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Systemic elicitation of defense related enzymes suppressing *Fusarium* wilt in mulberry (*Morus* spp.)

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Antagonist and organic amendments based bioconsortia (seri bed waste+Pf1+Bs4+Th1+neem cake) was found to lead the restriction of *Fusarium solani* pathogen in mulberry roots mainly by inducing inherent defense enzymes. Induction of defense enzymes such as peroxidase, polyphenoloxidase, phenylalanine ammonia lyase, phenols, catalase and superoxide dismutase, was studied in mulberry plants pretreated with bioconsortia and comparative fungicides challenged inoculation with *Fusarium solani* in glasshouse condition. There was increased expression of defense enzymes in mulberry plants treated with bioconsortia, when compared to control. The bio-chemical reaction of the above defense enzymes started to increase at the 3rd day, reached maximum on the 5th day and thereafter declined gradually. The native polyacrylamide gel electrophoresis (PAGE) experiment showed that one to four isoforms of the defense enzymes each with greater intensity were expressed in these treatments. This is indicating that the restriction of *F. solani* in mulberry plants was mainly due to application of microbial bio-consortia.

Key words: Bio-consortia, defense enzymes, *Fusarium* wilt, isoforms, mulberry.

INTRODUCTION

Mulberry (*Morus* spp.) is a fast growing deciduous woody perennial plant belonging to the genus *Morus* and grows under various climatic conditions ranging from temperate to tropical regions. Mulberry foliage is the best food for the silkworm (*Bombyx mori L.*). The total acreage of mulberry in India is around 282,244 ha. Cultivation of mulberry is one of the most important aspects in sericulture industry. The diseases have become more alarming because of its epidemic nature and propensity to kill the plant completely. Among the various diseases, *Fusarium* wilt of mulberry is considered as severe disease leading to death of entire plant. In India, the wilt is caused by soil borne fungal pathogen *Fusarium solani* (Siddaramaiah and Hegde, 1990).

Although fungicides are effective in the management of the diseases, they leave harmful residues in the soil, causing environmental pollution and other deleterious effects on the ecosystem (Misato and Yamayuchi, 1977). The biological control method has been considered as a promising approach

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for the management of soil borne diseases by enhancing the inherent systemic resistance. However the most of the approaches mainly biological control of plant disease have used a single organism. The application of a single antagonist is not likely to be better in all environmental conditions where it is applied. Thus more emphasis is laid on the combined used of two or more antagonist with preferred substrates, which has turned out to be more successful than either of them alone, as reported by several researchers (Thilagavathi et al., 2007; Sundaramoorthy et al., 2013). Antagonistic microorganisms *viz., Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated in host plants individually and in combinations for their resistance inducing ability against *F. solani* pathogen in mulberry crop. Plant has endogenous defense mechanisms that can be induced in response to attack by insects or pathogens. Defense reaction occurs due to the accumulation of PR-proteins, phytoalexins, chalconesynthase, phenylalanine ammonia lyase (PAL), peroxidase (PO), catalase (CAT) polyphenoloxidase (PPO), superoxide dismutase (SOD) and phenolics (Manikandan and Raguchader, 2014). Considering the importance of mulberry, destructive nature of the disease, the present studies were undertaken to study the induction of defense-associated enzymes against *F. solani* of mulberry using potential biocontrol agents (Choudhari et al., 2012).

MATERIALS AND METHODS

Collection and maintenance of biocontrol agents

Ten (10) isolates of *Bacillus* spp., 7 isolates of *Pseudomonas* spp*.,* and 10 isolates of *Trichoderma* spp., were isolated from the rhizosphere soil samples collected from mulberry fields by serial dilution (Pramer and Schmidt, 1956) using *Trichoderma* selective medium for *Trichoderma* spp., King's B (KB) medium for *Pseudomonas* spp. and nutrient agar (NA) for *Bacillus* spp. One isolate of *Pseudomonas fluorescens* (Pf1) and one isolate of *Trichoderma harzianum* (Th1) were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. Each of agents were further purified and sub-cultured in the selective medium. The efficient strains of *T. harzianum* and with plant growth-promoting rhizobacteria (PGPR) bacteria biocontrol agents, *P. fluorescens* and *B. subtilis* were evaluated for their antagonistic activity against *F. solani* by dual culture technique (Dennis and Webster, 1971).

Pot culture experiment

A pot culture experiment was laid out in completely randomized design to test the efficacy of fungicides (carbendazim, pre mixture fungicide carbendazim + mancozeb, tebuconazole) and selected compatible biocontrol agents (Pf1, Bs4 and Th1) along with seri-bed waste and neem cake. Potting medium (red soil: cow dung: manure at 1:1:1 w/w/w) was autoclaved twice and filled in pots. The culture of *F. solani* was mass multiplied separately in sand maize medium and inoculated separately in different pots grown with mulberry plants at 5% (w/w) around collar region. The pathogen alone inoculated served as control. Three replications (three pots per replication) were maintained and the pots were arranged in a randomized manner. The incidence of *F. solani* wilt disease was recorded on 60 days post inoculation (dpi). The treatments details were as follows.

Treatments

T_1: Soil drenching of consortia (seribed waste at 100 g+ Pf_1 at 10 g + Bs_4 at 10 g+ Th_1 at 50 g + neem cake at 30 g/plant) at 200 g/plant
T_2: Soil drenching of *Pseudomonas fluorescens* (Pf_1) at 10 g/plant.
T_3: Soil drenching of *Bacillus subtilis* (Bs_4) at 10 g/plant.
T_4: Soil drenching of *Trichoderma harzianum* (Th_1) at 50 g/plant
T_5: Soil drenching of tebuconazole at 0.1%
T_6: Soil drenching of carbendazim at 0.1%
T_7: Soil drenching of carbendazim + mancozeb at 0.1%
T_8: Control (untreated)

Collection of samples and enzyme extraction

Leaf samples were collected from bottom portion of the plant at two days interval up to 9 days. Two leaves from each replication of the treatment were collected for biochemical analysis. The leaf tissues were homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0). The homogenate was centrifuged for 20 min at 10,000 rpm at 4°C. The supernatant was used as enzyme extracts and used for the assay of phenylalanine ammonia lyase, peroxidase, polyphenoloxidase, superoxide dismutase and catalase enzymes.

Assay of defense related enzymes

Assay of phenylalanine ammonia lyase (PAL)

PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm. 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. Enzyme activity was expressed in fresh weight basis as nmol *trans*-cinnamic acid $min^{-1}mg^{-1}$ of sample (Dickerson et al., 1984).

Assay of peroxidise (PO)

Fresh mulberry 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. The supernate served as an enzyme source and was used within 2 to 4 h. To a spectrophotometric sample cuvette, 3 ml of sodium phosphate 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. The absorbance was recorded at 420 nm using spectrophotometer. The enzyme activity was expressed as changes in absorbance min^{-1} g^{-1} of fresh tissue (Hammerschmidt and Kuc, 1982).

Assay of polyphenoloxidase (PPO)

The polyphenoloxidase activity was determined as the procedure given by Mayer et al. (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as change in absorbance min^{-1} g^{-1} of protein.

Assay of phenol

One gram of the mulberry leaves was ground in 10 ml of 80% methanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernate was evaporated to dry and the residue was dissolved in 5 ml of

distilled water. 0.2 ml of solution was taken and was made up to 3 ml with distilled water and then 0.25 ml of (1 N) Folin-Ciocalteau reagent was added. After 3 min, 1 ml of 20% sodium carbonate was added and mixed thoroughly. Thus the tubes were placed in boiling water for one minute and cooled. The absorbance was measured at 725 nm against a reagent as blank. The phenol activity was expressed as µg of catechol g^{-1} of plant tissue (Zieslin and Ben-Zaken, 1993).

Assay of catalase (CAT)

CAT activity was assayed spectrophotometrically as described by Chaparro-Giraldo et al. (2000) using 3 ml assay mixture containing 100 mM potassium phosphate buffer (pH 7.5), 2.5 mM H_2O_2 prepared immediately before use and 100 µl enzyme extract. The activity was measured by monitoring the degradation of H_2O_2 using UV-visible spectrophotometer (Varian Cary 50) at 240 nm over 1min, against a plant extract-free blank. The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, activity was calculated using the extinction coefficient ($\varepsilon_{240\ nm}$ = 40 mM^{-1} cm^{-1}) for H_2O_2 and expressed in µmol min^{-1} mg^{-1} of sample.

Assay of superoxide dismutase (SOD)

The enzyme extracts were prepared by homogenizing 1 g of the mulberry leaf in 2 ml of 0.2 M citrate phosphate buffer (pH6.5) at 4°C. The homogenate was centrifuged at 15,000 rpm at 4°C for 30 min. The supernate served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT (Giannospolitis and Ries, 1977). The assay mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA, 100 µl of the enzyme extract and the riboflavin which was added at the end. Tubes were shaken and placed under a 40-W fluorescent at 25°C. The reaction was initiated and terminated by turning the light on and off respectively. The absorbance at 560 nm was measured against identical non illuminated in parallel to the sample tubes for blank. Each extract was subtracted from the blank and multiplied by 100 to obtain the percentage inhibition of NBT-photoreaction. The SOD activity was expressed in SOD units g^{-1}tissue (50% NBT inhibition=1 unit)

Native anionic polyacrylamide gel electrophoresis

Peroxidase (PO)

To study the expression pattern of different isoforms of peroxidases in different treatments, native anionic polyacrylamide gel electrophoresis was carried out. One gram of mulberry leaf tissue was homogenized in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0), centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used as enzyme source. For native anionic polyacrylamide gel electrophoresis (PAGE) resolving gel of 8% acrylamide concentration and stacking gel of 4% acrylamide concentration were prepared. After electrophoresis the gels were incubated in a solution containing 0.15% benzidine in 6% NH_4Cl for 30 min in darkness. Then a few drops of 30% H_2O_2 were added with constant shaking until the appearance of bands. After staining the gel was washed with distilled water and photographed (Sindhu et al., 1984).

Polyphenoloxidase (PPO)

One gram of mulberry leaf tissue was homogenized in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0), centrifuged at 10000 rpm for 15 min at 48°C and the supernatant was used as an enzyme source. After native electrophoresis, the gel was equilibrated for 30 min in 0.1% *p*-phenylenediamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in the appearance of dark brown discrete enzyme bands. After staining, the gel was washed with distilled water and photographed (Jayaraman et al., 1984).

Superoxide dismutase (SOD)

Electrophoresis was carried out under native condition in 8% polyacrylamide gels for SOD activity staining. Electrophoresis running conditions were as described by Vitoria et al. (2001) and buffers and gels were prepared as described by Laemmli (1970) lacking SDS. Equal amounts of enzyme (40µg) were loaded on to each lane. SOD activity was determined on native PAGE gels as described by Beauchamp and Fridovich (1971) and modified by Azevedo et al. (1998). The gels were rinsed in deionized water and incubated in the dark for 30 min at room temperature in an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3% (v/v) N,N,Ń,Ń-tetramethylethylenediamine (TEMED). At the end of this period, the gels were rinsed with deionized water and placed in deionized water and exposed on a light box for 5 to 10 min at room temperature until the development of colourless bands of SOD activity in a purple-stained gel was visible. The reaction was stopped by transferring the gels to 6% (v/v) acetic acid.

Statistical analysis

The data were statistically analyzed. The treatment means were compared by Duncan's multiple range test (DMRT). The software package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines.

RESULTS AND DISCUSSION

Induction of defense related enzymes in mulberry plants

Results of colorimetric assay revealed that bio-consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) significantly increased activities of PO (Figure 1), PPO (Figure 2), PAL (Figure 3), total phenol (Figure 4), CAT (Figure 5) and SOD (Figure 6) enzymes in mulberry plant against wilt disease. The increased activity was observed up to five days in all the treatments and thereafter declined. The mulberry treated with bio-consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) gave the highest anti-oxidative and defense related enzymatic activity. The mulberry treated with Pf1 gave the second highest enzymatic activity. The mulberry treated with pathogen alone recorded (changes in absorbance $min^{-1}g^{-1}$ of fresh tissue) only slightly increase in enzymes activity. The healthy mulberry without any treatments showed lesser activity. Accumulation of PO has been correlated with ISR in several crops (Ramamoorthy et al., 2001). Isolates of *Pseudomonas* systemically induced resistance against *Fusarium* wilt of chickpea and suppressed the disease by 34.45% when compared to control (Saikia et al., 2005). Ramamoorthy and Samiyappan (2001) observed accelerated PPO activity in chilli plants treated with *P. fluorescens* when co-inoculated with *Colletotrichum capsici*. Induction of PAL by

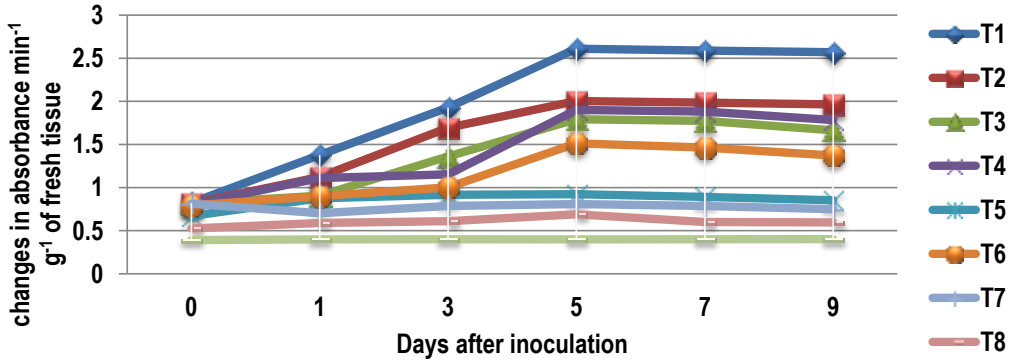

Figure 1. Induction of peroxidase (PO).

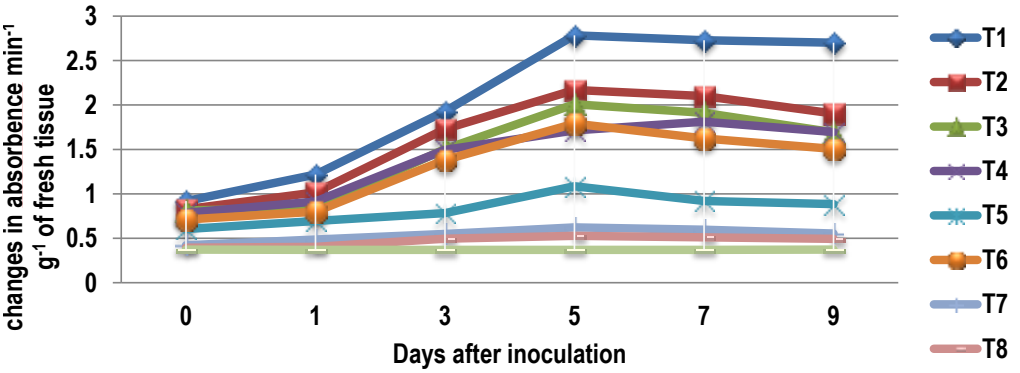

Figure 2. Induction of polyphenoloxidase (PPO).

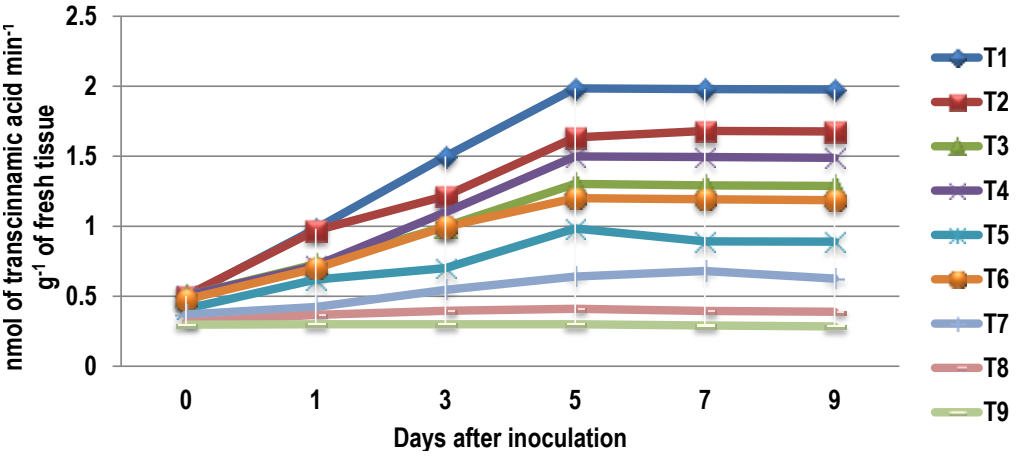

Figure 3. Induction of phenylalanine ammonia lyase (PAL).

fluorescent pseudomonads was reported in cucumber against *Pythium aphanidermatum* (Chen et al., 2000). Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou et al., 1997). Superoxide dismutase and catalase (which scavenges O^{-}_{2}) suppresses the oxidative burst during the pathogen infection (Vera-Estrell et al., 1993).

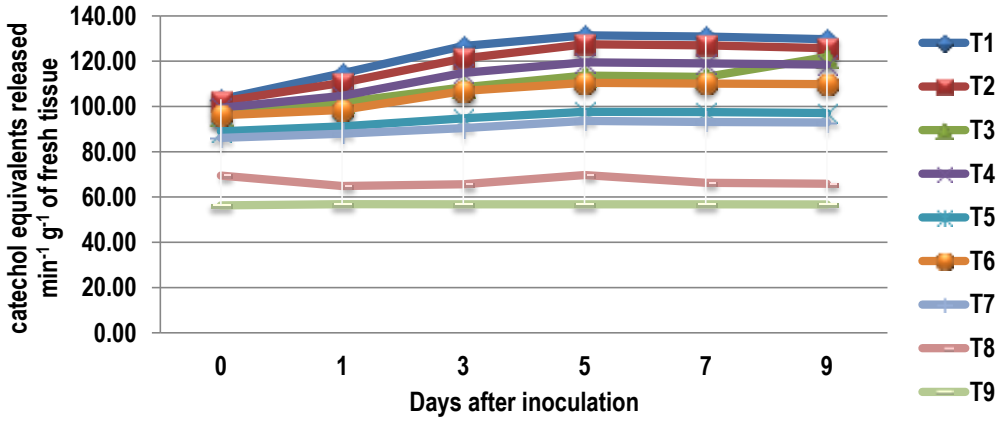

Figure 4. Induction of total phenol.

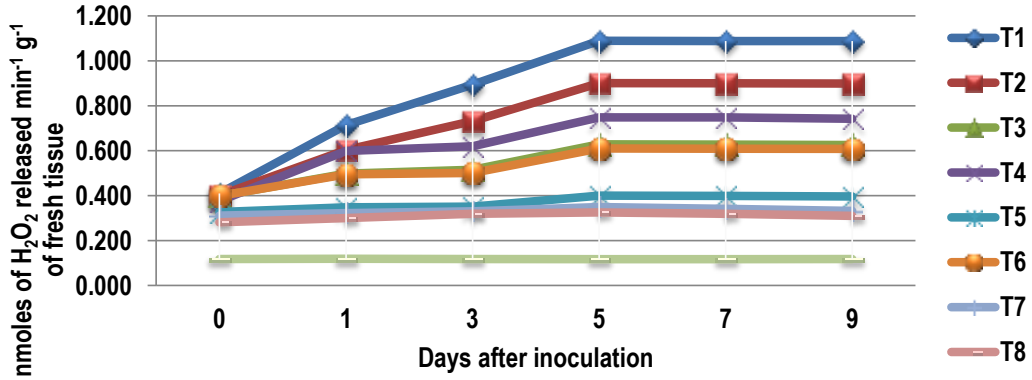

Figure 5. Induction of catalase (CAT).

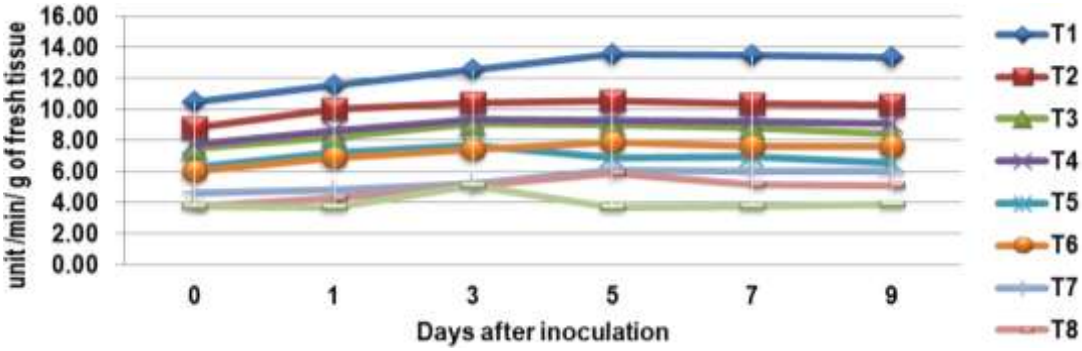

Figure 6. Induction of superoxide dismutase (SOD).

Detection of different isozymes by native gel

Isoforms pattern of peroxidase (PO)

In the present investigation, native polyacrylamide gel electrophoretic separation of enzyme extracts from biocontrol agents and fungicides treated plants were tested for the induction of PO and PPO isoforms separately. The results showed that plants were treated with bio-consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) and Pf1 showed four isoforms *viz*., PO1, PO2 PO3 and PO4 (Plate 1). The other treatments *viz.,* Th1, tebuconazole, carbendazim showed three isoforms *viz.,* PO1, PO2, and PO3. PO is a useful marker of plant development, physiology, infection and

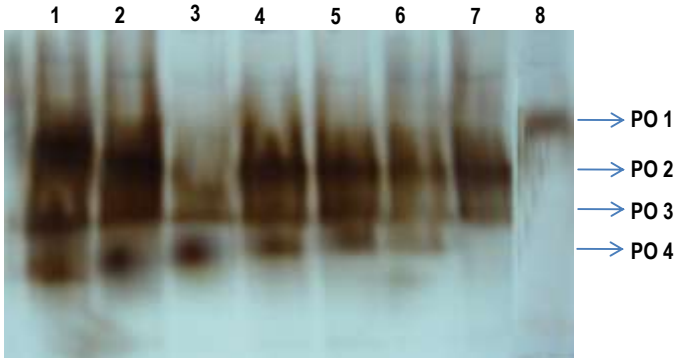

Plate 1. Native page profile of peroxidase (PO).

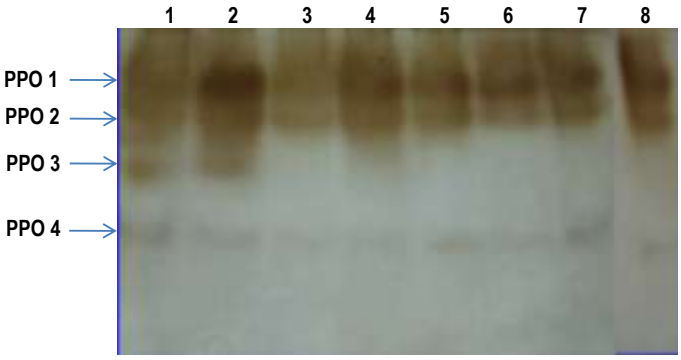

Plate 2. Native page profile of polyphenoloxidase (PPO)

stress (Welinder, 1992). Interestingly, increased peroxidase activity in leaf extracts of black gram infested with pathogens might be due to its utilization in cell wall lignifications (Parthasarathy et al., 2015). Therefore, induction of PO by bio-consortia in mulberry during wilt can be considered as one of the marker of disease resistance. Plants are equipped with well-organized and coordinated defense network of biochemical reactions, which are inducible in response to appropriate signals (Jones and Dangl, 2006). Inducing innate biochemical defense mechanisms in plants by treating them with biocontrol agents are thought to be novel plant protection strategies (Van Loon et al., 2008; Kashyap and Dhiman, 2009).

Isoforms pattern of polyphenoloxidase (PPO)

Similarly, the bio-consortia and Pf1 induced four isoforms of PPO *viz*., PPO1, PPO2, PPO3 and PPO4. The other treatments *viz.,* Pf1, Bs4, Th1, tebuconazole, carbendazim, and sprint showed three isoforms *viz.,* PPO1, PPO2, and PPO3 (Plate 2). The result indicates that the expression of PO4 and PPO4 in mulberry plants may be due to the treatment with bio-consortia, which expressed additional enzymatic activity.

The other treatments Bs4 and sprint revealed two isoforms and the control shown only one isoforms with less intensity. Increased PPO activity contributed to disease resistance due to its property to oxidize phenolic compounds to more toxic quinines which invade pathogenic micro-organisms (Vinale et al., 2008). It substantiated the role of PPO in disease resistance during pathogenesis. Our results are in affirmation with the study that reported a gradual increase in polyphenol content in red pepper (Sriram et al., 2009) and tomato (Nawrocka et al., 2011) treated with biocontrol agents.

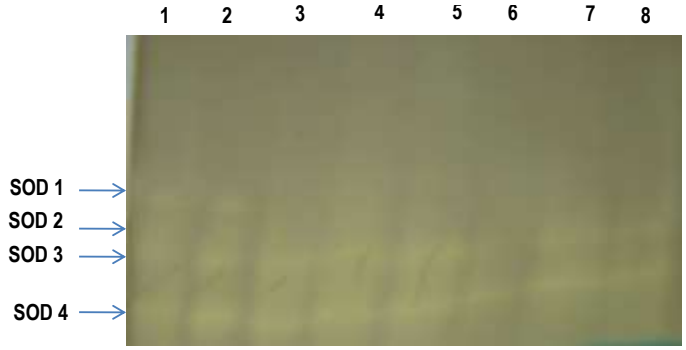

Plate 3. Native page profile of superoxide dismutase (SOD).

Isoform pattern of super oxide dismutase (SOD)

The results showed that plants treated with consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) and Pf1 showed four isoform *viz*., SOD1, SOD2 SOD3 and SOD4 (Plate 3). The other treatments *viz.,* Bs4, Th1, tebuconazole, carbendazim, and sprint showed two isoforms *viz.,* SOD and SOD2. The result indicates that the expression of SOD3 and SOD4 super oxide dismutase in mulberry plants may be due to the treatment with bio-consortia. Super oxide dismutase suppresses the oxidative burst (Vera-Estrell et al., 1993) and inhibits tissue necrosis.

Conclusion

The present study supports that the bio-consortium (seri bed waste+Pf1+Bs4+Th1+neem cake) effectively reduced the *Fusarium* wilt disease in mulberry plant. Antagonistically, at the same time bioconsortia triggers the activity of defense enzymes in mulberry roots during the infection with *Fusarium solani.* Hence, simultaneous and inherent induction of defense metabolites revealed that slowdown of the pathogen might be due to activation of phenylpropanoid metabolism. In this regard, it is recommended to use consortia based biocontrol agents as a promising alternate to chemical fungicides to minimize the adverse impact on the

environment and ensuring plant disease management.

Conflicts of Interests

The authors have not declared any conflict of interests.

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