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Induction of Systemic Resistance by Rhizobacterial and Endophytic Fungi against Foot Rot Disease of *Piper nigrum* L. by Increasing Enzyme Defense Activity

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***Abstract*— *Two strains of rhizobacteria, Bacillus subtilis and Pseudomonas fluorescens and two strains of endophytic fungi Trichoderma viride and Trichoderma asperellum were selected as inducers of systemic resistance against Phytophthora capsici and were tested individually for biological control of foot rot disease of pepper. Black pepper vines were grown under greenhouse and challenge inoculated with P. capsici were selected for analyzing the disease index, chitinase, peroxidase, phenylalanine ammonia lyase activity, polyphenol oxidase, β-1, 3-glucanase assay and estimation of phenol content. Treatments with B. subtilis and T. viride isolates provided protection to pepper plant against P. capsici infection as observed with reduced per cent disease incidence showed 20 and 18% as compared to untreated control. Increases in the enzyme level were detected in different strains of pepper vines at time intervals after challenge inoculation. The study reveals the potential of B. subtilis and T. viride as biocontrol agent for prevention of P. capsici infections in black pepper.*

Keywords*— *Endophytic fungi, Foot rot disease, Piper nigrum L., P. capsici, Rhizobacteria.

I. INTRODUCTION

Black pepper (*Piper nigrum* L.) is a commercial spice crop cultivated in India. It ranks first in the world in terms of production, consumption and exports. In India, it is grown in an area of 1,31,230 ha with an annual production of 55,500 tones (Indian Spice Board, India, Feb 2017). Foot rot disease of black pepper is caused by *Phytophthora capsici*. It was known to affect 90% of the yield in India (Nair and Gupta, 2003; Krishnamoorthy and Parthasarathy, 2011). The control of foot rot disease has been almost exclusively based on the application of chemical pesticides that effectively kill the *Phytophthora capsici*. Although, several effective pesticides have been recommended for use against this pathogen, they are not considered to be long-term solutions due to concerns of expense, exposure risks, fungicide residues, toxicity to non-target organisms and other health and environmental hazards. Therefore, recent efforts have been focused on developing eco-friendly safe, long lasting and effective against many plant pathogens for the management of plant diseases.

Now a day's many chemical fungicides are being used to control this disease. The control of plant diseases using antagonistic bacteria is now considered as a promising alternative method that could reduce the use of hazardous chemical fungicides or bactericides. Rhizobacteria, saprophytic bacteria that live in the plant rhizosphere and colonize the root system, have been studied as plant growth promoters for increasing agricultural production and as biocontrol agents against plant diseases (Kloepper and Beauchamp, 1992; Liu *et al*., 1995; Chen *et al*., 1996; Silva *et al*., 2004). Colonization of the plant root system can lead to reduced pathogen attack directly through the production of antimicrobial substances or competition for space, nutrients and ecological niches and indirectly through induction of systemic resistance (Liu *et al*. 1995; Kloepper and Beauchamp 1995). They are eco-friendly in nature, have a high cost-benefit ratio and do not pose the risk of the pathogen developing resistance. Many root-rot colonizing bacteria are known to promote plant growth by

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producing gibberllins, cytokinin and indole acetic acid. Several plant growth promoting rhizobacteria (PGPR) strains have been reported to control various fungal (Van Peer *et al*., 1991), bacterial (Liu *et al*., 1995) and viral diseases (Maurhofer *et al*., 1994; Raupach *et al*., 1996). The mechanisms of biological control by PGPR strains generally involve the production of siderophores (Kloepper *et al*., 1980), hydrogen cyanide (Ahl *et al*., 1986) and lytic enzymes (Sneh *et al*., 1984; Jones *et al*., 1986), the biosynthesis of antibiotics (Howie and Suslow, 1991) and competition for substances (Elad and Chet, 1987).

Accumulating evidences suggest that the organisms under most scrutiny for potential use in biological control of pest and diseases are bacteria belonging to the genera *Pseudomonas* and *Bacillus* (Ramamoorthy *et al*., 2001). Further, plant growth promoting endophytic (PGPE) bacteria, especially *Bacillus subtilis* and PGPR, especially *Pseudomonas fluorescens* (Pf1) strains have been developed commercially as a talc based formulation and tested against several crop diseases (Vivekananthan *et al*., 2004; Rajendran *et al*., 2007; Kavino *et al*., 2007; Harish *et al*., 2008). Sundaramoorthy *et al*. (2012) reported that combination of *P. fluorescens* strains and *B. subtilis* strain together resulted in significant growth promotion that was correlated with induced resistance in *Capsicum annum* L. Several approaches have been made to manage the foot rot of pepper. A large number of enzymes have been associated with induced systemic resistance (ISR), including peroxidase, phenyalalanine ammonia-lyase, lipoxygenase, β-1, 3 glucanase and chitinase (Ye *et al*., 1990; Koch *et al*., 1992; Schneider and Ullrich, 1994; Van Loon, 1997; Silva *et al*., 2004).

This work aimed to verify the specific protection in Black Pepper provided by rhizobacteria and endophytes against *Phytophthora capsici* pathogen. However, no attempts have been made for the management of *P. capsici* disease by using rhizobacteria and endophytic strain. *Trichoderma viride* were used to detect the increasing activity of enzymes involved with ISR such as lipoxygenase, peroxidase, phenylalanine ammonia lyase (PAL) activity, polyphenol oxidase (PPO), β-1, 3-glucanase assay and estimation of phenol.

II. MATERIALS AND METHODS

2.1. Plant materials and pathogen

The black pepper vines were obtained from Pepper Research Center, Appangala, Madikeri, Karnataka. The pathogen was isolated from the black pepper foot showing typical symptoms of *P. capsici* by using oat meal agar (OMA) medium and the fungal cultures were identified as *P. capsici* in the Department of Studies in Biotechnology, University of Mysore, Mysuru, India.

2.2. Biocontrol agents

Endophytic fungal strains of *Trichoderma viride* and *Trichoderma asperellum* were isolated from medicinal plant *Azadiracta indica*, respectively. In addition, the rhizobacterial strain *Bacillus subtilis* and *Pseudomonas fluorescens* isolated from soil was reported to contain many plant diseases and pest. Pure culture of *T. asperellum* and *T. viride* strains were maintained on potato dextrose agar (PDA) slants and *B. subtilis* and *P. fluorescens* strain was maintained on nutrient agar (NB) agar slants and King's B medium at 4°C.

2.3. Efficacy of rhizobacterial and endophytic fungal strains biocontrol agents on radial growth of *P. capsici*

Rhizobacterial strains *P. fluorescens* and *B. subtilis* and endophytic strains were *T. asperellum* and *T. viride* were tested against *P. capsici* by dual culture technique (Webster, 1971). The mycelial disc (9 mm) from seven days old culture of *P. capsici* was placed in one side of the petriplate containing 15 ml of PDA medium. After three days of pathogen inoculation, 24 h old bacterial strains *P. fluorescens* and *B. subtilis* strains were streaked on the opposite of the petriplate by the help of sterilized inoculation needle. Endophytic fungal strains *T. asperellum* and *T. viride* were streaked simultaneously against *P. capsici*. Three replications were maintained for each treatment. The plates were incubated at room temperature (22 ± 2°C) for three days and seven days, respectively, and inhibition zone was measured. The radial growth of the pathogen and per cent reduction over control was calculated by using the formula as follows.

$$\text{Per cent reduction over control} = \frac{\text{C-T}}{\text{C}} \times 100$$

where, C – mycelial growth of the pathogen in control (mm) and T – mycelial growth of the pathogen in dual plate (mm).

2.4. Preparation of individual and mixtures of PGPR and PGPE bio-formulations

A loopful of *P. fluorescens* and *B. subtilis* were inoculated into the sterilized KB and NB and *T. asperellum* and *T. viride*, respectively, and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (26 ± 2°C) for bacteria and seven days of incubation for endophytic fungi. Bacterial suspensions (9×10^8 cfu/ml) and fungal endophytic suspension (5×10^5/g inoculum) was used for the preparation of talc-based formulation. To the 400 ml of suspension, 1 kg of the talc powder (sterilized at 105°C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose (CMC) 10 g (adhesive) were

mixed under sterile conditions, following the method described by Nandakumar *et al*. (2001). After shade drying for overnight, it was packed in polypropylene bag and sealed. At the time of application, the population of bacteria and fungal endophytes in talc formulation was 3×10^5/g of the inoculum. Finally, strains that are going to make up the mixture were added equally (v/v) and mixed with talc powder, $CaCO_3$ and CMC.

2.5. Greenhouse studies

2.5.1. Effects of bio-formulation mixtures on the incidence of foot rot disease

To study the ISR against *P. capsici* of pepper veins, a pot culture experiment was conducted with rhizosphere bacteria *B. subtilis*, *P. fluorescens* and endophytic fungi *T. viride* and *T. asperellum.* Six months veins were grown in earthen pots (size-0.35 m diameter, 0.50 m height, volume of soil: 0.04 m^3) filled with sterilized potting soil per vein per pot. In all the treatments, the talc-based bio-formulation mixture was applied for vine treatment and soil application. The fungicide (**RIDOMIL GOLD® MZ**) was used as a positive control. For treatment, the pepper vine **cv.** Subhakara was used. Six months pepper vines were sprayed with bacterial suspensions (9×10^8 cfu/ml) for one vine per pot (size-0.35 m diameter, 0.50 m height, volume of soil: 0.04 m^3) containing sterilized soil mixture (cow dung: sand: soil in 1:1:1 ratio) and inoculated with the spore suspension (2×10^5 spores/g of inoculum) of *P. capsici*. In another set, *Trichoderma* inoculum (5×10^5/g) per pot was sprayed for six months old vines (40 - 45 cm height). Vine treated with **RIDOMIL GOLD® MZ** (2 g/litre) was considered as positive control. The pepper vine inoculated with the pathogen alone served as inoculated control and without any pathogen inoculation served as healthy control. The observation on the development of *P. capsici* symptoms was recorded after 30th day of the inoculation. Each treatment was replicated thrice in completely randomized block design (CRD). The per cent disease index (PDI) was estimated using the formula suggested by Mckinney (1923).

2.5.2. Inoculation preparation of *P. capsici* for enzyme study

For inoculation purpose, *P. capsici* was cultured on a series of 9 cm diameter petriplates containing OMA at $22 \pm 2^{\circ}$C until mycelia cover the media. Mycelia suspension was prepared in sterilized distilled water. Using haemocytometer, inoculum load was adjusted to 2×10^5 spores/g.

2.5.3. Induction of defense-related protein and experimental design

Organisms from rhizosphere, *B. subtilis, P. fluorescens* and endophytic fungal strains *T. viride* and *T. asperellum* were used in the induction of defense reactions in pepper. The bio-formulations treated vine were grown at the rate of one vien per pot (size-0.35 m diameter, 0.50 m height, volume of soil: 0.04 m^3) filled with sterilized potting soil containing the spore suspension (2×10^5 spores/g of soil) of *P. capsici*. Cultures of rhizosphere bacteria and spores of endophytic fungi treated plants were challenge inoculated with *P. capsici* in the first set and in the second set of experiment, treated plants were not challenged with the pathogen. Plants without prior biocontrol treatment was inoculated with the pathogen and kept as control. The plants neither treated with bio-formulation nor challenged by the pathogen were kept as healthy control. Three replications were maintained in each treatment. Each replicate consisted of three pots and in each pot one vine were maintained. The experiments were conducted using completely randomized block design in a greenhouse. The humidity in the greenhouse was maintained at around RH 70%. The temperature was adjusted to 26°C (day)/20°C (night).

2.5.4. Sample collection and assay of defense-related proteins

Vine leaf tissues were collected at different time intervals (0, 4, 8, 16, 24, 48 and 72 h after pathogen inoculation). Three pepper vines were sampled from each replication of the treatment separately and were maintained for biochemical analysis. Leaf samples were homogenized with liquid nitrogen in a pre-chilled mortar and pestle. One gram of leaf sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying PO (Hammerschmidt *et al*., 1982), polyphenol oxidase (PPO) (Mayer *et al*., 1965) and PAL (Dickerson *et al*., 1984). Enzyme extracted in 0.1 M sodium citrate buffer (pH 5.0) was used for the estimation of chitinase (Boller and Mauch, 1988) and β-1, 3-glucanase (Pan *et al*., 1991). The total phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993).

2.5.5. Chitinase activity

Chitinase assay was performed by grinding 1 g of leaf sample using a chilled pestle and mortar with 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged at 8000 rpm for 20 min. The supernatant was used as a crude enzyme extract for assaying chitinase activity. The changes in the chitinase activity were determined by colorimetric assays described by Boller and Mauch (1988).

2.5.6. Assay for peroxidase

Fresh vine leaves (1 g) were homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) with pre chilled mortar and pestle. The homogenate was centrifuged at 18,000 rpm at 4°C for 15 min and used within 2 h. Supernatant was served as an enzyme source. To a spectrophotometric sample cuvette, 3 ml of buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0. 03 ml H_2O_2 solution were added and mixed well. Absorbance was recorded at 470 nm using spectrophotometer (Hitachi, 2000, Japan). The enzyme activity was expressed as changes in absorbance/min/g fresh weight (Hammerschmidt *et al*., 1982). Three replicates were maintained for each treatment.

2.5.7. Determination of phenylalanine ammonia lyase activity

Leaf tissues (300 mg) from each of three replicates for each treatment were homogenized in the ice-cold 0.25 M borate buffer (pH 8.7) in an ice bath. The homogenate was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was then centrifuged at 15000 rpm for 15 min at 4°C. The resultant clear yellowish-green supernatant was used as crude enzyme extract. The reaction mixture contained 1 ml of enzyme extract, 0.5 ml of 0.2 M borate buffer (pH 8.7), 1.8 ml of distilled water and 0.2 ml of 1 M l-phenylalanine. Changes in absorbance at 290 nm were observed using spectrophotometer (Hitachi, Japan, 2000). Reaction mixture without substrate served as control. One unit of enzyme activity produced 3.37 nm of cinnamic acid/h (Singh and Prithiviraj, 1997). Three replicates were maintained for each treatment.

2.5.8. Assay of polyphenol oxidase

One gram fresh weight from biocontrol agents, endophytic fungal treated and control plants were ground to a fine powder in liquid nitrogen and extracted in 1 ml of extraction buffer containing 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000 g for 15 min at 4°C and the supernatant was used as enzyme source. The reaction mixture was started when 0.2 ml of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm at 30 sec intervals for 3 min. The mean change in absorbance was calculated for 1 min and the activity was expressed as changes in absorbance/min/mg of protein of the plant sample (Mayer *et al*., 1965).

2.5.9. **Assay for β-1, 3-glucanase**

The crude extracts was added to 62.5 µl of lamanarin and then incubated at 40°C for 10 min; the reaction was stopped by adding 375 µl of dinitrosalicylic acid and heated for 5 min on boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with lamanarin with zero time incubation served as blank. The activity was expressed as µg equivalent of glucose/min/mg of protein (Kavitha *et al*., 2005).

2.5.10. Estimation of phenol

One gram of fresh sample was homogenized with 10 ml of 80% methanol (Zieslin *et al*., 1993). One milliliter of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25°C. The absorbance of the blue was measured using a spectrophotometer (Hitachi, 2000, Japan) at 725 nm. Catechol was used as the standard (Kagale *et al*., 2004).

2.5.11. Statistical analysis

The data on effect of the treatments on the growth of pathogens, severity of diseases and activity of enzymes in pepper vine were analyzed by analysis of variance (ANOVA), and treatment means were compared by Duncan's multiple range test (DMRT). The data on disease severity was arcsine transformed before undergoing statistical analysis (Gomez and Gomez, 1984).

III. RESULTS

3.1. Compatibility among bacterial strains

PGPR strain of *B. subtilis, P. fluorescens* and PGPE strains of *T. asperellum* and *T. viride* were tested for their compatibility *in vitro*. None of the antagonistic bacteria were inhibited by each other, so the absence of inhibition zone suggesting that these biocontrol agents and endophytic fungal strains were compatible with each other.

3.2. Effect of biocontrol agents on radial growth of *P. capsici*

PGPR strain of *B. subtilis*, *P. fluorescens* and endophytic fungal strains of *T. viride* and *T. asperellum* were tested individually to assess the radial growth of *P. capsici*. All the treatments were effective in reducing the mycelial growth of the pathogen. However, *B. subtilis* had resulted with mycelial growth of 50 mm and inhibition zone of 25 mm and P. *fluorescens* showed 63 mm of mycelial growth and 18 mm inhibition zone, respectively. *Trichoderma viride* recorded the maximum inhibition zone of 26 mm, and *T. asperellum* showed 19 mm inhibition zone and 60 mm mycelia growth, respectively. The control plates recorded the highest mycelial growth of 91 mm (Table 1).

Table.1: Effect of biocontrol agents on the mycelial growth of P. capsici

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Sl. No	Treatments	Mycelial growth (mm)	Inhibition zone (mm)
1	*Bacillus subtilis*	50±0.32[de]	25±0.50[f]
2	*Psuedomonas fluorescens*	63±0.22[ef]	18±0.28[g]
3	*Trichoderma asperellum*	59±0.5[bc]	19±0.50[h]
4	*Trichoderma viride*	49±0.32[a]	26±0.50[a]
5	**RIDOMIL** GOLD	56±0.32[bcd]	17±0.50[e]
6	Control	91±0.12[h]	0.00

Values are mean of three replications. In a column, mean followed by a common letter (s) are not significantly different at the 5% level by DMRT

3.3. Efficacy of PGPR and PGPE strains on foot rot incidence under greenhouse conditions

Talc-based bio-formulation of PGPR strains *B. subtilis*, *P. fluorescens* and *T. asperellum*, *T. viride* strains individually were tested for their efficacy against *P. capsici* on pot method along with **RIDOMIL** GOLD as a chemical check. *Bacillus subtilis* significantly reduced the foot rot incidence by 20% and *P. fluorescens* showed 31% disease incidence compared to untreated plants upon control with 68% as shown in figure 1. Conspicuously, *T. viride* resulted in a significantly lower foot rot disease index (PDI) than any of the strains of 18%, and *T. asperellum* showed 33% disease incidence, respectively.

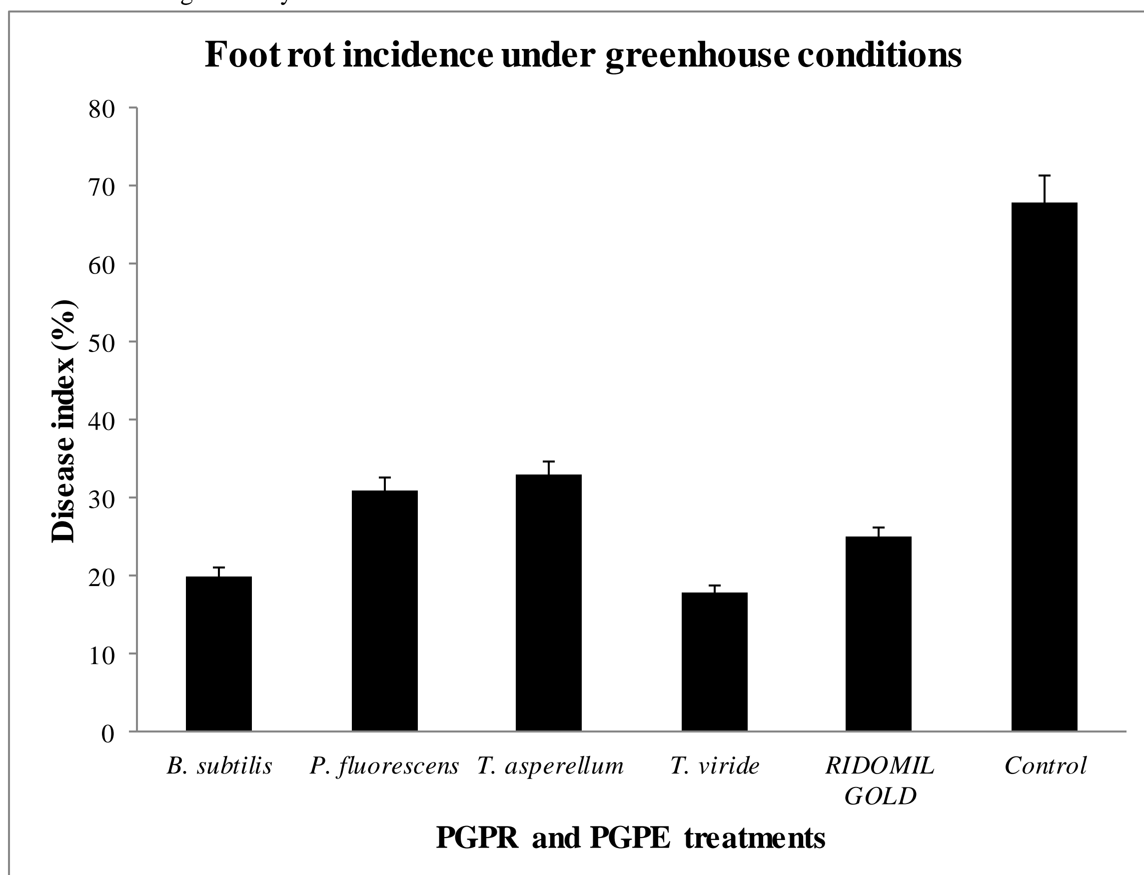

Fig.1: Efficacy of individual and mixture of biocontrol agents on foot rot incidence in black pepper under greenhouse conditions

Values are mean of three replications. The line on each bar represents ±SEM and the post test analysis was carried out using DMRT. The results suggest that $p>0.05$.

3.4. Effect of PGFR and PGPE treatment on Chitinase activity

Higher chitinase activities were observed in endophytic fungal strain *T. viride* pre-treated black pepper

challenge inoculated with pathogen (*P. capsici*) as seen in figure 2. Plants grown from different pre-treated vines presented the highest chitinase activity. *Trichoderma viride, T. asperellum* and *B. subtilis* were effective in yielding highest activity compared with other strains. The presence of pathogen (uninoculated control) alone not increased the chitinase activity compared to healthy control and inoculated, respectively.

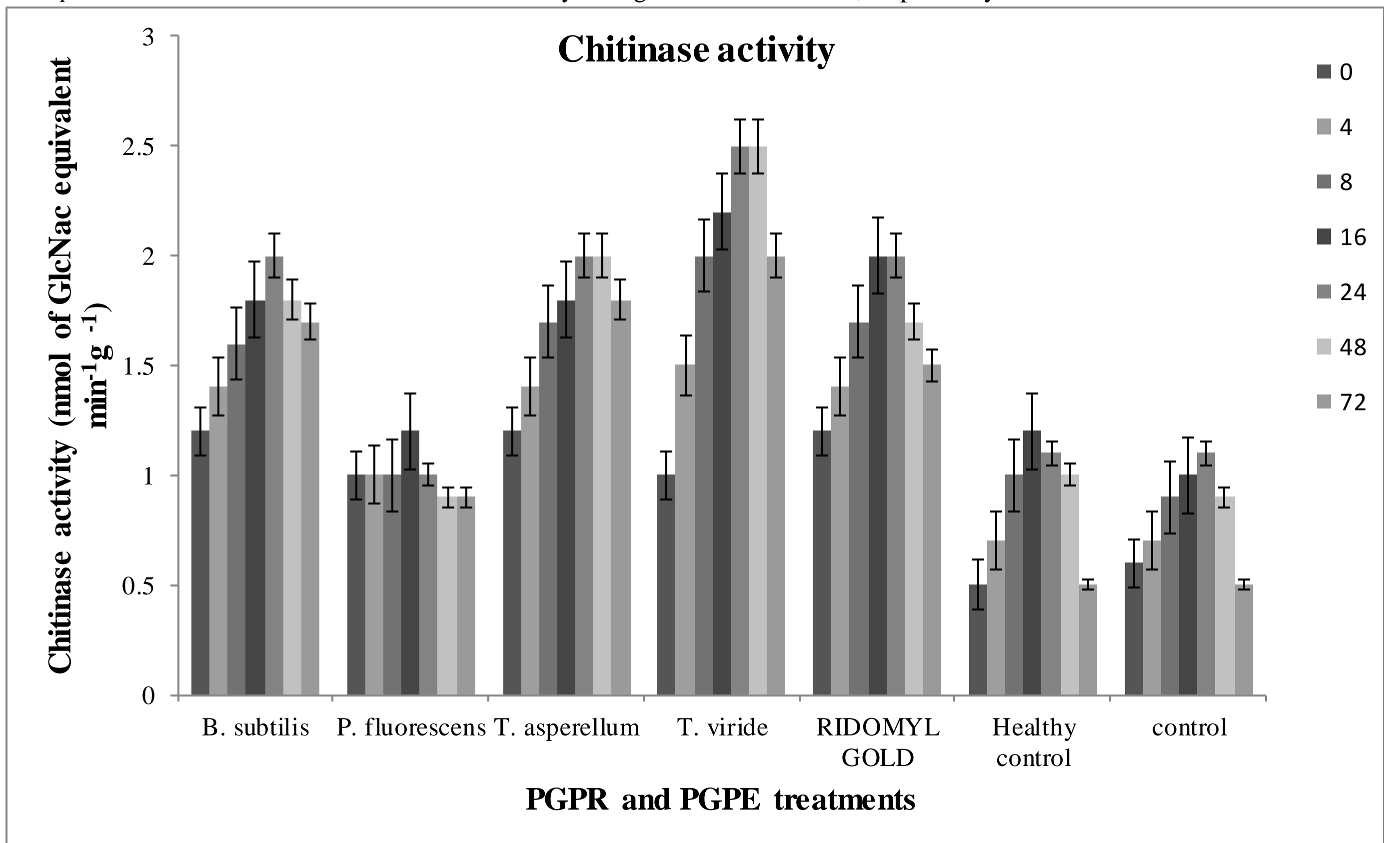

Fig. 2: Induction of chitinase in black pepper in response to PGPR and PGPE treatments against foot-rot disease

3.5. Effect of PGFR and PGPE treatment on peroxidase activity

Peroxidase assay indicated the increased peroxidase activity in the pepper vine inoculated with the target fungus *P. capsici*. After 48 h of challenge inoculation, and gradually decreases at 72 h this shows that peroxidase activity increased gradual persistence of resistance as shown in figure 3. *Trichoderma viride* were very effective in enhancement of peroxidase compared to *T. asperellum* and *B. subtilis* strains.

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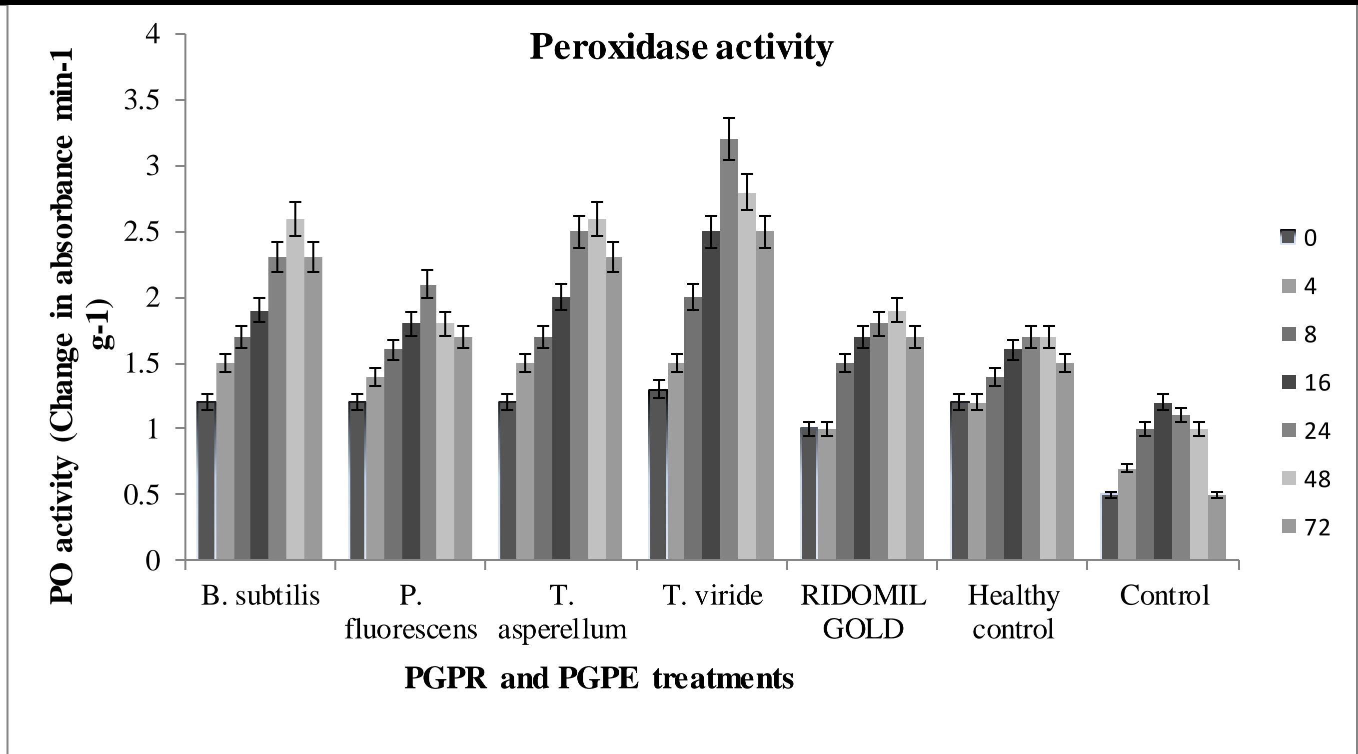

Fig.3: Induction of peroxidase activity in black pepper in response to PGPR and PGPE treatments against foot-rot disease

3.6. Effect of PGFR and PGPE treatment on polyphenols oxidase activity

Increase in polyphenol oxidase activities were observed in *T. viride*, *T. asperellum* and *B. subtilis* compared to *P. fluorescens* treated black pepper vines which showed a drastic increase from 24 to 48 h and decreases the activity at 72 h after inoculation as shown in figure 4.

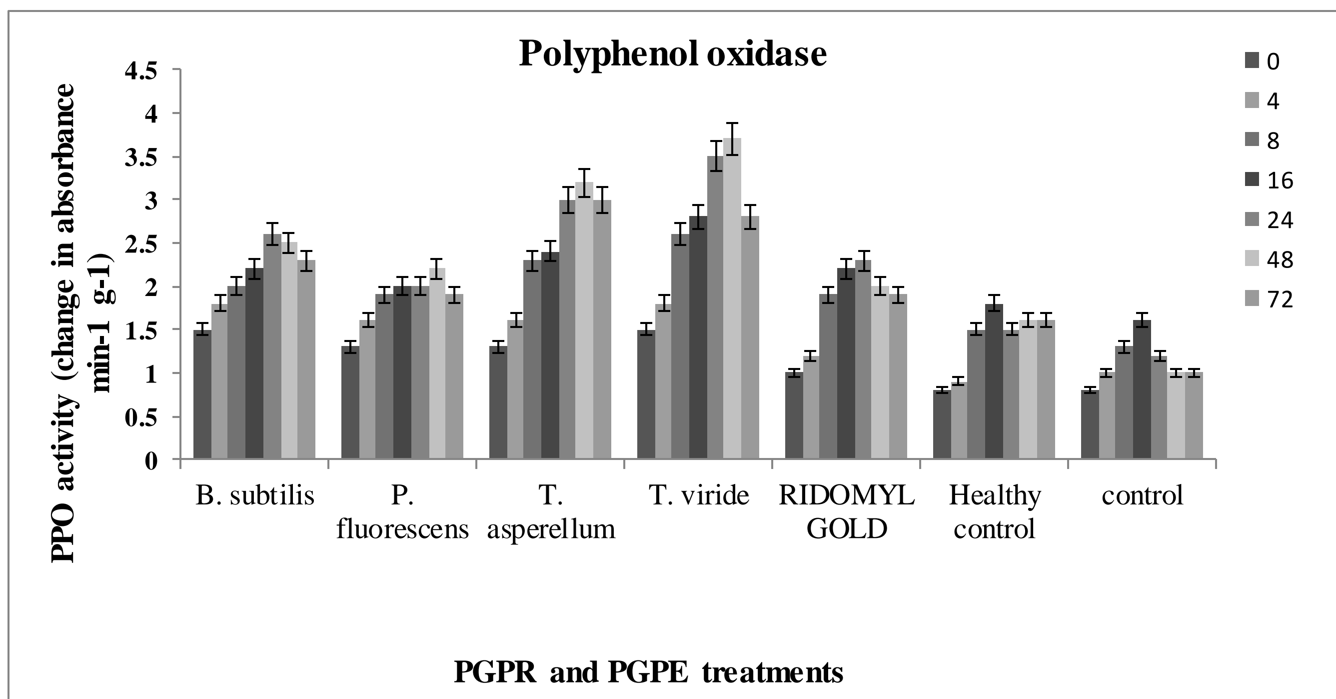

Fig.4: Induction of polyphenol oxidase in black pepper in response to PGPR and PGPE treatments against foot-rot disease

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3.7. Effect of PGFR and PGPE treatment on Phenylalanine ammonia lyase activity

Phenylalanine ammonia lyase (PAL) can be seen that microbiolization of vines with different PGPR and PGPE strains lead to increase in PAL activity. Also, changes in PAL activities were observed after challenge inoculation with the target pathogen *P. capsici* up to 48 h and drastic decrease at 72 h after challenge inoculation as shown in figure 5. *Trichoderma viride* was most effective in enhancing the PAL activity compared to other strains. *Trichoderma asperellum* and *B. subtilis* also showed higher activity when compared to *P. fluorescens,* healthy control and control.

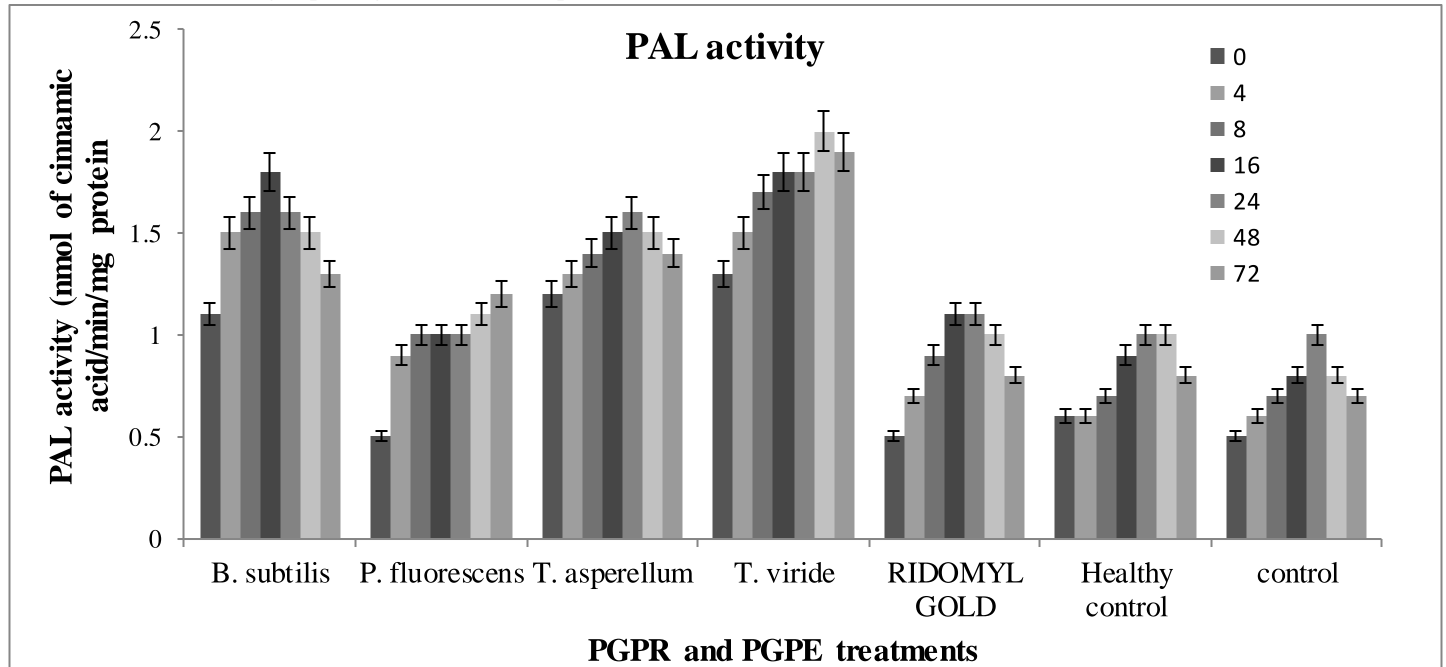

Fig. 5: Induction of phenylalanine ammonia lyase in black pepper in response to PGPR and PGPE treatments against foot -rot disease

3.8. Effect of PGFR and PGPE treatment on β 1,3-glucanase activity

The β 1, 3-glucanase activities were increased at 24 h up to 48 h after challenge inoculation. *Trichoderma viride*, *B. subtilis* and *T. asperellum* pretreated vines enhance their β1, 3-glucanase activity compared with other strain *P. fluorescens* as shown in figure 6.

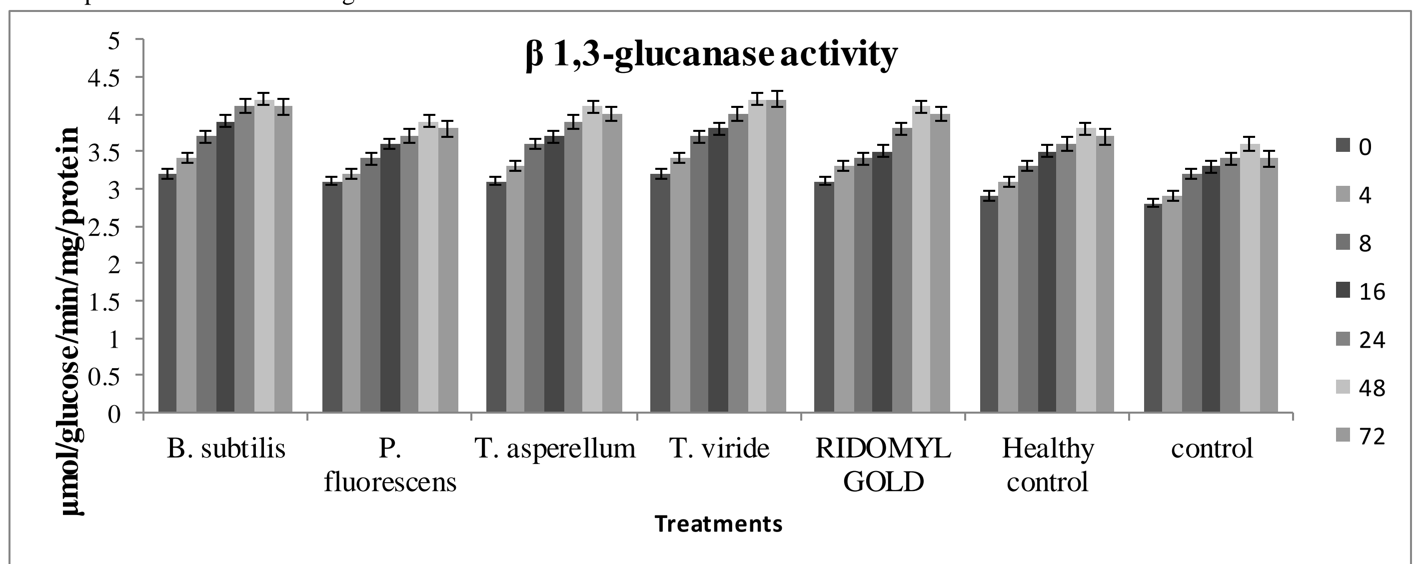

Fig. 6: Induction of β 1, 3-glucanase in black pepper in response to PGPR and PGPE treatments against foot-rot disease

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3.9. Effect of PGFR and PGPE treatment on phenol content

Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In the present study, vine treatments with *T. viride* and *B. subtilis* resulted in increased accumulation of phenolic substances in response to pathogen infection. Higher level of phenol activity was observed in *T. viride*, *B. subtilis* and *T. asperellum* treated vines when compared with *P. fluorescens*, healthy control and control treated vines as shown in figure 7.

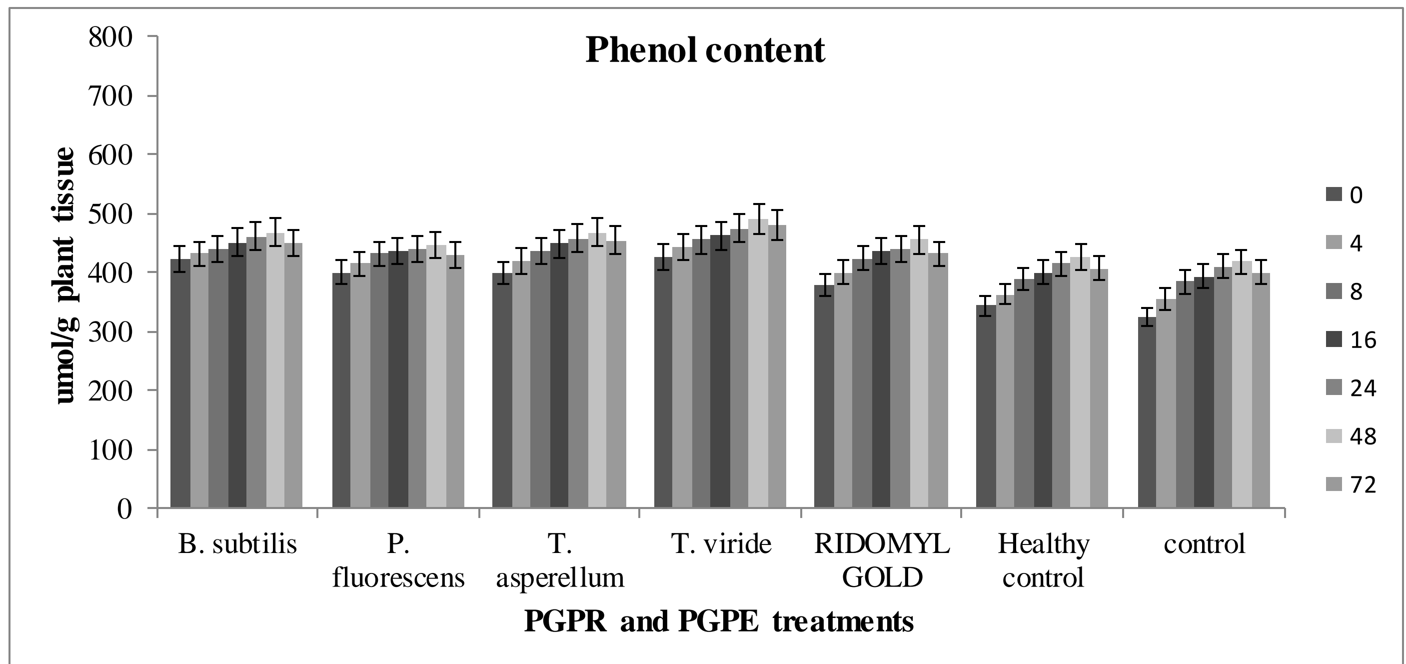

Fig. 7: Induction of phenol content in black pepper in response to PGPR and PGPE treatments against foot -rot disease

IV. DISCUSSION

Two strains of rhizobacterial isolates and two fungal endophytes were used for the efficacy study of foot rot disease. These isolates also induced the systemic resistance against *P. capsici* and in turn they enhanced the plant height. The massive accumulation of phytoalexins and phenolic compounds, the increased accumulation of PR proteins and peroxidase, increased the levels of mRNAs encoding phenylalanine ammonia-lyase (PAL), chalcone synthase and PR1a proteins and enhanced lignifications have been reported in plants following treatment with PGPR isolates (Hynes and Lazarovits, 1989; Van Peer *et al*., 1991; Zdor and Anderson, 1992; M'Piga *et al*., 1997). PGPR are free-living bacteria having a beneficial effect on plants as they enhance emergence, colonize roots and stimulate growth (Kloepper *et al*., 1988). In recent years, the concept of using PGPR and PGPE for the promotion of plant growth is gaining worldwide acceptance (Kloepper *et al*., 1991). PGPRs stimulate host plant growth through several possible mechanisms, including biological control (Pleban *et al*., 1995), induced systemic resistance to plant pathogens (Benhamou, 1996; Hallman *et al*. 1997), phytohormone production and the improvement of nutrient and water uptake (Pleban *et al*., 1995; Nowak and Lazarovits, 1997). They also improve plant performance in stress environments and consequently enhance yields (Frommel *et al*., 1991). The present findings are in agreement with these findings.

Increase in the chitinase activity was observed in PGPR treated pepper plants. Our results are in confirmation with the findings of Silva *et al*. (2004). Reports on the increase in chitinase activity in tissue expressing resistance in diverse pathosystems have been made (Croft *et al*., 1990). The mode of pathogen infection that excludes direct penetration in host cells (Romeiro, 1995), suggests the possibility that products of chitinase activity posses some antimicrobial activity (Sailaja *et al*., 1998).

The present study demonstrated that the isolates of rhizobacterial *B. subtilis,* endophytic fungi *T. viride* and *T. asperellum* consistently reduced the radial mycelial growth of *P. capsici* by producing various antibiotics and reduced the foot rot of pepper under green house and field conditions by inducing ISR compared to individual agents. Further, plant growth promoting rhizobacteria (PGPR),

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especially *B. subtilis* and plant growth promoting rhizobacteria (PGPR), especially *P. fluorescens* (Pf1) strains have been developed commercially as a talc-based formulation and tested against several crop of plant diseases (Vivekananthan *et al*., 2004; Rajendran *et al*., 2007). Sundaramoorthy *et al*. (2012) reported that the combination of *P. fluorescens* strains and *B. subtilis* strain together resulted in significant growth promotion that was correlated with induced resistance in *Capsicum annum* L. Several approaches have been made to manage the foot rot of pepper. A large number of enzymes have been associated with ISR, including peroxidase, phenyalalanine ammonia-lyase, lipoxygenase, β-1, 3 glucanase and chitinase (Koch *et al.,* 1992; Schneider and Ullrich, 1994; Van Loon, 1997; Ye *et al*., 1990; Silva *et al*., 2004). Furthermore, interactions among the bacterial strains may have synergistic effects that could induce ISR and promote the growth of the plants (Sundaramoorthy *et al*., 2012). Several literature have been documented that the use of biocontrol agents and endophytic fungal strains were more effective for management of plant diseases (Kavino *et al*., 2007; Harish *et al*., 2008; Sivakumar, 2012).

In the present study, *T. viride* and *B. subtilis* increased the peroxidase (PO) and polyphenol oxidase (PPO) activities, which increased the maximum activities on 48th h after challenge inoculation with *P. capsici*. The PO and PPO catalyse the last step in the biosynthesis of lignin and other oxidative phenols. Zdor and Anderson (1992) noticed that rhizosphere *B. subtilis* induced PO activity in bean. Chen *et al*. (2000) reported that the higher PO activity was observed in cucumber roots treated with *P. fluorescens* challenged with *P. aphadermatum*. Our results are in agreement with the results of Podile and Laxmi (1998) and Silva *et al*. (2004). The high peroxidase activities are usually associated with later stages of the infection process and are linked to generation of hydrogen peroxides that inhibit pathogens directly or generate other free radicles with antimicrobial effects (Hammerschmidt *et al*., 1982).

PAL activity was also increased at 48th h after challenge inoculation with target pathogen due to rhizobacterial and endophytic strains. In which, *B. subtilis* and *T. viride* and *T. asperellum* effectively enhanced the enzyme activity in the presence of pathogen when compared to other strains of *P. fluorescens*. Similar results were reported by Sundaramoorthy *et al*. (2012) in capsicum against *P. capsici*. The product of phenyl alanine trans-cinammic acid, is directly linked to cell lignifications processes and the highest levels of PAL activity usually about 24 h after initial infection (Podile and Laxmi, 1998). So, PAL generally shows increased activity during pathogen establishment in the host tissue (Bhattacharya and Ward, 1988). PAL plays an important role in the biosynthesis of phenolic phytoalexins (Daayf *et al*., 1997). The increase in PAL activity indicates the activation of phenyl propanoid pathway. In several host–pathogen interactions, increased levels have been shown to be correlated with incompatibility (Rathmell, 1973; Bhattacharya and Ward, 1988; Ralton *et al*., 1989). The product of PAL is trans-cinnamic acid which is an immediate precursor for the biosynthesis of SA, a signal molecule in systemic acquired resistance (SAR) (Klessig and Malamy, 1994).

β 1, 3-glucanase activity was increased in PGPR and PGPE treated black pepper after 48 h of challenge inoculation with target pathogen. Sundaramoorthy *et al*. (2012) have reported that seed treatment with PGPR and PGPE has produced hydrolytic enzymes such as chitinases and β1, 3-glucanases in capsicum. These host lytic enzymes accumulates at the site of penetration of the fungus *P. capsici* resulting in the degradation of the fungal cell wall (Benhamou *et al*., 1996). In a number of plant species, β1, 3-glucanase exists in multiple forms. These enzymes solubilize elicitor's active glucan molecules from the fungal cell wall (Mauch and Staechelin, 1989) and also induce defense enzymes. When the pathogen grows initially in the intercellular spaces, the fungus may come in contact with β1, 3-glucanase localized in the middle lamellae.

Increment of phenol was high in vine treated with *B. subtilis*, *T. viride*, *T. asperellum* and *P. fluorescens* resulted in increased accumulation of phenolic substances in response to infection by the pathogen among them *T. viride* and *B. subtilis* showed increased phenolic substance when compared to *T. asperellum* and *P. fluorescens*. M'Piga *et al*. (1997) reported that *B. subtilis* induced the accumulation of phenolic substances which exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. Ramamoorthy and Samiyappan (2001) have reported the accumulation of phenolic substances and PR proteins in response to infection by *F. oxysporum* f. sp. *lycopersici* in tomato. Our results substantiate the inhibition of various plant pathogen and disease management by using several biocontrol agents through the induction of ISR in plants reported by Van Peer *et al*. (1991), Kloepper (1993), Van Loon (1997), Chen *et al*. (2000). Thus, our findings provide evidence that the induction of defense enzymes and PR proteins by application of endophytic fungi and rhizosphere bacterial strains may strengthen the plants against various biotic stresses.

V. CONCLUSION

The present study demonstrates that PGPR and PGPE are due to isolates of *T. viride* and *B. subtilis* and their role in enhancing growth on foot rot disease of pepper. Moreover, it is concluded that *T. viride* and *B. subtilis* increase the plant growth and resistance to *P. capsici*. It is easily imagined that the different isolates of rhizobacterial strains and endophytic fungal strains produces antimicrobial products and defense enzymes restrict the development of challenging phytopathogenic fungi.

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