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RESEARCH ARTICLE

Trichoderma atrobrunneum: *In vitro* analysis of exoenzyme activity and antagonistic potential against plant pathogen from agricultural fields in the Patna region, India

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Abstract

Background: Fungi and oomycetes continue to be the primary source of plant diseases, which cause major crop loss factor in agriculture. *Trichoderma* is a genus of ascomycete fungus that lives in soil and is known for its ecological significance as a bio-fungicide.

Aim: The current study aimed to assess and contrast the antagonism and exoenzyme activity of *Trichoderma* isolates against *Pythium* sp., which causes damping-off and root rot diseases on tomato plants. *Trichoderma* spp. was isolated from rhizospheric soil of healthy plants in the agricultural fields of Patna, Bihar district, India.

Methodology: The *Trichoderma* isolates were evaluated for their suitability as antagonists by in vitro dual culture and non-volatile assay.

Results: The results showed that the isolated *Trichoderma* species were effectively reduced the growth of *Pythium* sp. in the dual culture assay and non-volatile metabolites (12.5%, 25% and 50% v/v concentration) assay. *Trichoderma atrobrunneum* effectively inhibited the mycelial growth of Pythium sp. both in the dual culture (81.48%) and non-volatile metabolite assay (71.37%). Followed by *Trichoderma harzianum* (79.01% and 58.82%), and *Trichoderma asperellum* (65.43% and 50.58%) respectively. Exoenzyme activities of the *Trichoderma* isolates were also investigated. The antagonistic *Trichoderma atrobrunneum* exhibited maximum activities of β-1,3-glucanase (41.39 U/ml) and chitinase (33.06 U/ml) in comparison to *T. harzianum* and *T. asperellum*. The capacity of antagonism is enhanced by the production of the enzyme. Based on the above results, it can be said that the non-volatile metabolites and the exoenzyme of the antagonist *T.* species act synergistically in inhibiting *Pythium* growth.

Conclusion: The secretion of enzymes by *Trichoderma* species shows that they can be used in plant disease control as novel biocontrol agents as well as to produce enzymes in biotechnological processes.

Keywords: *Trichoderma, phytopathogen*, *Pythium sp*., antagonist, biocontrol, exoenzyme.

Introduction

Plant diseases caused by soil borne phytopathogens are the most concerning factor since they significantly limit quality and yields of agricultural products. These losses pose a significant menace to the world's food production every year (O'Brien, 2017). Tomato plant (*Lycopersicon esculentum* Mill.) is the second-most widely grown vegetable crop worldwide (Sachdev & Singh, 2017) and has significant commercial value. It is one of the major economically important vegetables crops of India has numerous health benefits (Wang *et al*., 2023). The plants are susceptible to many diseases particularly soil borne phytopathogen Pythium spp. which causes damping off and root rot diseases are the major constraints in all the cultivated regions including India (Jeyaseelan *et al*., 2012). Synthetic pesticides are used extensively in modern agriculture for controlling plant diseases. However, concerns about public safety have been raised with the use of these Synthetic pesticides because their massive use produces adverse ecological effects. Such as the development of resistance pathogen strains (Lucas *et al*., 2015), leaves harmful residue in foods (Aktar *et al*., 2009), toxic effects on animals and fish (Gupta,

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2019), reduced beneficial soil microflora, and environmental pollution. (Sachdev & Singh, 2017; Dubey *et al*., 2007). Moreover, the accumulation of these chemicals in crops has also led to chronic poisoning effects on human health, domestic animals, and wildlife (Aktar *et al*., 2009; Gupta, 2019). At this perspective control of phytopathogens using biocontrol agents (BCA) has been considered a potential strategy. BCA are environmentally safe, sustainable, and efficient alternative for the managements of plant diseases (Sachdev & Singh, 2017). Among the BCAs, *Trichoderma* spp. is considered one of the most promising biocontrol agents for the control of soil borne diseases. Certain *Trichoderma* species are also effective against foliar (Qualhato *et al*., 2013), post-harvest pathogens (Thambugala *et al*., 2020) and nematodes (Saravanakumar & Wang, 2020; Vinale *et al*., 2008). They are ubiquitous, fast-growing fungi mostly found in soil and rotting wood (Druzhinina *et al*., 2006; Samuels, 1996). *Trichoderma* spp. is employed against wide variety of pathogens due to their multiprong mechanisms which include competition for nutrients and space (Benítez *et al*., 2004), mycoparasitism (Howell, 2003), antibiosis (Sivasithamparam K, 1998), induced resistance, secretion of enzymes (chitinase, glucanase, cellulase, protease etc.), enhancement of plant growth (Harman *et al*., 2004; Howell, 2003;). Thus, *Trichoderma* species has raised the interest to search the more efficient strain globally. Therefore, this research work was undertaken to evaluate the antagonistic efficiency along with the exoenzyme activity of *Trichoderma* isolates against *Pythium* sp., which causes Damping-off and root rot diseases in tomato and other crops.

Materials and methods

Soil sampling

The soil samples were collected from various agriculture fields near Patna (25.5941° N, 85.1376° E). All the samples were taken from healthy and diseased rhizospheric area (10 – 15cm deep) of pea, tomato, chickpea, and spinach. The samples were placed in sterile boxes and refrigerated at 4 ^{0}C until use (Meena *et al*., 2017; Naher *et al*., 2013).

Isolation of fungi

The collected soil samples were inoculated on Potato dextrose agar and Trichoderma selective media amended with chloramphenicol and streptomycin by using serial dilution plate technique (Aneja, 2007). Plates were incubated for seven days at 26±2 ^{0}C. Probable fungal colonies were subcultured on slant to obtained pure culture.

Morphological identification of the fungal isolates

The macroscopic and microscopic characters of the isolated fungal species were observed. For microscopic characteristics slide were prepared using lactophenol cotton blue and compared to the standard manuals (Bissett, 1991; Samuels *et al*., 2002; Siddiquee, 2017; Uzuhashi *et al*., 2010).

Molecular identification and phylogenetic analysis of *Trichoderma* isolate

Genomic DNA extraction was carried out from fungal culture, using spin column kit (HiMedia, India). ITS1 and ITS4 primers were used to perform a polymerase chain reaction of the 600 bp internal transcribed spacer (ITS) rRNA in a thermal cycler (Bruns *et al*., 1990). The amplification process consists of 2 minutes (95 °C) of initial denaturation then, thirty seconds (95 °C), thirty seconds (50 °C), sixty seconds (72 °C), and seven minutes at 72 °C as the final extension (Landum *et al*., 2016). Gel electrophoresis was used to purify and quantify the PCR product. PCR product was purified and quantified by gel electrophoresis. The Sanger method was used to sequence the purified PCR product using an ABI 3500xl genetic analyzer (Life Technologies, USA). Above procedure was performed in CSIR-NCL, Pune, India. The sequence was subsequently examined using the Basic Local Alignment Search Tool (BLAST), which relates the sequences to the nearest culture sequence found in the database of NCBI (National Center for Biotechnology Information) to recognize local regions of resemblance. Evolutionary relationships were analyzed by MEGA11. The phylogeny was constructed by using the method of Neighbor Joining cluster method. The tree is drawn to a scale of 0.0020, the evolutionary distance was computed using the p-distance method, and ambiguous positions were removed by pair-wise deletion approach (Meena *et al*., 2017; Kumar *et al*., 2012). The examine sequence was named with NCBI accession number: OR544402.1 (Figure 1).

Antagonistic test

Dual culture assay

To determine the antagonistic potential of the *Trichoderma* isolates against *Pythium* sp. a dual culture assay was used, as described by (Elshahawy & El-Mohamedy, 2019; Morton & Stroube, 1955). The *Trichoderma* and *Pythium* sp. were freshly grown for five days 26±2 ^{0}C. 5mm mycelial disc

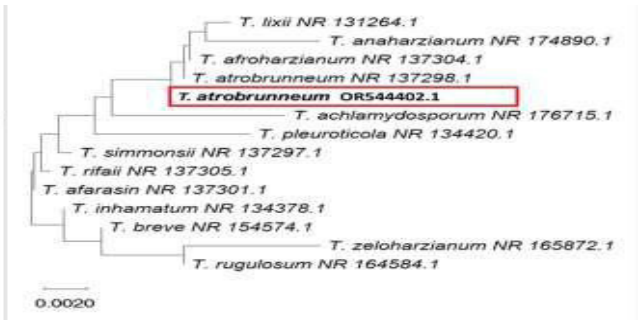

Figure 1: Evolutionary relationships of taxa Evolutionary relationships were analyzed by MEGA11. The phylogeny was inferred using the Neighbor-Joining clustering method. The tree is drawn to a scale of 0.0020, the evolutionary distance was computed using the p-distance method, and ambiguous positions were removed by pairwise deletion approach

each *Trichoderma* isolate and pathogen were aseptically transferred on the opposite side of sterile SDA plates with positive and negative control. Plates inoculated with commercial *Trichoderma* sp. and pathogen was used as a positive control, while plates inoculated with only pathogen were used as a negative control. Each experiment was done with three replicates. The plates were kept at a temperature of 26±2 ^{0}C for seven days. The radial growth of *Pythium* sp. on treated plates were compared with negative control plates, and the inhibition percentage of mycelial growth were computed using the following formula: I= [(A-B)/A] X 100, where A is radial growth of *Pythium* sp. on control, B is radial growth of *Pythium* sp. on treatment (Singh *et al*., 2002), (Figure 2).

Non-volatile metabolites assay

- *Production of non-volatile metabolites*

Selected *Trichoderma* isolates was separately cultured in Erlenmeyer flasks each comprising hundred ml of Sabouraud dextrose broth (SDB). A 5 mm mycelial disk of the young culture of each *Trichoderma* isolate was inoculated in a flask then incubated for 21 days (26±2 ^{0}C) in a shaker incubator at 150 rpm. The broth cultures were subsequently passed through Whatman No. 1 filter to remove *Trichoderma* spore and mycelia. The filtrates were centrifuge for 10 min at 10000 rpm and then sterilized by Millipore membrane filter and stored at 4^{0}C (Dennis & Webster, 1971; Meena *et al*., 2017), (Figure 2).

- *Poisoned food technique*

Culture filtrates of each *Trichoderma* spp. were added in molten SDA medium to obtain 12.5%, 25%, and 50% v/v concentrations. The mycelial block (5mm) of the pathogen was inoculated onto the center of SDA plates supplemented with culture filtrates (Meena *et al*., 2017). Culture filtrate of commercial *Trichoderma* sp. and distilled water (sterilized) was added in the medium for positive and negative control respectively, and incubated the plates at 26±2 ^{0}C for seven days. The pathogen's radial growth in treated plates was compared to negative control and inhibition percentage was calculated by previously mentioned formula (Singh *et al*., 2002).

Assay of exoenzymes activity

Primary screening

- *Chitinase activity*

The procedure suggested by Kamala & Indira, (2011), was used to measure the activity of the chitinase enzyme. Briefly *Trichoderma* isolates were grown in the chitinase detecting medium (CDM) supplemented with Bromocresol purple. 5mm mycelial disc of fresh culture of *Trichoderma* isolates were placed onto the center of CDM plates and incubated for 3 to 4 days at 25±2 ^{0}C. After incubation, the diameter

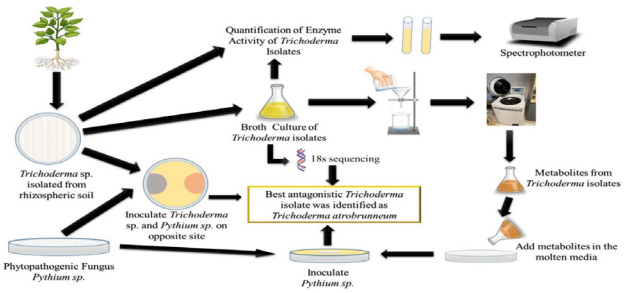

Figure 2: Graphic Representation of Current Research Work

of the purple color zone was measured for determination of chitinase activity. Because the medium is added with bromocresol purple (dye), a pH indicator, which changes the color of the medium yellow (at a pH of 4.7) into a purple when the pH rises. The pH increases due to Chitin is utilized by *Trichoderma* spp. and its subsequent degradation into N-acetyl glucosamine (Agrawal & Kotasthane, 2012).

- *β-1,3-glucanase activity*

The enzymatic activity of β-1,3-glucanase was evaluated by the procedure of Kamala and Indira, (2011). Plates were cultured with a mycelial disc (5mm) of *Trichoderma* isolates and kept for 3-4 days in an incubator at 25±2 ^{0}C After that the cultures were flooded with 1% aqueous solution of Congo red; then the plates were incubated for 15 to 20 minutes at room temperature. Distained with 1 N NaOH and 1 N NaCl for 15 minutes each, this was performed twice. The emergence of the clear zone indicates the presence of β-1,3-glucanase enzyme.

- *Secondary screening*

Growth condition of *Trichoderma* isolates: The broth medium containing colloidal chitin and laminarin was used to cultivate the selected Trichoderma isolates for their respective enzyme (chitinase and β-1,3-glucanase) activity. Trichoderma isolates were inoculated separately in Erlenmeyer flask then incubated for five days at 28 ^{0}C on rotatory shaker with 150 rotations per minute. Following the period of incubation the broth was passed through Whatman no. 1 filter paper and centrifuged for fifteen minutes (15000 rpm). Collected supernatant were used as enzyme source (Rao *et al*., 2016).

- *Chitinase*

The colorimetric method was used to estimate the activity of the chitinase enzyme described by Qualhato *et al*., (2013) with some modification. 500 µl of the enzyme supernatant and 1.5 millilitre of colloidal chitin (0.5%) in 0.05M acetate buffer (pH 5.2) were mixed and incubated for 1hour at 37 ^{0}C. The mixture was centrifuged for ten minutes at 5000rpm to stop the reaction. 3, 5-dinitrosalicylic acid (DNS) reagent method was used to assess the amount of reduced sugar (Miller, 1959). The absorbance at 540 nm was measured using

spectrophotometry to determine the released amount of N-acetyl glucosamine. Standard curves made from GlcNAc were used to calculate the quantity of released reducing sugar.

- *β-1, 3-glucanase*

β-1,3-glucanase's enzyme activity was determined using the method outlined by Rao *et al*., (2016). 1 ml of 0.5% laminarin in 50mM acetate buffer, pH-4.8 was added to 0.5 ml of enzyme supernatant and the mixture was incubated for 30 minutes at 40 ^{0}C. 2mL of DNS reagent was added and heated to a boil for 10 minutes. Once it had cooled, the absorbance was measured using spectrophotometer at 575nm. The reference curve made with glucose was used to determine the amount of reducing sugar released.

Data analysis

The presented data were the mean ± standard error. Statistical study was conducted with IBM SPSS Statistics software, and the data were analyzed by multifactor analysis of variance (ANOVA) using Duncan's multiple-range test.

Results and discussions

Identification of the fungal isolates

On the basis of morphological characteristics such as colony color, texture, margin, conidia, conidiophore, phialides, chlamydospores the fungal isolates were identified as *Trichoderma asperellum, T. harzianum, T. atrobrunneum, T. species,* and *Pythium* sp., using standard manuals described by (Bissett, 1991; Samuels *et al*., 2002; Siddiquee, 2017; Uzuhashi *et al*., 2010).

Molecular identification and phylogenetic analysis of Trichoderma isolate

The identification of potential antagonistic *T.* isolate was further confirmed by sequencing. The phylogenetic dendogram was constructed using Neighbor Joining cluster method by MEGA11. The dendogram was drawn to a scale of 0.0020. The BLAST analysis showed that the deposited sequence was 100 % similarity with T. atrobrunneum sequence available in the data base of NCBI. The examine sequence was named with NCBI accession number: OR544402.1 (Figure 1).

Antagonistic test

To determine the antagonistic potential of isolated *Trichoderma* spp. two methods were used, Dual culture assay and non-volatile metabolites assay. Both the assay showed significant reduction in the mycelial growth of tested *Pythium* sp. The percentage inhibition of mycelial growth (PIMG) in dual culture ranges from 35.8% to 81.48 %. The highest PIMG was recorded with *T. atrobrunneum* (81.48%) followed by *T. harzianum* (79.01 %) and *T. asperellum* (65.43 %) and the minimum PIMG was observed in *Trichoderma* sp.

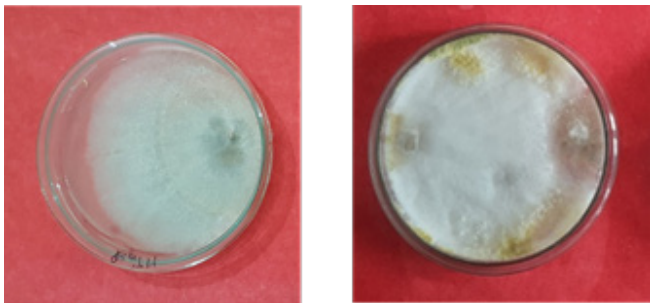

Figure 3: Dual culture assay of *Trichoderma atrobrunneum* against *Pythium* sp. a) *Pythium* sp. in negative control b) *Trichoderma atrobrunneum* and *Pythium* sp.

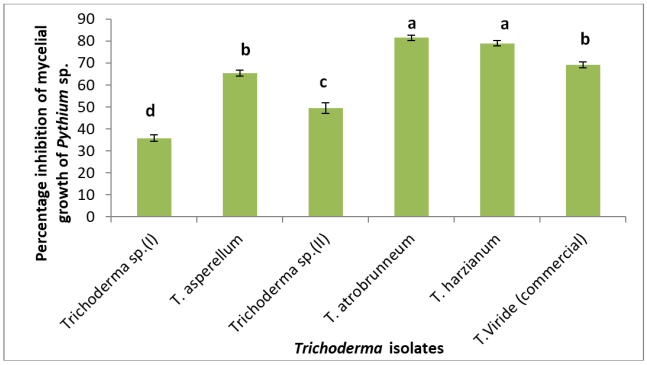

Figure 4: Percentage inhibition of mycelial growth of Pythium sp. in dual culture assay

(T14) 35.8% and *Trichoderma sp.* (T81) 49.38%. The mycelial inhibition was 69.13% observed with positive control (commercial *Trichoderma*) (Figures 3 and 4). In non-volatile metabolites assay, three concentrations viz., 12.5%, 25%, and 50% v/v of non-volatile sec metabolites of selected *Trichoderma* isolates were tested against *Pythium* sp. Three *Trichoderma* isolates considerably inhibited the growth of *Pythium* sp. by different inhibition rates, ranges from 21.17 % to 71.37 % among three different concentrations. Non-volatile sec metabolites of *Trichoderma atrobrunneum* inhibited mycelial growth of *Pythium* sp. was found most efficient by 71.37 % followed by *T. harzianum* 58.82 % and *T. asperellum* 50.58 %; and 55.29 % mycelial inhibition with positive control at 50% v/v concentration of metabolites (Figure 5). The collected data are in agreement with those published by (Elshahawy & El-Mohamedy, 2019; Jeyaseelan *et al*., 2012) they reported that *Trichoderma* species are significantly inhibited the growth of *Pythium* sp.in both the assays, dual culture and non-volatile.

Exoenzymes activity

The excretion of exoenzymes such as chitinase, β-1,3-glucanase, protease, cellulase, etc. and the production of toxic metabolites metabolites (nonvolatile and volatile compounds) are the strong weapons employed by

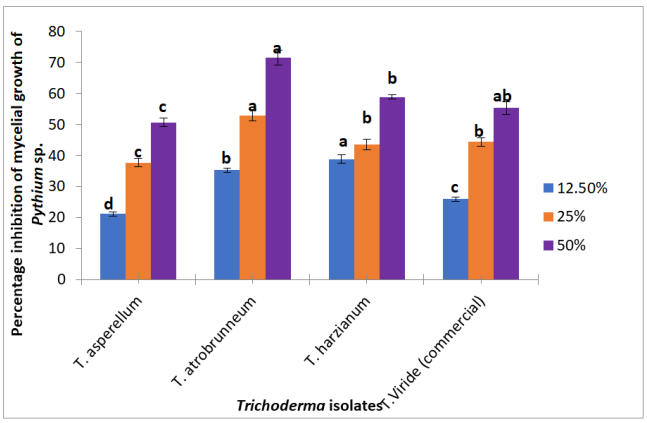

Figure 5: Percentage inhibition of mycelial growth of *Pythium* sp. in antifungal nonvolatile assay of *Trichoderma* isolates at 12.5%, 25%, and 50% v/v

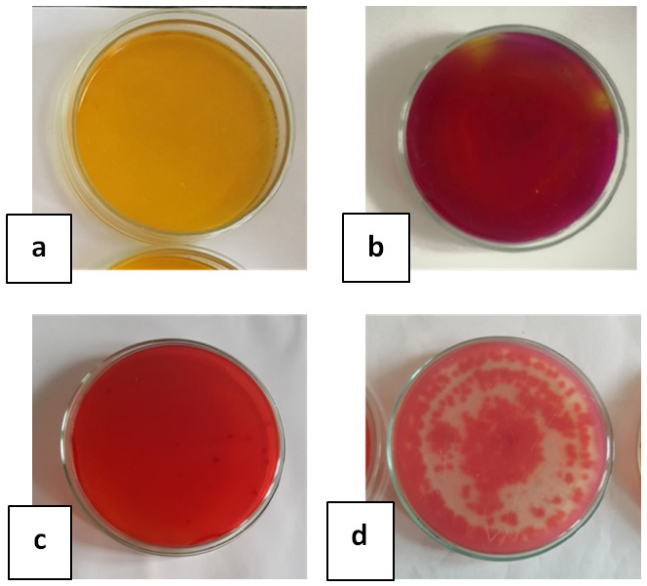

Figure 6: Isolates of *Trichoderma* showed chitinase and β-1,3-glucanase activity; a) uninoculated plate (control) supplemented with Bromocresol purple b) inoculated plate with *Trichoderma* isolate showing chitinase activity c) uninoculated plate (control) treated with Congo red d) inoculated plate with Trichoderma isolate showing β-1,3-glucanase activity

Trichoderma spp. to destroy the target pathogen (El-Katatny *et al*., 2000; Elad, 1999; Harman *et al*., 2004; Ramada *et al*., 2010).

These enzymes (Chitinase and β-1,3-glucanases) disrupt the integrity of the target pathogen's cell wall by dissolving their structural polysaccharides (chitin and β-glucans) (Howell, 2003). Therefore, in this study, enzymes secretion, including chitinase and β-1,3-glucanase, and effect of metabolites of *Trichoderma* isolates were assessed. In plate assay, maximum chitinase and β-1,3-glucanase activity was produced by *T. atrobrunneum* with a zone diameter of 85.7 and 83.3 mm, followed by *T. harzianum* (70 and 75.7 mm), while *T. asperellum*

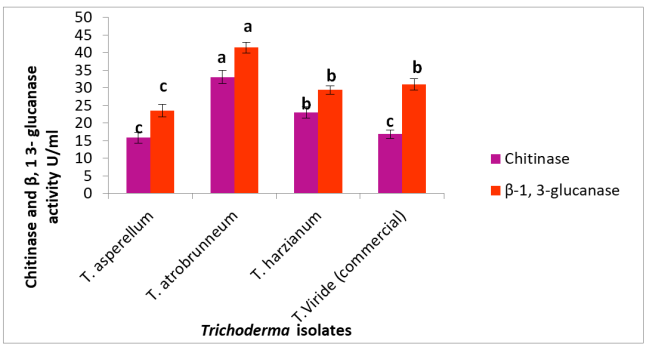

Figure 7: Chitinase and β -1, 3-glucanase activity of Trichoderma isolates (U/ml)

(37.6 and 52.3 mm) produced the least amount (Figure 6). In the secondary screening of enzymes, the *Trichoderma* isolate was cultivated in broth media that contained chitin (chitinase) and laminarin (β-1,3-glucanase) for carbon sources. *T. atrobrunneum* exhibited highest chitinase activity 33.06 U/ml while *T. harzianum* and *T. asperellum* were recorded with 22.98 U/ml and 15.78 U/ml respectively. The activity of β-1, 3-glucanase was also highest in *T. atrobrunneum* 41.39 U/ml and it was higher than chitinase activity, followed by *T. harzianum* 29.41 U/ml and T. asperellum 23.48 U/ml. In Positive control (commercial T*richoderma*) 16.81 U/ml of Chitinase and 30.97 U/ml of β-1, 3-glucanase activity were found (Figure 7). In this investigation, *T. atrobrunneum* was found to be the most effective producers of chitinase as well as β-1,3-glucanase activity, with *T. harzianum* coming in next. Similar results are also reported by (Chao & Wen-ying, 2019) who found that *T. atrobrunneum* had the highest chitinase activity. According to Kamala & Indira (2011), the *Trichoderma* isolates exhibit potent biocontrol activity under in-vitro and pot experiment against P. aphanidermatum, which causes damping-off disease in bean plants. Previous research also demonstrate that a variety of pathogens that cause plant diseases, such as *Fusarium oxysporum F. solani, Phytophthora capsica, Pythium aphanidermatum , P. ultimatum,R. solani , Botrytis cinerea, Curvularia lunata, S. rolfsii, A. alternria ,Bipolaris sorokiniana*, etc. are effectively combatted by the antimicrobial metabolites generated by *Trichoderma* spp., (Harman *et al*., 2004; Keswani *et al*., 2014; Saravanakumar & Wang, 2020).

Conclusion

Trichoderma is one of the most widely employed microbial fungal biocontrol agents (BCA) in the management of soil borne plant pathogens (Harman *et al*., 2004). According to certain theories, the mycoparasitic activity is one of the main mechanisms of *Trichoderma* species as antagonist. And it has also been reported that the enzymes and toxic metabolites from *Trichoderma* act synergistically in mycoparasitism (Monte, 2001). In the present investigation we found the three *Trichoderma* spp. considerably suppress the tested

phytopathogen's (*Pythium* sp.) mycelial growth in dual culture as well as non-volatile assay. *T. atrobrunneum* showed strong activity of β-1, 3-glucanase and chitinase. From this result, it is evident that metabolites and exoenzymes of *Trichoderma* spp. suppress the mycelial growth of *Pythium* sp. since *Trichoderma*-derived enzymes and metabolites can synergistically produce biocontrol activity against a variety of pathogens (Saravanakumar *et al*., 2016). In general, it can be concluded that *Trichoderma atrobrunneum* can prove as a good biocontrol tool against soil borne phytopathogens. It is obvious that commercial chemical-based control agents act as better control agents against phytopathogens. But humans' health and environment sustainability are to be considered in priority in this era. Keeping this in mind use of *Trichoderma* as biocontrol agent can be promoted with integrated pest management tactics.

Author Contribution

Conceptualization, Formal Analysis and Investigation, writing—original manuscript by FB; Project Supervision & Project Administration by RM; Review and editing by RM & SRA

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References

Agrawal, T., & Kotasthane, A. S. (2012). Chitinolytic assay of indigenous Trichoderma isolates collected from different geographical locations of Chhattisgarh in central India. *SpringerPlus*, *1*(1), 1–10. https://doi.org/10.1186/2193-1801-1-73

Aneja, K. R. (2005). *Experiment in microbiology, plant pathology and biotechnology*. New Age International Publishers, New Delhi.

Bell, D. K., Wells, H. D., & Markham, C. R. (1982). In vitro antagonism of Trichoderma species against six fungal plant pathogens. *Phytopathol*, *72*, 379–382.

Bissett, J. (1991). A revision of the genus Trichoderma . II. Infrageneric classification . *Canadian Journal of Botany*, *69*(11), 2357–2372. https://doi.org/10.1139/b91-297

Bruns, T. D., Lee, S. B., & Taylor, J. W. (1990). *White , T . J ., T . D . Bruns , S . B . Lee , and J . W . Taylor . Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics*. *May 2014*.

Chao, W., & Wen-ying, Z. (2019). Evaluating effective Trichoderma isolates for biocontrol of Rhizoctonia solani causing root rot of Vigna unguiculata. *Journal of Integrative Agriculture*, *18*(9), 2072–2079. https://doi.org/10.1016/S2095-3119(19)62593-1

Dennis, C., & Webster, J. (1971). Antagonistic properties of species-groups of Trichoderma. *Transactions of the British Mycological Society*, *57*(1), 41-IN4. https://doi.org/10.1016/s0007-1536(71)80078-5

Dickinson, C. H., & Skidmore, A. M. (1976). Interactions between germinating spores of Septoria nodorum and phylloplane fungi. *Transactions of the British Mycological Society*, *66*(1), 45–56. https://doi.org/10.1016/s0007-1536(76)80091-5

Druzhinina, I. S., Kopchinskiy, A. G., & Kubicek, C. P. (2006). *The first 100 Trichoderma species characterized by molecular data The first 100 Trichoderma species characterized by molecular data*. *April*. https://doi.org/10.1007/s10267-006-0279-7

Dubey, S. C., Suresh, M., & Singh, B. (2007). Evaluation of Trichoderma species against Fusarium oxysporum f. sp. ciceris for integrated management of chickpea wilt. *Biological Control*, *40*(1), 118–127. https://doi.org/10.1016/j.biocontrol.2006.06.006

El-Katatny, M. H., Somitsch, W., Robra, K. H., El-Katatny, M. S., & Gübitz, G. M. (2000). Production of Chitinase and β-1,3-glucanase by Trichoderma harzianum for Control of the Phytopathogenic Fungus Sclerotium rolfsii. *Food Technology and Biotechnology*, *38*(3), 173–180.

Elad, Y. (1999). The role of Trichoderma harzianum protease in the bio control of Botrytis cinerea. *Eur. J. Plant Pathol.*, *105*, 177–189.

Elshahawy, I. E., & El-Mohamedy, R. S. (2019). Biological control of Pythium damping-off and root-rot diseases of tomato using Trichoderma isolates employed alone or in combination. *Journal of Plant Pathology*, *101*(3), 597–608. https://doi.org/10.1007/s42161-019-00248-z

Goldman, G. H., Hayes, C., & Harman, G. E. (1994). Molecular and cellular biology of biocontrol by Trichoderma spp. *Trends in Biotechnology*, *12*(12), 478–482. https://doi.org/10.1016/0167-7799(94)90055-8

H.L. Barnett, B. B. H. (1972). *Illustrated Genera of Imperfect Fungi Fourth Edition*. Burgess Publishing Company, Minneapolis, p 241.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). Trichoderma species - Opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*, *2*(1), 43–56. https://doi.org/10.1038/nrmicro797

Howell, C. R. (2003). Mechanisms employed by Trichoderma species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Disease*, *87*(1), 4–10. https://doi.org/10.1094/PDIS.2003.87.1.4

Jeyaseelan, E., Tharmila, S., & Niranjan, K. (2012). Antagonistic activity of Trichoderma spp. and Bacillus spp. against Pythium aphanidermatum isolated from tomato damping off. *Archives of Applied Science Research*, *4*(4), 1623–1627.

Kamala, T., & Indira, S. (2011). Evaluation of indigenous Trichoderma isolates from Manipur as biocontrol agent against Pythium aphanidermatum on common beans. *3 Biotech*, *1*(4), 217–225. https://doi.org/10.1007/s13205-011-0027-3

Landum, M. C., Félix, M. do R., Alho, J., Garcia, R., Cabrita, M. J., Rei, F., & Varanda, C. M. R. (2016). Antagonistic activity of fungi of Olea europaea L. against Colletotrichum acutatum. *Microbiological Research*, *183*, 100–108. https://doi.org/10.1016/j.micres.2015.12.001

Mallikharjuna Rao, K. L. N., Siva Raju, K., & Ravisankar, H. (2016). Cultural conditions on the production of extracellular enzymes by Trichoderma isolates from tobacco rhizosphere. *Brazilian Journal of Microbiology*, *47*(1), 25–32. https://doi.org/10.1016/j.bjm.2015.11.007

Meena, M., Swapnil, P., Zehra, A., Dubey, M. K., & Upadhyay, R. S. (2017). Antagonistic assessment of Trichoderma spp. by producing volatile and non-volatile compounds against different fungal pathogens. *Archives of Phytopathology and Plant Protection*, *50*(13–14), 629–648. https://doi.org/10.1080/03235408.2017.1357360

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3), 426–428.

Morton, D. J., & Stroube, W. H. (1955). Antagonistic and stimulatory effects of soil microorganisms upon Sclerotium rolfsii. *Phytopath*, *45*(8), 417–420.

Nannipieri, P. (1994). *The potential use of soil enzymes as indicators of productivity, sustainability farming systems* (P. R. Pankhurst, C. E.; Doube, B. M.; Gupta, V. V. S. R.; Grace (ed.)). CSIRO Australia.

Qualhato, T. F., Lopes, F. A. C., Steindorff, A. S., Brandão, R. S., Jesuino, R. S. A., & Ulhoa, C. J. (2013). Mycoparasitism studies of Trichoderma species against three phytopathogenic fungi: Evaluation of antagonism and hydrolytic enzyme production. *Biotechnology Letters*, *35*(9), 1461–1468. https://doi.org/10.1007/s10529-013-1225-3

Ramada, M. H. S., Lopes, F. Á. C., Ulhoa, C. J., & Silva, R. do N. (2010). Optimized microplate β-1,3-glucanase assay system for Trichoderma spp. screening. *Journal of Microbiological Methods*, *81*(1), 6–10. https://doi.org/10.1016/j.mimet.2010.01.010

Samuels, G. J. (1996). Trichoderma: A review of biology and systematics of the genus. *Mycological Research*, *100*(8), 923–935. https://doi.org/10.1016/S0953-7562(96)80043-8

Samuels, G. J., Dodd, S. L., Gams, W., Castlebury, L. A., & Petrini, O. (2002). Trichoderma Species Associated with the Green Mold Epidemic of Commercially Grown Agaricus bisporus. *Mycologia*, *94*(1), 146. https://doi.org/10.2307/3761854

Samuels, G. J., Ismaiel, A., Bon, M. C., De Respinis, S., & Petrini, O. (2010). Trichoderma asperellum sensu lato consists of two cryptic species. *Mycologia*, *102*(4), 944–966. https://doi.org/10.3852/09-243

Saravanakumar, K., & Wang, M. H. (2020). Isolation and molecular identification of Trichoderma species from wetland soil and their antagonistic activity against phytopathogens. *Physiological and Molecular Plant Pathology*, *109*(December 2019), 101458. https://doi.org/10.1016/j.pmpp.2020.101458

Saravanakumar, K., Yu, C., Dou, K., Wang, M., Li, Y., & Chen, J. (2016). Synergistic effect of Trichoderma-derived antifungal metabolites and cell wall degrading enzymes on enhanced biocontrol of Fusarium oxysporum f. sp. cucumerinum. *Biological Control*, *94*, 37–46. https://doi.org/10.1016/j.biocontrol.2015.12.001

Siddiquee, S. (2017). Practical Handbook of the Biology and Molecular Diversity of Trichoderma Species from Tropical Regions. In *Fungal Biology* (Vol. 11).

Singh, D., Newburn, M., Smith, N., & Wiggins, M. (2002). SIngh 2002.pdf. In *British Journal of Midwifery* (Vol. 10, Issue 1).

Sivasithamparam K, G. F. (1998). Secondary metabolism in Trichoderma and Gliocladium. In C. P. Kubicek & G. E. Harman (Eds.), *Trichoderma and Gliocladium* (pp. 139–191). Vol I.Taylor and Francis Ltd, London pp.139–191.

Ulhoa, C. J., & Peberdy, J. F. (1992). Purification and some properties of the extracellular chitinase produced by Trichoderma harzianum. *Enzyme and Microbial Technology*, *14*(3), 236–240. https://doi.org/10.1016/0141-0229(92)90072-V

Uzuhashi, S., & Kakishima, M. (2014). *Phylogeny of the genus Pythium and description of new genera Phylogeny of the genus Pythium and description of new genera. March*. https://doi.org/10.1007/s10267-010-0046-7

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., & Marra, R. (2008). *Trichoderma – plant – pathogen interactions. June 2021*. https://doi.org/10.1016/j.soilbio.2007.07.002