and roots.

Among the various treatments, combination *viz*., Pf + FoL, Bc + FoL and Pf + BC + FoL were equally effective in increasing the Po activity in the leaves (2.21, 2.16 and 2.68 changes in absorbance/min/g of root tissue, respectively) compared to inoculated control (1.55 changes in absorbance/min/g of leaves tissue) on 5th day of the application of treatment. With respect to induction in roots, plants treated with Pf + Bc + FoL recorded maximum activity (2.56 changes in absorbance/min/g of tissue) which was on par with the following treatments such as Pf + FoL and Bc + FoL. In inoculated control, the activity noticed 1.44 changes in absorbance/min/g of root tissue on 4th day after the treatment.

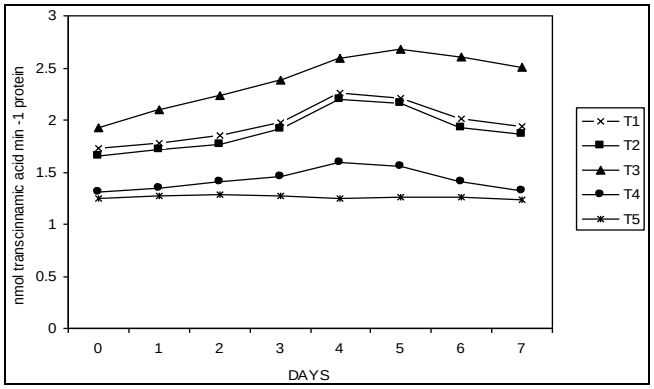

Fig 1: Peroxidase activity in tomato leaves in response to application of bio control agents and inoculation with *Fusarium oxysporum* (changes in absorbance min -1 mg Protein)

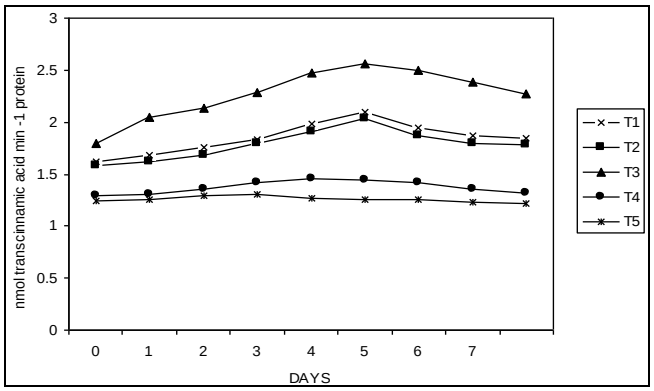

Fig 2: Peroxidase activity in tomato root in response to application of bio control agents and inoculation with *Fusarium oxysporum* (changes in absorbance min -1 mg protein)

Peroxidase (Po) Activity - *Fusarium* and *M. incogninta*

The bio control application in the fusarium wilt-nematode interaction system significantly increased the Po activity both in leaves and roots from first day after treatment and reached to a peak with 4 to 6 fold increase at 4th day after treatment compared to inoculated control. Thereafter, the Po activity decreased sharply but remained higher than the uninoculated control plants. Generally, root had higher activity of PPO than the leaves. In both the cases of leaves and roots the application of Pf + Bc + Pl along with FoL + RKN inoculation had registered higher level of Po activity than the other treatments (Fig. 3 and Fig.)

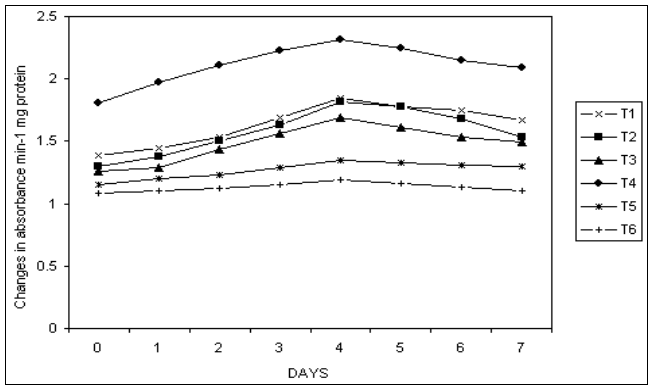

Fig 3: peroxidise acivity in tomato leaves in response to application with bio control agents and inoculation with Fusarium oxysporium and nematode (Changes in absorbance min-1 mg protein)

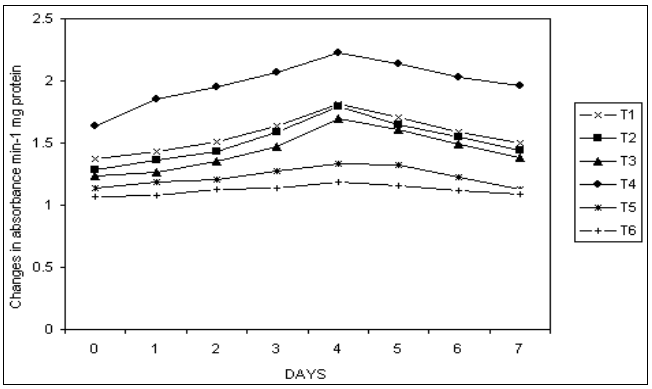

Fig 4: Peroxidase activity in tomato root in response to application with bio control agents and inoculation with *fusarium oxysporum* and Nematodes (Changes in absorbance min-1 mg protein)

The present study revealed that plants treated with combination of bio formulation and challenge inoculated with pathogen and nematode showed enhanced induction of peroxidase than individual antagonistic bio formulation treatments and pathogen and nematode alone inoculated plants. Peroxidases have been implicated in a number of physiological functions that may contribute to resistance including exudation of hydroxyl cinnamyl alcohol into free radical intermediates, phenol oxidation (Schmidt and Feucht, 1980).

Accumulation of peroxidase has been correlated with induced systemic resistance in several crops (Chen *et al.* 2000; Ramamoorthy and Samiyappan, 2001; Nandakumar *et al*. 2001; Bharathi, 2001; Vinod kumar *et al.* 2007) [7, 25 3, 37]. Thus the enhanced expression of peroxidase levels observed in the present study could be attributed as the reason for the suppression of *Fusarium* disease complex of tomato.

Generally biocontrol agents (fungal and bacterial antagonists) are known to induce resistance in plants against pathogens including nematodes; Oostendorp and Sikora, 1992; Devarajan and Rajendran, 2002; Sandeep, 2004; Kavitha, 2004; Krishnaveni, 2005; Shanthi and Rajendran, 2006) [10, 32, 18, 19, 35].

High level of expression of peroxidase was reported in *P.fluorescens* treated tomato plants challenged with *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al.* 2002) [30]. Suppression in the wilt incidence of cucumber and higher levels of defense enzymes Po and catalase were observed in plants treated with *T.viride* indicating that the production of phytoalaexin or lignin might be involved in disease suppression (Zhuang Jinghua *et al.* 2005) [43]. The increased PO activity has been correlated with resistance and these enzymes are involved in the polymerization of protein and lignin or suberin precursor into plant cell wall thus constructing a physical barrier that could prevent pathogen penetration of cell walls and movement through vessels (Bradley, 1992; Zacheo *et al.* 1993; Yedidia *et al.* 2003) [6, 41, 40].

Polyphenol Oxidase Activity (PPO)-*Fusarium* Alone

The effect of bio control agents on PPO activity in leaf and root were presented in fig. 5and fig.6 PPO activity was found to be induced in all the treatments compared to control. Integration of Pf + Bc + FoL resulted in the maximum induction of PPO activity in leaves on 4th day (3.23 changes in absorbance/min/g of leaf tissue), when compared to inoculated control (2.50 changes in absorbance/min/g of leaf tissue). Significant induction of PPO activity (3.19 changes in absorbance/min/g of root tissue) was observed in plants roots treated with Pf + Bc + FoL.

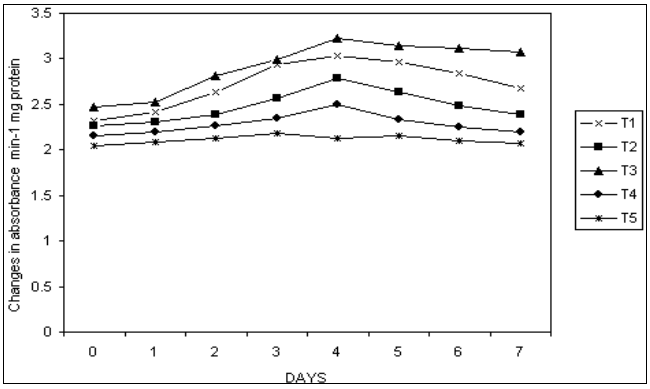

Fig 5: Induction of Polyphenol oxidase activity in tomato leaves in response application of biocontrol agents and inoculation with *Fusarium oxysporum* in tomato cv.

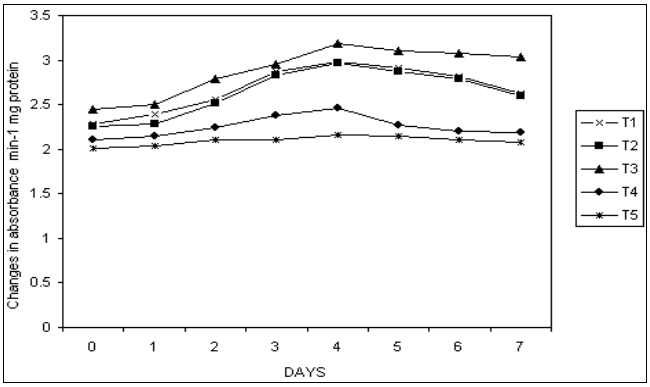

Fig 6: Induction of Polyphenol oxidase activity in tomato root in response application of biocontrol agents and inoculation with *Fusarium oxysporum* in tomato

Polyphenol oxidase (PPO) activity-*Fusarium* and *M. incogninta*

The effect of bio control along with FoL + RKN on PPO activity in leaf and root were presented in fig. 7 and fig 8. PPO activity was found to be induced in all the treatments compared to inoculated control. Integration of Pf + Bc + Pl + FoL + RKN resulted in the maximum induction of PPO activity on fourth day in both leaves and roots (3.25 and 3.23 changes in absorbance/min/g of tissue). Two fold increase in PPO activity was observed in both leaves and root tissue of tomato plants treated with the combination of Pf + Bc + Pl + FoL + RKN compared to inoculated control.

Enhanced PPO activity due to the application of bio control agents have been reported earlier by several workers., various rhizobacteria and *P. aphanidermatum* induced higher PPO activity in cucumber root tissues (Chen *et al*. 2000) [7]. *P. fluorescens* plus chitin bio formulation was found to enhance the activity of polyphenol oxidase and suppressed the incidence of anthracnose in mango leaves (Vivekananthan, 2004) [39]. Suppression in the wilt incidence of cucumber and higher levels of defense enzymes PAL, PO, PPO and catalase were observed in plants treated with antagonists indicating that the production of phytoalexin or lignin might be involved in disease suppression (Zhung Jinghuaea, 2005; Shanthi and Rajendran, 2006) [43, 35]. Thus in the present study also the combination of antagonists might have induced production of such phytoalexin or lignin which in turn resulted in the enhanced disease suppression.

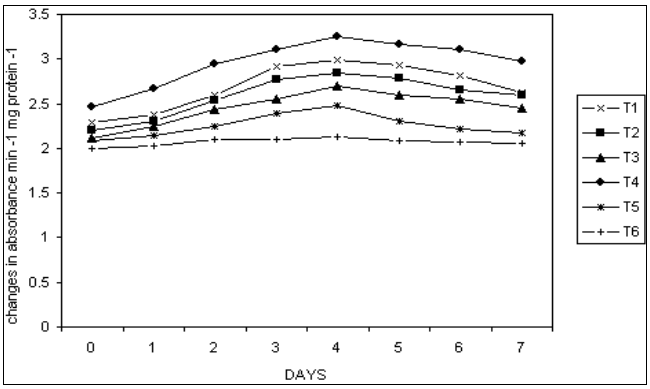

Fig 7: Polyphenol oxidase activity in tomato root in response application of biocontrol agents and inoculation with fusarium oxysporum and root-knot nematodes

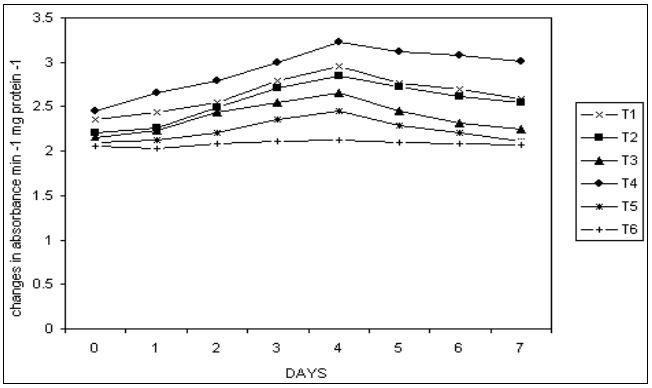

Fig 8: Polyphenol oxidase activity in tomato root in response application of biocontrol agents and inoculation with fusarium oxysporum and root-knot nematodes

Phenylalanine Ammonia Lyase (PAL)-*Fusarium* alone

All the treatments induced the PAL activity on 4th day compared to inoculated control and decreased thereafter. Combination of Pf + Bc + FoL exhibited significantly higher activity (21.89 n mol transcinnamic acid/min/g leaf tissue) which was followed by T_1 and T_2 (Fig. 9. Considering the PAL induction in roots, application of Pf + Bc + FoL recorded significantly maximum induction (21.83 n mol transcinnamic acid/min/g tissue) (10). This was followed by T_1 and T_2 when compared to inoculated control.

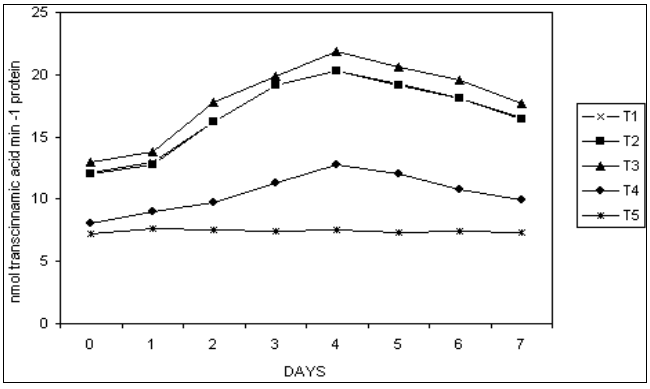

Fig 9: Phenylalanine ammonia lyases activity in tomato leaves in response to application with bio control agents and inoculation with *Fusarium oxysporium* (nmol transcinnamic acid)

Phenylalanine ammonia lyase (PAL)-*Fusarium* and *M. incogninta*

The activity of PAL increased sharply from 2nd day after treatment and reached the maximum on 4th day and thereafter declined in both leaves and roots of tomato plants applied with bio control agents and inoculated with FoL + RKN. In the leaves, the maximum increase was recorded in Pf + Bc + Pl applied plus FoL and RKN inoculated plants at 4th day after treatment. Thereafter the activity was decreased. In the case of roots also the maximum activity was observed in Pf + Bc + Pl + FoL and RKN inoculated plants which was followed by Pf + FoL + RKN, Bc + FoL + RKN and Pl + FoL + RKN, when compared to inoculated control. (Fig 11 and 12).

PAL activity could be induced in plant pathogen interactions and fungal ellicitor treatment (Ramanathan *et al.* 2000) [17]. Rhizosphere colonization of *P.aerunginoso* activated PAL in bean roots and increased the salicylic acid levels in leaves (De Meyer *et al.* 1997) [9]. Induction of PAL by fluorescent *Pseudomonas* was reported in sugarcane against *Colletotrichum falcatum* (Viswanathan and Samiyappan, 1999) [38] in cucumber against *P. aphanidermatum* (Chen *et al.* 2000) [7], in bean against Botrytis cinerea (Zdor and Anderson, 1992) [42] and in tomato against *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al.* 2002) [30]. Increased activity of PAL due to *P. fluorescens* treatment might have prevented the fungal invasion (Ramamoorthy *et al.* 2002) [30].

Higher activities of PAL were observed in *B.subtilis* and *P. chloroaphis* pretreated chilli plants challenge inoculated with *P. aphanidermatum* (Kavitha *et al.* 2005).

Thus the early and increased synthesis of PAL observed in the present study could be attributed as the reason for the increased growth parameters of tomato.

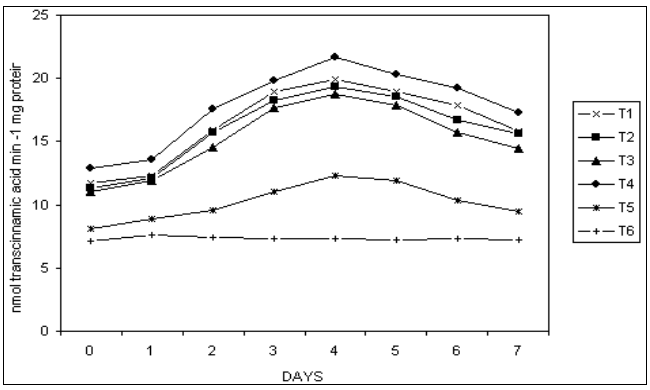

Fig 11: Phenylalanine ammonia lyase (PAL) activity in tomato leaves in response to application of biocontrol agents and inoculation with fusarium oxysporum and nematodes (nmol transcinnamic acid min -1 protein)

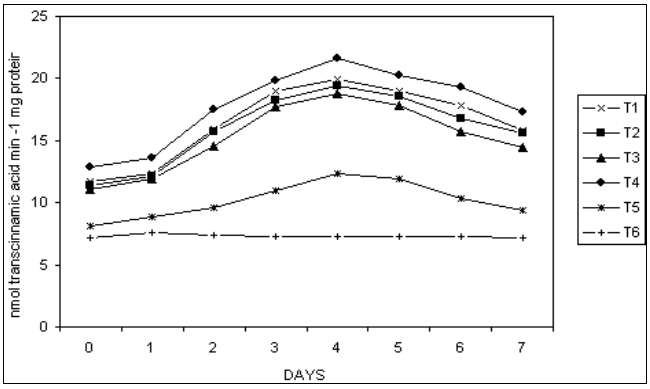

Fig 12: Phenylalanine ammonia lyase (PAL) activity in tomato root in response to application of biocontrol agaents andinoculation with fusarium oxysporum and nematodes nmol transcinnamic acid min-1 protein)

Phenolic content - *Fusarium* alone

The total phenolic content in leaves and roots of tomato

plants applied with antagonists increased significantly from 2nd day after treatment and reached maximum at 4th day and thereafter it decreased. The maximum phenolic accumulation (194.86µg cathechol mg protein -1) was observed in the fourth day in combined treatment which was followed by the treatment T_1 and T_2. The pathogen inoculated control also showed increased accumulation of phenolic but showed drastic decline after four days (Table1).

The results presented in (Table 28) revealed higher accumulation of phenolics in roots all the treatments challenge inoculated with the pathogen except healthy control. The maximum phenol content accumulation (194.72 µg cathechol mg protein^{-1}) was observed in combined treatment on the fifth day followed by the treatment with T_1 Pf (163.43 µg cathechol mg protein^{-1}) and T_2 (160.17 µg cathechol mg protein -1) alone. The pathogen inoculated control also showed increased accumulation of phenolics but showed drastic decline after fifth day.

Table 1: Effect of antagonists treatment and fusrium inoculation on the accumulation of total phenols in tomato root (µg cathechol mg protein-1)

T. No.	Treatments	No. of days								Mean
		0	1	2	3	4	5	6	7	
T_1	Pf + Fol	94.93	97.31	113.37	139.94	161.53	163.43	157.69	141.24	133.68
T_2	Bc + Fol	92.84	95.65	107.86	129.12	157.46	160.17	155.37	135.36	129.23
T_3	$T_1 + T_2$	97.48	99.56	117.51	133.12	180.46	194.72	165.64	149.43	142.24
T_4	Pathogen alone	93.63	95.54	100.07	112.02	127.09	122.63	118.56	114.64	110.52
T_5	Healthy control	94.57	97.02	98.32	103.43	102.18	100.31	99.23	99.84	99.36
	Mean	94.69	97.02	107.43	123.53	145.74	148.25	139.30	128.10	123.01

	T	D	TD
SE (d)	0.41186	0.52097	1.16492
CD (1%)	1.08757	1.37569	3.07613

Table 2: Effect of biocontrol agents and pathogens fusarium oxysporum and nematode inoculation on the accumulation of total phenol in tomato leaves (µg cathechol mg protein-1)

T. No	Treatments	No. of days								Mean
		0	1	2	3	4	5	6	7	
T_1	Pf + Fol + Nem	94.82	97.34	112.41	141.61	116.73	164.41	159.51	142.21	128.63
T_2	Bc + Fol + Nem	92.91	95.31	107.82	130.63	158.14	162.03	154.71	135.64	129.65
T_3	Pl + Fol + Nem	90.43	93.42	105.14	126.31	150.30	159.12	151.62	129.56	125.74
T_4	$T_1 + T_2 + T_3$	99.43	100.04	119.65	159.61	192.69	180.19	165.94	150.52	146.01
T_5	Fol + Nem	93.61	95.34	100.43	119.14	132.09	130.12	125.17	121.07	114.62
T_6	Healthy control	91.30	92.12	98.31	100.17	101.37	100.37	99.32	99.98	97.87
	Mean	93.75	95.60	107.29	129.58	141.89	149.37	142.71	129.83	123.75

	T	D	TD
SE (d)	0.64073	0.81047	1.81226
CD (1%)	1.69193	2.14015	4.78551

Total phenolics fusarium-*Fusarium* and *M. incogninta*

The phenolic content of both leaves and roots has increased markedly in response to the application of bio control agents and challenge inoculation with FoL + nematodes. The increase was noticed even on 2nd day after treatment and reached the maximum on 4th day and then decreased slightly at 5th days after treatment. In all these days of sampling 2 to 4 fold increase in the phenolic content was observed in both leaves and roots compared to uninoculated control. Either RKN + FoL inoculation also increased the phenolic content significantly compared to control but less than the bio control agents applied plants.

Among the different bio control agents used, the phenolic content of leaf was higher in Pf + Bc + Pl + FoL + RKN treatments followed by T_1 and T_2 treatments at 4th day of sampling. Whereas in the case of roots, the phenolic content was higher in Pf + Bc + Pl + FoL + nematode inoculated plants followed by T_1 and T_2 when compared to inoculated control. (Table 3 and 4)

Induced resistance in several crops is associated with enhancement of lignifications and also increased activities of enzymes involved in Phenyl Propanoid Pathway and PR protein synthesis (Hammerschmidt and Kue, 1995; Boller and Mauch, 1982).

Increase in the phenolics due to treatment with *P.fluorescens* and challenge inoculation with pathogen were reported in sugarcane against *C. falcatum* (Viswanathan and Samiyappan, 1999) [38], in tomato and hot pepper against *P. aphanidermatum* (Ramamoorthy *et al.* 2002) [30]. *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots against *P. ultimum* (Benhamou *et al.* 2000) [7]. Found that the per cent increase in phenolic compounds with respect to T. harzianum (Kan.) treated leaves over healthy and diseased plants in paddy brown leaf spot. The increase in the phenolics could also have played a major role in preventing infection by *Fusarium* and *M. incognita* which in turn resulted in reduced the disease incidence.

Table 3: Effect of biocontrol agents and pathogens fusarium oxysporum and nematode inoculation on the accumulation of total phenol in tomato root (µg cathechol mg protein-1)

T. No.	Treatments	No. of days								Mean
		0	1	2	3	4	5	6	7	
T_1	Pf + Fol + Nem	92.61	95.14	110.52	139.73	159.63	165.43	158.32	141.31	132.84
T_2	Bc + Fol + Nem	90.37	93.43	105.63	132.37	156.31	161.38	153.53	133.71	128.34
T_3	Pl + Fol + Nem	89.41	90.52	100.13	121.62	151.63	156.73	149.37	126.32	123.22
T_4	T_1 +T_2+ T_3	99.13	102.11	119.31	190.83	200.32	185.63	165.91	153.62	152.11
T_5	Fol + Nem	92.32	94.38	101.31	120.07	131.10	131.14	124.13	120.09	114.32
T_6	Healthy control	88.43	89.43	90.31	95.16	99.48	100.17	99.31	97.73	95.00
	Mean	92.05	94.17	104.54	133.30	149.75	150.08	141.76	128.80	124.30

	T	D	TD
SE (d)	0.63872	0.80793	1.80658
CD (1%)	1.68663	2.13344	4.77051

Table 4: Effect of biocontrol agents and pathogens *Fusarium oxysporum* and nematode inoculation on the accumulation of total phenol in tomato root (µg cathechol mg protein^{-1})

T. No.	Treatments	No. of days								Mean
		0	1	2	3	4	5	6	7	
T_1	Pf + FoL + RKN	92.61	95.14	110.52	139.73	159.63	165.43	158.32	141.31	132.84
T_2	Bc + FoL+ RKN	90.37	93.43	105.63	132.37	156.31	161.38	153.53	133.71	128.34
T_3	Pl + FoL + RKN	89.41	90.52	100.13	121.62	151.63	156.73	149.37	126.32	123.22
T_4	T_1 +T_2+ T_3	99.13	102.11	119.31	190.83	200.32	185.63	165.91	153.62	152.11
T_5	FoL + RKN	92.32	94.38	101.31	120.07	131.10	131.14	124.13	120.09	114.32
T_6	Healthy control	88.43	89.43	90.31	95.16	99.48	100.17	99.31	97.73	95.00
	Mean	92.05	94.17	104.54	133.30	149.75	150.08	141.76	128.80	124.30

	T	D	TD
SE (d)	0.63872	0.80793	1.80658
CD (1%)	1.68663	2.13344	4.77051

Chitinase activity - *Fusarium* alone

The chitinase activity was found to increase progressively in all the treatments (both leaves and roots) and the increase was observed up to four days in the plants treated with the bio control agents and challenge inoculated with the pathogen. Among the treatments, the maximum induction of chitinase (4.97 Nmol Glc Nac min-1 mg-1 protein) was observed in combined treatment on the fourth day followed by the treatment T_1 Pf (4.47 Nmol Glc Nac min-1 mg-1 protein) and T_2 (4.16 Nmol Glc Nac min-1 mg-1 protein). The pathogen inoculated control also showed increase in the activity of chitinase up to four day and thereafter showed decline (Fig. 13).

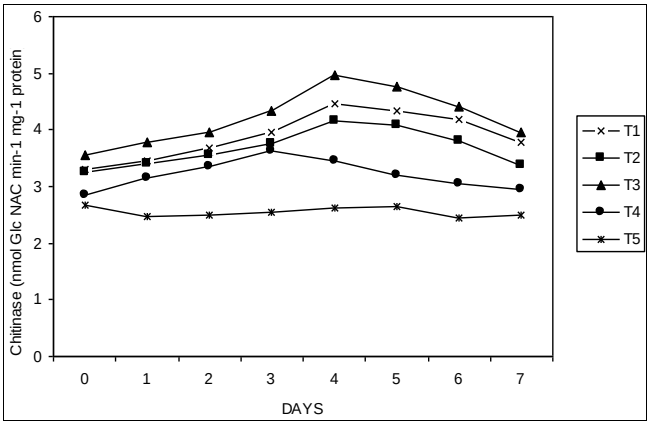

Fig 13: Introduction of chitinase in tomato leaves in response to treatment with biocontrol agent and inoculation with fusarium oxsporum (nmol Glc NAC min-1 mg-1 protein)

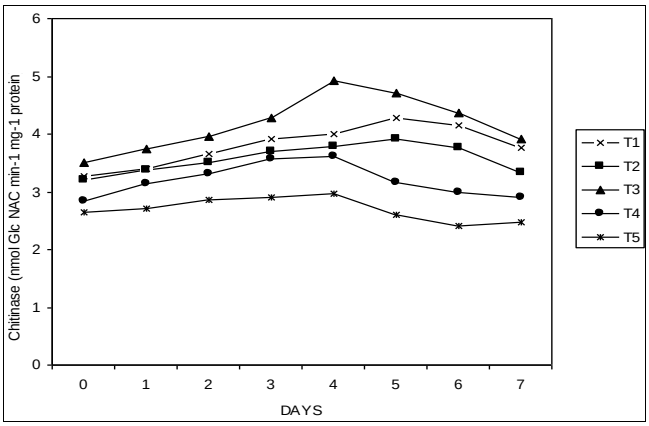

Fig 14: Introduction of chitinase in tomato root in response to treatment with biocontrol agent and inoculation with fusarium oxysporum (nmol NAC min-1 protein)

The expressions of chitinase activity in roots due to various treatments are furnished in fig. 14. The results revealed that Pf + Bc + FoL registered higher activity of chitinase 5th day after treatment followed by Pf + FoL and Bc + FoL which are equally effective. The enzyme activity increased up to 5th day and then it started decreasing.

Chitinase activity - *Fusarium* and *M.incogninta*

The activity of chitinase in both leaves and roots was increased from 2 days after application of treatments and reached a maximum of two fold at 4th day after treatment compared to control. Thereafter, the activity of the enzymes has declined. In the leaves, the maximum induction of chitinase was observed in Pf + Bc + Pl + FoL + RKN

treatment similarly in also roots the maximum activity of chitinase was observed in T_4 (Pf + Bc + Pl + FoL + RKN) applied plants at 4th day after the treatments **(**Fig15 and 16).

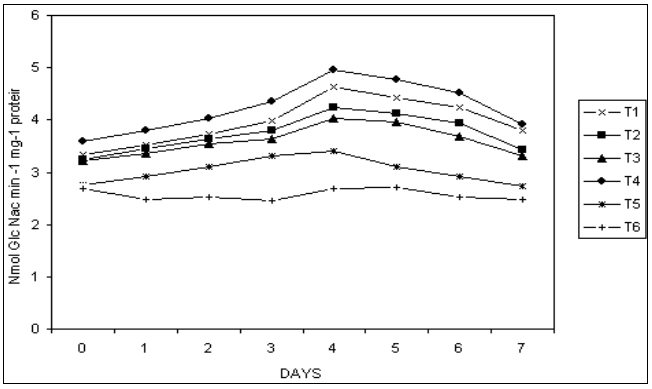

Fig 15: Induction of chitinase in tomato leaves in response application of bio control agents and inoculation with fusarium oxysporum and nematodes

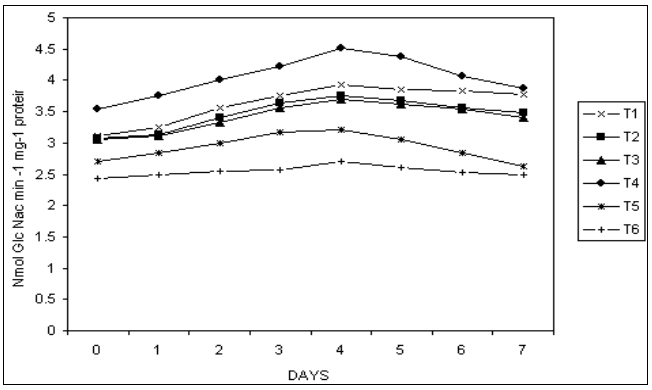

Fig 16: Induction of chitinase in tomato root in response application of bio control agents and inoculation with fusarium oxysporum and nematodes

β-1, 3 glucanase activity - *Fusarium* alone

Generally the activity of β-1, 3 glucanase increased significantly in all the treatments (both leaves and roots) and the increase up to fifth day in combined treatment and thereafter it declined. The combined treatment recorded higher β-1, 3 glucanase activity when compared to individual antagonist treatments. Maximum glucanase activity was observed in the combined treatment of Pf + Bc + FoL treated plants (20.77 µg glucose min-1) followed by T_1 (18.29 µg glucose min-1) on the fifth day and T_2 (18.25 µg glucose min-1) treated plants. The plants inoculated with the pathogen alone also showed increased glucanase activity up to fifth day and thereafter declined (Fig. 17).

Fig. 20 depicts the induction of β-1, 3 glucanase activity in root tissues of tomato in various treatments. The root of tomato treated with Pf + Bc + FoL expressed the maximum activity (20.65 µg glucose/min/g of fresh tissue) on fifth day after treatment and significantly differed from other treatments and this was followed by Pf + FoL (18.19 µg of glucose/min/g of fresh tissue). The expression of β-1, 3 glucanase observed in all the treatments was higher than in inoculated control. The activity reached its maximum on 5th day after the treatment of bio agents and then started declining.

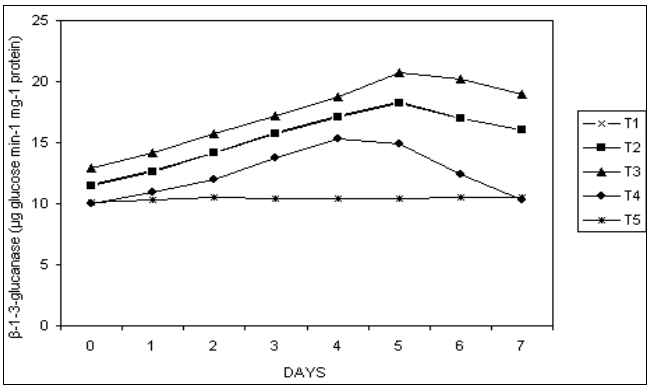

Fig 17: Induction of β-1-3-glucanase in tomato leaves in response to treatment with bio control agents and inoculation with Fusarium oxysporum (µg glucose min-1 mg-1 protein)

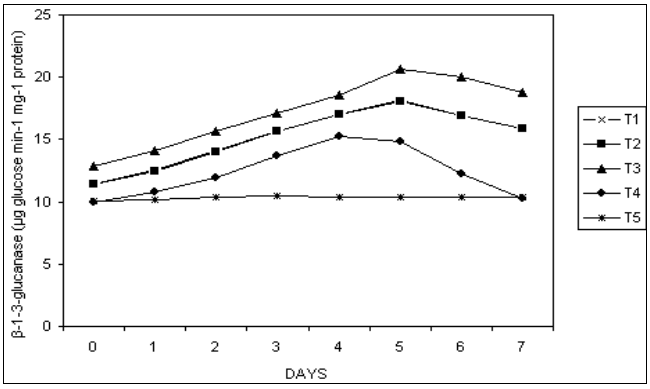

Fig 18: Induction of β-1-3-glucanase in tomato root in response to treatment with bio control agents and inoculation with fusarium oxysporum. (µg glucose min-1 mg-1 protein).

β-1, 3 glucanase activity - *Fusarium* and *M. incogninta*

Generally, challenge inoculation of FoL + RKN in the antagonists applied plants resulted in higher activity of β-1, 3 glucanase in both leaves and roots compared to uninoculated control plants (Fig. 19 and Fig. 20). In the leaves the maximum activity was observed in Pf + Bc + Pl + FoL + RKN inoculated plants which was followed by T_1 and T_2. In the case of roots more than 5 fold increase in β-1, 3 glucanase activity was observed by T_4 followed by T_1 and T_2, when compared to control. After 4th day of treatment, the β-1, 3 glucanase decreased sharply in both leaves and roots but 2 to 3 fold higher than the uninoculated control.

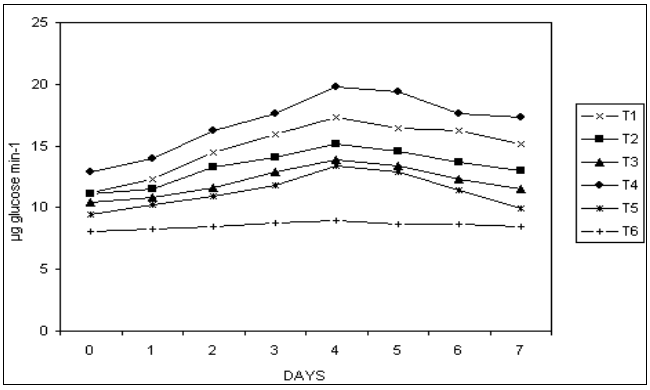

Fig 19: Induction of β-1, 3 – glucanase in tomato leaves in response to application of bio control agents and inoculation with fusarium oxysporum and nematodes

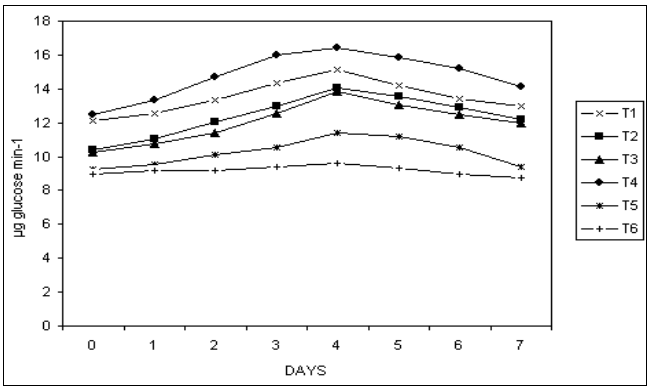

Fig 20: Induction of β-1,3 – glucanase in tomato root in respone to application of bio control agents and inoculation with *fusarium oxysporum* and nematodes agents and inoculation with *fusarium oxysporum* and nematodes

Synthesis and accumulation of PR proteins have been reported to play and important role in plant defense against attack of pathogens. Production of lytic enzymes like chitinase and β-1, 3 glucanase by certain antagonists forms the basis for control of plant pathogenic fungi in the rhizosphere (Mauch and Staetielin, 1998) [4]. Chitinases and β -1, 3 glucanases (which are classified under PR-3 and PR-2 groups of PR proteins, respectively) have been reported to be associated with plants resistance against fungal pathogens (Maurhofer *et al.* 1994).

Normally fungal cells contain chitin and glucan as their cell wall constituent. The main mode of action of antagonistic activity of microbes are production of lytic enzymes (chitinase and β -1, 3 glucanase) which act on cell walls of organisms which have chitin or glucan as their cell wall component (Singh *et al.* 1999) [29] and also through induced systemic resistance (ISR) in plant system. The results of the present study revealed enhanced levels of chitinase and β-1, 3 glucanase activities in plants treated with combination of bio control agents.

The results are in agreement with the findings of several earlier workers. In tobacco, induction of two PR proteins *viz.,* β -1, 3 glucanase and chitinase were noticed due to application of *P. fluorescens* in response to infection by tobacco necrosis virus (Maunhofer *et al.* 1994). Induction of these hydrolytic enzymes was also reported in BCA against *P. ultimum* and *F. oxysporum* f.sp. *pisi* (Benhamou *et al.* 1996) [2]. The enzymatic degradation of the fungal cell wall by hydrolytic enzymes may release non-specific elicitors (Ren and West, 1992) which in turn elicit various defense reactions. The fungal cell wall elicitors have been reported to elicit various defense reactions in greengram (Ramanathan *et al.* 2000) [17]. Viswanathan and Samiyappan (1999) [38] reported that ISR by fluorescent *Pseudomonas*, which was associated with induction of chitinase appear to be a promising technology for the management of red rot of sugarcane. Higher level of the activities of β -1,3 glucanase and chitinase was observed in *B. subtilis* and *P. chlorotaphis* pretreated chilli plants challenge inoculated with *P. aphanidermatum* (Kavitha *et al.* 2005). Plants triggered by inoculation with *Exobasidiem vexans* showed a general increase in the chitinase and β -1,3 glucanase activity within 24 h of inoculation (Vinod kumar *et al.* 2007) [37].

Chitnase plays an important role in controlling nematode as well as *Fusarium* infection. Induction of chitinase by soil micro-organism was reported to destroy eggs and egg masses of plant parasite nematodes (Gooday, 1983) [13]. The enhanced phenolic content and PR proteins in response to application of combination of antagonists in tomato might have acted directly on hyphae of the fungus and also on the juvenile development of nematodes within the egg during embryogenesis. These types of actions thus resulted in reduction of both *Fusarium* wilt incidence and root lesions and root - knot caused by nematodes.

Reference

1. Anonymous. Hort. Stat., Directorate of Horticultural and Plantation Crops, Chennai, 1997.
2. Benhamou N, Kloepper JW, Quadt-Hallman A, Tuzun S. Induction of defense-related ultra-structural modifications in pea root tissues inoculated with entophytic bacteria. Plant physoil. 1996; 112:919-929.
3. Bharathi R. Development of a rhizobacteria based bio-formulation for the management of major pests and diseases in chillies. M.Sc. (Ag.) thesis, Tamil Nadu Agricultural University, Coimbatore-3, India, 2001.
4. Boller T, Mauch F. Colorimetric assay for chitinase. Meth. Enzymol. Planta. 1998; 157:22-31.
5. Bora T. *In vitro* and *in vivo* investigation on the effect of some antagonistic fungi against the damping-off disease of eggplant. J Turkish Phytopath. 1977; 6:17-23.
6. Bradley DJ, Kjellborn P, Lomb C. Elicitor and wound induced oxidative cross-linking of a plant cell wall proline-rich protein: A novel, rapid defense response. Cell. 1992; 70:21-30.
7. Chen C, Belanger RR, Benhamou N, Paullitz TC. Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR). Physiol. Mol. Plant Pathol. 2000; 56:13-23.
8. Cook RJ, Baker KF. The nature and practice biological control of plant pathogens. The American Phytopathological Society, St. Paul, MN, 1983, 539.
9. De Meyer G, Hofte M. Salicylic acid produced by the rhizobacterium *Pseudomonas aervainosa* 7 NSK2 induces resistance to leaf infection by *Botrytis cinerea* on bean. Phytopathology. 1997; 87:588-593.
10. Devrajan K, Rajendran G. Biochemical alterations in resistant and susceptible banana clones due to the burrowing nematode. Indian Journal of Nematology. 2002; 32:159-161.
11. Dharamveer Duhan, Partap PS, Rana MK, Dudi BS. Growth and yield performance of genotypes in a line x tester set of tomato. Haryana Journal of Horticultural Science. 2005; 34:357-361.
12. Dubey SC, Patel B. Evaluation of fungal antagonists against *Thanetephorus cucumeris* causing web blight of urd and mung bean. Indian Phytopathol. 2001; 54:206-209.
13. Godoy G, Kabana Rodriguez R, Moraan-Jones G. Fungal parasites of *Meloidogyne arenario* eggs in an Alabama soil. A Mycological Survey and Green house studies. Nematropica. 1983; 13:201-213.
14. Hammerschmidt R, Nuckles EM, Kuc J. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol. Plant Pathol. 1982; 20:73-82.
15. Harman GE. Seed treatments for biological control of plant diseases. Crop Prot. 1991; 10:166-171.
16. Jain RK, Paruthi JJ, Gupta DC. Estimation of yield loses in tomato due to root-knot nematode. Indian J Nematol. 1994; 24:140-145.
17. Jayashree K, Shanmugam V, Raghuchandar J,

Ramanathan A, Samiyappan R. Evaluation of *Pseudomonas fluorescens* (Pf-1) against black gram and sesame root rot disease. J Biol. Control, 2000; 14:55-61.
18. Kavitha. Management of Root-knot nematode *Meloidogyne incognita* with plant growth promoting rhizobacteria in Tomato. M.Sc (Ag). Thesis TNAU, 2004.
19. Krishnaveni Management of spiral nematode *Helicotylenchus multicinctus* (Cobb, 1893) Golden 1956, in banana (Musa spp.) cv. nendran using plant growth promoting rhizobacteria. Ph.D Thesis TNAU, 2005.
20. Lukyanenko AN. Disease resistance in tomato in genetic improvement of tomato (Ed. Kalloo, G.). Monographs on theoretical and Applied Genetics-14, Springer Verlag, Berlin Heidelberge, 1991, 99-119.
21. Mai WF, Abawi GS. Interaction among root-knot nematodes and *Fusarium* wilt fungi on host plants. Ann. Rev. Phytopath. 1987; 25:317-318.
22. Maurhofer M, Hase C, Meuwly P, Metraux JP, Defago, G. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHAO: Influence of the gacA gene and of pyoverdine production. Phytopathology, 84: 139-146.
23. Mayer, A.M., Harel, E. and Shaul, R.B. 1965. Assay of catechol oxidase a critical comparison of methods. Phytochemistry. 1994; 5:783-789.
24. Meyer SLF, Roberts DP. Combinations of bio control agents for management of plant parasitic nematodes and soil borne plant pathogenic fungi. J Nematol, 2002; 34:1-8.
25. Nandakumar R, Babu S, Viswanathan R, Sheela J, Raguchander T, Samiyappan R. A new bio-formulation containing plant growth promoting rhizobacterial mixture for the management of sheath blight and enhanced grain yield in rice. Bio control. 2001; 46:493-510.
26. Oostendorp M, Sikova RA. Utilization of antagonistic rhizobacteria as a seed treatment for the biological control of *Heterodera schachtii* in sugar beet. Rev. Nematol. 1989; 12:77-83.
27. Padmodaya B, Reddy HR. Screening of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *lycopersici* causing wilt in tomato. Indian J Mycol. Pl. Pathol. 1996; 26:266-270.
28. Pan Q, Te YS, Kuc J. A technique for the detection of chitinases. β-1, 3-glucanase and protein patterns after single separation using PAGE or isoelectric focusing. Phytopathology, 1991; 81:970-974.
29. Pathack GR, Gupta ML, Singh HB, Kumar S. The influence of vesicular-arbuscular mycorrhizal fungi alone or in combination with *Meloidogyne incognita* on *Hyoscyamus niger* L. Bioresource Technol. 1999; 69:275-278.
30. Ramamoorthy V, Raguchander T, Samiyappan R. Enhancing resistance of tomato and hot pepper to *Pythium* disease by seed treatment with fluorescent pseudomonads. European J Plant Pathol. 2002; 108:429-441.
31. Ross WW, Sederoff RR. Phenylalanine ammonia lyase from loblolly pine: Purification of the enzyme and Isolation of Complementary DNA clones. Plant Physiol. 1992; 98:380-386.
32. Sandeep A. Bioefficacy of *Pseudomonas fluorescens* (Native Isolates) on *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 in Banana (*Musa* spp.) M.Sc. Thesis, Tamil Nadu Agricultural University, Coimbatore, 2004.
33. Sasser JN. Economic importance of *Meloidogyne* in tropical countries, In: Root-knot news (*Meloidogyne* spp.)-Systematics, biology and control. (Eds. F Lambertia and C.E. Taylor), Academic Press, New York, 1979, 477: 359-374.
34. Schonbeck F, Dehne HW, Beicht W. Untersuchungen Zur Aktivieruna Unspezifischer. Resistenz-Mechanismen in Pflanzen. Z. Pflanzenk. Pflanzen. 1980; 87:654-664.
35. Shanthi A, Rajendran G. Induction of systemic resistance in banana against lesion nematodes by biocontrol agents. International Journal of Nematology. 2006; 16:75-78.
36. Thompson DC. Evaluation of bacterial antagonists for reduction of summer patch symptoms in Kentucky blue grass. Plant Dis. 1996; 80:856-862.
37. Vinod Kumar VB, Chauhan and Srivastava JP. Pathogenic and biochemical variability in *Fusarium udum* causing pigeon pea wilt. Indian Phytopath. 2007; 60:281-288.
38. Viswanathan R. Induction of systemic resistance against Red Rot Disease in Sugarcane by plant growth promoting Rhizobacteria. Ph.D. Thesis, Tamil Nadu Agricultural University, Coimbatore, India, 1999, 175.
39. Vivekanandhan R, Ravi M, Sible GV, Prakasam V, Samiyappan R. *Pseudomonas fluorescens* (FP-7) amended with chitin bioformulation for the management of anthracnose pathogen in mango cultivation Alphonso. Madras Agric. J, 2004; 91:475-482.
40. Yedidia I, Shoresh M, Kerem Z, Benmou N, Kapulink Y, Chet I. Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* and accumulation of phytoalexins. Appl Environ. Microbiol. 2003; 69:7343-7353.
41. Zacheo G. Introduction in Nematode interaction. (ed. M.W. Khan). Chapman and Hall, London, 1993, 1-25.
42. Zdor RE, Anderson AJ. Influence of root colonizing bacteria on the defence responses in bean. Plant and Soil. 1992; 140:99-107.
43. Zhuang Jinghua, Gao Zenggui, Liu Xian, Chen Jie, Yang YV. Effect nutrition elements on bio control efficiency of *Trichoderma* against melon wilt. Acta Phytophylacica Sinca. 2005; 31(4):359-364.