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Research Article

Quantitative Changes in Defense System of Tomato Induced By Two Strains of *Bacillus* against *Fusarium* Wilt

***Waheed Akram and Tehmina Anjum**
Institute of Agriculture Sciences. University of the Punjab. Lahore Pakistan.
**Author for Correspondence*

ABSTRACT

Systemic induction of defense response in plants can be obtained by treatment with different microorganisms. Present study was aimed to investigate the effect of two strains of *Bacillus* viz *Bacillus fortis* 162 and *Bacillus subtilus* 174 for induction of systemic resistance in tomato against *Fusarium* wilt disease under lab conditions. For this purpose roots of tomato seedlings were primed with *Bacillus* strains and plants were challenged with pathogen. Biocontrol potential of test strains of *Bacillus* was studied along with quantification of total phenolic compounds and defense related proteins viz. Polyphenol Oxidase (PPO), Phenyl Ammonia Lyase (PAL) and Peroxidase (PO) by Calometric methods. *B. subtilus* 174 showed great biocontrol potential and significant reduction in disease severity. Higher levels of phenolic compounds, PPO, PAL and PO activities were observed in case of *B. subtilus* 174 in shoots of tomato plants as compared to *B. fortis* 162. This bacterial strain can be used as a biocontrol agent for sustainable management of *Fusarium* wilt of tomato.

Key Words: *Fusarium* wilt, *Bacillus fortis*, *Bacillus subtilus*, Phenolic compounds, Polyphenol Oxidase, Phenyl Amonia Lyase, Peroxidase.

INTRODUCTION

Fusarium oxysporum is soil-borne plant pathogens, and is distributed world wide in numerous types of soil (Fravel *et al.,* 2003). This pathogen is able to survive in soil for 10–15 years. It has wide host range and can infect more than hundred plant species including tomato (Chung *et al*., 2008). Susceptible plants is usually infected through the root during all stages of growth, and eventually resulting in plant wilting and yield losses as high as 100% (Sherf and MacNab., 1986). Chemical fungicides have long been used as active agents in controlling of soil-borne plant diseases. However, some chemicals used as soil fumigants, such as Metham sodium and Carbofuran, leads to environmental pollution and toxic effects on human health, and gives possibility to pathogens for building-up resistance to chemicals (Baysal et al., 2009). These treatments are only effective for a short time in the growing season. Therefore, the stratagem of biocontrol has become an important approach to create a more long lasting effect and facilitating sustainable agriculture (Nagorska et al., 2007).

Many studies have shown that members of bacterial genera can induce systemic resistance in different plants for control of soil-borne diseases (Nagorska *et al*., 2007). Some members of bacillus genre are able to produce various lytic enzymes (e.g. chitinase and b-1, 3-glucanase) and antibiotics, along with induction of systemic resistance of plants, such as increasing the activities of plant defense related enzymes of peroxidase (POX), polyphenol oxidase (PPO) and phenylalanine ammonialyase (PAL) (Jayaraj *et al*., 2004). Lytic enzymes produced by bacteria are considered to be most effective enzymes for degrading fungal cell walls (Leelasuphakul et al., 2006). Some oxidative enzymes such as POX and PPO, can catalyze the formation of lignin and other oxidative phenols, and contributes in formation of defense barriers by changing the cell structure defense system get actuated against pathogens (Thilagavathi et al., 2007). PAL is considered to be the key enzyme in the phenylpropanoid biosynthetic pathway, and also plays an important role in the flavonoid production and the lignin biosynthesis (Podile and Laxmi., 1998). These enzymes have been reported to correlate with the defense activities against pathogens in several plant species (Thilagavathi et al., 2007). Present study was carried out to evaluate the changes in defense system of tomato under influence of five strains of bacillus genera for management of Fusarium wilt of tomato.

MATERIALS AND METHODS

Treatments

Two isolates of bacillus i.e. *Bacillus fortis* 162, *Bacillus subtilus* 174 were procured from bacterial conservatory of Institute of Agricultural sciences, University of the

Punjab, Pakistan. Information about Bacterial strains is given below in table 1.0.

Table 1: Bacillus strains used in current study.

Accession No	Specie	Source
162	*Bacillus fortis*	Compost
174	*Bacillus subtilus*	Pharmaceutical effluent

Seeds of tomato were bought from market. These were surface sterilized with 1.0% solution of Sohiumhypochlorite for 1-2 minutes and then thoroughly washed with distilled water. Seeds were sown in sterilized sandy loam soil for seedling development under lab conditions. When seedlings were of two weeks old, these were transplanted in plastic pots of 10 inch diameter containing sterilized sandy loamy soil and were subjected to further experiment. There were three replicates with five plants in each replicate. Experimental design was as followed.

Group 1st: Supplied with distilled sterilized to serve as control

Group 2nd: Challenged with pathogenic strain of *Fusarium oxysorum* f.sp.Lycopersici to serve as disease control.

Group 3rd: Provided with test bacterial treatment alone to serve as bioagent control.

Group 4th: Provided with both bacteria land pathogenic fungi treatment.

Plants were kept under green house conditions. These were provided with distilled sterilized whenever needed.

Root priming of tomato seedlings with test bacterial genera

Test Bacterial strains were cultured onto conical flasks containing 100 ml of L.B. broth media. These were kept in incubator at 35°C for 24 hours with agitation. After incubation, material was taken out from flask and centrifuged at 4000 rpm. Supernatant was discarded and bacterial cells were collected from the pallet. Inoculum of bacterial cells was prepared in normal saline at concentration of 1000 bacterial cell per ml. Roots of seedlings were primed with bacterial inoculum by keeping then in inoculum for 30 minutes. After this these seedlings were transferred in pots prepared as discussed previously.

Inoculation with F. oxysporum f. sp. Lycopersici

Virulent strain of *Fusarium oxysporum* f. sp. Lycopersici causing wilt disease in tomato was obtained from First Fungal Culture Bank of Pakistan, Institute of Agriculture sciences, University of the Punjab, Pakistan. The pathogen inoculum was prepared by culturing the fungus on potato dextrose agar (PDA) medium for one week in petri-plates. Micro conidial suspension was prepared by pouring 30 mL of sterile distilled water in each petri-plate. Concentration of micro conidia was adjusted to 1000 conidia per mL. This spore suspension was then challenged to allotted pots at rate of 50ml in each pot.

Disease assessment under Lab conditions

Disease severity was recorded on a 0–4 visual scale of the shoots and root according to Rothrock (1987), which 0 = rhizome and root with no symptom, 1 = 25% damage 2 = 25–50% damage, 3 = 50– 75% damage, 4 = 75–100 damage %.

The disease ratio, disease index and biocontrol effect were calculated according to the method of Li et al., (2008):

$$\text{Disease ratio (\%)} = \frac{\text{Disease Plants}}{\text{Diseased plants + healthy plants}} \text{ X100}$$

$$\text{Disease index (\%)} = \frac{\sum \text{(Grade of disease severity + diseased plants of this grade)}}{\text{Total plants assed X Highest grade of disease severity}} \text{ X100}$$

$$\text{Biocontrol effect (\%)} = \frac{\text{(Disease index of pathogen control- diseased index of bacterial control)}}{\text{Disease index of pathogen control}} \text{ X100}$$

Estimation of total Phenolics

One gram shoot sample was extracted with 10mL of 80% methanol at 70°C for fifteen minutes. Reaction mixture was containing 1 mL of methanolic extracts and five mL of distilled sterilized water 250 µL of Folin Ciocalteau reagent (1 N). This solution was kept at 25°C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Gallic acid was used as the standard. The amount of phenolics was expressed as milligram gallic acid / gram plant material (Zieslin and Ben-Zaken, 1993).

Assay of defense enzymes

Leaf samples were taken at regular intervals from the plants for enzymes assays. One gram of leaf sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) in ice bath for enzyme assays. The homogenates were then centrifuged at 10,000 g for 10 min. Supernatants were used to analyze the POX, PPO and PAL activities.

Assay of PPO: PPO activity was determined according to method proposed by Mayer et al., (1965). The reaction mixture was containing 200 µL enzyme extract and 1.5 mL of 0.01 M catechol. Activity was expressed as changes in absorbance at 495 nm min-1mg-1 protein.

Estimation of PAL activity: PAL activity was determined according to method of Burrell and Rees (1974). The reaction mixture contained 0.03 M L-phenylalanine and 0.2 mL enzyme extract in a total 2.5 mL of sodium borate buffer (pH 8.8). This reaction mixture was kept in a water bath at 37 0C for 1 h and 0.5 mL of 1 M (trichloro acetic acid) TCA was added. The amount of trans-cinnamic acid formed from L-phenylalanine was measured spectrophotometerically at 290 nm. Enzyme activity was expressed as µg of trans-cinnamic acid h-1 mg-1 protein.

Estimation of PO activity: Method of Fu and Huang (2001) was used to estimate the peroxidase activity. For this purpose 50 ul of enzyme extract was added to 2.85 ml of 0.1 M phosphate buffer (pH 7.0) and mixed with .05ml of 20 mM guaiacol reagent. The reaction was started by the addition of .02 ml of 40 mM hydrogen peroxide to the mixture. Rate of increase in absorbance at 470 nm was measured over 1 min. One unite of enzyme activity was defined by the change in absorbance of 0.01 for 1 g fresh weight per minute.

Statistical analysis

The results represented are mean values of two independent experiments. Data were statistically analyzed by ANOVA and Duncan Multiple range test at significant level of 0.05.

RESULTS

The effect of two bacilli strains i.e. *Bacillus fortis* 162, *Bacillus subtilus* 174 was evaluated under green house conditions. Plants that were treated as pathogenic control first showed symptoms on stem then symptoms were spread all on aerial parts of plant also. However plant treated with *Bacillus subtilus* 174 exhibited greater biocontrol potential as compared to *Bacillus fortis* 162 as shown in Table 2. Plants treated with both of these strains exhibited significant difference from control also. The disease ratios and disease indexes were significantly decreased in case of both test bacterial species as compared to control. In this regard, plants treated with *B. subtilus* exhibited greater biocontrol potential in comparison with *B. fortis*. This study shows that *B. subtilus* has good control potential against Fusarium wilt of tomato.

Estimation of the total phenolic compounds

For further understanding of disease control phenolics compounds were quantified. High quantity of total phenolics were observed in case of *B. subtilus* root priming as compared to *B. fortis* (Fig 1).

Table 2: Effect of bacillus strains on control of Fusarium wilt of tomato.

Treatment	Disease ratio (%)	Disease index (%)	Biocontrol index (%)
Bacillus fortis+ F	14.48 A	13.25 A	53.75 A
Bacillus subtilus+ F	19.12 B	17.59 B	81.39 B
Bacillus fortis control	-	-	-
Bacillus subtilus control	-	-	-
Pathogen control	51.28 C	39.16 C	-
Control	-	-	-

Results are the mean of three replicates. Capita letters shows significant difference of LSD test at P<0.05. (F) Fusarium oxysporum. (–) no disease plants were recorded in the treatment.

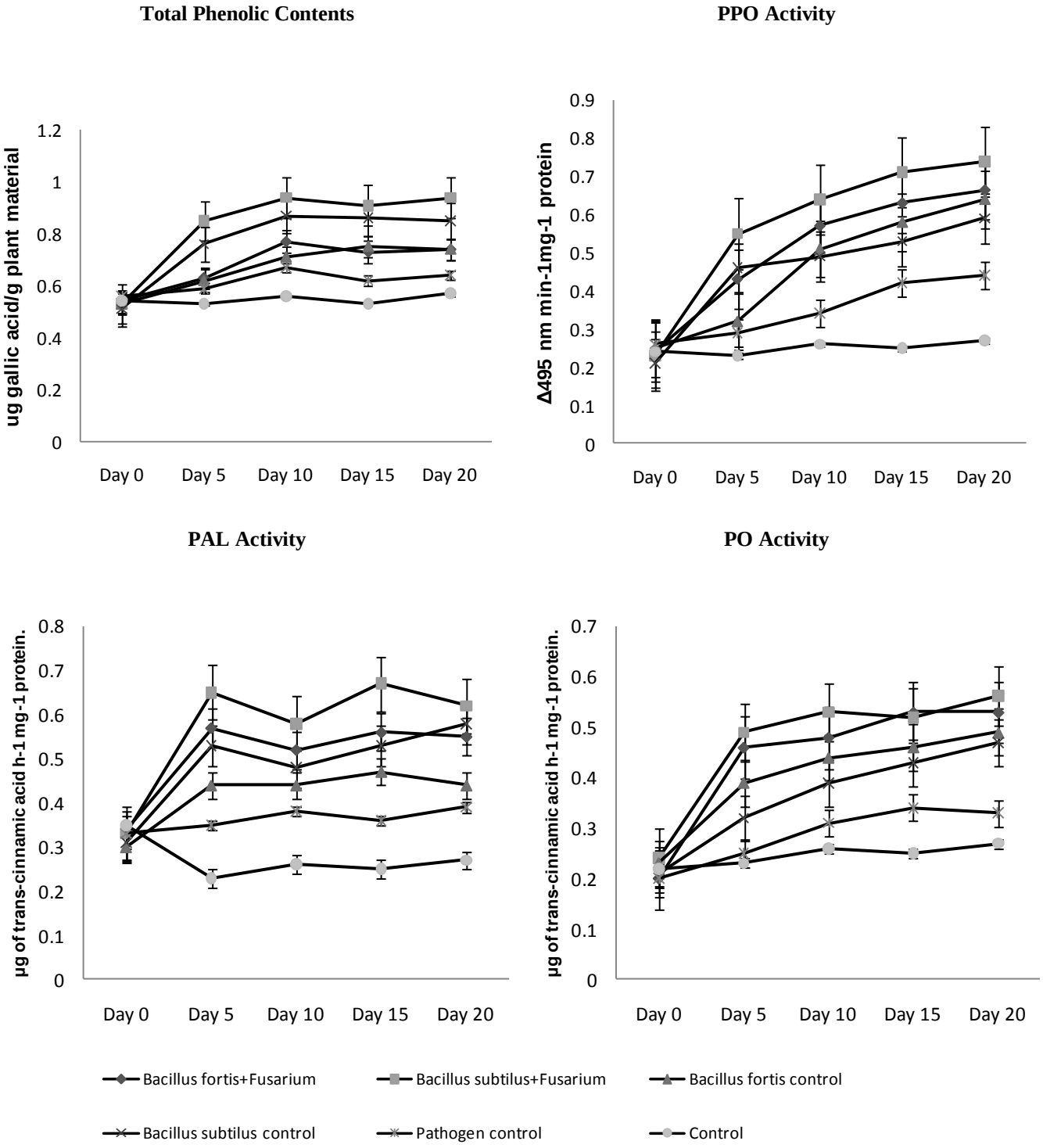

Fig 1: Changes in total phenolic contents and PPO, PAL and PO activity in tomato plants of different treatments. Mean values were three replicates. Bars represent standard error.

In the initial days, quantity of phenolics increased very rapidly up to 1.3 folds as compared to control. At day ten, maximum amounts of phenolics were observed in plants which gradually lowered down in next days. Least amounts of phenolic compounds were observed in untreated control. Pathogen control first showed little shift in quantity of phenolics compounds in initial days. No shift in quantity of phenolics was observed in untreated control (Fig 1).

Assay of defense enzymes

PPO activity: Quantitive changes in plant defense related were also observed in same regard as in previous section. In case of PPO activity, great amounts were observed in plants whose roots were primed by *Bacillus subtilus* (Fig 1). There was an increase of 1.7 folds as compared to control. *Bacillus fortis* was proved to be the second best treatment where increase of 1.2 folds was observed. Pathogen control and untreated control exhibited least amount of PPO activity. Increase in PPO activity of the plants also followed the same trend as like of phenolic compounds that increased rapidly up to day 10 then increased slowly. A slight increase in PPO activity was seen in pathogen control plant in the initial days that in rest of days was nearly flat. This result showed that *Bacillus subtilus* could induce higher amount of PPO activity as compared to *Bacillus fortis.*

PAL Activity: PAL activity was increased up to 1.9 folds in case of *B. subtilus*. In case of *Bacillus fortis* an increase of 1.4 folds was observed in PAL activity (Fig 1). PAL activity was noted in the form of increasing and decreasing trends like wave pattern. It increased rapidly at day five then, decreased at day 10. It increased again at day 15 followed by slight decrease at day 20. This trend of PAL quantity was observed in case of all treatments except untreated control.

PO activity: *B. subtilus* treated roots showed high amount of PO activity revealed by this study. Here increase of 1.6 folds was observed as compared to control. *B. fortis* proved itself to be second best. Plants incubated with pathogen also showed increase in PO activity by as compared to untreated control after five days of treatment (Fig 1).

DISCUSSION

Induction of systemic resistance in plats by application of any bioagent is thought to be the beast alternative for plant protection from nasty pathogens. Our studies revealed that application of bacterial bioagent can be used effectively to meet this task. Ahn., *et a l* (2002) reported that induction of systemic resistance in host plants is the main mechanism of disease suppression in crops by bacterial bioagents. This mechanism has proved its effectiveness in case of bacterial, fungal and viral plant pathogens (Akn., *et al* 2002; Perk., *et al* 2006a; Perk., *et al* 2006b). It was reported by Nakaho *et al*., (2000) that structural and biochemical modifications in tomato are main source of defense that reduce the severity of fugal wilt pathogen. Gou *et al.,* (2004) proposed that host defense modifications can slow down the pathogen spread in host plat.

Elicitation of induced systemic response by use of Bacillus strains has been documented on tomato against fungal and bacterial diseases. A starain of bacillus i.e. *B. vallismortis* strain EXTN-1 had been reported to induce systemic resistance against numerous pathogens (Perk *et al*., 2001). *B. subtilus* strain FZB-G was shown to be effective to produce defence related biochemical in tomato against Fusarium wilt disease (Gupta *et al.,* 2000). In a study carried out by Arfaoui *et al.,* (2007) it was seen that when chick pea seedlings were exposed to Rhizobium isolates, an increase in PAL activity was observed in comparison to water treated control. Our studies also showed a rapid increase in PAL activity under influence of pathogen and bacterial strains. Kang and Buchenauer (2000) reported increase in Phenolic contents in wheat under influence of a systemic defense inducer in response to Powdery mildew disease. Phenolic compounds have an important role in protection of plants against fungal pathogens. These inhibit growth of fungi in plants. In the present study it was seen that when roots of tomato seedlings were treated with bacterial strains, an increased level of phenolic compounds was observed in pant as compared to control. PAL is reported to have key role in synthesis of phenolic compounds in the plants via phenylpropeniod pathway (Hallbrock and Scheel 1989).

PPO is also an important contributor in plant defense pathway. Tomato transgenic plants producing large enhanced of PPO were seen to be resistant from some diseases (Li and Stiffnes 2002). In presented study increased amounts of PPO were also observed in plants that were challenged with pathogen or bacterial strains. PPO has a role in catalyzing phenolic oxidation in limiting disease development (Thipyapong and Stifness 1997). PPO may therefore be involved in induction of defense resistance against plant diseases.

Present study reveals the potential of *B. subtilus*, to suppress the Fusarium wilt of tomato. These strains can be used for biocontrol this disease. There is need of further investigations up to molecular level to understand the basics of induction of defense system under influence of this bioagent.

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