

Original Article

Isolation, morpho-physiological and molecular characterization, phylogenetic analysis of *Trichoderma asperellum* in Bangladesh

Isolamento, caracterização morfofisiológica e molecular, análise filogenética de *Trichoderma asperellum* em Bangladesh

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Abstract

The experiment was conducted at the Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh to identify *T. asperellum* in a countrywide screening program and to evaluate its antagonistic effect against several soil borne pathogens. Samples were collected from the rhizosphere soil of 49 different crops in 107 different locations in Bangladesh, especially, considering the several isolates of *T. asperellum* for purification. Based on morphological and physiological features, fifteen isolates were selected. Of these, the isolates of TR₂₇ and TR₄₅ were grown and sporulated at 40 °C except all the isolates with 35 °C, and particularly, showing a decrease of mycelial growth across all the isolates for increasing pH. Meanwhile, *T. asperellum* showed significant antagonistic effects against *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*, resulting in reducing foot and root rot, collar rot and damping off diseases, respectively. Four isolates were selected for molecular characterization among 15 isolates in terms of higher mycelial growth and spore density *in-vitro* condition, isolates of (TR₂₇) Sadar, Moulvibazar (Rice), (TR₄₅) Sadar, Mymensingh (Sweet gourd), (TR₇₀) Chapra, Chapai Nawabganj (Sesame) and (TR₈₅) Nayanpur, Lalmonirhat (Maize) were studied at ITS and TEF region. Isolates of TR₄₅, TR₇₀ and TR₈₅ were observed with 98% homology, and TR₂₇ exhibited 88% in their respective closest isolates at ITS sequences. Isolates of TR₂₇ and TR₈₅ also exerted their respective nearest homology (96%), while TR₄₅ showed 99%, and 93% homology with TR₇₀ in TEF sequences. Isolates TR₄₅, TR₇₀ and TR₈₅ were evidently determined as *T. asperellum* of 100% bootstrap value, and TR₂₇ isolate was also recognized with 72% bootstrap value in the phylogenetic tree. However, complementary effects of significant superior homology and the greatest bootstrap value in the identification of *T. asperellum* were found as noteworthy. In the phylogenetic analysis, magnificent differentiation among the *Trichoderma* isolates within and among the groups of closely related species was observed in Tef1 region than reflecting maximum variability in the isolates of rDNA at ITS region, whereas demonstrating a higher transversion ratio and evolutionary divergence.

Keywords: antagonism, ITS, pH, temperature, Tef1, *Trichoderma asperellum*.

Resumo

A experiência foi conduzida no Departamento de Fitopatologia da Universidade Agrícola de Bangladesh, Mymensingh, para identificar *Trichoderma asperellum* em um programa de rastreio em nível nacional e para avaliar o seu efeito antagonístico contra vários agentes patogênicos transmitidos pelo solo. Amostras foram coletadas do solo da rizosfera de 49 culturas diferentes em 107 locais diferentes em Bangladesh, especialmente considerando os diversos isolados de *T. asperellum* para purificação. Com base nas características morfológicas e fisiológicas, foram selecionados quinze isolados. Destes, os isolados de TR₂₇ e TR₄₅ foram cultivados e esporulados a 40 °C, exceto todos os isolados com 35 °C, e particularmente mostrando uma diminuição do crescimento micelial em todos os isolados para o aumento do pH. Enquanto isso, *T. asperellum* apresentou efeitos antagonísticos significativos contra *Fusarium oxysporum*, *Sclerotium rolfsii* e *Pythium aphanidermatum*, resultando na redução da podridão do pé e da raiz, podridão do colo e doenças de amortecimento, respectivamente. Quatro isolados foram selecionados para caracterização molecular entre 15 isolados em termos de maior crescimento micelial e densidade de esporos em condições *in vitro*, isolados de (TR₂₇) Sadar, Moulvibazar (arroz), (TR₄₅) Sadar, Mymensingh (cabaca doce), (TR₇₀) Chapra, Chapai Nawabganj (gergelim) e (TR₈₅) Nayanpur, Lalmonirhat (milho) foram estudados na região ITS e TEF. Isolados de TR₄₅, TR₇₀ e TR₈₅ foram observados com 98% de homologia, e TR₂₇ exibiu 88% em seus respectivos isolados mais próximos nas sequências ITS. Os isolados de TR₂₇ e TR₈₅ também exerceram sua respectiva homologia

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mais próxima (96%), enquanto TR₄₅ apresentou 99% e 93% de homologia com TR₇₀ em sequências de TEF. Os isolados TR₄₅, TR₇₀ e TR₈₅ foram evidentemente determinados como *T. asperellum* com 100% de valor de *bootstrap*, e o isolado TR₂₇ também foi reconhecido com 72% de valor de *bootstrap* na árvore filogenética. Entretanto, efeitos complementares de homologia superior significativa e maior valor de *bootstrap* na identificação de *T. asperellum* foram considerados dignos de nota. Na análise filogenética, foi observada uma diferenciação magnífica entre os isolados de *Trichoderma* dentro e entre os grupos de espécies intimamente relacionadas na região Tef1, refletindo a variabilidade máxima nos isolados de rDNA na região ITS, ao mesmo tempo que demonstrava uma maior taxa de transversão e divergência evolutiva.

Palavras-chave: antagonismo, ITS, pH, temperatura, Tef1, *Trichoderma asperellum*.

1. Introduction

Trichoderma spp. greatly emphasise the new dimension for modern agriculture due to their diversified metabolic function and their antagonistic habit against plant pathogens (Japanis et al., 2022). *Trichoderma* spp. are also soil inhabitants, act as biocontrol agents as well as controlling of plant diseases, and facilitate naturally and environmentally effective, and alternative to existing chemical treatments (Shalini and Kotasthane, 2007). Biological control asserts as the most important tool in reducing the risks and dangerous effects of indiscriminate use of fungicides, resulting in the notable success to protect the crops against the plant diseases (Ozkara et al., 2016; Rur et al., 2018). Application of *Trichoderma* spp. as in biofertilizers and biopesticides is also used as bioeffectors (Marra et al., 2019; Rivera-Méndez et al., 2020) and functions as bioremediation for heavy metals and other pollutions (Tripathi et al., 2013; Zhang et al., 2018).

Trichoderma has its significant attributes as biocontrol agents in controlling soil borne plant pathogenic fungi such as *Fusarium*, *Sclerotium* and *Rhizoctonia* (Begum et al., 1999; Sultana and Hossain, 2000). In recent years, *Trichoderma* exhibits its potential effects in controlling the diseases of damping off and root-rot (Mahmud et al., 2020; Khadka and Miller, 2021). *Trichoderma* spp. also exert competition with soil borne pathogens in the bio-control mechanisms for nutrients, spaces and root colonisation, and often it causes stimulation with resistance and immune system in plants (Yu et al., 2022).

Trichoderma spp. emerge mycoparasitism in antagonistic mechanisms while recognizing the pathogen, and its mycelium proceeds in confront with pathogen mycelium in a coiling fashion that results in dissolution with death of the pathogen. Consequently, cell wall-degrading enzymes (CWDEs) consist of chitinases, glucanase and proteases, that are produced due to the mycoparasitism, which penetrate the pathogen mycelium and nourishes its nutrients from dissolved pathogen (Sharon et al., 2007; Bailey et al., 2008), where secondary metabolites are released that inhibit the pathogen. Different kinds of enzymes such as indole acetic acid, lignins and phenolic products which are deposited in the host plants, offering defence in response to fungal infection (Reddy et al., 2014; Ozkara et al., 2016).

The growth and optimum sporulation of *Trichoderma* spp. have been influenced at 30-35 °C temperature (Sangle et al., 2003), ranging from 20 °C to 37 °C for the best growth and sporulation (Jayaswal et al., 2003). The highest growth and sporulation of *T. viride* are also linked with pH of 4.5 to 5.5 (Jayaswal et al., 2003), and potentiality

of *Trichoderma* species that has been greatly attributed to pH, the most favourable pH range of 6.5-7.5 which demonstrate the functional growth and sporulation of *Trichoderma* (Srivastava et al., 2014).

Molecular characterization focuses on *Trichoderma* taxonomy and also in fungal diversifications in RAPD analysis, characterizing the eleven isolates of *T. viride* and eight isolates of *T. harzianum* both with ITS-PCR of rDNA region of ITS1 and ITS4 primers that reveal genetic variability in constructing eight clusters with 600 bp products among the isolates (Chakraborty et al., 2010). Twelve isolates of *Trichoderma* spp. of different locations in South Andaman are characterised with ITS-PCR and the sequence analysis of these isolates differentiates seven different species of *Trichoderma* spp. (Kumar et al., 2012).

Significant effect of BAU-Biofungicide (*Trichoderma* based preparation) is critically observed in controlling the seed borne *Ralstonia solanacearum* of Brinjal and tomato (Dey et al., 2024), and also results in inhibiting the leaf blight pathogen of wheat (Hossain and Monjil, 2015). Notably, higher germination rate and plant stand, low disease severity and maximizing grain yield of rice have been greatly influenced by BAU-Biofungicide (Mahmud and Hossain, 2017; Mahmud et al., 2020).

Application of *Trichoderma* spp., an alternative to fungicide is needed as the development of efficient measures to combat the diseases of crops in order to grow healthy crop production. Although research on *Trichoderma* spp. has been led sporadically in Bangladesh but country-wise detailed research on rhizosphere soil of different crop species for *Trichoderma* based biocontrol agents, their characterization and antagonistic study have a confined focus in different Agro-Ecological Zones (AEZs). Therefore, *T. asperellum* reveals the resistance-related molecular mechanisms with physiological and molecular characters due to the higher mycelial growth, sporulation rate and strong inhibitory effects against the pathogens, releases the antimicrobial secondary metabolites that induces the plant resistance (Wu et al., 2017). Nevertheless, the present research has been designed to isolate, and to identify *T. asperellum* with physiological and molecular characterization collected from the rhizosphere soil samples of 49 different crops across 107 locations in different AEZs of Bangladesh, and to evaluate their antagonistic effects of identified *Trichoderma* isolates on *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*, causing foot and root rot, collar rot and damping off diseases in lentil, respectively.

2. Materials and Methods

2.1. Experimental site

The experiments were conducted during January 2015 to December 2018 at the Eco-friendly Plant Disease Management Laboratory, Molecular Plant Pathology Laboratory and Biosafety Laboratory of the Department of Plant Pathology, and Net house of Prof. Golam Ali Fakir Seed Pathology Center, Bangladesh Agricultural University, Mymensingh.

2.2. Isolation, purification and preservation of *Trichoderma* from rhizosphere soil samples in 20 agro-ecological zones (AEZs) of Bangladesh

2.2.1. Collection of soil samples, preparation of soil dilution and isolation

A total of 107 rhizosphere soil samples along with 49 different crops was collected for the isolation of *Trichoderma* species from 20 AEZ in 36 different districts of Bangladesh, consisting of 107 locations as presented in Table S1. The soil samples were kept in a refrigerator at 4 °C in the laboratory until further use. Afterward, one gram of soil was kept in a test tube with 9 mL of sterile water and stirred thoroughly for a few minutes to obtain a uniform at 10⁻¹ dilute soil suspension, representing as a stock suspension. Then 1 mL of the suspension was transferred with a sterile pipette into a second test tube containing 9 mL sterile water and it was shaken thoroughly, providing a 10⁻² dilute soil suspension, while a similar process offered the dilution of 10⁻³. Meanwhile, *Trichoderma* was isolated from soil samples using the soil dilution plate technique method (Askew and Laing, 1993).

2.2.2. Isolation of *Trichoderma* from soil and pure culture preparation

Twenty mL of warm melted PDA medium was (approx. 45 °C) poured in each sterile petri-plate. Half mL of diluted soil sample (10⁻³) was inoculated at the centre of a plate on PDA, spread with a glass rod and inoculated with 0.5 mL of diluted sample, while it was repeated with every soil sample. The inoculated PDA plates were incubated at 28 ± 2 °C for 4 days and plates were observed for purified *Trichoderma* colonies after incubation (Kumar et al., 2012). The growing margin of the *Trichoderma* colony was cut into 6 mm discs with a cork borer and was carefully laid in a new PDA plate to produce a pure culture of *Trichoderma*, and the plates were incubated at 28 ± 2 °C for 7 days (Kumar et al., 2012), exhibiting the growth of pure culture. The cultures were prepared as subcultures to PDA plates and were transferred to PDA slants for preservation as separate cultures and the slants were preserved in the refrigerator at 4 °C, while the isolates were identified (Kubicek and Harman, 1998).

2.2.3. Morphological and physiological characterization of collected *Trichoderma*

The isolates were characterized morphologically and were maintained on potato dextrose agar (PDA) medium at

28 ± 2 °C (incubation) for identifying cultural characteristics (Samuels et al., 2002; Kumar et al., 2012). Radial mycelia growth of the isolates was determined following the method (Sultana et al., 2006). After 24 hours of inoculation, colony diameter was measured up to 6 days, while the colonies were filled with the plate, and sporulation occurred within 6 days. Moreover, the radial mycelial growth was recorded with the mean of two perpendicular diameters after 24, 48 and 72 hrs of inoculation, and the mean of three replications was computed as growth of each isolate.

The number of colonies was observed per gram of soil, resulting in the colonies regular or irregular in shape, while colony colours were black/ white/ grey/ blackish/ whitish/ blackish white/ whitish black. After 7 days of incubation of a single culture on PDA, mean radial growth of the isolate was measured following the method (Sultana et al., 2001) (Equation 1).

$$\text{Mean radial growth} = \frac{\text{Length} + \text{Breadth}}{2} \quad (1)$$

Compact or loose characters were determined on the basis of compactness of the colony.

The surface of the culture was noted as smooth/ glistening/rough/wrinkled/ dull etc.

The opacity of the culture such as transparent (clear), opaque, translucent (like looking through frosted glass) etc. was ascertained.

Number of spores per mL: Conidia per mL after 7, 14 and 21 days of incubation were determined following the formula (Gampala and Pinnamaneni, 2010) (Equation 2).

$$\text{Sporulation} = \frac{\text{Number of spores counted} \times \text{Dilution}}{\text{Number of smallest square counted}} \times 4000 \quad (2)$$

2.3. Measurement of spore density of fifteen isolates

Eighty eight *Trichoderma* isolates obtained from the rhizosphere soil of 49 different crops in 107 locations that categorized viz., fast, medium, and slow growing on the basis of their growth rate, while the fast growing fifteen isolates were ascertained for further study across the isolates. The selected *Trichoderma* isolates were grown on PDA medium using the method (Gampala and Pinnamaneni, 2010) and the mycelial blocks (1 cm²) of pure cultures were transferred in the medium, and incubated the plates at 30 °C on 7, 14, and 21 days for growth and sporulation. Meanwhile, 7, 14 and 21 days old culture was scraped with a slide from a pure culture petri plate and kept in a different test tube with 100 mL water, making a *Trichoderma* suspension. Later on, 10 mL suspension was poured in each PDA plate and incubated at 30 °C for 7, 14 and 21 days. Afterwards, the colony was scraped smoothly with a glass rod to collect conidia from petri plate, and kept in beaker with 100 mL sterile water for conidial suspension and stirred with a glass rod continuously, and 1 drop of Tween-20 was added to it and dispersed well. One drop of suspension of the solution was taken on the center of the haemocytometer and a cover slip was kept on it.

The spores per mL as spore density were counted under the field of microscope at 40X with a haemocytometer using the block system. In case of spore density at 7 days old culture for 25 °C, 30 °C and 35 °C, the following works were done in the above method as the spore density of 30 °C for 7, 14 and 21 days old culture was determined.

2.4. Effects of temperature on mycelial growth of *Trichoderma* spp.

The effect of temperature on mycelial growth of *Trichoderma* spp. was evaluated in terms of six levels of temperature viz., 15, 20, 25, 30, 35 and 40 °C, as the experiment was conducted in three incubators (Froilabo, model no. 69330 MEYZIEU-FRANCE) with different temperatures. On the basis of colony diameter of mycelium, the influence of temperature levels on mycelial growth was determined, and the fungus was grown on PDA plates for radial colony growth.

2.5. Effects of pH on mycelial growth of *Trichoderma*

The effect of pH on mycelial growth of *Trichoderma* was tested against the four levels of pH at 6.0, 7.0, 8.0 and 8.5. Whilst the PDA was prepared, using three PDA plates for each treatment. The pH of the medium was adjusted to the required level with an electrode pH meter, while NaOH of 0.1N and HCl of 0.1N were added to increase and decrease pH levels, respectively. The pH level was also adjusted at 6.5 after extracting the potato, whereas the medium was boiled on a microwave oven for melting the agar powder. The media was kept in controlled conditions and inoculated the PDA plates with mycelial discs of the fungus. While the inoculated plates were incubated at 30 °C for its growth habit, and recorded the data on radial colony diameter.

2.6. Potentiality of *Trichoderma* against some important soil borne plant pathogens

2.6.1. Isolation of soil borne plant pathogens

Pythium aphanidermatum and *Fusarium oxysporum* were isolated from infected roots of lentil crops obtained from the field of Bangladesh Agricultural University, Mymensingh, following the method of Chittem et al. (2015) and isolation of *Sclerotium rolfsii* from infected chickpea roots was done in the method of Shirsole et al. (2018). Moreover, *Sclerotium rolfsii* was identified with morphological characteristics comprising of colour, branching and presence of sclerotia as described by Watanabe (2002) and *Pythium aphanidermatum* was determined morphologically following the method of Matsumoto et al. (1999) and *Fusarium oxysporum* was identified of its colony characteristics using morphological criteria of Leslie et al. (2006). Later on, the pure cultures of pathogens were preserved in PDA at 5 ± 1 °C as stock culture for further study.

2.6.2. Determination of antifungal properties of *Trichoderma* isolates

The *Trichoderma* was led to test their antifungal activity against the three soil borne fungi by dual culture

technique in *in-vitro* bioassays (Bastakoti et al., 2017). A 6 mm diameter mycelia disc from the margin of the *Trichoderma* with 7 days-old culture of isolates and the soil borne pathogens both were kept on the opposite side of the plate at equal distance from the periphery. The experiment was conducted in a completely randomized design with three Petri dishes for each isolate. In the control plate (without *Trichoderma*), a sterile agar disc was put at the opposite side of the soil borne inoculated isolates' of plates and incubated the plates at 30 °C until the end of the incubation period (6 days after inoculation). Radial growth of isolates of the pathogen was measured at 3 and 6 days after incubation period (DAI), and computed the inhibition percentage of average radial growth (Mahmud and Hossain, 2017).

2.7. Pot experiment

This study was conducted separately for each of the foot and root rot, collar rot and damping off causal pathogens by selecting the lentil seeds of BINA Mushur-3 for the pot experiment. The pot experiment led to the net house of Prof. Golam Ali Fakir seed pathology centre, BAU, Mymensingh, having soil application of *Trichoderma* against *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum* causing foot and root rot, collar rot and damping off diseases in lentil, respectively.

2.7.1. Soil preparation, sterilization and pot filling

Soil was collected from the field laboratory of the Department of Plant Pathology, BAU, Mymensingh and cow dung from the dairy farm of BAU, Mymensingh, was added to it, whereas soil was mixed uniformly with cow dung at 2:1 (Soil: cow dung) and was sterilized with formalin (40%) at the rate of 5 mL formalin diluted with 20 mL of water for 7 kg soil. The formalin treated soils were covered with polythene sheet for 48 hrs and then exposed to 78 hrs aeration before pot filling of 7kg soil/pot (Dashgupta, 1988; Hossain, 2000), and were filled the fifteen pots with the treated soil.

2.7.2. Preparation of inoculum of *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum* and inoculation

The experiment was laid out in RCBD with three replications, including control of sixteen different treatments for soil treatment, while control pots were inoculated with pathogens alone. The inoculum of *Fusarium oxysporum* was grown on chickpea bran (Khan et al., 1998). Whilst the chickpea bran was soaked in water at the ratio of 3:4 (w/v) in a 2000 mL beaker, taking two hundred and fifty gram of chickpea bran in a 2000 mL beaker, and wrapped the beaker tightly with cotton and brown paper and were autoclaved for 30 minutes in 121 °C temperature with 15psi pressure. The sterilized chickpea bran was inoculated with 15 blocks (6 mm) of *F. oxysporum* in previously grown of PDA and was incubated at 25 ± 1 °C for 15 days, while the beaker was shaken occasionally during incubation for uniform growth of the fungus. The isolate *F. oxysporum* of chickpea bran was applied in pot soil before 3 days of lentil seed sowing @ 5g/kg of soil (Sivan et al., 1984). In case of

other pathogens such as *S. rolfisii* and *P. aphanidermatum*, similar procedure was followed for inoculum preparation.

2.7.3. Culture of the isolates of *Trichoderma* and application in soil

The isolates of *Trichoderma* were grown on chickpea bran (Islam et al., 2002), and the chickpea bran was soaked in water at the ratio of 3:4 (w/v) in a 2000 mL beaker, taking the chickpea bran (500 g) in a 2000 mL beaker and the beaker was closed tightly with cotton, wrapped by brown paper and autoclaved for 30 minutes in 121 °C temperature with 15psi pressure. The sterilized chickpea bran was inoculated with 15 blocks (6 mm) of *Trichoderma* isolates that was previously grown on PDA and was incubated at room temperature for 15 days. The culture of *Trichoderma* grown on chickpea bran was applied in pot soil @ 5g/kg of soil at the time of sowing seeds in pots (Sivan et al., 1984).

2.7.4. Determination of foot and root rot, collar rot/damping off

Lentil seeds (20/pot) were sown in equal distances with 3 cm depth in the pot soil. As the soil was moistened and weeding was done whenever necessary. No chemical pesticides were applied in the pot for controlling the pests and diseases of the crop, inspecting the pots regularly and the diseased plants were collected and were taken to the laboratory, Department of Plant Pathology, BAU, Mymensingh for identifying the pathogens (Begum et al., 1998).

2.7.5. Recording of data

Disease incidence was observed regularly and recorded at 7, 14 and 30 days after sowing of seeds. Data were collected on the different parameters viz., seed germination (%), foot and root rot (%), collar rot (%) / damping off (%), plant stand (%), root length (cm) / plant, shoot length (cm) / plant and fresh root and shoot weight (g) / pot.

2.8. Statistical analysis

Data on different parameters were analysed statistically using Web Agri Stat Package (Jangam and Wadekar, 2004) computer program to find out the significant variation of experimental treatments. The difference between the treatments was evaluated with Duncan's Multiple Range Test following the procedure (Seal et al., 1993; Gomez and Gomez, 1984).

2.9. Molecular characterization of selected *Trichoderma*

Four, out of 15 isolates were selected based on higher mycelial growth, their better spore density and tolerance to high temperature (40 °C) *in-vitro* condition. These were characterized using molecular markers of ITS4, ITS5 and TEF1, TEF2.

2.10. Preparation of broth culture to harvest mycelia of *Trichoderma* spp.

Trichoderma isolates were cultured individually in 150 mL conical flasks with 100 mL liquid potato dextrose

culture medium for DNA extraction representing broth culture. Hyphae were collected from potato dextrose broth (PDB) with mycelia and incubated for 7 days in the incubator at 25 ± 1 °C. Afterwards, it was kept in filter paper in a Buchner funnel, and was washed with distilled water in order to remove debris, then kept in frozen, and lyophilized. After harvest, fresh mycelia were wrapped with an aluminium foil sheet for each isolate separately and kept at 4 °C until genomic DNA isolation.

2.11. Genomic DNA extraction and determination of DNA concentration

The genomic DNA of each *Trichoderma* isolate was extracted from harvested mycelia of 3 days old culture following the protocol of Wizard® Genomic DNA Purification Kit solution: pH 8.0 (Promega, Madison WI, USA) (Year 2018). Forty mg of mycelia was cut into small pieces and was kept in a 1.5 mL eppendorf tube and liquid nitrogen was added to grind with a micro pestle. Six hundred µl of Nuclei Lysis solution was mixed and was vortexed for 20 seconds for proper mixing and incubated at 65 °C for 15 minutes in a hot water bath for digestion. The solution of 3µl of RNase was added and incubated at 37 °C for 15 minutes. Then the sample was cooled at room temperature for 5 minutes. Protein precipitation solution of 200 µl was mixed and was vortexed gently, and centrifuged at 15,000 rpm for 3 minutes. The supernatant was transferred in a clean tube without disturbing the lower portion and 600 µl was put together at room temperature. Then isopropanol was added to the supernatant and shaken slowly. Mixed by inversion was done and centrifuged at 15,000 rpm for 3 minutes to produce precipitation of the cell debris. Six hundred µl of 70% ethanol was mixed in a blended supernatant at room temperature and centrifuged at 15,000 rpm for 2 minutes. The pellet was air dried in order to evaporate the ethanol. DNA rehydration solution of 25 µl was added and mixed gently by finger tapping, and preserved the DNA solutions overnight at 4 °C.

The spectrophotometer was set at 260 nm for quantification of DNA. A square cuvette (the zero or blank cuvette) was filled with 2 mL double distilled water and kept in the cuvette chamber. Then the absorbance reading was adjusted to zero for standardization. The test samples were prepared with 2 µl of each DNA sample in the cuvette of 2 mL sterile distilled water and mixed comprehensively by pipetting. As the absorbance reading was taken at 260 nm, then the cuvette was rinsed out with sterile water, stamped out on a paper wipe, and absorbance reading for each sample was recorded in the same way. The original concentration was determined by using the absorbance readings in the following formula (Equation 3):

$$\text{DNA concentration (ng / } \mu\text{l)} = \text{Absorbance} \times \frac{\text{Volume of distilled water (}\mu\text{l)}}{\text{Amount of DNA sample (}\mu\text{l)}} \times (3) \\ CF (0.05) \times 1000$$

2.12. PCR amplification of the ITS region (ITS4 and ITS5) and TEF region (TEF1-728F and TEF2-986R)

Specific primers such as ITS4, ITS5, TEF1-986R and TEF2-728F were employed for matching the template DNA of the *Trichoderma* to confirm the *Trichoderma* through PCR. PCR reactions were determined on each amplified DNA with the sequence of TCCTCCGCTTATTGATATGC (5'-3') and GGAAGTAAAGTCGTAACAAGG (5'-3') both in the primer of ITS4 and ITS5, respectively (Seal et al., 1993), whereas the primer of TEF1-728F and TEF2-986R revealed the sequence CATCGAGAAGTTCGAGAAGG (5'-3') and TACTTGAAGGAACCTTACC (5'-3'). The amplification was carried out using the T 100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) following the profile (Hermosa et al., 2000). The thermal cycling profile was started at 95 °C for 5 minutes in pre-denaturation followed by 35 cycles at 94 °C for 1.5 minute, 2 min annealing at 55 °C, extension at 72 °C for 3 minutes and 5 minutes for 72 °C in final extension for all amplified fragments in ITS region. Thermal cycling profile of TEF region was begun with 2 minutes for 94 °C in pre-denaturation followed by 30 cycles at 98 °C for 10 seconds in denaturation, 30 second annealing at 55 °C, extension for 35 second at 72 °C and 10 minutes for 72 °C in final extension of all amplified fragments. Amplified DNA of all isolates and total 7 µl of 100 bp DNA ladder were used in the gel electrophoresis at 80 Volt for 55 minutes. The gel was stained with ethidium bromide for 30 minutes at room temperature and was transferred from the Ethidium bromide tray and kept on the UV transilluminator for image documentation of DNA bands and photographed with the Gel Documentation System.

2.13. DNA sequencing, analyses of nucleotide sequences and Phylogenetic analysis

DNA sequencing was done following the standard protocols (dna.macrogen.com) for the ABI 3730×1 DNA genetic analyser (Applied Biosystems, Foster City, CA, USA) with BigDye® Terminator v1.1 and 3.1 Cycle Sequencing Kits. The nucleotide sequences were submitted to the basic local alignment search tool (BLAST) in the national centre for Biotechnology Information database (NIH, 2022) to identify the *Trichoderma* isolate. The sequences were aligned with the closest sequences identified by the BLAST algorithm using CLUSTALW.

Phylogenetic analysis was conducted using the neighbor-joining method in MEGA 7 program (Kumar et al., 2016). Confidence values were assessed from 1000 bootstrap replicates of the original data.

3. Results

3.1. Selection of isolates for morphological and physiological characterization

Eighty eight isolates of *Trichoderma* were grouped, resulting in categorizing the isolates as fast, medium and slow growing in terms of their growth habit. Fifteen isolates were selected from the fast growing ones based on crops and locations (Table 1).

3.2. Spore density of different isolates of *Trichoderma* at 30 °C

Fifteen fast growing isolates of *Trichoderma* were determined for the number of spores per mL soil at 30 °C temperature in 7, 14 and 21 days old culture (DOC), while the number of spores per mL increased with the increasing age of the culture. The spore density of the isolates showed significant variation and the highest number of spores was obtained in the isolates of the maize field of Nayanpur upazila, Lalmonirhat district at 14 and 21 days old culture. Another large number of spores was found to be in the isolate of T₂₇ at 21 DOC that was collected from rice fields of Sadar upazila, Moulvibazar district (Table 1).

3.3. Effect of temperature on radial mycelial growth of different *Trichoderma* isolates

It was noteworthy that radial mycelial growth and spore density of *Trichoderma* were increased, which were consequently attributed to increased temperature, but spore density was decreased at 35 °C (Table S2). Four isolates such as Sadar, Moulvibazar, Sadar, Mymensingh, Chapra, Chapai nawabganj and Nayanpur, Lalmonirhat produced higher mycelial growth and spore density on PDA at different temperatures. Only two isolates such as TR₂₇ and TR₄₅ of *Trichoderma* could grow at the higher temperature of 40 °C, which were collected from Sadar upazila, Moulvibazar district and Sadar upazila, Mymensingh, respectively (Figure 1 and Figure S1). The 15 isolates of *Trichoderma* were emerged at regular shaped colonies under different temperatures of 15 °C, 20 °C, 25 °C and 30 °C. *Trichoderma* isolates were also magnified in five different colonies of colour viz. whitish, whitish green, green, dark green and light green, and two types of colony consistency such as compact and loose were markedly observed at different temperatures (Table S3).

3.4. Influence of pH on mycelia of different isolates of *Trichoderma*

Remarkably, fifteen isolates of *Trichoderma* exhibited higher average radial mycelial growth rate between pH 6.0 and pH 7.0, while the radial mycelial growth rate was reduced with the higher value of pH at 8.00 and 8.5. Four isolates such as Sadar Moulvibazar, Sadar Mymensingh, Chapra, Chapai nawabganj and Nayanpur, Lalmonirhat had better mycelial growth on PDA at different pH values (Figure 2). The 15 isolates of *Trichoderma* signified regular and irregular shaped colonies in different pH values of 6.0, 7.0, 8.0 and 8.50, that were recognised to its four different colony colours viz., whitish, green, dark green and light green whereas colony consistency of compact and loose in two types was esteemed at different pH values (Table S4).

3.5. Antagonism of *Trichoderma* as determined by the dual culture against *F. oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*

The antagonism of the *Trichoderma* isolates was tested against three different soil borne plant pathogens viz., *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum*. Moreover, the

Table 1. Spore density of different isolates of *Trichoderma* at 30 °C on 7, 14, 21 days old culture (DOC).

Isolate of <i>Trichoderma</i>	Location	Name of crop & scientific name	No. of spore/mL		
			7 DOC	14 DOC	21 DOC
TR ₉	Dashmina, atuakhali	Brinjal (<i>Solanum melongena</i>)	4.74×10 ⁷ cd	5.83×10 ⁷ de	7.50×10 ⁷ c
TR ₁₁	Kawkhali, Pirojpur	Taro (<i>Colocasia esculenta</i>)	4.65×10 ⁷ d	5.58×10 ⁷ ef	5.71×10 ⁷ c
TR ₂₅	Zaflang, Sylhet	Mustard (<i>Brassica nigra</i>)	4.55×10 ⁷ d	5.43×10 ⁷ ef	5.79×10 ⁷ f
TR ₂₇	Sadar, Moulvibazar	Rice (<i>Oryza sativa</i>)	5.81×10 ⁷ a	7.35×10 ⁷ b	9.55×10 ⁷ a
TR ₄₅	Sadar, Mymensingh	Sweet gourd (<i>Cucurbita muschata</i>)	5.61×10 ⁷ ab	7.44×10 ⁷ b	9.37×10 ⁷ ab
TR ₅₂	Sadar, Mymensingh	Papaya (<i>Carica papaya</i>)	4.86×10 ⁷ cd	6.43×10 ⁷ c	6.33×10 ⁷ ef
TR ₅₆	Melando, Jamalpur	Brinjal (<i>Solanum melongena</i>)	4.38×10 ⁷ d	4.54×10 ⁷ g	7.15×10 ⁷ c
TR ₆₄	Shipganj, Bogra	Maize (<i>Zea mays</i>)	4.91×10 ⁷ cd	6.45×10 ⁷ c	7.25×10 ⁷ c
TR ₇₀	Chapra, Chapai nawabganj	Sesame (<i>Sesamum indicum</i>)	5.92×10 ⁷ a	7.56×10 ⁷ b	8.82×10 ⁷ b
TR ₇₇	Sadar, Rangpur	Rice (<i>Oryza sativa</i>)	4.99×10 ⁷ cd	5.59×10 ⁷ ef	6.05×10 ⁷ ef
TR ₇₈	Gobindoganj, Gaibanda	Tobacco (<i>Nicotiana tabacum</i>)	4.68×10 ⁷ cd	5.24×10 ⁷ f	6.30×10 ⁷ ef
TR ₇₉	Sadar, Kurigram	Wheat (<i>Triticum aestivum</i>)	5.34×10 ⁷ bc	6.30×10 ⁷ cd	7.47×10 ⁷ c
TR ₈₂	Sadar, Kurigram	Okra (<i>Abelmoschus esculentus</i>)	4.39×10 ⁷ d	5.79×10 ⁷ de	7.08×10 ⁷ cd
TR ₈₅	Nayanpur, Lalmonirhat	Maize (<i>Zea mays</i>)	5.85×10 ⁷ a	8.66×10 ⁷ a	9.05×10 ⁷ ab
TR ₈₇	Pirganj, Thakargaon	Natishak (<i>Brassica chinensis</i>)	4.90 ×10 ⁷ cd	5.56×10 ⁷ ef	6.43×10 ⁷ ed
CV (%)			8.12	5.47	5.37

Data in column having same letter(s) do not differ significantly at 1% level of significance. DOC = Days old culture.

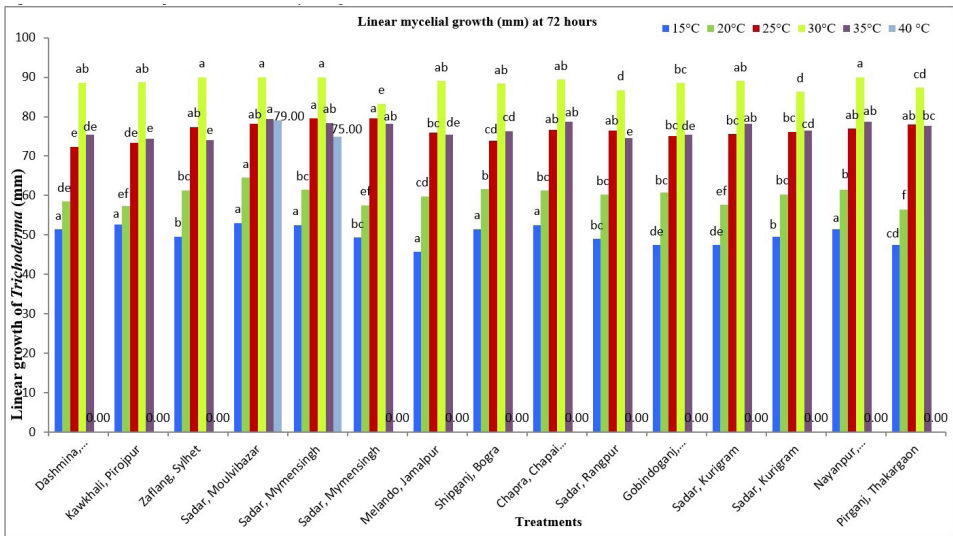


Figure 1. Effect of different temperatures on the radial mycelial growth of different isolates of *Trichoderma* in PDA at 72 hours. In Bar graph, dissimilar letter differ significantly at 1% level of significance as per DMRT. Linear mycelial growth at 40 °C only in Sadar Moulvibazar and Sadar Mymensingh.

four *Trichoderma* isolates of TR₂₇, TR₄₅, TR₇₀ and TR₈₅ inhibited the significant mycelial growth of *F. oxysporum* by 89.08-97.03% over control as presented in Table 2. The greatest (97.03%) reduction of mycelial growth of *F. oxysporum* was obtained in TR₂₇ over control at 9 days after inoculation and the lowest was in TR₇₉. Maximum inhibition of *S. rolfsii*

(87.61%) was found to have with TR₂₇ over untreated control followed by TR₄₅ (87.20%), TR₇₀ (87.16%) and TR₈₅ (85.98%) isolates. As the isolate TR₂₇ exhibited the highest reduction (89.33%) of *P. aphanidermatum*, and this was followed by the significant inhibition of isolates, TR₄₅ (86.26%), TR₇₀ (83.15%) and TR₈₅ (83.88%) (Table 2 and Figure S2).

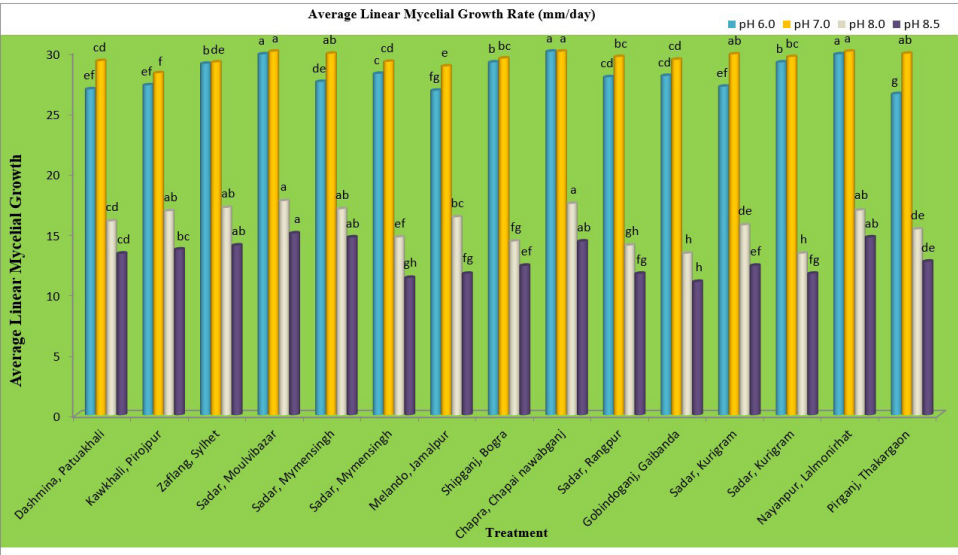


Figure 2. Effect of pH on the growth of different isolates of *Trichoderma*. In Bar Graph, dissimilar differ significantly at 1% level of significance as per DMRT.

Table 2. Antifungal activity of different isolates of *Trichoderma* against *Fusarium oxysporum*, *Sclerotium rolsii* and *Pythium aphanidermatum* (Dual culture).

Isolate of <i>Trichoderma</i>	<i>Fusarium oxysporum</i>		<i>Sclerotium rolsii</i>		<i>Pythium aphanidermatum</i>	
	9 DAI		6 DAI		9 DAI	
	Mycelial growth (mm)	% inhibition	Mycelial growth (mm)	% inhibition	Mycelial growth (mm)	% inhibition
TR ₉	3.16	89.22c	10.23	84.47cd	6.10	79.85d
TR ₁₁	5.16	82.38e	9.46	85.64bc	6.43	78.76d
TR ₂₅	2.88	90.27c	10.10	84.66cd	6.10	79.85d
TR ₂₇	0.88	97.03a	8.16	87.61a	3.23	89.33a
TR ₄₅	1.23	95.84a	8.43	87.20ab	4.16	86.26b
TR ₅₂	5.23	82.15e	11.23	82.95d	9.16	69.75g
TR ₅₆	4.16	85.10d	10.16	84.58cd	8.23	72.82f
TR ₆₄	3.30	88.73c	10.10	84.66cd	7.16	76.35e
TR ₇₀	3.20	89.08c	8.46	87.16ab	5.10	83.15c
TR ₇₇	2.23	92.46b	10.10	84.66cd	7.23	76.12e
TR ₇₈	7.62	73.99f	10.23	84.47cd	9.10	69.95g
TR ₇₉	8.10	72.35f	11.10	83.15d	8.46	72.06f
TR ₈₂	7.88	73.10f	10.16	84.58cd	9.23	69.52g
TR ₈₅	2.10	92.83b	9.23	85.98ab	4.88	83.88c
TR ₈₇	4.88	83.34de	9.88	85.00c	7.88	73.98f
CV (%)	-	1.29	-	1.27	-	1.57
Control	29.30	-	65.88	-	30.28	-

DAI = Days after Inoculation. Data in column having same letter(s) do not differ significantly at 1% level of significance.

3.6. Antagonistic effect of *Trichoderma* against *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum* on lentil

Seed germination (%), foot and root rot (%), plant stand (%), root length plant⁻¹ (cm), shoot length plant⁻¹ (cm), fresh root and shoot weight pot⁻¹ (g) of lentil were greatly influenced by *Trichoderma* treated soil inoculated with soil borne plant pathogen of *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum*. The highest (99.1%) germination of lentils was observed in the isolate TR₈₅, while the lowest germination (74.00%) was in untreated control. The control treatment resulted in maximum foot and root rot incidence (15.30%) and the lowest incidence (3.83%) in TR₈₅ treatment, showing an impressive reduction (74.97%) of foot and root rot disease over control. In terms of soil treatment, TR₈₅ stood out as the highest 95.28% plant stand which showed 62.31% higher increase over control. A maximum root length per plant (6.01 cm) was noted with TR₈₅ in soil treatment practices, where minimum root length per plant was in control. TR₈₅ isolate exhibited the highest (25.12 cm) shoot length per plant of lentil, and it was followed by TR₄₅ (24.40 cm) and TR₂₇ (23.29 cm). Notably, TR₈₅ displayed the maximum increase of fresh shoot and root weight per pot (118.65%) over control in treated soil followed by isolate of TR₄₅ (97.25%) and TR₂₇ (79.20%) (Table 3). In terms of *S. rolfsii*, the highest germination (98.10%) of lentil seed was observed with isolate TR₈₅ followed by TR₂₇, TR₄₅, TR₂₅ and TR₇₀. The lowest incidence 4.11% in TR₄₅ treatment marked 72.96% higher reduction of collar rot disease over control followed by TR₂₇ (72.04%), TR₈₅ (68.22%), TR₅₂ (66.12%) and TR₇₀ (64.21%). Maximum plant stand (94.12%) was observed in treated soil with the isolate TR₈₅, showing a potential increase of 63.83% over untreated control. Moreover, the isolate TR₂₇ exhibited the highest root length per plant (6.78 cm) in treated soil, while isolate TR₂₇ that was found to be maximum shoot length per plant (26.57 cm) of lentil followed by TR₄₅ (24.63 cm).

The greatest fresh shoot and root weight per pot (7.63 g) was estimated in treated soil with TR₂₇ followed by TR₈₅ (Table 4). In case of *P. aphanidermatum*, the highest (93.67%) germination of lentil with TR₇₀ isolate, while the lowest was found in untreated control as presented in Table 5. TR₂₇ isolate resulted in maximum 71.26% reduction of damping off disease over control followed by TR₄₅ and TR₇₀. The isolate of TR₇₀ marked the highest plant stand (89.27%) in terms of treated soil with a higher increase of 60.99% over untreated control. The maximum root length per plant (6.88 cm) was found to be noticed in isolate TR₇₀, followed by TR₈₅ (6.50 cm), TR₂₇ (6.14 cm) and TR₄₅ (6.12 cm) in case of soil treatment. While the lowest shoot length per plant (14.25 cm) in control, as the highest shoot length per plant (26.67 cm) of lentil was profound with TR₇₀. Fresh shoot and root weight per pot (7.94 g) was maximised in treated soil of TR₇₀ isolate followed by TR₄₅, TR₂₇ and TR₈₅ (Table 5).

Different isolates of *T. asperellum* exerted their varied antagonistic effects against the soil borne pathogens, while the isolates of *Trichoderma* inherited from various native sources with habitats showing potentiality of antagonistic habit. Moreover, diversified ecological conditions in interacting with biotic and abiotic factors might be the element, contributing to variable antagonistic effects.

3.7. Molecular features of selected *Trichoderma* isolates

Based on the radial mycelial growth, mycelial structure, spore density, colony colour, colony shape and colony consistency, only four isolates were considered for molecular characterization (Figure S3) that were characterized at molecular level in PCR amplification with ITS4, ITS5, TEF1 and TEF2 regions.

3.8. Molecular characterization

3.8.1. Characterization based on ITS and TEF region

Blast homology revealed that the *Trichoderma* isolate of Sadar, Moulvibazar (TR₂₇) showed significant 88% homology with *Trichoderma asperellum* in accession number (OM287548). In terms of pronouncing homology, the isolates of Sadar, Mymensingh (TR₄₅), Chapra, Chapai nawabganj (TR₇₀) and Nayanpur, Lalmonirhat (TR₈₅) exhibited the best homology of 98% in *T. asperellum* with accession number of OM287549, OM287550 and OM287551, respectively as presented in Table 6 and Figure 3. The impressive and significant homology (98%) of three isolates (TR₄₅, TR₇₀, TR₈₅) in the identification of *T. asperellum* at ITS region were found to be evident through molecular characterization, whereas the isolate of Sadar, Moulvibazar (TR₂₇) revealed the closest homology. Consecutively, isolates of Sadar, Moulvibazar, Chapra, Chapai nawabganj, Sadar, Mymensingh and Nayanpur, Lalmonirhat were defined as *T. asperellum* based on the sequence analyses of TEF1 of Blast program. Both the isolates of Sadar, Moulvibazar and Nayanpur, Lalmonirhat resulted in the best homology (96%) with *T. asperellum* in accession number of OM809398 and OM480709, respectively. Remarkably, *T. asperellum* was found to be the greatest homology (99%) in the isolate of Sadar, Mymensingh with accession number (OM480707), while the isolate of Chapra, Chapai nawabganj, exerted the significant percentage of homology (93%) in *T. asperellum* with accession number of OM480708 (Table 6 and Figure 4). Evidently, the four isolates were also found to show profound and superior homology in the determination of *T. asperellum* at TEF sequences in molecular study.

3.8.2. Phylogenetic tree of selected *Trichoderma* based on ITS and TEF sequences

The phylogenetic tree differentiated the four *Trichoderma* isolates based on the ITS sequences in two clusters (Figure 3). Cluster I consisted of 3 isolates, such as Sadar, Mymensingh, Chapra, Chapai nawabganj and Nayanpur, Lalmonirhat were ascertained as *T. asperellum* that was determined with maximal bootstrap value (100%) and the isolate of Sadar, Moulvibazar (Sub cluster II) also emerged as *T. asperellum* and it was supported with better bootstrap of 72%. Phylogenetic tree in the *T. asperellum* was also recognized with two clusters at TEF sequences whilst cluster I was grouped into two sub clusters. In cluster I, the isolate of Sadar, Mymensingh showed the significant bootstrap value (98%), and the superior bootstrap value (99%) was found to have with the isolate of Chapra, Chapai nawabganj. In sub-cluster II, the isolate in Moulvibazar district of Bangladesh was evident at 99% bootstrap value, whereas the isolate of Nayanpur, Lalmonirhat in cluster II amplified with the highest bootstrap value of 100% (Figure 4).

Table 3. Effect of soil treated with isolates of *Trichoderma* and inoculated with *Fusarium oxysporum* on seed germination (%), foot and root rot disease and growth parameters of lentil.

Treatments	Seed germination (%)	Incidence of foot and root rot (%)	Plant stand (%)	Root length plant ⁻¹ (cm)	Shoot length plant ⁻¹ (cm)	Fresh shoot and root weight pot ⁻¹ (g)
TR ₉	85.2 g (+15.13)	9.99 c (-34.71)	75.28 h (+28.24)	4.59 cde (+60.49)	18.02 h (+10.89)	4.11 g (+38.84)
TR ₁₁	87.0 fg (+17.57)	6.66 e (-56.47)	80.38 fg (+36.93)	4.05 f (+41.61)	20.22 ef (+24.43)	4.54 ef (+38.84)
TR ₂₅	91.1 d (+23.11)	6.33 e (-58.63)	84.78 cd (+44.43)	4.65 cd (+62.59)	20.21 ef (+24.36)	5.43 cd (+66.05)
TR ₂₇	95.4 bc (+28.92)	4.50 gh (-70.59)	90.94 b (+54.92)	5.30 b (+85.31)	23.29 b (+43.32)	5.86 c (+79.20)
TR ₄₅	96.6 ab (+30.54)	5.11 fg (-66.60)	91.55 b (+55.96)	5.80 a (+102.80)	24.40 a (+50.15)	6.45 b (+97.25)
TR ₅₂	90.4 de (+22.16)	5.89 ef (-61.50)	84.55 cd (+44.04)	4.63 cde (+61.89)	19.35 fg (+19.08)	5.27 d (+61.16)
TR ₅₆	87.5 efg (+18.24)	8.22 d (-46.27)	79.33 g (+35.14)	4.08 f (+42.65)	22.48 bc (+38.34)	4.56 ef (+39.45)
TR ₆₄	89.3 def (+22.30)	6.44 e (-57.91)	82.89 def (+41.21)	4.65 cd (+62.59)	20.39 e (+25.48)	4.40 efg (+34.56)
TR ₇₀	90.5 de (+24.73)	6.44 e (-57.91)	84.11 cde (+43.29)	4.91 bc (+71.68)	22.13 cd (+36.18)	5.80 c (+77.37)
TR ₇₇	92.3 cd (+17.57)	6.11 ef (-60.06)	86.22 c (+46.88)	4.18 ef (+46.15)	19.15 g (+17.85)	4.72 e (+44.34)
TR ₇₈	87.0 fg (+17.17)	12.44 b (-18.69)	74.56 h (+27.02)	4.18 ef (+46.15)	18.15 h (+11.69)	5.16 d (+57.80)
TR ₇₉	90.8 d (+22.70)	9.77 c (-36.14)	81.12 efg (+38.19)	4.04 f (+41.26)	21.35 d (+31.38)	4.20 fg (+28.44)
TR ₈₂	90.4 de (+22.16)	8.55 d (-44.12)	81.89 def (+39.50)	4.21 def (+47.20)	19.54efg (+20.25)	3.99 g (+22.02)
TR ₈₅	99.10 a (+33.92)	3.83 h (-74.97)	95.28 a (+62.31)	6.01 a (+110.14)	25.12 a (+54.58)	7.15 a (+118.65)
TR ₈₇	87.4 efg (+18.11)	6.77 e (-55.75)	80.67 fg (+37.43)	3.45 g (+20.63)	19.84efg (+22.09)	4.58 ef (+40.06)
Control (T ₀)	74.0 h	15.30 a	58.70 i	2.86 h	16.25 i	3.27 h
CV (%)	2.18	9.35	2.27	6.30	2.62	5.30

Data in column having same letter(s) do not differ significantly at 1% level of significance by DMRT at 30 days age of the plant. Data in parentheses indicate % increase (+) and % decrease (-) over control.

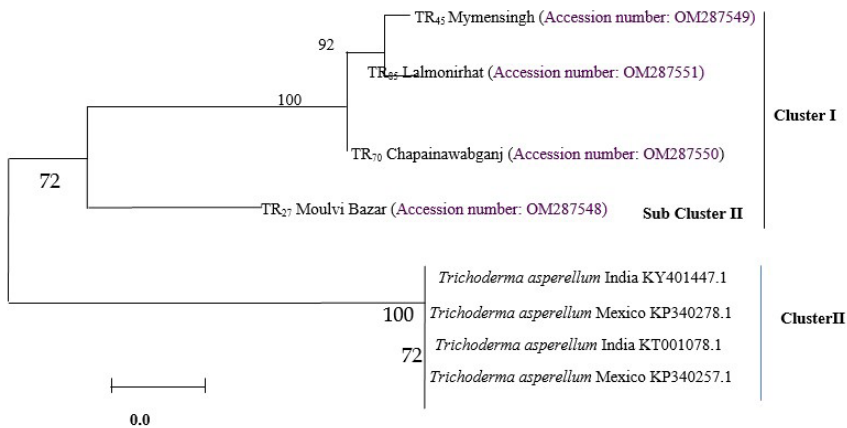


Figure 3. Phylogenetic relationships among the *Trichoderma* isolates in the analysis of ITS sequences.

Table 4. Effect of soil treated with isolates of *Trichoderma* and inoculated with *Sclerotium rolfsii* on seed germination (%), collar rot disease and growth parameters of lentil.

Treatments	Germination (%)	Collar rot (%)	Plant stand (%)	Root length plant ⁻¹ (cm)	Shoot length plant ⁻¹ (cm)	Fresh shoot and root weight pot ⁻¹ (g)
TR ₉	86.06fg (+19.03)	10.83b (-28.75)	75.23i (+30.95)	6.58abc (+213.33)	20.87de (+25.87)	4.58d (+81.75)
TR ₁₁	87.00efg (+20.33)	7.08d (-53.42)	79.92fg (+39.11)	6.64ab (+216.19)	23.21bc (+39.99)	3.54f (+40.47)
TR ₂₅	91.50bc (+26.56)	7.05d (-53.62)	84.45cd (+47.00)	5.96cd (+183.80)	21.63cd (+30.46)	5.76c (+128.57)
TR ₂₇	93.40b (+29.18)	4.25h (-72.04)	89.55b (+55.88)	6.78a (+222.86)	26.57a (+60.25)	7.63a (+202.78)
TR ₄₅	92.60b (+28.08)	4.11h (-72.96)	88.49b (+54.03)	5.49d (+161.43)	24.63b (+48.55)	5.29c (+109.92)
TR ₅₂	89.20b (+23.37)	5.15fgh (-66.12)	84.45cd (+46.99)	6.00bcd (+185.71)	18.45fg (+11.28)	4.50d (+78.57)
TR ₅₆	85.50gh (+18.26)	8.33c (-45.20)	77.40h (+34.72)	4.23ef (+101.43)	23.10bc (+39.32)	4.21de (+67.06)
TR ₆₄	85.30gh (+17.98)	6.44de (-57.63)	79.00gh (+37.51)	5.51d (+162.38)	20.18def (+21.71)	4.12de (+63.49)
TR ₇₀	90.00cd (+24.48)	5.44efg (-64.21)	85.10c (+48.13)	4.56e (+117.14)	22.94bc (+38.36)	5.30c (+110.32)
TR ₇₇	88.80de (+22.82)	6.11def (-59.80)	82.90de (+44.30)	5.47d (+160.48)	20.75de (+25.15)	5.32c (+111.11)
TR ₇₈	83.00h (+14.80)	10.44b (-31.32)	73.56i (+28.04)	4.81e (+129.05)	19.22ef (+15.92)	4.36de (+73.02)
TR ₇₉	87.80efg (+21.44)	8.77c (-42.30)	79.00gh (+37.51)	4.19ef (+99.52)	20.76de (+25.21)	3.92ef (+55.56)
TR ₈₂	88.40def (+22.27)	8.80c (-42.11)	79.86g (+39.01)	5.93d (+182.38)	19.51ef (+17.67)	4.10def (+62.69)
TR ₈₅	98.10a (+35.68)	4.83gh (-68.22)	94.12a (+63.83)	4.57e (+117.62)	21.20e (+27.86)	6.34b (+151.59)
TR ₈₇	88.50def (+22.41)	6.77d (-55.46)	81.67ef (+42.16)	3.79f (+80.48)	23.55b (+42.04)	4.55d (+80.56)
Control (T ₀)	72.30i	15.20a	57.45j	2.10g	16.58h	2.52g
CV (%)	1.71	9.45	1.32	7.43	4.92	7.23

Data in column having same letter(s) do not differ significantly at 1% level of significance by DMRT at 30 days age of the plant. Data in parentheses indicate % increase (+) and % decrease (-) over control. CV: Coefficient of variation.

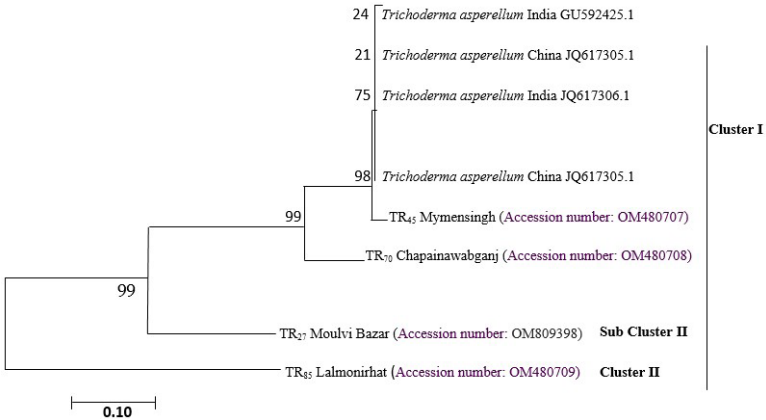


Figure 4. Phylogenetic relationships among the *Trichoderma* isolates by analysis of TEF sequences.

Table 5. Effect of soil treated with isolates of *Trichoderma* and inoculated with *Pythium aphanidermatum* on seed germination (%), damping off disease and growth parameters of lentil.

Treatments	Germination (%)	Damping off (%)	Plant stand (%)	Root length plant ⁻¹ (cm)	Shoot length plant ⁻¹ (cm)	Fresh shoot and root weight pot ⁻¹ (g)
TR ₉	88.33cd (+27.41)	9.83b (-31.26)	78.50gh (+41.57)	5.54def (+163.81)	20.25gh (+42.11)	4.70de (+76.03)
TR ₁₁	82.33hi (+18.75)	7.45c (-47.90)	81.65cde (+47.25)	5.63cde (+168.09)	24.29bc (+70.46)	4.82de (+80.52)
TR ₂₅	83.67ghi (+20.68)	5.25h (-36.71)	78.56h (+41.68)	5.08fg (+141.90)	21.21f (+48.84)	5.12cd (+91.76)
TR ₂₇	91.33ab (+31.73)	4.11j (-71.26)	87.44a (+57.69)	6.14bc (+192.38)	23.54d (+65.19)	5.45c (+104.12)
TR ₄₅	87.33cde (+25.96)	4.15j (-70.98)	83.25bc (+50.13)	6.12bc (+191.43)	24.42b (+71.37)	7.14b (+167.42)
TR ₅₂	87.67cde (+26.45)	6.15fg (-43.01)	81.52cde (+47.02)	5.92cd (+181.90)	19.95h (+40.00)	5.00cd (+87.27)
TR ₅₆	82.33hi (+18.75)	6.33ef (-55.73)	76.34i (+37.67)	4.95g (+135.71)	23.89cd (+67.65)	4.14efg (+55.06)
TR ₆₄	88.00cd (+26.93)	7.35c (-48.60)	80.69def (+45.52)	5.83cd (+177.62)	21.10f (+48.07)	3.59h (+34.46)
TR ₇₀	93.67a (+35.11)	4.47ij (-68.74)	89.27a (+60.99)	6.88a (+227.62)	26.67a (87.16)	7.94a (+197.38)
TR ₇₇	81.33i (+17.31)	6.55de (-54.19)	74.89i (+35.06)	4.42hi (+110.47)	20.35gh (+42.81)	4.18efg (+56.55)
TR ₇₈	84.67fgh (+22.13)	6.44def (-54.97)	78.85gh (+42.20)	5.12efg (+143.81)	19.12i (+34.18)	4.10efg (+53.56)
TR ₇₉	85.33efg (+23.08)	5.77g (-59.65)	79.56fgh (+43.48)	4.10i (+95.24)	22.12e (+55.23)	4.21efg (+57.68)
TR ₈₂	89.67bc (+29.34)	7.40c (-48.25)	82.65bcd (+49.05)	4.90gh (+133.33)	20.54g (+44.14)	4.69gh (+75.66)
TR ₈₅	89.00bcd (+28.37)	4.83i (-66.22)	84.17b (+51.79)	6.50ab (+209.52)	24.19bc (+69.75)	5.22cd (+95.51)
TR ₈₇	86.67def (+25.01)	6.77d (-52.66)	80.12efg (+44.49)	4.89gh (+132.86)	23.55d (+65.26)	4.32ef (+61.80)
Control (T ₀)	69.33j	14.30a	55.45j	2.10j	14.25j	2.67j
CV (%)	1.69	3.42	1.56	5.96	1.32	7.88

Data in column having same letter(s) do not differ significantly at 1% level of significance by DMRT at 30 days age of the plant. Data in parentheses indicate % increase (+) and % decrease (-) over control. CV: Coefficient of variation.

Table 6. Closest relatives of the selected *Trichoderma* isolates based on ITS and TEF sequences.

Isolates	Closest relatives	Sequences	Alignment	Homology
TR ₂₇	<i>Trichoderma asperellum</i> India (KY401447.1)	ITS	144/163	88
TR ₄₅	<i>Trichoderma asperellum</i> _Mexico KP340278.1)	ITS	578/588	98
TR ₇₀	<i>Trichoderma asperellum</i> _India (KT001078.1)	ITS	556/569	98
TR ₈₅	<i>Trichoderma sperellum</i> _Mexico (KP340257.1)	ITS	578/588	98
TR ₂₇	<i>Trichoderma asperellum</i> _India_JQ617306.1	TEF	178/185	96
TR ₄₅	<i>Trichoderma asperellum</i> _India_GU592425.1	TEF	299/303	99
TR ₇₀	<i>Trichoderma asperellum</i> _China_JQ617305.1	TEF	262/281	93
TR ₈₅	<i>Trichoderma asperellum</i> _China_JQ617305.1	TEF	178/185	96

4. Discussion

Eighty eight isolates were collected from rhizosphere soils of 49 different crops in 107 different locations throughout thirty six districts in Bangladesh. The identified isolates of *Trichoderma* spp. showed distinct variation in colony colour, radial growth, growth habit, consistency, conidia and spore density which findings were complemented with the researcher (Kucuk and Kivanc, 2003; Gezgin et al., 2023). Significant variations among different isolates, and mycelial growth, colony consistency and sporulation rate were also differentiated in morphological characteristics of *Trichoderma* spp. that were aligned with the findings (John et al., 2015). According to Lin et al. (2023), the morphological differences on PDA might be associated to nutritional factors, as well as environmental and genetic factors resulted in influencing fungal growth and development, where biological control agents were also sensitive to environmental conditions that had been cited for inconsistent performance (Guigon-Lopez et al., 2010).

We found that temperature and pH greatly influenced the mycelial growth and sporulation of the *Trichoderma* isolates. Previous studies revealed the highest radial mycelial growth at 30 °C temperature (Sharma et al., 2005; Singh et al., 2014), whereas our study highlighted that mycelial growth and sporulation were observed at the temperature ranging from 15 to 30 °C, perchance, the growth and sporulation was increased at the temperature of 40 °C. Conversely, the species of *Trichoderma* exhibited the best growth at the range of 25-30 °C (Singh et al., 2014; Srivastava et al., 2014). In terms of pH, our results in the studies showed that average radial mycelial growth was obtained between pH of 6.0-7.0, and reduced the fungal growth to some extent at higher pH 8.0. These findings were in agreement with the research of Shahid et al. (2011) and Singh et al. (2014), who observed that the highest radial mycelial growth was attained between pH 6.0 and pH 7.0. Potentiality of *Trichoderma* species which had been greatly influenced by pH 6.5-7.5 for the growth and sporulation as reported by Srivastava et al. (2014).

Significant inhibitory effects on the growth of the phytopathogenic fungi *Fusarium oxysporum*, *Pythium aphanidermatum* and *Sclerotium rolfsii* were observed using dual culture assay in our study. Similar findings were supported by Mishra et al. (2011) and Alamri et al. (2012). Alamri et al. (2012) who reported that 336 screened strains of *Trichoderma in-vitro* test by dual cultures were studied, resulting in the significant reduction of the growth of *F. oxysporum*, *P. ultimum* and *Rhizoctonia solani*. Moreover, the results in our study enlightened that the tested four *Trichoderma* isolates against *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum* reduced the highest mycelial growth of 97.03%, 87.61% and 89.33% over untreated control, respectively in dual culture. These findings were consistent with the researchers, led by Mishra et al. (2011) and Devi et al. (2012). As per previous results, several isolates of *Trichoderma* in dual culture against *Rhizoctonia solani*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, and *Fusarium solani* were found to show profound effect in the substantial growth reduction of 70%, 68.2%, 70.0% and 73.3%,

respectively as indicated in the study of Mishra et al. (2011). These findings were further supported by Choudary et al. (2007) and Hossain and Hossain (2013). Moreover, the antagonistic effect of *T. harzianum* against *S. rolfsii*, *R. solani* and *F. oxysporum* was evident in dual culture, showing an impressive inhibition of 83-92% (Choudary et al., 2007).

In the present study, 15 isolates were studied under pot conditions against foot and root rot, collar rot and damping off disease caused by *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum*, respectively. The findings in our study, clearly revealed that TR₈₅ isolate (*Trichoderma* sp.) showed the lowest (3.83%) disease incidence of foot and root rot disease, and maximum root length and shoot length per plant of lentil seedling were found to be 6.01 cm and 25.12 cm, respectively, additionally TR₈₅ emerged the highest increase of fresh shoot and root weight per pot (118.65%) over control in pots treated soil of *F. oxysporum* with *Trichoderma* sp.. The similar findings were investigated by the previous researchers (Kubicek et al., 2003; Kamala and Devi, 2012; Dubey et al. 2007). The lowest disease incidence due to the higher level of mycoparasitism has been observed, leading to released enzymes by these *Trichoderma* isolates (Kubicek et al., 2003). These findings were in line studied by Kamala and Devi (2012) and Dubey et al. (2007), who found that the treated plants of *F. oxysporum*, and *Trichoderma* sp. evidently exerted the lowest (9%) disease incidence, attributing to the highest shoot length (74.67 ± 1.45 mm), and root length (75.33 ± 1.45 mm) in maximal growth compared to controls. Nevertheless, the treated plant with *Trichoderma* spp. maximized 1.7 times of fresh shoot weight compared to an infected control plant, inoculated with the *Rhizoctonia* pathogen (Mahmoodian et al., 2022). From the current study, a maximum (71.26%) reduction was noted in the application of the *Trichoderma* isolate TR₂₇ against damping off disease of lentil seedling in treated soil with *P. aphanidermatum* compared to control. Kipngeno et al. (2015) also reported that *T. asperellum* was also used with seed as coating for management of seedling damping-off disease caused by *P. aphanidermatum* in tomato production, and exhibited the lower disease incidence. Similar results were also supported by Kamala and Devi (2012) and Dubey et al. (2007). Our studies resulted in the highest (98.10%) germination of lentil seedling with *Trichoderma* isolate TR₈₅, while 72.96% higher reduction of collar rot disease was found over control in case of *S. rolfsii* treated soil in pot culture. Similar observations were also reported on other host pathogen systems (Datta and Das, 2002; Liton et al., 2019), who studied *T. harzianum*, *T. viride* and *T. koningii* on disease incidence and growth parameters against collar rot of tomato and bush bean (*S. rolfsii*), resulting in reducing significant disease incidence and increasing dry mass weight of shoot and root (g/plant). Consecutively, fifteen isolates of *Trichoderma* spp. against *R. solani* (RS) and *F. oxysporum* f. sp. phaseoli (FOP), causing damping-off and wilt of bean, whilst *T. harzianum* (Th1), *T. viride* (Tv1) and *T. spirale* (Ts3) isolates implied different inhibitory effects against both of the tested pathogens (Sallam et al., 2008), showing the great reduction of damping-off and wilt diseases, and enhancing shoot length, root length, and vigour index of bean plants. These findings were also

supported by Faruk et al. (2002) and Benítez et al. (2004). Notably, *Trichoderma* exerted its superior attributes as mycoparasite, stronger opportunistic invader, prolific producer of spores and powerful antibiotics, antifungal compounds, secondary metabolites and enzymes as reported by Kubicek et al. (2003). In our study, *Trichoderma* prefaces profound results in reducing diseases of foot and root rot, collar rot and damping off diseases, and magnifying the growth parameters of lentil seedling in the pot experiment.

The molecular characterisation in the ITS region and TEF1 genes of the *Trichoderma* isolates exhibited that four *Trichoderma* isolates were partitioned in two clusters. Moreover, in our study, PCR based identification of ITS and TEF1 genes determined the evidence of genes at molecular level, whereas ITS and TEF1 revealed the highest homology, ranging from 88 to 99% homology. These findings were aligned with the researchers by Wu et al. (2017) and Gezgin et al. (2023). Wu et al. (2017) who indicated that the highest homology was observed with *T. asperellum* in phylogenetic analysis of ITS sequences in the NCBI BLAST programme. Consequently, at the higher percentage of homology, *Trichoderma* isolates might be determined within the species level based on the sequences of its gene region, contrasting with the sequences as registered in the NCBI GenBank. Similar observations were supported by Xue et al. (2021), Kumar et al. (2012) and Hoyos-Carvajal et al. (2009). Fifteen isolates of *T. asperellum* were found to be ascertained in DNA sequence data analysis of the ITS1-ITS2 and TEF1 region, and TEF1 showed the higher variation with greater transition than ITS1-ITS2 (Devi et al., 2012). In our findings, *Trichoderma* isolates were not fairly differentiated in ITS4 and ITS5 regions while these were clearly separated with TEF1 sequence analysis into different clusters, and the sequence analyses with TEF1 were amplified with better markers to distinguish *Trichoderma* species because of greater transition ratio and evolutionary divergence. However, the phylogenetic analysis in TEF1 extended at wider differentiation among the *Trichoderma* isolates within and among the groups of closely related species, resulting in the higher level of variability in TEF1 than in the rDNA of the ITS region as reported by Druzhinina et al. (2005) and Samuels (2006). While the twelve *Trichoderma* strains as *T. asperellum* were identified in phylogenetic analysis of the translation elongation factor 1 (TEF1), and were expanded with primers of TEF1-728F in TEF-1 gene (Xue et al., 2021). The translation elongation factor (TEF1) gene sequences also exerted 20 *Trichoderma* isolates in the phylogenetic analysis (Rai et al., 2016) which were in complementing the works (Al-Sadi et al., 2015; Hermosa et al., 2004). However, greater than 80% bootstrap support values were verified in the identification of *Trichoderma* species with the corresponding representative strain in phylogenetic analysis based on TEF1, while the isolate Sadar, Mymensingh in cluster I pronounced 98% bootstrap value, and 99% bootstrap value was also found in the isolate Chapra, Chapai nawabganj which were also evident as reported by Devi et al. (2012) and Dou et al. (2020). As we found the isolate of Moulvibazar district in sub-cluster II and the isolate Nayanpur, Lalmonirhat that were observed in cluster II with 100% bootstrap value.

Besides this, a well-supported bootstrap value of 87% was also categorized with the representative strain (TR 48) of the new species of *T. asperellum* (Hermosa et al., 2000) which was remarkably noted in similar observations (Devi et al., 2012; El Komy et al., 2015).

In our present study, mycelial growth and sporulation rate were found to remain at the temperature ranging from 15 to 30 °C, while the highest temperature (40 °C) also increased the growth and sporulation. Meanwhile, the species of *Trichoderma* resulted in the best growth at the range of 25-30 °C as reported in the study of Singh et al. (2014) and Srivastava et al. (2014). In this context, the wider variation of temperature in the convenient effect of fungal growth and sporulation due to the variable nutritional status and microclimatic conditions of *Rhizosphere* soil derived from different crop species. On the other hand, our findings marked the average radial mycelial growth from pH 6.0 to pH 7.0, and at higher pH 8.0, the fungal growth declined to some extent. It was noted that Shahid et al. (2011) and Singh et al. (2014), who observed the highest radial mycelial growth at pH ranging from (6.0-7.0). Notably, the inhibiting effect of *Trichoderma* at higher pH (8.0) was not consistently evident in the published form of report in recent years. The superior and impressive bootstrap value of displayed *T. asperellum* was contributed to the profound effect in magnifying the characteristics towards the development of *Trichoderma* strain vibrantly.

5. Conclusion

The fifteen *Trichoderma* isolates pronounced growing and sporulating in PDA medium with pH value and temperatures in physiological features, while *Trichoderma asperellum* was determined in the 4 isolates using molecular characterization across the isolates of different 107 locations, resulting in the composite representative of *Trichoderma* covering 20 Agro-Ecological Zones (AEZs). Four isolates such as Sadar, Moulvibazar (Rice), Sadar, Mymensingh (Sweet gourd), Chapra, Chapai nawabganj (Sesame) and Nayanpur, Lalmonirhat (Maize) also revealed *Trichoderma asperellum* at ITS region and TEF1 nucleotide sequencing. Nonetheless, the *Trichoderma* isolates were identified to native in Bangladesh as *Trichoderma asperellum* strains in four crops (Rice, Sweet gourd, Sesame and Maize) that were found to be rendered as tested in physiological with molecular characterisation, having potential antagonists in antagonism. Moreover, the various soil borne pathogens viz., *F. oxysporum*, *S. rolfii* and *P. aphanidermatum* pose the great threat to the pulse crops, resulting in the severe disease of foot and root rot, collar rot and damping off, respectively, whereas the maximum reduction of disease incidence has been indicated, when the *Trichoderma* is treated with pot soil. Preferably, our results of *Trichoderma* as antagonists also attribute to emerged plant stand, prolonged root length, shoot length and fresh shoot weight and root weight of lentil seedlings in pot experiment, which might be available as the biopesticide in controlling soil borne pathogens, substitute to indiscipline use of pesticides that can mitigate the ecological degradation, as well as environmental pollution. However, with the

great magnitude of our research, the characterisation of the vast samples of *Trichoderma* species throughout the country will be unique novelty outcomes until we get the further published research findings.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. Collection of rhizosphere soil samples from different locations and different crops in 20 AEZ of Bangladesh

Table S2. Spore density of different *Trichoderma* on PDA at different temperatures of 7 days old culture

Table S3. Effect of different temperatures on colony growth characters of different *Trichoderma* isolates on PDA after 72 hrs

Table S4. Effect of different pH levels on colony growth characters of different isolates of *Trichoderma* on PDA

Figure S1. Isolate of Sadar Moulvibazar and Sadar Mymensingh at 40 °C, DOC: Days old culture

Figure S2. Antifungal activity of different isolates of *Trichoderma* against *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum* (Dual culture)

Figure S3. Structure of different isolates of *Trichoderma asperellum*

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