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Induced Systemic Resistance by Rhizosphere and Phyllosphere Microorganism against Root Rot caused by *Sclerotium rolfsii* in Tropical Sugarbeet (TSB) Ecosystems

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A B S T R A C T

Phylloplane and rhizosphere microorganism were isolated from tropical sugarbeet. Phyllolplane fungi viz., *Cladosporum* sp., *Penicillium* sp., *Aspergillus niger*, *Aspergillus flavus*, and *Curvularia* and Bacteria of *B. subtilis* and *P. fluorescens* were isolated seven phylloplane microorganisms tested, *Pseudomonas fluorescens* significantly recorded maximum per cent mycelial growth reduction (41.90), sclerotial Number (70.65 per cent) and Sclerotial germination (58.67%) reduction over control. Among rhizosphere forty four *Pseudomonas fluorescens* and six isolates of *Bacillus subtilis* and *Trichoderma* sp 31 isolates tested for their antagonistic activity against *Scerotium rolfsii* by dual culture, one isolate namely *T.viride* (TVB 1) *P. fluorescens* (SBHRPF 2), *B. subtilis* (SBHRBS1) showed highest per cent mycelial growth inhibition allowed minimum of sclerotial production. Pot culture experiments The application of biocontrol agents triggered the activity of three defense related enzymes viz., peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were induced and the accumulation of phenol was also noticed in Tropical sugarbeet upon challenge inoculation soil application *S.rolfsii* the causal agent for root rot in tropical sugarbeet. The activities of defense enzymes reached a peak at four days after inoculation (DAI) with the pathogen. Native PAGE analysis revealed the expression of an additional isoforms of PO and PPO were observed in biocontrol agents treated soil application due to induced systemic resistance (ISR) induction.

Introduction

The world harvested 301 million metric tonnes of sugar beets (*Beta vulgaris* L. ssp. vulgaris var. altissima) in 2017. The world's largest producer was Russia, with a 51.9 million-metric-tonne harvest. The average yield of sugar beet crops worldwide was 58.2 tonnes per hectare. The root of the sugar beet is 20% sugar, 5% pulp, and 75% water. The sugar of the sugar beet is of primary value and the pulp left after sugar extraction is used as animal feed. The byproducts of this crop include molasses and pulp and this adds a further 10% value to the cash crop. Now India introduced tropical sugar beet varieties are gaining momentum in tropical and subtropical countries.

New crop introduced in climatic condition the severe Root rot caused by *Sclerotium rolfsiiis* a major problem in all the tropical sugar beet growing areas. Intensive cultivation of tropical sugar beet has resulted in the increased occurrence of root rot. seedling blight Utter Pradesh in India (Mukhopadhyay, 1971). Leach (1934) recovered the pathogen from the soils of sugar beet fields in California. Paramasivan *et al.,* (2014) reported that root rot caused by 30% yield loses and it management with *Trichoderma viride* . As an ecologically viable alternative, biological control has been a desirable strategy for controlling plant diseases (You *et al.,* 2015) and there are an increasing number of biocontrol agents (BCAs), such as *Bacillus* spp., *Pseudomonas* spp., *Trichoderma* spp. etc., being commercialized for various crops (Cha *et al.,* 2016) Plant has endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Bostock *et al.,* 2001). Defense reaction occurs due to the accumulation of PR-proteins, phytoalexins, chalcone synthase, PAL, PO, PPO and phenolics. The objective of the present study is to unravel the induction of various defense related genes encoding proteins implicated in strengthening of plant cell walls by biocontrol agents treatments in response to infection by *S.rolfsii* in tropical sugarbeet ecosystems.

Materials and Methods

Isolation of phylloplane and rhizosphere microorganism in tropical sugarbeet ecosystem

Phylloplane microorganisms were isolated from three different tropical sugarbeet varieties, viz., Indus, cavury and subraja. The active fresh leaves were collected and cut into small bits by mean of a sterile scalpel. The leaf bits were suspended in three ml of sterile distilled water and thoroughly shaken for five minutes and allowed to stand for five minutes. From this one ml of suspension was pipetted out into each sterilized Petri dish using a sterilized pipette. Fifteen ml of PDA medium was poured each of these plates, gently swirled and allowed to solidify. For bacteria, nutrient agar medium was used. Three replications were maintained for each treatments. Sterile water added plates served as control. The plates were incubated at room temperature. Forty eight hours after incubation, the bacterial colonies were sub cultured and subsequently purified by streak plate method (Rangaswamy and Soumini Rajagopalan, 1973). The fungal colonies were sub cultured after 3-5 days and purified by single hyphal tip method. (Rangaswami, 1972). The fungal and bacterial cultures isolated were maintained on PDA, King's B and nutrient agar slants respectively for further studies.

Isolation of native antagonists

Soil antagonistic microbes viz., *Trichoderma* sp., *Pseudomonas* sp. and *Bacillus* sp. were isolated from the rhizosphere soil using *Trichoderma* selective medium (TSM) (Elad and Chet, 1983), King's B medium (King *et al.,* 1954) and Nutrient agar medium (NA) (Difco manual, 1953) respectively. Morphological identification was made through the light microscope and pure cultures were maintained on respective agar slants at 4^{o} C.

Pseudomonas sp. = SBHRPF1 to SBHRPF 21, SBHPPF22 to SBHRPF30, SBDPPF31 to SBDPPF36 and SBDRPF 37 to 44 and *Bacillus* sp. = SBHRBS 1, SBHPBS 2, SBDRBS3, SBHPBS 4, SBHRBS 5 and BHRBS 6.

Efficacy of phylloplane organism against *Sclerotium rolfsii*.

A nine mm actively growing culture disc of the fungus was placed onto sterilized Petri dish containing previous plated and solidified PDA medium approximately 1.5 cm away from the edge of the plate. In the same way, a nine mm culture disc of the purified phylloplane test fungus was placed on the medium at the opposite side of the fungus causing root rot. For bacteria, the fungus alone was placed and the actively growing 48 h old bacterial culture was separately streaked onto the medium at the opposite side of the plate. Similarly three replications were maintained for each treatment. PDA medium inoculated with the pathogen alone served as control.

Effect of biocontrol agents on the induction of defense related enzyme in tropical sugar beet plants

Two months old tropical sugarbeet plants were treated with the biocontrol agents *T .viride* (TVB1), *T. harzianum* (THB1), *P. fluorescens* isolate (SBHRPF 2), *P. chlororaphis* isolate PA23 and *B. subtilis* isolate (SBHRBS 1) by soil application and challenged with *S. rolfsii* ($SBSR_3$) mass multiplied in sand maize medium.

Tropical sugarbeet plant along with tubers was carefully removed from the pots after 0, 2, 4, 6, 8, and 10 days after challenge inoculation with *S. rolfsii* and washed several times with sterile distilled water before enzyme extraction. The enzymes were extracted separately from both leaf and tubers at ice-cold condition (5˚C). The samples were homogenized with phosphate buffer (1 g of leaf or tubers with 1 ml of sodium phosphate buffer (0.1M) pH 7.0). The homogenates were centrifuged at 10,000rpm for 15 min. The supernatant was used as enzyme source for peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia–lyase (PAL), and phenol assay.

Peroxidase activity (PO)

One gram of fresh plant tissue was ground in 1 ml of 0.1M phosphate buffer with pH 7.0 in a pre-cooled pestle and mortar. The homogenate was centrifuged at 15,000 rpm at 4^o C for 15 min. The supernatant was used as enzyme source. The reaction mixture consists of 1.5 ml of 0.05M pyrogallol, 0.1 ml of enzyme extract and 0.5 ml of one per cent H_2O_2. The change in absorbance of the reaction mixture was recorded at 420 nm at 30 sec interval for 3 min. at room temperature (28 ± 2˚ C). The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in absorbance of the reaction mixture per min. per g of leaf or tuber (Hammerschmidt *et al.,* 1982).

Polyphenol oxidase (PPO)

One gram of fresh plant sample was ground in 1 ml of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000g for 15 min. at 4˚ C and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer pH 6.5 and 0.1 ml of the enzyme extract. The reaction was initiated by the addition of 0.2 ml of catechol (0.01M). The activity was expressed as change in absorbance at 495 nm at 30 sec interval for 3 min. The enzyme activity was expressed as change in absorbance per min. per g of leaf or tuber (Mayer *et al.*, 1965).

Phenylalanine ammonia lyase (PAL)

Five hundred mg of plant material was homogenized in 5 ml of cold 25mM borate HCl buffer (pH 8.8) containing 5mM mercaptoethanol 0.4 ml per l. The homogenate was centrifuged at 15,000g for 15 min. and the supernatant was used as enzyme source. The assay mixture consists of 0.2 ml of enzyme extract, 1.3 ml water and

0.5 ml borate buffer. The reaction was initiated by the addition of 1 ml of 12mM L-Phenylalanine. The reaction mixture was incubated for 1 h at 32˚C. The reaction was stopped by the addition of 0.5 ml of 2N HCl. A blank was run in which phenylalanine was added after adding 2N HCl. The absorbance was measured at 290 nm. The enzyme activity was expressed as µmol of cinnamic acid/ min/ g of leaf or tuber (Dickerson *et al.*, 1984).

Phenols

One gram of the sample was ground in a pestle and mortar in 10 ml of 80 per cent methanol. The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was evaporated to dryness and the residue was dissolved in 5 ml of distilled water. From this 0.2 ml was taken and the volume was made up to 3 ml with distilled water. To that 0.25 ml of Folin-Ciocalteau reagent (1N) was added. After 3 min. 1 ml of 20 per cent sodium carbonate was added and mixed thoroughly. Then the tubes were placed in boiling water for 1 min. and cooled. The absorbance was measured at 725 nm against a reagent blank. The phenol activity was expressed in µg of catechol per g of plant tissue (Zieslin and Ben Zaken, 1993).

Native gel electrophoresis

Plant samples were collected on the 4th day after challenge inoculation with pathogen at which the activity of PO and PPO will be higher.

The protein extract was prepared by homogenizing 1 g of plant sample in 1 ml of 0.1M sodium phosphate buffer (pH 7.0) and centrifuged at 16,000g for 20 min. at 4˚C. The protein content of the sample was determined by Broadford method (Bradford, 1976). Sample (50 µg protein) was loaded onto 8 % polyacrylamide gels for the analysis of PO and PPO isoforms. (Sigma, USA). After electrophoresis, PO isoforms were visualized by soaking the gels in 0.05 per cent benzidine in dark for 30min. (Sigma, USA) (Laemmli, 1970) in acetate buffer (20mM, pH 4.2). After incubation H_2O_2 was added drop wise (Nadlony and Sequira, 1980). For assessing PPO isoforms profile, the gels were equilibrated in dark for 30 min. in 0.1 per cent p-phenylenediamine in 0.1M phosphate buffer (pH 7.0) followed by the addition of 10mM catechol in the phosphate buffer (Jayaraman *et al.*, 1987).

Results and Discussion

Efficacy of phylloplane and rhizosphere biocides against *S. rolfsii in vitro*

Among the six phylloplane microorganisms tested, *Pseudomonas fluorescens* significantly recorded maximum per cent mycelial growth reduction (41.90) over control followed by *Aspergillus flavus* and *Bacillus subtilis* which recorded 39.18 and 39.00 per cent inhibition respectively and these were on par with each other. The minimum inhibition was exerted by *Curvularia* sp and *Penicillium* sp which recorded 21.48 and 21.32 per cent inhibition respectively (Table 1). Among seven rhizosphere microorganisms *Trichoderma viride* recorded maximum per cent mycelial growth reduction 66.81 per cent reduction over control, followed by *P. fluorescens* and *A. niger* which recorded 50.96 per cent inhibition and these were on par with each other. The minimum inhibition was noted in *Rhizopus* spp which showed 20.72 per cent inhibition (Table 1a).

Number of sclerotia

Among the six phylloplane microorganisms *P. fluorescens* showed the maximum reduction of sclerotial production which was recorded 70.65 per cent followed by *B.*

subtilis and *A. flavus* which recorded 58.05 and 56.30 per cent reduction respectively. (Table1). Among rhizosphere microorganisms *T.viride* significantly recorded 69.16 per cent sclerotial reduction over control (Table 1a) followed by rhizosphere *P. fluorescens* recorded 64.85 per cent reduction.

Sclerotial germination

Among the six phylloplane microorganisms *P fluorescens* recorded maximum sclerotial germination inhibition of 58.67 per cent followed by *B. subtilis* and *A. flavus* which recorded 52.67 and 51.67 per cent inhibition respectively and these were on par with each other (Table 1) Among rhizosphere microorganism *T.viride* was recorded minimum sclerotial germination 37.50 per cent when compared to control which recorded 100.00 per cent germinations (Table 1a)

Effect of fungal antagonists against *S. rolfsii in vitro* (Dual culture technique)

Among best four isolates of *T.viride* Viz., TVB1, TVB2, TVB3 and TVB31 and Two isolates of *T. harzianum* viz., THB1 and THB2 were tested under *in vitro* condition. Among the ten fungal antagonists tested T. *viride* (TVB 1) significantly exerted highest per cent mycelial growth inhibition of (73.03) over control. Followed by *T. harzianum* (THB1) and *T. viride* (TVB 2) recorded of 71.19 per cent mycelial growth inhibition over the control and on par with each other. (Table 2).

Sclerotial number and sclerotial germination

The sclerotial production of *S. rolfsii was* also assessed in dual culture technique with ten fungal antagonists of *Trichoderma* spp. Among the isolates screened TVB1 allowed minimum of sclerotial production (28.04/plate) and less germination (31.22 per cent) followed by THB1 which recorded 29.36 sclerotia / plate as against the control (204.93/palte) among the fungal antagonist *Chaetomium globosum* allowed maximum sclerotia production with less inhibition 129.69 followed by *T.reesei* 95.04 when compare to other *Trichoderma* isolates. (Table 2)

Screening of rhizosphere and phylloplane bacterial antagonist against the mycelial growth of *S. rolfsii*

Among the forty four isolates of *Pseudomonas fluorescens* and six isolates of *Bacillus subtilis* tested for their antagonistic activity against *S. rolfsii* by dual culture, one isolate namely *P. fluorescens* (SBHRPF 2) were found to be effective (Table 3). *P. fluorescens* (SBHRPF 2) showed highest per cent mycelial growth inhibition 66.74, over control. Among the isolates of *B. subtilis*, screened for antifungal activity against *Sclerotium rolfsii*, SBHRBS1 recorded 51.01 per cent reduction of mycelial growth over control (Table 3). The mycelial growth of *S.rolfsii* recorded in dual plates of SBHRBS 1 and SBHRBS 6 were 4.36 cm and 4.46 cm respectively as compared to control (8.90 cm).

Induction of systemic resistance by antagonists

Biochemical changes in tropical sugar beet plants treated with antagonists

The biochemical changes were assessed in tropical sugar beet plants treated with the antagonists on challenge inoculation with *Sclerotium rolfsii*. The activities of phenol a peroxidase, polyphenol oxidase were monitored upto10 days after challenge inoculation. In general the enzyme activities

were higher in treatments treated with the antagonists compared to healthy and inoculated control.

Effect of antagonists in peroxidase enzyme activity

Induction of peroxidase enzyme activity also in both the fungal and bacterial antagonists induced the activity of peroxidase in tropical sugar beet leaf and tuber. The enzyme activity was more in antagonists application and subsequently inoculated with *S. rolfsii* In all the treatments the enzyme activity showed an increasing trend up to four days after treatment and there after a decline in the enzyme activity was observed. The plants treated with *T. viride* (TVB1) and subsequently inoculated with *S.rolfsii* recorded the maximum induction of peroxidase activity (0.253 changes in absorbance/min/g/of tissue) in the leaves followed by *T. viride* (TVB1) alone treated plants (0.226) as against control (healthy) (0.173). A similar trend was observed in the activity of peroxidase in the tuber also (Fig. 1).

Effect of antagonists in Polyphenol oxidase enzyme activity

The sugar beet plants expressed higher activity of PPO when challenged with pathogen irrespective of pretreatment with biocontrol agents. Although an increase in PPO activity began from 2nd day after challenge inoculation, accumulation reached maximum on 4th day after inoculation with the *T. viride* (TVB1) and subsequently inoculated with *S.rolfsii* which recorded in sugar beet 0.283 changes in absorbance/min/g of tissues in leaves and 0.355 changes in absorbance/min/g of tissues in tuber) followed by *P.fluorescens* and subsequently inoculated with *S.rolfsii* (0.268 and 0.308) as against control (0.197 and 0.222) (Fig. 2).

Effect of biocontrol agents on Phenylalanine ammonia lyase (PAL) activity

The enzyme activity in the pretreated and challenged plants increased from the 2nd day after inoculation and maintained at higher-level upto 6th day after challenge inoculation. Whereas in healthy and inoculated (*S.rolfsii*) control plants the activity was decreased many folds lower than in pretreated plants with biocontrol agents with challenge inoculated plants Among the biocontrol agents, pretreatment of sugarbeet plants (leaves) with the *T. viride* (TVB1) and subsequently inoculated with *S.rolfsii* recorded the maximum PAL activity of 37.49 nmol transcinnamic acid on 2nd day after challenge inoculation and increased up to 6th day (53.29 nmol transcinnamic acid) followed by a slow decline after 6th day (Figure 3). But the activity of PAL in inoculated control was only 27.63 on 2nd day after challenge inoculation and 34.54 on 6th day in sugarbeet leaves similarly in sugarbeet plants root the pretreatment *Trichoderma viride* (TVB1) and subsequently inoculated with *S. rolfsii* recorded the maximum PAL activity of 40.09 on 2nd day after challenge inoculation and increased upto 6th day (57.55) followed by a slow decline after 6th day But the activity of PAL in inoculated control was only 30.45 on 2nd day after challenge inoculation and 38. 08 on 6th day in roots (Fig3).

Effect of antagonists on phenolics

In all the treatment there was an increasing trend in total phenol content of the leaf and tuber from zero to four days after treatment. *S. rolfsii* alone inoculated plants recorded the phenolic content of 0.638 mg/g in leaf and 0.672 mg/g in tuber. Sugar beet plants inoculated with *T. viride* (TVB1) and subsequently inoculated with *S.rolfsii*

recorded 0.850 mg/g in leaf and 0.915 mg/g in tuber of total phenols as against control (0.440 mg/g and 0.432 mg/g respectively) four DAT (Fig 4) these were followed by the plants treated with *P.fluorescens* isolate SBHRPF 2 (0.840 and 0.881 mg/g) and *T. harzianum* isolate THB1 and inoculated with *S.rolfsii* (0.780 and 0.713 mg/g respectively).

Native gel electrophoresis

Isoform pattern of peroxidase

Native gel electrophoretic separation of enzyme extract from biocontrol agents treated plants challenged with *S.rolfsii* showed different peroxidase (PO) isoform patterns in both sugarbeet leaves and tuber. In *T.viride* isolateTVB1 and *P.fluorescens* isolate SBHRPF 2 after challenge inoculation with *S.rolfsii* showed 3 isoforms PO1- PO2, but in the healthy control no isoform were expressed. The intensity of expression of PO2 was more pronounced TVB1 and SBHRPF2 alone and also challenge inoculation of *S.rolfsii* with an additional expression of PO3 (SBHRPF2) than healthy control. (Plate 1).

Native gel electrophoretic separation of enzyme extract from biocontrol treated sugarbeet tuber showed different peroxidase (PO) patterns in sugarbeet tubers. The plants treat with *P. fluorescens* (SBHRPF2) along with challenge inoculation *S. rolfsii* showed 5 isoforms PO1-PO5, but in control (without pathogen inoculation) plant revealed only two isoform. PO1 and PO2 induced in bacterized tubers as well as bacterized tubers followed by challenge inoculation with *S.rolfsii*

The expression of PO3-PO5 were absent in *T. viride* (TVB1) inoculated control and Healthy control, but isoform PO5 was induced when the pathogen inoculated with in SBHRPF2 along pathogen challenge inoculation (Plate 1a).

Isoform pattern of polyphenol oxidase

Isoform pattern of the PPO in the sugarbeet plants challenged with or without *S.rolfsii* were studied. The PPO isoform pattern of biocontrol agents treated sugarbeet plants (leaves) after challenge inoculation with pathogen revealed the presence of 3 isoforms in sugarbeet leaves. PPO1 was present in all the plants irrespective of the treatments, but PPO3 was detected only in plants treated with *P.fluorescens* isolate SBHRPF2 and *T. viride* isolate TVB1 challenged with *S.rolfsii*.

But PPO1 and PPO2 remain as dark band. But in the healthy control plants the isoforms PPO1 and PPO2 were observed as a faint band. The unique enzyme band of PPO2 was not observed in gels of extracts prepared from leaf tissues of biocontrol agents (TVB1) alone treated plants without challenge inoculation (Plate 2). But the same band (PPO2) appeared with higher intensity in biocontrol agents treated plants, challenged with pathogen than in uninoculated healthy plants.

Isoform pattern of the PPO was studied in sugarbeet root also, The PPO isozyme pattern of biocontrol agents treated tissues taken from sugarbeet tuber after challenge inoculation with pathogen revealed the presence of a unique isozyme PPO3 and PPO4 detected only in the fungal and bacterial bioagents treated plant and remains as dark band without change over time. But in the control plants the isozyme PPO3 and PPO4 are absent. The unique enzyme band (PPO 4 and PPO 5) was not visible in gels of extracts prepared from tuber tissues of control plant. But the intensity of the isoforms (PPO1 and PPO2) was comparatively faint when compared to fungal and bacterial bioagents alone treated sugarbeet tubers. The PPO3, PPO4 and PPO5 isoforms were induced in *P.fluorescens isolate* SBHRPF 2 and *T.viride* isolate TVB1treated tubers (Plate 2a).

Use of biocontrol agents are considered as an alternative to chemicals for the management of crop diseases. *P. fluorescens* from phylloplane and *T.viride* from Rhizosphere exerted the maximum reduction of pathogen. *Pseudomonas fluorescens* and *P. putida* were isolated from the spermosphere of sugarbeet in California (Osburn *et al.*, 1989). Fulkui *et al.,* (1994) reported that *Pseudomonas* from sugarbeet spermosphere system effectively controlled *P. ultimum.* Qin *et al.,* (2019) the efects of two diferent BCAs on the phyllosphere microbial community and further revealed the potential relationships between phyllosphere bacterial community and plant health.

The isolates *T. viride* (TVB1), *T.harzianum* (THB1) *P. fluorescens* (SBHRPF1) and *P. chlororaphis* (PA 23) caused drastic reduction in sclerotial production by the pathogen. This has a practical bearing in the control of this pathogen which survives in soil in the form of sclerotia. Therefore reduction in sclerotial production by the antagonist will reduce the inoculum potential and subsequently disease incidence. Rangeshwaran and Prasad (2000) reported eleven rhizobacteria antagonistic to *S.rolfsii* including *P. fluorescens, P. putida, Streptomyces* spp. and *Bacillus* spp. which completely inhibited *S.rolfsii* under dual culture by the production of antibiotics or lytic enzymes

Induced systemic resistance by antagonists

Plants have endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Heil, 2001). It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. Induced resistance by inducing agents in several crops is associated with enhancement of lignification and with increased activities of defense gene products that are involved in phenyl propanoid pathway and PR protein synthesis (Hammerschmidt and Kuc, 1995). The activities of the defense enzymes superoxide dismutase (SOD), peroxidase (POX) and glutathione reductase (GR) increased strongly in Dasht in response to *M. albescens* infection (Dariush Ebadi Almas and Atefeh Rahmani Kamrod., 2019)

The phenolic compounds may contribute to enhance the mechanical strength of host cell wall and may also inhibit the fungal growth as phenolics are fungitoxic in nature. In the present study, increased levels of phenol accumulation were observed in *T.viride* (TVB1), *T. harzianum* (THB1) *P. fluorescens* (SBHRPF2) *P. chlororaphis* (PA23) and *B. subtilis* (SBHRPBS1) pretreated plants challenge with pathogen inoculation. In all the treatments, accumulation of phenol was more up to four days after treatment and afterwards a sudden decline was recorded. (Fig 4) Seed treatment with *P. fluorescens* 63-28 induced the accumulation of phenolics in tomato root tissues (M' Piga *et al.,* 1997). The increased phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content of the pathogen. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported groundnut against *C. personata* (Meena *et al.,* 2000),

Induction of peroxidase and poly phenol oxidase

The antagonists along with pathogen inoculation induced more peroxidase and polyphenol oxidase activity in tropical sugarbeet plants leaf and tuber, biotic and abiotic inducers play an important role in activating the defense genes in plants (Van

Loon, 1997). In the present investigation, it was observed that the tropical sugarbeet plants applied with *T.viride* isolate (TVB1), *T. harzianum* (THB1) *P.fluorescens* isolate (SBHRPF2) *P. chlororaphis* isolate PA23 and *B. subtilis* isolate SBHRPBS1 significantly induced the defense compounds (peroxidase and polyphenol oxidase) compared to unsprayed control (Fig 1and 2)

Peroxidase represents another component of an early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread. Increased peroxidase (PO) has been observed in a number of resistant interaction involving plant pathogenic fungi, bacteria and virus (Nandakumar *et al.,* 2001). Increased activity of cell wall bound peroxidase has been elicited in different plants such as cucumber (Chen *et al.,* 2000), In the present study, peroxidase activity was significantly increased from 2nd day after challenge inoculation. The peroxidase activity was maximum on 4th day after challenge inoculation in tropical sugarbeet tuber and leaves pretreated with the *T.viride* isolate (TVB1) and *P.fluorescens* isolate SBHRPF2.

In the present study, the total of five isozymes (PO1-PO5) was detected in *T.viride* (TVB1) and *P. fluorescens* (SBHRPF2), treated plants with *S. rolfsii* challenge inoculation. The expressions of PO2, and PO3 were higher in leaves pretreated with the biocontrol agents (*T.viride* isolate TVB and 1 *P. fluorescens* isolate SBHRPF2) pretreated plants challenged with pathogen when compared with plants treated with biocontrol agents alone, pathogen alone and healthy. But in case of tropical sugarbeet tubers, PO4 and PO5 were induced in fungal and bacterized tubers as well as followed by challenge inoculation with *S. rolfsii PO* was found to accumulate with concomitant increase in the lignin in plants treated with biocontrol agents following pathogen infection. Peroxidase reduces H_2O_2 to water at the expense of electron from coniferyl alcohol and the process initiates the lignifications chain reaction in plants (Smith *et al.,* 1991). It has been established that lignin depositions from barriers limit fungal penetration. The early and increased expression of the peroxidase enzymes would have involved in the biochemical reaction necessary for lignification which protected the plants from *S. rolfsii* infection. In the present investigation, PPO activity was increased from 2nd day after challenge inoculation, but maximum activity was recorded on 4th day after challenge inoculation with pathogen in all the biocontrol agents treated tropical sugarbeet leaves and tubers , Meena *et al.,* (2000) reported that the *P. fluorescens* induced the activities of PPO in response to infection by *C. personata* in groundnut.

The present experiment demonstrated that the application of *T.viride* isolate TVB1 and *P.fluorescens* isolate SBHRPF2 induced the polyphenol oxidase (PPO) activity in leaves. Five isoforms were observed in tropical sugarbeet plants pre treated with biocontrol agents followed by challenge inoculation with pathogen. PPO3 was detected only in plants treated with SBHRPF2 and TVB1 challenged with *S. rolfsii*. The isoform PPO3 was not observed in plants treated with biocontrol agents alone without challenge inoculation.

But in tropical sugarbeet tubers, PPO4 and PPO5 isoforms are induced by TVB1 and SBHRPF 2. The isoform PPO4 and PPO5 was not observed in plants treated with biocontrol agents alone without challenge inoculation.The induction of PO and PPO might have resulted in cell wall thickening and would have suppressed the penetration of *S. rolfsii* into tropical sugarbeet plants.

Table.1 Antagonistic effect of phylloplane biocides against *S. rolfsii in vitro*

S No	Treatments	Mycelial growth*		Number of sclerotia*		Sclerotial germination* (%)	
		Mycelial growth (cm)	Growth inhibition (%)	No. of sclerotia/ plate	Per cent reduction	Sclerotial germination (%)	Per cent reduction
1	***Aspergillus flavus***	5.37	39.18	91.33	56.30	48.33	**51.67**
2	*Cladosporum*	6.33	28.31	181.33	13.23	67.33	**32.67**
3	*Curvularia*	6.93	21.48	192.33	7.97	65.33	**34.67**
4	*Penicillium* spp	6.77	23.32	189.67	9.25	61.66	**38.34**
5	*Pseudomonas* fluorescens	5.13	41.90	61.33	70.65	41.33	**58.67**
6	*Bacillus subtilis*	5.33	39.00	87.67	58.05	47.33	**52.67**
7	Control	8.83	-	209.00	-	100	
	CD (P=0.05)	**0.68**		**2.23**		**2.66**	

* Mean of three replications

Table.1a Antagonitic effect of rhizosphere biocides against *S. rolfsii in vitro*

S. No	Treatments	Mycelial growth*		Number of sclerotia*		Sclerotial germination* (%)	
		Mycelial growth (cm)	Growth inhibition (%)	No. of sclerotia/ plate	Per cent reduction	Sclerotial germination (%)	Per cent reduction
1	*Aspergillus niger*	4.33	50.96	66.33	54.87	59.20	**40.80**
2	*Aspergillus flavus*	5.47	38.05	78.00	46.93	67.10	**32.90**
3	*Penicillium* spp	6.67	24.46	99.67	32.19	69.07	**30.93**
4	*Rhizopus* spp	7.00	20.72	112.33	23.58	67.10	**32.90**
5	*Trichoderma viride*	2.93	66.81	45.33	69.16	37.50	**62.50**
6	*Pseudomonas fluorescens*	4.33	50.96	51.67	64.85	39.47	**60.53**
7	*Bacillus subtilis*	4.67	47.12	64.00	56.46	45.39	**54.61**
8	Control	8.83		147.00	-	100.00	-
	CD (P= 0.05)	**0.93**		**3.16**		**1.40**	

* Mean of three replications

Table.2 Effect of fungal antagonists against *S. rolfsii in vitro* (Dual culture technique)

S. No	Treatments	Mycelial growth (cm)		Number of sclerotia		Sclerotial germination (%)	
		*Mycelial growth of *S. rolfsii* (cm)	Per cent reduction over control	No. of sclerotia/ plate	Per cent reduction over control	Sclerotial germination (%)	Per cent reduction over control
1	*T. viride* (TVB1)	2.40	73.03	28.04	86.31	31.22	**68.78**
2	*T. viride* (TVB2)	2.50	71.19	31.34	84.70	34.88	**65.12**
3	*T. viride* (TVB3)	2.63	70.44	36.63	82.12	38.87	**61.13**
4	*T. viride*(TVB31)	2.86	67.86	34.65	83.09	40.86	**59.14**
5	*T. harzianum* (THB1)	2.50	71.19	29.36	85.67	31.55	**68.45**
6	*T. harzianum* (THB2)	2.96	66.74	37.748	81.58	43.18	**56.82**
7	Control	8.86	-	204.93	-	100	-
CD (P=0.05)		**0.32**	**-**	**0.95**	**-**	**1.66**	**-**

* Mean of three replications

Table.3 Effect of bacterial antagonists' against *S. rolfsii in vitro*

S. No	Treatments	Mycelial growth (cm)		Number of sclerotia		Sclerotial germination (%)	
		*Mycelial growth of *S. rolfsii* (cm)	Per cent reduction over control	No. of sclerotia/ plate	Per cent reduction over control	Sclerotial germination (%)	Per cent reduction over control
1	*P. fluorescens* (SBHRPF2)	2.96	66.36	22.69	84.66	34.65	**65.35**
2	*P. fluorescens* (SBHRPF4)	3.06	65.23	34.53	76.67	43.56	**56.44**
3	*P. fluorescens* (SBHRPF8)	3.50	60.22	39.46	73.33	41.58	**58.42**
4	*P. fluorescens* (SBHRPF14)	3.50	60.22	45.38	69.33	59.4	**40.60**
5	*P. fluorescens* (SBHRPF18)	4.50	48.86	34.53	76.33	57.42	**42.58**
6	*P. fluorescens* (SBHRPF38)	3.10	64.77	38.48	74.00	42.57	**57.43**
7	*P. chlororaphis* (PA 23)	3.50	60.22	42.42	71.33	51.48	**48.52**
8	*B. subtilis* (SBHRBS1)	4.36	50.45	54.27	63.33	40.59	**59.41**
9	*B. subtilis* (SBHRBS2)	4.48	49.09	52.29	64.67	69.3	**30.70**
10	Control	8.80	-	148.01	-	100	-
	CD (P=0.05)	**0.07**		**0.92**		**1.29**	

* mean of three replications

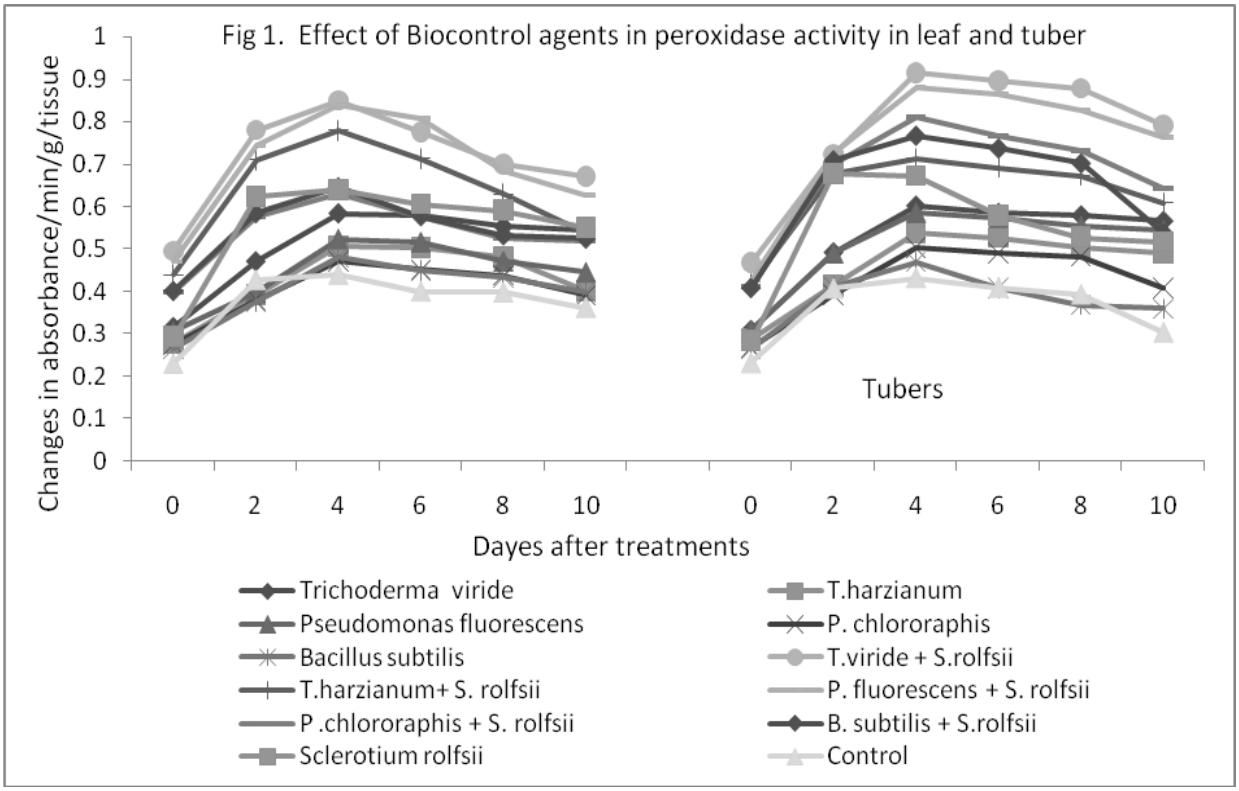

Fig.1 Effect of Biocontrol agents in peroxidase activity in leaf and tuber

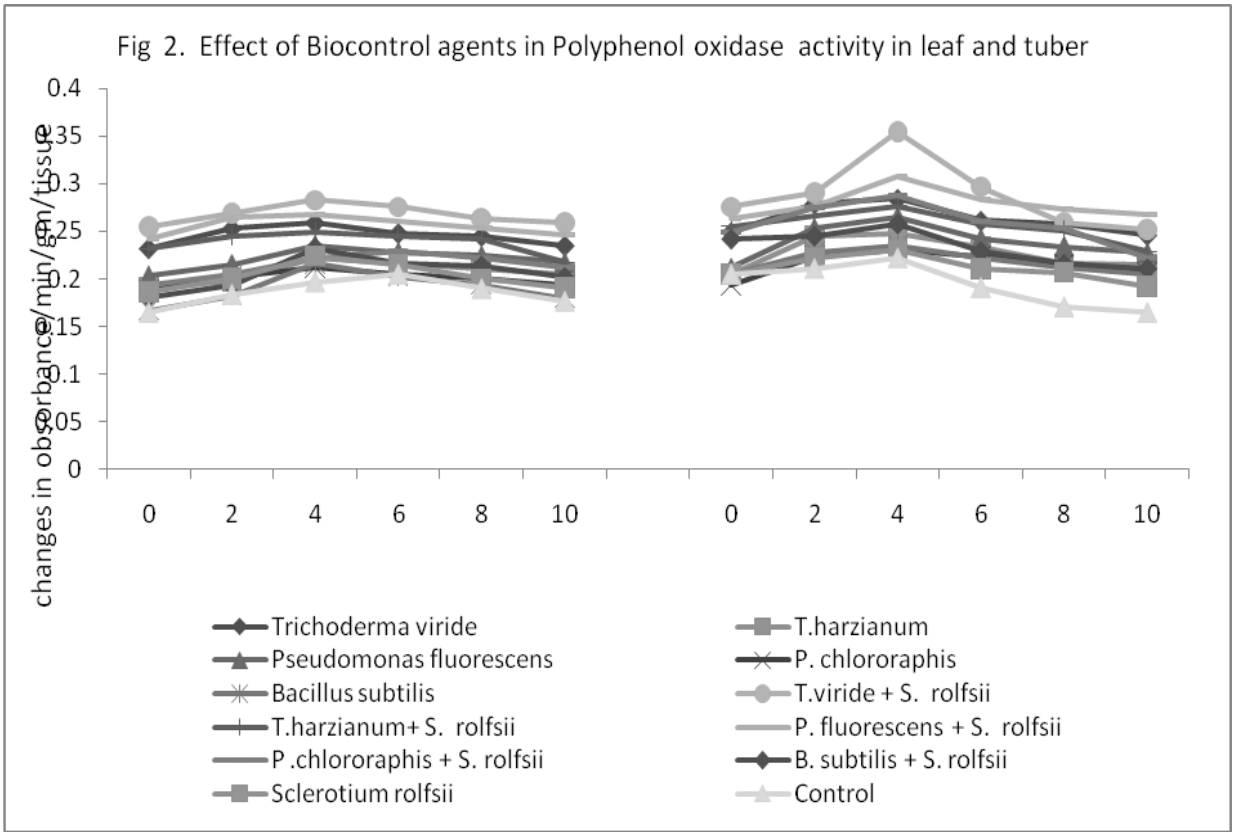

Fig.2 Effect of Biocontrol agents in Polyphenol oxidase activity in leaf and tuber

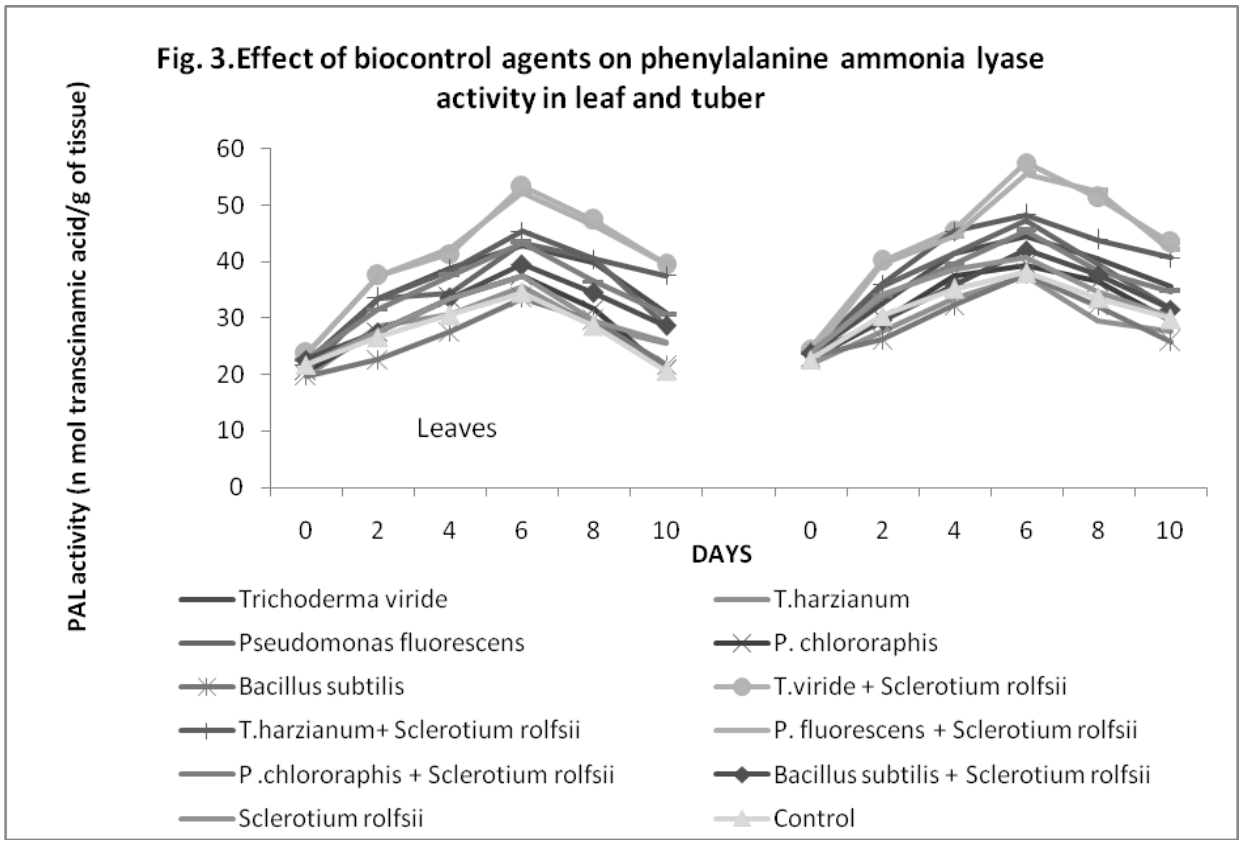

Fig.3 Effect of biocontrol agents on phenylalanine ammonia lyase activity in leaf and tuber

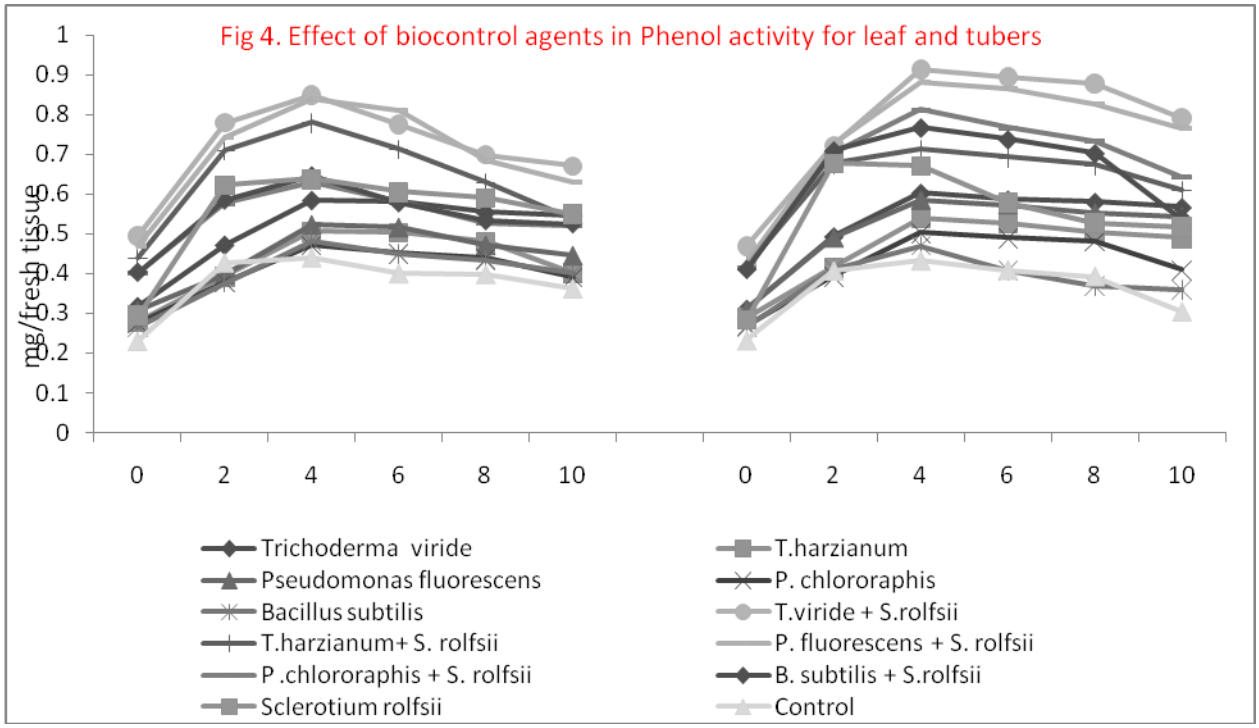

Fig.4 Effect of biocontrol agents in Phenol activity for leaf and tubers

Phenylalanine ammonia lyase 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 the establishment phase of a pathogen within the host tissues, The product of PAL activity is transcinnamic acid which is an immediate precursor for the biosynthesis of salicylic acid, a signal molecule in systemic acquired resistance (SAR) (Klessig and Malamy, 1994).

Phenyl propanoid metabolism starts with the conversion of L-phenylalanine into trans-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) (Massala *et al.,* 1980). In the present study, treatment with *T.viride* (TVB1), *T. harzianum* (THB1) *P.fluorescens* (SBHRPF2) *P. chlororaphis* (PA23) and *B. subtilis* (SBHRBS1) induced the plants to synthesize PAL when the plants were challenge inoculated with *S.rolfsii* . The activity reached the maximum level on four day after challenge inoculation in tropical sugarbeet leaf and tubers pretreated with the Soil application of *T. viride*, and *P. fluorescens* which was challenged with *S. rolfsii*. When groundnut plants were sprayed with *P. fluorescens*, increased activity of PAL was observed (Meena *et al.,* 2000). Cucumber plants treated with *P. corrugata* had initially higher levels of PAL and levels were lower after challenging the plants with *P.aphanidermatum* (Chen *et al.,* 2000). Prior treatment of anthurium seedlings with biocontrol agents triggered the plant defense mechanism in response to infection by *C. gloeosporioides*. Hence, it is speculated that among the various direct antagonistic tools, ISR is also the one indirect tool by which the tested biocontrol agents afforded resistance to anthurium against the pathogen (Thangeswari and Sankaralingam, 2013) addition to soil drench, foliar spray showed that PGPR and chemical defense inducers can be exploited to control take-all throughout the crop growing season. Often farmers seek products that prevent the disease from spreading after its appearance in the field. Most plant probiotic products are designed to be applied inthe soil. However, it is necessary to develop bacterial formulations to be applied under phyllosphere conditions (Ali Mahmood Jasem *et al.,* 2018).concluded that soil application of Rhizospher and phyllosphere microorganism induced systemic resistance against soil borne pathogen in tropical sugarbeet ecosystem.

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