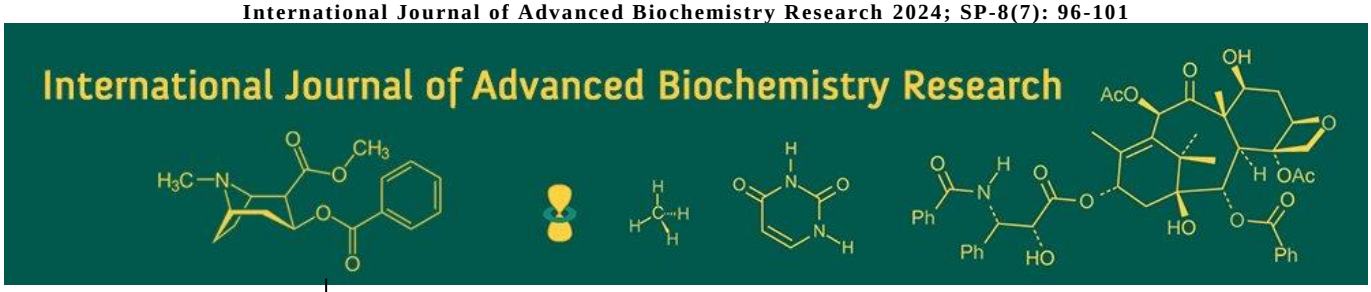

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Beauveria bassiana: A promising biocontrol agent for *Helicoverpa armigera*

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Abstract
Beauveria bassiana is a naturally occurring entomopathogenic fungus that infects and kills a wide range of insect pests. This study aimed to isolate and evaluate the pathogenicity of *Beauveria bassiana* against *Helicoverpa armigera*. *B. bassiana* was isolated from naturally infected insect cadavers collected from major field crop across various locations in Maharashtra. The cadavers were surface sterilized, and infected tissue bits were cultured on sabouraud dextrose yeast agar (SDAY) media. Out of 25 infected cadavers 16 samples showed positive growth of *B. bassiana*. Additionally, soil samples were collected from the rhizosphere of major crops, and *B. bassiana* was isolated using the *Galleria* bait and serial dilution methods. Out of 25 soil samples, 3 showed positive growth. The pathogenicity of twenty *B. bassiana* isolates was tested against third instar larvae of *H. armigera*. Larvae were treated with a spore suspension (1×10^8 spore/ml) and incubated at 25±1 °C and 90% relative humidity. Observations recorded after 10 days indicated significant variability in larval mortality rates among the isolates. Nine isolates exhibited high virulence with mortality rates between 70% and 85%, with Bb-6 isolate showed highest efficacy 85% on 3rd instar larvae of *H. armigera*. These findings highlight the potential of specific *B. bassiana* isolates as biocontrol agents against *H. armigera*. Further field studies are recommended to validate their efficacy.

Keywords: *Beauveria bassiana*, larval mortality, *Galleria* bait method

Introduction
Chickpea pod borer is a significant pest that causes substantial damage to chickpea crops. Finding effective and environmentally friendly methods to manage this pest is crucial for ensuring sustainable agriculture. *Beauveria bassiana* is an entomopathogen that infects a huge variety of insects. It has gained attention as a potential biocontrol agent due to its ability to provide long-term pest control and its minimal impact on non-target organisms and the environment. Understanding the characteristics of different *Beauveria bassiana* isolates will help to determine their efficacy and suitability for controlling the chickpea pod borer.

Fungal insect diseases are common, widespread and often decimate their host population. Entomopathogenic fungi (EPF) infect by breaching the insect host cuticle. Fungi secrete extracellular enzymes proteases, chitinases and lipases to degrade the major constituents of the cuticle (i.e. protein, chitin and lipids) and allow hyphal penetration (Cho *et al.*, 2006) [5]. Most of the studies of the fungi have been based on isolation from insect cadavers or soil (Brownbridge *et al*., 2010) [3].

Susceptible insect host and selective media have been utilized to the isolation of entomopathogenic fungi form soil (Shimazu and Sato, 1996) [20]. Soil is also the main source of fungal entomopathogens, and isolation of these organisms involves soil sampling since that is their natural habitat. Soil factors (temperature, pH or organic content, relative moisture or mineral, organic or biotic components) can affect fungal persistence and activity (Riba *et al.*, 1991; Charnley, 1997) [17, 4]. Use of insect bait is a very sensitive detection method where EPF can be selectively isolated. Baiting soil samples with *G. mellonella* larvae is a widely applied tool to screen for indigenous fungal species. However, some insect species may be selected for specific fungal pathogens and difficult to quantify inoculum levels. Although pathogenicity of the isolated fungi would be evaluated on target insect, by contrast, selective media have some advantages for the mass collection of positive EPF and quantitative data.

The objective of the present study was to isolate and identify the *Beauveria bassiana* as biocontrol agents, and to test their efficacy of the isolated native strains against chickpea pod borer (*H. armigera*) under laboratory conditions.

Materials and Methods

A. Collection and isolations of *Beauveria bassiana* from naturally mycosed insect cadavers

Beauveria bassiana was isolated from infected insect cadaver which were collected from different geographic locations of Maharashtra. The sample (dead larvae) showing typical whitish fungal massive growth on body of larvae in field crop. Collect infected insect cadavers using sterile tools like forceps or brushes to avoid contamination. Each cadaver was placed in a sterile container labelled with location and insect species information to maintain proper records.

The collected cadavers were surface sterilized with 0.1 percent mercuric chloride solution ($HgCl_2$) for five seconds and then rinsed three times with sterile distilled water to remove the traces of mercuric chloride. They were blot dried and placed on sterilized filter paper to absorb the excess water. The surface sterilized specimens were cut into small bits in a sterile petri plate and a bit of infected tissue were aseptically transferred to a sterile petri plate containing sabouraud dextrose yeast agar (SDAY) media and kept in BOD at 25 °C temperature and 90±5 percent relative humidity (RH). All above operations were carried out in sterilized condition (under laminar air flow unit). Enough growth of fungus was observed in petri plates on seventh day. The small portions of the appeared growth around bits were transferred to SDAY slant for further studies. Identification of the selected isolates was initially confirmed based on morphological studies (Debnath, 2015) [6].

B. Collection and isolation of *Beauveria bassiana* from soil

a) Soil sample collection and isolation of fungus by serial dilution

The soil sample were collected near the rhizosphere region of major crops including cotton, soybean, gram and castor etc. The collection was done from different geographic locations.

One gram of a rhizospheric soil sample and 9 ml of sterilized distilled water were mixed in test tubes and vortexed for 10 minutes to obtain a homogenous solution. A serial dilution from 10^{-1} to 10^{-7} was prepared for each soil sample in order to isolate a single fungal colony. One ml of suspension was spread on selective medium SDAY (sabouraud dextrose yeast agar) and incubated at 25±2^{0}C for 2 weeks. After the completion of the incubation period, developing single colonies were transferred to other SDAY plates to get pure culture (Gurlek *et al.*, 2018) [8].

b) Isolation of *B. bassiana* from soil by *Galleria* bait method

1. Rearing of *Galleria mellonella* for isolation of *Beauveria bassiana*

The moths of *Galleria mellonela* were procured from Department of Entomology, Akola. Adult greater wax moths from stock cultures were placed into one litre mason jars, where commonly mating of moths took place. Also, folded sheets of wax paper held together with paper clips were placed into the jars on which the moths deposited their eggs. The eggs were removed from the jars and used to initiate the next generation of wax moths. 115 g of the medium was placed into 500 ml sterilized mason jar, wax moth eggs were added to the surface of the medium (Metwally *et al.*, 2012) [11] and the jar was sealed with aluminum foil.

The jars were incubated in the dark at 28.5 °C. Second instar larvae are readily observed in 7-10 days by the extensive tunnelling at the periphery of the medium. The foil is then removed and replaced with wire screening covered with cheesecloth. This allows adequate gaseous exchange and prevents excessive moisture build-up. The larvae remain in the medium for an additional 8-10 days. By this time, the medium is reduced to a fine, dusty, dark powder and the larvae are readily separated from it by screening. The larvae were transferred into a one litre mason jar to which 175 g of the medium were added. Within 4-5 days, fourth instar larvae become available and in 7-8 additional days, fifth instars were available. If pupae are desired another 7-10 days are required in incubation.

Those larvae which are not in the prepupal stage in the mason jar may be transferred to fresh medium (100 g) or either in another jar. After adult emergence same procedure was repeated (Mohamed and Coppel, 1983) [13].

2. *Galleria* bait method for isolation of test fungus

The soil samples at the depth of 10 cm were collected from different fields and locations, sieved and filled in small disinfected plastics boxes. However, it was important that the larvae placed into these soil samples were forced to stay inside it and to move in the soil. This was achieved by turning around the test boxes from time to time or by filling them totally with soil up to the lid.

Larvae of greater wax moth were used for the detection of fungal entomopathogens. Depending on the size of the larvae and the amount of soil, 5, 10 or 15 larvae of *Galleria* were added to each of the moistened soil samples. The boxes were stored at room temperature and inspections were made after 1 week and again after 2 weeks. Finally, all specimens with signs and symptom of disease on larvae were placed in a moist chamber for outgrowth of fungi then mycelial growth transferred to petri plate containing SDAY media and kept at 25 ± 2ºC and 95 percent relative humidity (Zimmermann, 1986) [24].

C. Pathogenicity of *B. bassiana* against 3rd larval instars of *Helicoverpa armigera*

1. Collection and rearing of the test insect *Helicoverpa armigera* in the laboratory

Helicoverpa armigera (Hubner) was used as test insect for assessing the pathogenicity of *B. bassiana*. The larva of test insect was collected from different field crop sown in the field during Kharif and Rabi and reared in the Plant Pathology Section, COA, Nagpur.

To avoid the cannibalism, larvae were individually reared in the plastic bottles and provided water soaked chickpea seeds daily as a food. Full grown larvae about to pupate were transferred into gogglets containing sterilized soil and the gogglets were covered with muslin cloth.

The emerged ·adults were collected carefully from the gogglets and released in mating chamber. In each mating chamber five pairs of adults were released and folded black

papers were provided for egg laying. Ten percent aqueous honey solution was provided as a food for the adults with the help of cotton swab hanging in mating chamber. The mating chamber kept in the dark place during mating period as the insect is nocturnal in habit. The folded black paper with egg were removed every day and placed in another petri dish for hatching.

After hatching, the larvae were separated and kept individually in the sterilized plastic bottles and were supplied with fresh water-soaked chickpea seeds. Thus, the hatch wise larval population were reared in the laboratory to 3rd instar larvae for pathogenicity studies.

2. Preparation of spore suspension

The conidia were harvested from the two-week-old cultures of *B. bassiana* by washing the surface of the plates with 75-100 ml of sterile distilled water containing 0.02 percent tween-20 (Rombach *et al.* 1986) [18]. Spore suspensions of 1×10^8 spores per ml were standardized after assessing the number of spores in the suspension with an improved Neubauer hemocytometer by adopting standard protocols.

3. Pathogenicity study

All twenty isolates were used for testing the pathogenicity on 3rd instars larvae of *Helicoverpa armigera.* The 1×10^8 spore/ml suspension was used to study the pathogenicity of *B. bassiana* against third instar of *Helicoverpa armigera* by direct spray of spore suspension on the body of the insect. Two ml solution was treated topically (Gopalkrishnan and Narayanan, 1988) [9]. Before spraying of spore suspension on insect body 3rd instars larvae were treated with 1% sodium hypochloride solution and rinsed gently twice with distilled water.

The excess water was removed with blotting paper. The treated larvae of *H. armigera* were taken separately in separate plastic bottles due to cannibalism. Treated larva was kept in bottle having water-soaked chickpea seed. After 24 hours the first lot of food was given to each larva. Plastic bottles with larvae were incubated at constant temperature 25 ± 1 °C with 90 percent RH. Four such replication and five larvae per replications were maintained for pathogenicity. Four lots of five larvae were sprayed with two ml of sterilized distilled water served as control. Observations were recorded after 10 DAI (Prasad and Syed, 2010) [15]. The percentage larval mortality due to mycosis was calculated.

Results and Discussion

A. Collection and isolation of *B. bassiana* from infected cadavers and soil

a) Collection of infected cadavers and soil samples

Beauveria bassiana was isolated from naturally infected insect larvae, the dense coating of white fungus diseased cadavers was also collected from the various field locations of Maharashtra. The infected larvae brought from field were properly maintained for further studies. The standard culture of *Beauveria bassiana* (BB-1) were procured from Plant Pathology Section, College of Agriculture, Nagpur.

Isolation of *Beauveria bassiana* was also performed from the soil using the *Galleria* bait and serial dilution method. A soil survey was conducted at different locations which covering major crop area in Maharashtra and nearby places. Total 25 soil samples were collected.

b) Isolation of *B. bassiana* from infected cadavers

The fungus *B. bassiana* was isolated from infected larvae, collected from different locations by infected bit and streak plate method on PDA medium. Among the 25 infected cadavers collected, 16 cadavers exhibited growth of the *B. bassiana* fungus. The isolated fungus brought in pure culture by hyphal tip and single spore method. The colonies of *B. bassiana* grew relatively slow. They appeared wooly as well as powdery, whitish in colour. Septate, smooth, hyaline aerial hyphae was observed. Submerged hyphae were similarly structured. Conidiogenous cells were found in dense clusters or whorls which arise from short swollen stalk cells. The culture was identified as *Beauveria bassiana* (Bals.) Vuill through molecular basis and manuals microscopic observations. Morphological identification of the fungi can be done based on shape and size of spores in slide cultures. Conidia of *B. bassiana* were globose to subglobose. The conidiogenous structures formed sympodial to whorled dense clusters with flask-shaped to short-globose conidiogenous cells. Succession of hyaline, one-celled, holoblastic conidia were borne on a progressively elongating sympodially branched denticulate rachis. The denticles are equally wide as the rachis is a peculiar characteristic of *Beauveria spp*. The identified cultures were maintained in the PDA slant at 27 ± 2 °C temperature and used for further study.

c) Isolation of *B. bassiana* from Soil Samples

i) Isolation of *B. bassiana* from Soil Samples using serial dilution method

Isolation of *B. bassiana* from soil through by using the serial dilution method. Soil samples were inoculated directly onto the DOC2 media through serial dilutions. Soil samples were diluted upto 10^{-7}. These plates were incubated and observed white mycelial growth on the Petri plates within a span of ten days. This white mycelial growth observed plates were separated from the others and those fungal discs were inoculated onto the SDAY media plated Petri plates for further growth. Using serial dilution method, two fungal isolates from the Nagpur and Buldhana regions were identified as *Beauveria bassiana*. According to Table 2. these fungal isolates were designated as Bb-5 and Bb-14.

Table 1: *Beauveria bassiana* isolated from infected cadavers

Sr. No.	Common name of insect	Scientific name	Place of collection	Crop	Colour colour	Isolate code
1.	American Cotton bolloworm	*Helicoverpa armigera*	Kondhali	Cotton	Yellowish white	Bb-2
2.	American Cotton bolloworm	*Helicoverpa armigera*	Nagpur	Cotton	White	Bb-4
3.	Gram pod borer	*Helicoverpa armigera*	Chandrapur	Chickpea	-	-
4.	Tobacco caterpillar	*Spodoptera litura*	Darwha	Soybean	-	-
5.	Tobacco caterpillar	*Spodoptera litura*	Katol	Soybean	Yellowish white	Bb-6
6.	Fall armyworm	*Spodoptera frugiperda*	Deori	Maize	Yellowish white	Bb-7
7.	Castor semilooper	*Achaea janata*	Tumsar	Castor	Yellowish White	Bb-8
8.	Gram pod borer	*Helicoverpa armigera*	Manwath	Chickpea	-	-
9.	Tobacco caterpillar	*Spodoptera litura*	Anjangaon Surji	Soybean	White	Bb-9
10.	American cotton bolloworm	*Helicoverpa armigera*	Akot	Cotton	White	Bb-10
11.	Tobacco caterpillar	*Spodoptera litura*	Karanja	Soybean	-	-
12.	Fall armyworm	Spodoptera frugiperda	Hinganghat	Maize	White	Bb-11
13.	Gram pod borer	*Helicoverpa armigera*	Warora	Groundnut	White	Bb-12
14.	Gram pod borer	*Helicoverpa armigera*	Khamgaon	Chickpea	-	-
15.	Gram pod borer	*Helicoverpa armigera*	Mangrulpir	Chickpea	White	Bb-13
16.	Okra fruit borer	*Earias insulana*	Udgir	Okra	-	-
17.	American Cotton bolloworm	*Helicoverpa armigera*	Nashik	Cotton	White	Bb-15
18.	American Cotton bolloworm	*Helicoverpa armigera*	Kalmbeshwar	Cotton	White	Bb-16
19	Gram pod borer	*Helicoverpa armigera*	Bhusawal	Chickpea	White	Bb-17
20.	Tobacco caterpillar	*Spodoptera litura*	Dapoli	Soybean	-	-
21.	Gram pod borer	*Helicoverpa armigera*	Daryapur	Chickpea	White	Bb-18
22.	Tobacco caterpillar	*Spodoptera litura*	Lakhni	Soybean	-	-
23.	American Cotton bolloworm	*Helicoverpa armigera*	Murtijapur	Cotton	White	Bb-19
24.	Gram pod borer	*Helicoverpa armigera*	Arvi	Chickpea	White	Bb-20
25.	Tobacco caterpillar	*Spodoptera litura*	Umarkhed	Soybean	-	-

Table 2: *Beauveria bassiana* isolated from soil by serial dilution method

Sr. No	Place of collection of soil	Crop rhizosphere	Colony colour	Isolate code
1.	Karanja	Chickpea	White	Bb-5
2.	Umarkhed	Cotton	White	Bb-14

ii) Isolation of *B. bassiana* from Soil Samples using *Galleria* bait method

Galleria bait method was used to isolate the *B. bassiana* from rhizospheric soil samples (Zimmermann, 1986) [24]. About 25 soil samples were collected in the surveyed fields of Maharashtra and nearby areas were used for *Galleria* bait method. One isolate of *B. bassiana* were obtained by using this method. The isolates were designated as Bb-3 as listed in table 3.

Table 3: *Beauveria bassiana* isolated from soil by *Galleria* bait method

Sr. No	Place of collection of soil	Crop rhizosphere	Colony colour	Isolate code
1.	Chandrapur	Chickpea	White	Bb-3

The present research findings were similar to Sookar *et al.* (2008) who examined 224 soil samples from 19 locations in three climatic zones of Mauritius for the entomopathogenic fungi. Soil samples were baited with the wax moth larvae and they were able to isolate *B. bassiana* as a major entomopathogen. Vijayavani *et al.* (2009) [23] also reported *Beauveria bassiana* fungal isolates were originally isolated from the *Spodoptera litura,* collected in cotton growing regions of Guntur district, Andhra Pradesh. Safavi (2010) [19], *Beauveria bassiana* was isolated from soil using a DOC2 selective medium. Conidiophores of whorls and dense clusters of short conidiophorous cells with one-celled spherical conidia were the distinguishing feature of this isolate

Similar results were obtained by Moorthi *et al*. (2011) [14] from tobacco, cotton, and chili crops grown in various parts of the Tamil Nadu districts of Dindigul, Madurai, and Bb02, Bb09, and Bb10. Similarly, Patel (2015) [16] conducted intensive survey of forest areas, fields having different crops and plantation area belonging to Dindori, Mandla and Jabalpur districts of Madhya Pradesh (India). They collected the soil samples to isolate entomopathogenic fungi thought baiting *Galleria mellonella.* Six isolates of entomopathogenic fungi from sixty soil samples were isolated. However *Beauveria bassiana* were found to be the potential entomopathogenic fungi. Gurlek *et al*. (2018) [8] used the serial dilution technique to isolate *B. bassiana* from soil. *Beauveria* and *Metarhizium spp*. were isolated from 90 soil samples obtained from walnut farms using selective media.

With the present study, it can be confirmed that infected cadavers are the good source for isolation of *B. bassiana*, as compared to soil. Twenty different isolates of *B. bassiana* were isolated during the cropping season 2021-2022 out of which sixteen isolates were isolated from naturally infected cadavers and three by soil using *Galleria* bait and serial dilution method.

C. Pathogenicity studies

Pathogenic abilities of different isolates of *Beauveria bassiana* at 1×10^8 spore/ml concentration were assessed against third instar larvae of *H. armigera*. The larval mortality was recorded upto ten days.

1. Pathogenicity against third instar larvae of *H. armigera*

The pathogencity studies against third instar larvae of *H. armigera* showed varied degree of percent mortality with respect to *B. bassiana* isolates. The percent larval mortality of ranged between 5.00 to 85.00% for different isolates. Among all 20 isolates 9 isolates were most virulent which

recorded larval mortality ranged between 70.00 to 85.00 percent against third instar of *Helicoverpa armigera*. (Table 4). Maximum mortality was recorded by the isolate Bb-6 exhibiting 85.00 percent followed by Bb-7 (80.00%), Bb-1, Bb-8, Bb-17, Bb-18 (75%) and Bb-4, Bb-14, Bb-20 (70%) mortality respectively. The lowest mortality was noticed in the isolate Bb-2 (5.00%). No larval mortality was observed in Bb-3, Bb-11 and control.

Table 4: Virulence of different isolates of *Beauveria bassiana* to 3rd instar larvae of *Helicoverpa armigera* at 10 days after treatment

Sr. No.	Source / Host	Isolates strain 1×10^8 ml^{-1}	Number of larvae	Dead larvae	Cumulative mortality (%)
1.	Test isolate	Bb-1	20	15	75.00
2.	Cotton	Bb-2	20	01	5.00
3.	Soil	Bb-3	20	00	0.00
4.	Cotton	Bb-4	20	14	70.00
5.	Soil	Bb-5	20	2	10.00
6.	Soybean	Bb-6	20	17	85.00
7.	Maize	Bb-7	20	16	80.00
8.	Castor	Bb-8	20	15	75.00
9.	Soybean	Bb-9	20	4	20.00
10.	Cotton	Bb-10	20	3	15.00
11.	Maize	Bb-11	20	00	0.00
12.	Groundnut	Bb-12	20	04	20.00
13.	Chickpea	Bb-13	20	02	10.00
14.	Soil	Bb-14	20	14	70.00
15.	Cotton	Bb-15	20	75	15.00
16.	Cotton	Bb-16	20	05	25.00
17.	Chickpea	Bb-17	20	15	75.00
18.	Chickpea	Bb-18	20	15	75.00
19.	Cotton	Bb-19	20	06	30.00
20.	Chickpea	Bb-20	20	14	70.00

Similar observations were recorded by Vijayavani *et al*. (2009) [23], the strains SBT#11 and SBT#16 of *B. bassiana* caused 100% mortality at 1×10^8 conidia ml^{-1} on *S. litura* larvae under laboratory condition. Ummidi *et al*. (2013) [22], all the strains tested in the laboratory bioassay at concentration of 1×10^8 conidia ml^{-1} were found to be pathogenic to 2nd instar larvae. LT_{50} values for 2nd instar *S. litura* larvae varied from 3.93 to 6.4 days. *M. anisopliae* strains M20 and *B. bassiana* B55 originally isolated from *Nilaparvata lugens*, Homoptera demonstrated least LT_{50} value of 3.93 and 4.13 days respectively. Kirubakaran *et al*. (2014), the virulence of seven fungal isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to 3rd instar *C. medinalis* was tested in initial screening bioassay by application of conidia at the concentration of 1×10^8 conidia/ml. Among the tested *B. bassiana*, MTCC7690 produced 83% mortality and MTCC4104 of *M. anisopliae* produced 79% mortality. Aker and Tuncer (2016) [1], studied the virulence efficacy of *B. bassiana* against 4th instar larvae of *H. cunea*. 1×10^8 conidial suspension of *B. bassiana* were the most efficacious in controlling larvae of *H. cunea* with 100% mortality after 9 DAT as compared to 1×10^6 conidial suspension. Fite *et al*. (2019) [7], Isolates of both *B. bassiana* and *M. anisopliae* caused 20–91% mortality against 3rd instar larvae of *H. armigera* larvae at 10^8 conidia/ml.

Conclusion

In this study, *Beauveria bassiana* was successfully isolated from naturally infected insect larvae and soil samples from various field locations in Maharashtra. The isolates were identified through morphological characteristics and maintained in pure cultures. Among the 25 infected cadavers collected, 16 cadavers and from the 25 soil samples collected, 3 samples yielded positive growth of *B. bassiana* using the *Galleria* bait and serial dilution methods. The pathogenicity tests against third instar larvae of *Helicoverpa armigera* revealed significant variability in larval mortality rates among the *B. bassiana* isolates. Out of 20 isolates tested, 9 isolates exhibited high virulence with larval mortality rates ranging from 70% to 85%. The isolate Bb-6 demonstrated the highest efficacy with an 85% mortality rate, followed by Bb-7 at 80%, and Bb-1, Bb-8, Bb-17, and Bb-18 each at 75%. In contrast, the isolate Bb-2 showed the lowest mortality rate at 5%, and no mortality was observed in Bb-3, Bb-11, and the control. These findings indicate that several *B. bassiana* isolates possess significant potential for biological control of *H. armigera*, offering an environmentally friendly alternative to chemical insecticides.

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