Online Available at: https://www.sciplatform.com/index.php/wjb/article/view/1183

ISSN (Online) = 2522-6754 ISSN (Print) = 2522-6746

WORLD JOURNAL OF BIOLOGY AND BIOTECHNOLOGY

Research Manuscript www.sciplatform.com

Molecular characterization and induced systemic resistance of *Trichoderma asperellum* against bacterial wilt of tomato

[a] Tanjid Ahmed, [a] Md. Ratan Mia, [a] Tawseef Al Aff, [a] Sadia Afrin Shila, [a] Md. Sohel Tanvir, [a] Subarna Yasmin Sumi, [b] Sadia Afrin Shupta, [a] Mohammad Delwar Hossain*

[a] Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh. Bangladesh,
[b] Plant Physiology Division. Bangladesh Rice Research Institute (BRRI),

Authors' Contribution: **Ahmed, T., M. R. Mia, S. A. Shila, M. S. Tanvir, S. M. Sumi, S. A. Shupta, M. D. Hossain** conducted the research.

*Corresponding Author's Email Address: delwarmhossain@bau.edu.bd

Review Process: Peer review

ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating disease of tomato in Bangladesh and no significant effective control measure is available here till date. In this research work, Trichoderma from tomato rhizosphere is being utilized formulated with farm yard manure (FYM) to combat this pathogen attack. The pathogen was isolated from the infected tomato fields and characterised where *R. solancearum* isolate RsBAU1 was found most virulent, resulted 288 bp amplicon size with the species specific primers. Among 10 Trichoderma isolates, TR3 isolates (OM 480709) showed highest inhibition *in vitro* against *R. solancearum* isolate RsBAU1 that was resulted about 633 bp amplicon size with ITS primers, showed 99-100% blast homology with the other *T. asperellum* in phylogenic tree. *T. asperellum* (OM 480709) was formulated with FYM which significantly reduced and delayed Ralstonia wilt incidence and increased tomato fruit yield including other agronomic characters under both net house and field conditions. This formulated Trichoderma (FT) decreased the Ralstonia wilt disease incidence by 51.5% and increased 49.73% tomato yield when compared with the pathogen-treated plot during 2022-2023. Again, FT treated plants significantly increased peroxidase (POX), phenylalanine ammonium lyase (PAL), polyphenol oxidase (PPO), β-1,3-glucanase and total phenol activities in compared to non-treated plants and these activities were increased up to 8th days when challenged with *R. solancearum* (RsBAU1) and then declined. This indicates *T. asperellum* (OM480709) might have collectively contributed to induced resistance in tomato plants against *R. solanacearum* (RsBAU1 isolate). Therefore, *T. asperellum* (OM480709) formulated in FYM can be a sustainable and eco-friendly management strategy for controlling *Ralstonia* wilt of tomato in Bangladesh.

Keywords: *Trichoderma asperellum*, FYM, ralstonia wilt, induced systemic resistance (isr) and tomato.

INTRODUCTION: Tomato (*Lycopersicon esculentum*) is an important vegetable of the world as it has high nutritive value and diversified use (Bose and Som, 1985). Bacterial wilt caused by *Ralstonia solanacearum* is a destructive and prevalent soilborne disease that limits tomato production in the world (Ramesh *et al.*, 2014). This is also one of the major bacterial disease of tomato affecting its growth and destruction of yield in tomato ranged from 10.83% to 90.60% in Bangladesh (Khalequzzaman *et al.*, 2002). This bacterium persists in soils and water for several years to form latent infections in the crops and its eradication is difficult (Avinash et al., 2015). Farmers are using Resistant varieties, cultural practices and chemical pesticides those were found ineffectual to control this disease. Again, there are various bioagents viz. *Pseudomonas putida*, *P. fluorescens, Trichoderma* spp., Bacteriophages, *Streptomyces* spp., *Bacillus* spp. and *Paenibacillus macerans* have been implemented for inhibition of Ralstonia under laboratory conditions (Ling *et al.*, 2010). *Trichoderma* induces PR gene coding pathogenesis-related proteins (PR protein) such as: chitinase, peroxidase (PO), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) etc. and release secondary metabolites which were expressed by plant to defense pathogen infection (Keswani *et al.*, 2016). Several researchers found a decrease in the R. solanacearum population in soil amended with decomposed organic fertilizer/manure which provides nutrients to the microbes, thus increases the biocontrol agent's ability and makes them extra competitive in the rhizosphere soil and on roots (Liu *et al.*, 2013). *Trichoderma* spp. are now the greatest common fungal biocontrol agents that have been broadly studied and deployed throughout the world (Patil and Prajapati, 2017). *T. asperellum* was reported as very effective to control *Ralstonia* wilt in tomato when they are co-inoculated with Farm Yard Manure (FYM) (Konappa *et al.*, 2018). Bangladesh soil has many isolates *T. asperellum.* However, the induced systemic resistant property and decomposition ability of Farm yard manure by *T. asperellum* and effects of such decomposed FYM against *Ralstonia* wilt are still not carried out.

OBJECTIVES: This research work was designed to investigate potential *T. asperellum* responses in terms of defense enzymes against *Ralstonia* and to evaluate the effectiveness of *T. asperellum* decomposed FYM for suppression of Ralstonia wilt of tomato under field conditions.

MATERIAL AND METHODS: Isolation and identification of *Trichoderma* spp.: Rhizosphere soil samples were collected from healthy tomato plants. *Trichoderma* spp. were isolated using the soil dilution plate method and cultured on PDA medium (Monirul *et al.*, 2016). Morpho-molecular and microscopic studies (colony color, shape, size, arrangement and development of conidiophores and conidia or phialides) were confirmed as described by Monirul *et al.* (2016).

Isolation and identification of *R. solanacearum*: Infected plants were collected from the tomato growing fields of Mymensingh and surface sterilized with 1% sodium hypochlorite (NaOCl) solution for 2 min., followed by washings 3 times with distilled water and blot dried. The plant sections (0.5-1 cm) were placed onto a selective TTC (Triphenyl tetrazolium chloride) medium (glucose 10 g, peptone 10 g, casein hydrolysate 1 g, agar 18 g, distilled water 1000 mL, 5 mL of TTC solution filter sterilized was added to the autoclaved medium to give final concentration of 0.005%) (Murthy *et al.*, 2013). The plates were incubated at 28 ± 2°C for 24-48 h. Ten virulent *R. solanacearum* strains were isolated from infected tomato samples preserved in 10% skim milk kept at -20 ˚C refrigerator for further studies. For identification of *R. solanacearum*, gram staining and kovac oxidase tests were conducted (Hossain *et al.*, 2021). Pathogenicity/hypersensitivity reaction test, race and biovar tests were carried out (Popoola *et al.*, 2015)

Isolation and identification of *R. solanacearum*: Diseased tomato stem samples were washed with tap water, and surface sterilized with 70% ethanol for 2 min. and rinsed repeatedly in sterile water for 5 min. The samples were then suspended in the 5 mL sterile distilled water for 10 min. to make them turbid due to oozing of bacterial cells from cut ends of diseased tissue. The bacterial suspensions were prepared to appropriate dilutions from which, 1 mL of the bacterial suspension was spread onto the surface of solidified Triphenyl tetrazolium chloride agar (TTC) medium and incubated at 28±2°C for 48 h. (Alelign, 2020).

Streaming and potassium hydroxide (KOH) solubility test: To diagnose the presence of *R. solanacearum* in wilted tomato plants, stems of infected tomato were cut above the soil level and the cut surfaces were suspended in test tube containing Distilled water. A drop of KOH (3% aq., w/v) was placed on a microscope slide using a Pasteur pipette. A loop full Bacteria were mixed into KOH solution and slime threads were recorded (Chaudhry and Rashid, 2011).

Biovar determination: Ten ml each of 10% sugar (lactose, maltose, cellobiose, mannitol, sorbitol and dulcitol) was added in sterilized screw-capped test tubes and then heated at 100°C for 30 min. to sterilize these solutions. Two drops of *R. solanacearum* suspensions, prepared from 48h old cultures of individual isolates were added to each tube of sugar+ TTC medium. The tubes were incubated at 30°C and examined after 24 h., 48 h., 72 h. to one week for the presence of indicator change from olivaceous green to orange colour on the surface of medium (Chaudhry and Rashid, 2011).

Race determination and hypersensitive reaction (HR) test : For race determination, hypersensitive reaction (HR) test was done on tobacco leaf using the leaf infiltration technique (Lozano and

Sequeira, 1970). Sterile water served as a negative control. Leaf reactions were recorded from 24 h to 3wk (Horita and Tsuchiya, 2001).

Screening of *Trichoderma* isolates against virulent *R. solanacearum* isolate RSBAU1: After HR test, the most virulent *R. solanacearum* isolate (RsBAU1) was selected for challenging antagonistic Trichoderma isolates by in vitro techniques using Tryptic Soy Agar (TSA). About 100 µL cell free supernatants from one week old culture broths of *Trichoderma spp.* was grown in Potato Dextrose Broth (PDB) were tested by agar well diffusion method (Murthy *et al.*, 2013). Following incubation, the inhibition zone was recorded. *Trichoderma* isolate that showed the highest inhibition zone / antagonistic activity inhibiting the virulent strains of *R. solanacearum* (RsBAU1) compared to the other *Trichoderma* isolates selected for molecular characterization. The virulent strains of *R. solanacearum* (RsBAU1) was also identified by molecular markers.

Genomic DNA preparation : Genomic DNA of *T. asperellum* (isolate TR3) and *R. solanacearum* (isolate RsBAU1was extracted by using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, US) and DNA concentration (ng/µL) was determined following the formula of Monirul *et al.* (2016).

PCR detection of *T. asperellum* Isolate TR3 : For sequence, *T. asperellum* DNA was amplified with ITS4 (5′ TCC TCC GCT TAT TGA TAT GC- 3′) and ITS5 (5′ GGA AGT AAA AGT CGT AAC AAG G 3′). PCR mixture and thermal conditions were followed as described by Monirul *et al.* (2016).

PCR detection of *R. solanacearum* isolate RsBAU1: The virulent *R. solanacearum* isolate RsBAU1 was characterised based on using two 16S rDNA universal primers 27F (5′ AGA GTT TGA TCM TGG CTC AG 3′); 1518R (5′ AAG GAG GTG ATC CAN CCR CA 3′) (Giovannoni *et al.*, 1991) and specific primers Y2 (5′ CCC ACT GCT GCC TCC CGT AGG AGT 3′) and OLI1 (5′ GGG GGT AGC TTG CTA CCT GCC 3′) (Singh et al., 2018). The 16S rDNA was amplified through polymerase chain reaction (Bio-Rad). PCR reaction mixture was prepared (Hossain *et al.*, 2021). The amplification was performed by pre-heating at 95 °C for 5 min. followed by 35 cycles at 94 °C, 55°C and 72 °C for 1 min. The final extension time was 7 min at 72 °C. For species specific primer amplification.

Gel electrophoresis, documentation, sequencing and Phylogenetic analysis: The PCR products was analyzed by gel electrophoresis using 1.2% agarose in 1X TBE buffer containing ethidium bromide (0.5 µg/mL). After electrophoresis, gel was placed under UV transilluminator using Gel Documentation System for visualization of DNA bands. The DNA sequence of potential Trichoderma (TR3) was compared with other Trichoderma available in the NCBI database using Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted following the methods of MEGA Version 5.22.

Preparation of bacterial inoculum : Stock suspension of *R. solanacearum* (1mL) was added to casamino acid peptone glucose (CPG) broth (1-g casamino acid, 10-g peptone, 5-g glucose per liter) and incubated at 28 °C for 48 h on rotary shaker at 150 rpm (Kelman, 1954). Culture broth was centrifuged at 12,000 rpm for 10 min at 10 °C. The bacterial pellet was resuspended in sterile distilled water and final concentration of suspension was set to 1×10^8 cfu/ml, by spectro-photometrically adjusting to O.D 600 nm = 0.1 . About 1 mL suspensiob (1×10^8 cfu) of *R. solanacearum* stock suspension was prepared (Konappa *et al.*, 2018).

Preparation of *Trichoderma* inocula with FYM (Formulated Trichoderma, FT): The suspension of potential *T. asperellum* (TR3 isolate) was prepared from 7day-old culture on potato dextrose agar (PDA), using sterile distilled water. The fungal concentration (5×10^8 spores/mL) was mixed with 45 kg of farmyard manure (FYM) (3 layers) and incubated for 45 days before applying to each plot (Jahangir *et al.*, 2021).

Net house/pot experiment: The pot experiment was conducted at BAU, Mymensingh (Sain and Pandey, 2016). Here, suppression of bacterial wilt of tomato and enhancement of plant growth under net house conditions were evaluated using *T. asperellum* isolates TR3. Four-week-old tomato seedlings (BARI 16) were uprooted and transplanted to experimental pots. Soil was sterilized and fertilized with recommended doses of NPK. The treatments were as follows: T1= FT (FYM +*T. asperellum*), T2 = FT+ R.s (*R. solanacearum*), T3 = R. *solanacearum* alone, and T4= Control (untreated seedling). Three replications were maintained for each treatment with 10 plants. After 1 weeks of seedling transplantation, pot soil was inoculated by 50 ml 48h-old *R. solanacearum* suspension @10^8 spores/mL for each pot. Disease incidence and plant growth promotion was recorded (Murthy *et al.*, 2013). Data was recorded on the following parameters: Disease incidence (%), fresh weight (g), dry wt.(g), shoot length 9cm), and root length (cm).

Field experiment : Based on the previous in vitro and in vivo studies, under laboratory and greenhouse conditions (Murthy *et al.*, 2013), the most promising *T. asperellum* isolates TR3 was selected for trial against the virulent *R. solanacearum* (RsBAU1) under field conditions. The field experiment was conducted at, BAU farm, Mymensingh where it was naturally infested with *R. solanacearum*. Seeds of wilt susceptible tomato variety BARI 16 was procured from Horticulture Farm, BAU. Four-week-old tomato seedlings were uprooted from portrays and transplanted to experimental plots and treated with farmyard manure [(FYM + *T. asperellum* TR3) mixture] (FT) @ 20 g/seedling. The other three treatments were used as follows: Control (untreated seedlings), *R. solanacearum* alone (R.s), and FT+ R.s. The selected individual experimental plot area was 2.45 x 2.30 m^2 with 0.5 m spacing and 65 cm x 60 cm spacing between each plant and line. The field was fertilized once with NPK fertilizer and FYM@ 3.0 kg/m^2. After 2 weeks of seedling transplantation, they were challenge inoculated by 48-h-old R. solanacearum suspension, 5 mL per plant by soil drenching method (Konappa *et al.*, 2018). The field experiment used RCBD design with three replications. The soil around each seedling's base was ground up once it was established. Each growing plant was staked with a bamboo stick to keep it upright. In the plots, weeding, pruning, and watering were done as required. Buffer zones of 1 m without tomato seedlings were maintained between plots. The completely wilted tomato plants in each treatment were observed, 1 week after challenge inoculation up to 90 days. Percent disease incidence (%DI) was calculated as described below that had completely wilted. Plant height (cm), fresh weight (g), dry weight(g), No. of fruits/plant, Fruit wight / plant (g) and tomato yield (t/ha) in each treatment was recorded at this season. A total of four harvests were made at weekly intervals. The wilt incidence was evaluated when the infection emerged and calculated as the percentage of infected plants compared with the total number of growing plants in each plot. %DI = No. of wilted plants in a plot/total no. of plants in a plot × 100 (Konappa *et al.*, 2018).

Sample collection for biochemical analysis: The tomato leaf tissues of treated and untreated tomato plants were collected at different times of intervals (2, 4, 6, 8 and 10 days) after pathogen inoculation and stored in a deep freezer (−80 °C) until used for biochemical analysis (Konappa *et al.*, 2018). Leaf were homogenized by liquid nitrogen in a mortar and pestle. One gram of tomato leaf tissues was homogenized by 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C, and the homogenate was centrifuged for 20 min at 12,000 rpm. The supernatant was used as a crude extract for analyzing peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL). As for the estimation of β-1,3-glucanase, 1 g of tomato leaf tissues was homogenized by 2 ml of 0.1 M sodium citrate buffer (pH 5.0) in a pre-chilled mortar and pestle, centrifuged, and supernatant was used for the estimation (Pan *et al.*, 1991). The total phenol content was estimated as per the procedure given Zieslin and Ben-Zaken (1993). The treatments were same as described above for field experiments.

Statistical analyses: All data of field experiments will be statistically analyzed, using MSTATC program and the means will be analyzed, using Duncan's new multiple range post test at $p \leq 0.05$.

RESULTS AND DISCUSSION: Isolation and identification of *T. asperellum*: The isolated ten Trichoderma spp. produced different colony colour viz. light green, green, dark green with loose, compact or very compact colony consistency at 30^0C in pH 7.0 on PDA medium. Among the isolates, *Trichoderma* TR3 showed fast grow and sporulated 9.67×10^7 cfu/ml on PDA (table 1, figure 1). This finding is also in association with the results of Monirul *et al.* (2016). This Trichoderma TR3 isolate was selected for decomposing FYM and molecular characterization. DNA extracted from *Trichoderma* spp. TR3 isolate resulted 633 bp (Accession No. OM480709) when amplified with ITS primer pairs and showed 99-100% blast homology with the other *T. asperellum* of gene bank and 94% bootstrap value with the *T. asperellum* (OM 681185) (figure 1). This phylogenic tree revealed that *T. asperellum* TR3 isolate belongs to *Trichoderma asperellum.*.

 Digital Object Identifier: https://doi.org/10.33865/wjb.009.01.1183

Figure 1: Phylogenic tree of *T. asperellum* TR3 isolate (OM 480709) and its colony, a: phylogenic tree, b: mycelial growth of *T. asperellum* TR3 isolate on PDA medium.

Isolate Name	Colony color	Growth habit	Colony consistency	No. of colonies (1:1000)	No. of spore/mL
TR1	Green	Moderate	Loose	2.7	7.52×10^7
TR2	Light green	Slow	loose	2.1	5.72×10^7
TR3	Dark green	Fast	Very compact	8.6	9.67×10^7
TR4	Light green	Moderate	Loose	6.20	9.45×10^7
TR5	Dark green	Moderate	compact	5.8	9.37×10^7
TR6	Dark green	Fast grow	Very compact	4.8	8.82×10^7
TR7	green	Moderate	loose	2.9	6.33×10^7
TR8	green	slow	Loose	2.6	6.05×10^7
TR9	Whitish green	Fast	Compact	3.2	7.15×10^7
TR10	Whitish green	Fast	Compact	3.1	7.25×10^7

Table 1: Morphological characters of isolated *Trichoderma* spp. on PDA at 30 ^{0}C .

Isolation and identification of *R. solanacearum*: Ten virulent R. solanacearum strains were isolated from the infected tomato plants (figure 2a), observed with water streaming positive test (table 2, figure 2b) and cultured on TTC selective medium which produced pinkish creamy colony (figure 2c). The bacterial isolates subjected to KOH solubility test resulted slime thread (figure 2d) confirmed the Gram-negativity of the pathogens. Isolates also appeared rod-shaped under the microscope following simple staining procedure (figure 2e) and Kovac oxidase test gave positive reaction (figure 2f).

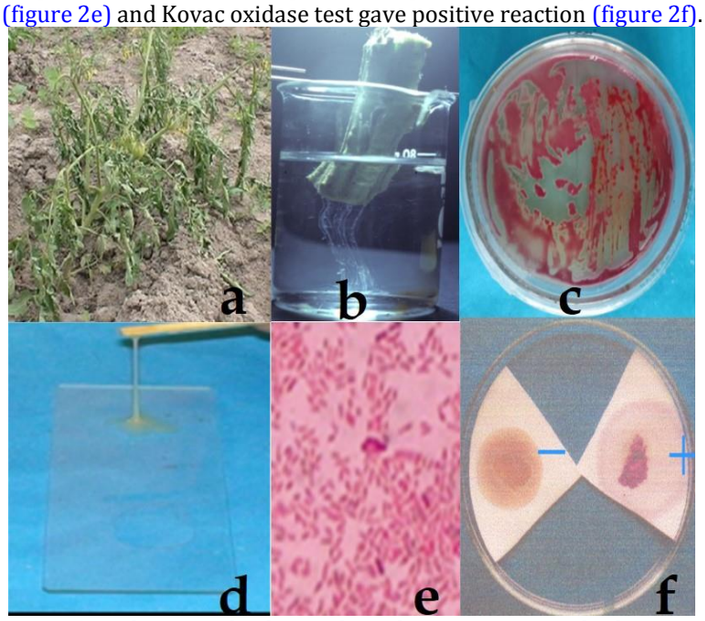

Figure 2: Isolation and culture of *R. solanacearum*. a: Wilted tomato plant, b: bacterial ooze streaming in water, c: pinkish creamy color on TTC medium, d: creamy threads on 3% KOH, e: rod shaped bacteria, and f: Kovac oxidase test.

Bacterial isolates in each location were indicated in (table 2). Based on the cultural and morphological appearance, the isolated pathogens were identified as *Ralstonia solanacearum*. Nine (9) bacterial isolates of *R. solanacearum* produced ashy/brownish symptoms in Tobacco hypersensitivity (HR) test in 24 hrs after inoculation indicating these strains belonged to Race 1. Tobacco leaves became yellow within 48 hrs when RsBAU1 isolate infiltrated suggesting it was Race 3. However, no symptoms of Race 2 strains were observed in any of the isolates (table 2, figure 3). *R. solanacearum* RsBAU1 isolate (race 3) developed wilt on tomato plant rapidly, resulting in complete wilting and death. The same pathogen was re-isolated from the inoculated plants, confirming pathogenicity of the isolates (data was not shown).

Figure 3: HR test for *R. solanacearum.* a: infiltration of *R. solanacearum* RsBAU1 isolate, b: slight localized chlorosis, and c: necrotic and yellowing of tobacco leaf.

Bacterial Isolates	Colony colour	KOH solubility test	Utilization of Sugars	Biotype	HR Test	Race
RsSK1	Pink	+	+	3	+	1
RsSK2	Pink	+	+	3	+	1
RsSK3	Pink	+	+	3	+	1
RsSK4	Pink	+	+	3	+	1
RsRSK5	Pink	+	+	3	+	1
RsRS1	Pink	+	+	3	+	1
RsRS2	Pink	+	+	3	+	1
RsBAU1	Pink	+	+	3	+++	3
RsBAU2	Pink	+	+	3	+	1
RsBAU3	Pink	+	3	3	+	1

Table 2: Determination of biotypes and races of *R. solanacearum* obtained from infected tomato plants.

Utilization of sugars /determination of biotypes: The results of the biovar test showed that 10 isolates of *R. solanacearum* oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol) and were classified as Biovar III (table 2). Bacterial wilt of Tomato caused by *R.solanacearum* (isolate RsBAU1) was a vascular infection in nature resulted water streaming that growth on TTC media. This TTC medium is used to distinguish *R. solanacearum* among other bacteria during isolation and showed the difference between avirulent colonies from the virulent. In this study, the colonies were found fluidal whitish with a pink center, indicating RsBAU1 isolate is a virulent species of *R. solanacearum* (Popoola *et al.*, 2015). The differences in reactions of tobacco leaves and susceptible tomato cultivars to inoculation with RsBAU1 isolate of R. solanacearum determined the race of virulent isolate of this pathogen. Here, nine *R. solanacearum* isolates belonged to Race 1 while one isolate (RsBAU1) belonged to Race 3. Race 3 causes wilting in potato, tomato and rarely other solanaceous plants. Gram staining of *R. solanacearum* resulted pink colour and straight rod-shaped structure. KOH solubility test produced viscous thread which is an indication of Gram-negativity (Popoola *et al.*, 2015; Hossain *et al.*, 2021). Sugar utilization tests revealed that R. *solanacearum* was able to utilize carbon as a source of energy and belonged to biotype III (Hossain *et al.*, 2021). Race 3 is highly virulent on potatoes, tomatoes and other solanaceous crops. Biovar characterization showed that most of the R. solanacearum isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol). The oxidation reaction was indicated by the change of colour (Plate was not shown). All ten isolates of R. solanacearum tested positive to Biovar III. With the species-specific primer, the PCR produced a single band of 288 bp from the strains of *R. solanacearum* isolated (Ito *et al.*, 1998).

Screening of *Trichoderma* isolates against *R. solanacearum*: *T. asperellum* (OM480709) exhibited the highest antagonistic activity of zone inhibition (33 mm in dia.) against the most virulent *R. solanacearum* (RsBAU1 isolate) compared to the other *Trichoderma spp*. and this *T. asperellum* (OM480709) TR3 isolate was selected for further molecular and field studies. The zone inhibition was ranged from 15-33 mm in diameter (figure 3 & 4). Similar observation was reported by Konappa *et al.* (2013) who found zone of inhibition was 24-29mm by *T. asperellum*.

The PCR detection of *R. solanacearum* RsBAU1 isolate: Extracted DNA from the virulent *R. solanacearum* RsBAU1 isolate resulted approximately 1500 bp and 288 bp amplicon size in PCR using by universal 16S rDNA primers, and Y-2 & OLI-1specific primers respectively which were shown on 1.2% agarose gel (figure 5). This banding pattern was in agreement with the result of Razia *et al.*

 Digital Object Identifier: https://doi.org/10.33865/wjb.009.01.1183

(2021) also reported that *R. solanacearum* yielded 288 bp band size when DNA of that pathogen was amplified with the specific primer Y-2/ OLI-1.

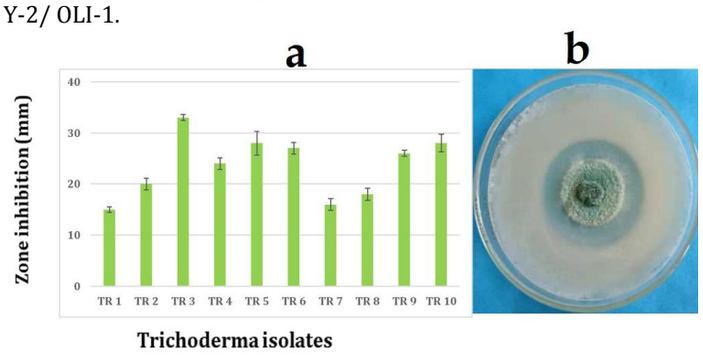

Figure 4: Screening of isolated *Trichoderma* spp. against virulent *R. solanacearum* (RsBAU1 isolate), a : zone inhibition in mm, b: agar well diffusion plate.

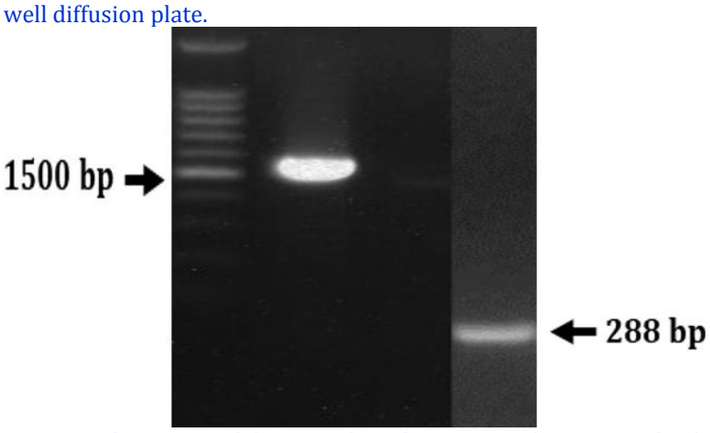

Figure 5: The PCR amplification of *R. solanacearum* (RsBAU1 isolate) DNA with 16S rDNA, Y-2 & OLI-1 specific primers. Lane 1: Mol. Marker, Lane 2: 1500 bp amplicon size, Lane 3: Water (contro) and Lane 4 : 288 bp amplicon size.

Preparation of *T. asperellum* (OM480709) inocula with FYM / formulated *Trichoderma* (FT) and its effect on net house experiment: FYM decomposed by *T. asperellum* (OM480709) (FT) was dark in color (figure 6) and *T. asperellum* (OM480709) (FT) colony was counted there (data was not shown) and used for pot and field experiment.

Figure 6: Formulated *Trichoderma* (FT) and its effect on bacterial wilt of tomato (net house expt.), a: FT, b: pot experiment.

Net house experiment revealed that that *T. asperellum* (OM480709) TR3 isolate reduced 90% wilt disease of tomato and increased significantly all the agronomic parameters of Tomato plants than the control (table 3, figure 6). This result is with parallel of the findings of Murthy *et al.* (2013).

Treatments	Disease incidence (%)	Plant height (cm)	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
T1 (FT)	0.0 a	18.2 c	14.6 c	9.0 c	22.2 d	6.6 d
T2 (FT +R.s)	10.0 b	14.0 b	11.2 b	5.6 b	16.4 b	4.2 b
T3 (R.s)	90.0 c	9.60 a	8.00 a	3.8 a	10.5 a	3.4 a
T4 (Control)	0.0 a	15.6 ab	12.2 b	8.1 c	18.8 c	5.2 c

Table 3: Effect of different treatments on plants growth promotion studied under net house using 30days-old tomato seedlings.

Means sharing different alphabetical (a, b, c, d) superscripts in a column significantly different (P<0.05). Here, R.s= *R. solanacearum* (RsBAU1 isolate), FT= formulated *Trichoderma*, FT+R.s = formulated *Trichoderma* + *R. solanacearum*.

Effect of FT against *Ralstonia* wilt under field condition: In *R. solanacearum* infected tomato plants, wilt incidence ranged from 4.0-87. Formulated Trichoderma (FT) treated plots showed the lowest incidence (4.0%) and Ralstonia (RsBAU1) inoculated plots resulted the highest disease incidence (87.0%). FT treatment decreased the disease incidence by 51.5% (FT + R.s) (table 4).

Treatments	Disease incidence (%)	Plant height (cm)	Fresh weight (g)	Dry weight (g)	No. of fruits /plant	Individual Fruit wt. (g)	Yield (t/ha)
Control	15.0 a	57 a	409 a	47 a	33 a	68.0 a	56.1 a
R.s	87.0 b	32 b	161 b	14 b	10 b	42.0 b	10.5 b
FT	4.00 c	76 c	672 c	51 c	42 b	80.0 b	84.0 c
FT+R.s	35.5 d	49 d	378 d	36 d	31 c	64.0 c	49.6 d
LSD	3.086	3.765	12.489	3.122	4.314	3.408	3.323

Table 4: Influence of *T. asperellum* (OM480709) on tomato plant growth, yield, and control of bacterial wilt under field conditions during 2022-2023.

Means sharing different alphabetical (a, b, c, d) superscripts in a column significantly different (P<0.05). Here, R.s= *R. solanacearum* (RsBAU1 isolate), FT= formulated *Trichoderma*, FT+R.s = formulated *Trichoderma* + *R. solanacearum*.

The tomato yield in non-treated control plot was recorded 56.1 t/ha, R. solanacearum infected plot was 10.5t/ha and FT treated plot was 84.0 t/ha. Again, FT plots challenge inoculated with R. solanacearum yielded 64.0 t/ha. Thus, FT treatment enhanced the tomato yield by 39.1 t/ha when compared with R.s treated plots and 27.9 t/ha in compared to non-treated plots. The FT treated plots showed significant increase in overall plant growth including plant height (cm), fresh weight (g), dry weight(g), no. of / plant, individual fruit wt.(g) and yield (t/ha) (tables 4). FT treatment increased plant height, fresh weight, dry weight, no. of fruits / plant, individual fruit wt./plant by 19 cm, 263 g, 4.0g, 9 fruits, and 12g/plant, respectively as compared with the control plot. The results showed the induction of plant growth, increased tomato yield and reduced wilt incidence under field conditions upon soil treated with FT. The root colonization is a successful major requirement for the useful effects of *Trichoderma* spp. on plants not only concerning antagonistic behavior and increase in plant growth but also for inducing systemic resistance (Murthy *et al.*, 2013). Studies on Trichoderma application in farming practices as biological control agents, biofertilizers, and soil amendments for the control of plant pathogens and crop development have been well established (Manjunatha *et al.* 2013). Trichoderma is adepted of colonizing farmyard manure, and therefore, application of colonized FYM to the soil is more suitable and helpful. This is the mainly successful method of application of Trichoderma, particularly for the control of soilborne diseases (Hamed *et al.*, 2015)

Induced resistance chamical/PR-Protein analysis: Here, it was found that *T. asperellum* TR3 isolate (OM480709) significantly induced the highest levels of defense enzyme activities in tomato leaves when challenged with *R. solanacearum* (RsBAU1) pathogen compared with non-treated tomato plants. In another words, T. asperellum TR3 isolate (OM480709) exhibited significantly induced POX, PPO, PAL, β-1,3-glucanase, and total phenolic contents in plants challenged with *R. solanacearum* in tomato plants (figure 7, 8 and 9).

Accumulation of systemic resistance enzymes and their activity depends on the plant genotype, physilogy and pathogenic characters. Synthesis of defense proteins is triggered by a series of morphological and biochemical changes initiated by specific pathogen (Surekha *et al.*, 2013). Therefore, treatment of tomato seedlings with formulated Trichoderma (T. asperellum ,OM480709) +FYM) induced POX, PAL, PPO, β-1,3-glucanase and total phenolic contents when inoculated with R. solanacearum (RsBAU1 isolate). It was observed that POX, PAL, PPO, β-1,3-glucanase activity and total phenolic contents significantly increased up to 8 (eight) days after FT treatment upon challenge inoculation with *R. solanacearum* and declined thereafter in all the treatments. Tomato plants treated with FT alone also significantly enhanced the activity of POX, PAL, PPO, β-1,3-glucanase and phenolics as compared with *R. solanacearum* treated and non-treated control (figure 7-9).

POX plays a key role in the biosynthesis of lignin that limits the invasion of pathogens (Vidhyasekaran, 2008). Increasing of POX level induced systemic resistance by quick synthesis of reactive oxygen derivatives which leads to cell death and inhibits pathogenic activities (Prasannath *et al.*, 2014). Therefore, increasing of POX level on 8th day in tomato plant leaves is considered to be the marker for the development of disease resistance against R. solanacearum. This results were supported by earlier researchers who found induction of POX in plants infected by pathogens resulting faster and stronger resistance against them i.e peroxidase plays a defense role against attacking pathogens (Caruso *et al.*,

 Digital Object Identifier: https://doi.org/10.33865/wjb.009.01.1183

2001). PPO is a copper cluster enzymes that catalyze oxidation of hydroxy phenols to quinone derivatives those have antimicrobial activity, accelerating cell death, and reduced bioavailability of cellular proteins to the pathogen (Chunhua *et al.*, 2001).

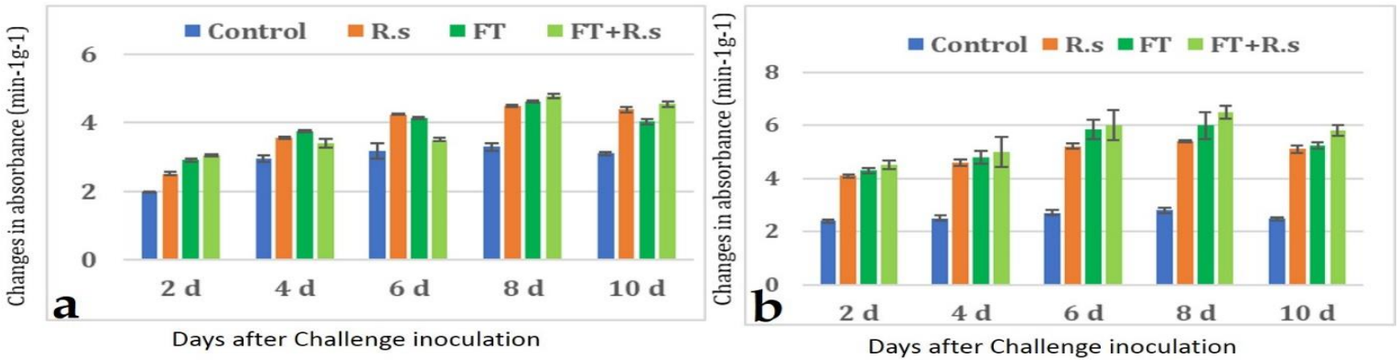

Figure 7: Changes of POX and PPO activity in tomato plants of different treatments.
Mean values were three replicates. Bars represent standard error. FT = formulated *Trichoderma*, R.S=plants inoculated with *R. solanacearum* (RsBAU1 isolate). a: POX activity, b: PPO activity

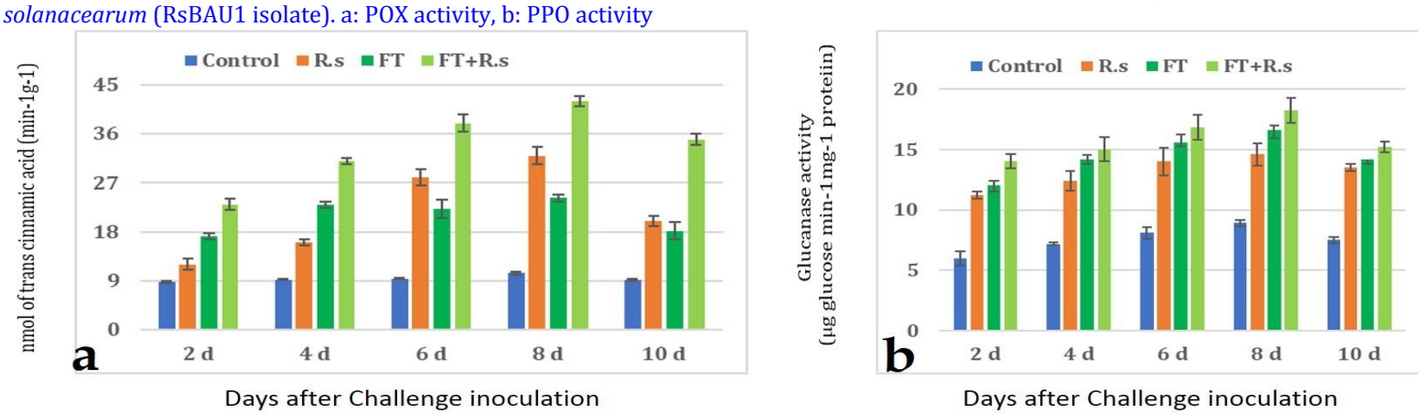

Figure 8: Changes in phenylalanine ammonia lyase (PAL) and β-1,3-glucanase activity in tomato plants of different treatments.
Mean values were three replicates. Bars represent standard error. FT = formulated *Trichoderma*, R.S=plants inoculated with *R. solanacearum* (RsBAU1 isolate). a: PAL activity ; b : β-1,3-glucanase activity .

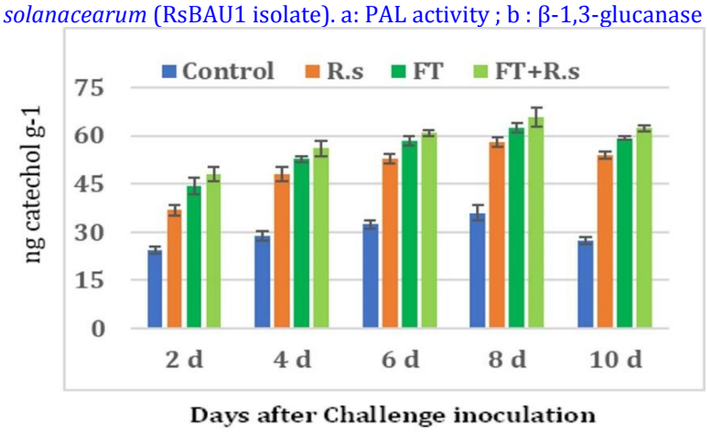

Figure 9: Changes in total phenols in tomato plants of different treatments.
Mean values were three replicates. Bars represent standard error. FT = Formulated Trichoderma, R.S =Plants inoculated with R. solanacearum (RsBAU1 isolate).

Oxidative enzymes such as POX and PPO can catalyze the formation of lignin and contribute in the formation of defense barriers by changing the cell structure against pathogens (Vinale *et al.*, 2008). PAL activity in tomato plants treated with FT was also induced (figure 8). PAL is the key enzyme that is responsible for metabolism of aromatic amino acids and secondary metabolic products (MacDonald and D'Cunha, 2007). It helps in synthesis of different phenolic compounds and lignin those provide mechanical strength to the plant cell wall and prevent from the establishment of plant pathogens (Konappa *et al.*, 2018). In this study, increased PAL activity and the accumulation of phenolic content was recorded highest in FT treated tomato plants infected with the *R. solanacearum*, may be due to prevention of pathogen attack. Again, increased level of β-1,3-glucanase activity was observed in *T. asperellum* (OM480709) formulated FYM treated tomato plants and leads to disease resistance against *R. solanacearum*. Moreover, β-1,3-glucanase can directly destroy pathogen cell walls. Improved β-1,3-glucanase activity was observed up to the 8th day after R.s inoculation and then, it starts decreasing leading to disease resistance in tomato plants against *R. solanacearum* (Saksirirat *et al.*, 2009). The maximum total phenols was obtained in FT treated tomato plants was induced upon challenge inoculation with *R. solanacearum*. Minimum amounts of phenolic compounds were observed in nontreated / control plants (figure 9). The enhancement of production of phenolics, known as defense molecules of plants against plant pathogens. FT treatment also resulted the highest agronomic parameters of Tomato plant may be T. asperellum TR3 decomposed FYM yard manure provides nutrients to the microbes, thus increases the biocontrol agent's ability and makes them extra competitive in the rhizosphere soil and on roots. Root colonization by biocontrol agents is considered a prerequisite and is directly connected to their effectiveness in controlling soil borne infections. The induction of PR proteins in non-treated plants may be due to the crucial role of SA and hormone regulates processes. SA could also contribute to maintain cellular redox homeostasis through the regulation of antioxidant enzymes activity (Slaymaker *et al.*, 2002).

CONCLUSION: The present study screened out one *T. asperellum* TR3 isolate (OM480709) which has antibacterial capability and characterised with molecular marker. The biochemical characterization of induced systemic resistance by *T. asperellum* TR3 isolate (OM480709) against *R. solanacearum* (RsBAU1 isolate) and the role of defense enzymes in developing tomato wilt resistance under field condition. *T. asperellum* TR3 isolate (OM480709) formulated with FYM proven an effective inhibition against a virulent *R. solanacearum (RsBAU1 isolate)* that significantly increased agronomic parameters including yield of tomatoes under both net house and field conditions. So, the role of *T. asperellum* TR3 isolate (OM480709) as BCA in the induction of a series of defense enzymes viz. of POX, PPO, and PAL and accumulation of phenolics may be involved in phenylpropanoid and PR-protein metabolism that responded as a potential biocontrol agent. Therefore, use of *T. asperellum* TR3 isolate (OM480709) formulated with FYM against Ralstonia wilt disease management strategy is reasonable in tomato field, and this could be a sustainable and eco-friendly approach.

CONFLICT OF INTEREST: The authors declared no conflict of interest.

ACKNOWLEDGEMENT: The authors are very much grateful to University Grants Commission (UGC), Agargaon, Sher-e-Bangla Nagar Dhaka, Bangladesh for funding this project (ID: 2022/19/UGC) work. Heartfelt thanks to Professor. Dr. Md. Rashidul Islam for supplying species specific primers for conducting PCR reactions.

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