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Role of beneficial fungi in managing diseases and insect pests of tea plantation

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Abstract

The effectiveness of *Trichoderma atroviride*, *T. asperellum*, *T. harzianum*, against targeted tea disease causing pathogen, *Fusarium solani* (dieback), *Beauveria bassiana* against tea mosquito (*Helopeltis theivora*), and *Metarhizium anisopliae* against the red spider mite, *Oligonychus coffeae* Nietner, and the live wood eating termite (*Microcerotermes beesoni Snyder*), respectively were evaluated under laboratory and field conditions. The *Trichoderma* isolates showed effective control of *F. solani* (64.6 to 71.7%) under laboratory conditions, while the wettable powder (WP) formulation could reduce dieback disease incidence (57.1 to 77.7%) over control, under field conditions. The post prune application of *Trichoderma asperellum* on light pruned (LP) and application of *T. asperellum* as well as *T. harzianum* on deep skiffed (DS) tea fields showed a significant growth promotional response. The treated bushes could produce an average number of 421.2 and 398.4 shoots when it was applied 7.5 and 5.0% (w/v), respectively, which was higher than the number of shoots recorded in the control plots (259.4). The antagonist also induced comparatively higher shoot length (6.85–7.99 cm) than the untreated control plots (5.13–6.38 cm). The *B. bassiana* isolates (2×10^9 conidia/ml) exhibited 71.5 to 93.0% control of 2nd instar nymphs of *H. theivora* under in vitro conditions. However, addition of different adjuvants resulted in enhanced mortality. Under field conditions, the highest reduction of tea mosquito population of 52.3% was noted when *B. bassiana* (1×10^9cfu/ml) was sprayed along with Tween 20 plus and crude sugar. However, the wettable powder formulation of this EPF along with jaggery reduced the insect incidence. The *M. anisopliae* effectively controlled the red spider mite to the tune of 46.3 to 63.8% under laboratory conditions, while the wettable powder formulation of this isolate was more effective against the termite, resulting in the reduction of infestation to the tune of 84.2% under field conditions.

Keywords: Darjeeling tea, *Trichoderma harzianum*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Fusarium solani*, *Helopeltis theivora*, *Oligonychus coffeae*, Termite, Control

Background

Tea (*Camellia* sp.) is one of economically important plantation crops of India. It is perennial in nature and hence provides favorable micro as well as macroclimate for thriving of different insect pests and fungal diseases, which together cause enormous crop loss in terms of quantity and quality. Synthetic agrochemicals have been used for the management of these pests and diseases. Certainly, they provide efficient control for longer period; however, their frequent and injudicious application had definitely invited numerous other associated problems such as environmental pollution, development of pesticide resistance in insects, and unwanted pesticide residues in made tea (Roy et al. 2011).

The biological control agents (BCAs) like the species of *Trichoderma*, *Beauveria*, and *Metarhizium* are reported to be safer and promising components of integrated pest and disease management (IPDM) strategies, which have been adopted in various crops including tea (Hall and Papierok 1982). Although

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research works have been carried out in this direction (Debnath 1996; Babu and Kumhar 2013, 2014), however very little information is available on the commercialization of such beneficial fungi as potential microbial pesticides.

The present study aimed to isolate *Trichoderma* spp., *B. bassiana*, and *M. anisopliae* from the tea soils and development of suitable formulations for in vitro as well as in vivo evaluation against targeted diseases and insect pests.

Materials and methods

Isolation and identification of fungal species

The species of *Trichoderma*, *Beauveria*, and *Metarhizium* were isolated from soil through standard technique (Askew and Laing 1993; Ghanbary et al. 2009 and Qazzaz 2012) during 2014–2015. Purification of isolates was done through hyphal tip culture technique and stored in refrigerator. Then, pure cultures were grown on potato dextrose agar (PDA) plates, followed by incubation at 25 ± 2 °C for 48–96 h. The pure cultures were identified based on their colony characters like colony color, growth pattern and formation of conidial rings, and color of conidia. The shapes of conidiophores and phialides were observed using microscope. Later on, the identity of isolates was re-confirmed from Indian Type Culture Collection, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, India.

For isolation of pathogen, i.e., *Fusarium solani*, die-back diseased tender tea shoots were collected from tea bushes during 2014–15. The diseased shoots were cut in to small pieces, followed by surface sterilization with mercuric chloride (0.1%) and subsequent 2 washing with distilled water. Then, these pieces were inoculated in to PDA plates, and plates were incubated at 26 ± 2 °C for 1 week. Colonies developed in plates were purified, using PDA plate. After colony development, they were identified on the basis of morphological characteristics.

In vitro bioefficacy of fungi

Trichoderma spp.

Bioefficacy of *Trichoderma* spp. was assessed by dual culture technique (Stack et al. 1986). Five-millimeter discs of both fungi (*Trichoderma* spp. and *F. solani*) were transferred in to plates at equidistance. In control, only *F. solani* was inoculated for comparison. Each treatment was replicated 5 times. Plates were incubated at 25 ± 2 °C for 1 week. Pathogen's mycelial growth was measured and its inhibition was worked out, using the following formula:

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

Beauveria bassiana

The in vitro bioassay of *B. bassiana* against *H. theivora* was carried out by employing the methodology of Amarsena et al. (2011) with slight modifications. To prepare spray suspension, 10 ml of distilled sterilized water was added in to 2 weeks old *B. bassiana* culture grown on potato dextrose agar (PDA) slant, and biomass was harvested, followed by filtration twice through muslin cloth. The conidial concentration (2×10^9) was determined, using hemocytometer and sprayed on the nymphs of *H. theivora* with the help of an atomizer. Each treatment was replicated 3 times. Insect mortality was recorded till 192 h, and percent mortality was corrected using Abbott's formula (Abbott 1925).

$$\text{Corrected mortality } (\%) = \frac{\text{No.of live insect in control} - \text{No.of live insect in treatment}}{\text{No.of live insect in control}} \times 100$$

Different adjuvants, namely, Tween 20 (2 ml/l of water), glycerol (5 ml/l of water), and crude sugar/molasses (5 g/l of water), were in vitro studied to find out their role in enhancing the efficacy of *B. bassiana* against 2nd instar tea mosquito. *B. bassiana* conidial suspension in combination with these adjuvants separately was sprayed on tea shoots, and known numbers of nymphs were released on to these shoots. Each treatment was replicated thrice times, and observations on insect mortality were recorded and 24-h interval for a period of 1 week.

Metarhizium anisopliae

The pathogenicity of *M. anisopliae* was carried out against the red spider mite (*O. coffeae*) employing leaf disc technique (Plate 1). The fungal culture was mass multiplied, using potato dextrose broth (PDB). The conidial concentration (1×10^8 to 2×10^8) was determined, using hemocytometer and sprayed on the red spider mite with atomizer. Twenty insects per treatment were taken, and each treatment was replicated 4 times. The insect mortality was calculated by the following formula.

$$\text{Insect mortality } (\%) = \frac{\text{Number of dead insect}}{\text{Total number of insect used}} \times 100$$

The dead insects were collected and re-inoculated in the PDA (potato dextrose agar) plates followed by

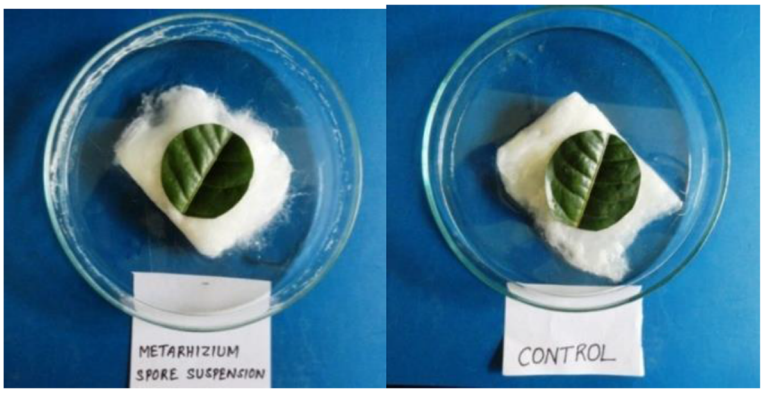

Plate 1 Leaf disc technique for in vitro bioassay of *Metarhizium anisopliae*

incubation at 26 ± 2 °C for 1 week to confirm insect mortality.

In vivo testing of fungi

The liquid fermentation technique was adopted with minor modifications for the mass production of *Trichoderma atroviride*, *T. asperellum*, *T. harzianum*, and *B. bassiana* using 25-l capacity fermenter (Bhat et al. 2009). The potato dextrose broth (Hi-media) was used as basal medium. The biomass was harvested after 11 days, and wettable powder formulations were prepared. *M. anisopliae* was mass multiplied on PDB medium. After 2 weeks, the medium and biomass was homogenized and used for field application. It was sprayed on tea stem and drenched uniformly in collar region during 2015–16.

Trichoderma spp.

Developed wettable powder (WP) formulation (2×10^8 cfu/g) of *Trichoderma* isolates and commercial formulation was tested against dieback disease in field during 2015–16. Two sprays at weekly interval were given immediately after plucking the shoots. Sixty bushes were taken per treatment, and each treatment was repeated four times. Pre- and post-spray observations on number of dieback shoots were recorded. One hundred shoots from plucking basket were taken, and infected shoots were counted. The disease reduction over control was calculated by the following formula.

$$\text{Disease reduction } (\%) = \frac{\text{No.of disease shoots in control} - \text{No.of disease shoots in treatment}}{\text{Number of disease shoots in control}} \times 100$$

The formulation was tested during cold weather in the first week of December on light pruned (LP) and deep skiffed (DS) teas at experimental plot of Tea Research

Table 1 In vitro bioefficacy of *Trichoderma* spp. against *Fusarium solani*

Trichoderma sp.	Growth inhibition (%) after 1 week*
T. atroviride (KBN-24)	67.3 (55.1 ± 0.5)
T. harzianum (KBN-1/14)	67.3 (55.2 ± 1.5)
T. harzianum (KBN-2/14)	64.6 (53.5 ± 1.1)
T. asperellum (KBN-29)	71.7 (57.9 ± 1.5)
T. harzianum (sample 1)	60.9 (51.3 ± 1.7)
T. viride (sample 2)	61.5 (51.7 ± 1.1)
C.D.	3.9
SE (m)	1.3
SE (d)	1.8
C.V.	4.8

*Mean of 5 replications, values in parenthesis are angular transformed with ± SE

Table 2 In vitro bioassay of *Beauveria bassiana* isolates against tea mosquito (*Helopeltis theivora*)

Beauveria bassiana strain	Spore conc./ml	% corrected mortality after 192 h*
Isolate I (IIHR)	2×10^9	93.0 (77.3 ± 6.3)
Isolate II (IIHR)	2×10^9	78.5 (62.9 ± 4.6)
Isolate III (IIHR)	2×10^9	89.3 (74.5 ± 8.3)
Isolate IV (IIHR)	2×10^9	71.5 (57.8 ± 2.1)
BB 3 (tea ecosystem)	2×10^9	85.6 (68.0 ± 3.1)
BB 4 (tea ecosystem)	2×10^9	82.2 (65.3 ± 2.7)
BKN 20 (TRA NBRRDC)	2×10^9	85.6 (68.0 ± 3.1)
C.D.		13.5
SE (m)		4.5
SE (d)		6.3
C.V.		13.1

*Mean of 3 replications, values in parenthesis are angular transformed with ± SE

Table 3 In vitro bioefficacy of *Beauveria bassiana* against *Helopeltis theivora* in combination with different adjuvants

Treatment	Mortality of 2nd instar nymphs of *Helopeltis theivora* when exposed to *Beauveria bassiana* after days of spray*	
	5 days	10 days
T1, *Beauveria bassiana* + surfactant (Tween 20)	67.5 (56.1 ± 5.7)	17.5 (21.6 ± 7.4)
T2, *B. baasiana* + humectant (glycerol)	70.0 (57.1 ± 4.3)	25.0 (29.3 ± 4.5)
T3, *B. bassiana* + surfactant + humectant	75.0 (60.1 ± 1.9)	27.5 (31.5 ± 1.7)
T4, *B. bassiana* + UV protectant (crude sugar)	77.5 (62.1 ± 3.5)	30.0 (33.0 ± 2.6)
T5, *B. bassiana*	62.5 (52.5 ± 3.9)	20.0 (26.2 ± 3.0)
CD	N.S.	NS
SE (d)	5.7	6.1
SE (m)	4.1	4.3
CV	14.1	20.4

*Mean of 3 replications, values in parenthesis are angular transformed with ± SE

Association North Bengal Regional Research and Development Centre. It was sprayed immediately after pruning operation. After 2 months, the number of bud break (five bushes) and shoot length (5 shoots of 5 bushes) was recorded from each treatment.

Beauveria bassiana

The *B. bassiana* formulation was evaluated during 2015–2016 at TRA NBRRDC plot in combination with different adjuvants, namely, Tween 20 (2 ml/l of water) and crude sugar (5 g/l of water). Randomized block design (RBD) with 3 replications was followed.

Metarhizium anisopliae

The in vivo bioefficacy of *M. anisopliae* was also assessed for the control of termite during 2015–2016. The broth culture at 5% v/w sprayed on the bush stem and drenched in the collar region properly two times at an interval of 3 months. Thiamethoxam was used as standard check for comparison. Pre- and post-treatment observations on number of bushes showed presence of earth runs, or live termite was calculated.

Statistical analysis of data

Statistical analysis of the data was carried out with the help of online statistical package OPSTAT of Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India.

Results and discussion

Isolation and identification of fungal species

On the basis of colony and morphological characters, the isolated antagonistic and EPFs were identified by the first author. Later on, Dr. T. Prameela Devi, Principal Scientist, Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, identified the isolates as *Trichoderma atroviride*, *T. harzianum*, *T. asperellum*, *B. bassiana*, and *M. anisopliae*. Two strains, i.e., *Trichoderma harzianum* (ITCC 7765) and *T. asperellum* (ITCC 7764), got accession number from ITCC, New Delhi.

Pathogen was identified as *Fusarium solani*, which produced cream-colored colonies with pinkish pigmentation on PDA. It produced sickle-shaped macro-conidia

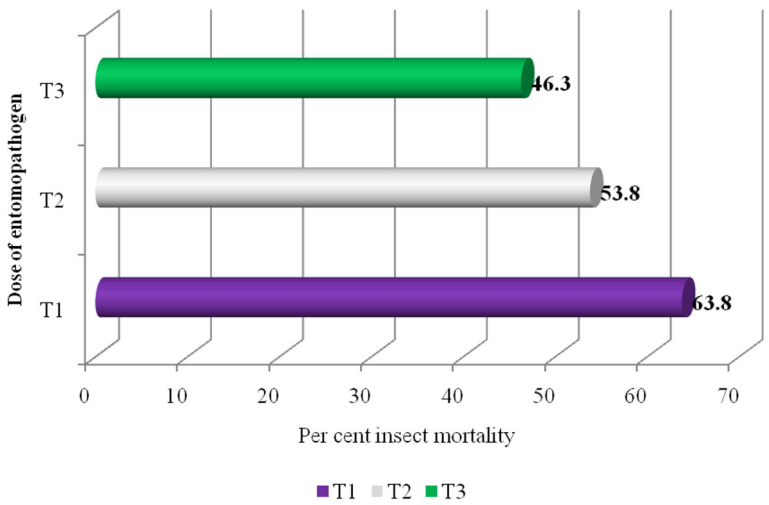

Fig. 1 Pathogenicity of *Metarhizium anisopliae* against red spider mite

Fig. 2 View of *Trichoderma* applied fields. 1 Foliar spray and 2 Post prune spray

(mostly mono, bi, tri, rarely tetra septate) and aseptate micro-conidia.

In vitro bioefficacy of fungi

Trichoderma isolates effectively controlled *F. solani* (64.6 to 71.7 %). Among tested isolates, the maximum growth inhibition was noted in case of *T. asperellum* (KBN-29), followed by *T. atroviride* (KBN-24) and *T. harzianum* (KBN-1/14) as indicated (Table 1). Vidhya Pallavi et al. (2010) found a very good control of gray blight (*Pestalotiopsis* sp.) and wood rot (*Hypoxylon* sp.) pathogens of tea through *Trichoderma* spp. Kumhar and Babu (2015a, b) noted control of *F. solani* (26.03–59.05%) by *Trichoderma* spp. Similarly, Naglot et al. (2015) reported efficacy of *Trichoderma* spp. against this pathogen.

B. bassiana isolate I caused the highest mortality (93.0%) of *H. theivora*, followed by isolate III; however, the performance of all isolates was statistically at par with each other (Table 2). Different strain of *B. bassiana* was reported to be pathogenic to shot hole borer beetle (*Euwallacea fornicatus*) of tea, *H. antonii* of guava, and *H. theivora* of tea (Selvasundaram and Muraleedharan 2000, Visalakshy and Mani 2011 and Babu and Kumhar 2014). Shophiya et al. (2014) noted that *B. bassiana* as an effective biological control agent of different larval stages of castor hairy caterpillar, *Pericallia ricini* Fab.

In this present investigation, it was observed that different adjuvant enhanced the efficacy of *B. bassiana* under laboratory conditions. The highest mortality (77.5%) was achieved when crude sugar was added with *B. bassiana.* Combination of surfactant plus humectant ranked 2nd when insect population was exposed to tea leaves, which were sprayed by the formulation 5 days prior to the initiation of the experiment. When insects were exposed to tea shoots sprayed 10 days prior to the experiment resulted in reduced insect mortality (17.5 to 30.0%). The sprayed shoots were supported with glass vial filled with water to keep the shoots without wilting (Table 3). The EPF *Lecanicillium lecanii* and NKAE in combination with jaggery resulted in to increase the insect mortality as high as 12.5% (Subramaniam Sankara Rama et al., 2011). Gatarayiha et al. (2010) observed 60.0 to 85.7% mortality rate of spotted spider mite due to application of *B. bassiana* (4.2×10^6 conidia per ml) in combination with Break-thru (polyether-polymethylsiloxane-copolymer, a silicone surfactant). In combination with oil emulsion, this fungus could cause 39.4 to 61.3% mortality of adult mites.

In the present investigation, the formulation of *M. anisopliae* could cause mortality of red spider mites to the tune of 46.3 to 63.8%. This mortality recorded was concentration dependent, and it was the highest at the concentration containing 2×10^8 and the lowest at 1×10^8 conidia/ml (Fig. 1) under laboratory conditions. Roobak

Table 4 In vivo bioefficacy of *Trichoderma* spp. against dieback disease

Trichoderma sp.	Dose (g/l of water)	Disease reduction over control*
Trichoderma asperellum (KBN-29)	2.5	66.7 (54.77 ± 1.314)
T. asperellum (KBN-29)	5.0	77.7 (58.9 ± 1.605)
T. atroviride (KBN-24)	2.5	57.1 (56.21 ± 4.811)
T. atroviride (KBN-24)	5.0	68.62 (50.6 ± 3.147)
Commercial (Tricho-H)	2.5	41.0 (56.76 ± 1.881)
Commercial (Tricho-H)	5.0	44.4 (39.42 ± 5.424)
C.D.		11.25
SE (m)		3.69
SE (d)		5.23
C.V.		14.02

*Mean of 3 replications, values in parenthesis are angular transformed with ± SE

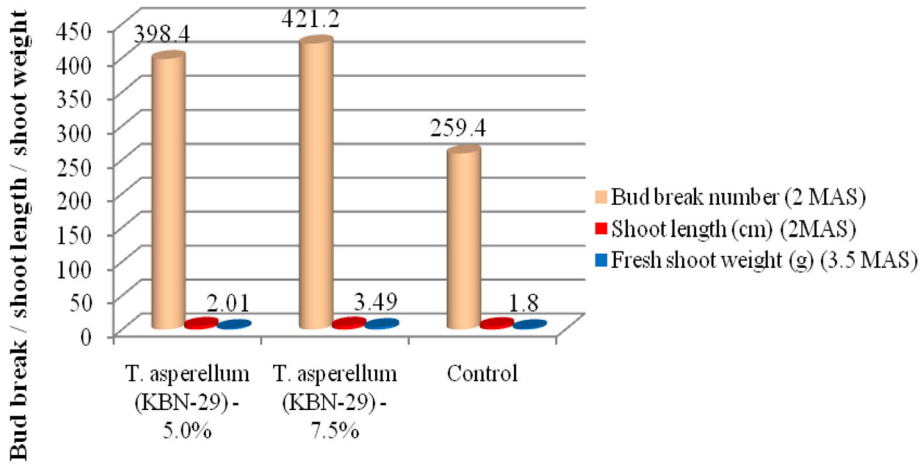

Fig. 3 Comparison of application of concentration of *Trichoderma asperellum* for light pruned (LP) tea

Kumar et al. (2011) observed that *Pseudomonas putida* was capable of controlling of red spider mites under laboratory conditions. The mites (both nymphs and adults) when exposed to this bacterium, it resulted in reduced mobility, cessation of feeding, and leading to the ultimate death. Sileshi et al. (2013) assessed in vitro bioefficacy of 4 isolates of *M. anisopliae* and *B. bassiana* against termite by spraying of 1×10^5 to 1×10^9 conidia per milliliter concentrations, and they found that *M. anisopliae* and *B. bassiana* could cause 60–100 and 25–95% mortality, respectively, at different concentration.

In vivo testing of fungi

In vivo testing of Trichoderma spp.

The results of the present investigation revealed that *Trichoderma* isolates could reduce the dieback incidence by 57.1 to 68.6 and 66.7 to 77.7%, when sprayed at 2.5 and 5.0 g per liter, respectively, which was found to be superior to the treatment with a commercial formulation (Table 4) which is evident from lush green appearance both in foliar spray and post- prune spray treatments (Fig. 2). Panwar et al. (2014) assessed bioefficacy of *T. harzianum* and *T. viride* against *Fusarium* head blight pathogen of wheat (*Fusarium graminearum*) under greenhouse conditions and found that foliar application of *T. harzianum* and *T. viride* alone and in combination significantly reduced the disease severity than the control. *T. harzianum* at 5 g/l resulted in better control of root disease in Sri Lanka (Balasuriya 2005). Species of *Trichoderma* were found to be potential in controlling the blister blight of tea under field conditions in North East India (Sarmah et al. 2005).

At the experimental plot of Tea Research Association, the applied formulation had promoted vegetative growth

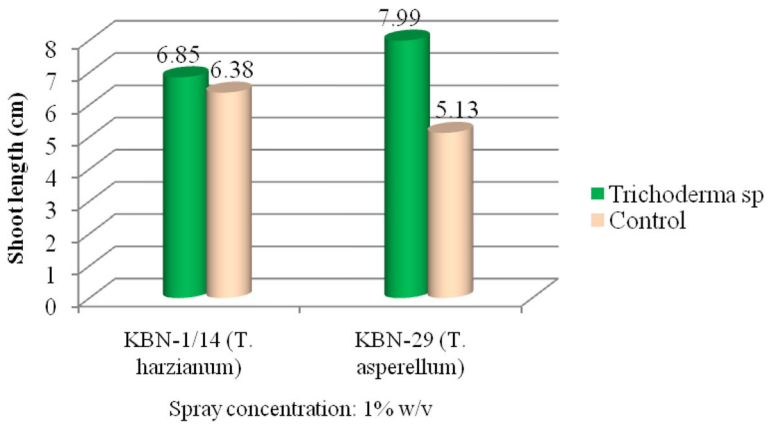

Fig. 4 Vegetative growth promotional effect of *Trichoderma* spp. on DS tea (2 MAS)

Table 5 In vivo evaluation of *Beauveria bassiana* in different combination of adjuvants

Treatment	Infestation reduction (%)*	Number of mycosed insect after 10 days*
T1, *Beauveria bassiana* at 5×10^7/ml + Tween 20 + crude sugar	47.6 (43.6 ± 3.6)	3.7
T2, *B. bassiana* at 1×10^8/ml + Tween 20 + crude sugar	52.3 (46.3 ± 3.1)	4.0
T3, *B. bassiana* at 5×10^7 + Tween 20	38.3 (38.1 ± 3.7)	3.3
T4, *B. Bassiana* at 1×10^8 + Tween 20	42.3 (40.4 ± 4.4)	4.3
T5, Tween 20 + crude sugar	12.2 (16.9 ± 8.5)	0.0
T6, Standard insecticide check (Thiamethoxam)	83.6 (66.2 ± 1.7)	0.0
T7, Untreated control	-	0.0
CD	15.9	
SE (d)	7.1	
SE (m)	4.9	
CV	20.6	

*Mean of 3 replications, values in parenthesis are angular transformed with ± SE

of tea bushes in terms of increased number of bud break, shoot length, and shoot weight than the untreated control. The average number of bud break was 398.4 and 421.2 at 5 and 7.5% concentration, respectively, which was higher than that recorded in untreated control (259.4) as shown in Fig. 3. Similar trend of promotion of vegetative growth was observed on deep skiffed tea plantation also (Fig. 4). In both cases, health of bushes was found better than control. Kumhar and Babu (2015a, b) noted that foliar spray of *Trichoderma* formulation could promote the vegetative growth of tea plantation in terms of increased number of shoots and their length.

In vivo testing of B. bassiana

The highest mortality of 52.3% was noted when *B. bassiana* (1×10^8 cfu/ml) was sprayed by Tween 20 plus crude sugar (Table 5) against tea mosquito bug. The infected insects were observed in almost all the treatments with varying numbers except in T5, T6, and T7. Selvasundaram and Muraleedharan (2000) established that *B. bassiana* in combination with 2 adjuvants like Triton AE and Teepol enhancing the mortality of shot hole borer beetles (*Euwallacea fornicatus*) of tea plants under field conditions. The formulated wettable formulation of *B. bassiana* exhibited a better control of tea mosquito (56.4–58.4%) at both locations, i.e., Kurti TE and North Tukvar TE (Fig. 5), than commercial formulation (38.0–40.5%). Ghatak and Reza (2007) observed that *B. bassiana* at different doses was found effective against tea pest under field conditions, and its efficacy was comparable with that of synthetic chemical insecticides. Annamalai et al. (2016) reported that *B. bassiana* and *L. lecanii* were effective for the control of thrips, *T. tabaci* on onion.

In vivo testing of M. anisopliae

Application of *M. anisopliae* formulation under field conditions resulted in significant reduction of termite population as indicated in Fig. 6. The field bioassay of *B. bassiana* and *M. anisopliae* was carried out (Singha et al. 2011) and was noticed that the application of these entomopathogens could reduce the number of termites per tea plant until the 5th week after treatment as compared to control.

Conclusion

It is concluded that, although *Trichoderma atroviride*, *T. asperellum*, and *T. harzianum* effectively controlled the dieback disease and promoted vegetative growth of tea plantation, the effectiveness was more pronounced by *T.*

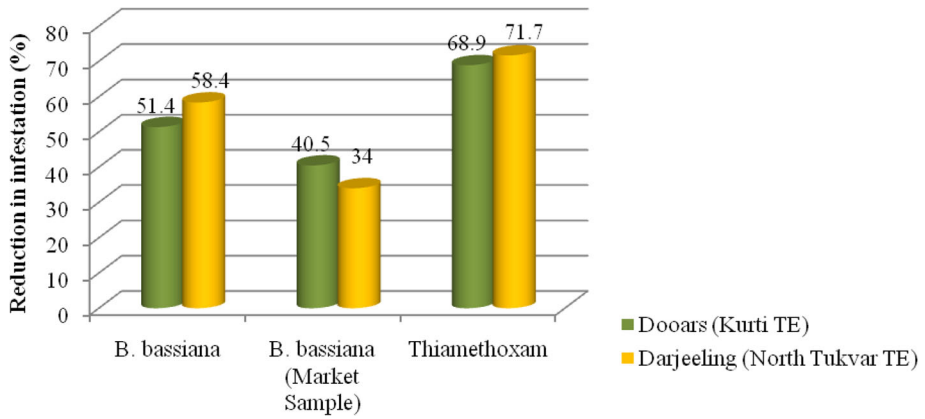

Fig. 5 In vivo bioefficacy of *Beauveria bassiana* against *Helopeltis theivora*

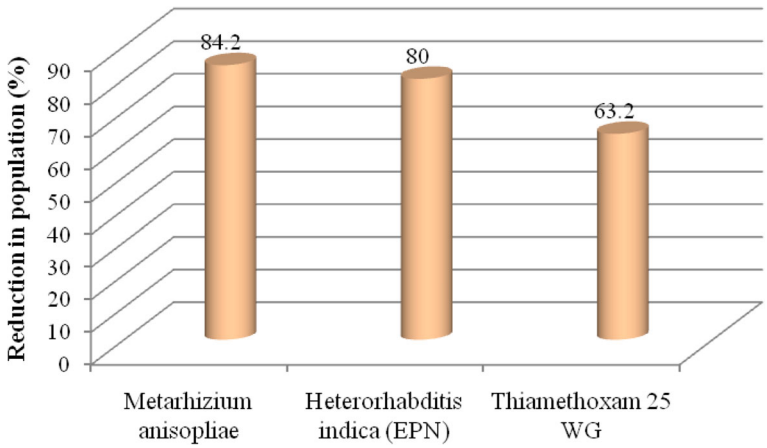

Fig. 6 In vivo bioefficacy of *Metarhizium anisopliae* against termite

asperellum than the other 2 species. The EPFs *B. bassiana* and *M. anisopliae* were found effective in controlling the tea mosquito and red spider mite, respectively. Hence, application of these fungal species may be an alternate measure to minimize the hazardous effect of synthetic agrochemicals for the management of selected tea pests and disease. Commercialization and utilization of these microbial formulations may be helpful in solving the problems associated with tea cultivation including the pesticide residues in made teas to a great extent.

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Authors' contributions
Kishor Chand Kumhar planned, executed, analyzed experimental data, and prepared the manuscript. Azariah Babu guided about entomopathogenic fungi and their bioefficacy tests under both lab and field conditions. John Peter finalized the protocol for formulation of these fungal species. Bhabesh Deka conducted in vitro experiments of *B. Bassiana* and *M. anisoplae*. Mitali Bordoloi conducted in vitro experiments of *Trichoderma* spp. Hirakjyoti Rajbongshi and Pritam Dey assisted in conduction of field trials of these fungal species. The authors read and approved the final manuscript.

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Availability of data and materials
All data generated and analyzed for this study are presented in the manuscript, and the corresponding author has no objection to the availability of data and materials.

Ethics approval and consent to participate
Not applicable. The study was conducted using local isolates of beneficial fungal species that are abundant in the ecosystem hence does not require ethical approval.

Consent for publication
The authors agree to publish this paper. The data has not been published partially or completely in any other journal.

Competing interests
It is declared that the authors have no competing interests.

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