Ann. Bangladesh Agric. (2020) 24 (2) : 107-120 *ISSN 1025-482X (Print)*
www.doi.org/10.3329/aba.v24i2.55788 *2521-5477 (Online)*

INHIBITORY EFFECT OF *TRICHODERMA ASPERELLUM* ISOLATE AGAINST *RALSTONIA SOLANACEARUM* CAUSING BRINJAL WILT

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ABSTRACT

A native *Trichoderma* isolate was collected from the agricultural soil of Gazipur. This isolate was identified as a *Trichoderma asperellum* through morphology and analysis of internal transcribed spacer (ITS) region of ribosomal RNA gene sequence and reconstruction of the phylogenetic tree. The antagonistic effects of the newly identified *T. asperellum* isolate were assessed against brinjal bacterial wilt caused by *Ralstonia solanacearum* both *in vitro* and *in planta.* Both qualitative and quantitative bioassays were conducted *in vitro*. For qualitative tests, dual culture and antibacterial activity were carried out, and pathogen growth was observed visually. The antagonism of *T. asperellum* cell free culture filtrate on the growth of *R. solanacearum* was conducted in a quantitative test. Successful antagonism was recorded after both *in vitro* qualitative tests. In addition, the lowest colony forming unit was recorded in 100% of CFC ($2.4\pm0.51 \times 10^3$ cfu/ml) in quantitative test. The *T. asperellum* inoculated plant showed low disease incidence (13.33%) when seedlings were challenged with *R. solanacearum in planta* experiment. Disease incidence was 100% for seedlings when treated with only *R. solanacearum.* The results showed that the isolated and identified *T. asperellum* isolate suppressed *R. solanacearum* growth *in vitro* and protected the seedling from wilting *in planta*. Therefore, this isolate could be considered as a potential isolate.

Keywords: Internal transcribed spacer (ITS), *Trichoderma*, *Ralstonia*, phylogeny, antagonism, bioassay.

Introduction

Bacterial wilt is caused by a soil-borne, bacterial pathogen *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* (formerly *Pseudomonas solanacearum*). The pathogen is distributed widely in tropical, subtropical, and some temperate region in the world (Yabuuchi *et al.,* 1995). This pathogen limits the production of solanaceous crops such as brinjal, tomato, pepper, tobacco, and potato (Hayward, 1995). For example, yield losses vary from 0 to 91% in the tomato, 33 to 90% in the potato, 10 to 30% in tobacco, 80 to 100% in the banana, and up to 20% in the groundnut (Elphinstone, 2005). Walker and Collin (1998) reported more than $950 million annual loss from about three million farm families in 80 countries due to bacterial wilt (Walker and Collin, 1998).

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R. solanacearum is difficult to manage due to its ability to grow endophytically, survive in soil, especially in the deeper layers, travel along water, and its relationship with weeds (Cook and Baker, 1983). Disease control is being attempted in different crops with rotation, intercropping, organic manuring, and use of resistant cultivars and chemical control. These control measures are often overcome by the pathogen due to its genetic diversity and wide adaptability (Xue *et al.,* 2009). Moreover, chemical control for wilt disease challenges its limited efficacy, and many of the chemicals have been deregistered worldwide for their detrimental environmental effects (Fujiwara *et al*., 2011). No specific chemical fungicide has been proved to be 100% efficient in controlling brinjal bacterial wilt caused by *R. solanacearum* till date. Due to the limited effectiveness of the current chemical control, the use of biological control agents against bacterial wilt has potential in integrated management strategies.

The members of the fungal genus *Trichoderma* (Ascomycota, Hypocreales) are free living, soil borne microorganism and being used as biological control agents (BCAs) (Mukhopadhyay and Kumar, 2020; Kumar and Khurana, 2021). The species of *Trichoderma* can colonize plant roots and stimulate plant growth by producing some metabolites (Vinale *et al*., 2012). Moreover, they suppress plant disease by producing extra-cellular enzymes like chitinases, glucanases, and proteases (Viterbo *et al*., 2002). Moreover, it can induce defence responses in plants (Harman *et al*., 2004). Due to their multiple benefits, many species of *Trichoderma* have been marketed as biopesticides or biofertilizers (Woo *et al.*, 2014; Kumar *et al.,* 2017). In Bangladesh, a number of *Trichoderma* species were well studied for their biological control efficacy against soil borne fungal diseases. For instance, Liton *et al*. (2019) demonstrated that *T. harzianum* isolates reduced seedling mortality of bush bean caused by *Fusarium oxysporum* f. sp. *phaseoli, Rhizoctonia solani and Sclerotium rolfsii* while applied with composts. Another field study showed that *Trichoderma* enriched compost reduced seedling mortality of chickpea and yield (Talukdar *et al.*, 2017). However, information regarding biocontrol efficacy of *Trichoderma* species against soil borne bacterial crop diseases is limited. One of the research findings noted that *T. harzianum* was effective against brinjal bacterial wilt, but it was not conclusive (Barua and Bora, 2008). The aim of this study was to isolate and characterize novel strains of *Trichoderma* from native soils in Bangladesh and its potential in controlling wilt bacterium *R. solanacearum* in brinjal.

Materials and Methods

Isolation and phylogeny of *Trichoderma*

Isolation of *Trichoderma* isolate

Soil samples were collected from agricultural fields of Bangabandhu Sheikh Mujiubur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh during the winter 2019 and brought to the Institute of Biotechnology and Genetic Engineering lab for further research work. Approximately, 300 g of soil was collected with a soil trowel to a depth of 10-15 cm. Soil samples were kept in individual zip lock bags, labelled, and stored at 4^0C in a cold room until all samples were analysed.

Soil samples were sieved through a 5-mm

mesh, and 5 g of soil was suspended in 50 mL of sterile 0.1% Tween 80, in a 50-mL screw cap plastic tube, and incubated at room temperature for 3 hours. All tubes were inverted five times at 30 minutes intervals. Following incubation, the tubes were kept for sedimentation for 20 seconds. A volume of 100 µL of supernatant from each tube was plated on a Petri plate with Sabouraud dextrose agar (SDA) medium (peptone 10g /L, agar 10g /L, dextrose 40g/L, with CTAB 60mg/L) with streptomycin (30 mg/L) to prevent bacterial contamination. Following inoculation, all plates were incubated at 22^0 C for two weeks. Plates were examined for characteristic dense, compact white mycelium growth every 2-3 days interval. Isolates likely to be Hypocreales were isolated and sub-cultured.

Morphological identification of fungal isolates

Immediately after sporulation of fungal colonies on SDA, microscopy was conducted to observe their vegetative and reproductive structure. Small plaques from the edge and the centre of each growing colony were transferred onto glass slides, and examined using a compound light microscope.

Sub-culture, DNA isolation, and sequencing

For DNA isolation and sequencing, the fungal isolates were sub-cultured on SDA agar plates without antibiotics. The DNA was extracted by the method described by Islam (2018).

Briefly, a small lump of fungal mycelium of 7-day-old culture was taken into an Eppendorf tube, homogenised using a sterile plastic pestle, resuspended in 1 mL lysis buffer (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, and 1% SDS) and incubated at 50^0C for 1 hour in a heat block. A volume of 150 µl of precipitation buffer (5 M potassium acetate 60.0 mL, glacial acetic acid 11.5 mL, distilled water 28.5 mL) was added and vortexed and incubated on ice for 5 minutes. Following the centrifugation, 500 µl of supernatant was transferred to a new tube, and an equal volume of isopropanol was added to precipitate DNA. The DNA pellet was collected after centrifugation at 18,000 rcf for 20 minutes and washed with 1 mL of 70% ethanol. The DNA pellet was air dried for 10 minutes and dissolved in 100 µl of Tris-EDTA (TE) buffer. The quality of the DNA was checked in nano-drop. The Internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using ITS1F: CTTGGTCATTTAGAGGAAGTAA and ITS4R: TCCTCCGCTTATTGATATGC (Islam *et al.,* 2018). The thermal profile was as follows: initial denaturation at 90°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, followed by a final extension at 72°C for 15 min.

PCR product was electrophoresis in 1% agarose in 1x TBE buffer at 120 volts with GelRed nucleic acid stain and visualized under UV light using a 'Molecular Imager® (Gel DocTM). The PCR products were purified using an 'Isolate DNA Kit' (Bioline) following the standard manufacturer protocol. The PCR purified product was sent to First BASE laboratories, Malaysia for both way sequencing.

Phylogenetic tree construction

Sanger's sequence data from the isolate was manually edited by using Geneious V10. The reference genome sequences were retrieved

from NCBI. Multiple-alignment was done in Geneious V.11 using MAFT plug-in and the final alignment was manually corrected. The phylogenetic tree was developed by maximum likelihood analysis using RAxML (Stamatakis, 2006) and to search the best-scoring tree under the GTR-GAMMA model with 1000 replicates. The tree was visualised by TreeGraph 2 (Stover and Muller, 2010).

***R. solanacearum* isolate**

The bacterial isolate was collected from the culture collection of the Department of Plant Pathology, BSMRAU, Bangladesh.

Antagonism of *Trichoderma* isolate against *R. solanacearum*

Qualitative and Quantitative bioassay

Qualitative bioassay for antagonism was conducted through the dual culture method and assessing antibacterial activity.

Dual culture bioassay

Dual culture was conducted according to Khatun *et al.* (2017). One mycelial disk from the periphery of a 5-day-old TA culture was placed on a PDA plate 3 cm apart from the *R. solanacearum* inoculation on the same plate. After inoculation, the Petri dish was kept at 25^0C in the dark for 5 days and was examined after 7 days from each plate. The antagonism activity was recorded on the basis of visual observation only after 7 days of pathogen inoculation.

Metabolite test (Furuya *et al*., 1997)

The antibacterial activity of *Trichoderma* isolate was done by following a method described by Furuya *et al.* (1997) with some modifications. A small lump of fungal mycelium was placed in the center of Petri dishes containing 20 mL of SDA medium. The plates were incubated at 28°C for one week so that the plate was covered with a mycelial mat. Then the plate was turned upside down. A sheet of filter paper was placed in the Petri dish lid with 0.5 mL chloroform. The dish was kept at room temperature (25±2˚C) for 2 h. After complete exclusion of chloroform vapor, the bacterial colonies were overlaid with 5 mL melted water agar (1.5%) at 50^0C containing a suspension of *R. solanacearum* which was prepared by inoculating into YP broth and kept overnight in agitating condition at 28^0C. The plate thus prepared was incubated at 30^0C for 2 days. The growth of RA on chloroform-killed fungus was observed at 48h of incubation visually.

Quantitative bioassay using in cell-free culture filtrate

To quantify antagonism of *Trichoderma* isolate on bacterial growth, cell-free culture filtrate (CFC) of fungus was used. The isolate of *Trichoderma* was inoculated into 100 mL of yeast extract broth (0.1% yeast extract in distilled water; YEB) and incubated at 25°C (Sasan and Bidochka, 2013). Culture filtrate was collected through filtration after 4 days of incubation, using Whatman filter paper no. 44. The culture filtrate was checked for the presence of fungus by plating a sample on SDA. The cell-free culture filtrate was diluted in 0.1% yeast extract, and 3 mL of 100%, 90%, 50%, and 10% cell-free culture filtrate were inoculated with 300 µL of RS (10^4 cell/ mL) in glass tubes. A concentration of 0.1% yeast extract was used as the control. The glass tubes were kept at 25°C in a shaker. The colony forming unit (CFU) per mL of *R. solanacearum* was estimated on SDA plate by serial dilution method after 48 hours.

***In planta* antagonism assay**

Seeds of brinjal variety BARI 10 were collected from Horticulture Research Centre of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. All seeds were soaked in sterile distilled water for 30 minutes to enhance germination. Afterward, seeds were immersed in 4% sodium hypochlorite for 10 minutes and washed with sterile distilled water for three times. Later, seeds were placed on autoclaved sand: soil (1:1) mixture for germination. The spores of *T. asperellum* were collected in water by scraping from 14 days old SDA culture plate and filtered through cheesecloth. A final concentration of spore 1×10^6 per ml in water was adjusted with a hemocytometer. The bacterial inoculum was prepared by growing in LB broth followed by filtration and centrifugation. The final concentration of RS suspension was made at 1×10^6 per ml of water with a hemocytometer.

The individual pot containing 300 gm of sterilized soil was inoculated with 10 ml of *T. asperellum* spore suspension (spore suspension, $1X10^6$ spore/ml). Following inoculation of fungus, soils in pot were kept for 7 days to develop as soil microbial population. Inoculated soils were occasionally pulverized for uniform the fungal growth. After 10 days of seed germination, the seedlings were transplanted in those *T. asperellum* inoculated pot individually. After 10 days of seedling transplantation (in the *T. asperellum* inoculated pot), all seedlings were inoculated with 10 ml of *R. solanacearum* suspension at the base of the stem.

There were four treatments applied to pot experiment: i) seedlings inoculated with *R. solanacearum* (RS), ii) seedlings inoculated with *T. asperellum* and *R. solanacearum* (TA + RS) iii) seedlings inoculated with *T. asperellum* (TA), and iv) seedlings inoculated with only water. Seedling pot soil inoculated with *R. solanacearum* suspension only was considered as the positive control, whereas seedling pot soil inoculated with water considered as a negative control. There were 15 seedlings per treatment. The number of total wilted seedlings was recorded after 15 days of *R. solanacearum* inoculation. Percentage of wilted seedling counted by using the following formula:

$$\%\text{Wilt incidence} = \frac{\text{Number of wilted seedling}}{\text{Total number of seedling}} \times 100$$

Growth of leaf shoot was observed visually and on photographed accordingly to provide pictorial evidence of this experiment.

Data analysis

Generalised linear model for the poisson data family was used to analyse the CFU data and mean values were compared by Tukey's post hoc test using 'lsmeans' package. All statistical analyses were performed in 'R'v3.3.1 (R core team, 2016).

Results and Discussion

Cultural characterization of *Trichoderma* sp.

The isolated *Trichoderma* sp. morphology was similar to the characteristic of the hypocrealean fungus observed on the SDA medium. One isolate viz. isolate G3 was recorded with white compact mycelium at the initial growth stage. This isolate formed green spores at 14 days of culture on SDA medium with characteristics concentric rings (Fig. 1). The morphology of isolate G3 was similar to *T. asperellum* T8a observed by Santos-Villalobos *et al.* (2012).

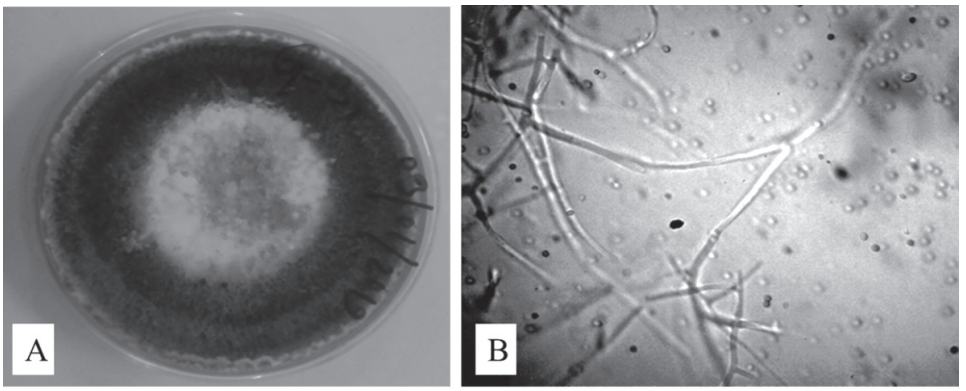

Fig. 1. *Trichoderma asperellum* isolate G3 culture on SDA medium. A) 14 days old culture plate with green mycelial growth, B) Mycelium and spore under light microscope

However, morphological characteristics are not the key features for Hypocrealean fungus to identify them at the species level (Waalwijk *et al.,*1996; Islam *et al.,* 2016). Because Hypocrealean fungus often subject to morphological transition due to environmental change/stress and can significantly vary from culture to culture (Seaby, 1996). Therefore, Islam *et al.* (2016) suggested the importance of molecular analysis to authenticate and to resolve the ambiguities regarding morphological identification.

Phylogeny of *Trichoderma* sp.

The ITS sequence data were processed and assembled through Geneious software and submitted to NCBI. The accession number of the isolate is MW052549. The sequence similarity search results showed that the isolate G3 had the closest similarity of >94% with *T. asperellum.* A maximum likelihood phylogenetic tree was reconstructed using the ITS sequences of isolate G3 with 46 *Trichoderma* species and one strain of *Hypomyces samuelsii* as out group. The phylogenetic analysis showed that the isolate G3 was placed within one *Trichoderma* clade of 23 species (Fig. 2) and closest to *T. asperellum*. Molecular identification of *Trichoderma* species using ITS region is a widely used method (Haque *et al*., 2020; Feitosa *et al*., 2019; Rukmana, *et al*., 2019). Both morphological characteristics and molecular sequence data of fungal strain suggested that the isolate G3 was a *T. asperellum.*

Qualitative and Quantitative bioassay

Profuse growth and sporulation of *T. asperellum* over *R. solanacearum* colony were observed visually in the dual culture on SDA medium and photographed accordingly (Fig. 3A). The bacterial growth was restricted by *T. asperellum* in duel culture plates. Bacterial growth was completely ceased on chloroform killed *T. asperellum* treated plates (Fig. 3B). The antagonism *T. asperellum* against *R. solanacearum*, was quantified and estimated on cell free culture filtrate (CFC) method (Fig. 4). The lowest colony forming unit (CFU) of *R. solanacearum* was in 100% of CFC (2.4±0.51 $\times 10^3$ cfu/ml) and highest

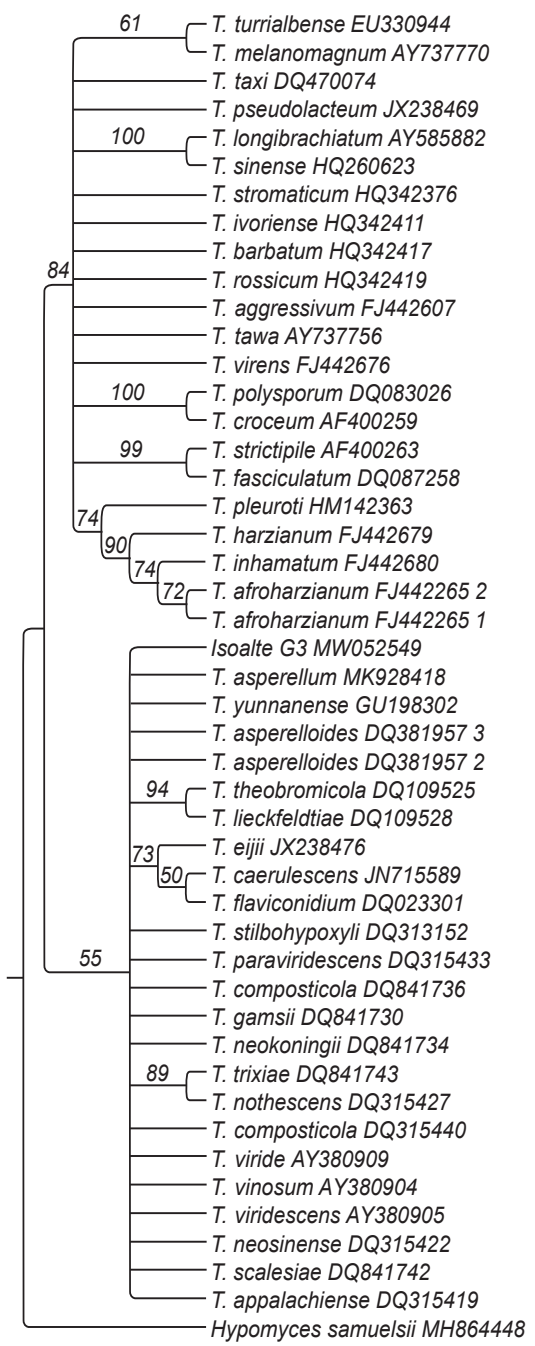

Fig. 2. Maximum likelihood phylogenetic tree of ITS data set with GTR-GAMMA model of 1000 bootstrap replicates. Value on node indicates bootstrap support value.

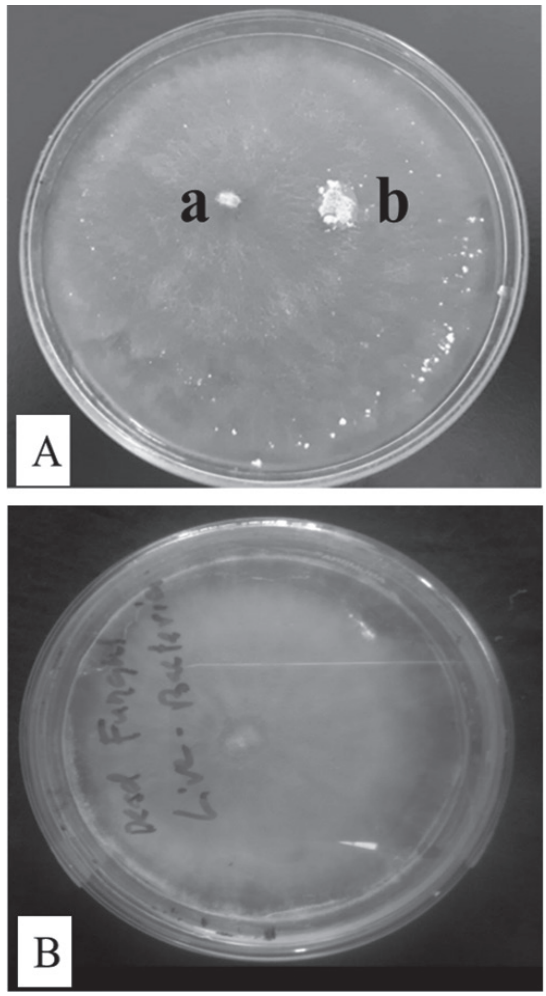

Fig. 3. *In vitro* qualitative bioassay. A) Dual culture bioassay on SDA medium. The inoculation position of *T. asperellum* sp. Isolate (a), profusing growth of *T. asperellum* over *R. solanacearum* colony (b). B) Metabolite test; growth of *R. solanacearum* on chloroform killed *T. asperellum*. isolate G3.

in 10% of CFC (1292.4±24.60 $\times 10^3$ cfu/ml). The bacterial growth was significantly reduced in higher concentration CFC of fungus (Fig. 5).

The present *in vitro* dual culture study

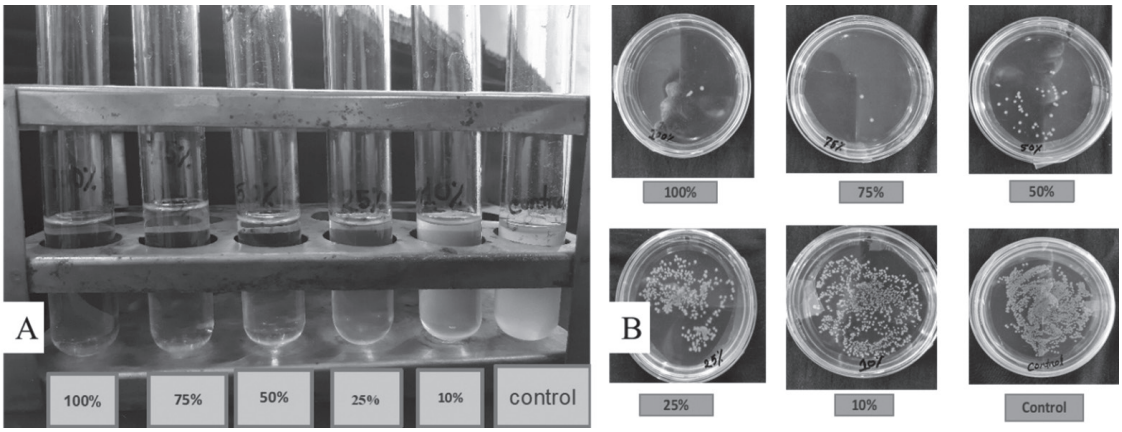

Fig. 4. ***In vitro*** **quantitative bioassay. A) Growth of** ***R. solanacearum*** **in different concentrations CFC of** ***T. asperellum*** **isolate G3 and B) CFU of** ***R. solanacearum*** **in different concentrations CFC of** ***T. asperellum*** **isolate G3, both after 48 hr of incubation.**

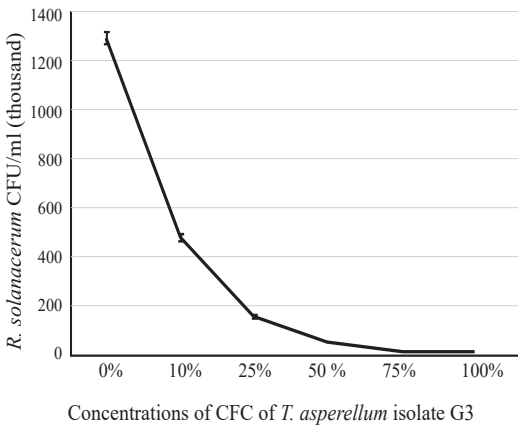

Fig. 5. Effect of different concentrations of CFC of ***T. asperellum*** **isolate G3 on (CFU) per ml of** ***R. solanacearum*** **after 48 h of incubation. (The mean±SEM value with lower case alphabet indicate statistical differences on Tukey's post hoc test, p < 0.05.)**

indicated successful antagonistic/inhibitory efficacy of *T. asperellum* isolate G3 against *R. solanacearum* of brinjal. Previous studies have addressed the promising potential of *T. asperellum* as it possesses strong inhibitory effects against various phytopathogens in different crops *in vitro*. For example, *in vitro* dual-culture analysis showed effective antagonism of *T. asperellum* against plant pathogenic fungi such as *A. niger* (Haque *et al*., 2020), *Fusarium. oxysporum* (f. sp. *cucumerinum* and f.sp. *capsici*) (Wu *et al.,* 2017; Islam *et al.,* 2016; Hewedy *et al.,* 2020), *F. circinatum*, *Rhizoctonia solani*, and *Phomopsis vexans* (Islam *et al*., 2016). Moreover, successful *in vitro* inhibition was also recorded against some spot and wilt causing bacteria such as *Xanthomonas perforans* (Chien and Huang, 2020) and *R. solanacearum* (Narasimha Murthy and Srinivas, 2012; Narasimha Murthy *et al.,* 2013).

In Bangladesh, Islam *et al.* (2016) reported that different strains of *T. asperellum* varied in their *in vitro* antagonistic effect against plant pathogenic fungi. Alternatively, a single strain of *T. asperellum* was potent against ten strains of *R. solanacearum in vitro* (Narasimha Murthy and Srinivas, 2012; Narasimha Murthy *et al.,* 2013).

Heavy sporulation against the *R. solanacearum*

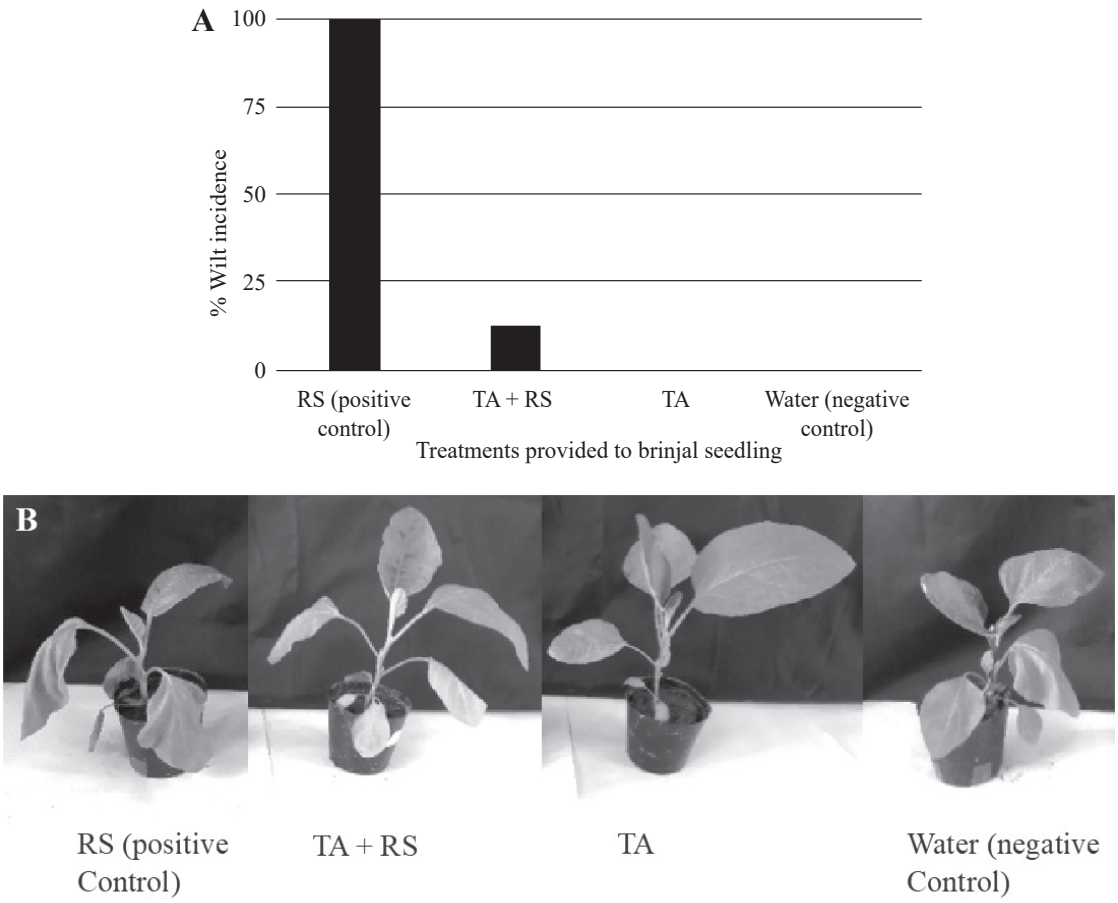

Fig. 6. A) Percent wilt incidence of 15 days after treatments. B) Photographs of brinjal seedlings. (RS: seedlings inoculated with *R. solanacearum*, TA + RS: seedlings inoculated with *T. asperellum* and *R. solanacearum,* TA: seedlings inoculated with *T. asperellum)*.

bacterium was recorded in the present dual culture plate during the qualitative antagonistic bioassay. This observation was similar to previous studies (Hermosa *et al.,* 2000; Patel and Saraf, 2017; Kilonzi *et al.,* 2020; Intana *et al.,* 2021), where fast growth and profuse sporulation of *Trichoderma* were observed against many plant pathogens (2018) recorded mycoparasitism of *T. asperellum* (isolate Ray1715) as it completely overgrew on *Phytophthora nicotianae* (*Phytophthora* foot and root rot of citrus). Additionally, the faster growth of *T. asperellum* T76-14 in the dual-culture assay against *F. incarnatum* on PDA suggested a competition mechanism (Intana *et al.,* 2021). According to Jiang *et al.* (2016) *T. asperellum* was able to collapse the mycelium of the colonies of the pathogen through dual culture tests by breaking down the pathogenic hyphae into fragments. (Jiang *et al.,* 2016). In antagonism, the sporulation behaviour and aggressiveness of *T. asperellum* were highly

dependable on type and concentration of target pathogen (Kilonzi *et al.,* 2020; Islam *et al.,* 2016). Therefore, the current study assumed that both competition and parasitism were the mode of action of *T. asperellum* against *R. solanacearum*.

Moreover, the culture filtrate of *T. asperellum* also inhibited the growth of *R. solanacearum in vitro*. This inhibition efficacy increased with the increase of the initial filtrate concentration of the mentioned antagonist. It was assumed that, this biocontrol agent produced secondary metabolites with effective antibacterial efficacy. Similarly, Wu *et al*. (2017) also demonstrated the inhibition rate of filtered fermentation liquor from *T. asperellum* GDFS1009 against *F. oxysporum* f. sp. *cucumerinum*. Successful inhibition against *F. oxysporum*, *R. solani*, *F. circinatum* and *Phomopsis vexans* through culture filtrate of *Trichoderma* strain also reported in Bangladesh (Islam *et al.,* 2016). However, both these studies examined a single culture filtrate concentration. Whereas, the present study indicated that the inhibition efficacy was highly dependent on filtrate concentration. Loc *et al.* (2020) demonstrated that the chitinases, produced by *Trichoderma* culture, inhibited the growth of *Sclerotium rolfsii*, and this inhibition was increased with the increase of chitinases concentration in the media. Previously Zhang *et al.* (2017) demonstrated the production and antibacterial effect of secondary metabolite by *Trichoderma* species. However, further research is important to identify the specific metabolites produced by this novel *T. asperellum* responisible for bacterial inhibition.

***In planta* antagonism assay**

In pot experiment, 13.33 % of seedling mortality was observed in the pot treated with *T. asperellum* + *R. solanacearum* in the *in planta*, whereas 100% seedling was observed with wilt symptom after 15 days of *R. solanacearum* inoculation (Fig. 6).

According to the present investigation, *T. asperellum* completely ceased wilt development by *R. solanacearum* in brinjal seedling. This result was supported by Kipngeno *et al*., (2015), who recorded significantly lower (24.07%) post-emergence seedling damping-off by *Pythium aphanidermatum* in tomatoes compared to control (65.89%). Similarly, Gurung (2018) revealed high efficacy (reduction of infection by 95-100%) of *T. asperellum* in preventing *P. nicotianae* infections in citrus seedlings in the greenhouse, but did not found any significant effect on the height of seedlings as compared to untreated control. A number of previous studies also reported the bactericidal efficacy of *T. asperellum*. Inoculation of *T. asperellum* Tc-Jjr-02 at six hours before and after and simultaneously with pathogens provided protection (reducing the symptom index by 56–63%) for young tobacco plants against tobacco bacterial wilt disease caused by *R. solanacearum* (Jalaluddin *et al*., 2021). Previous studies (Konappa *et al*., 2020, Konappa *et al*., 2018, Mohamed *et al*., 2020) also revealed a reduction of potato and tomato wilt, caused by *Ralstonia solanacearum* (Smith) by *T. asperellum* isolate under greenhouse and field conditions. Even leaf spray of *T. asperellum* also reduced the growth of bacterial spots on tomato caused by *Xanthomonas* (Chien and Huang, 2020). In addition to disease reduction, *T. asperellum* also enhanced tomato seed germination, fruit

yield, plant growth and increased the dry weight of plant biomass promotion under field conditions (Konappa *et al*., 2020, Jalaluddin *et al*., 2021).

The importance of bacterial wilt in brinjal production was mentioned earlier by different authors (Faruk, 2019, Rahman, 2010, Nahar, 2019). It may be mentioned that this is the first research showing suppression of bacterial wilt of brinjal in Bangladesh. It is always difficult to extrapolate the biocontrol activity of a given strain from the laboratory to natural environments (Hermosa *et al*., 2000). As this strain demonstrated potential in controlling the bacterial wilt of brinjal both *in vitro* and *in planta*, further field experiments should be conducted to confirm the efficacy in detail.

Acknowledgments

The authors acknowledged the Research Management Wing (RMW), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh for providing research fund.

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