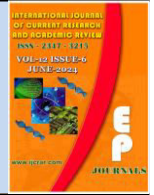
Int.J.Curr.Res.Aca.Rev.2024; 12(6): 94-103

International Journal of Current Research and Academic Review

ISSN: 2347-3215 (Online) Volume 12 Number 6 (June-2024)

Journal homepage: http://www.ijcrar.com



doi: https://doi.org/10.20546/ijcrar.2024.1206.011

Synergistic Effect of Biocontrol Agents on Inducing Systemic Resistance Against *Sclerotium rolfsii*

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Abstract

In the present study, bacterial and fungal biocontrol agents were tested individually and in combination for the induction of defence related enzymes or compounds against root rot disease under pot culture conditions. The results revealed that the combined application of *Pseudomonas fluorescens* and *Trichoderma asperellum* (Pf1 + TTH 1) as vermicompost-based bioformulations significantly increased the activities of phenylalanine ammonia-lyase (PAL), superoxide dismutase (SOD), catalase (CAT), β-1,3 glucanase, peroxidase (PO), and polyphenol oxidase (PPO) in sugarbeet plants against *S. rolfsii*. This effect was followed by treatment with Pf1 + EPCO 16 (*Bacillus subtilis*). These increased activities were observed up to 90 days after seed treatment (DAST). Other combination treatments, such as Pf1 + EPCO 16 + TTH 1 and EPCO 16 + TTH 1, also showed comparatively higher PAL, SOD, CAT, glucanase, PO, and PPO activities than their individual applications or the untreated control.

Article Info

Received: 10 April 2024
Accepted: 28 May 2024
Available Online: 20 June 2024

Keywords

Biocontrol agents, defense enzymes, root rot, *S. rolfsii*, sugarbeet, ISR.

Introduction

In plants, various types of genes play important roles in growth, development, and responses to the environment. These include structural genes, regulatory genes, signaling genes, defense-related genes, stress-responsive genes, and epigenetic genes. Defense genes in plants are specifically involved in the plant's defense mechanisms against pathogens, pests, and environmental stresses. Defense genes are activated by beneficial microbes involved in biological control. These biocontrol agents are antagonistic microbes capable of inducing resistance mechanisms in plants upon pathogen attack. This phenomenon is known as induced systemic resistance (ISR). The biocontrol efficacy of antagonistic microbes increase with the induction of systemic resistance in plants (Guetsky *et al.,* 2002). ISR in plants, which is a mechanism plants use to defend themselves against pathogen attacks, in addition to the direct antagonistic activity of the biocontrol agents (Van Loon *et al.,* 2003). When plants are treated with bacterial or fungal antagonists, they can trigger ISR by colonizing the plant's roots or leaves.

The secondary metabolites produced by PGPR strains induce systemic plant resistance in many crops (Gumede, 2008). ISR functions by reinforcing the physical and mechanical strength of the cell wall and altering the biochemical properties of host plants. This manipulation leads to changes in the physiological and biochemical

reactions of the host, ultimately resulting in the synthesis of defense chemicals (Ramos Solano *et al.,* 2008). Defense reactions in plants result in the accumulation of various proteins and compounds, including PR proteins (chitinase and β-1,3 glucanases), chalcone synthase, phenylalanine ammonia lyase, peroxidase, phenolics, callose, lignin, and phytoalexins. Mixture of *Bacillus* spp. consistently provides systemic protection against several diseases in various crops (Jetiyanon, 2007). Significant reductions in the incidence or severity of various diseases on sugarbeet, tomato, bell pepper, muskmelon, watermelon, tobacco, cucumber, etc., were observed due to the elicitation of ISR by several strains of *Bacillus* (Choudhary *et al.,* 2009). *Trichoderma* spp. are well-known for their potential as biocontrol agents against plant pathogens. Some *Trichoderma* strains establish long-lasting colonization of plant roots and produce or release compounds that induce localized or systemic plant resistance responses (Harman *et al.,* 2004).

Plants treated with these beneficial fungi exhibit enhanced resistance against a broad range of pathogens. PAL is the key enzyme in inducing the synthesis of salicylic acid (SA), which induces systemic resistance in many plants. Seeds treated with *Bacillus subtilis* showed a systemic increase in the phenylalanine ammonium lyase (PAL) activity in red gram and sugarbeet plants (Nijamol, 2006). Similarly, greengram plants treated with the combination of *Pseudomonas fluorescens* and *Trichoderma asperellum* showed enhanced induction of PAL activity against the root rot pathogen *Macrophomina phaseolina* (Thilgavathi *et al.,* 2007). Isolates of *Bacillus* spp. reduced Cercospora leaf spot of sugarbeet by inducing β-1,3glucanase activity (Bargabus *et al.,* 2004). A mixture of *Bacillus amyloliquefaciens* and *Bacillus pumilus* induced responses of superoxide dismutase (SOD) and peroxidase (PO) activities against different pathogens. These physiological changes were associated with disease protection in tomato against *Sclerotium rolfsii* and *Ralstonia solanacearum*, and in pepper against *S. rolfsii* and *Colletotrichum gloeosporioides* (Jetiyanon, 2007). Higher induction of peroxidase and polyphenol oxidase was observed in sugarbeet plants treated with *Bacillus subtilis* and *T. asperellum* challenged with the *S. rolfsii* root rot pathogen (Nijamol, 2006). Hence, the present study was aimed to assay the defense enzymes induced on the sugarbeet plants pre-treated with the mixture of bacterial (*P. fluorescens* or *B. subtilis*) and fungal biocontrol agents (*T. asperellum*) upon infection by *S. rolfsii* an incitant of root rot.

Materials and Methods

Biocontrol agents

Bacterial biocontrol agents such as *P. fluorescens* and *B.subtilis* and the fungal biocontrol agent, *Trichoderma asperellum* were obtained from the Department culture collection section, TNAU, Coimbatore.

Preparation of vermicompost bioformulation

Preparation of talc formulation of biocontrol agents

A loopful of *P.fluorescens*, Pf1 and *B. subtilis*, EPCO 16 strains were inoculated into the sterilized KB and nutrient broths respectively and incubated in a rotary shaker at 150 rpm for 72 h at room temperature (28±2°C). After 72 h, 400 ml of bacterial broth suspension containing 9 x 10^8 cfu/ml, 1 kg of the carrier material (talc powder), 15 g calcium carbonate (to adjust the pH to neutral) and 5 g CMC (adhesive) were mixed under sterile conditions by following the method described by Vidhyasekaran and Muthamilan (1995).

Fungal antagonist *T. asperellum* was multiplied in yeast molasses broth (30 ml molasses; 5 g yeast; made up to 1000 ml). For that, the sterilized broth (121°C, 15 psi for 30 min) was inoculated with actively growing mycelial disc of nine mm diameter and incubated for 15 days. The biomass containing 3 x 10^8 cfuml^{-1} along with the medium was incorporated into the sterilized talc powder carrier material at 50 ml suspension per 100 g and thoroughly mixed with addition of 500 mg CMC as reported earlier by Ramakrishnan *et al.,* (1994). The contents were shade dried for 12 h and stored in polythene bags for further use.

Preparation of 1:1 mixture of talc and vermicompost bioformulation

Vermicompost was collected from the TNAU farm house and it was autoclave sterilized for three consecutive days at 120 psi for 30 minutes. The biocontrol agents, *P. fluorescens* Pf1, *B. subtilis* EPCO 16 and *T. asperellum* TTH 1 were multiplied in their respective broths.

The talc based formulation of each biocontrol agent was mixed freshly (before drying) with vermicompost equally, talc powder:vermicompost (1:1). After mixing, it was incubated for seven days for the preparation of enriched vermicompost formulation and kept at room temperature for further experiments.

Pot culture experiment for studying induced systemic resistance in sugarbeet against *S. rolfsii*

Two separate pot culture experiments were conducted. Thirty centimeter dia pots were filled with autoclaved pot mixture (Red earth: sand: garden soil). The virulent isolate of *S. rolfsii*, SrSB 3 was used for challenge inoculation. One hundred sclerotial bodies were mixed with 100 g of soil and inoculated on the upper 5 cm soil. Each sowing hole was applied with enriched vermicompost formulation. For the application of individual biocontrol agent, one gram of vermicompost bioformulation per sowing hole was used. While combining more than one bioagent, one gram of each bioformulation was equally mixed and applied to single sowing hole. Soil application at 5g per pot for individual treatment and 10g for combination treatment (each 5 g). Finally, the susceptible sugarbeet *cv.* Indus was sown at 10 seeds per pot and after one week three plants per pot were maintained.

The treatments of pot culture experiments were as follows. T_1- Pf1 alone (SHA+SA) ; T_2- EPCO 16 alone (SHA+SA) ; T_3-TTH 1 alone (SHA+SA) ; T_4-Pf1+EPCO 16 (SHA+SA) ; T_5- Pf1+TTH 1 (SHA+SA) ; T_6- EPCO 16+TTH 1 (SHA+SA) ; T_{7-} Pf1+EPCO 16+TTH 1(SHA+SA) ; T_8- Difenoconazole (0.2% SD); T_9- Carbendazim (0.1% SD); T_{10}- Inoculated control ; T_{11}- Un inoculated control ; T_{12}- Healthy control; SHA – Sowing hole application ; SA- Soil application ; SD – Soil drench.

Soil applications were carried out at 30, 60 and 90 DAS respectively. The experiment was conducted in a completely randomized block design with twelve treatments and four replications.

Sample collection

Enzyme determinations were performed in biocontrol agents treated individual and combination treatments in response to infection by *S. rolfsii* on sugarbeet plants under glass house conditions. Leaves from different treatments were collected at 0 day, 5th day and upto 60 days after second treatment (DAST) at 10 days interval.

Enzyme extraction

Assay of phenylalanine ammonia-lyase (PAL)

One gram of leaf sample was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 50 mg of insoluble polyvinylpyrrolidone (PVP), filtered through cheese cloth and centrifuged at 20,000 g for 15 min at 4°C. The supernatant was used as the enzyme source. To determine PAL activity, 400 µl of sample 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. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm.

The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 $M^{-1}cm^{-1}$ (Dickerson *et al.,* 1984). Enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid min^{-1} mg^{-1} of sample.

Assay of superoxide dismutase (SOD)

Sample extract was prepared by homogenizing 1 g of sugarbeet leaf at 4°C in 2 ml of 0.2 M citrate phosphate buffer (pH 6.5), centrifuged at 15,000 g at 4°C for 30 min. The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT. The assay mixture (3 ml) contained of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin. 0.1 mM EDTA and 100 µl of the enzyme extract and the riboflavin was added at the end. Tubes were shaken and placed under a 40-W fluorescent lamp 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 mathematical difference was then divided by blank and multiplied by 100 to obtain the percentage inhibition of NBT photo-reduction. The SOD activity was expressed in SOD units mg^{-1} tissue (50% NBT inhibition = 1 unit) (Belid El-Moshaty *et al.,* 1993).

Assay of catalase (CAT)

Catalase activity was assayed spectrophotometrically as described by Chaparro-Giraldo *et al.,* (2000). The assay mixture (3 ml) containing 100 mM potassium phosphate buffer (pH 7.5) and 2.5 mM H_2O_2 was prepared immediately before use and added with 100 µl enzyme extract. The activity was measured by monitoring the degradation of H_2O_2 using UV-Visible Spectrophotometer at 240 nm over 1 min, against blank free of enzyme extract. The decrease in H_2O_2 was followed as the decline in optical density at 240 nm and

activity was calculated using the extinction coefficient (ε_{240nm} = 40 mM^{-1} cm^{-1}) for H_2O_2 and expressed in µmol min^{-1} mg^{-1} of sample.

Assay of β-1,3glucanase

One gram of leaf tissue was extracted in 5 ml of 0.05 M sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 20,000 g for 10 min at 4°C and the supernatant was used as enzyme source. The β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylic acid method (Pan *et al.,* 1991). The reaction mixture consisted of 62.5 µl of 4% laminarin (Sigma) and 62.5 µl of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid and heating for 5 min in boiling water, vortexed and its absorbance was measured at 500 nm. The enzyme activity was expressed as µg glucose released min^{-1} mg^{-1} of sample.

Assay of peroxidase (PO)

PO activity was carried out as per the procedure designated by Hammerschmidt *et al.,* (1982). The reaction mixture consisted of 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1 ml) was added to initiate the reaction, which was followed calorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme was used as blank and enzyme activity was expressed as the increase in absorbance at 470 nm min^{-1} mg^{-1} of protein.

Assay of polyphenol oxidase (PPO)

One gram of leaf sample was macerated in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 20,000 g for 15 min at 4°C. The supernatant was recovered and used to determine the PPO activity as per the procedure describe 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. The reaction was started by the addition of 200 µl of 0.01 M catechol and the activity was expressed as change in absorbance $min^{-1}mg^{-1}$ of protein.

Results and Discussion

The induced systemic resistance by biochemical analysis revealed the increased activities of the enzymes *viz.,* phenylalanine ammonia lyase, superoxide dismutase, catalase β-1,3glucanase, peroxidase, and polyphenol oxidase in the biocontrol agents treated sugarbeet plants against root rot disease. Enzyme activity was assayed in sugarbeet plants pretreated with an individual and combination of both bacterial and fungal biocontrol agents in the vermicompost based bioformulations and challenge inoculated with the *S. rolfsii.* Generally, the enzyme activity was increased in all the treatments (inoculated with the pathogen and biocontrol agents) when compared to control (inoculated with the pathogen only). While comparing an individual biocontrol agent, the combination treatments were recorded maximum induction of defense enzymes.

Phenylalanine ammonia lyase (PAL) activity was assayed in sugarbeet plants pretreated with an individual and combination of biocontrol agents and challenge inoculated with the *S. rolfsii.* The result indicated that the PAL activity was significantly increased in the combined application of Pf1 + TTH 1 and challenge inoculated with *S. rolfsii* followed by the treatment with Pf1+EPCO 16. The increased enzyme activity was observed upto 60 days after second treatment (DAST) which was given at 30 DAS. The other combination treatments *viz.,* Pf1+EPCO 16+TTH 1 and EPCO16+TTH 1 also showed comparatively higher PAL activity than they were applied alone and untreated control (Table1). Similar to PAL, SOD activity was appreciably increased in the combination of Pf1 + TTH 1 and challenge inoculated with *S. rolfsii* followed by the treatment with Pf1 +EPCO 16. The enhanced activity was observed upto 60 days after second treatment (DAST) which was given at 30 DAS. The other combination treatments *viz.,* Pf1+EPCO 16+TTH 1 and EPCO 16+TTH 1 also showed comparatively higher SOD activity than individual bioagent and control treatments (Table 2). The result revealed that similar to other enzymes, catalase activity was found to be increased in the combination of Pf1 + TTH 1 and Pf1 +EPCO 16 and challenge inoculated with *S. rolfsii*. The enhanced activity was observed up to 60 DAST. The other combination treatments *viz.,* Pf1+EPCO 16+TTH 1 and EPCO 16+TTH 1 also recorded comparatively higher catalase activity than individual and untreated control (Fig 1). Similar to PAL, SOD and catalase, variations in the glucanase activity in sugar beet plants challenge inoculated with the *S. rolfsii* and pretreated with the individual and combination of biocontrol agents were observed. Higher activity was observed in the treatment with Pf1+TTH 1 followed by Pf1+EPCO 16. The increased level of activity was observed upto 60 DAST.

Table.1 Induction of phenylalanineammonialyase (PAL) activity in sugarbeet plants treated with individual and combination of biocontrol agents against root rot disease under glasshouse conditions (nmol of transcinnamic acid min^{-1} g^{-1} of fresh tissue)

Treatments	Days interval							
	0 DAST	5 DAST	10 DAST	20 DAST	30 DAST	40 DAST	50DAST	60 DAST
Pf1 alone	7.21^{a}	7.22^{c}	7.24^{c}	7.25^{c}	7.27^{d}	7.33^{c}	7.4^{c}	7.35^{b}
EPCO 16 alone	7.1^{a}	7.19^{c}	7.25^{c}	7.26^{c}	7.29^{cd}	7.36^{c}	7.47^{c}	7.38^{b}
TTH 1 alone	7.14^{a}	7.28^{c}	7.44^{c}	7.44^{c}	7.59^{cd}	7.67^{bc}	7.8^{bc}	7.69^{b}
Pf1+EPCO 16	7.59^{a}	7.91^{b}	8.22^{b}	8.49^{b}	8.83^{b}	9.26^{a}	9.45^{a}	9.37^{a}
Pf1+TTH 1	7.32^{a}	8.74^{a}	9.09^{a}	9.36^{a}	9.52^{a}	9.66^{a}	9.68^{a}	9.6^{a}
EPCO 16+TTH 1	7.1^{a}	7.22^{c}	7.25^{c}	7.32^{c}	7.37^{cd}	7.56^{bc}	7.56^{c}	7.45^{b}
Pf1+EPCO 16+TTH 1	7.14^{a}	7.39^{bc}	7.56^{c}	7.82^{c}	7.88^{c}	8.04^{b}	8.18^{b}	7.91^{b}
Difenoconazole	5.45^{c}	5.46^{e}	5.4^{e}	5.46^{e}	5.49^{f}	5.43^{e}	5.44^{e}	5.35^{d}
Carbendazim	5.33^{c}	5.33^{e}	5.38^{e}	5.36^{e}	5.36^{f}	5.42^{e}	5.45^{e}	5.33^{d}
Inoculated control	6.14^{b}	6.15^{d}	6.15^{d}	6.13^{d}	6.13^{e}	6.22^{d}	6.33^{d}	6.34^{c}
Un-inoculated control	5.28^{c}	5.28^{e}	5.3^{e}	5.29^{e}	5.28^{f}	5.22^{e}	5.28^{e}	5.29^{d}
Healthy control	5.14^{c}	5.16^{e}	5.2^{e}	5.17^{e}	5.19^{f}	5.2^{e}	5.18^{e}	5.16^{d}

Values are mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT

Table.2 Induction of superoxide dismutase (SOD) activity in sugarbeet plants treated with individual and combination of biocontrol agents against root rot disease under glasshouse conditions (unit $min^{-1}g^{-1}$ of fresh tissue)

Treatments	Days interval							
	0 DAST	5 DAST	10 DAST	20 DAST	30 DAST	40 DAST	50DAST	60 DAST
Pf1 alone	2.45^{b}	2.52^{b}	2.64^{b}	2.70^{bc}	2.75^{bcd}	2.81^{cde}	2.87^{bc}	2.85^{b}
EPCO 16 alone	2.50^{ab}	2.55^{ab}	2.63^{b}	2.69^{bc}	2.72^{bcd}	2.79^{cde}	2.84^{bcd}	2.80^{bc}
TTH 1 alone	2.56^{ab}	2.61^{ab}	2.68^{ab}	$2.74a^{bc}$	2.79^{bcd}	2.83^{cde}	2.91^{b}	2.87^{b}
Pf1+EPCO 16 alone	2.61^{ab}	2.66^{ab}	2.72^{ab}	2.85^{ab}	2.91^{b}	3.15^{ab}	3.20^{a}	3.13^{a}
Pf1+TTH 1	2.67^{a}	2.74^{a}	2.86^{a}	2.92^{a}	3.22^{a}	3.30^{a}	3.38^{a}	3.30^{a}
EPCO 16+TTH 1	2.52^{ab}	2.59^{ab}	2.67^{ab}	2.75^{abc}	2.84^{bc}	2.93^{bc}	2.94^{b}	2.90^{b}
Pf1+EPCO 16+TTH 1	2.53^{ab}	2.57^{ab}	2.65^{b}	2.72^{abc}	2.80^{bcd}	2.84^{cd}	2.86^{bc}	2.82^{bc}
Difenoconazole	2.45^{b}	2.49^{b}	2.55^{b}	2.58^{c}	2.63^{cd}	2.64^{de}	2.65^{cd}	2.60^{cd}
Carbendazim	2.43^{b}	2.47^{b}	2.53^{b}	2.56^{c}	2.60^{d}	2.61^{e}	2.62^{d}	2.57^{d}
Inoculated control	1.94^{c}	1.99^{c}	1.23^{d}	1.25^{e}	1.28^{g}	1.30^{h}	1.32^{g}	1.29^{f}
Un-inoculated control	1.94^{c}	1.95^{cd}	1.95^{c}	1.98^{d}	2.00^{e}	2.20^{f}	2.24^{e}	1.95^{e}
Healthy control	1.78^{c}	1.77^{d}	1.78^{c}	1.79^{d}	1.76^{f}	1.77^{g}	1.76^{f}	1.76^{e}

Values are mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT

Table.3 Induction of β-1, 3 glucanase activity in sugarbeet plants treated with individual and combination of biocontrol agents against root rot disease under glasshouse conditions (µg of glucose released $min^{-1}g^{-1}$ fresh tissue)

Treatments	Days interval							
	0 DAST	5 DAST	10 DAST	20 DAST	30 DAST	40 DAST	50DAST	60 DAST
Pf1 alone	7.21^{a}	7.22^{c}	7.24^{c}	7.25^{c}	7.27^{d}	7.33^{c}	7.4^{c}	7.35^{b}
EPCO 16 alone	7.1^{a}	7.19^{c}	7.25^{c}	7.26^{c}	7.29^{cd}	7.36^{c}	7.47^{c}	7.38^{b}
TTH 1 alone	7.14^{a}	7.28^{c}	7.44^{c}	7.44^{c}	7.59^{cd}	7.67^{bc}	7.8^{bc}	7.69^{b}
Pf1+EPCO 16	7.59^{a}	7.91^{b}	8.22^{b}	8.49^{b}	8.83^{b}	9.26^{a}	9.45^{a}	9.37^{a}
Pf1+TTH 1	7.32^{a}	8.74^{a}	9.09^{a}	9.36^{a}	9.52^{a}	9.66^{a}	9.68^{a}	9.6^{a}
EPCO 16+TTH 1	7.1^{a}	7.22^{c}	7.25^{c}	7.32^{c}	7.37^{cd}	7.56^{bc}	7.56^{c}	7.45^{b}
Pf1+EPCO 16+TTH 1	7.14^{a}	7.39^{bc}	7.56^{c}	7.82^{c}	7.88^{c}	8.04^{b}	8.18^{b}	7.91^{b}
Difenoconazole	5.45^{c}	5.46^{e}	5.4^{e}	5.46^{e}	5.49^{f}	5.43^{e}	5.44^{e}	5.35^{d}
Carbendazim	5.33^{c}	5.33^{e}	5.38^{e}	5.36^{e}	5.36^{f}	5.42^{e}	5.45^{e}	5.33^{d}
Inoculated control	6.14^{b}	6.15^{d}	6.15^{d}	6.13^{d}	6.13^{e}	6.22^{d}	6.33^{d}	6.34^{c}
Un-inoculated control	5.28^{c}	5.28^{e}	5.3^{e}	5.29^{e}	5.28^{f}	5.22^{e}	5.28^{e}	5.29^{d}
Healthy control	5.14^{c}	5.16^{e}	5.2^{e}	5.17^{e}	5.19^{f}	5.2^{e}	5.18^{e}	5.16^{d}

Values are mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT

Figure.1 Induction of catalase in sugarbeet plants pretreated with individual and combination of biocontrol agents against root rot disease under glass house conditions.

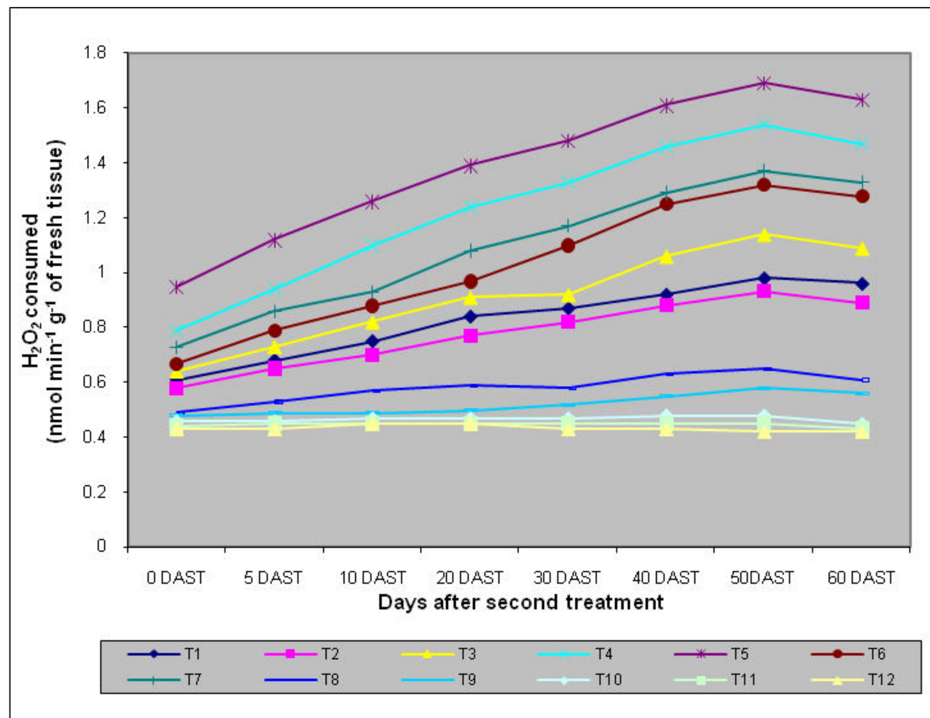


Figure.2 Induction of PO in sugarbeet plants pretreatedwith individual and combination of biocontrol agentsagainst root rot disease under glass house conditions.

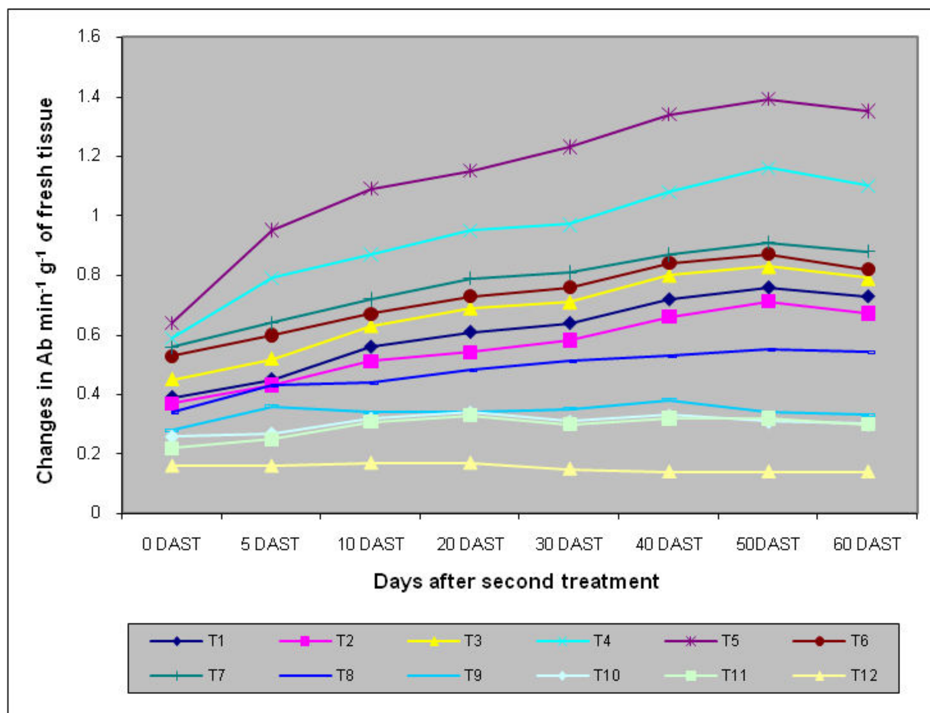
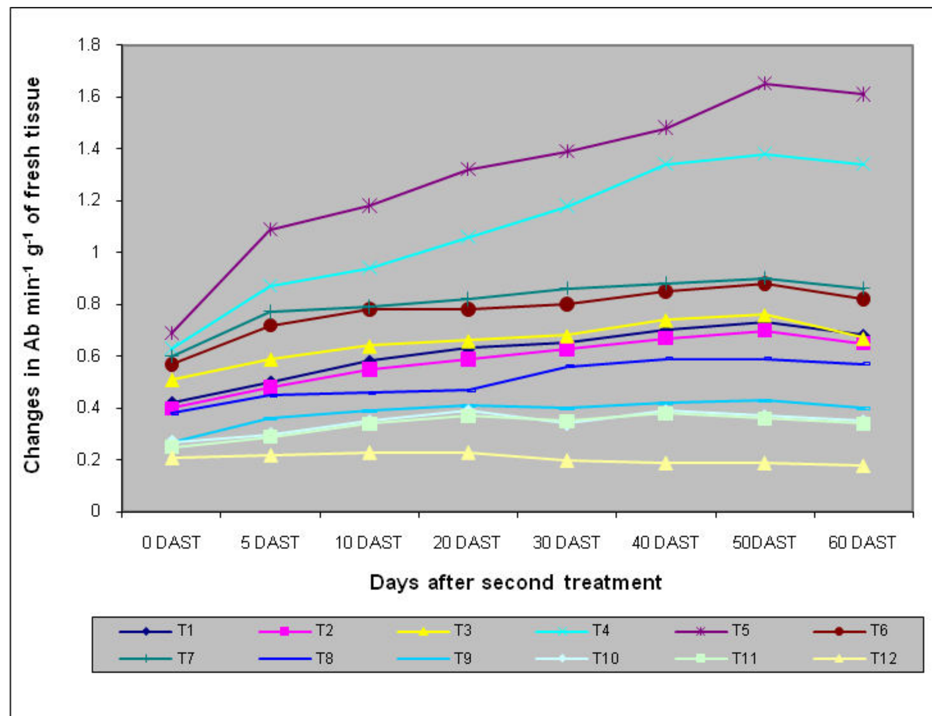


Figure.3 Induction of PPO in sugarbeet plants pretreatedwith individual and combination of biocontrol agents against root rot disease under glass house conditions.



Other combination treatments *viz.,* Pf1+EPCO 16+TTH 1 and EPCO 16+TTH 1 showed comparatively higher β-1, 3 glucanase activity than individual and untreated control treatments with lesser activity (Table 3). The PO and PPO activity was induced in sugar beet plants challenge inoculated with the *S. rolfsii* and pretreated with the individual and combination of biocontrol agents.

Higher activity was observed in the treatment with Pf1+TTH 1 followed by Pf1+EPCO 16. The increased level was observed upto 60 DAST. In other combination treatments *viz.,* Pf1+EPCO 16+TTH 1 and EPCO 16+TTH 1 comparatively higher PO activity was observed compared to individual and untreated control treatments (Fig 2 & 3).

Overall results of the present study revealed that the PAL, SOD, CAT, glucanase, PO, and PPO activities were significantly increased in the combined application of Pf1 + TTH 1 followed by treatment with Pf1 + EPCO 16. This increased activity was observed up to 90 days after seed treatment (DAST). Other combination treatments, such as Pf1 + EPCO 16 + TTH 1 and EPCO 16 + TTH 1, also showed comparatively higher PAL, SOD, CAT, glucanase, PO, and PPO activities than when applied alone or compared to the untreated control. Earlier and higher levels of expression of defense-related proteins and accumulation of chemicals at the infection site might certainly prevented the colonization of the pathogen *S. rolfsii* in sugarbeet plants treated with bioformulation mixture. The induction of phenolic compounds due to an increase in PAL activity was higher in PGPR-treated chickpea plants when the plants were inoculated with *S. rolfsii* (Singh *et al.,* 2003). The biological control agent *Bacillus mycoides* is capable of inducing resistance in sugarbeet (Bargabus *et al.,* 2002 & 2004). The activity of the defense-related PAL enzyme was significantly greater in greengram plants treated with a talc-based formulation containing a combination of *T.asperellum* and *P.fluorescens* (Tv1+Pf1) than in plants receiving other treatments or the untreated control (Thilgavathi *et al.,* 2007). Isolates of *B. pumilus* and *B. mycoides* induced β-1,3glucanase in sugarbeet (Bargabus *et al.,* 2004). Plants produce active oxygen species (AOS) such as superoxide anion (·O2-), hydrogen peroxide (H2O2), and hydroxyl radical (OH-) as one of the earliest responses to attempted infection by pathogens (Grant and Loake, 2000). Scavengers of active oxygen species like catalase (which catalyzes the decomposition of H2O2) (Scandalios, 1994) and superoxide dismutase (which scavenges O2-) (Bowler *et al.,* 1992) suppress the oxidative burst (Vera-Estrell *et al.,* 1993) and inhibit tissue necrotization. Catalase and peroxidase are of particular interest because of their role

in binding SA, which in turn plays an important role in induced resistance (Anderson *et al.,* 1998). The activity of the defense-related enzymes peroxidase and polyphenol oxidase was significantly greater in greengram plants treated with a talc-based formulation containing a combination of *T. asperellum* and *P. fluorescens* (Tv1+Pf1) than in plants receiving other treatments or the untreated control (Thilagavathi *et al.,* 2007). Hence, induced resistance of sugarbeet plant is increased synergistically while they were pre-treated with the combination of *T.asperellum* and *P.fluorescens* (Tv1+Pf1).

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How to cite this article:

Thilagavathi, R. and Shanmuga Priya, M. 2024. Synergistic Effect of Biocontrol Agents on Inducing Systemic Resistance Against *Sclerotium rolfsii*. *Int.J.Curr.Res.Aca.Rev.* 12(6), 94-103.
doi: https://doi.org/10.20546/ijcrar.2024.1206.011