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# *Beauveria bassiana*: as a potential microbial biocontrol agent for tea mosquito bug, *Helopeltis theivora* Waterhouse (Hemiptera: Miridae) in Dooars and Darjeeling, India

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## Abstract

**Background:** In the present study, the efficacy of two isolates of *Beauveria bassiana* namely, BKN20 and BKN1/14 was evaluated against the tea mosquito bug (TMB), *Helopeltis theivora*, Waterhouse (Hemiptera: Miridae) damaging harvestable shoots of tea plants in the Dooars and Darjeeling regions of West Bengal, India.

**Results:** Laboratory study revealed that, in both isolates, BKN20 was more pathogenic than BKN1/14, exhibiting 76% mortality of the test insect. The BKN20 isolate was formulated as an aqueous suspension (5%AS), and evaluated against TMB through micro-plot trials in tea plantations. The Micro-plot field study revealed a maximum of 72.19% reduction in the shoot damage due to TMB in plots sprayed with a 1000 ml/ha concentration of BKN20 5%AS containing  $2 \times 10^7$  conidia/ml, as compared to the synthetic insecticide (Thiamethoxam 25%WG), where a 63.12% reduction in the shoots was recorded. Furthermore, different concentrations of the formulated BKN20 5%AS were evaluated against the test insect at 2 locations of tea gardens in the Dooars and Darjeeling regions. The results from both locations revealed that 1000 and 1200 ml/ha concentrations of BKN20 5%AS (each concentration containing  $2 \times 10^7$  conidia/ml) significantly ( $p < 0.05$ ) reduced the TMB population and they were more effective than Thiamethoxam 25%WG (120 g/ha). However, non-significant differences in crop yields were recorded. The formulation BKN20 5%AS was found to be non-pathogenic to non-target insects, i.e. natural enemies present in the tea ecosystem. BKN20 5%AS had no phytotoxic effect on the tea leaves, with acceptable organoleptic attributes.

**Conclusion:** The BKN20 isolate could be commercialized as an alternative microbial insecticide to reduce the load of chemical insecticides in the tea ecosystem.

**Keywords:** Tea plant, *Helopeltis theivora*, Entomopathogenic fungi, Bio-formulation, Biological control

## Background

Tea [*Camellia sinensis* (L) O. Kuntze] is a perennial plantation crop that requires a warm, humid climate for

adequate growth and production. Such climatic conditions also favor the attack of various insect pests and diseases on this crop. Tea mosquito bug (TMB), *Helopeltis theivora* Waterhouse (Hemiptera: Miridae) is one of the major insect pests of tea plants in Indian tea plantations (Roy et al. 2015). It causes crop losses up to 25–40% (Hazarika et al. 2009) during favorable conditions. The TMB attacks the tender shoots of tea plants by inserting

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their proboscis into the tissues and sucking the plant sap that results in the dryness of the tissues around the punctured spot. The damaged tissues become brown and later become black where TMBs lay their eggs, multiply, and cause further damages of young shoots. The TMB remains active throughout the year, and unchecked infestation leads to 100% of the crop loss under favorable conditions if the appropriate management practices are not appropriately applied (Muraleedharan 1992).

In tea growing areas, planters use several chemical insecticides to reduce the populations of the pest below the Economic Threshold Level (ETL) of 5% (Mamun and Ahmed 2011). However, continuous and non-judicial use of these chemical insecticides leads to certain undesirable issues such as water pollution, degradation of valuable soil microbes, decline in biological control agents, resurgence, development of resistance in insect pests, and pesticide residues in the manufactured tea (Roy et al. 2008). To address these undesirable situations, exploring non-chemical control strategies has become essential. The application of the Microbial Control Agents (MCAs), especially entomopathogens plays a major role in the management of insect pests of tea plants in an eco-friendly way.

Entomopathogenic fungi (EPF), one of the MCAs, are the parasitic microorganisms that can infect and kill the arthropod pests (Rajab et al. 2020). These EPF are mainly used as biopesticides as a safe alternative to the toxic chemical insecticides (Lovett and Leger 2017). The fungus *Beauveria bassiana*, hyphomycetes, is extensively used for the management of insect pests of several crops in the different agro-ecosystems (Khashaba 2021). In tea plantations, several bio-pesticides based on MCAs and botanicals have been used (Gurusubramanian et al. 2008), but bio-insecticides based on the fungus *B. bassiana* has been less explored. Therefore, the present study was designed to isolate *B. bassiana* from the rhizospheric soils of tea growing areas of Dooars and Darjeeling regions, West Bengal, India, and to study their field efficacy against the TMB, which is a major threat for the tea industry.

## Methods

### Collection and laboratory culture of the TMB

The adults of TMB of mixed populations were collected from the experimental plots of North Bengal Regional R&D Centre (NBRRDC), Nagrakata (26° 54' 0" N, 88° 55' 0" E longitude), West Bengal, India. The culture of TMB was reared on a susceptible tea clone, TV1 in wooden cages containing glass chimneys under the laboratory conditions (25 ± 2 °C; 75 ± 5% RH; 16L: 8D photoperiod). The fresh tea leaves were provided to the adults every alternate day as food supplements. The cultures of the

insects were used for the efficacy assays maintained for more than 10 generations (from F1 to F10 without exposure to any pesticides).

### Isolation and identification of the fungus

Soil samples were collected with the help of auger from the rhizospheres of the tea plantations of Dooars and Darjeeling regions in pre-sterilized zip bags, separately in 2018 and were kept in a container with an ice bag (Ogunmwonyi et al. 2008). The collected samples were brought to the laboratory, and the fungus, *B. bassiana* was isolated through the serial dilution method (Morris and Rideout 2005). One ml aliquot of the soil sample from the 10<sup>-4</sup> dilution of the soil in the sterilized distilled water was plated on the Potato Dextrose Agar (PDA) plates in 3 replicates and these plates were incubated in a BOD (Biological Oxygen Demand) incubator at 28 °C for the isolation of target entomopathogen. After 6 days of incubation, the colony of *B. bassiana* was isolated from the plates and purified by single spore isolation method. The culture of the EPF was maintained on the agar slants at 4 °C for further use.

The fungal isolates BKN20 and BKN1/14 (collected from Dooars and Darjeeling regions respectively) were identified as *B. bassiana* by studying cultural and morphological characteristics, as well as by using a fungal key. The cultures of both isolates were sent to the Indian Type Culture Centre (ITCC), Indian Agriculture Research Institute New Delhi, India for long term preservation. The isolates BKN20 and BKN1/14 were also identified by sequencing the ITS regions (ITS1, 5.8S and ITS2) of the nuclear rDNA. The gDNA (genomic DNA) from each isolate was extracted, following the CTAB method of Moller et al. (1992) and quantified with the help of NanoDrop1000 spectrophotometer (Thermo Scientific). The rDNA gene cluster was amplified by PCR, using universal primer pairs ITS1/ITS4 (White et al. 1990). The amplified PCR products of each isolate were separated by electrophoresis on a 2% agarose gel, and the obtained bands were excised and purified (UniPro Gel extraction kit) for sequencing (Macrogen, Inc., Korea.). BLASTn was used to match *B. bassiana* sequencing results of each isolate with known sequences of *B. bassiana* strains accessible at the public database GenBank.

### Preparation of the fungal inoculum

For the preparation of the fungal suspensions of both isolates of *B. bassiana* (BKN20 and BKN1/14), each isolate was grown separately on the Potato Dextrose Broth (PDB) in conical flasks (500 ml) for 15 days at 28 °C in a BOD incubator. After the stipulated incubation, the fungal mycelial mat (conidia plus mycelia) was harvested

from each flask and ground in a grinder with 50 ml of double-distilled water under aseptic conditions to prepare the spore suspension. The homogenized spore suspension was filtered through a muslin cloth to make the stock suspension, and spore density of stock suspension was adjusted to  $2 \times 10^7$  conidia/ml using a hemocytometer (Singleton and Sainsbury 1981). Then, from the stock spore suspension of each isolate (BKN20 and BKN1/14), different concentrations such as 2.5, 5.0, and 10 ml (each concentration containing  $2 \times 10^7$  conidia/ml) were mixed in one liter of sterile water in the pre-sterilized glass containers to make the liquid formulations separately. Similarly for field trials, a stock solution of effective entomopathogen's spore suspension, i.e., isolate BKN20, was prepared and conidia were adjusted to  $2 \times 10^7$  conidia/ml. From this stock solution, different amounts such as 600 ml, 800 ml, 1000 ml, and 1200 ml (each concentration containing  $2 \times 10^7$  conidia/ml) were taken and mixed in the requisite amount of water for field applications to assess their bio-efficacy, separately.

#### **In vitro bio-efficacy of the spore suspensions of *B. bassiana* against TMB**

The bio-efficacy test was carried out against the second instar nymphs of TMB, collected from the laboratory reared culture, which were released on the tea leaves (TV 1 clone) kept inside a glass chimney (Roy et al. 2011). The spore suspensions of both isolates of BKN20 and BKN1/14 of different concentrations were sprayed on the tea leaves through a glass atomizer, separately. In control sets, tea leaves were sprayed with distilled water. A group of 20 TMBs of mixed populations were used for each treatment, and the experiments were carried out in 5 replicates in a complete randomized design (CRD). The mortality of TMB was recorded for 5 days at 24 h intervals. The number of dead insects was counted, and the percent mortality was calculated. During the laboratory experiment, the BKN20 isolate of *B. bassiana* was more pathogenic on TMB than the BKN1/14 isolate; hence it was selected for detailed field study.

#### **Micro-plot field study**

For the field experiment, a liquid formulation of BKN20 isolate containing 5% aqueous spore suspension was prepared, following the methodology of Godonou et al. (2000). A micro-plot field trial was conducted to evaluate the efficacy of BKN20 5%AS (aqueous suspension) against TMB. The trial was conducted in 3 replicates in a randomized complete block design (RCBD) in the experimental field of NBRRDC in 2018. Each plot ( $37.5 \text{ m}^2$ ) was consisting of 50 tea bushes. The treatments included BKN20 5%AS @ 1000 ml/ha (T1, containing  $2 \times 10^7$  conidia/ml), the recommended standard

insecticide (Thiamethoxam 25%WG) @ 120 g/ha (T2), and untreated control (T3). The plots with ETL above the 5% were selected and labelled for each treatment. Spraying was done, using a hand-operated knapsack sprayer (ASPEE Agro Equipment Pvt. Ltd.) using a spray volume of 1.5 l/plot. Care was taken to drench the bushes for better coverage and control. Plucking of the shoots was carried out before initiation of the treatment spray, and the percentage damage caused by TMB was computed from the randomly selected 100 shoots per replicate in each treatment. The first treatment spray (01.04.2018) was applied immediately after the harvesting. The second treatment spray (08.04.2018) was applied after plucking the leaves on the 7th day after the first spray. The percentage damage caused by TMB on 7th day after the 1st treatment spray, followed by the 7th day, 14th day and 21st day after the 2nd treatment spray (post-spray) was recorded from the randomly selected 100 shoots per replication. The percent shoot damage due to TMB was calculated, using the formula:  $C - T/C \times 100$ , where,  $C$  = Pre spray assessment and  $T$  = Post spray assessment (Sarmah and Bhola 2015).

#### **Large-scale field study**

The large-scale field trials were also conducted to evaluate the efficacy of the BKN20 5%AS in the 2 consecutive years of 2018 and 2019 in the tea gardens of 2 locations namely, Sungma Tea Garden, Darjeeling ( $26^\circ 94' 5'' \text{ N}$ ,  $88^\circ 17' 43'' \text{ E}$  longitude), and the experimental plot at the NBRRDC, ( $26^\circ 54' 0'' \text{ N}$ ,  $88^\circ 55' 0'' \text{ E}$  longitude). Each trial was conducted in a randomized complete block design (RCBD) in three replications. There were total 7 treatments [BKN 20 5%AS @ 600 (T1), 800 (T2), 1000 (T3), 1200 (T4) ml/ha (each treatment containing  $2 \times 10^7$  conidia/ml), *B. bassiana* 1.15%WP @ 2500 gm/ha (T5- a market product), recommended standard insecticide (Thiamethoxam 25%WG) @ 120 gm/ha (T6), and untreated control (T7)] per replication, and each plot ( $75 \text{ m}^2$ ) contained 100 tea bushes. The market product of *B. bassiana* (1.15% WP) was purchased from the local vendor. The pre-and post-treatment assessments were carried out as described earlier in the micro-plot study.

#### **Yield estimation of harvestable shoots from the large-scale field trials**

The yield estimation was also carried out during the experiments. By maintaining a standard plucking round (7 days interval), yield (green tea leaf) was recorded for the first 6 rounds of plucking. Average yield was expressed in kg/plot. The green leaf yield recorded at

every plucking was converted into processed tea for one hectare as described by Ponmurugan and Baby (2007), using the formula: Green leaf yield (kg)  $\times$  no. of bushes/ha  $\times$  conversion factor (0.225).

#### Effect of BKN20 5%AS on the non-target organisms

Laboratory bioassays were conducted to study the infectivity of the BKN20 5%AS the most common insect predator in the tea ecosystem. Larvae and adults of the predators were collected from the tea gardens at the NBRDC and reared in the laboratory. Experiments were carried out by spraying the different concentrations of BKN20 5%AS @ 600 (T1), 800 (T2), 1000 (T3), 1200 (T4) ml/ha (each treatment containing  $2 \times 10^7$  conidia/ml), and untreated control (T5) per replication. All the experiments were replicated 3 times, using 30 insects per treatment. Observations on larval mortality, larval period, pupation period and adult emergence were recorded by following the methodology of Leatemia and Isman (2004).

#### Phytotoxic effect and organoleptic evaluation

To evaluate the phytotoxicity effect (yellowing, stunting, necrosis, epinasty, hyponasty, etc.) of the BKN20 5% AS (at 'X' '2X' and '4X' concentration) on tea leaves, field experiments were carried out in three replicates at 84 square meters per replication in a RCBD design at Nagrakata, West Bengal. There were in total 3 treatments; 2.5 ml/l (T1), 5 ml/l (T2), 10 ml/l of water (T3), and an untreated control. The conidial density of each treatment solution, i.e., T1, T2 and T3 was  $2 \times 10^7$  conidia/ml. The spraying was applied by a hand-operated knapsack sprayer, using a spray volume of 400 l/ha. Observations were recorded on 0 day (pre-treatment) and day 3, 7 and 14 (post-treatment) on yellowing, stunting, necrosis, epinasty, hyponasty, etc., and the injury levels were graded, using the Phytotoxicity Rating Scale (PRS) as follows: no crop response/crop injury=0, 1–10%=1, 11–20%=2, 21–30%=3, 31–40%=4, 41–50%=5, 51–60%=6, 61–70%=7, 71–80%=8, 81–90%=9, 91–100%=10 (Babu et al. 2008).

#### Statistical analysis

Data obtained through laboratory and field experiments were analyzed through ANOVA (analysis of variance) using SPSS17. All the data were angularly transformed prior to the statistical analysis and the differences among the treatments in pre-spray percent incidence and percent reduction of shoot damage were assessed through Tukey's post-hoc Honestly Significant Difference (HSD)

test to separate the means at 95% confidence interval. For the green leaf yield, the treatments were compared with untreated control, using Tukey's post-hoc Honestly Significant Difference (HSD) test to separate the means at the 95% confidence interval. DMRT F-Test was carried out to compare the effect of different concentrations of BKN20 5%AS on non-target beneficial organism.

#### Results

Based on cultural, morphological and molecular studies, the isolated fungal isolates BKN20 and BKN1/14 were identified as *B. bassiana* (Dhar et al. 2019). The BLASTn sequencing analysis revealed the 99% similarity of sequences of isolated *B. bassiana* isolates with the nucleotide sequences of *B. bassiana* isolated from several sources, such as rice plant (MN337282), maize rhizosphere (AY531924.1), tomato plant (KX091133), cotton, rice, and vegetables rhizosphere (MG670098.1, MG670100.1, and MG670102.1), and rice leaf folder (JX122736.1) available in GenBank. Since the sequences of both isolates were identical, sequences of the BKN114 isolate were submitted to GenBank under accession number MZ734352.

#### In vitro bio-efficacy of the *B. bassiana* against TMB

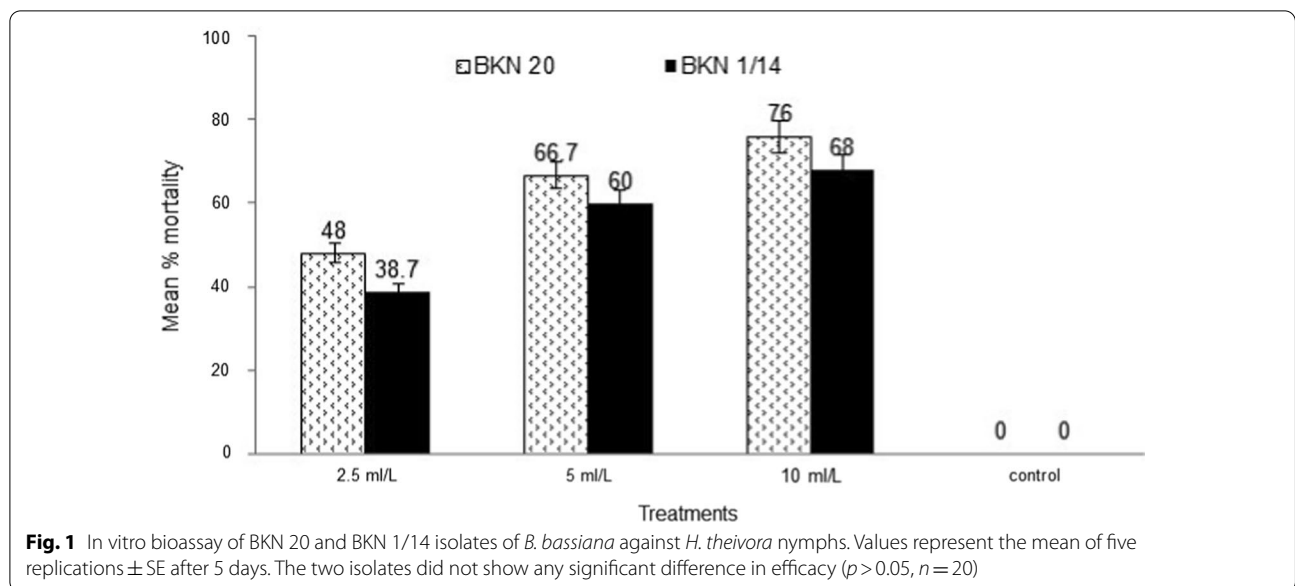
The results of the in vitro bio-efficacy test showed that both isolates BKN20 and BKN1/14 were pathogenic to the TMB and caused significant insect mortality (Fig. 1). The highest percentage of nymphal *H. theivora* mortality (76%) was recorded for BKN20 at 10 ml/l concentration ( $2 \times 10^7$  conidia/ml), while a similar concentration of BKN 1/14 isolate caused 68% mortality of the TMB after 5 days of treatments. In the case of control (water spray) set, no mortality of the nymphs was recorded. Although there was non-significant difference in the mortality between the 2 isolates ( $p > 0.05$ ), the BKN20 isolate was considered for its further formulations and field studies because of its higher pathogenicity on the TMB.

#### Micro-plot field study

The results of the micro-plot field study conducted against TMB are given in Table 1. After 21 days, a maximum of 72.19% reduction of shoots damage was recorded in the plots sprayed with BKN20 5%AS at 1000 ml/l concentration ( $2 \times 10^7$  conidia/ml), whereas 63.12% reduction of shoots damage was recorded in the plots sprayed with the standard insecticide Thiamethoxam 25%WG.

#### Large-scale field study

The field evaluation results (average of the season I and season II) of BKN20 5%AS against TMB at Doars region (TRA Experimental Plot), and Darjeeling region (Sungma Tea Estate) are presented in Table 2. At Darjeeling region,



**Table 1** Efficacy of BKN20 5%AS against TMB in micro-plot study

Treatments	Concentration (ha)	Pre-spray leaf damage incidence (%)*	Shoot damage reduction (%) after 21 days*
BKN20 5%AS	1000 ml <sup>§</sup>	29.11	72.19 <sup>b</sup>
Thiamethoxam 25%WG	120 g	31.18	63.12 <sup>a</sup>

\*Values represent the mean of three replications  $\pm$  SE, for pre-spray shoot damage incidence (before treatments) and shoot damage reduction (obtained from four rounds of leaf plucking after treatments at weekly interval)

<sup>§</sup> Containing  $2 \times 10^7$  conidia/ml. Values in the same column with different superscript are significantly different with each other at  $p < 0.05$  by Tukey's post-hoc Honestly Significant Difference (HSD) test

**Table 2** Bio-efficacy of BKN20 5%AS in controlling shoot damage of tea plants from TMB in large-scale field study

Treatments	Concentration (ha)	Darjeeling		Dooars	
		Pre-spray Shoot damage incidence (%) <sup>#</sup>	Shoot damage reduction (%) <sup>#</sup>	Pre-spray Shoot damage incidence (%) <sup>#</sup>	Shoot damage reduction (%) <sup>#</sup>
BKN20 5%AS	600 ml <sup>§</sup>	23.1	66.4 <sup>b</sup>	27.3	69.3 <sup>b</sup>
	800 ml <sup>§</sup>	21.5	68.9 <sup>b</sup>	27.0	73.0 <sup>bc</sup>
	1000 ml <sup>§</sup>	21.3	79.8 <sup>a</sup>	26.6	79.4 <sup>a</sup>
	1200 ml <sup>§</sup>	20.9	81.6 <sup>a</sup>	28.0	81.9 <sup>a</sup>
<i>Beauveria bassiana</i> 1.15%WP*	2500 g	23.1	62.5 <sup>c</sup>	28.5	66.6 <sup>b</sup>
Thiamethoxam 25%WG	120 g	20.4	66.7 <sup>b</sup>	28.5	70.1 <sup>b</sup>
F value	–	8.4	9.5	10.2	12.3
p value	–	< 0.0001	< 0.0001	< 0.0001	< 0.0001

<sup>#</sup> Pre-spray shoot damage incidence (%) and shoot damage reduction (%) values for each location are the mean of two consecutive seasons

<sup>§</sup> Containing  $2 \times 10^7$  conidia/ml. Pre-spray shoot damage incidence (%) are the mean of three replications  $\pm$  SE of leaf plucking before treatments and shoot damage reduction (%) are the mean of three replications  $\pm$  SE obtained from four rounds of leaf plucking after treatments at weekly interval for each season. Values in the same column with different superscript are significantly different with each other at  $p < 0.05$  by Tukey's post-hoc Honestly Significant Difference (HSD) test

\*Market product



pre-treatment observation on shoots damage caused by TMB was in the range of 20.4 to 23.1%. The ANOVA analysis revealed that all the treatments were found to be significantly ( $F=6.1$ ,  $df=1$  (between groups),  $df=5$  (within groups),  $p<0.05$ ) different between the treatments in minimizing the shoots damage caused by TMB. The reduction of mean shoots damage caused by TMB was significantly superior ( $p<0.05$ ) at the higher concentrations (1000 and 1200 ml/ha, each containing  $2 \times 10^7$  conidia/ml) of BKN20 5%AS, in comparison to 120 gm/ha of Thiamethoxam 25%WG ( $p<0.05$ ). The reduction of mean shoot damage was similar in plots treated with BKN20 5%AS at 600 and 800 ml/ha, and *B. bassiana* 1.15%WP at 2500 gm/ha as compared to the plots sprayed with Thiamethoxam 25%AS ( $p>0.05$ ) (Table 2).

At the Dooars region, the incidence of shoots damage caused by TMB before the initiation of spray ranged from 26.6 to 28.5%. The ANOVA analysis revealed that the mean percent reduction of shoots damage due to TMB was significantly higher ( $F=9.5$ ,  $df=1$  (between groups),  $df=5$  (within groups),  $p<0.05$ ) when the plots were sprayed by BKN20 5%AS at 1000 and 1200 ml/ha concentrations in comparison to the Thiamethoxam 25%WG. The 2 treatments resulted in superior control of TMB than the market product of *B. bassiana* 1.15%WP as well as untreated control ( $p<0.05$ ) (Table 2).

**Yield estimation of harvestable shoots from the large-scale field trials**

The average yields recorded from the first 6 pluckings (at 7 days intervals) during the experimental periods were presented in Table 3. The yield was significantly lower in untreated control, confirming the better

efficacy of different tested formulations ( $F=8.2$ ,  $df=6$ ,  $p<0.05$ ). Among the treatments, tea leaf yield was significantly higher ( $p<0.05$ ) in the plots sprayed at 1000 and 1200 ml/ha concentrations of BKN20 5%AS, and Thiamethoxam 25%WG at 120 gm/ha than in the plots sprayed with a market product of *B. bassiana* 1.15% WP at 2500 gm/ha, and 600 and 800 ml/ha concentrations of BKN20 5%AS (Table 3).

**Effect on non-target beneficial organisms**

Laboratory studies revealed that the formulation BKN20 5%AS did not cause any larval mortality of *Stethorus gilvifrons* M. (Coleoptera: Coccinellidae), the most common insect predator in the tea ecosystem (Table 4). There was non-significant variation ( $p<0.05$ ) in the larval period, pupal period and adult emergence after BKN20 5%AS treatment and untreated control, which revealed that BKN20 5%AS was harmless to *S. gilvifrons*.

**Phytotoxic effect and organoleptic evaluation**

The phytotoxic study was carried out separately at 2.5, 5.0, and 10 ml/l of water ( $2 \times 10^7$  conidia/ml). Observations recorded on the phytotoxicity symptoms indicated that none of the concentrations showed any type of phytotoxic effect on the tea leaves and harvestable shoots. There was no visible injury on the leaf tip, leaf surface, wilting, vein clearing, necrosis, epinasty and hyponasty. Similarly, tea shoots harvested 1, 3, 5, 7, 10 and 14 days after the application of BKN20 5%AS were processed in the miniature CTC unit at NBRDC,

**Table 3** Effect of BKN20 5%AS on the yield of harvestable shoots of tea

Treatments	Concentration (ha)	Green leaf yield* (kg/plot)	Processed tea (kg/ha/year)
BKN20 5%AS	600 ml <sup>§</sup>	2.51 <sup>cd</sup> ± 0.19	390.5 ± 4.25
	800 ml <sup>§</sup>	2.55 <sup>bc</sup> ± 0.18	396 ± 4.36
	1000 ml <sup>§</sup>	2.61 <sup>ab</sup> ± 0.16	405 ± 4.45
	1200 ml <sup>§</sup>	2.63 <sup>a</sup> ± 0.23	408.5 ± 4.21
<i>Beauveria bassiana</i> 1.15% WP <sup>¶</sup>	2500 g	2.50 <sup>de</sup> ± 0.29	388 ± 4.19
Thiamethoxam 25%WG	120 g	2.65 <sup>a</sup> ± 0.22	411 ± 4.37
Untreated control	–	2.43 <sup>e</sup> ± 0.14	378 ± 4.25
F value		8.2	15.5
p value		0.003	< 0.0001

\*Values represent the mean of three replications ± SE, for green leaf yield (obtained from six rounds of leaf plucking after treatments at weekly interval for two consecutive seasons)

<sup>§</sup> Containing  $2 \times 10^7$  conidia/ml. Values in the same column followed by the same letter are not significantly different from each other at the 95% confidence interval ( $p<0.05$ ) using Tukey's post-hoc Honestly Significant Difference (HSD) test

<sup>¶</sup> Market product

**Table 4** Effect of BKN20 5%AS on some biological parameters of non-target beneficial organisms (*Stethorus gilvifrons*) in tea

Treatment	Concentration (ml/ha)	Larval mortality (%)	Larval period (days)	Pupal period (days)	Adult emergence (%)
BKN20 5%AS	600 ml <sup>§</sup>	0.00 <sup>a</sup>	8.31 ± 2.12 <sup>a</sup>	4.64 ± 0.13 <sup>a</sup>	94.00 <sup>a</sup>
	800 ml <sup>§</sup>	0.00 <sup>a</sup>	8.32 ± 2.12 <sup>a</sup>	4.64 ± 0.13 <sup>a</sup>	95.00 <sup>a</sup>
	1000 ml <sup>§</sup>	0.00 <sup>a</sup>	8.32 ± 2.12 <sup>a</sup>	4.32 ± 0.11 <sup>a</sup>	94.00 <sup>a</sup>
	1200 ml <sup>§</sup>	0.00 <sup>a</sup>	9.33 ± 2.34 <sup>a</sup>	4.65 ± 0.13 <sup>a</sup>	100.00 <sup>a</sup>
Untreated control	–	0.00 <sup>a</sup>	9.12 ± 2.23 <sup>a</sup>	4.33 ± 0.11 <sup>a</sup>	100.00 <sup>a</sup>
CD (5%)	–		1.61	1.56	15.01
CV (%)	–		5.78	14.66	7.21

Means followed by similar letters in a column are not significantly different at 5% (DMRT F-Test,  $p > 0.05$ )

<sup>§</sup> Containing  $2 \times 10^7$  conidia/ml

and the report from professional tea tasters revealed that the processed tea samples had no taint (taste and odor foreign to the tea) and acceptable organoleptic attributes.

## Discussion

The tea production is threatened by many insect pests, and TMB is an important insect pest of tea plants worldwide (Roy et al. 2015). Although the management of this insect pest is achieved through cultural and chemical practices, however, chemical insecticides have their limitations as they cause several side effects as continuous application of synthetic pesticides causes different health hazards not only to the tea workers but also to the consumers, along with environmental pollution (Borkakati and Saikia 2019). In recent years, natural insecticides based on botanicals or bio-control agents have been attracted the attention of researchers to manage the insect pests of tea plants (Cheramgoi et al. 2016), and in this regard, EPF are no exception (Jensen et al. 2019). In the present study 2 isolates of *B. bassiana* such as BKN20 and BKN1/14 isolated from the rhizospheric soil samples of tea plantations of Dooars and Darjeeling regions were evaluated against TMB both in laboratory and in field. The laboratory study revealed that BKN20 was more pathogenic on the insect than that of BKN1/14, and hence BKN20 was evaluated at field level. In particular, the more pathogenic activity of the BKN20 isolate than the BKN1/14 may be due to its more endo-cutinase activity to penetrate the cuticle of the insect, which is the primary barrier.

Micro-plot and large-scale field evaluations of different concentrations of BKN20 isolate formulations revealed that the concentrations 1000 ml/ha was significantly reduced the populations of TMB in the tea plantations of both locations. Besides, the formulation of this isolate was superior over the market product of *B. bassiana*, and

also had non-significant impacts on the natural enemies present in the evaluated tea plantations. Earlier, the effectiveness of different isolates of *B. bassiana* was reported against TMB (Ekka et al. 2019), and other insect pests of tea plants such as: shot hole borer beetles (*Euwallacea fornicatus*) (Selvasundaram and Muraleedharan 2000), tea weevils (Cheramgoi et al. 2016), and termites (Gurusubramanian et al. 1999). Field application of *B. bassiana* ( $10^7$  and  $10^8$  spores/ml) against shot hole borer beetles damaging tea plants revealed that the higher concentrations were more effective in reducing insect populations than the lower concentrations (Selvasundaram and Muraleedharan 2000). In contrast, in the present study, low concentrations have been found to be also effective in the control of TMB.

Recently, Ekka et al. (2019) reported that the formulation (BPA/B7) of *B. bassiana* ( $1.68 \times 10^6$  spores/ml) reduced the shoots damage of tea plants up to 50% at a concentration of 21.87 ml/l in Assam India, however, in the present investigation more efficacy was reported, the concentration 1000 ml/ha with conidial concentration  $2 \times 10^7$  was able to reduce 77–79% of the damage of shoots from the TMB. The variation in the efficacy of *B. bassiana* may be due to different biotypes of insect pests from different locations or different origin of EPF used during the study. Remarkably, the efficacy of *B. bassiana* both in the laboratory, and in-field conditions may be due to the production of several toxins such as, beauvericin, enniatins, oosporein, and bassianolide during the infection, that had a major role in the pathogenic activity of *B. bassiana* against TMB (Vey et al. 2001),

As far as the impact of EPF on the natural enemies is concerned, Thungrabeab and Tonga (2007) found that *B. bassiana* ( $1 \times 10^8$  conidia/ml) was nonpathogenic on the non-target insects such as *Chrysoperla carnea* (Stephens), *Coccinella septempunctata* (Linnaeus), and *Dicyphus tamaninii* (Wagner) as well as *Heteromurus nitidus*

(Templeton), a beneficial soil insect. On the contrary, *Metarhizium anisopliae* was pathogenic toward *C. carnea* and *D. tamaninii*, with respective 4% and 10% mortalities. Similarly, in the present study, the evaluated bio-formulation BKN20 5%AS had a non-pathogenic impact on the populations of natural enemies such as *S. gilvifrons* present in the tea ecosystem. These results are also supported by observations of earlier investigators (Dromph and Vestergaard 2002) who reported that some EPF such as, *B. bassiana*, *Hirsutella* spp., and *M. anisopliae* did not affect the population of natural enemies. This shows that EPF could be quite specific and might infect only a certain type of host.

Previously, researchers reported that the formulations of *B. bassiana* were more effective than the synthetic insecticides against *H. antonii* damaging cashew (Navik et al. 2015), hairy caterpillar, *Pericallia ricini* damaging castor crop (Shophiya et al. 2014), and other insect pests of tea plants (Ghatak and Reza 2007). Similar results were reported in the present investigation, and the used formulation BKN20 5%AS was more effective than the Thiamethoxam against TMB. Besides, some investigators reported that *B. bassiana* was compatible with synthetic insecticides such as imidacloprid (Feng and Pu 2005), and Bifenthrin (Liu et al. 2012). Therefore, due to the eco-friendly nature of EPF, this will be compatible with the other insect management components in the integrated pest management program.

In the present study, the plots sprayed by BKN20 5%AS showed significantly higher yield than the untreated plots. The yield of harvestable shoots of tea was also high from the plots sprayed by our formulated BKN20 5%AS, than the *Beauveria* formulation purchased from the market. In addition, observations on the phytotoxicity on tea leaves revealed that BKN20 5%AS was non-phytotoxic to tea, and teas made from the leaves tested by professional tasters had no taint. In contrast, phytotoxic effect and insecticide residue level above the EU recommendations was reported for various insecticides in harvestable shoots of tea plants (Aktar et al. 2009), which showed that natural bio-pesticides could be an alternative to reduce the pesticide load in tea plantations. In particular, the compatible use of insecticides with EPF may reduce the pesticide residues' level in the harvestable shoots of tea plants (Liu et al. 2012). Thus, based on the percentage reduction of shoot damage, phytotoxicity, nonpathogenic on non-target organisms' population and green leaf yield recorded during the experimental periods, it can be concluded that BKN20 5%AS can be commercialized as a natural eco-friendly insecticide for the management of TMB in the tea plantations and can be an important component of IPM strategies for future.

## Conclusions

The present study revealed that the formulation of BKN20 isolate, i.e. BKN20 5%AS efficiently controlled TMB with a significant reduction in the harvestable shoot damage at both locations in multi-seasons. The BKN20 5%AS was also superior over the synthetic insecticide and bio-insecticides based on the *B. bassiana* purchased from the market. Besides, the formulated BKN20 5%AS did not show any phytotoxic effect on the harvestable shoots and had acceptable organoleptic attributes among the selected consumers.

## Abbreviations

AS: Aqueous suspension; PPC: Plant Protection Code; BCA: Biological Control Agents; PDB: Potato Dextrose Broth; NBRDC: North Bengal Regional Research and Development Centre; L: Litre; ml: Millilitre.

## Acknowledgements

Authors are thankful to the Biotechnology Industry Research Assistance Council (BIRAC) New Delhi-110003, Govt. of India, for the financial support rendered in the form of a Research Project.

## Authors' contributions

BD: Performed laboratory and field trials, analyzed and interpreted the data of the work and prepared the original manuscript. AB: designed and guided the laboratory as well as the field trials, reviewed and edited the writing, KCK: collected soil samples and isolated *B. bassiana*. AJP: finalized the protocol for the formulation of *B. bassiana*. AKP, SS, HR and PD: assisted in conduction of field trials of *B. bassiana*. ELA and VRT: prepared *B. bassiana* 5% AS liquid formulation. All authors read and approved the final manuscript.

## Funding

This study was performed as a part of externally funded research project under the CRS scheme of Biotechnology Industry Research Assistance Council (BIRAC), New Delhi.

## Availability of data and materials

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

## Declarations

### Ethics approval and consent to participate

Not applicable. The study was conducted using local isolates of fungal antagonists that are abundant in the ecosystem hence does not need ethical approval.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they do not have competing interests.

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Received: 15 July 2021 Accepted: 25 September 2021  
Published online: 05 October 2021



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