INDUCTION OF RESISTANCE IN TOMATO PLANTS AGAINST *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* MEDIATED THROUGH SALICYLIC ACID AND *TRICHODERMA HARZIANUM*

*Suprakash Ojha, Narayan Chandra Chatterjee[1]**

Mycology and Plant Pathology Laboratory, University Grants Commission Centre for Advanced Study
Department of Botany, The University of Burdwan
Burdwan – 713104, West Bengal, India

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Abstract: The effect of a soil application of salicylic acid (SA) and a biocontrol agent, *Trichoderma harzianum* (TH) on the induction of phenolic accumulation content and defense enzymes in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* (*F. oxysporum*) was investigated. The phenolic content was recorded to be higher in all the treatments viz. *F. oxysporum*, *F. oxysporum* + TH, *F. oxysporum* + SA and *F. oxysporum* + TH + SA than that of the healthy plants and reached its maximum level in the plants treated with *F. oxysporum* + TH + SA. Tomato plants treated with different concentrations of SA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) showed significant increases in the activities of both peroxidase and polyphenol oxidase where a prominent enhancement was observed at a 1.5 mM concentration of SA. *F. oxysporum* infection resulted in the induction of both of the enzyme activities but at a lower level. The activities of both peroxidase and polyphenol oxidase reached their maximum on the 28th day, when the plants were treated with *F. oxysporum* and SA (1.5 mM). The combined application of SA (1.5 mM) and TH in *Fusarium* infected tomato plants, also enhanced the activities of both of the enzymes.

Key words: fusarial wilt, phenol content, peroxidase, polyphenol oxidase, enzyme activity

INTRODUCTION

Plants in their natural habitats are exposed to a number of organisms, and they respond to pathogen attack by activating a wide variety of protective mechanisms. One potential method to reduce the severity of disease caused by the pathogen is the induction of a systemic acquired resistance (Abo-Elyousr *et al*. 2008). Biochemical resistance depends upon some pre-existing or induced substances synthesized by plants in response to fungal infection. According to Surplus *et al*. (1998), salicylic acid is a major phenyl propanoid compound that influences plant resistance to pathogens and probably to other stress factors.

Synthesis of aromatic substances is a major defense mechanism of plants. Sukand and Kulkarni (2006) reported that biochemicals and their oxidation products are implicated in disease resistance. Das *et al*. (2003) has pointed out that peroxidase enzyme is a key enzyme of the phenyl propanoid pathway, activated in response to pathogen infection. Changes in the activity of phenol-oxidizing enzymes including peroxidise, may play a role in the regulation of metabolic pathways in diseased or injured tissues (Mehrotra and Aggarwal 2003). Pradeep and Jambhale (2002) suggested that phenolic compounds and related oxidative enzymes are mostly considered as one of the important biochemical parameters for disease resistance. They differentiate resistant and susceptible genotypes and as such, would be helpful in discriminating the genotypes biochemically, in addition to their cytological status.

Trichoderma spp. are non-pathogenic rhizosphere colonizing fungi. Several *Trichoderma* spp. suppress the soil-borne pathogens by diversified mechanisms viz., production of a wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with the pathogen for nutrient and for occupation of infection court, induced resistance, production of protease and fungal cell wall degrading enzymes (Perello *et al*. 2003).

Wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* is quite common in different vegetable fields in India. Induction of defense responses against the pathogen through the application of *Trichoderma* or salicylic acid individually is well documented. But, there is scarce information about the mechanism of defense or synergism when they are used together. Thus, the present study was carried out to determine the induction of phenolic compounds and defense enzymes in *F. oxysporum* infected tomato plants in response to the application of *Trichoderma harzianum* in combination with salicylic acid.

*Corresponding address:
nc_chatterjee@rediffmail.com

MATERIALS AND METHODS

Host plants

In a greenhouse, tomato (*Lycopersicon esculentum* Mill.) plants were grown in large pots containing sterilized sandy loamy soil (2:1 w/w). The seedlings were transplanted after 20 days into clay pots (35 cm diameter) containing sterilized sandy loamy soil (1:2 w/w).

The microorganisms

The pathogen, *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*) was isolated from wilted tomato plants cultivated in the Sadarghat area of the Burdwan district, West Bengal, India. Biocontrol agent, *Trichoderma harzianum* Rifai (TH) was procured from the Indian Agricultural Research Institute, New Delhi. Stock cultures were stored on Potato Dextrose Agar (PDA) medium at 4°C.

Inoculation of plants

Tomato plants (45-days-old) were inoculated separately with a combination of *F. oxysporum* and TH. Transplanted tomato plants were inoculated with the pathogen by making a longitudinal slit (2 cm) with a sterile knife on the lower portion of the shoot. A piece of sporulating mycelial mat from 7-day-old actively growing culture of *F. oxysporum* was inserted through the slit. The inoculated portion was covered with a piece of sterile moist absorbent cotton and tied with twine. Mycelial suspension ($3.6x10^7$ colony forming units c.f.u./ml) of *F. oxysporum* (50 ml) was also poured into the soil of each earthen pot.

TH was grown in conical flasks containing 250 ml Czapek's synthetic medium at 28±1°C for 18 days. Then, the mycelial mats were harvested and the culture filtrates were collected. The culture filtrates thus obtained were stored at 4°C and directly applied to the soil, 14 days before transplanting the tomato seedlings. The mycelial mats were multiplied by growing on a substrate consisting of 1:1 wood saw dust and wheat bran with a small quantity of malt extract in sterilized plastic bags (30x26 cm) for 14 days at 28±1°C. This mass inoculum (150 g) of TH ($4.5x10^7$ c.f.u./g) was applied to each pot.

Application of salicylic acid

Different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) of salicylic acid (SA) were prepared in sterilized distilled water. SA was applied three times (100 ml/pot) at an interval of 5 days and the first application was done when tomato plants were 35-days-old.

Determination of total phenolic content

Total phenol content of tomato leaves was determined using the Folin-Ciocalteau reagent (Singleton and Rossi 1965). Freshly collected leaves (2 g) were homogenized in 80 % aqueous ethanol with a pinch of neutral sand to facilitate crushing and the mixture was passed through a clean cloth to filter the debris. The filtered extract was centrifuged at 10,000 g for 15 min and the supernatant was saved. The residue was re-extracted twice with 80% ethanol and supernatants were collected, put into evaporating dishes and evaporated to dryness at room temperature. Following evaporation the residue was dissolved in 5 ml of distilled water. Extract was diluted to 3 ml with water and 0.5 ml of Folin-Ciocalteau reagent was added. After 3 min, 2 ml of 20% of sodium carbonate was added and the contents were mixed thoroughly. The colour was developed and absorbance was measured at 650 nm in a spectrophotometer (Systronics, uv-vis, 117) after 60 min. Catechol as a standard was used. The phenolic content was expressed as mg catechol/100 g of fresh weight of tomato leaves.

Determination of polyphenol oxidase (EC 1.14.18.1)

Tomato leaves (2 g) were collected and washed thoroughly with running tap water followed by distilled water. The surface was wiped off with filter paper. To extract the enzyme, healthy and infected leaves were ground separately with a pinch of neutral sand in 6.0 ml of sodium phosphate buffer (0.1 M at pH 7.0) at 0°C. The extracts were obtained by filtering off the debris with a clean cloth and centrifuging at 3,000 rpm for 15 min in a refrigerated centrifuge. The supernatants were recovered and kept in a tube, in an ice bath until assayed.

Enzyme assay was done following the method of Sadasivam and Manickam (1996). Sodium phosphate buffer (0.1 M) at pH 7.0 (3.0 ml) and 2.0 ml of the enzyme extract were mixed in a cuvette in a spectrophotometer (Systronics, uv-vis, 117). The mixture was immediately adjusted to zero absorbance. Catechol of 0.01 M (1.0 ml) in 0.1 M phosphate buffer (0.4 mg/ml) was added to the above mixture and the reactants were quickly mixed. Enzyme activity was recorded as the change in absorbance at 495 nm up to 30 minutes after the addition of catechol.

Determination of peroxidase (EC 1.11.1.7)

Tomato leaves (4.0 g) were homogenized in 20 ml of chilled distilled water at 0°C. A pinch of neutral sand was added to facilitate crushing. The extracts were obtained by filtering off the debris with a clean cloth and centrifuged at 3,000 rpm for 15 min in the cold. The supernatants were collected and used as an enzyme source and kept in an ice-bath until assayed.

A peroxidase enzyme assay was done following the method of Mahadevan and Sridhar (1982). Freshly prepared 5 ml of pyrogallol reagent (prepared by mixing 10 ml of 0.5 M pyrogallol and 12.5 ml of 0.66 M phosphate buffer at pH 6.0) and 1.5 ml of enzyme extract were mixed in a tube of a spectrophotometer (Systronics, uv-vis, 117) and the mixture was immediately adjusted to zero absorbance, and H_2O_2 solution (0.5 ml of 1%) was added to it. The content was mixed by inverting the tube, and adding of H_2O_2 initiated the reaction. Enzyme activity was recorded as the change in absorbance at 430 nm after the addition of the substrate.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests using a statistical software package (SPSS 10.0 Inc.). There were five replications for each of the treatments.

RESULTS

Effect of TH and SA on phenol content

Phenol content (Table 1) was significantly higher in *F. oxysporum* infected tomato plants treated with either SA or TH. The phenol production was recorded to be highest when *Fusarium* infected tomato plants were treated with a combination of TH + SA (1.5 mM). Untreated tomato plants showed no noticeable changes in phenol level.

Effect of SA on polyphenol oxidase activity

A significant increase in polyphenol oxidase activity was noticed in *F. oxysporum* infected tomato plants following the application of SA (Table 2). The activity increased steadily with an increase of SA concentration and reached the highest level on the 28th day after 3.0 mM of SA treatment. Regarding the treatments, the enhancement in enzyme activity was more prominent at 1.5 mM of SA. In infected tomato plants, polyphenol oxidase activity increased sharply on the 14th day after inoculation.

Effect of SA on peroxidase activity

The application of SA (Table 3) significantly increased the activity of the peroxidase from the 7th day up to the 28th day after *F. oxysporum* inoculation. The 1.5 mM of SA exhibited a marked enhancement in the peroxidase at different periods of infection. A noticeable increase in peroxidase activity was also observed on the 14th day in the infected tomato plants, at all the concentrations of SA.

Table 1. Contents of total phenolic compounds in leaves of tomato at different stages of growth, infected with *F. oxysporum* f. sp. *lycopersici* and treated with *T. harzianum* (TH) and salicylic acid (SA)

Treatments	Total phenol [mg/100 g fresh weight of tomato leaves]*				
	days after *F. oxysporum* infection				
	0	7	14	21	28
F. oxysporum	70.18 cd	85.24 d	97.10 d	106.94 cd	112.68 b
F. oxysporum + TH	68.64 d	93.14 c	109.26 c	113.36 c	114.64 b
F. oxysporum + SA (1.5 mM)	72.28 b	98.32 b	115.66 ab	124.72 b	135.14 a
F. oxysporum + TH + SA (1.5 mM)	74.50 a	106.78 a	124.08 a	136.86 a	142.32 a
Control	71.20 c	68.40 e	74.12 e	70.24 e	72.84 c

*mean of the five replications

In a column, means followed by the same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test

Table 2. Effect of salicylic acid (SA) on activity of polyphenol oxidase in tomato leaves at different stages of growth

Treatments SA [mM]	Polyphenol oxidase activity [min/g fresh weight of tomato leaves]*				
	days after *F. oxysporum* infection				
	0	7	14	21	28
0.0	0.41 bc	0.85 f	1.03 h	1.32 f	1.52 f
0.5	0.36 d	1.05 de	1.80 ef	2.15 de	2.21 de
1.0	0.44 b	1.25 d	2.01 e	2.41 d	2.62 d
1.5	0.42 bc	1.84 c	2.70 c	3.01 c	3.46 c
2.0	0.54 a	2.00 b	2.91 bc	3.36 b	3.87 bc
2.5	0.40 a	2.01 b	2.98 bc	3.51 ab	4.12 b
3.0	0.45 b	2.53 a	3.47 a	3.71 a	4.63 a

*mean of the five replications

In a column, means followed by the same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test

Table 3. Effect of salicylic acid (SA) on activity of peroxidase in tomato leaves at different stages of growth

Treatments SA [mM]	Peroxidase activity [min/g fresh weight of tomato leaves]*				
	days after *F. oxysporum* infection				
	0	7	14	21	28
0.0	0.53 d	1.47 f	1.70 e	2.04 f	2.19 fg
0.5	0.56 bc	1.51 ef	1.83 de	2.27 ef	2.53 f
1.0	0.64 a	1.60 e	2.23 d	2.61 e	2.94 e
1.5	0.58 b	2.26 c	2.93 c	3.25 d	3.43 d
2.0	0.60 b	2.64 b	3.35 b	3.63 cd	3.94 c
2.5	0.59 b	2.94 a	3.45 b	3.85 c	4.32 b
3.0	0.63 a	3.02 a	3.97 a	4.55 a	4.83 a

*mean of the five replications

In a column, means followed by the same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test

Effect of combined treatment on polyphenol oxidase activity

Polyphenol oxidase activity (Table 4) increased considerably with the increase in the period of inoculation as compared to the control set. Regarding the treatments, the enzyme activity gradually increased in pathogen infected plants as the infection progressed. While in the host plants inoculated only with the antagonistic fungi, *T. harzianum*, the enzyme activity reached a peak on the 14th day of infection. A combined treatment with *F. oxysporum* and SA (1.5 mM) resulted in a significantly higher accumulation of the enzyme throughout the 28 day experimental period. However, application of TH, SA and *F. oxysporum* together showed the highest polyphenol oxidase activity on the 14th day, and then the activity gradually declined.

Effect of combined treatment on peroxidase activity

Peroxidase activity (Table 5) was more in *Fusarium* infected tomato leaves than in the healthy ones. The activity increased gradually from the 7th day up to the 28th day in plants infected with *F. oxysporum* and SA (1.5 mM) treatment. Enhancement of the peroxidase was also observed up to the 14th day in *F. oxysporum* infected tomato plants, upon treatment with TH and SA (1.5 mM).

Table 4. Polyphenol oxidase activity in tomato leaves at different stages of growth, infected with *F. oxysporum* f. sp. *lycopersici* and treated with *T. harzianum* (TH) and salicylic acid (SA), alone or in combination

Treatments	Polyphenol oxidase activity [min/g fresh weight of tomato leaves]*				
	days after *F. oxysporum* infection				
	0	7	14	21	28
F. oxysporum	0.56 a	0.93 e	1.44 d	1.88 e	1.95 de
TH	0.39 d	0.47 f	0.81 f	0.78 fg	0.72 gh
F. oxysporum + TH	0.53 a	1.33 d	1.84 c	0.96 f	0.92 g
F. oxysporum + SA (1.5 mM)	0.49 b	2.02 ab	3.10 b	3.54 a	3.82 a
F. oxysporum + TH + SA (1.5 mM)	0.52 ab	2.21 a	3.30 a	3.41 ab	3.27 bc
Control	0.39 d	0.42 fg	0.59 g	0.62 g	0.62 gh

*mean of the five replications

In a column, means followed by the same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test

Table 5. Peroxidase activity in tomato leaves at different stages of growth, infected with *F. oxysporum* f. sp. *lycopersici* and treated with *T. harzianum* (TH) and salicylic acid (SA), alone or in combination

Treatments	Peroxidase activity [min/g fresh weight of tomato leaves]*				
	days after *F. oxysporum* infection				
	0	7	14	21	28
F. oxysporum	0.56 bc	1.04 e	1.73 de	2.07 d	1.83 e
TH	0.38 d	0.41 g	0.84 g	0.98 g	0.98 gh
F. oxysporum + TH	0.62 b	1.89 de	2.35 c	1.83 e	1.42 f
F. oxysporum + SA (1.5 mM)	0.67 a	2.41 c	3.62 a	3.92 a	4.01 a
F. oxysporum + TH + SA (1.5 mM)	0.59 bc	3.91 a	3.72 a	3.58 b	3.52 b
Control	0.30 e	0.32 h	0.60 gh	0.61 h	0.62 h

*mean of the five replications

In a column, means followed by the same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test

DISCUSSION

It was evident from the results (Table 1) that there was a more or less steady increase in the level of the total phenols in all the treatments. Accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development, since such compounds are toxic to pathogens. Also, phenolic compounds may impede pathogen infection by increasing the mechanical strength of the host cell wall (Benhamou *et al*. 2000). In the present study SA individually or plus TH, significantly increased total phenolic content in *F. oxysporum* infected tomato plants as compared to the non infected or infected. So induction of total phenol accumulations in the host plant treated with SA and TH might play an important role in resistance and defense against *F. oxysporum*. These results agree with the general speculation, that when plant cells are recruited into infection, there is a switch from the normal primary metabolism to a multitude of the secondary defense pathway, and activation of novel defense enzymes and genes take place (Tan *et al*. 2004).

The data presented in table 2 and 3 demonstrated that soil application of SA resulted in enhancement of peroxidase and polyphenol oxidase activity in *F. oxysporum* infected tomato leaves. SA and related compounds have been reported to induce significant effects on vari-

ous biological aspects. SA is a major phenylpropanoid compound that influences plant resistance to pathogens, probably to other stresses (Durner *et al*. 1997; Surplus *et al*. 1998). There is also evidence that SA can alter the antioxidant capacity in plants (Chen *et al*. 1997; Fodor *et al*. 1997). Schonbeck *et al*. (1980) found that certain biochemical changes occurring after application of the inducing agents can act as markers for induced systemic resistance.

The results (Tables 4, 5) indicate that as a consequence of infection, both the polyphenol oxidase and peroxidase enzyme activity was found to be much higher, and gradually increased as the infection period progressed. Maximum activity was observed at the end of the incubation period. Earlier researchers observed enhanced activity of these enzymes in host tissues in response to pathogenic infection (Das *et al*. 2003; Ojha *et al*. 2005; Chakraborty and Chatterjee 2007; Abo-Elyousr *et al*. 2008). Ramadoss (1991) was of the opinion that toxic metabolites of the fungus may activate the production of phenol-oxidizing enzymes. *Fusarium* species are known to produce such metabolites which also play a vital role in the tissue browning by their ability to oxidize phenols to quinones.

On the other hand, development of an antioxidant defense system in plants protect them against oxidative stress damage by either partial suppression of reactive oxygen species production or the scavenging of reactive oxygen species which are generated during plant pathogen interactions (Ye *et al.* 2006; Cavalcanti *et al*. 2007). Thus, various antioxidant enzymes like peroxidases and polyphenol oxidases can participate in reactive oxygen metabolism of the species during infection. Morkunas and Gmerek (2007) stated that peroxidases may be some of the elements of the defense system in response to pathogen like *F. oxysporum*. Furthermore, the phenol oxidizing enzymes oxidize phenols to quinones, which are known to be more reactive and have more antimicrobial activity than the phenols, already exist in plants. Therefore, these enzymes may be directly involved in stopping pathogen development (Melo *et al.* 2006; Shimzu 2006), accelerating the cellular death of cells close to the infection site, preventing the advance of infection, and /or by generating a toxic environment inside the cells which will inhibit the pathogen growth (Bi and Felton 1995).

In the present study (Tables 4, 5), when plants were inoculated with only *T. harzianum* there was an increase in the activity of polyphenol oxidase and peroxidase up to the 14th day and the 21st day, respectively. After that, the activity decreases or remains more or less stable. Probably at the initial stage of infection, the host plant secretes more defense enzymes as a general rule. But after that, there was no toxic metabolite production by the antagonist, and thus the activity slows down or becomes steady. On the other hand, in the plants infected with *F. oxysporum* and TH treated, the activity of peroxidase and polyphenol oxidase was higher than their individual treatment. This might be due to the fact that in tomato plants, when challenged with the pathogen and the antagonist, the host plant ought to secrete more phenol oxidase enzyme for defense but at the later stages of infection the antagonist itself deters the activity of the pathogen – resulting in a falling off of enzyme activity.

Our results also indicate that soil application of SA following inoculation with *F. oxysporum* resulted in maximum peroxidase and polyphenol oxidase activity in tomato leaves on the 28th day, compared to other treatments. But *F. oxysporum* + SA + TH treated plants showed higher enzyme activities up to the 14th day, and after that the activities become lower. This might be due to deterring potential of TH on the pathogen. Kamalakannan *et al.* (2004) demonstrated that in addition to direct antagonism, the biocontrol agents also increase the activity of various defense-related enzymes and chemicals in response to pathogen infection

It seems to be quite natural that all plants are enriched with defense genes. These genes are quiescent in nature and require the appropriate stimulation signals to activate them. It has been reported that biocontrol agents activate latent plant defense mechanisms in response to pathogen infection (Kamalakannan *et al*. 2004). Thus, in conclusion, the current study indicates that inducing the plant's own defense mechanism by applying chemical inducer and biocontrol agents can be a novel strategy in plant disease management.

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