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Biocontrol potency of *Trichoderma* isolates against tea (*Camellia* sp.) pathogens and their susceptibility towards fungicides

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Abstract
A total of eleven *Trichoderma* species were isolated from the tea ecosystem of West Bengal, India and their *in vitro* evaluation was carried out against *Fusarium solani* and *Pestalotiopsis theae*, causing dieback and grey blight diseases of tea, respectively. Results of dual culture study revealed that, these indigenous isolates were found efficient in suppressing the growth of both the phytopathogens, *F. solani* (54.3-77.1%) and *P. theae* (49.3-65.8%). Of these, three *Trichoderma* strains (code KBN-1/14, KBN-2/14 & KBN-29) were chosen further, to study their susceptibility towards fungicides, which are generally used for the management of these phytopathogens. Results indicated that tested *Trichoderma* strains were highly susceptible to all three fungicides at their recommended dose of application, exhibiting very high level of growth inhibition (86.4 per cent). Thus the local *Trichoderma* isolates were found effective in managing the targeted phytopathogens. However, use of such fungicides either in combination or soon after the application of *Trichoderma* formulation should be discouraged to minimize the harmful impact on beneficial BCAs.

Keywords: Tea (*Camellia* sp.), *Trichoderma* spp., *Fusarium solani*, *Pestalotiopsis theae*, bioefficacy, compatibility

1. Introduction
Genus *Trichoderma*, a natural mycofungicide, is one of the renowned candidates amongst the known biological control agents, which has widely been used in different agro-ecosystems to protect crops from the attack of various phytopathogens (Naseby *et al*., 2000; Tondje *et al*., 2007; Houssien *et al*., 2010 and Pradeepa *et al*., 2008) [13, 19, 5, 15]. It controls the phytopathogens by different modes of actions such as competition for nutrients, antagonism, antibiosis etc. (Mendez-Vilas, 2010 and Valencia *et al*., 2011) [10, 20]. Its unique features such as wide distribution, fast growth, excellent conidiation, minimal nutritional requirement, wide adaptability in varying climates, broad host spectra, plant growth promotional activity (Hermosa *et al*., 2012) [4] and induction of systemic acquired resistance (SAR) in to plant system against phytopathogens (Sriram *et al*., 2009) [17] made this genus as one of the successful candidates amongst the other popular biological control agents (BCAs). Amongst various foliar diseases of tea (Chen and Chen, 1990) [2], dieback (pathogen: *Fusarium solani*) and grey blight (pathogen: *Pestalotiopsis theae*) are the destructive fungal diseases in entire tea growing regions of North East as well as West Bengal. These diseases infect tender shoots as well as mature leaves, moreover their incidence have been increasing every year, possibly due to change in climatic conditions, pathogenic variability and or introduction of newer agronomic practices like mechanized harvesting, which facilitates the easy entry to these phytopathogens (Sanjay and Ponmurugan, 2008) [16].
For the manufacturing of good quality tea, the young leaves or tender shoots, comprising of two leaves and bud, is the primary requirement. Although, the maintenance or older leaves do not use directly for tea manufacturing, yet it is equally important because it helps in promoting the vegetative growth in form of tender shoots. Hence, protection of tender as well as older foliage from these phytopathogens becomes an important concern for the growers, which could be achieved by applying a range of fungicides. However, their frequent and injudicious use may certainly leads to numerous adverse effects. Biological control of such pathogens by utilizing *Trichoderma* spp. could be a better option to minimize the use of chemical fungicides.

In addition, these are safer to human being, animals as well as ecosystem, too (Vidhya *et al*., 2011) [22]. Therefore, the present study has been undertaken to develop possible *Trichoderma* isolates, their *in vitro* screening against foliar pathogens and susceptibility to common fungicides.

2. Materials and Methods

2.1 Isolation of *Trichoderma* Spp: *Trichoderma* spp were isolated from different soil samples collected from tea ecosystem by serial dilution and plating technique (Askew and Laing, 1993) [1] with minor modifications using potato dextrose agar medium. Two hundred microliter suspension from 5th and 6th dilutions was plated in to PDA plates, followed by incubation for 2-3 days in BOD incubator at 25±2 ^{0}C. Developed colonies were identified morphologically and microscopically, and then purified by hyphal tip culture technique. Pure cultures (PDA slants) were stored in refrigerator for further studies.

2.2 Isolation of phytopathogens, *Fusarium solani* & *Pestalotiopsis theae:* Dieback disease pathogen, *F. solani* was isolated from infected tender tea shoot samples, collected from experimental plots of our research centre. The grey blight pathogen, *P. theae*, was isolated from older leaves i.e. maintenance foliage which were infected with this disease. For isolation of *P. theae*, diseased samples were also collected from same location. Standard technique was applied to isolate the pathogens (Joshi *et al*., 2009) [8]. Diseased samples were washed in running tap water followed by subsequent washing with distilled water. Then they were cut in to small pieces of convenient size, surface sterilized with mercuric chloride solution (0.1% w/v) followed by two subsequent washing with distilled sterilized water, dried by placing them in between the filter papers and inoculated in to agar plates amended with antibiotic, streptomycin sulphate (100 ppm) to avoid the bacterial contamination. Plates were sealed with parafilm and incubated 25±2 ºC for 3-5 days in BOD incubator. Developed colonies were purified by "hyphal tip culture technique" using potato dextrose agar.

2.3 Screening of *Trichoderma* isolates for antagonism: The *Trichoderma* isolates were screened against *F. solani* and *P. theae*, to assess their antagonistic potency by employing dual culture technique (Stack *et al*., 1986) [18]. Five millimetre mycelial discs of actively growing pathogens as well as *Trichoderma* spp. were inoculated in to PDA plates, at an equidistance of 30 millimetres and incubated at room temperature for one week. Three replications per treatment were kept and completely randomized design was followed for experimentation. During experiment period, the average morning and evening room temperature was in the range of 27.7 to 30.4 ºC, and average relative humidity was 74.1 to 80.6 per cent. After one week, the colony diameter was measured in dual cultured as well as control plates. Finally, per cent growth inhibition was worked out by applying following formula.

$$\text{Mycelial growth inhibition (\%)} = \frac{\text{Colony dia. in control} - \text{Colony dia. in treatment}}{\text{Colony dia. in control}} \times 100$$

2.4 Susceptibility of *Trichoderma* isolates towards fungicides: The Poisoned food technique (Nene and Thapliyal, 1993) [14] was employed to evaluate the susceptibility of antagonistic isolates towards common fungicides. Three *Trichoderma* strains i.e. KBN-1/14, KBN-2/14 and KBN-29 were chosen to for this purpose, because their exact identity was established by Indian type culture collection centre, IARI, New Delhi. The required quantity (0.5 to 2.5 g or ml/L media) of copper oxychloride 50 WP, copper hydroxide 77 WP and hexaconazole 5 EC were measured and added in to PDA medium at its lukewarm state followed by proper homogenization. Prepared poisoned medium was then poured in to sterilized plates (90 mm diameter) immediately and plates were allowed to get solidify. Five millimetre discs of antagonists were inoculated in to the centre of plates and incubated at 25±2 ºC in BOD incubator. Three replications per treatment was maintained and completely randomized design was followed for experimentation. Colony diameter was measured when antagonist's mycelia covered the entire surface area of plates in control (after 4-6 days). To find out critical difference among the treatments, data were statistically analysed using an online package OPSTAT of Chaudhary Charan Singh, Haryana Agricultural University, Hisar, Haryana, India (www.hau.ernet.in).

3. Results and Discussion

3.1 Isolation of *Trichoderma* spp:

In totality, eleven different *Trichoderma* spp. were isolated from various top soil samples in present study, which exhibited rapid mycelial growth and rich conidiation; however, slight variations with respect to mycelial growth rate, conidial initiation time and its visible quantity were clearly observed. The *Trichoderma* isolates were identified on the basis of their colony characteristics, conidiophore branching pattern, shape of phialides, colour of asexual spores (conidia) in culture etc. (fig. 1). Their identity was reconfirmed from Indian Type Culture Collection Centre, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, Pusa, New Delhi, India.

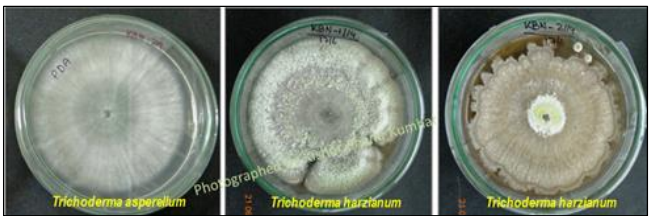

Fig 1: *Trichoderma* isolates growing on potato dextrose agar (PDA) medium

3.2 Isolation of phytopathogens, *Fusarium solani* & *Pestalotiopsis theae*: Two fungal phytopathogens were isolated in this study, based on their colony characteristics as well as microscopic observations of conidial anatomy; they were identified as *F. solani* and *P. theae.* The colony of *F. solani* on PDA was light pinkish colored (fig. 2A). It produced micro- as well as macro-conidia on conidiogenous cell. The micro-conidia were smaller in shape with zero to one transverse septum; however, the macro-conidia were bigger in size with 3-5 transverse septa (fig. 2B).

The colony of *P. theae*, was light pinkish in color with numerous concentric rings, which turned little darker with advancement of time (fig. 2C). It produced spindle or clavate shaped conidia. The conidia were 5 celled, three central cells were dark coloured while the upper and lower most cells were hyaline. The upper most cells beared 2-3 cellular appendages i.e. setulae, however the lower most cells beared a short hyaline pedicel (fig. 2D).

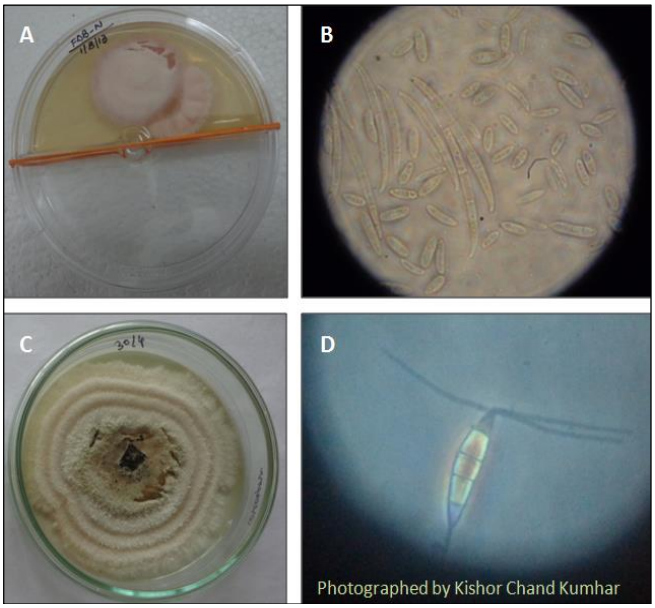

Fig 2: Mycelial colonies and conidia of tea pathogens (A: F. Solani colony, B: micro and macro conidia of F. Solani, C: P. theae colony & D: conidium of P. theae

3.3 Screening of *Trichoderma* spp. for antagonism

The *Trichoderma* isolates showed control of both targeted tea pathogens i.e. *F. solani* as well as *P. theae* by suppressing their mycelial growth significantly in dual culture, however, they were slightly more effective against dieback pathogen, where they controlled it in the range of 54.3 to 77.1 per cent. In case of grey blight pathogen, they exhibited its control from 49.3 to 65.5 per cent (table 1). Out of these, none of the isolate formed inhibition zone around the pathogens' colonies. Vidhya *et al*. (2011) [22] assessed the *Trichoderma* sp. against *Pestalotiopsis* sp. and achieved significant control of phytopathogen under laboratory conditions. Similarly, a few *Trichoderma* isolates were tested against tea pathogens viz. *F. solani* and *P. theae* and found effective against them under *in vitro* conditions (Naglot *et al*., 2015) [11]. It was observed by Joshi *et al*. (2012) [8] that metabolites of *T. harzianum* and *T. viride* could successfully control the *F. oxysporum.* It has already been reported that *T. viride* and *T. harzianum* had strong mycoparasitism against *F. solani* f. sp. *pisi* (Hamid *et al*., 2012) [3]. Earlier researchers also noted that the local strains of *T. viride* and *T. harzianum* could perform better than outsourced ones (Jagtap *et al*., 2013) [6], all these findings are in close conformity to our present findings.

Table 1: Influence of *Trichoderma* isolates on tea pathogens

Trichoderma isolate	Pathogens' growth inhibition (%) after one week*		Inhibition zone development	
	P. theae	*F. solani*	*P. theae*	*F. solani*
Trichoderma sp. (KBN-33)	49.3 (44.6±1.8)	54.3 (47.4±1.2)	-	-
Trichoderma sp. (KBN-35)	55.8 (48.3±2.6)	69.5 (56.5±1.6)	-	-
Trichoderma sp. (KBN-38)	57.9 (49.6±1.7)	69.5 (56.5±2.1)	-	-
Trichoderma sp. (KBN-37)	49.4 (44.7±0.2)	66.3 (54.5±1.6)	-	-
Trichoderma sp. (KBN-34)	59.2 (50.3±0.7)	62.7 (52.3±1.8)	-	-
T. harzianum (KBN-1/14)	65.8 (54.2±1.7)	77.1 (61.5±1.9)	-	-
Trichoderma sp. (KBN-11/14)	65.4 (54.0±0.6)	75.7 (60.5±1.3)	-	-
T. harzianum (KBN-2/14)	62.6 (52.3±0.4)	75.7 (60.5±1.3)	-	-
Trichoderma sp. (KBN-3)	62.1 (52.0±1.0)	67.4 (55.2±1.7)	-	-
Trichoderma sp. (KBN-4)	59.2 (50.3±0.9)	66.3 (54.5±1.6)	-	-
T. asperellum (KBN-29)	52.4 (49.6±1.7)	69.5 (56.5±2.1)	-	-
C.D.	4.3	4.7		
C.V.	5.0	4.9		

*Mean of three replications, figures in brackets are angular transformed values with ±Standard Error,
- indicates no formation of inhibition zone

3.4 Susceptibility of *Trichoderma* isolates towards fungicides:

While assessing the susceptibility of *Trichoderma* isolates towards commonly used three fungicides, it was noted that copper oxychloride, copper hydroxide as well as hexaconazole had adverse effect on the antagonists even at lower concentrations. Copper oxychloride showed the highest inhibition followed by copper hydroxide and hexaconazole, irrespective of tested concentrations. Tested fungicides inhibited the growth of *Trichoderma* spp. in the range of 53.8 to 86.4 per cent as evident from table 2. The results of this study explained clearly that *Trichoderma* isolates were highly susceptible to these fungicides. Narayana and Srivastava (2003) [12] have reported that different triazole grouped fungicides namely; hexaconazole, propiconazole and penconazole had high inhibitory influence on *T. harzianum* at their varying concentrations. Similarly, it has been noted that hexaconazole was highly inhibitory to *T. viride and T. harzianum* even at 0.5 ppm concentration (Johnson, 2001) [7]. Vidhya *et al*. (2012) [21] found that *Trichoderma* efficiently controlled the grey blight pathogen of tea and its growth was hampered by copper oxychloride. All these findings are in favour of our present results.

Table 2: Influence of fungicides on selected *Trichoderma* isolates

Fungicides	Conc.(ppm)	Growth inhibition (%) of *Trichoderma* strain*		
		T. harzianum (KBN-1/14)	*T. harzianum* (KBN-2/14)	*T. asperellum* (KBN-29)
Copper oxychloride 50 WP	1500	80.2 (63.5±1.2)	82.4 (65.2±1.3)	79.4 (63.0±1.0)
Copper oxychloride 50 WP	2000	82.5 (65.3±1.8)	86.3 (68.4±1.6)	81.0 (64.1±0.6)
Copper oxychloride 50 WP	2500	84.1 (66.5±0.8)	85.6 (67.7±0.9)	83.4 (66.0±0.9)
Copper hydroxide 77 WP	1500	73.8 (59.2±0.8)	77.6 (61.8±0.8)	72.7 (58.5±1.1)
Copper hydroxide 77 WP	2000	75.4 (60.3±0.9)	79.3 (62.9±1.2)	74.4 (59.6±1.1)
Copper hydroxide 77 WP	2500	83.3 (65.9±1.2)	86.4 (68.3±0.8)	82.6 (65.4±1.3)
Hexaconazole 5 EC	500	65.1 (53.8±1.00	68.8 (56.0±0.7)	63.5 (52.9±1.4)
Hexaconazole 5 EC	750	70.6 (57.2±0.70	74.4 (59.6±0.7)	69.4 (56.4±0.7)
Hexaconazole 5 EC	1000	74.6 (59.7±1.6)	78.4 (62.3±1.1)	73.4 (59.0±1.8)
C.D.		3.4	3.1	3.5
C.V.		3.3	2.9	3.3

*Mean of three replications, figures in brackets are angular transformed values with ±Standard Error,

4. Conclusion

It can be concluded that indigenous *Trichoderma* isolates had promising efficiency to control both tea phytopathogens within a period of one week. Hence, these antagonists could easily be find place in to integrated disease management (IDM) program for tea crop sustainability. In addition, field application of such microbials especially in tea cropping system, certain pesticide originated problems such as pesticide residues, resistance development, environmental pollution, imbalanced ecosystem and health hazards etc. would automatically be tackled to the great extent. All three fungicides inhibited the growth of *Trichoderma* spp. even at lower concentrations, indicated the high susceptibility towards chemical fungicides, which means *Trichoderma* spp. are could not be used in combination with incompatible with current recommended doses of fungicides.

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