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An assessment of biological control of the banana pseudostem weevil *Odoiporus longicollis* (Olivier) by entomopathogenic fungi *Beauveria bassiana*

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ABSTRACT

Banana (*Musa sp.*) is the most imperative staple food crop for all types of people worldwide, which is commonly grown in Southeast Asia. Banana plantain can be severely affected by the devastating pest *Odoiporus longicollis* that results in severe economic losses in India. Management of weevil pests using chemical methods is harmful to the environment, and cultural methods are also partially successful. Therefore, an alternative approach of plant defense mediated by endophytic fungi to control banana stem borer larvae is necessary, which could affect the extracellular enzyme chitinase and protease. Among four isolates, *Beauveria bassiana* isolate KH3 is the most virulent entomopathogenic fungus compared with other isolates, and species identification was achieved using molecular phylogenetic characteristics. The *B. bassiana* isolate KH3 (1×10^8 conidia/mL^{-1}) is more bioeffective against *O. longicollis* larvae, causing > 90% significant mortality in 12 and 18 days. Adult weevils were treated with a higher concentration (1×10^8 conidia/mL^{-1}) of the endophytic *B. bassiana* isolates KH3 and KH9, which resulted in a maximum mortality of 76.6% at 27 days of post inoculation compared to that with other isolates ($p > 0.05$). Based on our results, *B. bassiana* isolate KH3 (MN165867) can be exploited as a potential biocontrol agent for reducing insect population and stem damages in banana by lure and kill method.

1. Introduction

Banana (*Musa sp.*) is the fourth most important agricultural food crop in India, which is the leading country in the production of bananas and plantains around the world. Bananas constitute a major staple food crop for millions of people and also provide a valuable source of income through local and international trade (Dwivany et al., 2016). The banana stem weevil *Odoiporus longicollis* (Coleoptera: Curculionidae) is one of the most destructive pests that is widely distributed all over the world, particularly in tropical and subtropical countries. This pest causes extensive stem damage because the banana plantain is propagated by suckers, which are often infected by these pests from the corm region to the entire stem portion of the plants. This results in reduced fruit and bunch size and reduced uptake of nutrients from the entire stem portion, leading to toppling of plants (Alagesan et al., 2016; Padmanaban et al., 2001). Adult weevils often confine themselves within the pseudostem and also in the decomposing tissues of the infested banana plant. All the life stages of the weevils are present throughout the year, and adult weevils are strong fliers moving from one plant to another for feeding. Through feeding, the larvae move athwart horizontally or in an accumbent direction boring into the core stem along with the inner leaf sheaths (Azam et al., 2010). This pest infestation has a severe impact on banana production, leading to higher yield losses.

Insect pest management of *O. longicollis* has been reported via cultural methods, host plant resistance, and biological control. Of these, cultural methods include mulching, intercropping, and stem cuttings to ensure pest-free planting material. Chemical pesticides are more effective for controlling several arthropods but are prohibitively expensive and also cause adverse effects on human health and the environment (Bellotti, 2008; Holguin and Bellotti, 2004). Recently, biological control approaches possessing more potential towards the control of phytopathogens have been reported using entomopathogenic fungi like *Beauveria bassiana* (Akello et al., 2008), *Metarhizium anisopliae* (Panyasiri et al., 2007) and *Verticilium lecanii* (Aqueel and Leather, 2013). Earlier, biocontrol agents such as neem oil, pongamia oil and synthetic pyrethroids were used for the control of *O. longicollis*; however, it became resistant due to continuous applications. To overcome

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these problems, an alternative approach for the control of *O. longicollis* using the endophytic fungi *B. bassiana* is recommended as an eco-friendly pest management strategy.

Entomopathogenic fungi (EPF) that infect pests have been receiving considerable research attention due to their potential for the biological control of pests. *B. bassiana* has naturally occurring endophytes in most of the plants against several phytopathogens (Vega, 2008; Zimmermann et al., 2013). It is also considered as the most appropriate biocontrol agent in temperate regions (Glare, 2004). Moreover, *B. bassiana* and *M. anisopliae* produce several enzymes, which are primarily involved in the degradation of the cuticle layer of insects. Tharani et al. (2018) demonstrated the isolation of the chitinase gene from the resistant genotype *Pisang lilin* that was able to degrade the exoskeleton layer of nematodes as well as their eggs. An artificial inoculation method of *B. bassiana* has been successfully demonstrated against arthropods of some agricultural crops like banana (Akello et al., 2008), coffee (Posada and Vega, 2006), cotton (Gurulingappa et al., 2010; Lopez and Sword, 2015; Ownley et al., 2008), maize (Bing and Lewis, 1991), common bean (Parsa et al., 2013), cacao (Posada and Vega, 2005), fava beans (Akello and Sikora, 2012), date palm (Gomez Vidal et al., 2006), radiata pine (Brownbridge et al., 2012), opium poppy (Quesada Moraga et al., 2014), and tomato (Ownley et al., 2008). The present study was conducted to evaluate the pathogenicity of *B. bassiana* isolates and their extracellular activities against the banana stem weevil and the larvae of *O. longicollis* by feeding or social contact with phytopathogens.

2. Materials and methods

2.1. Insect rearing

Reproductively active male and female *O. longicollis* were collected from Kolli hills (11°2′N latitude and 78°3′E longitude), Namakkal, for rearing and maintained in an insect breeding chamber at 25 ± 2^{0}C under 16L: 8D photoperiod and 65% relative humidity in a culture room for fecundity development for bio-assay.

2.2. Isolation of entomopathogenic fungi

Banana leaves were collected from four different commercial farms in the state of Tamil Nadu, India. A total of 10 leaves were randomly collected per location, and a total of 40 samples were used for the isolation. Each leaf was collected into the respective polyethylene bag using sterile forceps, cut into 6–8 mm^2 foliar pieces, and placed onto a solid selective medium containing Sabouraud dextrose agar supplemented with yeast (SDAY) extract (200 g glucose, 20 g peptone, 5 g yeast extract and 15 g agar/L distilled water) and containing antibiotics (0.1 g penicillin, 0.2 g streptomycin and 0.05 g tetracycline/L SDAY) in separate petri dishes (90 × 15 mm). The adaxial part of the leaf was first firmly pressed onto one side, then the leaf was turned over, and the abaxial part of the foliar piece was pressed onto the other side of the Petri dish. The fungus was cultured on petri dishes and incubated in the laboratory at 27^{0}C for 18 days, after which it was subcultured for further bioassay (Akello et al., 2008).

2.3. Morphological identification of endophytes

Morphological studies were conducted to identify conidial structures under the stereo microscope (Model No. M125, Leica, Wetzlar, Germany). Slides were prepared for light microscopy by staining with lacto phenol cotton blue and identified with the description given in standard manuals (Domsch et al., 1980).

2.4. Conidial suspension preparation

A total of 40 isolates, the best 4 isolates, were selected based on a preliminary evaluation assay against *O. longicollis*. Conidia obtained from the best of these four isolates, *B. bassiana* isolate TP23, *B. bassiana* isolate TP32, *B. bassiana* isolate KH3 and *B. bassiana* isolate KH9, were grown at 27 ± 2 °C in dark on SDAY, consisting of peptone 10 g/L, glucose 20 g/L, and agar-agar 20 g/L (constant volume of 17 ml), in standard petri-dishes (90 mm diameter). Fungal conidiospores were harvested from 18-day-old culture plates by scrapping into tubes containing sterile Tween-20 (0.01%). The suspension was vortexed for 2 min and agitated for 1.5 h on a flask shaker at room temperature before filtering through four layers of sterile muslin cloth. The conidial concentration of the stock suspension was estimated using Neubauer counting chamber, and the spore suspension was adjusted to 1×10^5,1×10^6, 1×10^7, and 1×10^8 conidia/mL^{-1}. These spore suspensions were kept overnight on ice at 4 °C for conidial germination, and they were checked routinely before use in bioassays as described by Gabarty et al. (2014).

2.5. Efficacy of endophytes against pseudostem weevil

Second-instar larvae of *O. longicollis* were treated with the four isolates at four different concentrations of conidial suspensions consisting of three replicates for larval bioassay. Each replicate contained 3 larvae with a total of 36 larvae for each isolate. The viable spore suspensions at each concentration were sprayed on the preferable host plant Poovan (AAB) stem discs (4–5 cm diameter) and the released second instar larvae for the evaluation of larval efficiency. Poovan genotypes (AAB) were more susceptible and were characterized by the method of Alagesan et al. (2016, 2018). The EPF-inoculated stem discs were transferred individually to a insect culture container containing a sterile filter paper along with the control. Accordingly, 10-cm long stem pieces were taken and swabbed with *B. bassiana* on the outer surface of the stem, and four adult weevils were released into each container for the bioassay at 27 °C, according to the method of Duarte et al. (2016).

2.6. Number of cadavers

Dead larvae and adult weevils were collected from each treatment assay and incubated in the laboratory for 7–12 days for the observation of mycelial growth on the insects, after which they were subjected to molecular characterization.

2.7. Isolation of genomic DNA

Fungal genomic DNA was isolated from the collected insect cadavers from *B. bassiana* isolate KH3 using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A final volume of 80 μl DNA extract was eluted and stored at −20 °C for further use.

2.8. Polymerase chain reaction

Specific primers of *BbF*-5′-GGTTGAAATGACGCTCGGAC-3′ and *BbR*-5′-TCCCTCTGTTGGTGAACCTG-3′ were designed based on the 18S rRNA gene sequences of *Beauveria bassiana* using the Fast PCR software. PCR amplification was performed in a final volume of 25 μl, with each reaction mixture containing 2 μl of DNA template, 8.5 μl of nuclease-free water, 12.5 μl of 1.5 mM $Mgcl_2$ (Taq 2X Master mix, Red Ampliqon) and 1 μl of each primer at 10 pmol. Target amplification was carried out by the following PCR steps: 35 cycles of three steps each, comprising initial denaturation at 95 °C for 30 s, primer annealing at 56 °C for 30 s and product extension at 72 °C for 1 min. At the end of the amplification, a final extension step was achieved at 72 °C for 10 min. A total of 12 μl of the PCR products from each PCR reaction was electrophoresed on 1% (w/v) agarose gel containing 5 mg/ml of ethidium bromide in a 1X TAE buffer (pH 8.4). 100-bp DNA ladder (Sigma-Aldrich, Bangalore) was used as a template. Gel electrophoresis was performed at 100 V, and it was characterized by the Gel Documentation system (BioRad, California, USA).

2.9. Sequencing

The amplified products were sequenced at Xcelris genomics Pvt. Ltd, Bangalore. The obtained sequences were further analyzed by the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Species identification was performed using the lowest expected value (E-value) of the BLAST output, and the percentage of homology was analyzed.

2.10. Chitinase assay

Chitinase activity was determined using spectrophotometry method by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The culture filtrate was composed of 0.5 ml of 1% colloidal chitin suspended in 0.02 M phosphate buffer (pH 7.0) and 0.5 ml of the enzyme solution. After 30 min of incubation at 37 °C, 0.75 ml of 3,5-dinitrosalicylic acid reagent (DNSA) was added to stop the reaction. The suspension was heated for 10 min at 100 °C and centrifuged at 8000 rpm for 10 min. The absorbance of the supernatant was measured at 530 nm wavelength and a standard curve was obtained using N-acetylglucosamine as a standard. One unit of the chitinase activity was defined as the amount of enzyme that yields 1 μmol of reducing sugar as N-acetyl-D-glucosamine (Glc NAC) equivalent per minute (Toharisman et al., 2005).

2.11. Protease assay

A supplementation of 1% gelatin with minimal broth was used as the growth medium. Erlenmeyer flasks with a capacity of 150 mL were dispensed with 25 mL of broth. These flasks were inoculated with the spore suspension of *B. bassiana* and were incubated at 27^{0}C. The enzyme activity was measured at the 14 days of post incubation. Aliquots were aseptically removed from the flasks and centrifuged at 5000 rpm for 5 min. The supernatant was used as the crude enzyme. To determine the extracellular protease activity, the reaction mixture was prepared by mixing 1 mL of each crude enzyme and buffered casein stock solution (1 g casein in 100 mL of 0.1 M PO_4 buffer). This reaction mixture was incubated in a water bath set at 36^{0}C for 60 min. At the end of the incubation period, the reaction was terminated by adding 3 mL of trichloro acetic acid, which precipitated the unhydrolysed casein. The precipitate was removed by centrifugation, and 0.4 M 5 mL Na_2CO_3 and 0.5 M Folin's reagent were added to the supernatant. This was incubated at room temperature for 20 min, and enzyme activity was read as absorbance at 660 nm in a UV-VIS spectrophotometer (Shimadzu, Japan). Control was prepared by adding 1 mL of distilled water instead of the crude enzyme extract (Hasan et al., 2013).

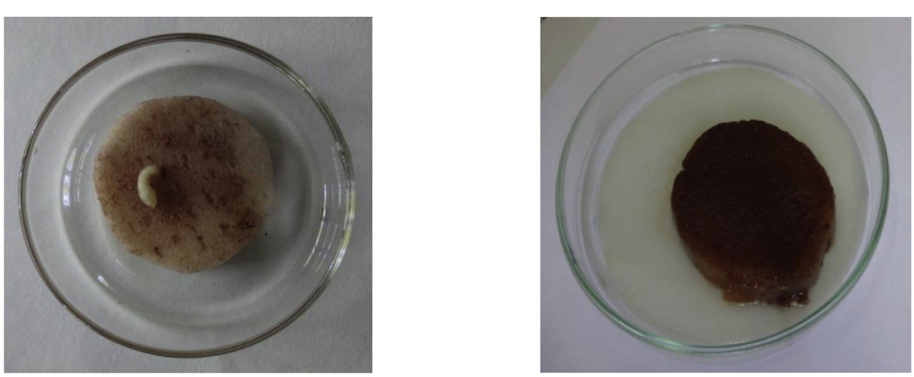
2.12. Statistical analysis

Data were described as mean values using the SPSS software (Version 16.0). The differences between various concentrations in terms of mortality ranges were evaluated by analysis of variance (ANOVA), and bars with the same letters indicate not statistically significantly different according to the Least Standard Deviation (LSD) test at $p < 0.05$ (Hurley et al., 2014).

3. Results and discussion

The four endophytic fungal strain *B. bassiana* was isolated from banana leaf samples at Kolli hills, Namakkal, Tamil Nadu, India, and it was characterized by microscopic studies. The banana stem weevil and the larvae of *O. longicollis* were treated with different isolates of *B. bassiana* by swabbing and spraying on the stem discs, respectively. Entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* are the most important biocontrol agents used in integrated pest management practices against arthropods (Faria and Wraight, 2001). In general, *B. bassiana* plays a potential biological role in the control of banana corm weevil (Akello et al., 2008), sugarcane stem borers (Easwaramoorthy and Santhalakshmi, 1993) and larvae of *Polyphylla fullo* (Erler and Ates, 2015). Nowadays, agricultural crops are being affected by plant pathogens and it was successfully controlled by *B. bassiana.* In this regard, the present research primarily focused on the efficiency of entomopathogenic fungi for the control of the stem weevil. Interestingly, a total of 15 fungal isolates were obtained from 40 leaf samples in *Musa* and 7 isolates of *B. bassiana* were identified based on their morphological characterization. Among these, only four fungal isolates of *B. bassiana,*i.e., TP23, TP32, KH3 and KH9, were selected for the bioassay to determine their virulence against the banana stem weevil and its larvae, according to the method of Dhawan and Joshi (2017).

Accordingly, stem weevil larvae were collected from the infested stem pieces and segregated into five different stages of larvae. First instar larvae were more susceptible to any microbial agents, and mortality occurred under normal conditions. Third and fourth instar larvae were selected for the bioassay, which can develop into pupal stages during the incubation period. Moreover, third and fourth instar larvae are voracious feeders that cause severe infestation on the banana stem. Therefore, second instar larvae were selected as they were more suitable for the accuracy of bioassay. Pest infestation was higher in the preferable host Poovan (AAB), which was infested between 5 and 7 months after plantation due to volatiles released from the core stem development in banana. Allegrucci et al. (2017) reported that leaf spraying is the most effective inoculation technique that achieved the highest percentage of colonization after 7 days of inoculation. Based on this finding, the four concentrations of conidial suspensions of 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/mL^{-1} were prepared for different



a b

Fig. 1. Bio-efficacy in larval treatment; a) Pre-inoculation b) Post-inoculation.

treatments and characterized against stem weevil larvae under *in vitro* conditions (Fig. 1). The growth rates of conidia increased on the post-inoculated stem disc, and simultaneously the mortality rates also increased when compared with the control. Similarly, the efficacy of EPF increased at high concentration, and the mortality rates of insects also increased (Kocacevik et al., 2016). There was no significant difference in mortality between the treatment and control assays of *O. longicollis* larvae. The same concentration of the fungal inoculum was used for the adult weevil *O. longicollis*.

The bioefficacy of the *B. bassiana* isolate KH3 revealed that the conidial concentration (1×10^8 conidia/mL^{-1}) exhibited minimum larval mortality rates of 46.6% at 6 days of post inoculation (dpi) and a maximum of 100% mortality was achieved on 24 dpi. The least larval mortality of 23.3% was observed at the lowest concentration (1×10^5 conidia/mL^{-1}) on 6 dpi and a maximum mortality of 70% was observed on 24 dpi. However, for larvae treated with the *B. bassiana* isolate KH9, a higher concentration (1×10^8 conidia/mL^{-1}) resulted in a minimum mortality rate of 40% on 6 dpi and a maximum mortality rate of 93.3% on 24 dpi. Similarly, the least concentration (1×10^5 conidia/mL^{-1}) resulted in a minimum mortality rate of 16.6% on 6 dpi and a maximum mortality rate of 66.6% on 24 dpi. In contrast, the other isolates of *B. bassiana,* TP23 and TP32, showed mild reduction in weevil population, and there were no chances of developing biocontrol agents with these fungal isolates. In addition, a higher concentration of the isolates KH3 and KH9 resulted in 93.3% mortality rates on 18 and 24 dpi, respectively (Table .1).

Similarly, the same treatment was conducted for adult weevils and followed by incubation for up to 27 days. The stem weevil treated with the *B. bassiana* isolate KH3 (1×10^8 conidia/mL^{-1}) resulted in a minimum mortality rate of 40% on 9 dpi and a maximum mortality rate of 76.6% on 27 dpi. However, the *B. bassiana* isolate KH9 (1×10^8 conidia/mL^{-1}) resulted in a minimum mortality rate of 30% on 9 dpi and a maximum mortality rate of 76.6% on 27 dpi, compared with other isolates (Table .2). In the control, there was no mortality in all the three replicates. The other isolates of *B.bassiana,* TP23 (1×10^8 conidia/mL^{-1}) and TP32 (1×10^8 conidia/mL^{-1}), resulted in maximum mortality rates of 60% and 66.6%, respectively. Analysis of the results indicated that higher mortality rates were observed from 18 to 27 dpi, and there was a significant difference in the fungal inoculum against *O. longicollis* larvae and adults compared to that at different concentrations ($p > 0.05$).

The present study demonstrated that the larval mortality was higher at a higher concentration (1×10^8 conidia/mL^{-1}) of conidial suspensions. The least larval mortality was observed with the lowest concentration (1×10^5 conidia/mL^{-1}) of conidial suspensions. Oliveira et al. (2002) reported that a higher concentration (1×10^8 conidia/mL^{-1}) of *B. bassiana* treated with red mite achieved a mortality rate of 77%–98%. Murerwa et al. (2014) also observed that *B. bassiana* achieved a pathogenic effect against *M. dirhodum*, and mortality rates of 84%–90% were recorded after 10 dpi. In addition, the *B. bassiana* isolates KH3 and KH9 confirmed that both are efficient strains in suppressing the larval as well as adult population development in *O. longicollis* at 1×10^8 conidia/mL^{-1} concentration compared with other isolates. Several studies have reported the isolation and their pathogenicity of EPFs, including *B. bassiana* and *M. anisopliae,* against the larvae of economic pests (Zimmermann et al., 2013). Moreover, the larvae and adult weevils treated with *B. bassiana* showed a maximum mortality at a higher concentration (1×10^8 conidia/mL^{-1}) at 12–18 days and 18–27 days, respectively. Tefera and Pringle (2004) reported that treatment with *B. bassiana* and *M. anisopliae* against the target pest *Chilo partellus* resulted in 100% mortality after 10 days. Similar

Table 2
Percentage mortality of adult stem weevil *Odoiporus longicollis* treated with aqueous conidial suspensions of *B. bassiana* isolates.

S. No	Isolate	CFU (Conidia/mL)	Days (Percentage Mortality)		
			9	18	27
1	*B.bassiana* isolate TP23	1×10^5	$6.66^a \pm 3.33$	$20.00^{ab} \pm 5.77$	$30.00^b \pm 5.77$
		1×10^6	$16.66^a \pm 3.33$	$33.33^b \pm 3.33$	$33.33^b \pm 3.33$
		1×10^7	$16.66^a \pm 3.33$	$26.66^b \pm 3.33$	$30.00^b \pm 5.77$
		1×10^8	$23.33^a \pm 3.33$	$50.00^b \pm 5.77$	$60.00^b \pm 5.77$
2	*B.bassiana* isolate TP32	1×10^5	$10.00^a \pm 5.77$	$23.33^b \pm 6.66$	$33.33^b \pm 8.81$
		1×10^6	$16.66^a \pm 3.33$	$33.33^b \pm 6.66$	$40.00^b \pm 5.77$
		1×10^7	$26.66^a \pm 8.81$	$33.33^{ab} \pm 6.66$	$46.66^b \pm 8.81$
		1×10^8	$20.00^a \pm 5.77$	$46.66^b \pm 6.66$	$66.66^b \pm 8.81$
3	*B.bassiana* isolate KH3	1×10^5	$16.66^a \pm 3.33$	$33.33^{ab} \pm 3.33$	$43.33^b \pm 8.81$
		1×10^6	$26.66^a \pm 8.81$	$43.33^b \pm 6.66$	$46.66^b \pm 3.33$
		1×10^7	$20.00^a \pm 5.77$	$33.33^{ab} \pm 3.33$	$53.33^b \pm 3.33$
		1×10^8	$40.00^a \pm 5.77$	$66.66^b \pm 3.33$	$76.66^b \pm 6.66$
4	*B.bassiana* isolate KH9	1×10^5	$20.00^a \pm 0.00$	$26.66^b \pm 6.66$	$36.66^b \pm 8.81$
		1×10^6	$26.66^a \pm 3.33$	$40.00^b \pm 5.77$	$40.00^b \pm 6.66$
		1×10^7	$30.00^a \pm 5.77$	$36.66^{ab} \pm 6.66$	$50.00^b \pm 5.77$
		1×10^8	$30.00^a \pm 0.00$	$56.66^b \pm 3.33$	$76.66^b \pm 3.33$

*Three replicates were used per treatment and each replicate contained 10 adult weevils of *O. longicollis*.
* Mean values followed by same letters in the table indicate not significantly different ($p > 0.05$) by LSD.

Table 1
Percentage mortality of *Odoiporus longicollis* larvae (Second instar) treated with aqueous conidial suspensions of *B. bassiana* isolates.

S. No	Isolate	CFU (Conidia/mL)	Days (Percentage Mortality)			
			6	12	18	24
1	*B.bassiana* isolate TP23	1×10^5	$16.66^a \pm 3.33$	$33.33^a \pm 6.66$	$53.33^b \pm 6.66$	$66.66^b \pm 6.66$
		1×10^6	$26.66^a \pm 3.33$	$50.00^{ab} \pm 5.77$	$63.33^{bc} \pm 3.33$	$80.00^c \pm 5.77$
		1×10^7	$36.66^a \pm 8.81$	$53.33^b \pm 6.66$	$66.66^c \pm 3.33$	$83.33^d \pm 3.33$
		1×10^8	$56.66^a \pm 3.33$	$63.33^{ab} \pm 3.33$	$73.33^{bc} \pm 6.66$	$86.66^c \pm 3.33$
2	*B.bassiana* isolate TP32	1×10^5	$6.66^a \pm 3.33$	$20.00^{ab} \pm 5.77$	$36.66^{bc} \pm 8.81$	$53.33^c \pm 3.33$
		1×10^6	$20.00^a \pm 5.77$	$33.33^{ab} \pm 3.33$	$50.00^{bc} \pm 5.77$	$63.33^c \pm 6.66$
		1×10^7	$50.00^a \pm 5.77$	$60.00^a \pm 5.77$	$66.66^{ab} \pm 6.66$	$80.00^b \pm 0.00$
		1×10^8	$56.66^a \pm 3.33$	$66.66^a \pm 3.33$	$80.00^b \pm 5.77$	$86.66^b \pm 3.33$
3	*B.bassiana* isolate KH3	1×10^5	$23.33^a \pm 3.33$	$40.00^{ab} \pm 5.77$	$56.66^{bc} \pm 3.33$	$70.00^c \pm 5.77$
		1×10^6	$26.66^a \pm 3.33$	$56.66^b \pm 3.33$	$73.33^{bc} \pm 8.81$	$86.66^c \pm 6.66$
		1×10^7	$43.33^a \pm 3.33$	$46.66^a \pm 6.66$	$83.33^b \pm 3.33$	$93.33^b \pm 3.33$
		1×10^8	$46.66^a \pm 8.81$	$83.33^b \pm 6.66$	$93.33^b \pm 3.33$	$100.00^b \pm 0.00$
4	*B.bassiana* isolate KH9	1×10^5	$16.66^a \pm 8.81$	$36.66^b \pm 6.66$	$50.00^{bc} \pm 0.00$	$66.66^c \pm 3.33$
		1×10^6	$20.00^a \pm 5.77$	$46.66^b \pm 6.66$	$60.00^{bc} \pm 5.77$	$73.33^c \pm 6.66$
		1×10^7	$36.66^a \pm 3.33$	$56.66^{ab} \pm 6.66$	$73.33^{bc} \pm 8.81$	$83.33^c \pm 8.81$
		1×10^8	$40.00^a \pm 5.77$	$66.66^b \pm 3.33$	$76.66^b \pm 6.66$	$93.33^c \pm 3.33$

*Three replicates were used per treatment and each replicate contained 10 larvae of *O. longicollis*.
* Mean values followed by same letters in the table indicate not significantly different ($p > 0.05$) by LSD.

observations were noticed in the third and fourth instar larvae of *O. longicollis* treated with the *B. bassiana* isolate KH3 on 24 dpi. According to Akbari et al. (2014), the percentage of mortality increased after 12 days of post inoculation. Wright et al. (2005) also reported that the efficacy of EPF against the target insect is dependent on the concentration, and the mortality also increases over time. A similar concept has been reported in aphids by Akmal et al. (2013).

The EPF-treated larvae and adult weevils were collected and stored at 27 °C for assessing fungal colonization on insects from 7 to 12 days. After incubation, genomic DNA was isolated from the infested cadavers and characterized by molecular confirmation. The PCR product of approximately 379 base pairs (bp) was amplified from the genomic DNA of *B. bassiana* strain 18S rRNA gene sequences using the specific primers *BbF*-5′-GGTTGAAATGACGCTCGGAC-3'and *BbR*-5′-TCCCTCTGTTGGTG AACCTG-3′, and then the amplified PCR product was sequenced (Xcelris genomics Private limited, Ahmedabad). Sequences were analyzed by basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). Molecular evolutionary relationship was analyzed through the neighbour joining method by the MEGA software (version 6.0), and bootstrap values were derived from the DNA STAR program. The *B. bassiana isolate* KH3 (MN165867) sequence showed 98% homology with those of *B. bassiana* strain JAU-BbP-2 (MH283877.1), *B. bassiana* isolate 1 (MH233321.1), *B. bassiana* strain Bb153 (KU702661.1) and *B. bassiana* strain NBAIR Bb93 (KU578004.1).

Phylogenetic analysis of nine deduced nucleotide sequences in GenBank was used to compare the relationship between *Beauveria* strain and various species using the MEGA software. This analysis revealed that *Beauveria* sp. was grouped into two major clusters. It was observed that all the sequences obtained in this study, namely those of *B. bassiana* isolate KH3 (MN165867) and *B. bassiana* isolate olrim524 (AY805547.1), were clustered together exclusively in cluster II with a sequence similarity of 99% homology (Fig. 2). Other strains of *Beauveria* belonged to various subspecies and were grouped into cluster I. Moreover, *Beauveria* strain isolate of morphologically related species was clustered into both subclusters I and II. Molecular characterization of the *B. bassiana* isolate KH3 (MN165867) was similar to the Gurlek et al. (2018).

In the present study, the bioassay evaluation indicated that *B. bassiana* could infect *O. longicollis* either by direct contact with conidia or indirectly by intake of nutrients from the banana stem disc. In the majority of cases, the fungus has defense-related enzymes such as chitinase and protease that affect the chitinous components of the cuticle present in the exoskeleton layer of arthropods. This could affect the target insects through extracellular enzymes that degrade the cuticle layer of chitin. Chitinases are a class of ubiquitous proteins that are widely distributed in plants and can be induced in response to a pathogen attack. In addition, chitinases synergically act with proteases to degrade the insect's cuticle (Fang et al., 2009). Batta (2018) and Boomsma et al. (2014) observed that the majority of EPFs infected arthropods by direct penetration of the host through its cuticle and spread with ease. St. Leger et al., (1987) also reported that the primary infection of the fungal conidium contact with larvae is by adhesion process; the spore suspensions penetrate the cuticle by degrading the enzyme chitinase. Similarly, *B. bassiana* secretes extracellular enzymes such as protease and chitinase to degrade the major constituents of the cuticle of larvae and insects. These enzymes play a vital role in pathogenesis and other physiological processes of *B. bassiana* (Wang et al., 2005). Protease has been considered as an important enzyme in the infective process of entomopathogenic fungi (Mustafa and Kaur, 2009), and extracellular protease produced by *B. bassiana* is suspected to be involved in the process of pathogenesis.

Our study findings suggested that the larvae and adult weevils treated with EPF exhibited mortality caused by the enzyme chitinase and protease. Pelizza et al. (2012) reported that the fungal isolate *B. bassiana* producing high levels of chitinase was more pathogenic against *Tropida criscollaris*. Perinotto et al. (2014) also reported that the most virulent strain of EPF resulted in the maximum chitinase activity against tick species of *R. microplus,* and this chitinase can be correlated with the virulence of *B. bassiana*. Accordingly, after 6 days of fungal inoculation, chitinase activity was recorded in the *B. bassiana* isolate KH3 at a maximum of 0.61 U/mL, followed by that in the *B. bassiana*

Table 3
Estimation of chitinase and protease activity from *B. bassiana* isolates against *O. longicollis*.

S.No	*Beauveria bassiana* isolates	Chitinase activity (U/mL)	Protease activity (U/mL)
1	TP23	0.48	0.41
2	TP32	0.43	0.19
3	KH3	0.60	0.39
4	KH9	0.57	0.35

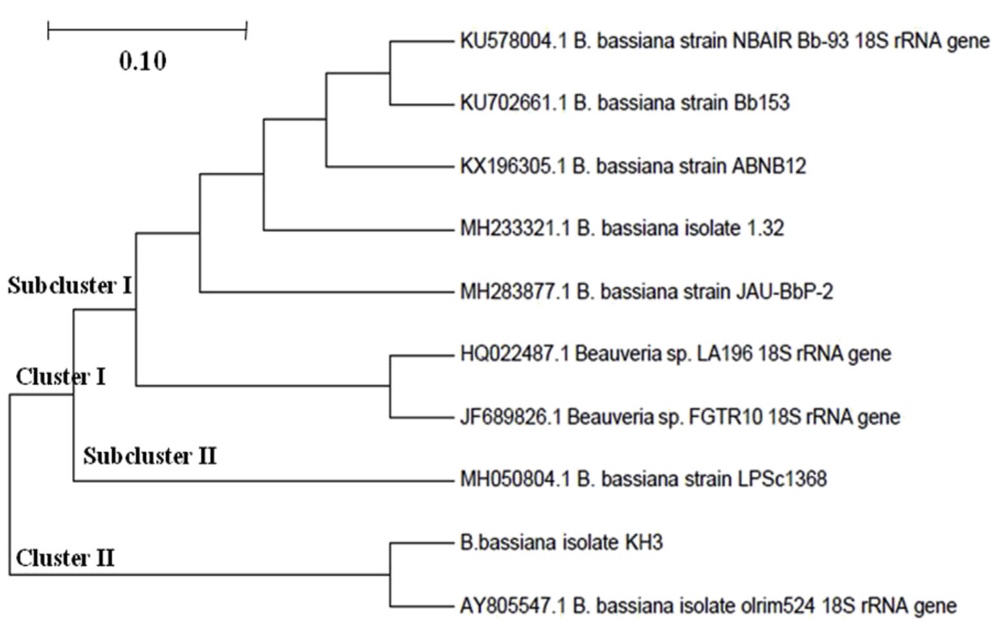


Fig. 2. Phylogenetic tree derived from maximum likelihood analysis of 18S rRNA gene sequences belonging to the *Beauveria* isolates and their closely related species. Bootstrap values based on 1000 replicates are indicated above nodes. Bootstrap values C ≥ 85 are labelled. The scale on the top of the phylogram indicates the degree of dissimilarity.

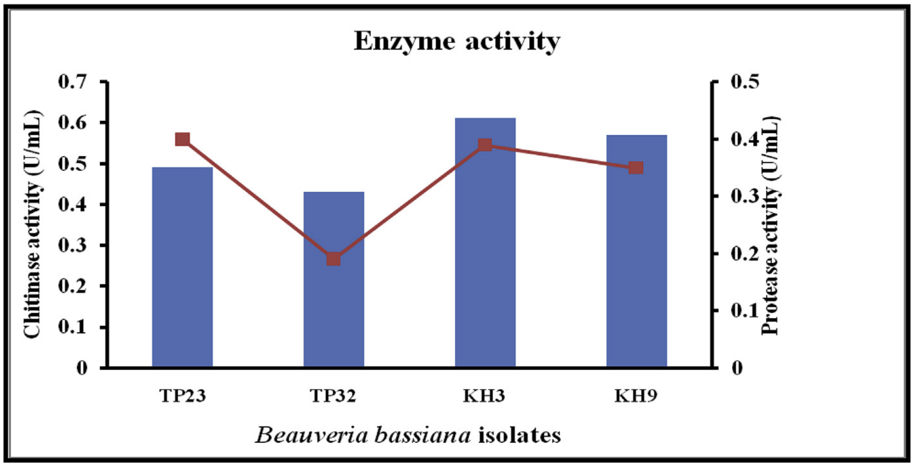


Fig. 3. Effect on chitinase and protease activity at 7 days of post inoculation from different isolates of *Beauveria bassiana*.

isolate KH9 (0.57 U/mL). In contrast, minimum chitinase activities were observed in the *B. bassiana* isolates TP23 and TP32, at 0.49 U/mL and 0.43 U/mL, respectively (Table .3). The maximum chitinase activity (0.61 U/mL) was recorded at 7 days that was significantly better than that of other isolates (Fig. 3). However, the maximum protease activity was observed in the *B. bassiana* isolate TP23, at recorded 0.40 U/mL, followed by that in the *B. bassiana* isolate KH3 (0.39 U/mL) on the 7th day. Both are significantly no difference but, slight differences in the *B. bassiana* isolate KH9 (0.35 U/mL). The least protease activity was recorded at 0.19 U/mL in the *B. bassiana* isolate TP32. Among the four isolates of *B. bassiana*, the highest chitinase activity was recorded in *B. bassiana* KH3, which exhibited the maximum mortality against third and fourth instar larvae of *O. longicollis*.

4. Conclusion

In conclusion, *in vitro* study demonstrates the effectiveness of four different strains of *B. bassiana* (TP23, TP32, KH3 and KH9) against *O. longicollis*, a coleopteran pest of bananas worldwide. The *B. bassiana* isolate KH3 exhibited 93% of larval mortality on 24 dpi and weevil mortality occurs at a maximum of 76.6% mortality on 27 dpi than other isolates. Fungal entomopathogens infect insects, which can produce functional metabolites of defense-related enzymes such as chitinase and protease involved in the degradation of the exoskeleton layer of insects, leading to death. However, further molecular characterization of these fungal isolates and their potential role of defence related enzymes were active against these pests. The entomopathogenic strains of *B. bassiana* isolate KH3 can be successfully used as a biocontrol agent against the banana stem weevil and larvae of *O. longicollis*. The persistence of *B. bassiana* specifically in the corm and stem portion of banana plants provides a promising delivery system for the management of *O. longicollis*. Moreover, resistance cannot be developed against this insect and they can be used for prolonged pest control. *B. bassiana* is an alternative tool for chemical control against the coleopteran insects. This strategy could result in a greater response to reduce stem weevil population and could be used to develop a pheromone trap with *B. bassiana* to control these pests by the lure and kill method.

Conflicts of interest

The authors declare that they have no competing interests.

Authors' contributions

This study is a part of the Ph.D thesis of the first author AA, who carried out the research under the guidance of Assistant Professor SM. GT, BP, and SJ helped during sample collection. The manuscript was drafted by AA. Revisions were made by SM, BP, SJ and GT. All authors have read and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101262.

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