Indian Phytopathology
https://doi.org/10.1007/s42360-021-00344-0

RESEARCH ARTICLE

Bio-control potential of *Trichoderma* spp., against *Fusarium* spp., the incitants of *Pokkah boeng* disease of sugarcane under in-vitro conditions

Raghvendra Tiwari[1] · S. K. Shukla[1] · V. P. Jaiswal[1] · Lalan Sharma[1] · Deeksha Joshi[1] · Kajal Chandra[2] · Asha Gaur[1] · Abhay Srivastava[1] · Rajesh Kumar Tiwari[3]

Received: 31 December 2020 / Revised: 17 February 2021 / Accepted: 15 March 2021

Abstract
Fifty one *Trichoderma* isolates were isolated from sugarcane rhizosphere soil using selective culture media and characterized for their cultural and morphological characteristics. *Trichoderma* isolates were evaluated in-vitro conditions for their bio-control activity against *Fusarium* spp., the causal agent of *Pokkah boeng* disease of sugarcaneby dual culture method, an assay for production of volatile inhibitory metabolites and mycoparasitism. Morphological and genetic characterization was carried out. There was not much variability in colony characters and growth rate among fifty-one isolates of *Trichoderma* spp. Among the *Trichoderma* isolates, twenty promising *Trichoderma* isolates were molecularly identified as *T. harzianum*, *T. afroharzianum*, *T. atrobrunneum*, *T. aureoviride*, and *T. asperellum*. It can be concluded that predominantly *Trichoderma harzianum* and other allied spp., are abundant in the sugarcane rhizospheric ecosystem. In dual culture assay, twenty isolates showed > 70% linear growth inhibition against *F. fujikuroi* and *F. proliferatum.* Twenty promising isolates were further screened for the production of inhibitory volatile metabolites. Among them, nine isolates showed growth inhibition > 7% against both *Fusarium* spp. Mycoparasitism was observed in eleven isolates, among them four isolates viz., T28, T38, T49, and T41 showed parallel mycoparasitism against *F. fujikuroi* while seven isolates (T9, T17, T26, T28, T41, T49, and T40) showed coiling around *F. proliferatum* hyphae. Thus, the four most promising strains viz. T28, T38, T41, and T49 were identified as potential *Trichoderma* spp., and need to screen further for disease management in field conditions. On the basis of the antagonistic study, it can be concluded that *T. harzianum* strain T28, *T. aureoviride* strainT38, *T. harzianum* strain T41, and *T. harzianum* strain T49 are potential candidates for being explored further as biocontrol agents for the management of *Pokkah boeng* disease.

Keywords *Trichoderma* · *Fusarium* · *Pokkah boeng* · Mycoparasitism · Antagonisms

Introduction

Sugarcane (*Saccharum officinarum* L.) belongs to C_4 plant crop that accounts for almost 75% of the world's sugar production. Worldwide, it is grown between latitude 36.7º N and 31.0º S of the equator extending from tropical to sub-tropical zones of the earth. In India, the largest sugarcane producing states are Uttar Pradesh, Maharashtra, Karnataka and Tamil Nadu. Uttar Pradesh is alone contributing approximately 30% of the total sugarcane produced in the country and having around 23 lakh hectare area under sugarcane production. At present, sugarcane is being cultivated almost 5.0 mha in the country. Various disease constraints in sugarcane production, fungi, bacteria, viruses, phytoplasma, and nematodes reported from India resulting in significant direct and indirect losses to sugarcane production indifferent regions (Viswanathan and Rao 2011). Management practices like use of resistant varieties, healthy seed programs, etc. have been recommended as an effective method to manage various dreaded diseases like red rot, wilt and smut of sugarcane to a large extent. However, with the changing varietal

✉ S. K. Shukla
sudhirshukla151@gmail.com

[1] ICAR-Indian Institute of Sugarcane Research, Lucknow, Uttar Pradesh, India

[2] Birbal Sahni Institute of Palaeosciences, Lucknow, Uttar Pradesh, India

[3] Amity University, Lucknow, Uttar Pradesh, India

Published online: 23 March 2021

scenario over the recent years, *Pokkah boeng*, is a disease which was earlier considered of minor economic importance but now emerged as a major threat to sugarcane cultivation in sub-tropical region of the country (IISR 2018–19). *Pokkah boeng* was first described in Java by Walker and Went in 1896. The disease is caused by *Fusarium* spp., with various workers reporting *F. moniliformae*, *F. sacchari*, *F. verticillioides*, *F. fujikuroi*, *F. proliferatum* and *F. andiyazi* as some of the species associated with this disease in different sugarcane growing regions (Lin et al. 2014; Vishwakarma et al. 2013; Viswanathan et al. 2011; Singh et al. 2006). This disease has assumed major importance in recent years and is causing considerable yield losses in some prominent sugarcane producing countries such as India, South Africa, Malaysia and China (Lin et al. 2014). In addition to yield loss, *Pokkah boeng* can also cause a significant quality reduction in high sugar yielding varieties, reducing the sugar by approximately 40.8–64.5% in infected crops (Siti Nordahliawate et al. 2008). In India, during recent years the disease has assumed alarming proportions in the major sugarcane producing states of Uttar Pradesh and Maharashtra, with disease incidence ranging from 5 to 90% in different sugarcane cultivars (Vishwakarma et al. 2013).

Symptoms of *Pokkah boeng* have been observed in various prevalent varieties. The disease is typically characterized by symptoms such as young leaves possessing chlorotic patches near its base (preliminary symptom), stalk distortion, rotting of stalk apical part, and knife-like cut on stalks (acute symptom). Maximum damage to crop occurs when fungus attacks on the whole pinhead of the plant causing the death of the plant and it identifies as a top rot symptom. Knife cut symptom is also reported in different sugarcane cultivars by Vishwakarma et al. (2013). To date, the use of chemical fungicides is the predominantly explored method for the management of this disease. Vishwakarma et al. (2013), suggested spraying of fungicides like Bavistin, Blitox, Copper oxychloride, or Dithane M-45 for management of *Pokkah boeng*. Shiqiang et al. (2019) also suggested spraying of Carbendazim to manage *Pokkah boeng* disease. With the deleterious short-term and long-term impacts of fungicides on the environment and human health, eco-friendly options like biological control agents need to be explored for the management of this disease. *Trichoderma* spp. are one of the most extensively explored and commercialized bio-control agents worldwide and have been effectively used for control of several plant diseases in diverse crops including sugarcane (Sharma et al. 2014). In addition to being exploited as bio-control agents, this fungus also acts as effective growth promoters, solubilize nutrients, produce various hydrolytic enzymes and induce resistance in plants to various abiotic and biotic stresses (Harman 2011). *Trichoderma* relies on various mechanisms to inhibit their target pathogen like mycoparasitism, antibiosis, competition, and induction of disease resistance in plants (Vinale et al. 2008). *Trichoderma* spp. have been used effectively for the management of various diseases caused by *Fusarium* spp. in several crops which include diseases like wilt, neck rot, and fusarium head blight. In the case of sugarcane, to date *Trichoderma* spp. have been explored and used effectively primarily for the management of red rot of sugarcane caused by the fungus *Colletotrichum falcatum* (Joshi et al. 2019). However, there are no studies exploring the potential of this fungus as a biocontrol agent for the management of *Pokkah boeng* disease of sugarcane. Keeping this in mind, the present study was undertaken to isolate and characterize *Trichoderma* spp., from the sugarcane agro-ecosystem and to assess them for their antagonistic activity against the two species of *Fusarium* (*F. fujikuroi, and F. proliferatum*) associated with *Pokkah boeng* disease of sugarcane in India.

Materials and methods

Isolation of *Trichoderma* spp.

Ten rhizospheric soil samples collected from different sugarcane cultivation fields at Grand Growth stage. The collected soil samples were processed by removing root particles and further used in serial dilution plating method (Johnson and Curl 1972) for the isolation of *Trichoderma* on *Trichoderma* Selective Agar medium. After incubation of Petri plates at 28 ºC for 5–7 days, emerging colonies showing distinct morphological features of *Trichoderma* were isolated, purified and cultures maintained on potato dextrose agar (PDA) slants for further studies.

Pokkah boeng pathogen isolation

The plant samples of highly susceptible sugarcane variety Co-0238 showing typical symptoms of *Pokkah boeng* were collected from farm of ICAR-IISR during month of June–August for pathogen isolation. The infected parts were cut in small pieces and surface sterilized with 4% sodium hypochlorite (NaOCl), followed by 70% ethanol, and then washed with sterile distilled water sequentially and inoculated on Petri plates containing PDA. Single spore cultures were derived and maintained on PDA and their preliminary identity was confirmed by morphological characterization using microscope according to different keys of identifications. Further, pathogen species was identified by sequencing ITS region as *Fusarium fujikuroi* strain F2 (Accession no.-MG965881) and *Fusarium proliferatum* strain F7 (Accession no.-MG965882). The purified pathogen cultures were preserved at 4 ºC till further use.

Cultural and morphological characterization of *Trichoderma* isolates

Trichoderma isolates were characterized on the basis of sporulation time, colony colour, presence or absence of coconut odour, concentric ring formation, chlamydospore formation, and growth rate in terms of colony diameter at different intervals on potato dextrose agar medium (PDA) [ingredients—potato (peeled) 200 g, dextrose 20 g, agar 20 g/L.) at 28 ºC ± 1. A 6 mm mycelial disc of *Trichoderma* isolate taken from margin of actively growing 3 days old culture, was inoculated in the center of a 90 mm Petri plates containing PDA and incubated at 28 ± 1 °C for 5 days with three replications for each isolate. Colony characteristics and growth as mentioned above was recorded in 3 days old cultures.

Antagonistic potential of *Trichoderma* isolates

Dual culture assay

Trichoderma isolates were evaluated for their antagonistic activity against *Fusarium fujikuroi* strain F2 and *Fusarium proliferatum* strain F7 by dual culture plate method (Dennis and Webster 1971b). A 6 mm mycelial discs of fresh culture of the pathogen was inoculated at one end of Petri plate having PDA, and *Trichoderma* isolates were inoculated at opposite end. The Petri plate was incubated at 28 ± 1 °C for 7 days with three replications for each *Trichoderma* isolate. Control plate was inoculated with *Fusarium* spp. alone. Observations on growth inhibition percentage of *F. fujikuroi* and *F. proliferatum* were recorded at 7 days of incubation. Most promising 20 *Trichoderma* isolates (Fig. 1) showing

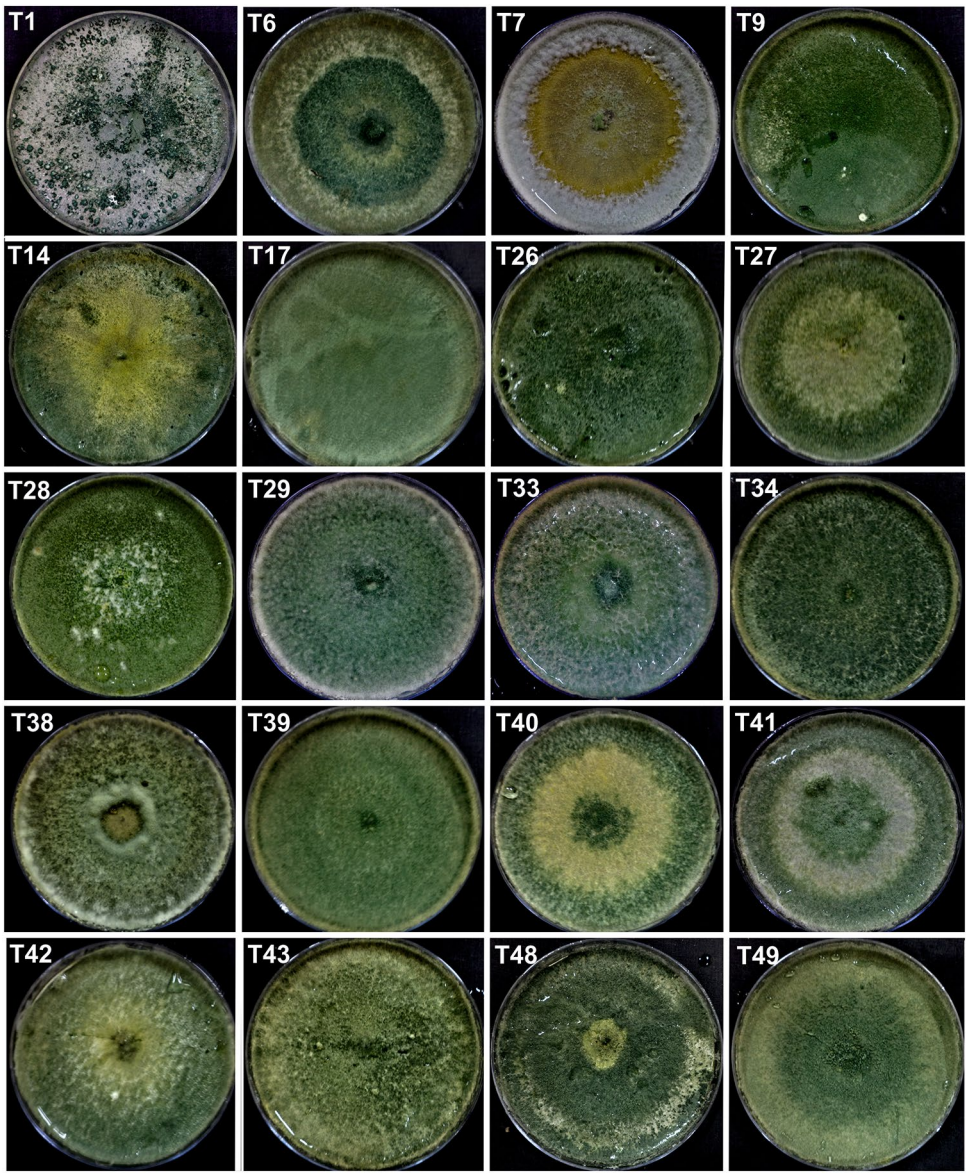

Fig. 1 Promising 20 *Trichoderma* strains (left–right)

highest inhibition in *Fusarium* growth were selected and further evaluated for their mycoparasitic activity and production of inhibitory volatiles against *F. fujikuroi* and *F. proliferatum*.

Mycoparasitic activity of *Trichoderma* isolates

The mycoparasitic activity of selected promising 20 *Trichoderma* isolates was observed by the method of Ojha and Chatterjee (2011). A small piece of mycelium was gently lifted from the inhibition zone of interaction between *Trichoderma* and *Fusarium* in dual culture. Mycelium was placed on slide, spread with needle, stained with lactophenol cotton blue and observed for coiling of *Trichoderma* around pathogen mycelium under microscope.

Production of inhibitory volatiles

Twenty selected promising *Trichoderma* isolates were evaluated for production of inhibitory volatiles against the test pathogens *F. fujikuroi* and *F. proliferatum* following the method (Dennis and Webster 1971b) with slight modification. A 6 mm mycelial disc of *Trichoderma* was inoculated in one Petri dish and similarly *Fusarium* culture in another Petri dish. After inoculation, lid of both inoculated Petri plates was removed and the base plates containing mycelial disc culture were packed together with cellophane adhesive tape in a manner to look like Petri dish. Further, inoculated Petri dish was kept for incubation a way that *Trichoderma* inoculated plate, was at bottom side. In control, Petri dish was only inoculated with *Fusarium* culture. Observations on the radial growth inhibition percentage of the test pathogen were recorded at 7 days of incubation period and inhibition percentage was calculated.

Molecular identification of promising *Trichoderma* isolates

Mycelia culture of twenty promising *Trichoderma* isolates was prepared by inoculating 6 mm mycelium disc on potato dextrose broth medium and kept them on shaker at 120 rpm at 26 ºC for 15 days. The culture was filtered using Whatman filter paper and further used for genomic DNA extraction. The total genomic DNA was extracted from mycelium mat through modified CTAB method described by Rogers and Benedich (1988). PCR reaction was performed by using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) at PCR conditions (94 ºC—3 min, followed by 30 cycles at 94 ºC—30 s, 48 ºC—30 s, 72 ºC—1 min, 72 ºC—7 min, and 4 ºC—forever). The PCR amplified purified product was used for ITS region sequencing through Sanger Technology. The nucleotide sequences of ITS region submitted to GenBank, NCBI for receiving accession number.

Results

Cultural characterization of *Trichoderma* isolates

A total of 51 *Trichoderma* isolates were isolated from rhizospheric soil samples, purified and maintained on PDA slants. The isolates were confirmed as *Trichoderma* spp. based on their cultural and morphological characters, and designated as T-1 to T-51. In cultural characterization studies, it was observed that in almost all isolates green conidia were visible within 48 h with the exception of isolates T1, T9, T34, and T38 which showed green conidia formation after 72 h of incubation period. Not much variation was observed in colony colour with colonies of all isolates in varying shades of green. Coconut odour was not observed in any *Trichoderma* isolate. Most of the isolates showed chlamydospore formation except T1, T9, T15, T21, T25, T34, T35, T38 and T44. In growth studies, however, some variation was noted among the 51 isolates. The diameter of the isolates after 24 h of incubation ranged from 32 to 42 mm among the isolates. At 48 h, the diameter was between 42 and 59 mm across the 51 isolates while at 72 h the diameter ranged from 67 to 81mm (Table 1).

Antagonistic activity of *Trichoderma* spp.

Considerable variability was observed in the inhibitory activity of 51 *Trichoderma* isolates against both of the pathogen species. Against *F. fujikuroi*, the inhibition percentage accorded by the *Trichoderma* isolates ranged from 53 to 78% while against *F. proliferatum*, inhibition percentage in range of 51–83% (Table 2; Fig. 2). Against *F. fujikuroi* strain F2, highest percentage of inhibition was observed in *Trichoderma* strain T28 (78%) followed by 76% inhibition by *Trichoderma* isolates T38, T49 and T41. The lowest inhibition percentage was recorded in *Trichoderma* strain T44 (53%). Overall, out of the 51 isolate 20 isolates showed > 70% inhibition in growth of *F. fujikuroi*, 30 isolate showed inhibition in range of 60–70% and one isolate showed < 60% inhibition. Against *F. proliferatum* strain F7, the highest percentage of inhibition was recorded in *Trichoderma* strain T28 (83%) followed by 80% pathogen inhibition by three isolates namely T38, T41, and T49 while the lowest inhibition was recorded in *Trichoderma* isolates T25 and T44 (51%). Among the 51 *Trichoderma* isolates, inhibition of > 70% was recorded in 20 isolates, 60–70% inhibition was recorded in 18 isolates while in 13 isolates < 60% inhibition of *Fusarium* growth was recorded. Similarly, Reddy

Table 1 Cultural and morphological characteristics of *Trichoderma* strains

Trichoderma isolates	Time for sporulation (h)	Colour	Concentric ring formation (+/−)	Chlamydospore	Colony diameter		
					After 24 h of incubation (mm)	After 48 h of incubation (mm)	After 72 h of incubation (mm)
T-1	48–72	Light green		–	35	44	72
T-2	24–48	Green	–	Present	36	46	70
T-3	24–48	Yellowish green	+	Present	32	42	67
T-4	24–48	Green	–	Present	32	43	67
T-5	24–48	Green	–	Present	36	46	71
T-6	24–48	Green	+	Present	32	42	70
T-7	24–48	Yellowish green	+	Present	33	42	72
T-8	24–48	Green	–	Present	36	45	70
T-9	48–72	Green	–	–	38	47	70
T-10	24–48	Green	–	Present	35	46	70
T-11	24–48	Green	–	Present	40	49	70
T-12	24–48	Green	–	Present	40	49	70
T-13	24–48	Green	–	Present	38	46	68
T-14	24–48	Yellowish green	–	Present	38	46	71
T-15	24–48	Green	–	–	38	46	71
T-16	24–48	Green	–	Present	38	46	71
T-17	24–48	Green	–	Present	40	51	72
T-18	24–48	Light green	–	Present	40	52	70
T-19	24–48	Light green	–	Present	40	52	71
T-20	24–48	Green	–	Present	40	52	71
T-21	24–48	Green	–	–	38	50	70
T-22	24–48	Green	–	Present	38	50	70
T-23	24–48	Green	–	Present	36	47	70
T-24	24–48	Green	–	Present	40	58	72
T-25	24–48	Green	–	–	39	56	70
T-26	24–48	Green	–	Present	40	58	76
T-27	24–48	Green	+	Present	35	51	72
T-28	24–48	Green		Present	36	51	76
T-29	24–48	Green		Present	42	57	76
T-30	24–48	Green	+	Present	40	58	74
T-31	24–48	Green	–	Present	39	58	74
T-32	24–48	Green	+	Present	38	59	72
T-33	24–48	Green		Present	38	54	74
T-34	48–72	Green	–	–	38	53	72
T-35	24–48	Green	+	–	38	53	71
T-36	24–48	Green	–	Present	36	51	71
T-37	24–48	Green	–	Present	34	53	72
T-38	48–72	Green	–	–	39	53	74
T-39	24–48	Green	+	Present	37	52	74
T-40	24–48	Green	+	Present	37	52	70
T-41	24–48	Green	+	Present	39	53	71
T-42	24–48	Green		Present	39	53	72
T-43	24–48	Green		Present	41	56	79
T-44	24–48	Green	–	–	39	50	72
T-45	24–48	Green	–	Present	39	51	71
T-46	24–48	Green	+	Present	41	57	81
T-47	24–48	Green	+	Present	37	55	71

Table 1 (continued)

Trichoderma isolates	Time for sporulation (h)	Colour	Concentric ring formation (+/−)	Chlamydospore	Colony diameter		
					After 24 h of incubation (mm)	After 48 h of incubation (mm)	After 72 h of incubation (mm)
T-48	24–48	Green		Present	38	52	68
T-49	24–48	Green	+	Present	42	57	81
T-50	24–48	Green	−	Present	42	57	81
T-51	24–48	Green	+	Present	38	52	70

et al. (2014) identified antagonistic potential of *Trichoderma* spp. against soil borne plant pathogens.

Studies on mycoparasitic nature of 20 selected promising *Trichoderma* isolates against the *Fusarium* pathogen revealed two types of hyphal interaction. *Trichoderma* growing parallel and or in contact with *F. fujikuroi*, this hyphal interaction was observed in four *Trichoderma* isolates (T28, T41, T49 and T40) (Fig. 3a). Coiling hyphal interaction was observed in seven *Trichoderma* isolates (T9, T17, T26, 28, T41, T49 and T40) against the hyphae of *F. proliferatum* (Fig. 3b). Mycoparasitism is considered an important mechanism employed by *Trichoderma* for inhibiting its target pathogens also reported by Kumar et al. (2007).

The potential *Trichoderma* isolates were evaluated for inhibitory volatile metabolite production against *Fusarium* culture. The results revealed that against *F. fujikuroi*, inhibitory volatiles produced by *Trichoderma* isolates resulted in inhibition in range of 55.6–77.8% whereas against *F. proliferatum* the growth inhibition was in range of 61.1–83.3% (Table 3; Fig. 4). Highest inhibition percentage was recorded in *Trichoderma* strain T28 (77.8%) followed by T38 and T41 (75.6%) and T49 (74.4%) against *F. fujikuroi* while lowest inhibition percentage of 55.6% was recorded in *Trichoderma* isolates T26, T29 and T33. Results revealed that similar inhibition trends against *F. proliferatum* with *Trichoderma* isolate T28 (83.3%) followed by T41 and T49 (80.0%) and T38 (75.6%) whereas lowest inhibition percentage was recorded in *Trichoderma* isolate T48 with 61.1% of inhibition.

Molecular identification of *Trichoderma* isolates

Twenty potential strains of *Trichoderma* spp., were molecularly identified by sequencing ITS region (ITS1 and ITS4). The ITS region sequences were submitted to GenBank NCBI and allotted accession numbers (Table 4). The nucleotide sequences were aligned using online available software Phylogeny.fr. The ITS sequences were clustered in two group based their nucleotide similarity. Except *Trichoderma asperellum*, all 19 *Trichoderma* strains come under single group. The phylogeny tree indicates that there is not more variation in isolated *Trichoderma* strains from sugarcane rhizosphere. The identified *Trichoderma* strains were *T. harzianum*, *T. afroharzianum*, *T. atrobrunneum*, *T. aureoviride*, and *T. asperellum*. The efficiency of *T. afroharzianum*, *T. atrobrunneum* and *T. aureoviride*, in controlling plant diseases are numerously reported.

Discussion

Pokkah boeng is one of the most serious fungal disease in the sugarcane growing regions of the world. Disease significantly reduces the yield and quality of sugarcane, which is found in susceptible cultivars ranging from 40 to 60%. In the present study, we designed an in-vitro system to identify isolates of *Trichoderma* with potential to reduce the *Pokkah boeng* disease incidences. *Trichoderma* sp. can act as biocontrol agent by means of various synergistic pathways. It is difficult to forecast, however, in a natural pathosystem, the degree of synergism and the action of BCA.

All the fifty one *Trichoderma* strains inhibited mycelial growth of the pathogen. *T. harzianum* strain T28 inhibited maximum mycelial growth of both the pathogen *F. fujikuroi* (F2) and *F. proliferatum* (F7). The mechanism of inhibition may be competition for food and space. Coiling of antagonists hyphae around hyphae of *Fusarium* and lysis was observed (Kumar and Dubey 2001). The mycoparasitic activity of *Trichoderma* spp. against *Fusarium* is usually attributed to a combination of successful competition in nutrient and rhizosphere colonisation, cell wall degrading enzymes such as harzianic acid, alamethics, tricholine, peptaibols, 6-penthyl-alpha-pyrone, massoilactone, viridine, gliovirin, glisoprenine, heptilide acid and antibiosis (Sharma 2011; Brunner et al. 2005). Therefore. As alternatives to chemical fungicides, several *Trichoderma* isolates are being evaluated. However, the use of *Trichoderma* is not yet widespread for the biological control of sugarcane *Pokkah boeng* disease. *T. harzianum* and *T. viride* were reported by several workers as the best antagonists for growth inhibition of several soil and seed borne pathogens (Dubey 2003; Joshi and Mishra 2013). Growth of pathogen was also inhibited by the production of volatile substances. Initially, at 3 days it was

Table 2 Evaluating antagonistic effects of *Trichoderma* spp. against pathogen *F. fujikuroi* (F2) *and Fusarium proliferatum* (F7)

Strain. no.	Pathogen (F2) diameter (cm) after 7 days	% inhibition of pathogen (F2) after 7 days	Antagonistic effect of *Trichoderma* spp. after 7 days	Pathogen (F7) diameter (cm) after 7 days	% inhibition of pathogen (F7) after 7 days	Antagonistic effect of *Trichoderma* spp. after 7 days
T1	1.5	75	+++	1.3	79	+++
T2	2.2	66	++	2.0	67	++
T3	2.1	67	++	2.0	67	++
T4	2.2	66	++	2.0	67	++
T5	2.2	66	++	1.9	69	++
T6	1.9	70	+++	1.4	77	+++
T7	1.5	75	+++	1.5	75	+++
T8	2.0	69	++	2.7	56	+
T9	1.8	72	+++	1.5	75	+++
T10	2.2	66	++	2.3	62	++
T11	2.2	66	++	2.8	54	+
T12	2.1	67	++	2.3	62	++
T13	2.5	61	++	1.9	69	++
T14	1.9	70	+++	1.4	77	+++
T15	2.1	67	++	1.9	69	++
T16	2.2	66	++	1.9	69	++
T17	1.9	70	+++	1.4	77	+++
T18	2.0	69	++	2.3	62	++
T19	2.1	67	++	2.6	58	+
T20	2.2	66	++	2.5	59	+
T21	2.0	69	++	2.2	64	++
T22	2.1	67	++	2.4	63	++
T23	2.0	69	++	2.6	58	+
T24	2.3	64	++	2.2	64	++
T25	2.4	63	++	3.0	51	+
T26	1.8	72	+++	1.5	75	+++
T27	1.9	70	++	1.5	75	+++
T28	1.4	78	+++	1.0	83	+++
T29	1.9	70	+++	1.4	77	+++
T30	2.1	67	++	2.5	59	+
T31	2.0	69	++	2.4	63	++
T32	2.0	69	++	2.5	59	+
T33	1.9	70	+++	1.5	75	+++
T34	1.8	72	+++	1.5	75	+++
T35	2.0	69	++	2.0	67	++
T36	2.3	64	++	2.4	63	++
T37	2.3	64	++	2.5	59	+
T38	1.5	76	+++	1.2	80	+++
T39	1.8	72	+++	1.5	75	+++
T40	1.9	70	+++	1.5	75	+++
T41	1.5	76	+++	1.2	80	+++
T42	1.9	70	+++	1.5	75	+++
T43	1.9	70	+++	1.5	75	+++
T44	3.0	53	+	3.6	51	+
T45	2.2	66	++	2.0	67	++
T46	2.3	64	++	2.8	54	+
T47	2.2	66	++	2.4	63	++
T48	1.9	70	+++	1.5	75	+++

Table 2 (continued)

Strain. no.	Pathogen (F2) diameter (cm) after 7 days	% inhibition of pathogen (F2) after 7 days	Antagonistic effect of *Trichoderma* spp. after 7 days	Pathogen (F7) diameter (cm) after 7 days	% inhibition of pathogen (F7) after 7 days	Antagonistic effect of *Trichoderma* spp. after 7 days
T49	1.5	76	+++	1.2	80	+++
T50	2.2	66	++	2.8	54	+
T51	2.0	69	++	2.5	59	+
Control	9.0	00	–	9.0	00	–

≥ 70 (+++) high level, 60–70 (++) medium level, ≤ 60 (+) low level, of antagonism against the pathogen

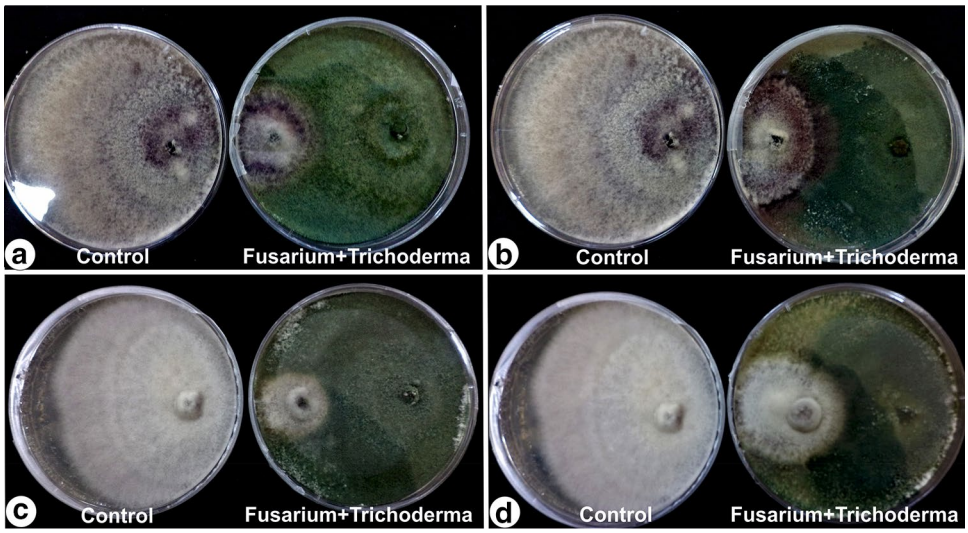

Fig. 2 Dual Culture Plate Technique against *Fusarium* strain F2 and F7. **a** Highest inhibition of pathogen strain F2 by *Trichoderma* strain T28. **b** Least inhibition of pathogen strain F2 by *Trichoderma* strain T44. **c** Highest inhibition of pathogen strain F7 by *Trichoderma* strain T28. **d** Least inhibition of pathogen strain F7 by *Trichoderma* T25. A control was performed for comparison in left side of each figure

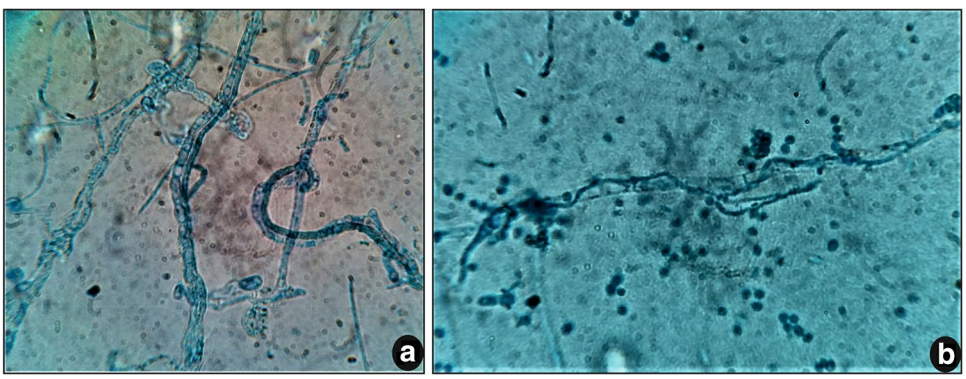

Fig. 3 Two types of mycoparasitism showed by *Trichoderma* strains. **a** Coiling against *F. fujikuroi* strain F2. **b** Coiling against *F. proliferatum* strain F7

recorded there was not much effect of volatile compounds on *Fusarium* strains but later on at 7 days 83.3% growth inhibition of *F. proliferatum* (F7) were recorded by *T. harzianum* (T28) which may be due to production of higher amount of volatile compounds upon ageing. *F. fujikuroi* (F2) isolate comparatively less affected by the volatile compounds of *T. harzianum* (T28) recorded 77.8% of inhibition. By the production of secondary metabolites, *Trichoderma* spp. inhibit the growth of soil borne plant pathogen, reported by Vinale et al. (2008). 2-methyl-1-propanol volatile metabolites produced by *Trichoderma* have antimicrobial activity against wide range of plant pathogens (Li et al. 2018) and production of some volatile compounds in *T. harzianum* (Zhang et al. 2014). Many volatile substances like dibutyl phthalate (DBP) have also been identified from *T. virens*, have antifungal activity (Tabarestani et al. 2016).

Trichoderma harzianum and other allied spp., are abundant in sugarcane rhizospheric ecosystem. Currently, genus *Trichoderma* consists more than 100 phylogenetically defined species. All the fifty one isolated strains were morphologically identified as *Trichoderma* spp. However, the taxonomic characterization of *Trichoderma* spp., based

Table 3 Volatile mediated inhibition of pathogen *F. fujikuroi* (F2) *and F. proliferatum* (F7) by *Trichoderma* strains

S. no.	*Trichoderma* strains	Growth (mm) of pathogen F2 after 7 days	% inhibition of pathogen (F2)	Volatile mediated effect of *Trichoderma* spp. after 7 days	Growth (mm) of pathogen F7 after 7 days	% inhibition of pathogen	Volatile mediated effect of *Trichoderma* spp. after 7 days
1	T1	25	72.2	+++	25	72.2	+++
2	T6	25	72.2	+++	26	71.1	+++
3	T7	26	71.1	+++	28	68.9	++
4	T9	28	68.9	++	26	71.1	+++
5	T14	26	71.1	+++	27	70.0	+++
6	T17	35	61.1	++	25	72.2	+++
7	T26	40	55.6	+	25	72.2	+++
8	T27	35	61.1	++	28	68.9	++
9	T28	20	77.8	+++	15	83.3	+++
10	T29	40	55.6	+	25	72.2	+++
11	T33	40	55.6	+	27	70.0	+++
12	T34	25	72.2	+++	25	72.2	+++
13	T38	22	75.6	+++	22	75.6	+++
14	T39	28	68.9	++	30	66.7	++
15	T40	28	68.9	++	25	71.1	+++
16	T41	22	75.6	+++	18	80.0	+++
17	T42	25	72.2	+++	27	70.0	+++
18	T43	25	72.2	+++	28	68.9	++
19	T48	35	61.1	+	35	61.1	++
20	T49	23	74.4	+++	18	80.0	+++
21	+Ve control	90	00	–	90	00	–

≥ 70 (+++) high level, 60–70 (++) medium level, ≤ 60 (+) of volatile inhibition against the pathogen

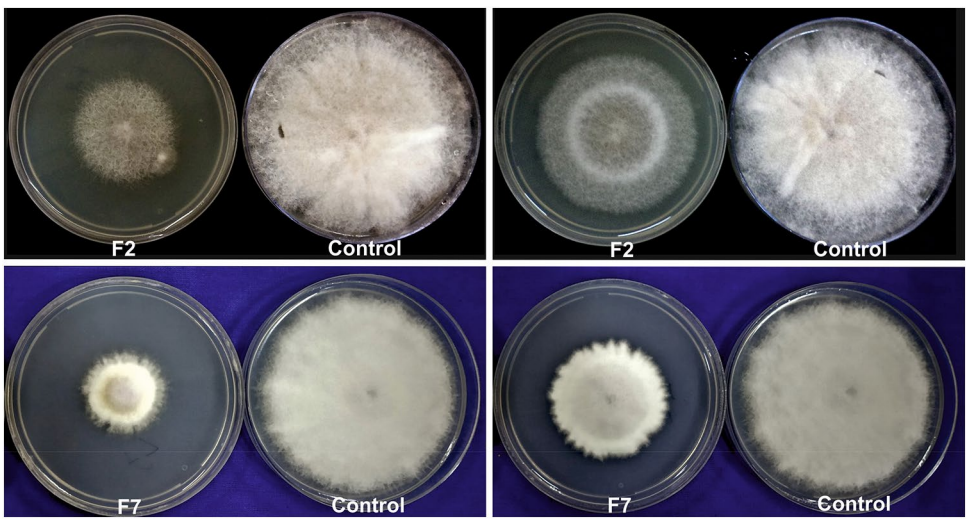

Fig. 4 Volatile mediate inhibition against *Fusarium* strain F2 and F7. **a** Highest inhibition of pathogen F2 by *Trichoderma* strain T28. **b** Least inhibition of pathogen F2 by *Trichoderma* strain T26. **c** Highest inhibition of pathogen F7 by *Trichoderma* strain T28. **d** Least inhibition of pathogen F7 by *Trichoderma* strain T48. A control was performed for comparison in right and left side showed pathogen inhibited in each figure

on only morphological characteristics, can be considered limited and of low accuracy, due to variation in its characteristics (Hebert et al. 2003). In addition, morphological features are influenced by cultural conditions (Diguta et al. 2011). The use of molecular technologies to compensate for the limitations of morphological characterization is therefore necessary. DNA sequences of the 5.8 s-ITS region was done in this study. According to Kullnig-Gradinger et al. (2002), the ITS region is one of the most reliable loci for species-level detection of a strain. Hermosa et al. (2000), adopted the internal transcribed spacer rDNA (ITS) based molecular technique for identification of *Trichoderma* species. Similarly, Brazilian *Trichoderma* isolates have been characterized based on genetic and morphological criteria

Table 4 ITS region sequencing of twenty potential *Trichoderma* isolates and GenBank Accession number

S. no.	Strain no.	Strain name	GenBank Accession no.
1.	T1	*Trichoderma asperellum*	MH150937
2.	T6	*Trichoderma harzianum*	MH151122
3.	T7	*Trichoderma harzianum*	MH151158
4.	T9	*Trichoderma afroharzianum*	MH155303
5.	T14	*Trichoderma harzianum*	MH151203
6.	T17	*Trichoderma harzianum*	MH156051
7.	T26	*Trichoderma harzianum*	MH156054
8.	T27	*Trichoderma harzianum*	MH156055
9.	T28	*Trichoderma harzianum*	MH156058
10.	T29	*Trichoderma harzianum*	MH156141
11.	T33	*Trichoderma harzianum*	MH156143
12.	T34	*Trichoderma atrobrunneum*	MH156193
13.	T38	*Trichoderma aureoviride*	MH156197
14.	T39	*Trichoderma harzianum*	MH156203
15.	T40	*Trichoderma harzianum*	MH161377
16.	T41	*Trichoderma harzianum*	MH156214
17.	T42	*Trichoderma harzianum*	MH156225
18.	T43	*Trichoderma harzianum*	MH156422
19.	T48	*Trichoderma harzianum*	MH156423
20.	T49	*Trichoderma harzianum*	MH156424

(Sharma et al. 2009). By comparing the 5.8 s-ITS region with the sequences deposited in GenBank, it is possible to classify all *Trichoderma* isolates with homology percentage at the species level. Thus, potential 20 *Trichoderma* spp. were successfully identified by integrating morphological and molecular approaches.

Conclusions

The genus *Trichoderma* has been identified as biological control agents for controlling large group soil borne plant pathogens as well as plant growth promoting attributes. *Pokkah boeng* is an emerging major disease in sugarcane, caused by various *Fusarium* spp. Number of fungicides as well as resistant sugarcane genotype are adopted to minimize incidence of disease. Application of fungicide against *Pokkah boeng* pathogen is neither effective nor eco-friendly safe. For its effective and eco-friendly management, diverse group of *Trichoderma* strains were isolated and characterized. In in-vitro studies, it was observed that *Trichoderma* isolates have the potential to effectively suppress the growth of *Pokkah boeng* pathogen—*F. fujikuroi and F. proliferatum* associated with sugarcane. Based on the antagonistic assays, four promising isolates *T. harzianum strain* T28, *T. aureoviride strain* T38, *T. harzianum strain* T41 and *T. harzianum strain* T49 were identified showing > 70% inhibition in *Fusarium* growth. The promising isolates identified need to be evaluated further in field conditions for management of this disease, and the work is under progress.

Acknowledgements We express our sincere thanks to Director, ICAR-Indian Institute of Sugarcane Research, Lucknow (U.P.) for continuous support and providing us necessary facilities to conduct the studies. We also express our gratitude to AIB, Amity University, Lucknow (U.P.) for giving me an opportunity to take up research work for Ph.D.

Declarations

Conflict of interest The authors declare that they have no conflict of interest that could have appeared to influence the work reported in this paper.

Ethical approval This article does not contain any studies with human participants oranimals performed by any of the authors.

Informed consent This manuscript is new and not being considered elsewhere. All authorshave approved the submission of this manuscript.

References

Brunner K, Zeilinger S, Ciliento R, Woo SL, Lorito M, Kubicek CP, Mach RL (2005) Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. Appl Environ Microbiol 71:3959–3965

Dennis C, Webster J (1971b) Antagonistic properties of species groups of *Trichoderma* III. Hyphal interactions. Trans Br Mycol Soc 57:363–369

Diguta CF, Vincent B, Guilloux-Benatier M, Alexandre H, Rousseaux S (2011) PCR ITS-RFLP: a useful method for identifying filamentous fungi isolates on grapes. Food Microbiol 28:1145–1154

Dubey SC (2003) Integrated management of web blight of urd/mung bean by bio-seed treatment. Indian Phytopathol 56:34–38

Harman GE (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol 189:647–649

Hebert PD, Cywinska A, Ball SL, de Waard JR (2003) Biological identifications through DNA barcodes. Proc Biol Sci 270:313–321

Hermosa MR, Grondona I, Iturriaga EA, Diaz-Minguez JM, Castro C, Monte E (2000) Molecular characterization and identification of bio-control isolates of *Trichoderma* spp. Appl Environ Microbiol 66:1890–1898

IISR (2018–19) Annual report. Indian Institute of Sugarcane Research, Lucknow, pp 23

Johnson LF, Curl EA (1972) Methods for research on the ecology of soil borne plant pathogens. Burgess publishing company, Minneapolis

Joshi D, Misra S (2013) Characterization of *Trichoderma* isolates from sugarcane agro-ecosystem and their efficacy against *Colletotrichum falcatum* causing red rot of sugarcane. Sugar Tech 15:192–196. https://doi.org/10.1007/s12355-013-0208-y

Joshi D, Singh P, Holkar SK, Kumar S (2019) *Trichoderma*-mediated suppression of red rot of sugarcane under field conditions in subtropical India. Sugar Tech 21:496–504

Kullnig-Gradinger CM, Szakacs G, Kubicek CP (2002) Phylogeny and evolution of the genus *Trichoderma*:a multigene approach. Mycol Res 106:757–767

Kumar D, Dubey SC (2001) Management of collar rot of pea by the integration of biological and chemical methods. Indian Phytopathol 57:62–66

Kumar M, Shahi SK, Shahi MP, Choudhary S (2007) Mycoparasitism of *Trichoderma harzianum* against *Fusarium oxysporum* and *Aspergillus niger* in in-vitro conditions. Plant Archit 7:741–743

Li N, Alfiky A, Wang W, Islam M, Nourollahi K, Liu X, Kang S (2018) Volatile compound-mediated recognition and inhibition between *Trichoderma* biocontrol agents and *Fusarium oxysporum*. Front Microbiol 9:2614

Lin Z, Xu S, Que Y, Wang J, Comstock JC, Wei J, McCord PH, Chen B, Chen R, Zhang M (2014) Species-specific detection and identification of *Fusarium* species complex, the causal agent of sugarcane *Pokkah boeng* in China. PLoS One. https://doi.org/10.1371/journal.pone.0104195

Ojha S, Chatterjee NC (2011) Mycoparasitism of *Trichoderma* spp. in bio-control of Fusarial wilt of tomato. Archi Phytopathol Plant Prot 44:771–782

Reddy BN, Saritha KV, Hindumathi A (2014) In-vitro screening for antagonistic potential of seven species of *Trichoderma* against different plant pathogenic fungi. Res Rev Res J Biol 2:29–36

Rogers SO, Benedich (1988) Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual, vol A6. Kluwer Academic Publishers, Botson, pp 1–10

Sharma P (2011) Complexity of *Trichoderma-fusarium* interaction and manifestation of biological control. Aust J Crop Sci 5:1027–1038

Sharma K, Mishra AK, Misra RS (2009) Morphological, biochemical and molecular characterization of *Trichoderma harzianum* isolates for their efficacy as bio-control agents. J Phytopathol 157:51–56

Sharma P, Sharma M, Raja M, Shanmugam V (2014) Status of *Trichoderma* research in India: a review. Indian Phytopathol 67:1–19

Shiqiang X, Wang j, Wang H, Bao Y, Li Y, Govindaraju M, Yao W, Chen B, Zhang M (2019) Molecular characterization of carbendazim resistance of Fusarium species complex that causes sugarcane *Pokkah boeng* disease. BMC Genom 20:115

Singh A, Chauhan SS, Singh A, Singh SB (2006) Deterioration in sugarcane due to Pokkah boeng disease. Sugar Tech 8:187–190

Siti Nordahliawate MS, Nur Ain Izzati MZ, Azmi AR, Salleh B (2008) Distribution, morphological characterization and pathogenicity of *F. sacchari* associated with Pokkah boeng disease of sugarcane in Peninsular Malaysia. Pertanika J Trop Agric Sci 31:279–286

Tabarestani MS, Rahnama K, Jahanshahi M, Nasrollanejad S, Fatemi M (2016) Identification of volatile organic compounds from *Trichoderma virens* (6011) by GC–MS and separation of a bioactive compound via nanotechnology. Int J Eng Trans A Basics 29:1347–1353

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) *Trichoderma*-plant–pathogen interactions. Soil Biol Biochem 40:1–10

Vishwakarma S, Kumar P, Nigam A, Singh A, Kumar A (2013) Pokkah boeng: an emerging disease of sugarcane. J Plant Pathol Microbiol. https://doi.org/10.4172/2157-7471.1000170

Viswanathan R, Rao GP (2011) Disease scenario and management of major sugarcane diseases in India. Sugar Tech 13:336–353

Vishwanathan R, Poongothai M, Malathi P (2011) Pathogenic and molecular confirmation of *Fusarium sacchari* causing wilt in sugarcane. Sugar Tech 13:68–76

Zhang F, Yang X, Ran W, Shen Q (2014) *Fusarium oxysporum* induces the production of proteins and volatile organic compounds by *Trichoderma harzianum* T-E5. FEMS Microbiol Lett 359:116–123