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Exploration of *Trichoderma* spp. as an Effective Bio Control Agents against the Sclerotial wilt Caused by *Sclerotium rolfsii* Sacc.

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A B S T R A C T

Jasmine is the most attractive flower crop being cultivated throughout the tropical and sub-tropical countries. In Tamil Nadu jasmine cultivation is highly interrupted by the soil borne wilt pathogen *Sclerotium rolfsii* Sacc. In the current scenario of disease management of soil borne pathogens *Trichoderma* spp can be effectively exploited for the effective containment of the disease. The isolate SR1 was found to be the most virulent during the surveillance programme and it produced the maximum amount of host non-specific toxin oxalic acid. The *Trichoderma* species such as *T. harzianum* (TspT), *Trichoderma* sp. (TspK) and *T. viride* 1 performed well and inhibited the mycelial growth effectively. The best isolate TspT excelled in toxin degradation. The ITS region of TspT was amplified with primers ITS1 and ITS4 and the product (650 bp) showed homology sequences (98%) with *T. harzianum* in the NCBI-Blast search.

Introduction

India is having favourable agroclimatic conditions for the production of flower crops. Floriculture is a fast emerging and highly competitive industry in our country. Jasmine is one of the ancient flowers grown in India. It comes under the family Oleaceae, order Oleales and genus *Jasminum*. Jasmine is a queen of flowers and is called the "Queen of fragrance" or “Queen of the Night”. The word jasmine is derived from an Arabic word “Jessamine” called as “Yasmin” or yasmyn” which means fragrance. Scented oil is mainly extracted from jasmine. India exports the scented oil to England, United States of America, Sweden, Japan, Holland, European Union and Norway. Jasmine crop is susceptible to many fungal and phytoplasma diseases among these root rot and collar rot disease or *Sclerotial* wilt incited by *Sclerotium rolfsii* Sacc. (Telomorph: *Atheliarolfsii*) cause serious losses to jasmine plant. It has caused complete destruction of jasmine fields.

The pathogen *S. rolfsii* causes a variety of diseases such as damping off of seedlings, collar rot or stem rot, foot rot, crown rot,

Sclerotium wilt and blight (Tang *et al.,* 2015). This pathogen is having a wide host range which include alfalfa, banana, bean, beet, brussels sprouts, cabbage, carrot, cauliflower, celery, chrysanthemum, coffee, cotton, cucumber, garlic, ginger, gourd, lettuce, mango, muskmelon, mustard, onion, southern pea, peanuts, pineapple, potato, pumpkin, radish, soybean, squash, tobacco, tulip, turf, turnip, and yam (Aycock, 1966).

The annual loss to world crops has been estimated as about 30,000 million dollars due to this fungal pathogen *S. rolfsii* (Chaurasia *et al.,* 2014). Rolfs (1893) first noticed the unnamed fungus as tomato blight in Florida. Saccardo (1911) named the fungus as *Sclerotium rolfsii* which was sterile consisting of hyphae and sclerotia. It is a well-known polyphagous and most destructive soil borne fungus.*S. rolfsii* forms brownish sclerotia that can survive in soil for long period tolerating biological and chemical degradation due to the presence of melanin in the outer membrane (Chet, 1975) (Fig. 1a, b and c).

S. rolfsii secrete non-host specific phytotoxin, oxalic acid and tissue degrading enzyme cellulase during infection process and it is corrosive to plant tissues. Oxalic acid combine with calcium in plant tissues, removing it from association with the pectic compounds in plant cell walls, lowering cell wall pH, and thereby favouring the activity of the cell wall-degrading enzymes polygalacturonase and cellulase. Oxalic acid and tissue degrading enzymes work together to break down the cell walls and resulting in tissue maceration. The pathogens then absorb nutrients from the macerated tissue. Maceration interrupts transport of water and nutrients in plant tissues, thereby causing wilting, yellowing and necrosis (Billah, 2017).

Usage of bio control agents and their antibiotics, is an alternative method for chemical control. It is a safe, effective and ecofriendly approach in plant disease management(Wang *et al.,* 1999).The biocontrol mechanism involved in *Trichoderma* spp. were competition for nutrients and space, mycoparasitism, antibiosis, production of volatile metabolites and hydrolytic enzymes such as β-13 glucanase and chitinase which partially degrades the cell wall of the pathogen and leads to parasitization (Kubicek *et al.,* 2001). *Trichoderma* spp. came into contact with the pathogenic fungi, they attached and coiled around the pathogens and formed appressoria for infection. Holes are produced at the site of appressorial contact and *Trichoderma* entered into the lumen of the target fungi and killed the target fungi (Harman *et al.,* 2004).

Materials and Methods

Source of the pathogen

A survey was conducted in jasmine growing areas of southern districts of Tamil Nadu and it was noticed that highest disease incidence (70.00 per cent) was recorded at Kalvai, Srivaikundam, Thoothukudi district (SR1). The pathogen was isolated and named as SR1. The pathogenicity experiment was conducted to test the virulence of the isolates and prove the Koch's postulates. In this test the isolate SR1was found highly virulent by recording the highest per cent disease incidence (93.50%) as compared with other isolates and was forwarded for doing other experiments. This isolate produced the typical wilt symptoms on the artificially inoculated jasmine plants in pot culture.

Isolation of bacterial and fungal antagonists from the rhizosphere region of Jasmine plants

Ten gram of rhizosphere soil was transferred to 250 ml Erlenmeyer flask containing 100 ml

of sterile distilled water. The antagonist in the suspension was isolated by serial dilution plate method after thorough shaking (Pramer and Schmidt, 1956). From the final dilutions of 10^{-3}, $10^{-5}$and 10^{-6} one ml of each aliquot was pipetted out, poured in sterilized Petri plate containing *Trichoderma* selective medium (TSM), King's B medium and Nutrient agar medium separately and they were gently rotated clockwise and anti-clockwise for uniform distribution and incubated at room temperature (28 ±2°C) for 24 hours in case of bacteria and seven days in case of fungi. The colonies were viewed under UV light. Colonies with characteristics of *Bacillus* spp. and *Pseudomonas* spp. were isolated individually and purified by "streak plate method" (Ranaswami, 1993) on Nutrient agar medium and King's B medium respectively. *Trichoderma* sp. isolated from TSM was purified and maintained on PDA medium. The pure cultures were maintained on respective agar slants at 4°C. *Trichoderma* spp. isolated from Thovalai, Kanyakumari Dt. and Kalvai, Srivaikundam, Thoothukudi Dt. were named shortly as Tsp_T (TOV) and Tsp_K (KAL). Like this *Pseudomonas* spp. as Psp_T (TOV), Psp_K (KAL) and *Bacillus* spp. as Bsp_T (TOV) and Bsp_K (KAL). In addition to this the standard bio control agents being maintained in the department of Plant Pathology, AC and RI Killikulam such as *Trichoderma viride* (Tv1), *Pseudomonas fluorescens* (Pf1) and *Bacillus amyloliquefaciens* were used against *S. rolfsii*.

Efficacy of fungal and bacterial bio control agents against the growth of *S. rolfsii in vitro* Dual culture technique

The antagonistic effect of three isolates of *Trichoderma sp.* (Tsp_K, Tsp_T, Tv_1), *Bacillus* spp. (Bsp_K, Bsp_T, *B. amyloliquefaciens*) and *Pseudomonas* spp. (Psp_K, Psp_T, Pf1) were tested against the pathogen by dual culture method(Dennis and Webster, 1971). As the pathogen is a fast growing it was inoculated against the pathogen simultaneously. The Petri plates containing medium inoculated with the pathogen alone were served as control. The radial growth of the pathogen was measured in all the treatments when full growth of the fungus was seen in the control plate. The results were expressed as per cent inhibition over control by using the formula described by Pandey and Upadhyay (2000).

Molecular characterization of *Trichoderma* sp. (Tsp_T)

Genomic DNA extraction from *Trichoderma* sp.

Isolation of fungal genomic DNA was done by the method described by Chakraborty *et al.*, (2010). To verify the quality of isolated DNA, 2.5µl of total DNA solution was resolved in the 1% agarose gel electrophoresis.

Agarose gel electrophoresis

One gram of agarose was added to the 100 ml of 1X TAE buffer in a 250 ml conical flask and melted until it turned to a clear, transparent solution in microwave oven. When the molten agarose gel was cooled to 55°C, ethidium bromide was added to a final concentration of 0.5µg/ml (5µl of 10mg/ml ethidium bromide stock is added to 100 ml solution) and the solution was mixed by gentle swirling. Warm agarose solution was poured into gel casting tray to a thickness of 5-10 mm and the comb was placed to make wells to load the DNA samples in the gel. The gel was kept at room temperature for 30-45 minutes to set completely. Gel along with the casting tray was placed into electrophoresis tank filled with 1X TAE buffer. The comb was carefully removed from the gel. The samples were mixed with 6X loading dye. Slowly the samples were loaded into the wells of the submerged gel using a micropipette. DNA ladder was added to the left or right side of the

gel as a marker. The lid of the gel tank was closed and electrical leads were attached to the tank. A voltage of 5-8 V/cm was applied. The gel electrophoresis was run until the bromophenol blue and xylene cyanol FF have migrated to 3/4th distance from the top of the gel. The gel was examined under a UV light and the image was documented using Gel Doc apparatus.

ITS Sequencing of *Trichoderma*

A PCR was performed in a total volume of 50 µl using Emerald Amp® GT PCR master mix using genomic DNA of *Trichoderma* as a template. The intermediate 5.8S ribosomal gene along with ITS1 and ITS2 region were amplified using the primers ITS1 and ITS4 with the PCR conditions initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 70 °C for 2 min and the final extension at 72 °C for 7 min. The reaction was carried out in a Eppendorf master cycler gradient PCR machine. The PCR products were resolved by electrophoresis in 1% agarose gel. The PCR products were purified using Favor Prep GEL/ PCR purification kit and sequenced at Eurofins genomics India Pvt. Ltd. Bangalore.

The Primers used for amplification of ITS region were

ITS1 - 5′ TCCGTAGGTGAACCTGCGG 3′ (forward primer)

ITS4 - 5′ TCCTCCGCTTATTGATATGC3′ (reverse primer)

Sequencing of ITS and identification of *Trichoderma* sp. by bioinformatics analysis

The obtained DNA sequences were trimmed at 5'and 3' region where the sequencing chromatogram were not clear. Then DNA sequence, in which clear chromatogram obtained, was made in Fasta format. This was used as input sequence (Query sequence) in nucleotide blast analysis program at NCBI database. The output data retrieved from the bioinformatics were analysed and, the organism showing major score was considered as the closely related species to the test fungus used in the study.

Efficacy of volatile metabolites produced by the bio control against *S. rolfsii in vitro*

PDA culture discs of 9 mm, diameter of the fungal antagonists were kept at the centre of the Petri plates containing sterilized PDA medium. In case of bacterial antagonists 48 hour old culture of *Pseudomonas* spp. and *Bacillus* spp. were streaked at the centre of the Petri plates containing King's B and Nutrient agar medium respectively. The plates were incubated at room temperature. After incubation period, the lid of each Petri plates was replaced by another bottom plate containing PDA medium inoculated with 9mm actively growing *S. rolfsii*. The two plates were sealed together and in case of control only the pathogen had been maintained in the upper lid along with the absence of antagonist. Five replications were maintained for each treatment. The colony diameter of the pathogen was measured on third day after inoculation. Data were expressed in per cent reduction over control.

Degradation of oxalic acid production by bio control agents

Czapek-Dox broth was prepared and 100 ml was poured into 250 ml conical flask and autoclaved at 121° C for 15 min. For evaluating the effect of antagonists against oxalic acid production by *S. rolfsii*, the flasks were inoculated with *S. rolfsii* culture disc in one side and on other side the flasks were inoculated with the culture discs of fungal

antagonist. For bacterial antagonists, cell suspension was prepared and inoculated with a loopful of culture. Czapek-Dox broth inoculated with *S. rolfsii* alone served as a control. The mycelial mat were removed by filtering the broth through Whatman No. 1 filter paper and the aliquots were centrifuged at 5000 rpm for 10 min to remove the mycelial fragments. 8 ml of calcium chloride-acetate buffer (pH 4.5) was added to 10 ml of culture filtrate and mixed thoroughly.

Allowed the mixture to stand overnight and centrifuged at 5000 rpm for 10 min, supernatant was discarded and the residue was washed with 10 ml of H_2SO_4. The solution was transferred to 100 ml conical flask and heated at 80°C on water bath. While hot it was then titrated with 0.02N potassium permanganate until faint pink colour persisted. 1 ml of 0.02 N potassium permanganate reacted with 1.2653 mg of oxalic acid. The oxalic acid present in the culture filtrate was calculated and expressed as mg/ml (Mahadevan and Sridar, 1986).

Results and Discussion

Dual culture technique

Among these biocontrol agents, *Trichoderma* sp. Tsp_T (TOV) performed better and resulted maximum mycelial growth reduction of *S. rolfsii* (81.27 per cent) over control followed by Tsp_K (KAL) (71.91 per cent reduction) and Tv1(66.29 per cent reduction). The isolate Tsp_T grows over the pathogen and completely inhibit the sclerotial production. *Trichoderma* sp. attacked the *S. rolfsii* by adopting various mechanisms like coiling of hyphae, formation of holes and appressoria on the pathogenic cell wall. Due to the production of β-1,3 glucanase and chitinase enzymes it produces holes on the cell wall of the pathogen. All the above said mechanism have been observed in the dual culture technique (Elad *et al.,* 1983). In the present investigation our best antagonist *T. harzianum* (Tsp_T) grew faster than the *S. rolfsii* in dual plate method. This is highly advantage of the antagonist and effectively utilize the space and nutrients more than the pathogen (Table 1; Fig. 2).

Efficacy of volatile metabolites produced by the biocontrol agents against the pathogen *in vitro*

The bacterial and fungal bio control agents were tested for the production of volatile compounds using inverted plate technique. Among the tested isolates, *T. viride* (Tv1) and Tsp_K were on par with each other and reduced the mycelial growth to 1.23 and 1.33 cm against the control (9.00 cm). The second best isolate is *Trichoderma* sp. (Tsp_T) (Table 2; Fig. 3). Besides mycoparasitism, *Trichoderma* spp. produces diffusable metabolites which are volatie in nature. Humpty number of volatile secondary metabolites produced by *Trichoderma* sp. such as ethylene, hydrogen cyanide, aldehydes and ketones which play an important role in controlling the plant pathogen (Bhagat *et al.,* 2014).

Identification and confirmation of *Trichoderma* sp. by molecular technique

The ITS region of *Trichoderma* sp. (Tsp_T) isolatewas amplified with primers ITS 1 and ITS 4 using a thermocycler and the products produced were visualised as a single band in agarose gel stained with Ethidium bromide. The size of the PCR fragment was approximately 650 bp length. When the ITS sequence of the *Trichoderma* sp. (Tsp_T) was BLAST searched in the NCBI data base, the output data showed matching sequences of *Trichoderma harzianum*. So the *Trichoderma* sp. (Tsp_T) isolate used in the present study was confirmed as *Trichoderma harzianum* (Fig. 4). This is agreement with findings of Chakraborty *et al.,* (2010).

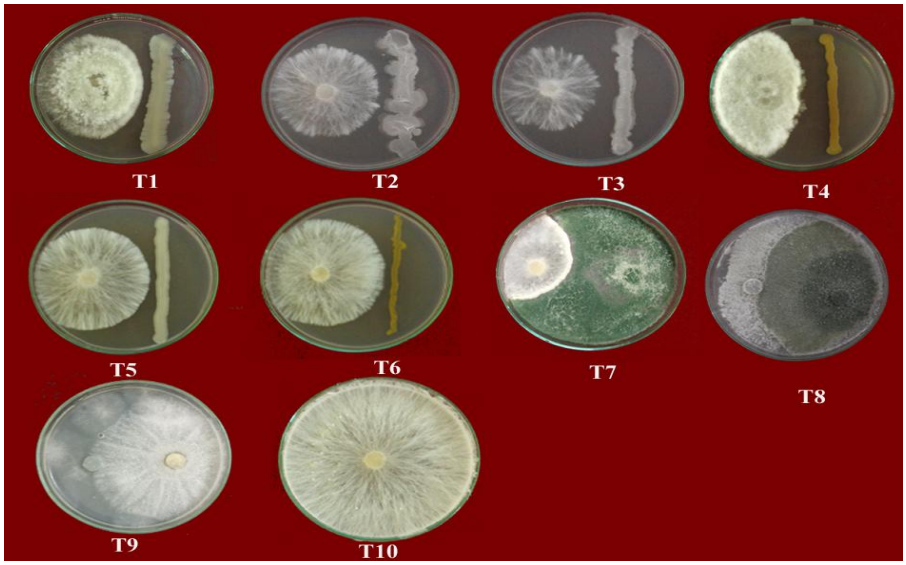
Plate.1Field view of jasmine affected by *Sclerotium rolfsii*

1a

1b. Sclerotia and fluffy mycelium on plant debris

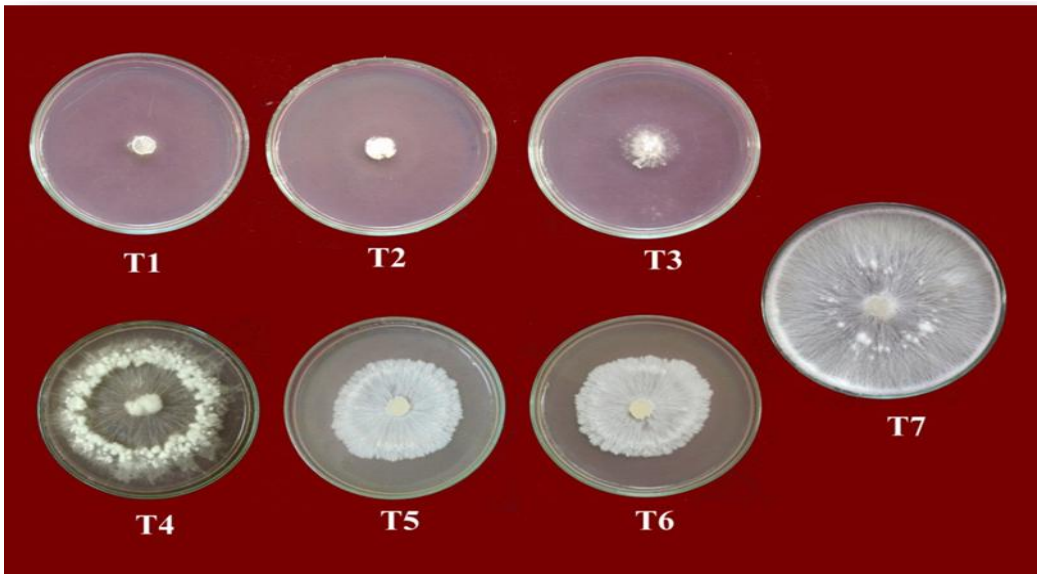
1c. Wilted plant

Fig.2 Effect of bio control agents on the mycelia growth of *S.rolfsii* (Dual plate method)



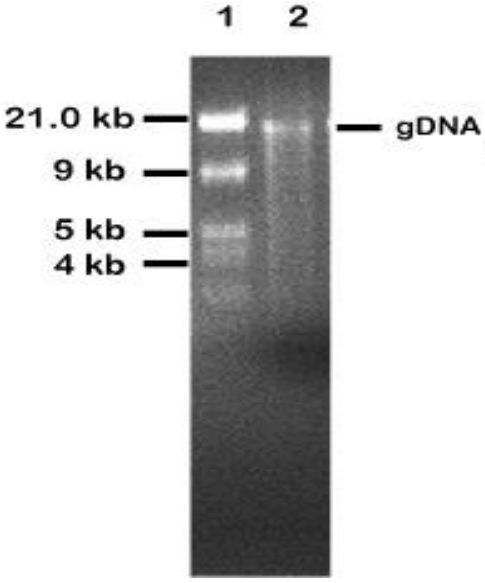
T1 -*B. amyloliquefaciens*
T2 -Bsp_T
T3 -Bsp_K
T4 – *P. fluorescens* (Pf1)
T5 -Psp_T
T6 -Psp_K
T7 - *T. viride*(Tv1)
T8 -Tsp_K
T9 -Tsp_T
T10 - Control

Fig.3 Effect of volatile metabolites produced by the bio control agents against the mycelial growth of *S. rolfsii*

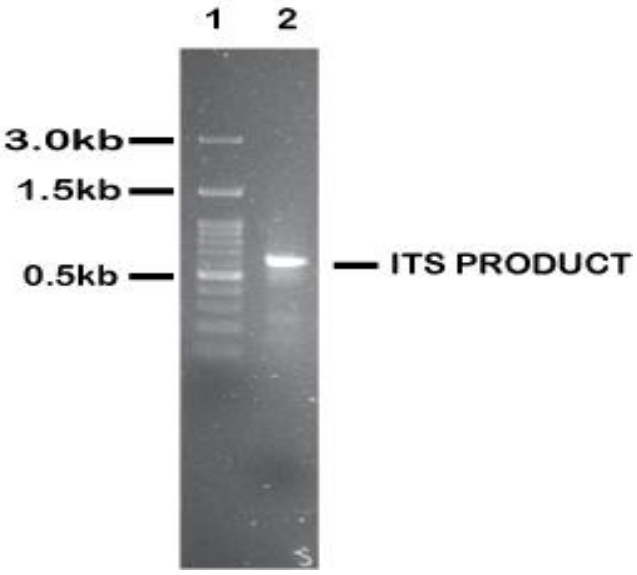


T1. ***T. viride*** **(Tv1)**
T2. **Tsp_K**
T3. **Tsp_T**
T4. ***P. fluorescens*** **(Pf1)**
T5. ***B. amyloliquefaciens***
T6. **Bsp_K**
T7. **Control**

Fig.4 Genomic DNA (gDNA) and PCR product of *Trichoderma sp.* Tsp_T (Thovalai) isolate



Lane 1: Lambda ladder
Lane 2: gDNA of *Trichoderma* sp.



Lane 1: 100 bp ladder
Lane 2: ITS of *Trichoderma* sp.

Table.1 Effect of bio control agents against the mycelial growth of *S. rolfsii* (Dual culture technique)

Treatment	Bio control agents	*Mycelial growth of the pathogen (cm)	Per cent inhibition over control*
T_1	*Bacillus amyloliquefaciens*	5.00	43.82 (41.45)^{d}
T_2	Bsp_T(TOV)	5.24	41.09 (39.86)^{d}
T_3	Bsp_K(KAL)	5.10	42.70 (40.80)^{d}
T_4	*Pseudomonas fluorescens*(Pf1)	5.05	43.22 (41.10)^{d}
T_5	Psp_T(TOV)	6.33	28.84 (32.46)^{e}
T_6	Psp_K(KAL)	7.27	18.35 (25.32)^{f}
T_7	*Trichodermaviride* (Tv1)	3.00	66.29 (54.51)^{c}
T_8	Tsp_K(KAL)	2.50	71.91 (58.07)^{b}
T_9	Tsp_T(TOV)	1.67	81.27 (64.41)^{a}
T_{10}	Control	8.90	0.00
CD (p=0.05)			2.56

*Mean of five observations
The treatment means are compared using Duncan multiple range test (DMRT)
Values in parentheses are arcsine transformed
In a column, mean followed by a common letter (s) are not significantly different (p=0.05).

Table.2 Effect of volatile metabolites produced by bio control agents against the mycelial growth of *S. rolfsii in vitro*

Treatment	Bio control agents	*Mycelial growth of the pathogen (cm)	Per cent inhibition over control*
T_1	*Trichodermaviride* (Tv1)	1.23	86.30 (68.28)[a]
T_2	Tsp_T(TOV)	2.23	75.19 (60.30)[b]
T_3	Tsp_K(KAL)	1.33	85.19 (67.38)[a]
T_4	*Pseudomonas fluorescens*(Pf1)	6.60	26.67 (30.86)[d]
T_5	*Bacillus amyloliquefaciens*	4.50	50.00 (45.00)[c]
T_6	Bsp_K(KAL)	7.07	21.48 (27.61)[d]
T_7	Control	9.00	0.00
CD (p=0.05)			1.79

*Mean of five observations
The treatment means are compared using Duncan multiple range test (DMRT)
Values in parentheses are arcsine transformed
In a column, mean followed by a common letter (s) are not significantly different (p=0.05).

Table.3 Degradation of oxalic acid by bio control agents

Treatment	Isolates	*Oxalic acid production (mg/ml)
T_1	Tv1	2.50[b]
T_2	Tsp_K	2.53[b]
T_3	Tsp_T	1.05[a]
T_4	Pf1	2.53[b]
T_5	Bsp_K	3.32[d]
T_6	*Bacillus amyloliquefaciens*	2.7[c]
T_7	Control	3.7[e]
CD (p=0.05)		0.10

*Mean of five replications
The treatment means are compared using Duncan multiple range test (DMRT)
In a column, mean followed by a common letter (s) are not significantly different (p=0.05)

They isolated and maintained eight *Trichoderma harzianum* isolates and eleven *T. viride* isolates from various rhizosphere soil of plantation crops, forest soil and agricultural fields collected from Northern West Bengal region. These isolates were analysed by ITS amplification using the primers ITS 1 and ITS 4. The amplified product is of 600 bp in length which is seen in all the isolates.

Degradation of oxalic acid by bio control agents

The result indicated that *T. harzianum* reduced the oxalic acid production of the pathogen to 1.05 mg/ml in culture filtrate as against the control of (3.7 mg/ml) followed by Tv1 and Tsp_K (2.50 and 2.53 mg/ml respectively) and these two were on par with each other. Oxalic acid production was not degraded by *Bacillus* sp. (Bsp_K) (Table 3). Paramasivan *et al.,* (2013) discovered that while testing the culture filtrate containing both *T. viride* (TVB1) and *S. rolfsii*, TVB1 very much reduced the oxalic acid (0.79 mg/ml culture filtrate) compared to control 3.07 mg/ml. In this current study the best antagonist reduced the oxalic acid concentration upto 1.05 mg/ml culture filtrate compared to control (3.75 mg/ml). Oxalic acid degradation by *Trichoderma* spp. is the very effective approach in biological control of wilt disease.

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 groundnut plants challenge with *S. rolfsii* (Paramasivan *et al.*, 2013).When these *Trichoderma* spp are applied in the plant ecosystem by seed treatment and soil application, these antagonists contain the pathogen by mechanisms like mycoparasitism, production of antibiotics, degradation of toxins of pathogens and production of volatile compounds. So, exploration of *Trichoderma* spp. and external application of these antagonists to the field ecosystem by various formulations are highly needed to check many soil borne pathogens causing heavy yield loss in various crops.

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