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Characterization and evaluation of native *Trichoderma* isolates for antagonistic activity against *Fusarium oxysporum* f. sp. *ciceris*

DEEPAK KUMARI, N. K. YADAV*, NARENDER SINGH, MAHENDRA KUMAR SARAN AND RAHUL

Department of Plant Pathology, CCS Haryana Agricultural University, Hisar, Haryana-125004
**E-mail: yadavnk67@gmail.com*

ABSTRACT

Chickpea wilt incited by *Fusarium oxysporum* f. sp. *ciceris* is the most destructive seed and soil borne disease which appears every year and causes heavy losses in yield. Present investigation was carried out for *in vitro* evaluation of *Trichodema* isolates against *F. oxysporum* f. sp. *ciceris* (Foc-49) and to characterize them on morphological basis. Thirteen *Trichodema* isolates were isolated from rhizosphere samples collected from chickpea growing areas of Haryana. *Trichoderma* isolates exhibited fastest growth on corn meal agar (5.0-5.9 cm) medium while, slowest growth was recorded on czapek-dox agar (1.1-3.2 cm) medium. Conidial and mycelial size varied with different *Trichoderma* isolates. Conidial size varied from 7.23 to 18.86 µm whereas, width of mycelia of different isolates of *Trichoderma* varied from 2.29 to 5.49 µm. Eight isolates showed positive results for HCN production. IAA production levels ranged from 11.48 to 21.78µg/ml for different isolates. The efficacy of these antagonists was investigated by employing the dual plate confrontation assay technique against Foc-49. The mycelial growth inhibition zone varied from 62.2 to 88.1% against *F. oxysporum* f. sp. *ciceris* and the highest inhibition (88.1%) was exhibited by HST-1 isolates. Further, the isolates which performed best under *in vitro* namely HST-1 (ITCC No. 9071) and HMT-2 (ITCC No. 9072) were identified as *Trichoderma asperellum* on morphological basis.

Key words: Biocontrol, chickpea, *F. oxysporum* f. sp. *ciceris, Trichoderma*

Chickpea (*Cicer arietinum* L.) is the world's third most important pulse crop, after dry beans and dry peas. It is self-pollinated legume crop that has been originated from south-eastern Turkey and the adjoining part of Syria. Global area of chickpea is about 13.72 million hectares with production of 14.25 million metric tonnes, in which, India contributes 70 per cent of global production with an annual production of 112.29 lakh tonnes from area of 105.61 lakh hectare (FAOSTAT, 2021). In India, the crop is grown mainly in the states of Madhya Pradesh, Rajasthan, Maharashtra, Uttar Pradesh, Haryana, Karnataka and Andhra Pradesh. Chickpea is a rich source of lysine rich protein that makes it a highly valuable crop (Jukanti *et al*., 2012). It contributes to sustainable production by reducing the need for nitrogenous fertilization by fixing atmospheric nitrogen.

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Biotic and abiotic stresses significantly reduce the chickpea production as well as productivity. Chickpea is prone to many diseases including Fusarium wilt caused by *F. oxysporum* f. sp. *ciceris*, which is considered as most important factor for low production of chickpea in India. The disease is most widespread in chickpea growing areas of India and can cause yield loss up to 60 per cent under favourable conditions (Singh *et al*., 2007). Fusarium wilt epidemics can cause up to 100 per cent loss under favourable environmental conditions (Haware and Nene, 1982; Halila and Strange, 1997). The pathogen is both seed and soil borne. Frequent use of chemicals for management of diseases causes environmental pollution and development of resistance in plant pathogens. Hence, biological control is considered as a substitute for disease management. *Trichoderma* spp. has gained immense importance since last few decades due to its biological control ability against several deadly plant pathogens (De Medeiros *et al*., 2017). Genus *Trichoderma* includes more than 300

morphologically and molecularly described species (Bissett *et al*., 2015; Marik *et al*., 2019; Tamandegani *et al*., 2020). Several mode of actions proposed to explain the bio control of plant pathogens by *Trichoderma* includes production of antibiotics, cell wall degrading enzymes, competition for key nutrients and space, synthesis of antifungal metabolites, parasitism, stimulation of plant defense mechanisms, antibiosis, mycoparasitism, rhizosphere competent and combination of these possibilities (Larkin and Fravel, 1998; Harman *et al*., 2004). *Trichoderma* spp. generally grows in their natural habitat particularly at rhizospheric region and plays key role in control of root diseases. Therefore, considering the importance of the disease and its destructive nature, the study of isolation and characterization of native *Trichoderma* isolates for antagonistic activity against *F. oxysporum* f. sp. *ciceris* was undertaken.

MATERIALS AND METHODS

The experiments were conducted in biological control laboratory of College of Agriculture, Chaudhary Charan Singh Haryana Agricultural University, Hisar using Completely Randomized Design (CRD) with three replications. *F. oxysporum* f. sp. *ciceris* isolate Foc-49 used in the study was received from ICAR-Indian Institute of Pulses Research (IIPR), Kanpur, India (Maharshi *et al*., 2021; Maharshi *et al*., 2023).

Isolation of *Trichoderma* isolates

Thirteen isolates of *Trichoderma* were isolated from soil samples collected from rhizosphere of chickpea from different chickpea growing regions of Haryana (Table 1) using the serial dilution method (Johnson, 1957). After diluting upto eighth dilution, 0.5 ml of the suspension from the eighth dilution was then spread on Petri plates containing PDA media. These plates were incubated in BOD incubator at 26 ±1˚C for 5-7 days. Preliminary screening of *Trichoderma* species was done by looking at both microscopic and macroscopic characteristics of the fungal colonies. After *Trichoderma* was identified, a pure culture of the fungus was obtained using the hyphal tip method (Rangaswami, 1972) and it was stored at 4±1°C for further investigation.

Table 1. Locations of soil sample collection and their corresponding *Trichoderma* isolates obtained

S.N.	Location/Districts	*Trichoderma* isolate
1.	RRS Bawal (Rewari)	HBT*
2.	Shikohpur (Gurugram)	HGT
3.	Nathusarichopta (Sirsa)	HST-1
4.	Badrai (CharkhiDadri)	HCdT
5.	CICR (Sirsa)	HST-2
6.	KVK Rohtak	HRT
7.	Bhopani (Faridabad)	HFrT
8.	Gorakhpur (Fatehabad)	HFtT
9.	KVK Jhajjar	HJT
10.	Nandpura (Bhiwani)	HBhT
11.	Satnali (Mahendergarh)	HMT-1
12.	KVK Mahendergarh	HMT-2
13.	New Delhi (IARI)	HDT

*1st letter (H) stands for Haryana; 2nd letter (B) stands for Location from where samples were collected and 3rd letter 'T' indicates *Trichoderma*

Characterization of *Trichoderma* isolates

To study growth characteristics of various *Trichoderma* isolates, five culture media were used *viz.* Potato dextrose agar (PDA); Malt extract agar (MEA); Corn meal agar (CMA); Sabouraud dextrose agar (SDA); Czapek-Dox agar (CDA).

All the media were obtained from Hi-media laboratory and prepared as per their procedure by sterilizing at 121.6℃ and 15 psi for 20 minutes in autoclave. To carry out cultural study, 15 ml of each medium was poured in Petri plates and replicated thrice. Each plate was inoculated with 5 mm bits of actively growing culture of *Trichoderma* and incubated in BOD at 26±1 ℃. Colony colour and colony diameter were recorded after 48, 72 and 96 hours of inoculation.

Cultural and morphological characterization of *Trichoderma* isolates

This experiment was conducted to study the possible morphological variations in different *Trichoderma* isolates. For this, 15 ml of sterilized PDA media was poured in Petri plates and these plates were then inoculated with 5 mm mycelial disc of *Trichoderma* isolates that was cut with the help of corkborer from the margin of the actively growing colony and then incubated in BOD incubator at 26±1

℃ for 5 days. Colony characters were recorded. For mycelial and conidial characters, a loopful culture of *Trichoderma* isolates was taken from five days old culture and then placed on glass slides mounted with a drop of water and covered with coverslip. The slides were observed under microscope with 40X magnification and observations were recorded.

Screening of *Trichoderma* isolates for plant growth promoting traits

The retrieved *Trichoderma* isolates were screened for plant growth promoting activities *viz.* Indole Acetic Acid (IAA) and Hydrogen Cyanide (HCN) production

Indole Acetic Acid production: IAA production was assayed by using modified protocol described by Brick *et al*. (1991). Fungal cultures of *Trichoderma* isolates were grown on potato dextrose broth using one gm/l of L-tryptophan as precursor for 72 hours at 26±1˚C. After incubation, the cultures of different isolates of *Trichoderma* were centrifuged at 3000 rpm for 30 minutes and the supernatant was collected. Two drops of orthophosphoric acid were added to two ml of supernatant followed by four ml of Salkowski reagent (50ml: 35% perchloric acid, 1 ml 0.5% M $FeCl_3$). Pink colour development in the solution indicated production of IAA.

Hydrogen Cyanide Production: HCN production was assayed according to protocol described by Bakker and Schippers (1987). HCN production is result of cyanogenesis of glycine which is performed by microorganisms. HCN is volatile in nature and when it reacts with picric acid in presence of Na_2CO_3, it gives a colour change from deep yellow to orange and finally gives dark brown colouration. The *Trichoderma* isolates producing HCN changed the colour of filter paper dipped in 0.5% picric acid solution in 0.2% (w/v) Na_2CO_3, placed in the test tubes. The test tubes were sealed with parafilm and then incubated for 72 hours at 28˚C. The change in the colour of strips from deep yellow to brown was considered as positive test and no colour change indicated negative results.

Antagonistic activity of *Trichoderma* isolates against *F. oxysporum* f. sp. *Ciceris*

Using the dual culture technique (Johnson and Curl, 1972), the degree of antagonistic activity of several isolates of *Trichoderma* against *F. oxysporum* f. sp. *ciceris* was investigated under *in vitro* conditions. Each treatment was replicated three times in CRD and appropriate controls were maintained. The per cent growth inhibition was calculated as per following formula given by Vincent (1947):

$$\text{Mycelial growth inhibition, I (\%)} = \frac{\text{C-T}}{\text{C}} \times 100$$

Where

I = Per cent mycelial growth inhibition

C = Radial mycelial growth of *F. oxysporum* f. sp. *ciceris* in control

T = Radial mycelial growth of *F. oxysporum* f. sp. *ciceris* in treatment

Collected data from *in vitro* assays were analyzed by using OPSTAT software (http://hau.ernet.in/about/opstat.php). Each treatment consisted of three replicates and the experiments were conducted in a completely randomized design.

RESULTS AND DISCUSSION

Cultural characterisitics of native *Trichoderma* isolates

Growth of retrieved *Trichoderma* isolates was observed on different media *viz*., SDA (Sabouraud dextrose agar), MEA (Malt extract agar), CMA (Corn meal agar), CDA (Czapek-Dox agar) and PDA (Potato dextrose agar) after 48 hours of inoculation. The colony colour was observed as whitish green on PDA, greenish yellow colour on MEA, whitish on CDA, dark green on SDA and light green on CMA. Data recorded on mycelial growth have been presented in Fig. 1 and it was observed that *Trichoderma* isolates exhibited fastest growth on CMA (5.0-5.9 cm) media followed by PDA (4.4-5.9 cm), MEA (4.0-5.6 cm) and SDA (3.8-5.7 cm) whereas, slowest growth was observed on CDA (1.1-3.2 cm) media. Native *Trichoderma* isolates exhibited fastest growth on Corn meal agar media and slowest on Czapeck dox agar which was similar to results of Chaithra and Pankaja (2022) who observed effect of different culture media on rhizospheric and non-rhizospheric *Trichoderma* isolates and recorded that all the seven *Trichoderma* isolates exhibited maximum mycelial growth rate on corn meal agar and oat meal agar media.

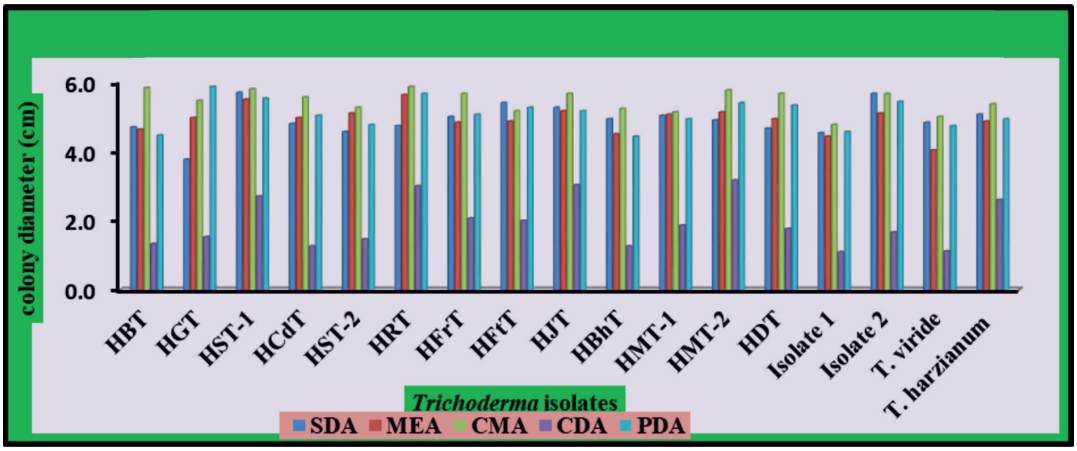

Fig. 1. Effect of different culture media on mycelial growth of *Trichoderma* isolates

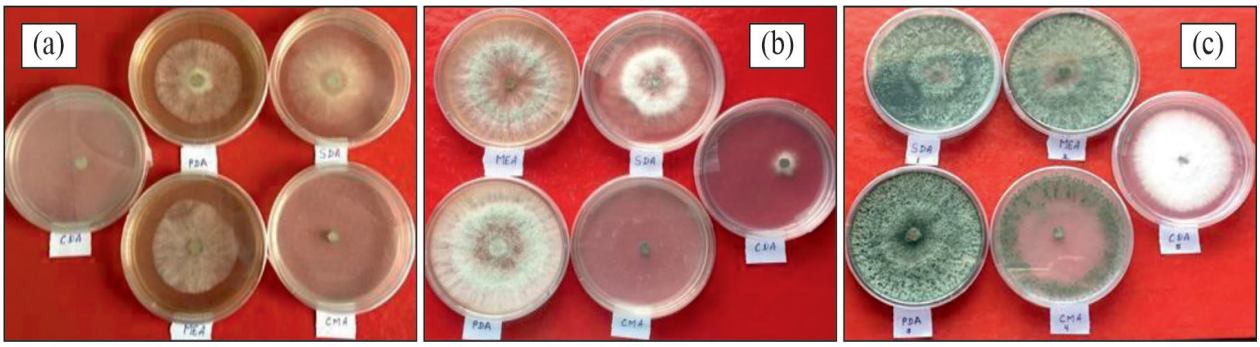

Plate 1. Effect of different culture media on growth of *Trichoderma* a) 48 h after inoculation, b) 72 h after inoculation and c) 96 h after inoculation

Morphological characteristics of retrieved *Trichoderma* isolates

The conidial shape of retrieved *Trichoderma* isolates varied from oval to round. Conidial colour varied from olive green to green. The length of conidia varied from 2.78 to 4.83 µm as presented in Table 2. The longest conidia was observed in HMT-2 with a conidial length of 4.83 µm followed by HRT (4.29 µm), HCdT (4.18 µm) whereas, the shortest conidial length of 2.78 µm was recorded in HST-2. Width of conidia of different isolates varied from 2.56 to 3.90 µm (Table 2). The maximum width was observed in case of HMT-2 (3.90 µm) followed by HBhT (3.71 µm) and HDT (3.61 µm) whereas, the minimum breadth was recorded in HJT (2.56 µm). The size of conidia varied with different isolates of *Trichoderma.* Conidial size varied from 7.23 to 18.86 µm. Among the isolates studied, the biggest conidial size was recorded in HMT-2 (18.86 µm) followed by HBhT (15.78 µm) and HCdT (14.46 µm) while, the shortest conidial size was recorded in HST-2 (7.23 µm). Width of mycelia of different isolates of *Trichoderma* varied from 2.29 to 5.49 µm (Table 2). The maximum width was recorded in HMT-2 (5.49 µm) whereas, the minimum width of 2.29 µm was recorded in HMT-1. The results obtained were found similar to observations recorded by Soesanto *et al*. (2011), where the average size of conidia for *Trichoderma* isolate 3 was 5 µm × 2 µm and 7.2 µm × 7.2 µm for *Trichoderma* isolate 5. Kumar *et al*. (2010) also reported that length and breadth of conidia for different *Trichoderma* isolates varied from 2.50–3.50 × 4.00–5.50 µm, respectively.

Screening of *Trichoderma* isolates for plant growth promoting traits

Isolates HST-1, HCdT, HST-2, HFtT, HMT-1, HMT-2, HDT and Isolate-2 (KBN-29) showed positive results for HCN production whereas, the remaining isolates showed negative results for HCN production (Table 3). Table 3 also revealed that all the isolates exhibited positive results for IAA production.

Table 2. Measurement of mycelia and conidia of different isolates of *Trichoderma*

Trichoderma isolate	Width of mycelia (µm)	Conidial length (µm)	Conidial width (µm)	Conidial size (μm^2)
HBT	2.35	3.17	2.90	9.20
HGT	4.15	3.40	3.21	10.89
HST-1	3.75	3.49	2.98	10.40
HCdT	4.38	4.18	3.46	14.46
HST-2	4.49	2.78	2.60	7.23
HRT	3.05	4.29	2.74	11.76
HFrT	4.91	3.17	2.90	9.22
HFtT	3.29	3.23	3.13	10.09
HJT	2.62	3.37	2.56	8.64
HBhT	3.72	4.25	3.71	15.78
HMT-1	2.29	3.71	3.06	11.35
HMT-2	5.49	4.83	3.90	18.86
HDT	2.34	3.94	3.61	14.22
Isolate-1 (RKTV)	3.69	3.94	3.31	13.05
Isolate-2 (KBN-29)	5.40	3.70	3.37	12.46
T. viride (commercial formulation)	2.75	4.03	3.44	13.85
T. harzianum (commercial formulation)	4.03	3.22	2.74	8.83
CD (p=0.05)	(0.27)	(0.16)	(0.06)	(0.5)
SE(m)	(0.09)	(0.06)	(0.02)	(0.17)

Table 3. Potentiality of different *Trichoderma* isolates for production of HCN and IAA

Trichoderma isolate	HCN production	IAA production (µg/ml)
HBT	-	13.78
HGT	-	12.74
HST-1	+	21.78
HCdT	+	16.13
HST-2	+	21.48
HRT	-	11.57
HFrT	-	15.35
HFtT	+	17.22
HJT	-	12.35
HBT	-	16.57
HMT-1	+	12.35
HMT-2	+	16.26
HDT	+	20.74
Isolate-1(RKTV)	-	12.39
Isolate-2 (KBN-29)	+	15.30
T. viride (commercial formulation)	-	11.48
T. harzianum (commercial formulation)	-	12.00
CD (p=0.05)		0.67
SE(m)		0.24

But the maximum IAA was produced by isolate HST-1 (21.78 µg/ml) followed by HST-2 (21.48 µg/ml) and HDT (20.74 µg/ml) while, the lowest IAA production was recorded in case of *T. viride* (11.48 µg/ml) and HRT (11.57 µg/ml). The inferences reported in the present study are similar to the findings of Yijayan *et al*. (2015) who examined twenty-nine isolates that produced varying amounts of HCN (0.8 to 180.6 ppm) and IAA was produced by 19 isolates ranging from 2.5 to 57.5 µg/ml. Mohiddin *et al*. (2017) tested the efficacy of 20 native *Trichoderma* isolates and observed that all native isolates produced IAA (1.54-6.61 µg/ml). Only three *Trichoderma* isolates namely, AT3, AT5 and AT7 were tested as positive for HCN production. Prasad *et al*. (2017) studied the plant growth promoting activities of 24 native *Trichoderma* isolates isolated from tomato rhizosphere and revealed that all bioagents showed positive results for HCN production and only two isolates showed positive results for IAA production.

Antagonistic activity of *Trichoderma* isolates against *F. oxysporum* f. sp. *ciceris*

Different *Trichoderma* isolates exhibited a wide range of antagonistic activity against *F. oxysporum* f. sp. *ciceris* (Table 4). Isolate HST-1 had the highest mycelial growth inhibition (88.1%), followed by HBhT (83%) whereas, HCdT had the lowest mycelial growth inhibition (62.2%). Native isolates were found to be more efficient than commercial formulations. Earlier studies by Rudresh *et al*. (2005) in which nine *Trichoderma* isolates were tested for their ability to inhibit growth of *F. oxysporum* f. sp. *ciceri* and concluded that isolate *T. virens*- PDBCTVs 12 exhibited maximum growth inhibition of 86.6%. Kumar *et al*. (2019) examined the bioefficacy of *Trichoderma* spp. and found that *T. harzianum* produced the significant level of inhibition against *F. oxysporum* f. sp. *ciceri*. Sallam *et al*. (2019) evaluated effect of seven *Trichoderma* isolates (T_1 to T_7) on Fusarium wilt disease of tomato and observed that *Trichoderma* isolate T_7 (67.8%) showed the maximum mycelial growth inhibition of *F. oxysporum* f. sp. *lycopersici*. Mao *et al*. (2020) evaluated that MHT1134 showed an inhibition of 81.80% against *F. oxysporum*. Moutassem *et al*. (2020) reported that *T. harzianum* and *T. polysporum* exhibited the higher inhibition against *F. oxysporum* while, *T. viride*, *T. atroviride* and *T. virens* resulted quite effective growth inhibition.

Plate 2. HCN production by different *Trichoderma* isolates

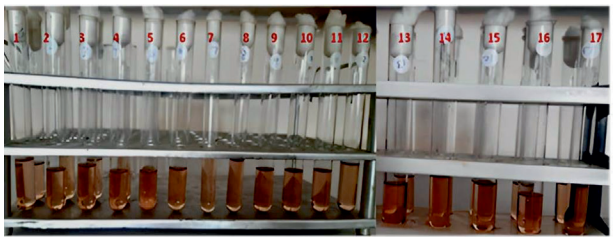

Plate 3. IAA production by different *Trichoderma* isolates

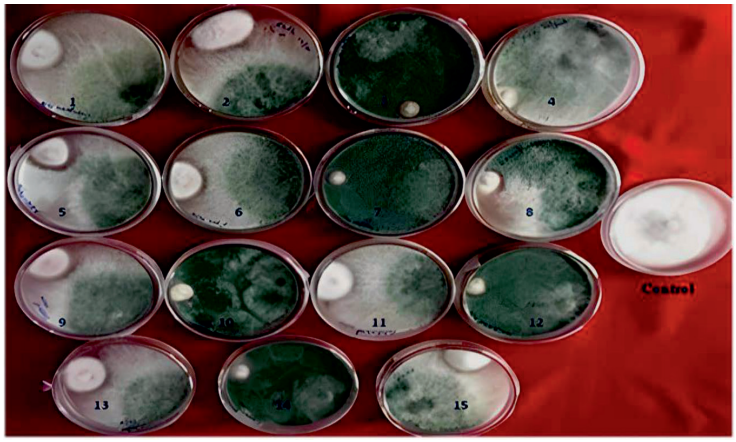

Plate 4. Mycelial growth inhibition of Foc-49 by various *Trichoderma* isolates

Table 4. Per cent mycelial growth inhibition of *F. oxysporum* f. sp. *ciceris* by different *Trichoderma* isolates

Trichoderma isolate	Radial mycelial growth (mm)	Per cent mycelial growth inhibition (%)
HBT	22.33	75.2
HGT	27.00	70.0
HST-1	10.67	88.1
HCdT	34.00	62.2
HST-2	30.67	65.9
HRT	33.67	62.6
HFrT	21.00	76.7
HFtT	24.33	73.0
HJT	16.67	81.5
HBhT	15.33	83.0
HMT-1	21.33	76.3
HMT-2	18.00	80.0
HDT	23.67	73.7
Isolate 1(RKTV)	26.00	71.1
Isolate 2 (KBN-29)	30.00	66.7
T. viride (Commercial formulation)	27.00	70.0
T. harzianum (Commercial formulation)	28.33	67.7
C.D. (p=0.05)	(2.15)	(2.49)
SE(m)	(0.75)	(0.86)

Based on the results of potentiality of HCN production, IAA production and antagonistic activity of different *Trichoderma* isolates under *in vitro* conditions, the promising *Trichoderma* isolate was HST-1 followed by HMT-2. Characterization and identification of these two isolates, HST-1 (ITCC No. 9071) and HMT-2 (ITCC No. 9072) was done by Division of Plant Pathology, IARI, New Delhi and identified as *Trichoderma asperellum* and submitted to ITCC for open access to researchers. The study identified *Trichoderma asperellum* (HST-1 and HMT-2) as most effective antagonists as compared to all other isolates.

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