Role of defense enzymes activity in tomato as induced by *Trichoderma virens* against *Fusarium* wilt caused by *Fusarium oxysporum* f sp. *lycopersici.*

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ABSTRACT

Tomato (*Lycopersicon exculentum* L.) is a popular vegetable widely grown in the tropics, wthich is mainly attacked by *Fusarium* wilt incited by *Fusarium* o*xysporum* f sp. *lycopersici.* In this present scenario, ecofriendly alternative strategies such as use of fungi from rhizosphere and endophytic bacteria are being explored. Fungal antagonistic *Trichoderma* spp. are effective for the management of soil borne plant pathogens. Efficacy of various isolates of *T.virens* were evaluated under green house condition for efficacy in suppressing incidence of *Fusarium* wilt disease and promoting plant growth in tomato. Among the various isolates tested, native isolates of *T.virens* (Tv_1) increased the plant growth and highly inhibited the mycelial growth of the pathogen under *in vitro* condition. In green house studies seed treatment plus soil application of talc based formulation of *T.virens* (Tv_1) significantly reduced incidence of the diseases (54.66% more efficient than control), compared to the other isolates of *T.virens*. Expression of various defence related enzymes was found involved in the induction of systemic resistance against pathogen infection. Tomato plants treated with seed @ 4gkg^{-1} plus soil application of 4kg ha^{-1} of talc based formulation of *T. virens* (Tv_1) with challenge inoculation of *Fusarium* enhance the maximum induction of defense enzyme such as Peroxidase (PO), Polyphenol Oxidase (PPO) and Phenylalanine Ammonia Lyase (PAL) rather than the other isolates of *T.virens*. The enzyme activity increased from 7th day of sampling and the maximum was observed on 14th day of sampling and then it slightly decreased.

Key words: Defense enzyme, *Trichoderma virens*, Tomato.

INTRODUCTION

Tomato (*Lycopersican exculentum* L.) is a popular vegetable widely grown in the tropics which is an excellent source of vitamin A and vitamin C, minerals like iron and phosphorus. In tropical Asia it is an important cash crop from small farmers (Villareal, 1979). It tops the list of industrial crops because of its outstanding processing quantities. Total area under tomato in India is about 3.56 lakhs hectare with an average yield of 15.7 tonnes per hectare. In Tamil Nadu the area under tomato cultivation is 21055 hectare with an average yield of 11.04 tonnes per hectare (Vakes and Singhal, 2005). The crop is affected by several fungal pathogens, of which *F. oxysporum* f sp. *Lycopersici* inciting wilt disease is a major constraint in the production of Tomato. *Fusarium* sp. are essentially soil borne (Berkley, 1925; Horsfall, 1930) The wilt incidence was reported to an extent of 25 percent in Tamil Nadu in various cultivars of tomato (Kapoor *et al.,* 1981). The most common method to check the disease is by using fungicides, but frequent and indiscriminate use of fungicide leads to atmospheric pollution and development of fungicide resistance in pathogens. In recent years, biological control has become a promising alternative to chemical control in the management of soil borne disease (Harman *et al.,* 2004). The Biocontrol agents bring about induced systemic resistance (ISR) fortifying the physical and mechanical strength of cell wall and changing physiological and biochemical reaction of host leading to synthesis of defense chemicals against challenge inoculation of pathogens. Defence reaction occurs due to accumulation of PR proteins such as chitinase, â-13-glucanes, phenylalanine ammonia lyase, peroxidase, phenolics and phytoalexins (Kloepper *et al.,* 1992). The aim of the present study was to evaluate the induction of defense enzyme by seed and soil application of antagonistic nature against *Fusarial* wilt

MATERIALS AND METHODS

Diseases incidence and vigour index

The efficacy of seed and soil application of *T. virens* was evaluated in pot culture experiment laid out in Randomized block designs with ten treatments at Faculty of Agriculture, Annamalai University, Annamalai Nagar as

detailed below and were replicated thrice. The treatments were T_1= Seed treatment with *T.virens* (Tv_1) @ 4gm kg^{-1} seed. T_2 =Seed treatment with *T. virens* (Tv_2) @ 4gm kg^{-1} seed, T_3 = Seed treatment with *T.virens* (Tv_3) @ 4gm kg^{-1} seed, T_4 = Seed treatment with *T.virens* (Tv_4) @ 4gm kg^{-1} seed, T_5 = Seed treatment @ 4gm kg^{-1} seed plus soil application @ 6 kg ha^{-1} of *T.virens* (Tv_1), T_5 = Seed treatment @ 4gm kg^{-1} seed plus soil application @ 6 kg ha^{-1} of *T.virens*(Tv_1), T_6 = Seed treatment @ 4gm kg^{-1} seed plus soil application @ 6 kg ha^{-1} of *T.virens* (Tv_2), T_7 = Seed treatment @ 4gm kg^{-1} seed plus soil application @ 6 kg ha^{-1} of *T.virens*(Tv_3), T_8 = Seed treatment @ 4gm kg^{-1} seed plus soil application @ 6 kg ha^{-1} of *T.virens* (Tv_4), T_9-=Carbendazim 50 % WP @ seed treatment @ 2gm kg^{-1} seed plus soil application @ 0.02%. T_{10} =Control. *T. virens* (Tv_1), *T. virens* (Tv_2) and *T. virens* (Tv_3) were isolated from Cuddalore, Tindivanam and Aduthurai respectively and *T. virens* (Tv_4) was isolated from Annamalai University experimental farm, Annamalainagar. Which was formulated with talc powder and the formulation containing 3×10^8 cfu ml^{-1} was used for present study.

Eastern pots of 1 ft dia were filled with 5kg soil and inoculum *F. oxysporum* f sp. *Lycopersici* multiplied in sand maize medium, was mixed with soil @ 50 g $kg.^{-1}$ five seedlings were transplanted in the each pots with mentioned above treatment. The percent disease incidence was recorded periodically at 15 days interval using the formula

$$\text{Percent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total no. of plants}} \times 100$$

The germination percentage and vigour index were calculated by using the formula Germination (%) =

$$\frac{\text{Number of seeds germinated}}{\text{Total no. of seeds sown}} \times 100$$

Vigour index = shoot length + root length × germination percentage.

Enzyme studies

Collection of plant samples: The tomato seeds pre-treated with talc based formulation of *T. virens* were sown in earthen pots. The pots were challenge inoculated with the pathogen and also treated with bio control agents. The samples were collected starting from 0 to 15 days after challenge inoculation of the pathogen. Four plants samples were collected from each replication of the treatment separately and used for analysis.

For enzyme extraction one g of root sample was homogenized with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 2.0 minutes at 10000 rpm. The supernatant was used as a crude extract for enzyme 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 Pheny lalanine Ammonia Lyase (PAL). Enzyme extract was stored in deep freezer (-70°C) utilized for biochemical analysis.

Assay of peroxidase (PO) 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 butter, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1 ml) 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

Table 1. Effect of various isolate of *T. virens* on germination, vigour index and disease incidence of tomato on Pot culture experiment

T. No.	Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour index	Disease incidence (%)		
						30Days	60Days	90Days
T_1	Seed treatment with Tv_1	84(66.42)	6.8	3.8	890.0	16(23.57)	8.80(7.25)	6.50(14.77)
T_2	Seed treatment with Tv_2	85.2(66.81)	7.5	4.5	1034.43	15.10(22.80)	8.4(16.92)	6.10(14.29)
T_3	Seed treatment with Tv_3	84.5(66.81)	7	4	929.46	16.50(23.96)	8.80(17.25)	6.72(15.20)
T_4	Seed treatment with Tv_4	86.2(68.19)	8.1	4.8	1148.06	14.2(12.13)	8.1(16.53)	5.9(14.05)
T_5	Seed + Soil application of Tv_1	89(70.63)	8.05	5.0	1161.45	10.62(19.10)	7.6(15.81)	4.7(12.52)
T_6	Seed + Soil application of Tv_2	90(71.56)	8.1	5.0	1179.0	10.96(19.12)	7.2(15.10)	3.89(11.38)
T_7	Seed + Soil application of Tv_3	89.8(70.71)	8.2	4.93	1194.53	10.98(19.35)	7.4(15.78)	4.6(12.34)
T_8	Seed + Soil application of Tv_4	93(94.65)	8.4	5.11	1215.0	9.7(18.12)	6.93(13.26)	3.71(11.33)
T_9	Carbendazim 50 % WP	93.1(74.68)	6.8	4.0	1005.48	9.9(18.09)	7.10(13.41)	3.68(11.21)
T_{10}	Control	71.0(54.33)	4.6	4.1	617.7	41(90.14)	17.20(24.50)	10.10(18.53)
	SE.D	0.7075	0.0923	0.5555	14.1822	1.0340	0.3121	0.3671
	CD (p = 0.05)	1.4221	0.1855	0.1165	28.5062	2.2031	0.6272	0.7271

Figures in parenthesis are arcsine transformed value

Table 2. Induction of peroxidase activity in tomato crop treated with *T. virens* against *F.oxysporum* f.sp. *Lycopersici* under greenhouse condition

Treatments	Change in absorbance/min/g^{-1} units					
	0 day	3st day	6rd day	9th day	12th day	15th day
T_1 - S.T. + S.A of *T. virens* (Tv_1)	0.53cd	0.74bc	0.81c	0.92c	0.76d	0.54d
T_2 - S.T. + S.A of *T. virens* (Tv_2)	0.65b	0.73bc	0.98b	1.27b	0.95b	0.76bc
T_3 - S.T. + S.A of *T. virens* (Tv_3)	0.57c	0.77b	0.81c	0.91c	0.86c	0.79b
T_4 - S.T. + S.A of *T. virens* (Tv_4)	0.76a	0.96a	1.23a	1.63a	1.33a	1.00a
T_5 - Inoculated control	0.33e	0.43d	0.46d	0.52d	0.40c	0.31e

Values are mean of three replications; In a column, means followed by a common letter are not significantly different at the 5 % levels by DMRT; S.T. – Seed treatment; S.A. – Soil application

absorbance units/min. The boiled enzyme preparation served as blank. Activity was expressed as the increase in absorbance at 480 nm min^{-1} mg^{-1} of protein.

Polyphenol oxidase (PPO) 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 butter (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 change in absorbance (in units) min^{-1} mg^{-1} of protein.

The Phenylalanine ammonia lyase (PAL) assay was carried out 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 1 mM L-phenylalanine 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 in µ moles of cinnamic acid min^{-1} mg^{-1} of protein. The data gathered were analysis the method suggested by Gomez and Gomez (1984).

RESULTS

Disease incidence and vigour index

The effect of various strains of *T. virens* on the seed germination and vigour index of tomato plant was studied and the data are furnished in table 1. Maximum germination percentage of Tomato seeds (93 percent,) was absorbed in seed plus soil application of native isolates of *T. virens* (T_4). The same treatment recorded the maximum vigour index (1215.0) also. It was followed by seed plus soil application of (T_2).

Incidence of *Fusarial* wilt was recorded at regular intervals. Among the various isolates of *T. virens* tested, the minimum diseases incidence of 9.7,6.93 and 4.6 pre cent were observed on 30th,60th and 90th day respectively due seed plus soil application of native isolate of *T. virens* (Tv_4) (T_8).which was on par with chemical treatment (T_9). Generally seed plus soil application of bio agents significantly reduce the diseases incidence rather than the seed treatment alone. All the treatments significantly decrease diseases incidence rather than the control.

Table 3. Induction of polyphenol Oxidase activity in tomato crop treated with *T. virens* against *F.oxysporum* f.sp. *Lycopersici*under greenhouse condition

Treatments	Change in absorbance/min/g^{-1} units					
	0 day	3st day	6rd day	9th day	12th day	15th day
T_1 - S.T. + S.A of *T. virens* (Tv_1)	0.57c	0.64c	0.72c	0.80c	0.62c	0.59d
T_2 - S.T. + S.A of *T. virens* (Tv_2)	0.60bc	0.77a	0.85bc	0.95b	0.83b	0.79b
T_3 - S.T. + S.A of *T. virens* (Tv_3)	0.50d	0.55d	0.64d	0.69d	0.64d	0.59cd
T_4 - S.T. + S.A of *T. virens* (Tv_4)	0.69a	0.75ab	1.13a	1.52a	1.31a	0.96a
T_5 - Inoculated control	0.42e	0.43d	0.57e	0.69d	0.56d	0.52d

Values are mean of three replications; In a column, means followed by a common letter are not significantly different at the 5 % levels by DMRT, S.T. – Seed treatment;S.A. – Soil application

Table 4. Induction of phenylalanine Ammonia Lyase activity in tomato crop treated with *T. virens* against *F.oxysporum* f.sp. *Lycopersici* under greenhouse condition

Treatments	Change in absorbance/min/g^{-1} units					
	0 day	3st day	6rd day	9th day	12th day	15th day
T_1 - S.T. + S.A of *T. virens* (Tv_1)	56.32[b]	79.85[b]	93.63[b]	122.43[b]	114.33[b]	74.63[c]
T_2 - S.T. + S.A of *T. virens* (Tv_2)	54.42[b]	76.23[c]	84.90[c]	109.33[c]	89.97[c]	96.41[d]
T_3 - S.T. + S.A of *T. virens* (Tv_3)	38.46[d]	45.23[d]	47.42[d]	65.44[e]	61.08[e]	59.86[e]
T_4 - S.T. + S.A of *T. virens* (Tv_4)	59.92[a]	86.52[a]	98.43[a]	136.33[a]	120.14[a]	99.26[a]
T_5 - Inoculated control	41.32[c]	53.54[d]	72.62[d]	89.63[d]	72.33[d]	64.68[d]

Values are mean of three replications; In a column, means followed by a common letter are not significantly different at the 5 % levels by DMRT
S.T. – Seed treatment; S.A. – Soil application

Peroxidase (PO)

In greenhouse condition the activities of peroxidase was observed in root sample of Tomato at different day's interval, Among the various treatments, application of *T. virens* (Tv_4) (seed + soil) followed by challenge inoculation of *F. oxysporum* f.sp. *Lycopersici* (T_4) recorded maximum induction of peroxidase activities (1.63 min/g^{-1}) on 9th day of germination. The enzyme activity was significantly increased upto 9th day from the inoculation and then it declined gradually in all the treatments. *T. virens* untreated tomato plants inoculated with *F. oxysporum* f.sp. *Lycopersici* recorded minimum induction of peroxidase activity compared to all other treatments (Table 2).

Polyphenol oxidase (PPO)

In green house condition application of *T. virens* (Tv_4) (seed + soil) followed by challenge inoculation of *F. oxysporum* f.sp. *Lycopersici* (T_4) induced higher level of PPO activity upto 9th day of germination (1.52 min/g^{-1}) there after it decreased. It was followed by seed and soil application of *T. virens* (Tv_2) with challenge inoculation of *F.oxysporum* f.sp. *Lycopersici*(T_2). The plants treated the pathogen *F.oxysporum* f.sp. *Lycopersici* alone recorded minimum PPO activity as compared to all other treatments (Table 3).

Phenylalanine ammonia lyase (PAL)

PAL activity was significantly increased in plants treated with *T. virens* (Tv_4) (seed and soil) followed by challenge inoculation with *F.oxysporum* f.sp. *Lycopersici* (T_4), than the other treatments. PAL induction reached its maximum at 9th day of the germination and thereafter it decreased. Inoculated control recorded minimum PAL induction. Generally enzyme activities gradually increased up to 9th day from the germination and thereafter it declined gradually in all the treatments (Table 4).

DISCUSSION

Many soil borne fungal disease have been successfully controlled by use of antagonistic micro organism (Chet *et al.*, 1987) The result of the experiment revealed that seed plus soil application of *T. virens* was found more effective in enhancing the growth and suppress the wilt disease incidence and also seed plus soil application of native isolates of *T. virens* was most effective and significantly improved plant growth and reduced the disease incidence when compared to other isolates. There findings are in agreement with those of several workers (Chet, 1987; Chang *et al*. 2002).

From this study it has been concluded that tomato plants treated with native bioagents of *T.virens* followed by challenge inoculation of *F.oxysporum* fsp. *LycopIersici* enhance induction of defence related enzyme such as PO, PPO and PAL than other isolates which could be very effective in the control of *Fusarial* wilt of tomato. These findings are in agreement with those of several workers. Bio formulation of *T. virens* sprayed on leaves and flowers increased the induction of peroxidase activity in cucumber (Wei *et al.*, 1996). Nandakumar *et al.* (2001) reported that two peroxidase isoforms have been induced in PGPR treated rice plants inoculated with sheath blight pathogen *R. solani*. Radja commare (2000) reported that *P. fluorescence* (PF) induced PPO isoenzymes in rice against *R. solani*. Chen *et al*. (2000) reported that various rhizobacteria and *P. aphanidermatum* induced PPO activity in cucumber root tissues. Chen *et al.* (2000) who reported that the increased activity of PAL can also be contributed for enhancing the resistance in tomato plants against fungal pathogen, *F. oxysporum* f.sp. *Lycopersici* and induction of PAL by *Fluorescent pseudomonas* in cucumber against *P. aphanidermatum*.

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