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Original Research Article

Efficacy of *Trichoderma asperellum* against *Ralstonia solanacearum* under greenhouse conditions

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Abstract: The rhizosphere soil samples of healthy tomato plants were used to isolate *Trichoderma* spp and identified based on morphological and molecular characterization as a *Trichoderma koningii* (T1) *T. flavofuscum* (T2), *T. harzianum* (T3), *T. asperellum* (T4), *T. harzianum* (T5 & T7), *T. koningii* (T6), *T. asperellum* (T8), *T. harzianum* (T9), *T. viride* (T10). The isolates screened for antagonistic activity against ten virulent strains of *Ralstonia solanacearum (*Rs*).* Two isolates of *Trichoderma* (T4 and T8) exhibiting high antagonistic activity (24-29mm and 20-27mm respectively) and also studied for several biocontrol mechanisms under greenhouse conditions. These strains were found to be positive to protease, β-1, 3-glucanase, Cellulase, Chitinase, Xylanase, Amylase, Pectinase and lipase activity. Germination percentage increased by 48% and 45% by Trichoderma with pathogen treated seeds and also increased root length, shoot length, fresh weight, dry weight and vigour index. Efficacy of T4 and T8 isolate were evaluated under green house conditions in suppressing disease and promoting tomato plant growth. The disease incidence was significantly reduced by about 50% in tomato plants raised under green house conditions.

Keywords*:* Antagonistic, *Trichoderma asperellum, Ralstonia solanacearum,* enzymes, Tomato wilt*,* Plant growth promotion.

Introduction

Bacterial wilt caused by *R. solanacearum* is deemed to be one of the most important plant diseases in tropical agriculture. It has a large host range of more than 200 species in 50 families. The disease also affects other economically important crops such as potato, eggplant, chilly and non *Solanaceous* crops such as banana and groundnut in India [1]. In India, a study showed 10 to 100% incidence of bacterial wilt during the summer [2]. Infested soil and water are the primary sources of inoculum. The pathogen infects roots of susceptible plants, usually through wounds and colonizes within the xylem preventing the water movement into upper portion of the plant tissue.

Chemical control is most effective, especially when multiple treatments are applied. Yet this method presents a range of negative side effects such as environmental pollution, detrimental health effects for farmers, consumers and the risk of emergence of resistant pathogen strains. In view of these serious drawbacks, the development of more environment friendly control methods, such as biological control using antagonistic microorganisms, can help to complement current strategies for integrated management of the disease [3].

One of the major biocontrol agents which reduce soil borne diseases of various crops includes isolates of *Trichoderma* spp. [4]. The genus *Trichoderma* is widespread in soil and on decaying wood and vegetable matter. As saprophytic organisms, *Trichoderma* spp. are able to use a wide range of compounds as carbon and nitrogen sources and secrete a variety of enzymes to break down recalcitrant plant polymers into simple sugars for energy and growth. The high degree of ecological adaptability shown by strains within the genus *Trichoderma* is reflected in its worldwide distribution, under different environmental conditions, and its survival on various substrates. This considerable variation, coupled with their amenability of cultivation on inexpensive substrates, makes *Trichoderma* isolates attractive candidates for a variety of biological control applications [5].

The ability of certain *Trichoderma* species to induce plant resistance against some plant pathogens, promote plant growth and improve photosynthetic activity of plants

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greatly boost these microorganisms biological arsenal [6]. There are several mechanisms involved in *Trichoderma* antagonism namely antibiosis; competition for nutrients; and mycoparasitism whereby *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as Cellulase, Chitinase, β-1, 3 glucanase and protease.

The objective of this study was to isolate and screen for the potential *Trichoderma sp.,* capable of inhibiting the growth of *R. solanacearum* and screening of enzymes such as Protease, β-1,3-glucanases, Cellulase, Chitinase, Xylanase, Amylase, and lipase in the presence of corresponding substrates.

Materials and Methods

Isolation and Identification of *Ralstonia solanacearum*:

Collected tomato plant materials were surface sterilized with 1% Sodium Hypochlorite (NaOCl) solution for 1 to 2 min, followed by three repeated washings with distilled water and blot dried. Then the plant sections (0.5–1 cm) were plated onto 2, 3, 5 triphenyl tetrazolium chloride (Kelman's TZC agar) medium (glucose 10 g, peptone 10 g, casein hydrolysate 1 g, agar 18 g, distilled water 1000 ml, 5 ml of TZC solution filter sterilized was added to the autoclaved medium to give final concentration of 0.005%) [7]. The plates were incubated at 28 ± 2°C for 24–48 h. Isolation from rhizosphere soil samples was done by dilution plate technique on TZC medium. The plates were incubated at 28 ± 2°C for 24–48 h. The strains were subjected to different biochemical, physiological, hypersensitive and pathogenicity tests for confirmation of the identity of the pathogen [8, 9].

Isolation and Identification of *Trichoderma* spp.:

Rhizosphere soil samples of healthy tomato plants were collected from different agro climatic regions of Karnataka in India. *Trichoderma* spp, were isolated using the soil dilution plate method. Morphological and microscopic studies in slide culture were adopted for identification of *Trichoderma* spp. technique was used by examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *Trichoderma* spp. The *Trichoderma* spp. sent to National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, further confirmation. Two strains of highly antagonistic *Trichoderma* spp (T4 and T8) were further characterized by molecular identification based on the ITS region sequencing. NCBI-BLAST search was performed and the top 12 hit sequences were multiple aligned and Neighbor-Joining (NJ) bootstrap, phylogenetic tree was constructed using CLUSTAL X2 2.1 (Windows version) software.

Screening of *Trichoderma* isolates against *Ralstonia solanacearum*:

Antagonistic activity of *Trichoderma* spp. was tested against ten highly virulent strains of *R. solanacearum* by *in vitro* techniques using Tryptic Soy Agar (TSA). 100µl cell free supernatants from one week old culture broths of *Trichoderma* spp. grown in Potato Dextrose Broth (PDB) were tested by agar well diffusion method [10]. Following incubation, the zone of inhibition was observed. There were four replicates for each treatment. *T. asperellum* exhibited highest anatagonistic activity inhibiting all the test strains of *R. solanacearum* compared to the other *Trichoderma* spp. and selected *T. asperellum* for further studies.

Assay of enzyme activity:

Enzyme assay of *T. asperellum* was carried out as plate assay on the respective solid media for screening of extracellular enzymes.

a) Cellulase assay:

For Cellulase assay, the *T. asperellum* strains were grown on yeast extract peptone agar medium supplemented with 0.5% carboxymethyl cellulose (CMC). After incubation, the plates were flooded with 0.2% aqueous Congo red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the Cellulase activity [11].

b) β- 1, 3 Glucanase assay:

For screening of ß-1,3-glucanase activity, carboxymethyl cellulose agar (CMC agar) medium amended with laminarin was used according to El-Ketatny et al. 2001 [12]. A culture of *T. asperellum* disc was placed at the center of the plate. Plates were incubated at 25°C for three days. β-1,3-glucanases activity on the plates were observed by flooding with 0.1% congo red dye for 15 to 20 min followed by destaining with 1 N NaCl and

then with 1 N NaOH for 15 min. The destaining was repeated twice. β-1, 3-glucanase activity was recorded by measuring the clear zone around the colonies.

c) Protease assay:

Protease activity of *T. asperellum* isolate was determined according to the modified method of Berg et al 2002 [13]. Skim milk agar medium (51.5g/lt) was used for detection of protease activity. Culture disc from 5-6 days old *Trichoderma* cultures were inoculated on skim milk agar medium and incubated at 28°C ± 2°C for three to four days. Positive *T. asperellum* strain gave a clear zone indicating the production of protease enzyme.

d) Chitinase assay:

20 g agar was sterilized in 1 litre of distilled water at 120°C for 15 minutes, then 1 g/lt colloidal chitin, prepared according to the method of Hsu and Lockwood, 1975 [14], was added to the medium. Plates were inoculated with mycelial discs and degradation zone diameter was measured following incubation at 28°C for 2-3 days.

e) Pectinase test:

Hankin's media was used for the screening of pectinase activity. From the actively growing edge of the *T. asperellum* colonies mycelial disks were inoculated onto the medium and pectinase activity was observed after 2 days of incubation at 28°C by flooding the plates with 5 ml of 1% (10 g/lt in distilled water) hexadecyltrimethylammonium bromide (C-TAB), previously sterilized at 120°C for 20 minutes**.** After 2 hours of incubation the reagent was discarded and the diameter of the colony and the degradation halo was measured [15].

f) Xylanase assay:

The *T. asperellum* was cultured on xylan agar medium (xylan 1g, rice bran 5g, yeast extract 1g, agar 16g, in 1000 ml distilled water). After incubation, dilute iodine solution was used to stain the agar plates and a yellow-opaque area around colonies indicated the xylan degradation while the reddish purple color indicated for the undegraded xylan. Xylan utilization on the medium was determined by measuring the clear zone [16].

g) Amylase activity assay:

Amylase activity was assessed by growing the selected strains on glucose yeast extract peptone (GYP) agar medium (glucose 1g, yeast extract 0.1g, peptone 0.5g, agar 16g, in 1000 ml of distilled water) with 2% soluble starch. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was considered positive for amylase activity [11].

h) Lipase assay:

The medium consisted of peptone 10g , NaCl 5g , $CaCl_2$ x 2 H_2O 0.1g , agar 20g in 1000ml of distilled water, pH 6.10 previously sterilized Tween 20 was added to the medium after sterilization. After inoculation, plates were incubated at 28°C for 3 days. The diameter of the colony and the degradation zone was measured [15].

Effect of *Trichoderma asperellum* on tomato seed germination and seedling vigor index:

The effect of *T. asperellum* strain on seed germination and vigor index of seedlings was evaluated under laboratory conditions. Bacterial wilt susceptible tomato cultivar (Arka Megali) was procured from IIHR Bangalore, India. The germination tests were carried out according to the paper towel method [17]. After 10 days of growth, germination percentage, root length, shoot length, fresh weight and dry weight were measured and vigor index was calculated by using the formula VI = (mean root length + mean shoot length) × Germination percentage [18].

Green House Studies

a) Preparation of bacterial inoculums:

Inoculum of the pathogen was prepared by culturing it in Casamino acid Peptone Glucose (CPG) broth (1 g of Casamino acids, 10 g of peptone, 5g of glucose in 1000 ml of distilled water) [7]. Cultures were centrifuged at 12000g for 10 min at 10°C. The pellet was resuspended in distilled water and was adjusted spectrophotometrically to 10^8 CFU (colony forming unit) /ml.

b) Evaluation of *T. asperellum* isolates against *R. solanacearum*:

Suppression of bacterial wilt of tomato and enhancement of plant growth under green house conditions was evaluated using

T. asperellum isolates. Potting soil (containing soil, sand and farmyard manure at 2:1:1 proportion) was autoclave-sterilized for 1h on two consecutive days and was filled into pots (20cm diameter). Five seedlings per pot were used. 200ml of Potato Dextrose broth (PDB) was prepared, inoculated with a loop full of spore suspension of *T. asperellum.* It was incubated at room temperature on a rotary shaker for 7 days. Twenty days old tomato seedlings were root dipped in *T. asperellum* suspension having 3.7×10^8 spores /ml for half an hour and planted in the respective pots.

After one week, the treated seedlings were challenge inoculated with the highly virulent *R. solanacearum* suspension of 25ml per pot. The percent disease incidence was recorded and thirty-day-old-seedlings were sampled to analyze the shoot length, root length, fresh weight, and dry weight. Untreated healthy plants were used as control.

Percent disease:

$$\text{Incidence} = \frac{\text{Total no. of plants}}{\text{Number of diseased plants}} \times 100$$

Statistical analysis:

Data were analyzed using SPSS for windows (SPSS Inc.) by means of a univariate and multivariate ANOVA and subsequently differences between treatments were determined using least significant differences (LSD at α 0.05).

Results

Isolation and identification of *Ralstonia solanacearum*:

After incubation pink centers with white fluid colonies were selected and a total of 50 strains of *R. solanacearum* were isolated and identified (Fig.1). Microscopic studies revealed that bacterial isolates were Gram negative, rod shaped, non spore forming, strictly aerobic bacteria and it was confirmed by standard biochemical tests. Pathogenicity was confirmed by the development of wilt symptoms on tomato plants after 7 days of inoculation followed by reisolation and identification of the causal organism from diseased plants. The pathogen was tolerant to sodium chloride concentrations up to 2% beyond which growth was inhibited. All strains were grown at 37°C and failed at 40°C.

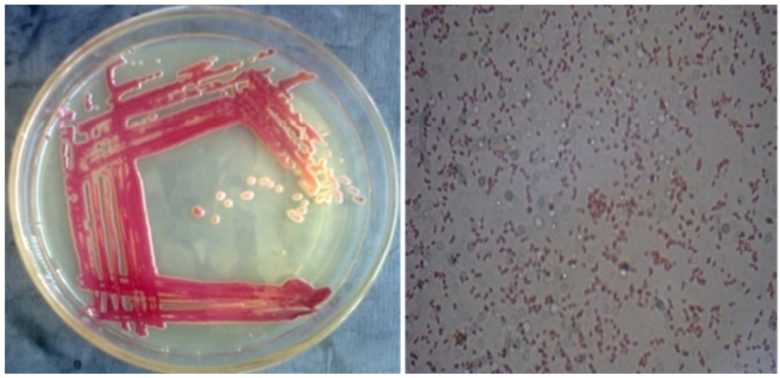

Figure.1: Pink centred virulent colonies of *R. solanacearum* on TZC agar medium and Microscopic view

Motility of each isolate was confirmed by performing hanging drop method. 10 highly virulent *R. solanacearum* (Rs1-Rs10) strains were selected to further studies based on pathogenicity assay.

Isolation and identification of *Trichoderma isolates*:

Total 10 isolates of *Trichoderma* spp. were isolated from soil dilution plates. Based on morphological characteristics, the isolates were identified as *Trichoderma koningii* (T1) *T. flavofuscum* (T2), *T. harzianum* (T3), *T. asperellum* (T4), *T. harzianum* (T5), *T. koningii* (T6), *T. harzianum* (T7), *T. asperellum* (T8), *T. harzianum* (T9), *T. viride* (T10) and was confirmed by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India. The BLAST search of the ITS sequence and the multiple alignment showed 97% similarity to *T. asperellum* strains indicating T4 and T8 to be *T. asperellum* (Fig. 3 & 4) and phylogenetic tree was constructed on the basis of a neighbor-Joining (NJ) analysis with 1,000 bootstrap replications.

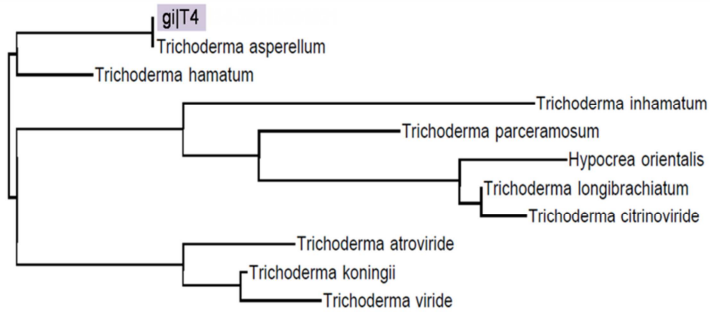

Figure.3: Phylogenetic relationships of *Trichoderma asperellum* (T4) isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of ITS sequences. Sequences used for this comparison were obtained GenBank.

Screening of *Trichoderma* spp. against *Ralstonia solanacearum:*

Ten isolates of *Trichoderma* spp. were selected for antagonistic studies. Among the ten strains, *T. asperellum* (T4 and T8) (Fig. 2) exhibited highest antagonistic activity inhibiting all the test strains of *R. solanacearum* (Fig. 5). The zone of inhibition was 24-29mm and 20-27mm respectively (Table. 1).

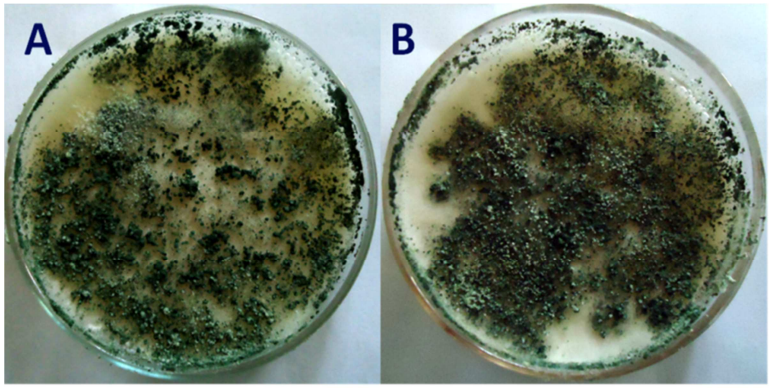

Figure.2: *Trichoderma asperellum* on PDA agar medium. A: T4 and B: T8.

Assay of enzyme activity:

Trichoderma asperellum was evaluated for the production of different enzymes. Initially screening was carried out by plate assay measuring the zone of clearance (Fig. 6). T4 strain exhibited highest enzyme activity for Protease, β-1,3-Glucanase, Cellulase, Lipase, Amylase, xylanase, and Pectinase as compared to T8 strain whereas both T4 and T8 strains exhibited same level of activity for chitinase enzyme (Fig.7).

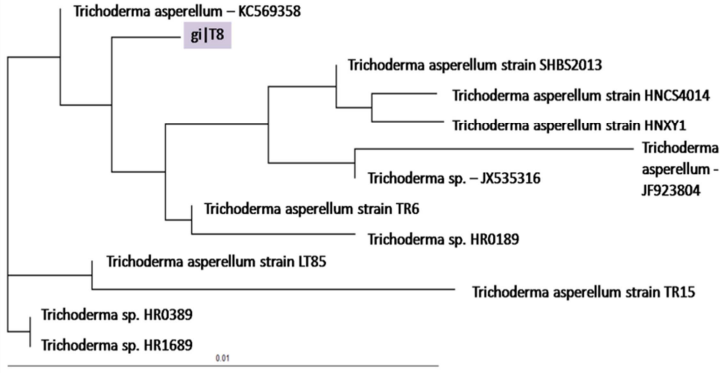

Figure.4: Phylogenetic relationships of *Trichoderma asperellum* (T8) isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of ITS sequences. Sequences used for this comparison were obtained from GenBank.

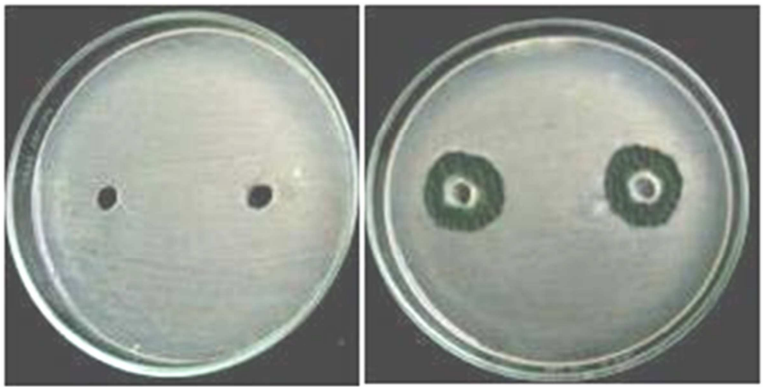

Figure.5: Screening of *Trichoderma* isolates against *Ralstonia solanacearum.*

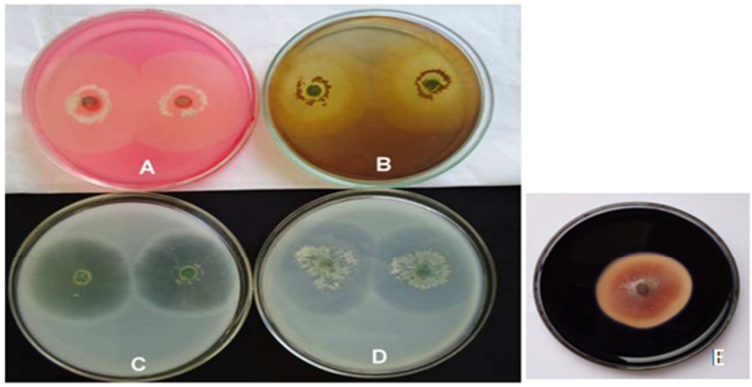

Figure.6: Plate Assay of enzyme activity A) Cellulase, B) Xylanase, C) Pectinase, D) Protease, E) Amylase

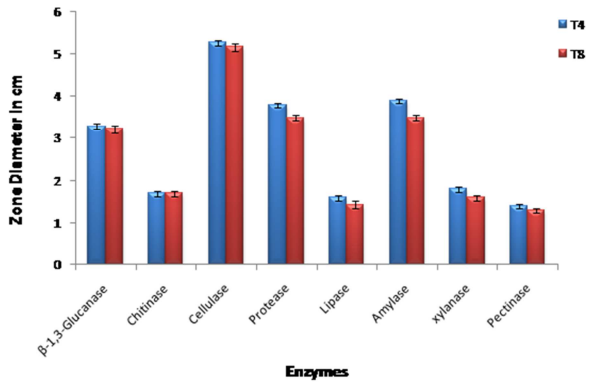

Figure.7: Activity of enzymes from selected *T. asperellum* isolates. Each bar represents the average of three independent measurements.

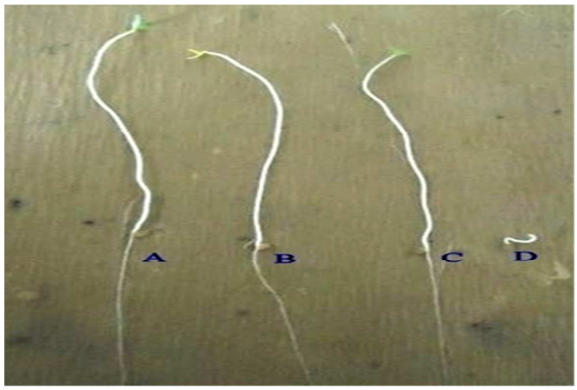

Figure.8: Effect of *T. asperellum* on tomato seed germination and seedling vigor index. Seed germination of tomato seeds A & B: *T. asperellum* treated**,** C: control respectively and D: Pathogen, treatments

Figure.9: Root growth of tomato plants A, B and C: Pathogen treated Control and *Trichoderma asperellum* treated respectively.

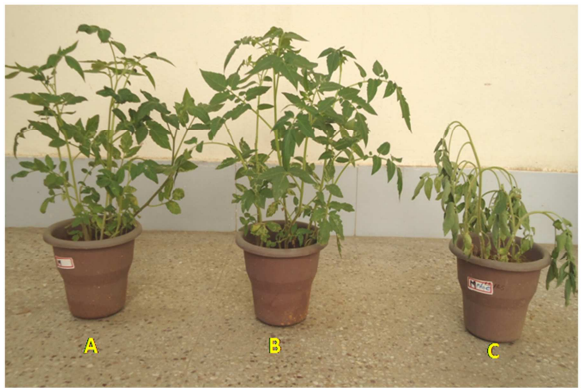

Figure.10: Study of Plant growth promoting effect of *Trichoderma asperellum* for tomato plant compare with control, control (A), seed treatment with *Trichoderma asperellum* (B) only *R. solanacearum* treated (C). Plants were harvested after 30 days old, challenge inoculated seedlings growth.

Table. 1: Screening of *Trichoderma* spp. against *Ralstonia solanacearum,* Zone of inhibition in mm.

SL. No.	Rs 1	Rs 2	Rs 3	Rs 4	Rs 5	Rs 6	Rs 7	Rs 8	Rs 9	Rs 10	Mean
T. koningii	18.00^{d}	15.66^{a}	18.00^{b}	17.66^{bc}	19.00^{cd}	13.33^{a}	14.66^{ab}	11.33^{a}	14.66^{a}	13.0^{a}	15.33
T. flavofuscum	15.00b^{cd}	13.66^{a}	11.00^{a}	12.66^{ab}	15.00^{abc}	11.33^{a}	15.66^{b}	12.66^{ab}	13.66^{a}	13.33^{a}	13.21
T. harzianum	10.00^{a}	13.33^{a}	14.00^{ab}	14.00^{ab}	11.66^{a}	14.66^{a}	16.0^{b}	13.33^{ab}	15.0^{a}	12.33^{a}	13.43
T. asperellum	25.33^{e}	24.00^{b}	27.00^{c}	26.00^{d}	24.00^{e}	26.0^{b}	26.66^{c}	27.33^{c}	26.33^{b}	25.33^{b}	25.79
T. harzianum	11.00^{ab}	12.33^{a}	14.33^{ab}	14.00^{ab}	12.00^{a}	12.33^{a}	13.33^{ab}	15.0^{b}	14.0^{a}	12.66^{a}	13.09
T. koningii	13.66^{abc}	16.00^{a}	15.66^{ab}	15.66^{ab}	17.00^{bc}	13.0^{a}	14.66^{ab}	15.33^{b}	15.0^{a}	13.33^{a}	14.93
T. harzianum	14.00^{a}^{bcd}	14.66^{a}	15.66^{ab}	13.33^{ab}	13.00^{ab}	11.33^{a}	10.33^{a}	14.33^{ab}	13.33^{a}	15.33^{a}	13.53
T. asperellum	23.00^{e}	21.33^{b}	24.33^{c}	22.66^{cd}	22.33^{de}	25.66^{b}	23.0^{c}	27.33^{c}	22.66^{b}	23.0^{b}	23.53
T. harzianum	15.66^{cd}	16.00^{a}	15.66^{ab}	15.33^{ab}	15.00^{abc}	11.66^{a}	13.0^{ab}	11.1^{a}	12.33^{b}	14.0^{a}	13.97
T. viride	13.00^{abc}	13.00^{a}	13.00^{a}	11.33^{a}	12.00^{a}	12.66^{a}	14.0^{ab}	13.0^{ab}	12.0^{a}	12.33^{a}	12.63

Rs- *Ralstonia solanacearum*, Scheffe post hoc test: Means sharing different alphabetical (a, b, c, d, e) superscripts in a column are significantly different (P<0.05).

Table.2: Effect of *Trichoderma asperellum* on tomato seed germination and seedling vigor index

Treatments	Germination (%)	Shoot Length	Root Length	Fresh Weight	Dry Weight	Vigor Index
Control	87.66^{c}	3.58^{b}	5.44^{b}	0.98^{b}	0.217^{ab}	791.08^{b}
Rs	33.0^{a}	2.99^{a}	3.35^{a}	0.44^{a}	0.12^{a}	209.29^{a}
T4	92.^{d}	5.76^{d}	8.55^{d}	1.25^{c}	0.36^{b}	1321.34^{d}
T8	90.00^{cd}	5.63^{d}	8.36^{cd}	1.267^{c}	0.27^{ab}	1259.18^{d}
T4 + Rs	81.33^{b}	5.15^{c}	7.60^{c}	1.17^{bc}	0.26^{ab}	1037.39^{c}
T8+Rs	78.33^{b}	5.03^{c}	7.75^{cd}	1.09^{bc}	0.22^{ab}	1001.32^{c}

Rs- *Ralstonia solanacearum*, T4 and T8- *Trichoderma asperellum* strains MRL - Mean Root Length; MSL - Mean Shoot Length; VI - Vigour index. Scheffe post hoc test: Means sharing different alphabetical (a, b, c) superscripts in a column significantly different (P<0.05).

Table.3: Plants growth promotion studied under green house conditions were done using 30-day-old–seedlings grown from *Trichoderma asperellum* treated seeds.

Treatment	Plant height	Shoot length	Root Length	Fresh weight	Dry weight	Disease Incidence (%)
Control	15.33^{d}	12.76^{c}	8.30^{c}	18.73^{c}	4.93^{c}	0.0000^{a}
Rs	8.23^{a}	7.90^{a}	4.33^{a}	9.16^{a}	2.43^{a}	86.83^{c}
T4	16.53^{c}	13.30^{c}	9.10^{c}	19.76^{c}	5.53^{d}	0.00^{a}
T8	15.66^{c}	12.83^{c}	8.83^{c}	18.70^{c}	5.26^{c}	0.00^{a}
T4 + Rs	13.30^{b}	10.30^{b}	6.76^{b}	15.53^{b}	4.06^{b}	35.33^{b}
T8+Rs	12.86^{b}	10.00^{b}	6.50^{b}	15.23^{b}	3.93^{b}	38.0^{b}

Distilled water treated seeds served as control. Scheffe post hoc test: Means sharing different alphabetical (a, b, c) superscripts in a column significantly different (P<0.05).

Effect of *Trichoderma asperellum* on tomato seed germination and seedling vigor index:

There was an improvement in seed germination (increased by 48% and 45%) and seedling vigour (increased by 828 and 792 VI) upon T4 and T8 seed treatment whereas *R. solanacearum* inoculated tomato seeds showed reduction in seed germination (Fig. 8). The maximum germination was recorded in T4 and T8 treated seeds (92% and 90%) respectively. (Table.2). The *T. asperellum* seed treatment enhanced the vigour index of tomato seedlings when compared to control.

Evaluation of *T. asperellum* isolates in greenhouse:

The efficacy of *T. asperellum* for the control of *R. solanacearum* wilt in tomato plants was evaluated under greenhouse conditions. *T. asperellum* significantly increased plant growth promotion compared to the control under greenhouse conditions. Analysis of plant height, fresh weight and dry weight of 30 days old, challenge inoculated seedlings revealed that *T. asperellum* treated seedlings showed increased growth over control. However, the plant growth characteristics significantly differed in response to T4 and T8. The Fresh weight, shoot length, root length, dry weight, root growth and disease incidence were tabulated (Fig. 9 & 10) and (Table. 3).

Discussion and Conclusion

Among the fungal Biocontrol agents, the genus *Trichoderma* was one of the most commonly used organisms for the control of soil borne pathogens. The antagonistic fungi, *Trichoderma* spp. are able to control various soil-borne plant diseases. It affects plant pathogens with different mechanisms such as competition, antibiosis and parasitism. Biological control, the use of specific microorganisms that interfere with plant pathogens as nature-friendly, ecological approach to overcome problems caused by the standard chemical methods of plant protection. Novel biocontrol agents have therefore been looked for, and *Trichoderma* isolates have been the preferred choice [19].

The aim of the current study was isolation, screening and selection of the potential indigenous *Trichoderma asperellum* strains to be utilized as a potential antagonist against the *R. solanacearum* associated with bacterial wilt of tomato.

Present study, we assessed the potential of two *T. asperellum* isolates were used as biological agents to control *R. solanacearum.* The results indicated that these antagonistic fungi were effective against the tomato wilt pathogen. Different enzymatic activities such as amylase, lipase, protease, pectinase, chitinase, cellulase, xylanase and β-1,3-glucanases of the

selected *T. asperellum* isolates were determined *in vitro* condition. *Trichoderma* directly attacks the plant pathogen by excreting the lytic enzymes such as chitinase, β-1, 3-glucanases and proteases.

Our present result exhibited correlation to other works that the *Trichoderma* spp. can induce resistance in tomato. Benitez *et al.* 2004 [20] demonstrated that *Trichoderma* strains that over produce chitinases have been shown to be effective biocontrol agents against various pathogens. Simmoms, 1994 [21] described that glucanases are among the plant defense responses to pathogen attack. *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1,3 glucanases and proteases. Because of the skeleton of pathogenic cell walls contains chitin, glucan and proteins, enzymes that hydrolyze these components have to be present in a successful antagonist in order to play a significant role in cell wall lysis of the pathogen [22]. Filamentous fungal cell wall also contains lipids and proteins. Therefore, it was expected that antagonistic fungi synthesized proteases which may act on the host cell-wall. The application of the *T. asperellum* isolates in our study against *R. solanacearum*, in tomato showed reduction in the disease symptoms. *Trichoderma* sp. are found to decrease wilt incidence in chickpea plants and increase root development in numerous other plants [5].

From the current study, it can be postulated that there is maximum disease reduction in the application of *T. asperellum* against the wilt disease of tomato. Therefore, *T. asperellum* isolates can be an excellent candidate as biocontrol agent against *R. solanacearum*, with the aim of reducing the use of chemical pesticides. Our results agree with numerous studies on the beneficial impact of *T. asperellum in vivo* [23]. The *in vivo* tests showed that the two *T. asperellum* strains reduced severity of tomato wilt. Indeed, pretreatment of the tomato seedlings with pure cultures of these two *T. asperellum* strains reduced infection by more than 50%, confirming the *in vitro* results where these strains were found to be the most efficient. It can be concluded that, this study signifies the antagonistic effect of *T. asperellum* against *R. solanacearum c*ausative agent for tomato wilt. However, it will be necessary to undertake field trials in order to determine the ability of these strains to protect tomato plants against the bacterial wilt disease under natural conditions.

Trichoderma asperellum has shown a significant antagonistic activity against bacterial wilt pathogen *Ralstonia solanacearum* both laboratory and under green house conditions. Thus from the results obtained *Trichoderma asperellum* can be considered as a potential biocontrol agent to control the wilt of tomato plants caused by *R. solanacearum*. Further studies on Induced Systemic Resistance (ISR), formulations and field trials are required to establish *T. asperellum* as successful biocontrol agent.

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