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CONSORTIA OF *PSEUDOMONAS, BACILLUS* AND *EUCALYPTUS* EXTRACT MEDIATED SYSTEMIC RESISTANCE IN RICE IS DRIVEN THROUGH AN ELEVATED SYNTHESIS OF DEFENSE ENZYMES

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ABSTRACT

Sheath rot of rice caused by *Sarocladium oryzae* is one of the most important diseases worldwide, causing considerable yield losses. The estimation of losses due to sheath rot of rice in India has been reported to be up to 20-85%. In the present study, the combined application of antagonistic microorganism and plant extract showed significant bio control activity and enhances plant growth by induced systemic resistance. (ISR) in rice. In this study, *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis* (*BsA1-ST*) + *E. globule*s extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*)+ *E. globule*s extract (FA) and challenge inoculated with *S. oryzae* showed enhanced activity of peroxidase (PO), polyphenol oxidase (PPO), phenyl alanine ammonia lyase (PAL) and phenol compared to control.

Key words : Rice, Sheath rot, Defense related enzymes.

Introduction

For management of sheath rot of rice using chemical fungicides cause severe threat to the environment and public health. Earlier days, chemical fungicides are used for control ofseed borne fungal pathogens, but these chemical fungicides persist in the agriculture ecosystem and cause toxicity to beneficial microbes and develop resistance to the plant pathogens. Hence, we need to find out alternative approach for control of plant disease through bio control methods without causing any environmental problems and health hazards (Shanmugaiah *et al*., 2010). In recent days, biological control is one of the best choices and extensively documented as both safe and consistent clarification for sustainable agriculture. Many findings coated several microorganisms considered as potential biocontrol agents such as *Pseudomonas aeruginosa* MML2212, *Burkholderia*, *Ceratobasidium*, *Bacillus pumilus* MTCC7615 and *Streptomyces aurantiogriseus* VSMGT10143,5 (Padaria *et al*., 2016; Harikrishnan *et al*., 2014). *Pseudomonas* and *Bacillus* were reported to be major associated bacteria. These bacteria have the ability to produce IAA, ACC deaminase, siderophore, hydrogen cyanide and lytic enzymes. Previous report showed that FPs strains facilitate to raise seed germination, plant growth and yield (Anjaiah *et al*., 2003). *Pseudomonas* spp are activates systemically in the plant system through induced systemic resistance (ISR). Recent report demonstrated that, plant growth promoting rhizobacteria (PGPR) activating defence genes encoding chitinase, POX, PPO and PAL in plants (Chen *et al*., 2000). *P. fluorescens* is providing plant growth promotion against plant diseases such as sheath blight, sheath rot, blast of rice, bacterial blight of cotton, ground nut, Pythium disease of tomato and hot pepper (Commare *et al*., 2002; Meena *et al*., 2000; Ramamoorthy *et al*., 2002). The ISR induced by *Pseudomonas* sp was established in bean, carnation, rice, cucumber and raddish (Nandakumar *et al*., 2001; Alstrom *et al*., 1991). The objectives of the current study is to carry out greenhouse experiment for sheath rot of rice with consortium of

bacterial bio agents plus plant product and their cell free culturefiltrate against *S. oryzae*. After 1-3 weeks of *S. oryzae* inoculation, we examine PAL, PO, PPO activity and total phenol content.

Materials and Methods

Isolation and identification of pathogen

The pathogen was isolated from the diseased rice sheaths showing the typical lesions of sheath rot. The edge of the lesions was cut into small pieces by means of a sterile knife. Then the pieces were surface sterilized in 0.1 percent sodium hypochlorite solution for 30 seconds and washed in three repeated changes of sterile distilled water and then plated into sterile petri dishes containing PDA medium. The plates were then incubated at room temperature 28±2°C. The tip of the hyphal growth radiating from the infected tissue was transferred in to PDA slants (Rangaswami, 1972). The fungus was purified again by single hyphal tip method and maintained on PDA slants for the further studies. A total of 10 isolates (So1 to So 10) were obtained from infected sheath region of rice plants collected from different districts of Tamil Nadu. Based on the pathogenicity studies, the highly virulent isolate of So_5 was alone taken for further studies.

Consortium of bacterial bioagents and *Eucalyptus* extract on the induction of systemic resistance in rice seedlings

A pot culture experiment was conducted during *Rabi* (Oct-March) 2021-2022 at Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalainagar to test the efficacy of consortium of *Fluorescent pseudomonads* (PfS5), *B. subtilis* (BsA1) and *Eucalyptus* leaf extract for assessing their influence on the incidence of sheath rot of rice. Seed treatment with *P.fluorescens* (PfS5) and *B. subtilis* (BsA1) were described in earlier methods. Whereas, two gram of *Eucalyptus* powder was suspended in 20 ml sterile distilled water in a 50 ml conical flask to obtain a 10% (w/v) concentration (Ernest R. Mbega *et al.*, 2012). Treated seed were sown in nursery beds as per the treatment given below:

Treatment schedule

T_1 - *Fluorescent pseudomonads* (*PfS5*-ST)

T_2 - *B. subtilis* (*BsA1-ST*)

T_3 - *E. globule*s extract (ST)

T_4 - *Fluorescent pseudomonads* (*PfS5*-FA)

T_5 - *B. subtilis* (*BsA1-FA*)

T_6 - *E. globules* extract (FA)

T_7 - $T_1 + T_2 + T_3$

T_8 - $T_4 + T_5 + T_6$

T_9 - $T_7 + T_8$

T_{10} - Carbendazim @0.1% (FA), 2g/kg (ST)

T_{11} - Control

Rice seeds (variety RNR15048) treated with consortium of *Fluorescent pseudomonads* (PfS5) and *B. subtilis* (BsA1) along with *Eucalyptus* powder on 30days old rice seedlings were transplanted in cement pots @ six hills per pot. The upper most flag leaves of tillers at the booting and the panicle emerging stages were inoculated with *S. oryzae* isolate (So_5) by the standard grain inoculum technique (Sakthivel and Gnanamanickam, 1987). The crop was maintained in a poly house with frequent spraying of water to provide adequate moisture and relative humidity to enable successful infection by the pathogen. The experiment was conducted in a Randomized Block Design with three replications for each treatment and a suitable control. The seeds treated with fungicide Carbendazim 50% WP @ 2g/kg of seed; 0.1 per cent for foliar application was used for comparison and the standard agronomic practices as recommended by the TNAU were followed.

Assay of defense-related enzymes and compounds

Sample Collection

Samples were collected from selected treatments to study the induction of defense enzymes in response to pathogen infection in rice plants under glass house conditions. Leaf tissues from bacterial bio-control (*Fluorescent pseudomonads* and *B. subtilis*) and plant extract treated plants inoculated with and without pathogen inoculated control but maintained under the same conditions. The samples were collected from 0 to 6 days after inoculation.

Enzyme extraction

1 g of leaf samples was homogenized with 2ml of 0.1 M sodium citrate buffer (pH 5.0) at 4ºC. The homogenate was centrifuged for 20 minutes at 1000 rpm. The supernatant was used as crude enzyme extract for enzyme activity. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of peroxidase (PO), polyphenol oxidase (PPO), phenyl alanine ammonia lyase (PAL) and total phenolics content. Enzyme extract was stored in deep freezer (-70ºC) until used for biochemical analysis. Assay of peroxidase activity (PO)

Assay of peroxidase activity (PO)

The reaction mixture consisted of 1.5 ml of 0.05M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of one

per cent hydrogen peroxide and the reaction mixture was incubated at 28±3°C. The changes in absorbance were recorded at 420 nm at 30 sec. intervals for three min. and the boiled enzyme preparation served as a blank. The enzyme activity was expressed as min/g of fresh tissue (Hammerschmidt *et al*., 1982).

Assay of polyphenoloxidase (PPO)

PPO activity was determined as per the procedure given by Mayer *et al*. (1965). The reaction mixture consisted of 0.2 ml of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). The reaction was started with the addition of 0.2 ml of 0.01 M catechol and the activity was expressed as changes in absorbance at 495 nm at 30 sec. interval for three min. The enzyme activity was expressed as min/g of fresh tissue.

Assay of phenylalanine ammonialyase PAL)

Samples (1g) were homogenized in one ml of 25 m M borate HCl buffer (pH 8.8)(Annexure-I) containing 0.4 ml of five m M mercaptoethanol L^{-1}. The homogenate was centrifuged at 15, 000 rpm for 15 min. and the supernatant was used as the enzyme source. The assay mixture consisted of enzyme extract (0.2 ml), water (1.3 ml) and borate buffer (0.5 ml), (pH 8.8). The reaction was initiated of 1ml of M L-phenylalanine and the reaction mixture was incubated for one h. at 32ºC. The reaction was stopped by the addition of 0.5 ml of 2 N HCl. A blank was run in which phenylalanine was added after adding 2N HCl. PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm. The enzyme activity was expressed as µmol of cinnami acid /min/g of fresh tissue (Dickerson *et al*., 1984).

Estimation of total phenol

The phenolic content of rice plants was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). Rice leaves (1g) were homogenized in pestle and mortar, ten ml of 80 per cent methanol and agitated for 15 min. at 70ºC. The supernatant was evaporated to dryness and the residue was dissolved in five ml of distilled water. From this, 0.2 ml was diluted to three ml with distilled water and 0.25 ml of Folin-Ciocalteau (1N) was added. One ml of 20 per cent(w/v) sodium carbonate was added after three min. and mixed thoroughly. The tubes were placed in boiling water for one min. and cooled. The absorption of the development of blue colour was measured using a spectrophotometer at 650 nm against a reagent blank. Catechol was used as a standard and the phenol was expressed as µg catechol per g of leaf tissue.

Statistical analysis

The data were statistically analyzed using the Wasp version 2.0 developed by the Indian Council of Agricultural Research, Goa (Gomez and Gomez, 1984). Prior to statistical analysis of variance (ANOVA) the percentage values of the disease incidence were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT). Per cent values were transformed by arcsine or square root transformation.

Results

Peroxidase (PO)

In the present study, peroxidase activity was observed in all the treatments including control with pathogen. In addition, it was observed that the activity of peroxidase was slowly increased from 0 days and attained maximum level on 4th days after inoculation there after the level was declined drastically in all thetreatments. Two to five fold increased in the activity of peroxidase was observed with treatment T_1- *Fluorescent pseudomonads* (*PfS5*-ST) + *B.subtilis* (*BsA1-ST*) + *E. globules* extract (ST) plus *Fluorescent pseudomonads (PfS5*-FA) + *B. subtilis* (*BsA1-FA*) + *E. globules* extract (FA) and challenge inoculated with *S. oryzae* (Fig. 1).

Polyphenol oxidase (PPO)

The present study showed that the activity of PPO was slowly increased from 0 days and attained maximum level on 4th day after inoculation thereafter the level was declined drastically in all the treatments. Among the treatments, combined application of *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis* (*BsA1-ST*) + *E. globules* extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*) + *E. globule*s extract (FA) –T_1 increased the polyphenol oxidase activities was recorded significantly maximum level of changes in absorbance min/g of fresh tissue (2.80) on 4th day after inoculation followed by- T_2 recorded 2.60 changes in absorbance /min/g of fresh tissue. The inoculated control recorded 0.90 changes in absorbance /min/mg of fresh tissue (Fig. 2).

Phenyl alanine ammonia lyase (PAL)

The combined application of *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis* (*BsA1-ST*) + *E. globule*s extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*) + *E. globules* extract (FA) recorded significantly maximum level of changes in absorbance /min /g of leaf tissue (3.92)

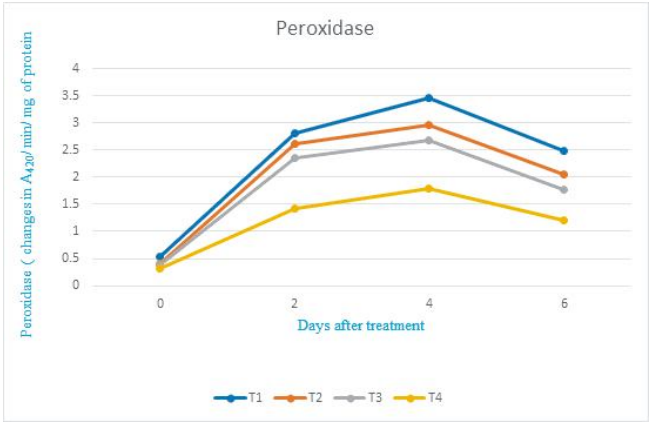

Fig. 1 : Induction of Peroxidase (PO) activity in rice plants treated with *Fluorescent pseudomonads* plus *B. subtilis* formulation and *E. globules* extract against *S. oryzae*. $\mathbf{T_1}$-*Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST) + *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_2}$- *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_3}$- *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST), $\mathbf{T_4}$- Control.

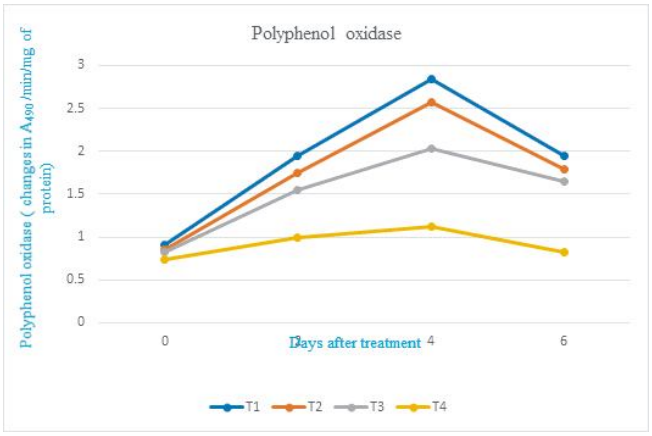

Fig. 2 : Induction of polyphenol oxidase (PPO) activity in rice plants treated with *Fluorescent pseudomonads* plus *B.subtilis* formulation and *E. globules* extract against *S. oryzae*. $\mathbf{T_1}$- *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST) + *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_2}$- *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_3}$- *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST), $\mathbf{T_4}$ – Control.

on fourth day after inoculation when challenged with the pathogen (Fig. 3).

Phenol

The changes in total phenol content were similar to changes in the polyphenol oxidase (PPO), peroxidase (PO) and phenyl alanine ammonia lyase (PAL). The

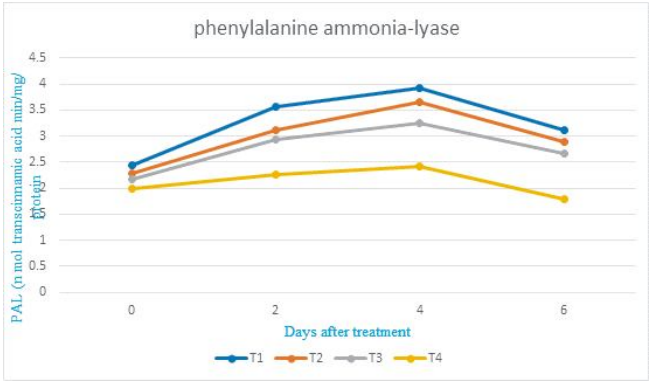

Fig. 3 : Induction of phenylalanine ammonia-lyase (PAL) activity in rice plants treated with *Fluorescent pseudomonads* plus *B.subtilis* formulation and *E. globules* extract against *S. oryzae*. $\mathbf{T_1}$- *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST) + *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_2}$- *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_3}$ - *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST), $\mathbf{T_4}$– Control.

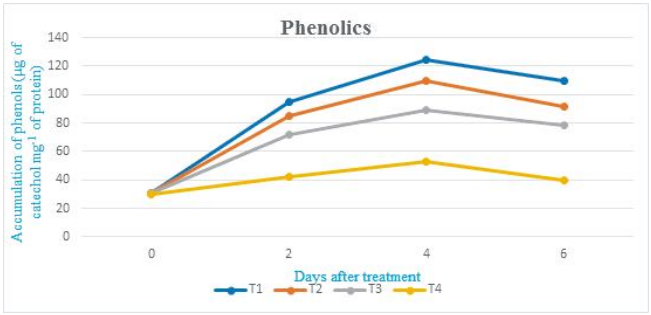

Fig. 4 : Induction of total phenolic contents in rice plants treated with *Fluorescent pseudomonads* plus *B.subtilis* formulation and *E. globules* extract against *S. oryzae*. $\mathbf{T_1}$- *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST) + *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*)+ *E. globules* extract (FA), $\mathbf{T_2}$- *Fluorescent pseudomonads* (*PfS_5*-FA) + *B. subtilis* (*BsA_1-FA*) + *E. globules* extract (FA), $\mathbf{T_3}$- *Fluorescent pseudomonads* (*PfS_5*-ST) + *B. subtilis* (*BsA_1-ST*) + *E. globules* extract (ST), $\mathbf{T_4}$ – Control.

maximum phenol accumulation (124.31 µg/g) was observed on the 4th day by the treatment-T_1 *Fluorescent pseudomonads* (*PfS5*-ST) +*B. subtilis* (*BsA1-ST*) + *E. globules* extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*) + *E. globules* extract (FA) which was followed by the treatment-T_2 *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*) + *E. globules* extract (FA) and treatment T_3 *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis* (*BsA1-ST*) + *E. globules* extract (ST) in the decreasing order of merit. The phenolic content slowly increased

from 0 to 4th days after pathogen inoculation and started to decline after 6th day of observations (Fig. 4).

Discussion

In the present study, the seed treatment and foliar application with combination of bioformulation *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis* (*BsA1-ST*) + *E. globules* extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*)+ *E. globules* extract (FA) and challenge inoculated with the pathogen showed enhanced induction of peroxidase than individual antagonistic bioformulation treatments and pathogen alone inoculated plants. Plant peroxidases are involved in the lignifications, suberification, polymerization of hydroxy proline-rich glycoproteins, regulation of cell wall elongation, wound healing and resistance against plant pathogens (Deborah *et al*., 2001; Yoshida *et al*., 2003; Maksimov *et al*., 2014). Prabhukarthikeyan *et al*. (2018) reported that the combined application (RD+SD) of liquid formulation of *P. fluorescens* strain-FP7 induced higher activities of PO in turmeric plants against *P. aphanidermatum*. Vinitha (2019) reported that combined application of Pf15 + *Ipomoea* leaf extract+ Sheep urine recorded increased activities of PO up to 72h of *S. oryzae* inoculation in control plants and there after a drastic reduction in enzyme activities were documented. After challenge inoculation with pathogen (*R. solani*), the maximal POX activity was seenat 72 h after pathogen inoculation in all *Pseudomonas*- treated plants and the activity was sustained at higher levels throughout the experiment. POX activity was lower inplants inoculated with the pathogen alone (Elsharkawy *et al*., 2022). All these earlier results lend support to the present findings.

The present study showed that the polyphenoloxidase activity was also enhanced progressively in all the treatments and the induction was higher in plantst reated with combined application of antagonists *P. fluorescens* (*PfS5*-ST) + *B. subtilis* (*BsA1-ST*)+ *E. globule*s extract (ST) plus *P. fluorescens* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*) + *E. globule*s extract (FA) recorded significantly maximum level of changes in absorbance /min/g of leaf tissue (2.84) on fourth day after inoculation when challenged with the pathogen.

PPO catalyzes the oxidation of phenolics to free radicals that can react with biological molecules, thus creating an unfavorable environment for pathogen development (Jockusch, 1966; Mohamed *et al*., 2012). Enhanced PPO activity due to the application of consortium of bio-control agents have been reported earlier by several workers (Muthukumar *et al*., 2011; Muthukumar and Venkatesh, 2014; Suthin Raj *et al*., 2016; Patel and Saraf, 2017; Vigila and Subramanian, 2018; Shoba *et al*., 2019). Rais *et al*. (2017) reported that the antagonistic *Bacillus* spp. significantly induced antioxidant defense enzymes like superoxide dismutase (1.7±1.9-fold and polyphenol oxidase (3.0±3.8- fold in rice leaves and roots under hydroponic and soil conditions respectively. Recently, Elsharkawy *et al*. (2022) that (*R.solani*), bacterized rice plants challenged with pathogen showed an enhanced polyphenol oxidase (PPO) activity, with activity higher at 72h after pathogen inoculation than at 24h after pathogen inoculation in all *Pseudomonas*-treated plants. In comparison to infected control plants, PPO activity was the highest in the leaves of rice plants inoculated with *P. putida*. The increase in PPO activity might be due to activation of latent host enzyme, solubilization of host PPO which is normally particulate or due to de novo synthesis (Manibhushan Rao *et al*., 1988). The induced PPO might have involved in the resistance of tomato damping-off through oxidation of phenolic compounds to fungi toxic quinones. These earlier reports corroborate with the present observations.

The present experiment, the maximum PAL activity was observed in plants treated with antagonist and *E. globules* extract. Among these, the combined application of *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis (BsA1-ST)* + *E. globule*s extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis* (*BsA1-FA*)+ *E. globule*s extract (FA) recorded significantly maximum level of changes in absorbance /min /g of leaf tissue (3.92) on fourth day after inoculation when challenged with the pathogen PAL catalyzes the first step of the phenyl propanoid pathway in the conversion of L-phenylalanine to trans-cinnamic acid, resulting in the biosynthesis of phytoalexin and phenolic compound (Garcion *et al*., 2014). These compounds had a vital role in the protection of plants against a wide range of pathogens (Mierziak *et al*., 2014). PAL activity could be induced in plant-pathogen interactions and fungal elicitor treatment (Ramanathan *et al*., 2000). Application of *Sargassum wightii*, *P. fluorescens* and Annamalai mixture followed by challenge inoculated with *R. solani* recorded maximum induction of PAL at 7th day, which decreased further (Suthin Raj *et al*., 2016). Kalaiselvi *et al*. (2019) reported that the combination treatment (T_6) involving *P. fluorescens* as seed and soil application (@10g/kg of seed and 2.5 kg/ha) plus neem oil cake as soil application @ 0.25 t/ha recorded induction of higher levels of PAL in cowpea plants challenge inoculated with *M. phaseolina*. The above results lend support to the present findings.

The present experiment, the maximum phenolic

activity was observed in plants treated with the combined application of *Fluorescent pseudomonads* (*PfS5*-ST) + *B. subtilis (BsA1-ST)* + *E. globule*s extract (ST) plus *Fluorescent pseudomonads* (*PfS5*-FA) + *B. subtilis (BsA1-FA)*+*E.globule*s extract (FA) recorded significantly maximum level of changes in absorbance / min/g of leaf tissue (3.92) on fourth day after inoculation when challenged with the pathogen. The production of phenol due to treatment with *P. fluorescens* and challenged with pathogen was reported in sugarcane against *C. falcatum* (Viswanathan and Samiyappan, 1999) in tomato and hot pepper against *P. aphanidermatum* (Ramamoorthy *et al*., 2002a). The total phenol content activity was significantly increased with *Pseudomonas* sp VSMKU2 (148.27µg catechol/ mg/g of protein) and cell free culture filtrate of VSMKU2 (137µg catechol/mg/g of protein) treated in rice seedlings on 7th day after challenged inoculation of *R. solani* compared to control (Nithya *et al*., 2019). Vinitha (2019) reported that combined application of Pf15 + *Ipomoea* leaf extract + Sheep urine and challenge inoculated with *S. oryzae* showed enhanced activities of phenol in rice seedlings. These earlier reports corroborate with the present observations.

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