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PLANT PATHOLOGY

Trichoderma spp. of West Bengal Tea Cropping System: Study on Cultural Characteristics and Conidia Production Potency

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ABSTRACT

Fungi belonging to the genus *Trichoderma* possess a tremendous antagonism in managing the pathogens causing various diseases in crops. The rate of mycelial growth and the number of spores of antagonists have a direct impact on the control of phytopathogens. Six *Trichoderma* strains developed at Tea Research Association, North Bengal Regional Research and Development Centre, Nagrakata were evaluated for their rate of mycelial growth and sporulation under laboratory conditions through test tube and Petriplate culture methods. Investigations revealed that all the isolates were fast-growing, they started to grow after 6 hours of inoculation on an artificial medium (Potato dextrose agar). Isolates exhibited small variation in their growth rate, initiation of conidiation, and color of conidia. The mycelial growth rate of isolate KBN-24 and KBN-34 was very fast, however, it was slower in isolate KBN-29 and KBN-32. They produced light yellow, light green, and dark green conidia. Diffusion of yellow pigment was also noticed in isolate KBN-33. The earliest conidiation was observed in isolate KBN-33. Isolate KBN-32 and KBN-24 could produce conidia after 72 hours, however; isolate KBN-34, KBN-29 and KBN-35 produced after 96 hours of inoculation. In Petri plates, isolates produced dull-white, light cream, and white-colored mycelia and also showed variations in the number of concentric rings of conidia. Three isolates were found to be fast-growing and covered the entire surface area of the plate within 72 hours. All the isolates produced 78.6 to 310.7 × 10^8/ mL conidia after a month of inoculation indicating their suitability as promising antagonists.

HIGHLIGHTS

- *Trichoderma* strains could be applied to manage soil-borne fungal pathogens of tea plantations.

Keywords: Tea, *Trichoderma* spp., mycelial growth, sporulation

Plants, in their natural surroundings, encounter numerous micro-organisms in different ways and many of them cause serious diseases that account for severe crop loss if not controlled by adopting appropriate control measures. Tea (*Camellia* sp.), is one of the most important plantation crops being attacked by different diseases (Lehmann-Danzinger, 2000) responsible for huge crop losses.

The most effective way to manage these diseases is the application of different fungicides. However, their continuous and injudicious use may certainly

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invite numerous problems such as environmental pollution, ecological imbalance, human health hazards, development of resistance, etc. apart from resulting in undesirable residues in made tea. Biological control of fungal phytopathogens is one of the alternative methods to manage these diseases in a comparatively safer way, which ensures crop sustainability more safely. Among various biological control agents (BCAs), the genus *Trichoderma* is considered one of the most promising ones, which has gained substantial importance during the last few decades due to its potential as a biological control agent against a wide range of phytopathogens (Eke *et al.* 2021; Kumhar *et al.* 2020; Khalid 2017).

Both *Trichoderma harzianum* and *T. viride* have been found successful in inhibiting the growth of soil-borne phytopathogens as well as in the improvement of overall plant health (Vinale and Sivasithamparam 2020; Syam *et al.* 2021; Cangi *et al.* 2020; Miftakhurrohmat and Sutarman 2021). The *Trichoderma* species could manage the phytopathogens in numerous ways like competition for nutrients and space (Mukhopadhyay and Kumar 2020) and other ways (Ghosh *et al.* 2017), modifying the environmental conditions (Benítez *et al.* 2004), secretion of antifungal metabolites (Li *et al.* 2019), mycoparasitism or hyperparasitism involving the production of lytic enzymes (Elad *et al.* 1982), promotion of plant growth and plant defensive mechanisms leading to induced resistance in the host (Benítez *et al.* 2004; Ozbay and Newman 2004) and production of siderophores (Dutta *et al.* 2006) a microorganism competes for Fe.

Research work on the biological control of tea phytopathogens with local antagonists has not yet been exploited to a significant level. Therefore, the present study had been planned to identify the native *Trichoderma* isolates having fast growth attribute with a very fabulous sporulation potency that could ultimately transform into a suitable qualitative product for the eventual application in tea plantations as an alternative to synthetic fungicide, especially for organic tea gardens.

MATERIALS AND METHODS

Isolation of *Trichoderma* species

Soil samples at a depth of 6-8 inches were collected systemically from different tea gardens of the Dooars region and experimental fields of Tea Research Association, North Bengal Regional Research and Development Centre, Nagrakata (88° 55′ 0″ East, 26° 54′ 0″ North longitude) West Bengal, India. The collected soil samples were kept in sterilized zipper polyethylene bags with proper labeling and stored in the refrigerator until processing.

Species of *Trichoderma* were isolated from soil samples by adopting the method of earlier researchers with slight modifications (Askew & Laing 1993; Cherkupally, Amballa & Bhoomi 2017; Awad *et al.* 2018) using potato dextrose agar medium (Hi-Media). Two hundred microliters from the 5^{th} and 6^{th} serial dilutions were transferred followed by uniform spreading in PDA plates and incubated at 26 ± 2 °C for 4 days. Appeared fungal colonies in plates were located and purified as per the slightly modified method of earlier workers (Wei *et al.* 2017) and incubated at 26 ± 2 °C for 7-8 days. Green-colored conidia forming colonies were identified, further, from these colonies, slides were prepared and viewed under the light microscope to identify *Trichoderma* sp. based on cultural and morphological characteristics (Rifai 1969; Bissett 1991). The pure cultures were maintained on potato dextrose agar slants as well as plates (Cherkupally *et al.* 2017).

Comparative cultural characteristics

The mycelia characteristics mainly growth rate and color, the pattern of conidial initiation their color, and conidial quantum of *Trichoderma* isolates were studied through slant and plate culture technique. In the slant culture technique, loopful inocula were inoculated in the test tube, whereas in the plate culture technique, five mm mycelial bit of actively growing cultures (3 days old culture) were cut with the help of a sterilized cork borer and inoculated at the center of plates, and the plates were sealed properly with parafilm strips. Test tubes and plates were then incubated at room temperature in the laboratory with alternate light and darkness. The average morning and evening temperature during the study period was 21.8 and 24.7 ^{0}C, respectively. Four replications were maintained per isolates in both methods. Observations on colony linear growth/diameter, colony color, conidiation initiation, and their color were recorded at an interval of 24 hours.

Assessment of conidia production potentiality of *Trichoderma* spp.

The desired quantity/concentration of spores/ conidia and or mycelial bit available in the product formulation determines the success of a particular product while applying to the crops to solve fungal phytopathogen. Therefore, the product registering apex / nodal agency i.e. Central Insecticide Board and Registration Committee (CIB&RC) of Directorate of Plant Protection, Quarantine & Storage, Ministry of Agriculture & Farmers Welfare, Department of Agriculture, Cooperation & Farmers Welfare, Government of India has set certain standards for a particular product and active ingredient in form of colony-forming units (CFU) per unit volume is decided as 2×10^6 to 2×10^8 CFU per ml or gram for the antagonists. For assessment of conidia production potency, an experiment was carried out. The plates were inoculated with different isolates separately and were incubated at room temperature, continuously for 30 days. After that 50 mL of distilled water was added to each plate and conidial biomass was harvested with the help of a sterilized stainless steel spatula, and the conidial suspension was collected into conical flasks. It was serially diluted up to the 7th dilution. Then 100 µL was taken from each sample with the help of a micropipette (Riviera™) and was transferred to PDA plates followed by uniform spreading and incubation at 26±2 °C for 24 hours. Three replications for each sample were kept. Developed colonies were assessed visually slide and were analyzed statistically.

RESULTS AND DISCUSSION

Isolation of *Trichoderma* species

A total of six *Trichoderma* isolates were collected from soil samples (Table 1). Based on colony characteristics *i.e.* growth pattern and colony color they were initially identified as *Trichoderma* sp. Then, microscopic observations were made to visualize the conidiophores and phialides architect as well as the shape of conidia (Seaby 1996). Later, their identity was get re-confirmed from the Indian Type Culture Collection, Division of Plant Pathology, ICAR- Indian Agricultural Research Institute, New Delhi, India.

Table 1: Details of *Trichoderma* isolates

Sl. No.	Trichoderma isolate	Sample used for isolation
1	KBN-34 – *T. harzianum*	Soil
2	KBN-32 – *T. harzianum*	Soil
3	KBN-29 – *T. asperellum*	Soil
4	KBN-35 – *T. harzianum*	Soil
5	KBN-33 – *T. harzianum*	Soil
6	KBN-24 – *T. atroviride*	Soil

T. harzianum formed 1-2 concentric rings with green-colored conidial production. Conidial production was denser in the center than margins. Conidia were globose to subglobose, light green colored, its phialides were flask-shaped, arranged in a divergent group of 2-4.

T. atroviride colonies exhibited a fast-growing nature. Conidiation was granular or crusty at the periphery; conidia were initially glaucous, rapidly turned dark green color. The reverse was usually colorless, otherwise dull yellowish or drab in age. Phialides were solitary, or 2–4- verticillate, more or less lageniform, and often curved. Conidia were dark green-colored, smooth, subglobose at maturity. Seven *Trichoderma* strains were isolated from the tea cropping system of southern India and their cultural morphology was investigated (Kuberan *et al.* 2012). Earlier researchers had isolated *Trichoderma* strains from different tea rhizosphere (Ahmad *et al.* 2013; Karaoglu and Ulker 2006).

A few *Trichoderma* isolates were recovered from mature tea plantations and soils of Bangladesh Tea Research Institute (BTRI) which started their growth after 24 hrs of inoculation and after 120 hrs they revealed more than 80 percent control of the branch canker phytopathogen (Ahmad *et al.* 2013), *T. asperellum* which was tolerant to acid and aluminum was isolated from tea fields (Kawai *et al.* 2000). *Trichoderma* sp. and *Gliocladium virens* were isolated from soil in Turkey (Karaoglu and Ulker 2006). Several *Trichoderma* isolates sp. were achieved from rhizosphere soils of different locations of districts of Uttarakhand state in our country (Sharma and Singh 2014).

Comparative cultural characteristics

Studies revealed that all *Trichoderma* isolates started their mycelia growth after 6 hours of inoculation. In slants, cultures showed variations in mycelial

Table 2: *In vitro* comparative mycelial growth of *Trichoderma* isolates on potato dextrose agar slants

***Trichoderma* isolate**	**Linear mycelial growth (mm) after days of inoculation***		
	24 hr	**48 hr**	**72 hr**
KBN 34 – *T. harzianum*	0.0	22.50 ± 1.04	60.75 ± 0.85
KBN 32 – *T. harzianum*	0.0	30.50 ± 1.04	39.75 ± 0.85
KBN 29 – *T. asperellum*	0.0	38.75 ± 1.31	40.50 ± 0.65
KBN 35 – *T. harzianum*	0.0	39.00 ± 1.22	50.50 ± 0.65
KBN 33 – *T. harzianum*	0.0	49.75 ± 0.85	55.50 ±0.65
KBN 24 – *T. atroviride*	3.0	45.75 ± 0.85	60.75 ± 0.85
C.D.		3.20	2.27
C.V.		5.67	2.95

** Values represent mean of 4 replications, ± Standard Error*

Table 3: Developmental events of mycelial growth and conidiation of *Trichoderma* isolates on PDA slant

***Trichoderma* isolate**	**Developmental events**	
	After 24 hr	**After 120 hr**
KBN 34	Light white mycelia	Light green colored conidiation
KBN 32	Light white mycelia	Dark green-colored conidiation
KBN 29	Light white mycelia	Light yellowish green colored conidiation
KBN 35	Light white mycelia	Light green colored conidiation
KBN 33	Light white mycelia	Yellowish green colored conidiation
KBN 24	Light white mycelia	Dark green-colored conidiation

Table 4: *In vitro* comparative mycelial growth of *Trichoderma* isolates on potato dextrose agar plate

***Trichoderma* isolate**	**Colony diameter (mm) after hours of inoculation***		
	24 hr	**48 hr**	**72 hr**
KBN 34 – *T. harzianum*	13.50 ± 0.65	44.75 ± 1.03	82.75 ± 1.11
KBN 32 – *T. harzianum*	13.00 ± 0.91	50.50 ± 0.65	86.25 ± 1.11
KBN 29 – *T. asperellum*	12.00 ± 0.91	45.25 ± 0.85	73.25 ± 0.85
KBN 35 – *T. harzianum*	11.75 ± 0.95	52.25 ± 0.85	88.75 ± 0.75
KBN 33 – *T. harzianum*	14.25 ± 0.85	55.25 ± 0.85	88.25 ± 1.03
KBN 24 – *T. atroviride*	20.00 ± 1.08	65.75 ± 0.85	87.50 ± 1.19
C.D.	2.70	2.56	3.05
C.V.	12.80	3.27	2.41

**Values represent mean of 4 replications, ± Standard Error.*

Table 5: Developmental events of mycelial growth and conidiation of *Trichoderma* isolates on PDA plate

***Trichoderma* isolates**	**Hours after incubation**	
	24	**240**
KBN-34	*Creamish mycelia*	Light yellowish green spores formed in the central rings. White masses outside these rings, some converted in light green color.
KBN-32	*Creamish mycelia*	White profuse and areal mycelial cottony growth which was raised on rings. Two outermost rings converted into a slightly green color.
KBN-29	*Light white mycelia*	Bigger and scattered white masses on inner rings, which converted to light green color. Green colored spore masses on the outermost ring on plate's edges
KBN-35	*Hyaline mycelia*	Raised mycelial growth in the inner portion. Its ring was provided with smaller white masses. Cottony growth had green spore masses.
KBN-33	*Hyaline mycelium*	White masses developed, starting from the periphery inwards but absent in the center. From the periphery, these were converted to light green.
KBN-24	*Creamish mycelium*	Uniform off-white growth with raised rings.

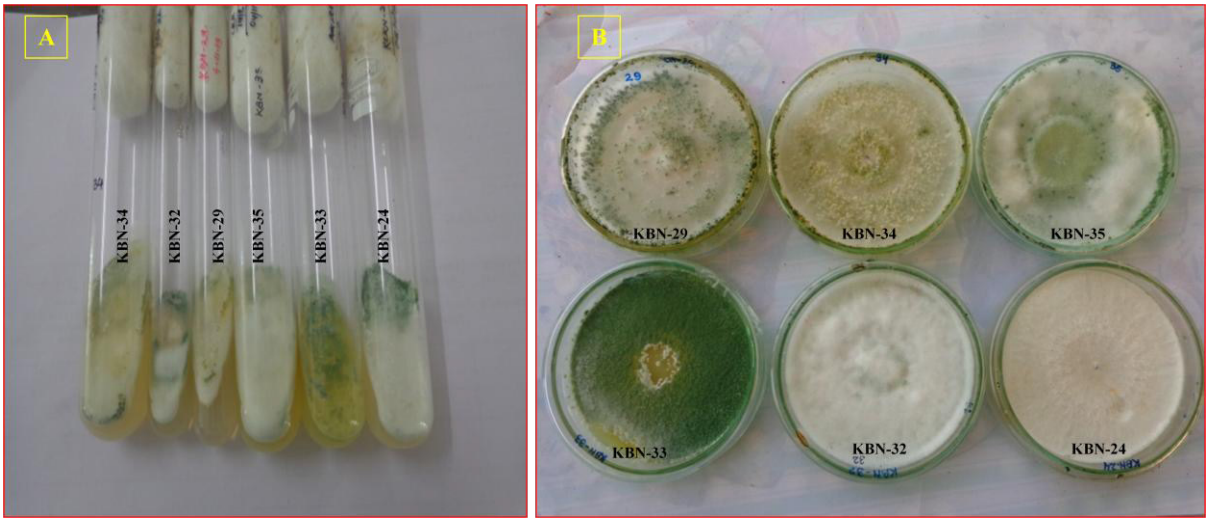

Fig. 1: Comparative mycelial growth and conidiation performance of *Trichoderma* isolates on PDA medium ((**A)** Slant culture & **(B)** Plate culture)

growth rate, mycelial growth pattern, color, initiation of conidiation, conidial color quantity, and diffusion of yellow pigmentation into the medium (Fig. 1A, Table 2 & 3). However, in PDA plates, they could produce hyaline, light cream, light white, or white-colored mycelia. The cultures produced either aerial or smooth linear mycelial growth. The majority of the isolates attained full growth after 72 hours of inoculation. Conidia were produced in a variable number of concentric rings in various patterns. Diffusion of yellow pigment was observed in the case of isolates KBN-33 and KBN-35 (Fig. 1B, Table 4 & 5).

The *Trichoderma* isolates were identified as *T. virens* and *T. harzianum* based on cultural features such as linear growth of mycelia, colony colour, pigmentation, and growth pattern of mycelia. Their morphological characterization was based on the structure, shape, and arrangement of conidiophores, phialides, and conidia (Sharma and Singh 2014). *T. harzianum T. aureoviride, T. viride,* and *T. crassum* were grown on malt extract agar, potato dextrose agar, and oatmeal agar media and identified based on colony appearance, mycelium growth rate, shape of conidia, phialides, and conidiophores (Carvalho *et al.* 2018).

Cultural and morphological characteristics like mycelial growth rate, colony appearance, the shape of conidia and conidiophores, and branching pattern of phialides are used to identify the *T. harzianum*, *T. viride* and *T. pseudokoningii* (Shah *et al.* 2012). Similar characteristics were applied to identify *T. harzianum*, *T. koningii* and *T. viride* isolated from different rhizospheres in Egypt (Hassan *et al.* 2015) cowpea, cucumber, wheat and faba bean plants. Based on morphological and cultural characteristics, the *Trichoderma* isolates were identified as *T. harzianum* isolates. In addition, several earlier workers also used such characteristics for the identification of various isolates of the genus *Trichoderma* (Mohiddin *et al.* 2018; Shamoli *et al.* 2017; Tiwari *et al.* 2021; Park *et al.* 2005; Tkalenko *et al.* 2020).

Assessment of conidia production potentiality of *Trichoderma* spp.

In slants, the isolates produced visible light yellow, light green, and dark green colored conidia at the variable time i.e. after 48 to 96 hours of incubation. The early conidiation was observed in the case of isolate KBN-33 after 48 hours. Isolate KBN-32 and KBN-24 produced conidia only after 72 hours; however isolate KBN-34, KBN-29 and KBN-35 initiated conidiation after 96 hours (Fig. 2).

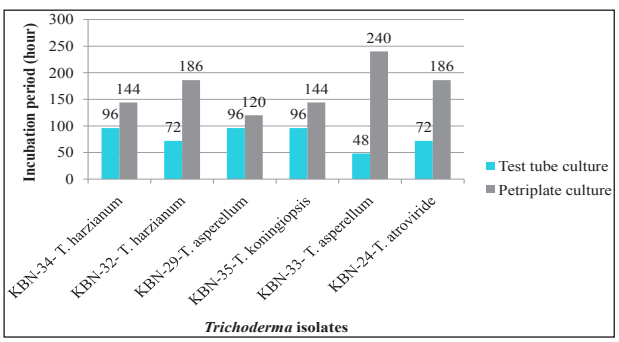

Fig. 2: Incubation period for conidial initiation of *Trichoderma* isolates in test tube and Petriplate culure

In plate culture, the tested isolates could initiate conidiation a little late when compared with slant culture (Fig. 2). All isolates produced plenty of conidia after 30 days in PDA plates, ranging from 78.7 to 310.7 × 10^8 per ml. Isolate KBN-34 ranked the first (310.7) followed by KBN-29 (303.7) and KBN-33 (246.3) as represented in Fig. 3. Research work related to number as well as quantification of conidia produced by *Trichoderma* spp. is limited. Some of the media and substrates and media were used to quantify the conidia mass of this antagonist (Hasan 2015; Chand Kumhar 2014).

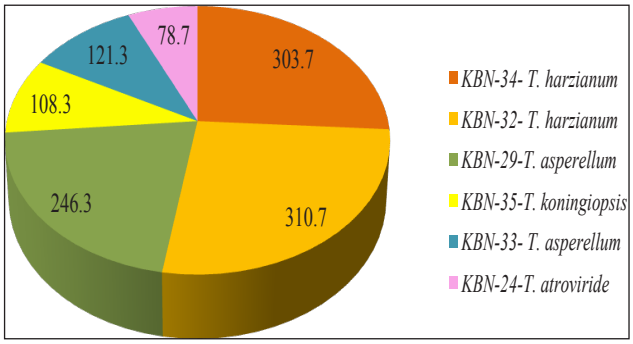

Fig. 3: Conidia producing strength (x108) of *Trichoderma* isolates

CONCLUSION

The rate of mycelial growth, its pattern, and conidial color varied amongst the isolates irrespective of the method employed. Conidiation took place at the variable time in both methods, however, it was initiated a little earlier in slant culture. Cultures, in the plate, showed conidiation at the variable time with varying numbers of concentric rings and conidial quantities. *Trichoderma* isolates had a variation for vegetative growth and conidiation; isolates KBN-32 and 34 could produce a comparatively higher number of conidia and therefore such strains can be exploited and promoted as a promising strategy for the managing the fungal phytopathogens of tea plantations which are needed for tea industry to get healthy tea produces free from residual problems especially for organically grown tea in Darjeeling and rest of area in West Bengal.

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