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Assessing the defense role of phenoloxidase in *Spodoptera litura* (Fab.) challenged with entomopathogenic fungi

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ABSTRACT: The study was conducted to evaluate the bioefficacy of two entomopathogenic fungi (EPFs), *Metarhizium anisopliae* (Metchnikoff) Sorok (Ma-NCIPM) and *Beauveria bassiana* (Balsamo) Vuill. (Bb-NCIPM), and the effect of these EPFs on the host defense related enzyme, phenoloxidase (PO) in *Spodoptera litura* (Fabricius) using two different bioassay methods, namely larval immersion and topical spray. The results revealed that the bioefficacy of Ma-NCIPM and Bb-NCIPM at 1×10^8 spores/ml against the third instar larvae of *S. litura* significantly differed. Ma-NCIPM recorded the highest ($68\pm0.49\%$) mortality, and Bb-NCIPM showed the lowest ($56\pm0.9\%$) mortality with larval immersion method at 168 h of application. The highest mortality was reported by Ma-NCIPM ($98\pm2.62\%$), followed by the Bb-NCIPM ($70\pm0.32\%$) with topical spray method at 168 h after treatment. Compared to control, the LT_{50} of Ma-NCIPM and Bb-NCIPM diminished by 3.41 and 1.03 folds, respectively, through larval immersion method. However, through topical spray method, LT_{50} of Ma-NCIPM and Bb-NCIPM diminished by 4.05 and 1.2 folds, respectively. The level of PO specific activity in the host, *S. litura* larvae also fluctuated by the exposure of Ma-NCIPM and Bb-NCIPM compared to the control.

KEYWORDS: *Spodoptera litura*, entomopathogenic fungi, bioassay methods, phenol-oxidase enzyme, median lethal time

INTRODUCTION

Chemical pesticides were designed to protect agri-horticultural crops and to combat negative effects wielded by different dreaded pests, resulting in enhancing the yield and efficiency of crops. These molecules are very toxic and able to harm non-targeted organisms [1]. According to a recent report, around 385 million agricultural workers fell ill globally with acute pesticide poisoning every year [2]. On the other hand, dependence on chemical insecticides to manage insect pest leads to resurgence of pests and also the environment hazards. Therefore, it is necessary to adopt a sustainable approach to overcome the unfavourable effects of synthetic pesticides. The microbial entomopathogens offers a good, sustainable lead in this direction. Several studies have demonstrated the potential use of microbial entomopathogens as a good alternative to chemical insecticides [3]. Microbial entomopathogens are a group of living organisms which are innately capable of killing and controlling the insect pest population. This category of bio-pesticides includes entomopathogenic viruses (Nuclear polyhedrosis viruses (NPVs)), entomopathogenic bacteria (*Bacillus thuringiensis*, Berliner), entomopathogenic fungi (EPFs) (*Metarhizium anisopliae* (Metsch.) Sorok), *Beauveria bassiana* (Bals.) Vuill, *Isaria fumosorosea* (Wize), and entomopathogenic nematodes (*Steinernema carpocapsae* Weiser). EPFs play a vital role in the biological control of insect pests because of their vast host range, specific mode of action, relatively low cost, and lower risk to ecosystem [4]. Over 700 different fungal species from 90 different genera have been discovered after being isolated from different insect species. As active ingredients in mycoinsecticides, at least 12 fungal species or subspecies (varieties) have been employed [5]. In order to combat a variety of agricultural insect pests, 176 mycopesticides have been devised globally employing *B. bassiana*, *M. anisopliae*, *I. fumosorosea*, and *Beauveria brongniartii*, Saccardo [6]. Out of total 10 mycopesticides, 7 from *B. bassiana*, 2 from *M. anisopliae*, and one combination of 2 or more species have been employed to suppress noctuid pests commercially [7]. EPFs such as *B. bassiana* and *M. anisopliae* have been reported to infect a variety of economically significant insect pests and thus considered promising biological control agents [8, 9]. Differences in host specificity and virulence have been reported among their strains [10]. The primary pathway to infect host is *via* host cuticle, which is achieved by conidia adherence to the integument, sprouting in hemocoel, after breaching the external integument [11]. The mycelia expand and travel throughout the entire host body after accessing the hemocoel, where they eventually develop into hyphae and release blastospores. Host mortality will be the result of a combination of bio-active toxins released by EPFs, physical blood flow obstructions, nutrient loss, and tissue mechanical damage [12]. The insect immune system always works to eliminate the pathogens

while the attack process is in progress. The insect immune system and the detoxification enzymes play a significant role in defending them from the damaging effects caused by pathogens and their bioactive toxins [13]. The phenoloxidase enzymes (PO) in insects are specifically associated with 3 crucial physiological processes that are biochemically substantial: the sclerotization of the insect cuticle, wound healing, and the encapsulation of melanin (a defensive substance) after exposure to foreign molecules. As a result, it is believed that PO plays a number of significant roles in the development of insects and their innate immunity to pathogens [14–16].

The tobacco caterpillar, *Spodoptera litura* (Fab.), is a notorious polyphagous insect pest with cosmopolitan presence which can lead to complete defoliation of crops during severe infestation [17]. Throughout the season, farmers and plant protectionists have major concerns about losses inflicted by this insect infestation. EPFs like *M. anisopliae*, *B. bassiana*, and *Lecanicillium lecanii* (Zimm.) Zare and Gams are innately capable of controlling noctuid insect pests [18]. Bioefficacy of the *M. anisopliae* (Ma-NCIPM: a strain from *Locusta migratoria* L. collected from local field) and *B. bassiana* (Bb-NCIPM: a strain from *Helicoverpa armigera* Hub. collected from the cotton field, Panjab) was examined. Larval immersion and topical spray exposure methods were followed. The activity of PO enzyme was also analyzed as one of the parameters of humoral immunity response of host insect against the EPFs because this enzyme has been reported to play a crucial role in the detoxification mechanisms against fungal infections. The relationship between bioassay methods and virulence is also discussed.

MATERIALS AND METHODS

The study was conducted in the Biological control laboratory, Division of Entomology, Indian Council of Agriculture Research-Indian Agricultural Research Institute (ICAR-IARI) Pusa, New Delhi. Tobacco caterpillar, *S. litura* culture was maintained at 25 ± 2 °C and 65 ± 5% RH with a 16 h of photoperiod in growth chamber throughout the course of investigation. Bioefficacy experiments of *M. anisopliae*, Ma-NCIPM and *B. bassiana*, Bb-NCIPM were carried out on the 7-day-old freshly moulted third instar larvae of *S. litura* in laboratory conditions. All the bioassay trials were performed in UV-radiated laminar air flow chamber (Ultra Scientific Instruments Co. (H-761) Delhi, India).

Insect culture and maintenance

Nucleus culture of tobacco caterpillar, *S. litura* was collected from the cabbage field (*Brassica oleracea* Capitata), Division of Floriculture, ICAR-IARI, Pusa New Delhi, and subsequent generations were then used for the experimental trials. The field collected larvae were reared on fresh castor (*Ricinus communis*, Linn.) leaves individually in small sized rearing dish. Pupae were kept separately, and emerged adults were shifted to mating jar in 1:1 (male:female) ratio and fed on 10% honey solution fortified with vitamin E. Larvae were reared on chickpea based artificial diet, as per the previous report [19]. In order to retain reproductive competence after 4–5 generations, the laboratory and field population of *S. litura* was mixed.

Fungal strains

EPFs, *M. anisopliae* (Ma-NCIPM) and *B. bassiana* (Bb-NCIPM), were procured from the ICAR-National Centre for Integrated Pest Management (NCIPM), Pusa New Delhi, and multiplied in the Biological control laboratory, Division of Entomology ICAR-IARI, New Delhi, for conducting bioassay experiments on *S. litura*. For mass production of Ma-NCIPM, potato dextrose agar (PDA) was used at 27 ± 2 °C and 80 ± 5% RH in complete darkness, while Sabouraud dextrose agar fortified with 0.5% yeast extract (SDAY) was used to subculture the Bb-NCIPM. After 15–25 days of incubation, the fungal spores were collected from the surface of the culture medium using a sterile blade (spatula).

After preparing the fungal suspensions, the spore concentration was estimated employing an improved Neubauer hemocytometer (Marienfeld, Cologne, Germany) under a compound microscope at 400× magnification, and spore density was adjusted to 1×10^8 spores/ml with 0.02% Tween 80.

Bioassay methods

Larval immersion

Pre-starved (3 to 4 h) third instar larvae were used for the experimentation. Fifty *S. litura* larvae per treatment (10 larvae per replication) were used. Ten third instar larvae were immersed into spore suspension (1×10^8 spores/ml) of Ma-NCIPM and Bb-NCIPM for 5–10 s and allowed to dry for 2 min on a sterile filter paper under laminar air flow chamber [20]. In brief, 500 µl of spore suspension (2.5 ml for 50 larvae) was used to immerse 10 *S. litura* larvae. The same procedure was followed for the control group (sterile distil water containing 0.02% Tween 80 instead of conidial suspension). After treatment, treated larvae were kept separately in rearing dish, and fresh, untreated artificial diet was provided as food. Mortality of the larvae was measured up to 168 h after treatment at each 24 h interval or up to adult emergence.

Topical spray

In topical spray methodology, 10 third instar *S. litura* larvae were placed on fungal (Ma-NCIPM or Bb-NCIPM) coated castor leaf disc (5 cm θ and 10 larvae per leaf disc). After that, 1 ml respective fungal suspension of 1×10^8 spores/ml concentration was sprayed directly using an atomizer [21, 22]. Total 5 replications with 10 larvae per replication were kept.

The control batch of larvae was treated with 500 µl of sterile distil water containing 0.02% Tween 80 only. Fresh, untreated artificial diet was provided as food after 24 h of fungal exposure. Mortality and other fungal infection associated symptoms were noted at every 24 h interval up to 168 h or adult emergence.

At every 24 h interval up to 168 h after treatment, the number of dead larvae was counted, and mortality rate of the EPF infected larvae was calculated by summing the number of dead larvae across all exposed replicates and then expressing this as a percentage of the total number of exposed larvae. Mortality rate = number of dead larvae/total number of larvae × 100. Percent mortality was correct using following formula:

$$\text{Corrected percent mortality} = \left(1 - \frac{\text{Ta} \times \text{Cb}}{\text{Tb} \times \text{Ca}}\right) \times 100$$

where Ta = number of larvae after treatment, Tb = number of larvae before treatment, Ca = number of larvae in control after treatment, and Cb = number of larvae in control before treatment.

The results of the bioefficacy experiments were subjected to probit analysis, and the median lethal time (LT_{50}) was calculated from the mortality data over the time. LT_{50} is the period of exposure to a pathogenic stimulus which will produce death in half the test insects. It is time dependent bioassay procedure.

To confirm Koch's postulates, dead and diseased cadavers were kept in Petri plate lined with damp sterile filter paper to facilitate mycosis and sporulation. Only phenotypic features (conidia shape and size) were used to study the morphological characteristics of the fungal strain, and fungal colonies (colony growth pattern and texture) were noted for identification (Fig. S1) [23, 24].

Estimation of PO enzyme activity

PO activity was estimated by the protocol depicted from a previous report [25] with some modifications. The PO enzymatic assay was carried out by *S. litura* larvae collected at 12, 24, 72, 120, 144, and 168 h of Ma-NCIPM and Bb-NCIPM exposure. There were 3 replicates under each treatment including control for every time interval. The whole bodies of treated larvae (head removed) for each treatment were homogenized for 2–5 min with a handheld motorized homogenizer (using teflon cordless pestle) in 5-fold volume ice-cold 0.1 M sodium phosphate buffer (PBS, pH 7.4). The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant under fat layer was shifted to new Eppendorf tubes and again centrifuged at 12,000 rpm for 10 min at 4 °C. Obtained supernatant was collected and used to assess the PO enzyme activity. The enzymatic activity was measured at 490 nm for 3 min at each 15 sec interval, and L-Dopa (L-3, 4-dihyroxyphenyl alanine) was used as the reaction substrate. The PO specific activity was expressed as µmol/min/mg of protein. All procedures related to enzymatic assay were carried out at 4 °C.

Protein estimation

The total protein content of the samples (enzyme) was measured according to the method described in a previous report [26] using bovine serum albumin as standard at 595 nm wavelength.

Data analysis

The experiment was conducted in a completely randomized design (CRD). Each bioassay experiment was repeated thrice. Every treatment had 5 replications and 10 larvae per replication. The data related to the mortality rate were calculated based on probit analysis to evaluate the LT_{50} value at 95% fiducial limit [27], and mortality data was analyzed using one-way ANOVA (using WASP 2.0 online statistical tool), as indicated in Tables 1 and 2. The values were expressed as mean with standard error of the mean. Differences among the treatments were considered significant at $p \leqslant 0.05$. The enzymatic data were analyzed by two-way ANOVA (Bonferroni post hoc test) to determine the differences between the treatments as well as bioassay methods, where significant difference was set at 0.05 using IBM SPSS statistics software (version 21) (Table 3) [28].

RESULTS

Evaluated EPFs, Ma-NCIPM and Bb-NCIPM, caused pathogenicity against the *S. litura* larvae, but their potency differed. Percent mortality rate was observed to increase proportionally with the time interval. The data pertaining to the LT_{50}, fiducial limits (LL-UL), p-value, percent mortality rate are summarized below for both the larval immersion and topical spray methods. Lower LT_{50} value corresponds with the higher bioefficacy of EPFs. Infection symptoms which were prominent in infected larvae are sluggish mobility, intense melanization and body stiffing at the time of death, prolonged larval duration, and deformed live pupae and adults with deformed wings.

Bioefficacy of Ma-NCIPM and Bb-NCIPM on *S. litura via* larval immersion method

The bioefficacy of Ma-NCIPM and Bb-NCIPM towards *S. litura* larvae was compared with that of control through larval immersion method. The results exhibited that the mean mortality of *S. litura* larvae exposed with Ma-NCIPM ($68 \pm 0.49\%$, $\chi^2 = 11.04$, $p \leqslant 0.05$) and Bb-NCIPM ($56 \pm 0.25\%$, $\chi^2 = 2.090$, $p \geqslant 0.05$) was significantly higher than that of the control ($18.06 \pm 0.38\%$, $\chi^2 = 1.87$, $p \geqslant 0.05$) after 168 h of treatment. Ma-NCIPM caused $52 \pm 0.38\%$ mortality after 96 h of treatment which was only $38 \pm 0.38\%$ for Bb-NCIPM and $12 \pm 2.02\%$ for control (Fig. 1).

Table 1 Efficacy of *M. anisopliae* and *B. bassiana via* larval immersion method against *S. litura*.

Treatment	% mortality (mean ± SEM)	LT_{50} (h)	Slope ± SEM	(LL-UL)	χ^2
Ma-NCIPM	$68^{*}\pm 0.49^{a}$	50.17	2.52 ± 0.159	38.76–62.180	11.04
Bb-NCIPM	$56^{*}\pm 0.25^{b}$	165.70	2.33 ± 0.26	145.4–198.05	$2.09^{\#}$
Control	18.06 ± 0.38^{b}	171.14	2.34 ± 0.98	94.4–330.23	$1.87^{\#}$

Values are presented in mean ± SEM (standard error of the mean); every % mortality value represents the mean of the 5 replications; percent mortalities followed by the same superscripted letter in columns are not significantly different at 5% level of significance by Duncan's multiple range test (DMRT); df = 2; * = corrected % mortality; lower LT_{50} equivalent to higher efficacy; LT_{50}, median lethal time (in h); fiducial limit at 95%; LL, lower limit; UL, upper limit; χ^2, chi square value; and $^{\#} = p$ value $\geqslant 0.05$ and non-significant differences among the treatment.

Table 2 Efficacy of *M. anisopliae* and *B. bassiana via* topical spray method *S. litura*.

Treatment	% mortality (mean ± SEM)	LT_{50} (h)	Slope ± SEM	(LL-UL)	χ^2
Ma-NCIPM	$98^{*}\pm 0.2^{a}$	32.17	2.68 ± 0.26	12.24–47.92	17.025
Bb-NCIPM	$70^{*}\pm 0.32^{b}$	108.09	1.915 ± 0.162	95.99–123.72	6.07
Control	18 ± 0^{c}	130.38	1.35 ± 0.25	85.59–208.42	$0.152^{\#}$

Values are presented in mean ± SEM; every % mortality value represents the mean of the 5 replications; percent mortalities followed by the same superscripted letter in columns are not significantly different at 5% level of significance by DMRT; df = 2; * = corrected % mortality; lower LT_{50} equivalent to higher efficacy; LT_{50}, median lethal time (in h); fiducial limit at 95%; LL, lower limit; UL, upper limit; χ^2, chi square value; and $^{\#} = p$ value $\geqslant 0.05$ and non-significant differences among the treatment.

The observed LT_{50} were 50.17 and 165.7 h for Ma-NCIPM and Bb-NCIPM, respectively, which were equal to 3.41- and 1.03-fold decrease, respectively, compared with the control (171.14 h) (Table 1). In addition, after 168 h of fungal exposure, Ma-NCIPM was more virulent with the 3.02-fold diminished LT_{50} (50.17 h) ($p \leqslant 0.05$) compared to that of Bb-NCIPM (165.7 h) ($p > 0.05$) (Table 1).

Bioefficacy of Ma-NCIPM and Bb-NCIPM on *S. litura via* topical spray method

Significant mortality was measured for Ma-NCIPM and Bb-NCIPM on *S. litura* under topical spray method ($p \leqslant 0.05$). The topical spray method witnessed the highest mortality of *S. litura* larvae exposed to Ma-NCIPM ($\chi^2 = 17.025$, $p \leqslant 0.05$) compared to that of Bb-NCIPM ($\chi^2 = 6.07$, $p \leqslant 0.05$) and control ($\chi^2 = 0.152$, $p \geqslant 0.05$) (Fig. 2, Table 2) after 168 h of treatment. The mean mortality rate at recommended conidial concentration (1×10^8 spores/ml) was 98 ± 2.62 and 70 ± 1.42% by Ma-NCIPM and Bb-NCIPM, respectively, with 18 ± 2.46% for the control. The LT_{50} of Ma-NCIPM and Bb-NCIPM was 32.17 and 108.09 h ($p \leqslant 0.05$), respectively, which was 4.05- and 1.2-fold decrease, respectively, compared to the control (130 h, $p > 0.05$) (Table 2).

Impact of Ma-NCIPM and Bb-NCIPM exposure on the PO enzyme in *S. litura*

The results exhibited that the exposure of Ma-NCIPM and Bb-NCIPM on *S. litura* triggered non-significant changes in PO activity up to 24 h (82.93 ± 1.25 µmol/min/mg of protein) of treatment as compared to the control (85.27 ± 0.01 µmol/min/mg of protein) ($F_{2,30} = 2.26$, $p > 0.05$). After 72 h *via* larval immersion, Ma-NCIPM infection led to drastic decline in the activity of PO from 75.24 ± 0.231 to 20.89 ± 1.16 µmol/min/mg of protein ($p < 0.05$) as compared to the control (81.94 ± 0.215 µmol/min/mg of protein). Similarly, activity of PO subsequently decreased from 81.42 ± 0.664 to 70.82 ± 0.809 *via* larval immersion

Table 3 Phenoloxidase (PO) activity of *S. litura* larvae upon exposure to Ma-NCIPM and Bb-NCIPM.

Effect	SS	DF	MS	*F*-value	*p*-value
Treatment	47	2	238	13.327	0.000
Bioassay method	72	1	72	40.3	0.000
Treatment X bioassay method	154	2	773	4.33	0.022
Residual	536	30	172		
Total	809	35			

SS = sum of squares, DF = degree of freedom, MS = mean square, p = probability of significance ($\alpha = 0.05$), and $p \leqslant 0.05$ indicates significant differences among the treatment.

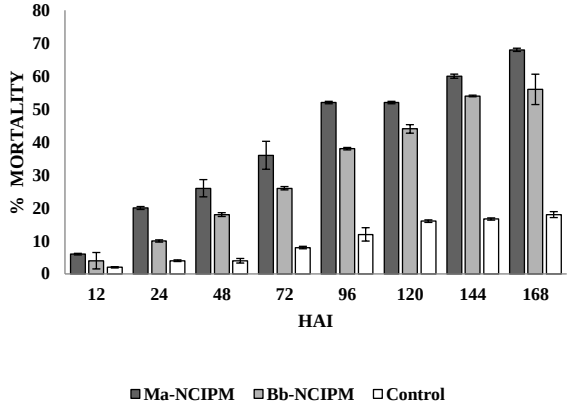

Fig. 1 Mortality of *S. litura* larvae treated with Ma-NCIPM and Bb-NCIPM *via* larval immersion. Black bar (35%) shade, gray bar (50%) shade, and white bar represent Ma-NCIPM, Bb-NCIPM and control, respectively. Each bar represents the mean of 5 independent replications, the error bars indicate SEM, different superscripted letters at the top of each bar show significant differences using Duncan's multiple range test (DMRT) at 5% level, and HAI = hours after infection.

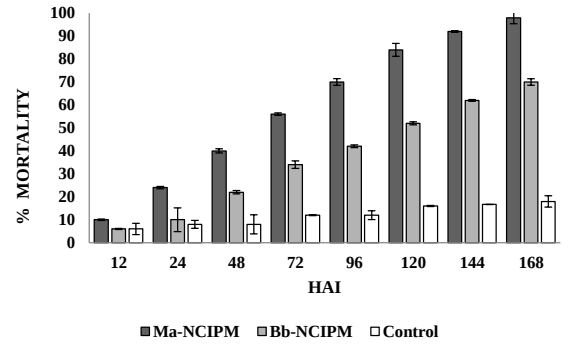

Fig. 2 Mortality of *S. litura* larvae treated with Ma-NCIPM and Bb-NCIPM *via* topical spray. Black bar (35%) shade, gray bar (50%) shade, and white bar represent Ma-NCIPM, Bb-NCIPM and control, respectively. Each bar represents the mean of 5 independent replications, the error bars indicate SEM, different superscripted letters at the top of each bar show significant differences using DMRT at 5% level, and HAI = hours after infection.

and from 34.2 ± 1.49 to 20.08 ± 1.06 µmol/min/mg of protein through topical spray method (Fig. 3). However, treatment with Bb-NCIPM led to a noticeable increment in the PO activity from 93.9 ± 0.693 to 109.87 ± 1.48 µmol/min/mg of protein after 120 h of treatment ($p < 0.05$) compared to the control (52.14 ± 1.64 µmol/min/mg of protein) (Fig. 3). At 168 h of fungal exposure, a sharp rise was noted in PO activity of all treatments compared to the control.

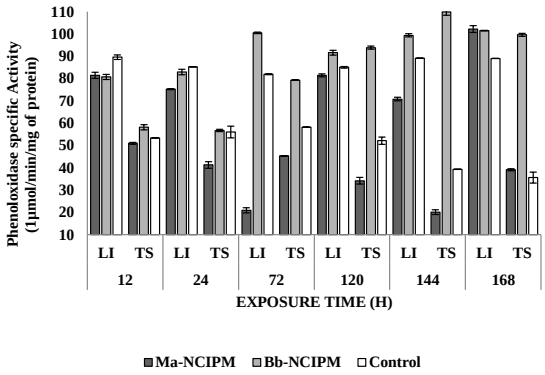

Fig. 3 Changes in the activity of phenoloxidase in the *S. litura* larvae as treated by Ma-NCIPM and Bb-NCIPM. Black bar (35%) shade, gray bar (50%) shade, and white bar represent Ma-NCIPM, Bb-NCIPM and control, respectively. LI = Larval immersion and TS = Topical Spray. Each bar represents the mean of 3 different replicates, mean values are specific activity of PO enzyme expressed as µmol/min/mg of protein, the error bars indicate SEM, and different superscripted letters at the top of each bar show significant differences using DMRT at 5% level.

Moreover, the factorial two-way analysis of variance demonstrated a significant effect of all treatments ($F_{2,30} = 13.327$, $p = 0.000$) for both bioassay methods ($F_{1,30} = 40.3$, $p = 0.000$) and the interaction between treatments and bioassay methods on specific activity of PO enzyme ($F_{2,30} = 4.323$, $p = 0.022$, Table 3).

DISCUSSION

The present study highlighted the pathogenic activity of Ma-NCIPM and Bb-NCIPM against third instar larvae of *S. litura* using 2 different exposure methods at recommended conidial dose (1×10^8 spores/ml), and both assessed EPFs exhibited lethal effects against *S. litura*. Our result exhibited that larval dipping of *S. litura* in Ma-NCIPM and Bb-NCIPM showed 68 ± 0.38 and 56 ± 0.25% mortality, respectively. However, through topical spray method, 98 ± 2.62 and 70 ± 1.42% mortality of third instar larvae of *S. litura* was observed by the exposure of Ma-NCIPM and Bb-NCIPM, respectively, at 1×10^8 spores/ml. The effective management of an insect population *via* EPFs hinges upon the equilibrium of host-pathogen-environment interactions [7]. Importantly, the utilization of recommended conidial densities (1×10^7 and 1×10^8 spores/ml) does not raise concerns, as these concentrations are deemed environmentally and biologically safer for animals, including humans. Hence, we assessed the virulence of Ma-NCIPM and Bb-NCIPM at 1×10^8 spores/ml concentration. A number of studies have reported significant lethal (bioefficacy) effects of *M. anisopliae* and *B. bassiana* against different species of *Spodoptera* [12, 17, 18, 24]. Han et al [12] evaluated the bioeffi-

cacy of *M. anisopliae* against *Spodoptera exigua* (Hub.). *M. anisopliae*, showing 100% mean mortality after exposure with 1×10^8/ml conidial concentration in topical spray method. The cumulative mortality of *S. litura* treated with *M. anisopliae* and *B. bassiana* was 91.4% and 63.56%, respectively, and the LT_{50} were 94.34 and 154.4 h, respectively in larval dip method [17]. El Husseini et al [18] also examined the pathogenicity of *B. bassiana* against *S. exigua*. *B. bassiana* caused 75% mortality after 168 h of application at 2×10^7/ml conidial concentration. By the same token [24], *B. bassiana* was screened for bioefficacy, and this EPF caused 43% significant mortality in *S. frugiperda* larvae at 1×10^8 per ml of spore concentration after 72 h of fungal inoculation. Our findings contradicted with the previous study by Lin et al [29], which suggested that the pathogenicity of *B. bassiana* (71.4%) was higher than that of *M. anisopliae* (63.3%). We observed that the mortality rate of the *S. litura* was increased proportionally with the inclination of exposure time. The least LT_{50} value of 32.17 h in case of Ma-NCIPM *via* topical spray method was observed while the highest LT_{50} value of 165.17 h was observed for Bb-NCIPM *via* larval immersion method. Present results contradict the findings by Ummidiet al [17] reporting 94.32 and 99.12 h of LT_{50} value against the *S. litura* by *M. anisopliae* and *B. bassiana*, respectively.

At the ground level, EPFs are one of the most prominent biological control agents. Over 700 different fungal species belonging to about 90 genera have been reported to be pathogenic to insects [30]. The mortality by anti-insect substances is often being related to the changes of some immune related defensive enzymes in the host insects [31]. Secondary metabolites are considered to accelerate oxidative stress and free radicals in the infected host, thus leading to host death [32]. It is known that these secondary metabolites are responsible for the induction of immune-reactions and modulate their activity. We found that in response to exposure with Ma-NCIPM and Bb-NCIPM, PO enzyme activity undulated. PO plays a key role in normal insect development, so if the enzyme activity is impeded or disturbed, insects could compromise their typical defense ability [16]. Our results indicated that the PO activity suddenly dropped with the treatment of Ma-NCIPM after 72 h of application and then gradually rose after 120 h of application. However, PO activity was inclined after the exposure of Bb-NCIPM after 72 h of treatment. Several studies have reported the effects of fungal infection on the PO activity in many economically important insect pests. Exposure with *M. anisopliae* has been reported to significantly increase in the level of PO after 24 h of treatment and then led to drastic drop in the PO activity against the *L. migratoria* [15]. Application of *M. anisopliae* and *B. bassiana* led to a significant increment in PO enzyme level in *S. litura* larvae [33]. Results of present study contradict with the findings of Bali et al [34] reporting a drop in the level of PO in *S. litura* with the exposure of *B. bassiana* after 72 h of treatment.

Bioassay methods deliver the spores directly to the host cuticle facilitating the spread of the fungus into the hemolymph of the host. We found that the topical spray method significantly affected the bioefficacy of Ma-NCIPM and Bb-NCIPM; however, larval immersion method was found less effective compared to the topical spray method. It is possible that a greater number of spores of the tested EPFs could attach to host larvae in the topical spray method since the ability of conidia to adhere to the host cuticle is substantially linked with virulence [35]. The actual dose of conidia per host was higher when the larvae move across fungal treated surface (foliage + topical spray) in comparison to the larval immersion method. We selected newly moulted third instar larvae for the bioassay purpose; however, the early larval instars are still more susceptible, and thus, intermediate instar (third or fourth) stages may be managed more effectively with microbes before reaching the adult stage [36]. Innate immune responses of the last larval stages and pupae render them less susceptible to microbial pathogens [37]. Both exposure methods deemed to be acceptable to evaluate the virulence of EPFs [38, 39]. The larval immersion method was used to investigate the susceptibility of various lepidopteran insect pests because the host body was equally covered by tested conidial suspension using this method better than the direct spray method. However, the topical spray and leaf dip methods have practical field implications and utility for testing biopesticides over the immersion method [40].

CONCLUSION

Based on the findings, we concluded that the Ma-NCIPM was more pathogenic than the Bb-NCIPM against the *S. litura*, and 48 to 120 h of application of EPFs was the most important time for the pathogenesis. These findings would offer information which could be exploited to boost the virulence of EPFs against the insect pests. Future research would be needed to examine the effectiveness of these EPF strains and there bio-formulations against other major insect pests of economic importance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.079.

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Appendix A. Supplementary data

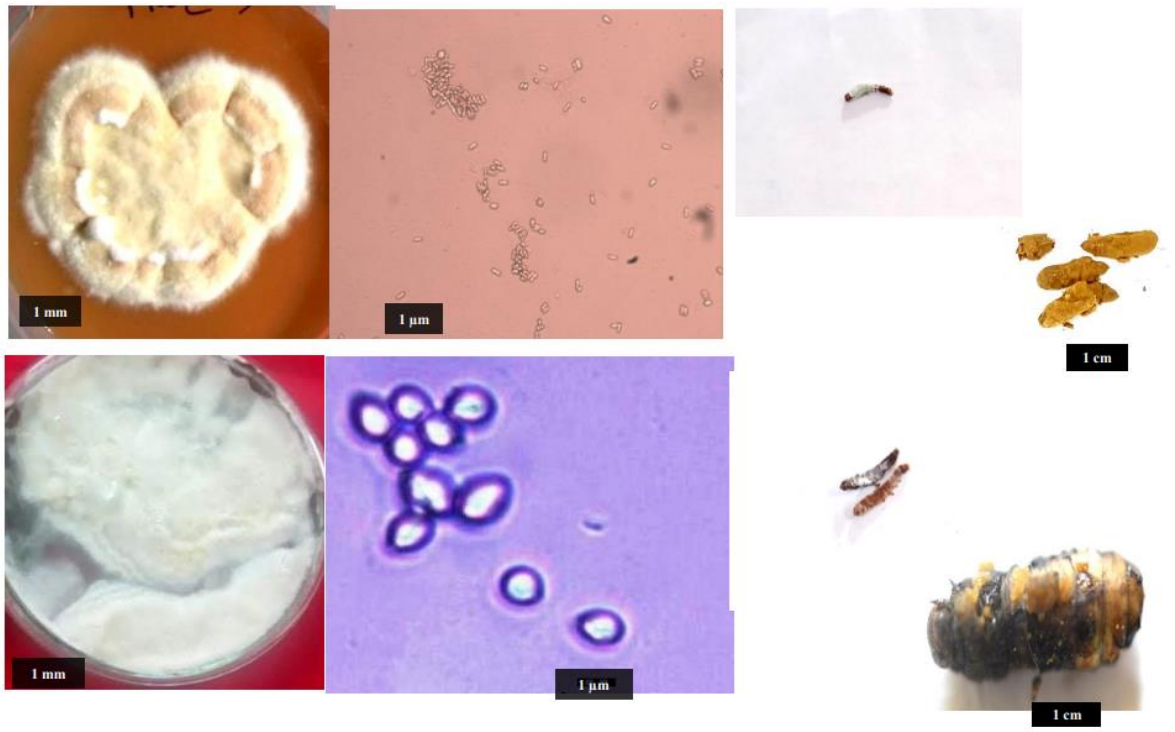

Fig. S1 *S. litura* showing symptoms due to Ma-NCIPM and Bb-NCIPM exposure. Upper one left to right: Ma-NCIPM 10-day-old colony, Ma-NCIPM spores @ 40×, *S. litura* cadaver showing symptoms of Ma-NCIPM, and Pupae of *S. litura* showing sporulation due Ma-NCIPM. Lower one left to right: Bb-NCIPM 10–12-day-old colony, Bb-NCIPM spores @ 100×, *S. litura* showing mycosis due to Bb-NCIPM, and Dead and deformed *S. litura* pre-pupa due to Bb-NCIPM, respectively.